Effects of beta-alanine supplementation on cardiac function and health

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Declaration

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Statement

The work presented in this thesis was part of a PhD studentship, match-funded (50/50) by Nottingham Trent University (UK) and Natural Alternatives International (San Diego, USA).

Published work

The content within section **1.2** of **Chapter 1** of this thesis has previously been published. Details of the publication can be found on **page xxi** and in section **1.1**. The content within **Chapter 4** has been accepted for publication. Details of this can be found on page **xxi** and in section **4.1**.

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Dedication

This thesis is dedicated to the loving memory of my dad Steven, my brother Liam, and my grandparents Chris and Janet. Although you never got to witness this academic adventure, I felt your presence with me every step of the way. I miss you more than words can say. I hope you are all proud. Liam, keep that celebratory drink cold for me.

List of abbreviations

2-D	2-dimensional
AEE	Activity energy expenditure
AGEs	Advanced glycation end products
AGXT2	Alanine-glyoxylate aminotransferase 2
ALE	Advanced lipid end product
ALP	Alkaline phosphatase
ALT	Alanine transaminase
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
AP2CH	Apical 2 chamber view
AP4CH	Apical 4 chamber view
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
BMI	Body mass index
BMP2	Bone morphogenetic protein-2
BSA	Body surface area
CARNMT1	Carnosine-N-methyltransferase
CARNS1	Carnosine synthase
CARNS1^{-}/$^{-}$	CARNS1-knockout
cDNA	Complementary DNA
CI	Cardiac index
CK	Creatine kinase
CK-mB	Creatine kinase isoenzyme
CNAP	Continuous non-invasive blood pressure
CNDP1	Carnosine dipeptidase 1
CNDP2	Carnosine dipeptidase 2
CO	Cardiac output
Ct	Cycle threshold
cTnT	Cardiac troponin T

CVD	Cardiovascular disease
DIO	Diet-induced obesity
DIT	Dietary-induced thermogenesis
DMEM	Dulbecco's modified eagle's medium
DMEM-F-12	Dulbecco's modified eagle's medium/nutrient mixture F-12 Ham
DNA	Deoxyribonucleic acid
DOPAL	3,4-dihydroxyphenylacetaldehyde
DOX	Doxorubicin
EC	Excitation-contraction
ECG	Electrocardiogram
EDV	End diastolic volume
EF	Ejection fraction
ESV	End systolic volume
FBS	Foetal bovine serum
GABA-T	4-aminobutyrate-2-oxoglutarate transaminase
GASE	General assessment of side effects
GATA4	GATA binding protein 4
GGT	Gamma-glutamyl transpeptidase
GP	General Practice
H & E	Haematoxylin and eosin
HbA1c	Glycated haemoglobin
HCD	Histidine-containing dipeptide
HDL	High-density lipoprotein
HF	High-frequency
HFD	High-fat diet
HHE	4-hydroxy-2-hexenal
HNE	4-hydroxy-2-nonenal
HRA	NHS Health Research Authority
hs-CRP	High sensitivity C-reactive protein
I/R	Ischemia reperfusion
IBI	Inter-beat interval

IMS	Industrial methylated spirit
IVSd	Interventricular septal end diastole
IVSs	Interventricular septal end systole
LDH	Lactate dehydrogenase
LDL	Low-density lipoprotein
LF	Low-frequency
LVIDd	Left ventricular internal diameter end diastole
LVIDs	Left ventricular internal diameter end systole
LVOT	Left ventricular outflow tract
M-mode	Movement mode
MSA	Malonate semi-aldehyde
MYCD	Myocardin
NBP	Non-invasive blood pressure
NFATc4	Nuclear factor of activated T cells 4
NFW	Nuclease-free water
NHS	National Health Service
NTU	Nottingham Trent University
OCT	Optimal cutting temperature compound
OPA	o-phtaldialdehyde
PAL	Physical activity level
PAT1	Proton-coupled amino acid transporter
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEPT1	Peptide transporter 1
PEPT2	Peptide transporter 2
PFA	Paraformaldehyde
PHT1	Peptide-histidine transporter 1
PHT2	Peptide-histidine transporter 2
PWd	Posterior wall thickness end diastole
PWs	Posterior wall thickness end systole
REE	Resting energy expenditure

RER	Respiratory exchange ratio
RMSSD	Root mean square of the successive differences of IBIs
RNA	Ribonucleic acid
RNA-Seq	RNA sequencing
ROS	Reactive oxygen species
RT-qPCR	Real-time quantitative polymerase chain reaction
RVOT	Right ventricular outflow tract
RyR	Ryanodine receptor
SD	Standard deviation
SEM	Standard error of the mean
SERCA2	Sarco(endo)plasmic reticulum Ca^{2+} ATPase
SV	Stroke volume
SVI	Stroke volume index
SVR	Systemic vascular resistance
SVRI	Systemic vascular resistance index
TauT	Sodium and chloride-dependent taurine transporter
TEE	Total energy expenditure
TTE	Transthoracic echocardiography
$\dot{V}CO_2$	Carbon dioxide production
$\dot{V}O_2$	Oxygen consumption
WAT	White adipose tissue

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List of publications, abstracts, and community engagements

Publications

Matthews, J.J*., **Creighton, J.V***., Donaldson, J., Swinton, P.A., Kyrou, I., Bellary, S., Idris, I., Santos, L., Turner, M.D., Doig, C.L., Elliott-Sale, K.J., Sale, S. β-alanine supplementation in adults with overweight and obesity: a randomised controlled feasibility trial. **The manuscript was accepted for publication in Obesity (23/10/2024). * Joint first authorship.**

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Wadley, A.J., Roberts, M.J., **Creighton, J.**, Thackray, A.E., Stensel, D.J., Bishop, N.C., 2021. Higher levels of physical activity are associated with reduced tethering and migration of pro-inflammatory monocytes in males with central obesity. Exerc Immunol Rev 27, 54-66.

Templeman, I., Smith, H.A., Chowdhury, E., Chen, Y.C., Carroll, H., Johnson-Bonson, D., Hengist, A., Smith, R., **Creighton, J.**, Clayton, D., Varley, I., Karagounis, L.G., Wilhelmsen, A., Tsintzas, K., Reeves, S., Walhin, J.P., Gonzalez, J.T., Thompson, D., Betts, J.A., 2021. A randomized controlled trial to isolate the effects of fasting and energy restriction on weight loss and metabolic health in lean adults. Sci Transl Med 13, eabd8034. https://doi.org/10.1126/scitranslmed.abd8034

Tan. A., Younis, A.Z., Evans, A., **Creighton, J.V.**, Coveny, C., Boocock, D.J., Sale, C., Lavery, G.G., Coutts, A.S., Doig, C.L., 2023. PARP1 mediated PARylation contributes to myogenic progression and glucocorticoid transcriptional response. Cell Death Discovery 9, 133. https://doi.org/10.1038/s41420-023-01420-2

Published abstracts and conferences

Creighton, J.V. (2022). The metabolic pathway of carnosine and beta-alanine in the heart. Presented at Nottingham Trent University STAR PGR Conference 2022. **This presentation won 1st prize in the 10-minute oral communication category.**

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Creighton, J.V., Matthews, J.J., Donaldson, J., Swinton, P.A., Kyrou, I., Bellary, S., Idris, I., Santos, L., Turner, M.D., Elliott-Sale, K.J., Doig, C.L., Sale, C. (2024). Three months of beta-alanine supplementation on haemodynamic and biochemical markers of myocardial

function and health in adults with overweight or obesity. Presented at Dietary Manipulations for Health and in the Prevention and Management of Disease Conference, Physiological Society, Manchester, UK. **This poster won 1st prize in the early career researcher poster competition. Travel funding received from the Physiological Society.**

Community engagement

Seminar presented to the U3A West Bridgford community group (Nottinghamshire) titled “Beta-alanine supplementation in adults with overweight/obesity” (April 2023).

Abstract

Carnosine, a pleiotropic histidine-containing dipeptide (HCD), is synthesised from beta-alanine and L-histidine; the intact dipeptide and constituent amino acids can be obtained through dietary sources (e.g., meat, poultry, and fish) or via supplementation. Beta-alanine is the rate-limiting precursor for intramuscular carnosine synthesis. The therapeutic potential of carnosine in cardiac tissue is promising due to its involvement in calcium handling and sensitivity and oxidative stress, and potential improvements on haemodynamic and histological outcomes. The overall aim of this research programme was to investigate the metabolism of carnosine and beta-alanine in the heart, and the physiological role(s) of beta-alanine supplementation on cardiac function and health in a metabolic disease model with an associated risk of cardiovascular disease.

The research programme had several specific objectives, utilising both *in vitro* and *in vivo* experimental models. Initially, the uptake and storage of exogenous carnosine and beta-alanine in rat cardiomyocyte cells was explored. This was followed by pre-clinical (*in vivo* mouse) and clinical (*in vivo* human) interventions, conducted to assess the effects of beta-alanine supplementation on markers of cardiac function and health in a model of metabolic stress (overweight and obesity).

The *in vitro* experiment suggests that rat cardiomyocyte cells have the enzymes and transporters in place to take up and store exogenous carnosine and beta-alanine; although, the accumulation of beta-alanine in the cardiomyocytes did not increase the synthesis of carnosine within the 72-hour treatment timeframe. In the pre-clinical experiment, beta-alanine concentration was increased in cardiac tissue of mice

supplemented with 1.2 % (w/v) of beta-alanine for 12 weeks. Additionally, carnosine concentration in cardiac tissue was increased with beta-alanine supplementation, indicating the synthesis of beta-alanine with L-histidine to form carnosine. An elevation in cardiac anserine concentration, a methylated form of HCD dependent on carnosine synthesis, was also shown. Both *in vivo* studies did not show any influence of beta-alanine supplementation on cardiac function, health, or morphology in overweight and obesity models. Longer beta-alanine supplementation and/or studies in experimental models with established cardiovascular disease comorbidities may be necessary to further explore any therapeutic potential of beta-alanine supplementation on markers of cardiovascular function and health.

Chapter 1: Introduction

1.1 Overview of the chapter

The content of section **1.2** has been previously published as an open-access narrative review (**Creighton, J.V.,** Gonçalves, L.D.S., Artioli, G.G., Tan, D., Elliott-Sale, K.J., Turner, M.D., Doig, C.L., Sale, C., 2022. Physiological roles of carnosine in myocardial function and health. Adv Nutr 13, 1914–1929. https://doi.org/10.1093/advances/nmac059). This section provides a review of the current literature relating to the effects of carnosine and beta-alanine on cardiac function and health, providing an overview of the metabolic pathway of carnosine and beta-alanine in cardiac tissue and the potential physiological roles they may play in the heart in both healthy and diseased environments. This is followed by a critical analysis of the existing literature, focusing on the effects of exogenous carnosine and beta-alanine on the potential physiological roles described. The review also identifies gaps in the literature and provides suggestions for future research to address these gaps. This narrative review has been included in the thesis as published, with only minor adjustments to heading, table, and figure numbers, and the referencing style changed to align with the thesis format.

Section **1.3** has been added to this chapter to include literature published since the review and to expand on literature that was outside the scope of the narrative review. The chapter concludes with the research rationale, aims, objectives, and hypotheses of the programme of work (section **1.4**).

1.2 Published narrative review

1.2.1 Introduction

Carnosine (β-alanine-l-histidine) is a pleiotropic histidine-containing dipeptide (HCD) abundant in skeletal muscle and the central nervous system—specifically, the olfactory bulbs [for a comprehensive review, see Boldyrev et al. (2013)]. Carnosine was first isolated in the early 1900s (Gulewitsch and Alikhanov, 1900) and has become well known for its role as an intracellular pH buffer, particularly in skeletal muscle, and subsequently, as an ergogenic aid to exercise capacity and performance (Hobson et al., 2012; Saunders et al., 2017b).

Various determinants of mammalian muscle carnosine content have been previously identified: diet, species, muscle fiber type, sex, age, training, and exercise (Harris et al., 2012). One of the key determinants of muscle carnosine content, particularly pertinent to this review, is the availability of carnosine and its constituent amino acids from the diet. The availability of dietary β-alanine is a key limitation to the in situ synthesis of carnosine (Harris et al., 2006), which can be largely obtained through the ingestion of meat/fish or via supplementation. In vegans, or in some vegetarians, however, the supply of β-alanine for carnosine synthesis is limited to its synthesis in the liver from uracil degradation or from supplemental sources if deemed acceptable. To highlight this, Everaert et al. (2011) reported low skeletal muscle carnosine contents in vegetarian participants when compared with individuals consuming a fairly typical omnivorous Belgian diet. This makes sense given that the de novo synthesis of β-alanine in the liver would be augmented by the hydrolysis of dietary supplied HCDs from meat in omnivores

but not in vegetarians, which would explain the significant differences in skeletal muscle carnosine content (Harris et al., 2007). The dietary availability of β-alanine might also be significantly influenced by cooking procedures, which can account for significant reductions in β-alanine availability.

Over the last 30 y, research has explored carnosine's therapeutic potential across various pathological conditions, including neurodegenerative diseases, diabetes, and in aging populations [for a review on this topic, see Artioli et al. (2019)]. Emerging evidence has highlighted a promising effect of carnosine on healthy and diseased cardiovascular systems. This growing research suggests that the physiological roles of carnosine in myocardial tissue could involve the regulation of calcium handling and sensitivity, quenching of reactive oxygen species (ROS), detoxification of reactive aldehydes [including lipid peroxidation products and advanced glycation end products (AGEs)], chelation of transition metal ions, and improvements in histological and hemodynamic parameters (Boldyrev et al., 2013). This narrative review explores the influence of carnosine and its physiological and potentially therapeutic roles in the heart, critically examining data from studies concerning both healthy and diseased myocardial models. We have focused specifically on human models where literature is available, but data are limited in this regard as it is challenging to access human myocardial tissue. Therefore, literature involving animal myocardial tissue and other experimental models has been included. In cases where these data do not exist, we have extrapolated from other relevant tissues (i.e., skeletal muscle). This review also suggests some future directions for research in this area. Due to the heterogeneity of experimental designs (e.g., species,

supplementation dosage, supplementation period, and experimental model), it was impossible to conduct a systematic review and meta-analysis.

1.2.2 Myocardial carnosine concentration

Table 1.1. Carnosine content of skeletal muscle and the brain in various species

Study	Tissue	Species	Concentration
Skeletal Muscle			
Harris et al. (1990)	Vastus Lateralis	Human	16.0 ± 7.2 mmol.kg^{-1} dry muscle (mean ± SD)
Flancbaum et al. (1990)	Muscle	Rat	19.07 ± 6.77 μg/g (mean ± SEM)
		Mouse	8.03 ± 4.27 μg/g (mean ± SEM)
		Guinea Pig	5.15 ± 0.41 μg/g [1] (mean ± SEM)
Mannion et al. (1992)	Quadriceps Femoris	Human	20 ± 4.7 mmol.kg^{-1} dry muscle (mean ± SD)
Chan et al. (1994)	Leg	Rat	874.1 ± 88.1μg/g (mean ± SEM)
Brain			
Margolis. (1974)	Olfactory Bulb	Mouse	2.2 mM
	Whole brain (excluding olfactory bulb)	Mouse	Not detected
Flancbaum et al. (1990)	Olfactory Bulb	Rat	11.20 ± 5.04 μg/g (mean ± SEM)
		Mouse	31.10 ± 4.85 μg/g (mean ± SEM)
		Guinea Pig	5.28 ± 2.49 μg/g (mean ± SEM)
	Hypothalamus	Rat	3.91 ± 0.71 μg/g (mean ± SEM)
		Mouse	25.97 ± 3.83 μg/g (mean ± SEM)
		Guinea Pig	3.11 ± 0.95 μg/g (mean ± SEM)
	Pituitary	Rat	14.22 ± 3.41 μg/g (mean ± SEM)
		Guinea Pig	1.56 ± 1.08 μg/g (mean ± SEM)
	Cerebrum	Mouse	7.60 ± 2.17 μg/g (mean ± SEM)

Carnosine is found in high concentrations within skeletal muscle and the brain (**Table 1.1**). The intracellular concentration of carnosine within myocardial tissue has been suggested to be 0.1 mmol/kg wet weight, with the total concentration of HCDs being as high as 10 mM/kg wet weight (O'Dowd et al., 1988). This is a plausible assumption in

myocardial tissue since HCD concentrations are typically higher in fast-twitch glycolytic fibers and lower in slow-twitch oxidative fibers (Xie et al., 2013), meaning that we would not expect concentrations in the oxidative myocardial tissue to exceed those measured in the more glycolytic skeletal muscle, and concentrations of other amino acids in myocardial tissue fall within this low millimolar range (Heger et al., 2013; Weitzel et al., 2013). Studies that have measured HCD concentrations within myocardial tissue are scarce, and there are discrepancies between those that have. Flancbaum et al. (1990) identified the presence of carnosine in murine (mean ± SEM: 10.94 ± 3.12 μg/g), rat (mean ± SEM: 25.11 ± 3.22 μg/g), guinea pig (mean ± SEM: 17.39 ± 3.74 μg/g), and human (mean ± SEM: 10.12 ± 1.23 μg/g) myocardial tissue, whereas Jackson and Lenney (1996) only confirmed the presence of carnosine within rat hearts and not human hearts using immunoreactivity. Chan et al. (1994) and Liu et al. (2016) measured endogenous carnosine in rat hearts to be 189.2 ± 3.5 μg/g (mean ± SEM) and 36.9 ± 13.61 μg/g (mean ± SEM). Interspecies variation and differences in the sensitivity and accuracy of the methodological techniques used may explain the inconsistencies between studies (Crush, 1970). In contrast to skeletal muscle, n-acetylcarnosine (an n-acetyl derivative of carnosine) may be the predominant HCD in mammalian myocardial tissue, with carnosine being present at a relatively low concentration (Johnson and Hammer, 1992; O'Dowd et al., 1988), although it is unclear why this may be the case. The additional n-acetyl group may allow n-acetylcarnosine to be more resistant to hydrolysis by carnosinase (Hipkiss, 2009), or it may have a more fundamental role within myocardial tissue. The effect of supplementation on n-acetylcarnosine content has not been investigated; it would be interesting to identify whether its concentration can be increased via β-alanine or carnosine supplementation and, if so, whether this exerts any

benefit to myocardial tissue. Therefore, a complete profile of the intracellular concentration of carnosine, and its associated derivatives, in myocardial tissue is warranted across a range of species, including humans.

1.2.3 Myocardial carnosine metabolism

The major pathways of carnosine metabolism consist of hydrolysis to, and synthesis from, its constituent amino acids (**Figure 1.1**) and the activities of peptide transporters, β-alanine transporters, and transaminases.

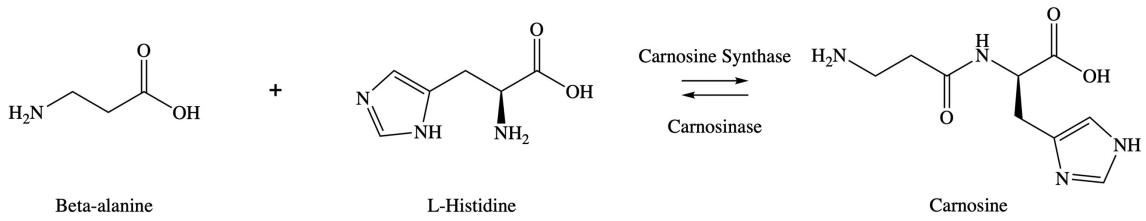


Figure 1.1. The chemical structure of β-alanine ($C_3H_7NO_2$), L-histidine ($C_6H_9N_3O_2$), and carnosine ($C_9H_{14}N_4O_3$), and the metabolic pathway between carnosine and its constituent amino acids. Created using Chem Draw (PerkinElmer).

1.2.3.1 Carnosine synthase

The metabolic pathway of carnosine is regulated by carnosine synthase (CARNS1) and carnosinase activity (CN1 and CN2) (Bellia et al., 2014). CARNS1, a member of the adenosine triphosphate (ATP)-grasp family, catalyzes β-alanine, l-histidine, and ATP into carnosine, adenosine diphosphate, and inorganic phosphate (Drozak et al., 2010). Carnosine synthesis is dependent upon the availability of its constituent amino acids from the diet, either directly or from the hydrolysis of HCDs. The content of β-alanine in cells is substantially lower than l-histidine and the affinity for CARNS1 is also lower for β-alanine compared with l-histidine (Km values of 1.0 to 2.3 mM and ∼17 μM) [rat brain

(Skaper et al., 1973); mouse olfactory pathway (Horinishi et al., 1978); rat central nervous system (Ng and Marshall, 1978)], meaning that β-alanine availability is considered the rate-limiting precursor for carnosine synthesis in skeletal muscle cells (Harris et al., 2006). Although these data do not relate specifically to myocardial tissue, β-alanine may also be rate-limiting in the myocardium due to the abundance of l-histidine.

As mentioned previously, β-alanine is produced in the liver through the degradation of uracil and released into the bloodstream (Fritzson and Pihl, 1957). The demand for β-alanine is assumed to far exceed this endogenous supply (Boldyrev et al., 2013). Figure 2 in Artioli et al. (2010) provides a detailed diagram of the β-alanine pathway and synthesis rate in the liver. Carnosine synthesis is dependent upon the availability of exogenous β-alanine ingested through the diet (meat extracts, poultry, and fish) (Everaert et al., 2011; Harris et al., 2007) or via supplementation. A typical Belgian omnivorous diet provides approximately 300 mg/d of β-alanine and a vegetarian/vegan diet will provide almost no β-alanine (Baguet et al., 2009). Sufficient availability of β-alanine is considered essential for myocardial carnosine synthesis (Blancquaert et al., 2016). Once synthesized, it is assumed that carnosine does not readily exit the cell in its intact form and can accumulate in the cell (Wu, 2020); nevertheless, carnosine content may wash out over time due to degradation.

1.2.3.2 Carnosinases

Carnosine is degraded into its constituent amino acids by two isoforms of carnosinase: CN1 and CN2. CN1 is primarily expressed in human plasma with high specificity for

carnosine and homocarnosine, whereas CN2, known initially as tissue carnosinase, has a broad substrate specificity and is ubiquitously expressed throughout central and peripheral human tissues (Jackson et al., 1991; Lenney et al., 1985; Teufel et al., 2003). CN1 is present in animal and human myocardium (Drozak et al., 2010; Margolis et al., 1987), although it is unclear whether this is simply a result of cross-contamination of circulating blood within myocardial tissue rather than it being directly expressed in the tissue (Jackson et al., 1991; Lenney et al., 1985). The role of CN2 in the myocardium has not been confirmed given that there are inconsistencies in the literature regarding myocardial CN2 expression in humans. Lenney et al. (1985) discovered CN2 activity in the human heart, whereas Teufel et al. (2003) showed mRNA expression but no protein expression. Carnosinases optimally function at a pH of 7.5 to 8.5 (CN1) and 9.5 (CN2) (Bellia et al., 2014; Boldyrev et al., 2013). Even though mRNA transcripts of CN2 are expressed in skeletal muscle (Everaert et al., 2013; Saunders et al., 2017a), the pH of skeletal [~7.1 (Matthews et al., 2019)] and cardiac [~7.2 (Vaughan-Jones et al., 2009)] muscles are not optimal for CN2 activity. This suggests that, even if the enzyme is present in the tissue, it is not guaranteed to participate in the regulation of tissue HCD concentrations. This suggestion is consistent with the very low rates of carnosine degradation shown in human skeletal muscle washout studies. These results indicate that carnosine is a highly stable metabolite and carnosinases do not play a role within skeletal muscle myocytes (Baguet et al., 2009).

1.2.3.3 β-alanine transaminases

Alanine-glyoxylate aminotransferase 2 (also known as AGXT2 or β-alanine-pyruvate transaminase) and 4-aminobutyrate-2-oxoglutarate transaminase (also known as GABA-

T or β-alanine-2-oxoglutarate transaminase) have been reported to be responsible for catalyzing the degradation of excess exogenous β-alanine into keto-acid malonate semi-aldehyde (MSA). MSA may be converted into acetyl-coenzyme A and subsequently enter the citric acid cycle (Blancquaert et al., 2016), although this hypothesis requires further investigation. Oxidative muscles (e.g., myocardial tissue and soleus) have a higher expression of β-alanine transaminases when compared with more glycolytic muscles (e.g., gastrocnemius and tibialis anterior muscles). This may explain the lower concentrations of carnosine in myocardial tissue when compared with the high concentration found in skeletal muscle. The transaminase degradation process may limit the quantity of β-alanine available for carnosine synthesis. The importance of transaminases in myocardial β-alanine and carnosine homeostasis is unclear and requires further investigation, although the contribution of AGXT2 to overall carnosine content is likely to be small. Plasma and skeletal muscle β-alanine and carnosine concentrations were unaffected in AGXT2-knockout mice and in humans with a decreased AGXT2 activity genotype (Stautemas et al., 2020), and a nonsignificant elevation in circulating β-alanine was shown with Vigabatrin (Sabril; Lundbeck, Deerfield, IL, USA), a GABA-T inhibitor (Blancquaert et al., 2016). This suggests that β-alanine homeostasis is either maintained by a tightly regulated system between GABA-T and AGXT2 (i.e., when one enzyme's activity is altered, the other enzyme can compensate) or AGXT2 activity in β-alanine and carnosine homeostasis is low and the system is dependent upon GABA-T activity. When both GABA-T and AGXT2 were inhibited with amino-oxyacetate, total HCD content in striated muscles (including myocardial and skeletal muscles) was increased 10-fold (Blancquaert et al., 2016), suggesting that GABA-T, and not AGXT2, regulates endogenous β-alanine homeostasis. It would be interesting

to investigate transaminase activity in response to exogenous β-alanine provision and whether they influence its availability and uptake into myocardium for a potential contribution to carnosine synthesis.

1.2.3.4 Amino acid and nonspecific peptide transporters

Proton-coupled amino acid transporter (PAT1) and sodium and chloride-dependent taurine transporter (TauT) are likely to facilitate β-alanine transportation, and the proton-coupled oligopeptide transporter family (peptide transporter 1 (PEPT1), peptide transporter 2 (PEPT2), peptide-histidine transporter 1 (PHT1) and peptide-histidine transporter 2 (PHT2); collectively known as the solute carrier family 15 are responsible for nonspecific cellular transportation of peptides (Smith et al., 2013). The degree of their involvement in the cellular transportation of amino acids and peptides into the myocardium is unknown. β-alanine and taurine regulate the expression of the transporter TauT within skeletal muscle and decreased β-alanine uptake was shown in an immortalized mouse skeletal muscle cell line (C2C12) exposed to hypotaurine (a TauT inhibitor), suggesting that TauT may be the primary transporter of β-alanine in skeletal muscle (Santos et al., 2021). Due to the abundance of taurine in myocardial tissue (Ito et al., 2008), it can be postulated that β-alanine would also enter myocardial tissue via this transporter. Rodent studies have demonstrated a significant decrease in myocardial taurine content with β-alanine supplementation (Abebe and Mozaffari, 2003; Allo et al., 1997; Harada et al., 1990; Jong et al., 2012, 2010; Pansani et al., 2012), suggesting competition between these two amino acids to use TauT for transportation into the myocardium, although this remains to be directly confirmed. The role of PAT1 in the myocardium may be primarily associated with cell growth regulation rather than amino

acid transportation (Jensen et al., 2014), suggesting that TauT may be the preferred transporter for the uptake of β-alanine into the myocardium.

1.2.4 Physiological roles of carnosine in myocardial tissue

The proposed physiological roles of carnosine include intramuscular pH buffer, the regulation of intramuscular calcium handling and improved contractile apparatus sensitivity to calcium, improvements in histopathological and hemodynamic parameters, the quenching of ROS, metal ion chelation, and protection against lipid peroxidation and AGEs (Boldyrev et al., 2013) (**Figure 1.2**). Much of the research into carnosine has focused on the implications in exercising human skeletal muscle [for a comprehensive review on this topic, see (Matthews et al., 2019)]. The first study looking at the physiological effect of carnosine on cardiovascular function demonstrated a decrease in systemic pressure in canines injected with carnosine (Razenkov et al., 1926). It has been suggested that carnosine plays different roles within different tissues, the same role in different tissues, and even multiple roles in the same tissue depending upon the health of that tissue. This section of the review will focus on the mechanistic action of endogenous and exogenous carnosine in both healthy and diseased myocardial models.

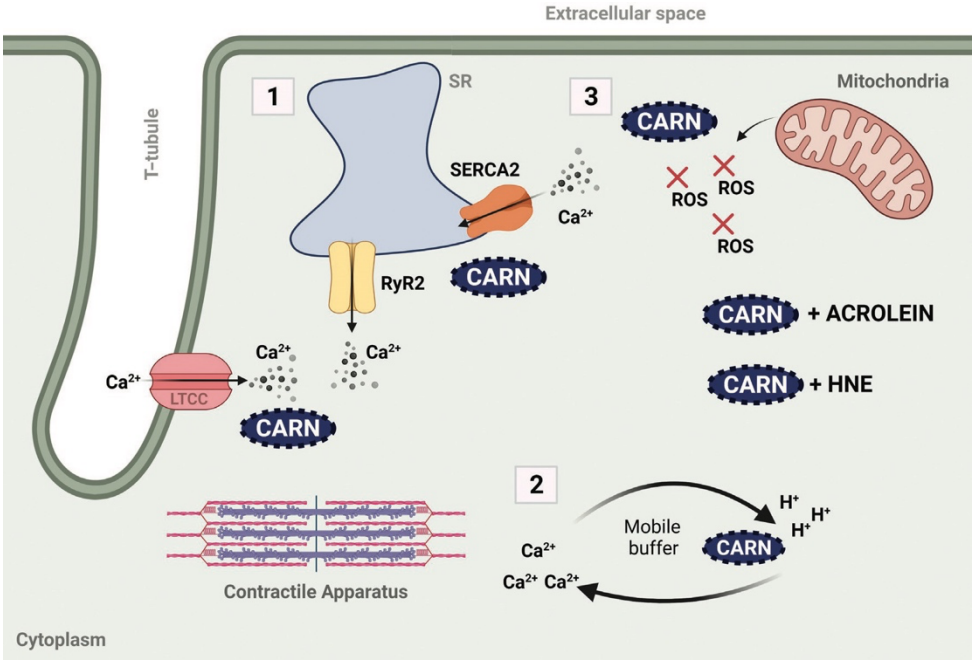


Figure 1.2. The main physiological roles of carnosine in myocardial function and health: an overview. (1) Carnosine (CARN) regulates EC coupling by influencing calcium (Ca^{2+}) release from the SR via the RyR2 and Ca^{2+} reuptake via SERCA2. (2) Carnosine acts as a mobile Ca^{2+}/H^{+} buffer, transporting Ca^{2+} across the cytosol in an H^{+}-coupled manner. (3) Carnosine prevents excessive accumulation of oxidative stress products (e.g., ROS) and acts as a scavenger to form covalent adducts with reactive aldehydes (e.g., acrolein and HNE) (created using BioRender.com).

EC, excitation-contraction; HNE, 4-hydroxy-2-nonenal; LTCC, L-type calcium channel; ROS, reactive oxygen species; RyR2, ryanodine receptor; SERCA2, sarco(endo)plasmic reticulum Ca^{2+} ATPase; SR, sarcoplasmic reticulum.

1.2.4.1 Intramuscular pH buffer and energy metabolism

Carnosine is recognized as an intracellular pH buffer due to the imidazole ring on the histidine residue of carnosine having a pKa of 6.83 (Smith, 1938). Although carnosine's buffering role is highly relevant to skeletal muscle, its role in myocardial tissue remains unclear. The absence of hydrogen ion accumulation, alongside the dependence on mitochondrial oxidative metabolism to maintain homeostasis (Doenst et al., 2013), suggests that any role of carnosine within healthy myocardial tissue may not primarily relate to intracellular pH buffering and energy metabolism. In healthy CARNS1-knockout

(CARNS1$^{-/-}$) rats, the absence of endogenous HCDs (such as carnosine) did not impair mitochondrial function or oxygen consumption and hydrogen peroxide release was not different between the CARNS1$^{-/-}$ rats and wild-type controls (Gonçalves et al., 2021). This may be attributed to the fact that oxidative stress, which can lead to mitochondrial dysfunction, was not increased in the absence of carnosine. Although carnosine may not exert these effects in healthy myocardial tissue, it could have a protective role under diseased conditions. When ATPGD1-transgenic mice overexpressing CARNS1 were exposed to myocardial ischemia, an increase in endogenous carnosine attenuated ischemia/reperfusion-induced changes in intracellular pH (Zhao et al., 2020). In isolated rat hearts exposed to cardioplegia (a pharmacological solution used to cease cardiac activity temporarily), an HCD supplement (consisting of carnosine, l-histidine, and acetylcarnosine) resulted in reduced lactate content and increased oxygen consumption (Bokeriya et al., 2008). This suggests that the hearts may have experienced increased oxidative energy metabolism and relied less on glycolysis when supplemented with HCDs.

1.2.4.2 Calcium handling and contractility

Carnosine has been described as an inotropic agent that can potentiate myocardial contractility (Zaloga et al., 1997). Excitation-contraction (EC) coupling and systolic (contraction) and diastolic (relaxation) functions are regulated by the dynamics of cytoplasmic concentrations of calcium ions within cardiomyocytes (Dewenter et al., 2017; Lou et al., 2012; Morgan, 1991). Myocardial contractility depends upon the availability of calcium and the sensitivity of myofilaments to calcium (Morgan, 1991).

Carnosine has been suggested to influence intracellular calcium concentrations and myocardial contractility. Gonçalves et al. (2021) recently developed a novel CARNS1$^{-/-}$ rat strain to investigate the absence of HCDs (including carnosine) on myocardial function. In isolated cardiomyocytes, the lack of carnosine and HCDs reduced sarcomere's maximal shortening, re-lengthening, and velocity measurements, affecting the overall contractility of the cardiomyocytes. These results, alongside impairments shown in in vivo functional data [i.e., electrocardiogram (ECG) and echocardiogram], indicate that the absence of carnosine in these animals could result in impairments to calcium handling. In addition, decreased calcium amplitude was shown, although there was no change in calcium release or sarco(endo)plasmic reticulum Ca^{2+} ATPase (SERCA2) expression. The study showed increased isovolumetric contraction time and reduced left ventricular ejection fraction and shortening fraction in CARNS1$^{-/-}$ rats, indicating an impairment in systolic function, whereby the left ventricle has difficulty pumping blood out of the heart, increasing the volume of blood remaining in the ventricle at the end of the contraction. Despite this, cardiac output was unaffected, suggesting that the heart might be able to compensate. CARNS1$^{-/-}$ rats display a reduced e' wave and an increased E:e' ratio, suggesting left ventricular relaxation impairments and a diminished diastolic function. This indicates that endogenous HCDs (since we cannot be sure from rodent studies that the effect is limited to carnosine or whether this relates to anserine or their acetylated analogues) are essential for myocardial calcium handling and contractility under healthy conditions (i.e., with no cardiometabolic disease), given that the myocardium was unable to function as well in these rats when compared with wild-type controls.

Exogenous carnosine has been shown to benefit calcium handling and myofilament sensitivity regulation (Lamont and Miller, 1992) in a dose-dependent manner in healthy rat hearts and ventricular pig tissue (Zaloga et al., 1997). This regulation occurs at two cellular sites of action: the sarcoplasmic reticulum and the ryanodine receptors (RyR) (Nelson et al., 1998). RyR2 (the myocardium isoform) is a significant ion channel responsible for calcium release from the sarcoplasmic reticulum to the sarcoplasm during myocardial contraction (Bers and Perez-Reyes, 1999). Carnosine may influence the opening of the RyR2; procaine, an anesthetic that blocks calcium release from RyR2, blocks the tension response to 40 mM carnosine (Zaloga et al., 1997). The addition of two physiological concentrations of carnosine (8 and 16 mM) improved RyR1 (the skeletal muscle isoform) calcium sensitivity and increased calcium release in mechanically skinned human skeletal muscle, directly enhancing contractile apparatus force production (Dutka et al., 2012). Even though this was in skeletal muscle, we may see similarities in myocardial tissue as the myocardial muscle is also striated, contains calcium, and is involved in muscle contractility. In chemically skinned cardiomyocytes, carnosine increased calcium release from the sarcoplasmic reticulum, but did not affect the rate of calcium reuptake (Nelson et al., 1998), and enhanced myofibrillar calcium sensitivity (Lamont and Miller, 1992). Chemically skinned tissue lacks a functional sarcoplasmic reticulum. Still, when the chemically skinned skeletal muscle was compared with mechanically skinned skeletal muscle, the effects on contractile apparatus properties was not detectably different (Dutka et al., 2012). Overall, in healthy models, carnosine may induce calcium release from the sarcoplasmic reticulum, increasing intracellular calcium availability and contractile apparatus sensitivity for calcium, subsequently affecting myocardial contractions. Supplementation with

carnosine or its intracellular synthesis may be essential for these effects in a myocardial model; no alterations in myocardial contractility were noted when isolated rat hearts were independently treated with β-alanine or l-histidine (Zaloga et al., 1997) and no functional changes in ECG parameters were shown when healthy rats were supplemented with β-alanine only for 6 wk (Lake and De Marte, 1988).

Altered calcium homeostasis is a primary contributor to myocardium pathophysiology, resulting in contractile dysfunction (Hipkiss, 2009; Zaloga et al., 1996). Therefore, carnosine's role in calcium handling and responsiveness has potential therapeutic applicability in myocardial tissue (Morgan, 1991). Two weeks of carnosine supplementation (10 mg $\cdot$ kg^{-1} $\cdot$ d^{-1}) attenuated detrimental ECG changes in rats exposed to adriamycin (Dursun et al., 2011; Özdoğan et al., 2011), although 15 mM of carnosine did not improve contractility in isolated rat hearts exposed to hypoxia and reoxygenation (Prokop'eva et al., 1992). Interestingly, an impairment in intracellular calcium homeostasis has been shown in doxorubicin (DOX)-treated rats supplemented with β-alanine for 3 to 4 weeks (Harada et al., 1990). The β-alanine–treated rats at 48 h post-DOX displayed an increase in calcium concentrations that exceeded the increase seen in the nonsupplemented controls. This study also showed a decrease in calcium uptake into the sarcolemmal vesicles, enhancement in the inhibition of sarcolemmal calcium uptake and inhibition in ATP-dependent calcium uptake in isolated vesicles. Although interesting, these results should be interpreted with caution, since sarcoplasmic reticulum membranes and the lack of oxalate-facilitated calcium transportation in the myocardial preparations do not represent a physiological myocardial model.

The role of β-alanine supplementation may not be related to myocardial contractility in a diseased model. Allo et al. (1997) showed, using rat hearts exposed to an ischemic occlusion and reperfusion model, that a decrease in infarct size–risk area ratio (calculated by dividing infarct zone volume by risk zone volume) with β-alanine supplementation did not correlate with changes in contractile function. The authors attributed this to the reduction in myocardial taurine concentration rather than a direct effect of β-alanine on the myocardium, although they did not measure or consider the potential roles of carnosine and n-acetylcarnosine. Therefore, exogenous carnosine and β-alanine may not influence calcium handling or myocardial contractility in a diseased myocardial model.

The ability of carnosine to buffer intramuscular pH may indirectly improve calcium handling. Carnosine can regulate calcium gradients by acting as a diffusible cytoplasmic calcium/hydrogen ion exchanger in isolated rat ventricular cardiomyocytes. This “pump” creates functional calcium gradients in response to local pH changes in the cardiomyocytes and transports calcium across the cytosol in a hydrogen ion–coupled manner. Calcium is released from the sarcoplasmic reticulum and dissipated across the cell to the sarcomeres, and hydrogen ions are released from the sarcomeres and dissipated across the cell (Swietach et al., 2013). Many cellular processes activated by calcium, such as EC coupling, are inhibited by hydrogen ions. Calcium and hydrogen ions compete at the troponin binding site, limiting the ability of the contractility machinery to operate effectively (Bolitho Donaldson et al., 1978). It should be noted that this experiment was conducted at room temperature rather than physiological temperature, which may have affected the behavior of the contractile apparatus. Nevertheless,

cytoplasmic calcium/hydrogen ion-coupling can help sustain calcium activation during a metabolic challenge. Therefore, an increase in carnosine availability may increase calcium delivery to, and hydrogen removal from, the sarcomeres, potentially improving contractile function (Swietach et al., 2013).

1.2.4.3 Oxidative stress

Carnosine has been characterized as an antioxidant and antiglycator (Boldyrev, 1993; Trexler et al., 2015) as it can neutralize and reduce oxidative reactivity. Many components of the myocardium are redox (oxidation-reduction reaction) sensitive; ROS are required to maintain homeostatic regulatory functions in cardiomyocytes, including cardiomyocyte development and maturation, calcium handling, EC coupling, and vascular tone (Peoples et al., 2019; Prokopieva et al., 2016). Mitochondria and the sarcoplasmic reticulum produce ROS as by-products of myocardial oxidative metabolism (Ray et al., 2012; Schieber and Chandel, 2014). Uncontrolled and excessive accumulation of oxidative stress, due to endogenous and exogenous stress, leads to an imbalance between these products and the endogenous antioxidant defense mechanisms, subsequently causing oxidative damage to lipids, proteins, and DNA (Ayala et al., 2014; Braunwald, 2008; Ramana et al., 2013; Schieber and Chandel, 2014). The low molecular weight (226 g/mol) and water solubility of carnosine make this dipeptide a “model” antioxidant (Prokopieva et al., 2016). Dupin et al. (1984) provided the first evidence of the antioxidant properties of carnosine: in skeletal muscle, 25 mM of carnosine inhibited the accumulation of lipid peroxidation end products in the sarcoplasmic reticulum and decreased malonic dialdehyde concentrations. Additionally, carnosine can form complexes with first-transition metals (e.g., $Cu^{2+,}$ $Zn^{2+,}$ and Fe^{2+}), preventing the metal

ions from contributing to the production of free radicals through the Fenton reaction, and thus reducing oxidative stress (Prokopieva et al., 2016; Reddy et al., 2005). Intracellular AGEs further increase oxidative stress by reacting with transition metal ions (Reddy et al., 2005). By buffering these metal ions, carnosine may also act as an AGE inhibitor, preventing AGE formation and glycoxidation (Reddy et al., 2005; Teufel et al., 2003). Indeed, carnosine has recently been shown to prevent 65–90% of AGE and advanced lipid end product (ALE) protein adduct formation in skeletal muscle cells under metabolic stress (Lavilla et al., 2021), suggesting the potential for a similar role in cardiomyocytes.

Carnosine is considered a more effective scavenger of singlet oxygen than histidine, suggesting the intact dipeptide is required to improve the functional recovery of ischemic myocardium (Lee et al., 1999). The intact dipeptide is also necessary to achieve the most effective scavenging response of lipid peroxidation products [e.g., 4-hydroxy-2-nonenal (HNE) and trans-2-hexenal] (Zhou and Decker, 1999). This suggests that radical scavenging ability can mainly be attributed to the N-terminus on the l-histidine residue (the imidazole ring); non-histidine-containing amino acids had limited interaction with the aldehydes (Zhou and Decker, 1999). l-Histidine supplementation produces a greater scavenger response when compared with β-alanine, and β-alanine is relatively ineffective. Carnosine supplementation produced the most reactive response, suggesting that the β-alanine amino group is also needed to maximize aldehyde scavenging (Aldini et al., 2002; Zhou and Decker, 1999); the constituent amino acids must work synergistically to achieve this. The lack of B-alanyl residue in n-acetylcarnosine may explain its low reactive scavenging ability compared with carnosine (Aldini et al., 2002).

Carnosine can form covalent adducts with reactive aldehydes. The presence of aldehyde conjugates has been confirmed in humans. Baba et al. (2013) and Bispo et al. (2016) showed that carnosine can form stable and structurally distinct conjugates with HNE, 4-hydroxy-2-hexenal (HHE), and acrolein, and these aldehyde conjugates are subsequently excreted in the urine. An increase in carnosine-acrolein adduct concentrations in exercising skeletal muscle was shown in humans supplemented with β-alanine. This indicates that skeletal muscle is a site for adduct formation and increasing carnosine content can enhance reactive-aldehyde scavenging by carnosine (Carvalho et al., 2018). The ability of carnosine to block the formation of catechoaldehyde protein adducts has been demonstrated in myocardial tissue taken from patients undergoing elective heart surgery. Pretreatment of mitochondria with 1 mM carnosine showed a concentration-dependent reduction in catechol-modified adducts and attenuated the decrease in state 3 respiration seen with 3,4-dihydroxyphenylacetaldehyde (DOPAL) (Nelson et al., 2019). Similar results have been shown in animal models. In ATPGD1-transgenic mice overexpressing CARNS1, an increase in endogenous carnosine protected the heart from damage caused by aldehydes (Zhao et al., 2020), and in isolated adult mouse cardiomyocytes, the pretreatment of 1 mM carnosine protected the myocytes from aldehyde-induced hypercontracture (Zhao et al., 2019).

Under healthy conditions, endogenous carnosine is unlikely to fulfil an antioxidant role in cardiomyocytes. Oxidative stress was not increased when myocardial tissue was depleted of endogenous carnosine and HCDs in a cardiometabolically healthy rat model; there was no change in hydrogen peroxide release or protein carbonyl concentrations in the myocardial tissue of CARNS1$^{-/-}$ rats (Gonçalves et al., 2021). The physiological

endogenous antioxidant defense mechanism is sufficient to maintain homeostatic redox; additional exogenous antioxidants, such as carnosine, provide no capacity for improvement. If carnosine quenched oxidative stress mediators in a healthy environment (where appropriate levels of oxidation products are required for normal function), the homeostatic balance might move towards an excess of antioxidants, and myocardial function and regulation may be disrupted. In a diseased myocardial model, an excess of ROS decreases the capability of the antioxidant system— an effective cellular antioxidant response can no longer be produced and additional antioxidant resources are required to improve defense mechanisms (Ray et al., 2012).

A summary of the literature investigating the effect of exogenous carnosine and β-alanine on oxidative stress markers in healthy and diseased myocardial models is presented in **Table 1.2**. There is still a significant gap in the literature for clinical studies in human participants supplemented with β-alanine or carnosine. The bulk of the support for carnosine as an antioxidant has been conducted in in vitro and experimental animal models (Sale et al., 2013), making the translation of beneficial findings into humans challenging (Peoples et al., 2019). If carnosine can ameliorate myocardial oxidative stress, we need to establish the direct effect this has on myocardial function and address why it is necessary to improve oxidative stress in pathological conditions. The potential link between ROS and myocardial calcium transients and contractility is of great interest to future research and raises several critical questions regarding carnosine's role. Anti-

Table 1.2. A summary of studies assessing the effect of exogenous carnosine, and its constituent amino acids, on oxidative stress parameters

Study	Species	Experimental Model	Supplementation Protocol		Main Results
			Dosages(s)	Duration	
Harada et al. (1990)	Rat	Doxorubicin (5 mg.kg^{-1} for 1 or 48 hours)	3% beta-alanine	3 – 4 weeks	Beta-alanine supplementation increased tissue MDA in doxorubicin treated rats but did not change tissue GSSG.
Lee et al. (1999)	Rat	40-minute ischaemia + 30-minute reperfusion	1 mM Carnosine, 1 mM L-histidine or 10 mM L-histidine	20-minutes	1 mM of carnosine was more effective at scavenging singlet oxygen compared to 1 mM or 10 mM of L-histidine.
Parildar et al. (2008)	Rat	Ageing	3% beta-alanine	6 weeks	MDA and DC levels, and AA- and NADPH-induced lipid peroxidation were increased in the hearts of aged rats but there were no changes in GSH, vitamin E or vitamin C levels, nor in SOD, GSH-Px or GST activities. Cardiac MDA and DC levels and the antioxidant system did not further change in the hearts of beta-alanine treated aged rats. AA- and NADPH-induced lipid peroxidation increased in the heart of aged rats treated with beta-alanine.
Aydın et al. (2010)	Rat	Ageing (young vs. old)	250 mg.kg^{-1}.day^{-1} carnosine	1 month	Aged rats experienced an increase in MDA and DC levels but no differences in enzymatic and non-enzymatic antioxidant elements when compared to the young rats. Carnosine supplementation had no effect on cardiac oxidative stress parameters in young or aged rats.
Dursun et al. (2011)	Rat	Adriamycin (single dose of 16 mg.kg^{-1} on day 14)	10 mg.kg^{-1}.day^{-1} carnosine	2 weeks	Carnosine supplementation increased plasma CAT activity in the rats not treated with adriamycin. Adriamycin decreased plasma SOD, GSH-Px and CAT activities; the addition of carnosine supplementation was able to maintain these activities at normal levels. Carnosine supplementation prevented the increase in plasma MDA that was seen with adriamycin.
Özdoğan et al. (2011)	Rat	Adriamycin (four doses over 8 days)	10 mg.kg^{-1}.day^{-1} carnosine	2 weeks	Carnosine supplementation prevented the increase in plasma lipid peroxidation that was seen with adriamycin. Plasma SOD, GSH-Px and CAT activity were decreased with adriamycin. Carnosine supplementation was able to maintain normal levels of these antioxidants when added to the adriamycin group.
Pansani et al. (2012)	Rat	Healthy	3% beta-alanine	30 days	Beta-alanine supplementation showed a higher concentration of tissue LH, and lower activity of tissue CAT and GSH-Px.
Kalaz et al. (2012)	Rat	Healthy or stress protocol (immobilisation and 4°C cold room for 1 hour/day for 5, 7 or 21 days)	250 mg.kg^{-1}.day^{-1} carnosine	30-minutes prior to stress protocol	Cardiac levels of MDA, PC, DC and NT, and non-enzymatic and enzymatic antioxidants were not affected by the stress protocol. The addition of carnosine in the stress group did not have any effect on these markers – carnosine only caused a decrease in GSH-Px. Carnosine supplementation had no effect on these markers in physiologically healthy hearts.
Evran et al. (2014)	Rat	Isoproterenol	250 mg.kg^{-1}.day^{-1} carnosine	2 or 12 days	Carnosine pre-treatment had no effect on plasma MDA and PC level but did increase FRAP values. 12 days of carnosine pre-treatment decreased cardiac MDA, DC and PC levels and increased GSH levels and the activities of SOD and GSH-Px.
Kumral et al. (2016)	Rat	Doxorubicin (single dose of 30 mg.kg^{-1} on day 8)	250 mg.kg^{-1}.day^{-1} carnosine	12 days	Carnosine supplementation decreased doxorubicin-induced oxidative stress (TBARS, PC and DC levels) in cardiac tissue. GSH decreased with doxorubicin but this was increased with the addition of carnosine supplementation. GSH-Px activity remained unchanged.
Hou et al. (2020)	Rat	30-minutes coronary artery occlusion	100 mM beta-alanine	30 days	The increase in cardiac MDA and intracellular ROS induced by the occlusion model was decreased with the addition of beta-alanine supplementation. The decrease in cardiac SOD and CAT activity, and GSH and GSH-Px levels induced by the occlusion model was increased with the addition of beta-alanine supplementation.

AA – ascorbic acid; CAT – catalase; DC – diene conjugate; FRAP – ferric reducing ability of plasma; GSH – (reduced) glutathione; GSH-Px – glutathione peroxidase; GSSG – oxidised glutathione; GST – glutathione transferase; LH – lipid hydroperoxide; MDA – malondialdehyde; NT – nitrotyrosine; PC – protein carbonyl; ROS – reactive oxygen species; SOD – superoxide dismutase; TBARS – thiobarbituric acid reactive substances.

ischemic activity may be influenced by the effects of carnosine on ROS concentrations (Stvolinsky and Dobrota, 2000). An increase in ROS may disrupt calcium handling and myofilament sensitivity to calcium, impair myocardial contractile function, and have detrimental effects on overall myocardial metabolism (Giordano, 2005; Seddon et al., 2007). These findings pose several questions, including: Does this link explain why improving oxidative stress is vital to help maintain the regulatory functions of a diseased cardiovascular system? Furthermore, a correlation has been shown between singlet oxygen quenching ability in vitro and myocardial functional recovery with carnosine; can the improvements in hemodynamic parameters be explained by the scavenging of ROS (Lee et al., 1999)? A large-scale study that measures all associated mechanisms of carnosine in the myocardium in both healthy and diseased models is warranted to further look at these questions and establish the exact roles carnosine plays in these myocardial models.

1.2.4.4 Hemodynamic parameters

Endogenous HCDs may directly impact, or be influenced by, cardiovascular risk factors such as hypertension. Lower levels of HCDs (a 35% decrease) were shown in the left ventricular myocardial tissue of hypertensive rats compared with normotensive rats (Johnson and Hammer, 1992). This may explain why supplementation has a potential beneficial effect on hemodynamic parameters in diseased myocardial models (i.e., when there is the capacity to increase HCD content) and no impact upon these parameters when the myocardial tissue is healthy and contains an adequate concentration of HCDs. Johnson and Hammer (1992) found no detectable levels of carnosine in either rat model, suggesting that carnosine may not be the predominant HCD in myocardial tissue.

There are contradictory findings regarding the vasodilatory properties of exogenous carnosine. The dose-dependent, vasodilatory effect of exogenous carnosine was demonstrated on isolated rat aortic rings (Ririe et al., 2000). Carnosine provoked significant, sustained contractures in rabbit saphenous vein rings. This effect was specific to carnosine, as its constituent amino acids were ineffective, and is considered to be attributed to the zinc/carnosine complex (Miller and O'Dowd, 2000; O'Dowd et al., 1996). Other mechanisms of the antihypertensive action of carnosine may be mediated via the histamine H1 receptors (O'Dowd and Miller, 1998) and the carnosine-histamine-histidine pathway (Greene et al., 1984). A summary of the research investigating the effect of exogenous carnosine, and its constituent amino acids, on hemodynamic parameters in animal models is displayed in **Table 1.3**. There is very little knowledge concerning the influence of carnosine on hemodynamic parameters in humans. Midoh and Noguchi (2009) showed that both a single intake and a 2-wk daily intake of chicken soup increased peripheral blood flow. No changes were noted in heart rate, systolic blood pressure, or diastolic blood pressure, and the authors did not suggest other mechanisms that may have induced this increase. Although this study was not directly focused on carnosine, we know that meat extracts are rich in amino acids and peptides, including carnosine. Therefore, these results may indicate a potential role of carnosine in hemodynamic regulation in humans.

1.2.4.5 Myocardial injury markers, morphological and histological parameters

A summary of the literature investigating the effect of carnosine and β-alanine supplementation on myocardial injury markers and morphological and histological

characteristics are displayed in **Tables 1.4** and **1.5**. Under healthy conditions, β-alanine and carnosine supplementation do not affect these parameters in the heart. The lack of myocardial injury may explain this; parameters are within their physiological range, and, therefore, there is no capacity or need for improvement.

Table 1.3. A summary of studies assessing the effect of exogenous carnosine, and its constituent amino acids, on haemodynamic parameters

Study	Species	Experimental Model	Supplementation Protocol		Main Results
			Dosages(s)	Duration	
Allo et al. (1997)	Rat	45-minutes coronary artery occlusion + 120-minute reperfusion	3% beta-alanine	4 – 28 days	Beta-alanine supplementation did not affect haemodynamic parameters in a diseased model.
Lee et al. (1999)	Rat	40-minute ischaemia + 30-minute reperfusion	1 mM carnosine, 1 mM L-histidine or 10 mM L-histidine	20-minutes	The LVDP recovery of 1 mM carnosine treated ischemic hearts improved more than untreated and 10mM histidine treated ischemic hearts. 1 mM carnosine and 10 mM histidine improved dP/dt recovery but did not improve coronary flow or HR. 1 mM histidine improved HR recovery but did not improve dP/dt, coronary flow or LVDP recovery.
Ririe et al. (2000)	Rat	Healthy	0.625 – 20 mM carnosine, L-histidine or beta-alanine	30-minutes	Carnosine increased vasodilation in a dose-dependent manner. L-histidine and beta-alanine had no vasodilatory effect. Beta-alanine increased vascular smooth muscle tone in a dose-dependent manner.
Niijima et al. (2002)	Rat	Normotensive or hypertensive (2 x a week DOCA + NaCl)	0.1 mg or 1 mg carnosine	5 weeks	Carnosine had no effect on systolic blood pressure in normotensive rats (116 mmHg vs. 112 mmHg at 0 and 5 weeks). Systolic blood pressure increased in the untreated hypertensive rats (114 mmHg vs. 198 mmHg at 0 and 5 weeks). Carnosine supplementation decreased the rise in systolic pressure seen with hypertension (0.1 mg carnosine: 115 mmHg vs. 163 mmHg at 0 and 5 weeks; 1 mg carnosine: 113 mmHg vs. 148 mmHg at 0 and 5 weeks).
Zieba et al. (2003)	Rabbit	Doxorubicin (2 $mg.kg^{-1}.week^{-1}$ for 7 weeks)	100 $mg.kg^{-1}.day^{-1}$ carnosine	9 weeks	MAP, CI and SI decreased with doxorubicin but the addition of carnosine with doxorubicin increased the levels to similar values seen in the untreated and treated groups not exposed to doxorubicin. There was no change in HR or TPR in any of the groups. Carnosine had no effect on haemodyanmic parameters in healthy rabbits not exposed to doxorubicin.
Abebe and Mozaffari (2003)	Rat	Endothelial-intact or endothelial-denuded	3% beta-alanine	3 weeks	Beta-alanine supplementation impaired the relaxation responses of blood vessels to adenosine agonists.
Dursun et al. (2011)	Rat	Adriamycin (single dose of 16 $mg.kg^{-1}$ on day 14)	10 $mg.kg^{-1}.day^{-1}$ carnosine	2 weeks	The MAP and LVDP decrease seen with adriamycin was maintained by the addition of carnosine. Similar was seen with dP/dt, however this was not significant.
Özdoğan et al. (2011)	Rat	Adriamycin (four doses over 8 days)	10 $mg.kg^{-1}.day^{-1}$ carnosine	2 weeks	The decrease in LVDP and ±dP/dt induced by adriamycin was increased with the addition of carnosine.
Pansani et al. (2012)	Rat	Healthy	3% beta-alanine	30 days	Beta-alanine supplementation decreased LVSD, HR, EF and %FS and increased E/A ratios compared to the untreated group.
Stefani et al. (2020)	Rat	Coronary heart failure induced by myocardial infraction surgery	250 $mg.kg^{-1}.day^{-1}$ beta-alanine + 55 – 75 $mg.kg^{-1}.day^{-1}$ L-histidine	8 weeks	No change in haemodynamic parameters with supplementation.

CI – cardiac index; dP/dt – first derivative of left ventricular pressure; EF – ejection fraction; FS – fractional shortening; HR - heart rate; LVDP – left ventricular developed pressure; MAP – mean arterial pressure; NaCl – sodium chloride; SI – stroke index; TPR – total peripheral resistance.

Table 1.4. A summary of studies assessing the effect of exogenous carnosine and beta-alanine on myocardial injury markers

Study	Species	Experimental Model	Supplementation Protocol		Main Results
			Dosages(s)	Duration	
Alabovsky et al. (1997)	Rat	40-minutes ischemia + 12-minutes reperfusion	2 mM carnosine, 5 mM carnosine, 10 mM carnosine or 10 mM acetyl-carnosine	15-seconds prior to ischemia and during the 40-minute ischemic period	Carnosine and acetyl-carnosine supplementation reduced the level of myoglobin released from the heart during ischemia/reperfusion. 10 mM carnosine supplementation decreased the release of myoglobin and nucleosides during ischemia/reperfusion but 2 mM carnosine had no effect.
Dursun et al. (2011)	Rat	Adriamycin (single dose of 16 $mg.kg^{-1}$ on day 14)	10 $mg.kg^{-1}.day^{-1}$ carnosine	2 weeks	Carnosine supplementation reduced the increase in plasma CK induced by adriamycin.
Özdoğan et al. (2011)	Rat	Adriamycin (four doses over 8 days)	10 $mg.kg^{-1}.day^{-1}$ carnosine	2 weeks	Carnosine supplementation reduced the increase in plasma markers (CK, AST, ALT and LDH) induced by adriamycin.
Pansani et al. (2012)	Rat	Healthy	3% beta-alanine	30 days	Beta-alanine supplementation had no effect on MMP-2 and MMP-9 values.
Evran et al. (2014)	Rat	Isoproterenol	250 $mg.kg^{-1}.day^{-1}$ carnosine	2 or 12 days	12 days of carnosine supplementation did not affect plasma cTnT or CK activities but did decrease plasma LDH and AST activities. 2 days of carnosine supplementation did not affect these plasma markers.
Al-Rasheed et al. (2015)	Rat	TiO_2 (600 $mg.kg^{-1}$ or 1 $g.kg^{-1}$)	200 $mg.kg^{-1}.day^{-1}$ carnosine	3 weeks	Carnosine supplementation decreased blood measurements (myoglobin, troponin, CK-mB and CRP) and cardiac capase 3 when compared to control and TiO_2 only groups.
Kumral et al. (2016)	Rat	Doxorubicin (single dose of 30 $mg.kg^{-1}$on day 8)	250 $mg.kg^{-1}.day^{-1}$ carnosine	12 days	Carnosine supplementation reduced the increase in serum cTn1 induced by doxorubicin.
Keskin et al. (2017)	Rat	30-minute ischemia + 60-minute reperfusion of the infrarenal abdominal aorta	250 $mg.kg^{-1}.day^{-1}$ carnosine	10-minutes prior to the end of ischemia	Carnosine supplementation improved AST and LDH levels in the ischemia/reperfusion model.

ALT - alanine aminotransferase; AST - aspartate aminotransferase; CK – creatine kinase; CK-mB – creatine kinase isoenzyme; CRP – c-reactive protein; cTn1 – cardiac troponin 1; cTnT - cardiac troponin T; LDH – lactate dehydrogenase; MMP - matrix metalloproteinases; TiO_2 - titanium dioxide.

Table 1.5. A summary of studies assessing the effect of exogenous carnosine, and its constituent amino acids, on myocardial histological and morphological parameters

Study	Species	Experimental Model	Supplementation Protocol		Main Results
			Dosages(s)	Duration	
Allo et al. (1997)	Rat	45-minutes coronary artery occlusion + 120-minute reperfusion	3% beta-alanine	4 – 28 days	Beta-alanine supplementation reduced the infarct size area ratio by 57%.
Zieba et al. (2003)	Rabbit	Doxorubicin (2 $mg.kg^{-1}.week^{-1}$ for 7 weeks)	100 $mg.kg^{-1}.day^{-1}$ carnosine	9 weeks	A smaller degree of damage was noted in the doxorubicin hearts treated with carnosine when compared to doxorubicin hearts not treated with carnosine. No differences were shown between control hearts (no doxorubicin) and control hearts treated with carnosine.
Dursun et al. (2011)	Rat	Adriamycin (single dose of 16 $mg.kg^{-1}$ on day 14)	10 $mg.kg^{-1}.day^{-1}$ carnosine	2 weeks	Carnosine supplementation improved the edema, organisational disturbance and myocardial injury abnormalities induced by adriamycin.
Pansani et al. (2012)	Rat	Healthy	3% beta-alanine	30 days	Beta-alanine supplementation decreased LVWT, LVWT/LVDD and myocardial cross-sectional area.
Kumral et al. (2016)	Rat	Doxorubicin (single dose of 30 $mg.kg^{-1}$on day 8)	250 $mg.kg^{-1}.day^{-1}$ carnosine	12 days	Carnosine supplementation decreased the histological damage seen with doxorubicin (mild degree of interstitial edema and lymphocyte infiltration, irregular clusters of myocardial fibers and necrotic changes).
Keskin et al. (2017)	Rat	30-minute ischemia + 60-minute reperfusion of the infrarenal abdominal aorta	250 $mg.kg^{-1}.day^{-1}$ carnosine	10-minutes prior to the end of ischemia	Carnosine supplementation had no effect on the histological parameters.
Hou et al. (2020)	Rat	30-minutes coronary artery occlusion	100 mM beta-alanine	30 days	Beta-alanine supplementation reduced the increase in infarct size induced by ischemia/reperfusion. Apoptosis increased with ischemia/reperfusion but was decreased with beta-alanine (not fully recovered).
Stefani et al. (2020)	Rat	Coronary heart failure induced by myocardial infraction surgery	250 $mg.kg^{-1}.day^{-1}$ beta-alanine + 55 – 75 $mg.kg^{-1}.day^{-1}$ L-histidine	8 weeks	Beta-alanine supplementation did not affect structural and morphological parameters or infarct size in the diseased model.

LVDD – left ventricular end-diastolic diameter; LVWT – left ventricular posterior wall thickness.

1.2.4.6 Methodological limitations

The cardioprotective effects of carnosine have primarily been researched within animal models; there is little evidence in human myocardial tissue. It is therefore difficult to extrapolate these findings to the human population. First, CN1, the enzyme responsible for the hydrolysis of carnosine into β-alanine and l-histidine, is absent in non-primate mammals (Jackson et al., 1991). The results in the studies described in this review have been obtained from animals lacking this enzyme (e.g., rodents). These studies primarily supplement with carnosine; therefore, elevated plasma carnosine may be partly responsible for any beneficial effects shown. Rodents have low endogenous concentrations of l-histidine, potentially explaining why these studies chose to supplement with carnosine as there may be inadequate levels of l-histidine available for synthesis with β-alanine. Second, the anatomical, metabolic, and functional differences between non-primate mammals and humans must be considered. Despite these limitations, animal models are critical to study myocardial tissue and function due to the extreme difficulty in obtaining human myocardial tissue and the obvious ethical implications of inducing myocardial dysfunction in humans.

1.2.4.7 Summary

The literature suggests that carnosine fulfills different roles within the heart, depending upon the environment (healthy or diseased). Many of the studies in the literature have exposed animal myocardial tissue to either a healthy or diseased environment. The cumulative findings from these studies indicate that carnosine improves calcium handling and, therefore, muscle contractility in a healthy model and improves oxidative stress, myocardial injury markers, and morphological and histological parameters in a

diseased model. Future research should include supplementation protocols in both healthy and diseased myocardial models to determine the specific roles of carnosine in both of these conditions.

β-alanine supplementation is commonly used to increase myocardial carnosine concentrations; however, it could also be implemented to potentially decrease myocardial taurine concentration. A concentration of >3% β-alanine supplementation has been suggested to be required to reduce myocardial taurine concentration (Dolan et al., 2019). There is a lack of consensus in the literature regarding the effect of taurine depletion on myocardial function and health, with rodent studies reporting beneficial (Allo et al., 1997), harmful (Harada et al., 1990; Jong et al., 2012; Pansani et al., 2012), and even no effects (Parildar et al., 2008). The decrease in myocardial taurine concentration is likely dependent upon a combination of the dose and the length of time of β-alanine supplementation. Lake and De Marte (1988) showed no difference in myocardial taurine concentrations between control rats and rats supplemented with β-alanine for 6 wk, and Allo et al. (1997) showed myocardial taurine concentrations reached a steady state after 2 wk of β-alanine supplementation. The fact that no changes in human skeletal muscle taurine content have been shown, despite downregulation of TauT after 24 wk of β-alanine supplementation, suggests that skeletal muscle has some way of maintaining taurine homeostasis during periods of elevated β-alanine availability (Saunders et al., 2017a). Further investigations into the potential complications of β-alanine supplementation on human myocardial taurine concentrations are required, although limited data suggest that the recommended doses of β-alanine are safe for human consumption (Trexler et al., 2015).

1.2.5 Future perspectives

The need for long-term randomized controlled clinical trials to confirm, or not, the beneficial effects of carnosine on myocardial function and health has been highlighted in recent literature (Cicero et al., 2020; Hopper et al., 2020). Lombardi et al. (2015) are the only group to have conducted a clinical human trial on carnosine supplementation and heart function. They investigated 6 mo of l-carnosine supplementation in patients with stable chronic heart failure and impaired left ventricular function. The participants were supplemented with 500 mg/d l-carnosine in an open-label approach. The control group received their regular, routine treatment. l-carnosine improved quality of life and exercise capacity, but no improvements were shown in myocardial function measurements. The authors suggested that the buffering effect of l-carnosine improved exercise capacity, although this was not directly measured. The potential underlying mechanisms that might support a role for carnosine in the myocardium were not measured. The supplementation protocol contains several limitations. The lack of blinding and placebo supplement in the control group means that the study is at a high risk of bias. Participants were supplemented with intact l-carnosine, which has low bioavailability and stability in circulation due to the presence of CN1 and, in turn, will limit the uptake of carnosine into myocardial tissue. In addition, it is uncertain, and highly unlikely, that carnosine can be directly taken up intact by the myocardium. Intact carnosine is likely to be hydrolyzed into β-alanine and l-histidine, with these constituent amino acids being taken up into the tissue and resynthesized to carnosine. An appropriate supplementation dose would be required to ensure adequate metabolite is available to the myocardium. Harris et al. (2006) showed that a high dose of l-carnosine (13 g/d) is required to be able to obtain a similar increase in skeletal muscle carnosine

content to that shown with 6.4 g/d of β-alanine. This suggests that the level of substrate available to the myocardium from a dose of 500 mg of l-carnosine may not be sufficient to have an overall effect on myocardial function. The recommended daily amount of β-alanine is 3.2 to 6.4 g/d for improvements in exercise capacity and performance (Dolan et al., 2019; Saunders et al., 2017b; Trexler et al., 2015), although potential therapeutic effects could be realized with lower doses.

Given these limitations and the insufficient evidence in human populations, higher quality clinical trials are warranted (Sale et al., 2013) . Future clinical studies should incorporate a placebo-controlled, double-blinded, randomized controlled protocol. Although the impact of an increased dietary intake of β-alanine and/or carnosine through the consumption of dietary rich sources on myocardial tissue carnosine stores and health would be interesting to examine, it could also mean an increased meat intake, which might have other health implications for some. As such, it might be that a supplementation approach is preferred—with supplementation doses typically being 3.2–6.4 g/d of β-alanine in a sustained-release formula across current studies, although many of these studies were targeting improved skeletal muscle performance rather than wider health implications. From a therapeutic perspective, it is possible that lower doses (1.6 or 2.4 g/d) would be equally effective, particularly if supplementation was being considered over a longer duration than used during most studies, but all of this remains to be determined. A sustained-release formula may increase supplementation adherence as participants will be less likely to experience the common side effect of paraesthesia (i.e., tingling), although this might also be achieved at lower doses of a non-sustained-release formula. Implementing a supplementation intervention in a heart

failure population is exceptionally challenging. There are many factors to consider, including feasibility, patient access, and side effects. Therefore, future human studies may evaluate the effectiveness of carnosine on myocardial function in clinical populations with an associated cardiovascular disease risk comorbidity (e.g., a prediabetic or aging population) before conducting interventions in higher-risk individuals.

1.2.6 Conclusions

Carnosine has the potential to improve healthy and diseased myocardial function, although there is still a significant gap in our understanding and application of carnosine within the cardiovascular system. More research is needed to identify the exact roles carnosine plays in the myocardial tissue in healthy and diseased models. It is possible that carnosine functions differently depending upon the tissue's environment, although further research is needed to clarify the distinct differences between these environments in cell, animal, and human experiments. The translation of findings into the human population is limited, primarily due to rodent models lacking the enzyme that degrades carnosine in humans (CN1). Practical recommendations for β-alanine supplementation cannot yet be given concerning myocardial function and health.

1.3. Additional literature

1.3.1 Anserine

As previously mentioned (section **1.2.2**), N-acetylcarnosine was initially suggested to be the predominant HCD in the heart. Since the publication of the narrative review, new research related to identifying HCD concentrations in the heart has reported the absence of N-acetylcarnosine in mice, rats, and human cardiac tissue (Van der Stede et al., 2023). There has been an interest in anserine and its presence in the heart, with this HCD being shown to be present in the cardiac tissue of mice, rats, and humans (Gonçalves et al., 2023; Van der Stede et al., 2023).

Anserine is a methylated derivative of carnosine, composed of the amino acids beta-alanine and 3-methylhistidine. It is synthesised via the carnosine-N-methyltransferase enzyme (*CARNMT1* gene), where a methyl group from S-adenosylmethionine is transferred onto a carnosine molecule, subsequently forming anserine (Drozak et al., 2015; McManus, 1962). This suggests that the synthesis of anserine is dependent upon the prior synthesis of carnosine, and in line with this, anserine was not found to be present in tissues of CARNS1^{-}/$^{-}$ rats who lack the ability to synthesise carnosine (Gonçalves et al., 2021). It has been suggested that anserine also exhibits effects similar to carnosine on sarcoplasmic reticulum calcium channels and calcium release (Batrukova and Rubtsov, 1997), antioxidant activity (Kohen et al., 1988), and buffering capacity (Abe et al., 1985); however, the specific physiological functions of anserine, and its comparative role to carnosine, remain largely unknown and requires further exploration.

1.3.2 Carnosine and anserine concentration

Since the publication of the narrative review, additional content has been published regarding the cardiac concentrations of HCDs. Heidenreich et al. (2021) measured endogenous carnosine and anserine in the hearts of C57BL/6N wild-type mice to be 7136 ± 5048 fmol/mg and 115762 ± 40282 fmol/mg (mean ± SD). Van der Stede et al., (2023) conducted an extensive profiling of intracellular HCD concentrations across a range of tissues, including the heart, in mice, rats and humans. In this study, tissue samples were obtained from an unspecified region of mouse and rat hearts, containing both atrial and ventricular tissue, and from the right atria appendage in humans. The presence of both endogenous carnosine and anserine was shown in mice (mean ± SD: 13.6 ± 6.39 µmol/kg and 4.79 ± 2.23 µmol/kg), rats (mean ± SD: 27.4 ± 6.54 µmol/kg and 14 ± 7.83 µmol/kg) and humans (mean ± SS: 26.4 ± 20.9 µmol/kg and 1.26 ± 1.55 µmol/kg). The human heart samples were collected from patients undergoing open heart surgery, though the pathologies of these patients were not specified, leaving it unclear how different pathologies might influence HCD distribution. Additionally, only one compartment of the heart was analysed, and it is unclear how HCD content differs across different regions of the tissue within the same species. Gonçalves et al. (2023) identified the presence of anserine in the ventricular wall of deceased humans, with concentrations ranging from approximately 1.5 to 45 µmol/kg of dry tissue. Although they identified large interindividual variation, no significant differences in cardiac anserine concentration were shown based on sex, age, or body mass. The causes of death in these individuals included cardiac failure, and, similar to Van der Stede et al. (2023), it remains unclear if, or how, different pathologies might affect HCD concentrations. Regardless, these studies

have begun to contribute to the growing evidence that carnosine and anserine are the predominant HCDs in cardiac tissue, with this finding being consistent across species.

1.3.3 Physiological roles of carnosine in cardiac tissue

In the past few years, advances have been made to determine carnosine's roles in the cardiovascular system, albeit in animal models. Newly published literature has contributed to supporting the beneficial effects of carnosine on myocardial injury markers. Rats pre-treated with carnosine (200 mg/kg/day) for 1 month exhibited an attenuation in elevated cardiac troponin T (cTnT) and creatine kinase isoenzyme (CK-mB) levels when exposed to an acute dose of cobalt chloride, a hypoxia mimetic (Kadry and Ali, 2023). Harris et al. (2022) observed an increase in beta-alanine and carnosine concentrations in the hearts of Ogfod1-knockout mice, with a subsequent improvement in infarct size area (% of the ventricular area) and improved recovery of cardiac function (determined by heart rate and left ventricular developed pressure) after *ex vivo* ischemia/reperfusion (I/R) injury when compared to wild-type mice. Additionally, wild-type mice also exhibited an improvement in infarct size and recovery of cardiac function when supplemented with 1 mM carnosine before ischemia and during the reperfusion period. These studies suggest a potential cardioprotective effect of both carnosine and beta-alanine. In a model using a high-fat, high-sucrose diet to induce cardiac dysfunction, Berdaweel et al. (2024) showed that supplementation with 80 mM of carnosine had a beneficial effect on myocardial fibrosis, as measured by Masson's trichrome staining. This metabolic model, however, did not alter cardiac function parameters, including cardiac output (CO), stroke volume (SV) and ejection fraction (EF). Consequently, the

administration of carnosine did not influence these physiological measures, likely because there was little to no scope for improvement with the addition of carnosine.

Yan et al. (2022) adopted a multi-omics approach, utilising RNA-sequencing (RNA-Seq), global proteomics and untargeted metabolomics, to investigate HCD-mediated responses in cardiac tissue from both wild-type and CarnsTg [transgenic overexpression of Carns1 (Zhao et al., 2020)] mice exposed to physiological and pathological conditions. The CarnsTg mice showed significantly higher levels of HCDs, including carnosine, anserine and homocarnosine, in the heart compared to wild-type mice. This multi-omic analysis suggests that the increase in HCDs through transgenic overexpression influenced cardiac function by optimising myocardial fuel utilisation (fatty acid and glucose) under both basal and ischemic conditions, indicating a cardioprotective effect of HCDs.

Beta-alanine is commonly used to induce taurine deficiency; however, as mentioned previously, its reported effects on cardiac function and health are inconsistent. Gates et al. (2022) showed a decrease in cardiac taurine concentration in rainbow trout supplemented with 3 % beta-alanine (by mass) for eight weeks. The taurine-deficient hearts demonstrated an impairment in the regulation of cardiac contractility and calcium cycling, exhibiting a downward shift in the force-frequency and extracellular calcium sensitivity relationship, as well as a decrease in maximum pacing frequency. This taurine reduction was also demonstrated in another fish model (brook char) supplemented with 5 % of beta-alanine (Dixon et al., 2023), where taurine-deficient hearts showed a dysfunctional cardiac response under acute thermal and hypoxic stress. The

concentrations of beta-alanine used in these studies are at or above the concentration that has been reported as having a significant effect on taurine concentration (Dolan et al., 2019). This reinforces the recommendation that research needs to supplement beta-alanine below a concentration of 3 % to prevent adverse effects.

1.3.4 Overweight and obesity

The future perspectives section (**1.2.5**) of the narrative review concludes by recommending that carnosine and beta-alanine studies need to be conducted in clinical populations with an associated cardiovascular disease (CVD) risk comorbidity. Overweight and obesity are major public health issues, costing the National Health Service (NHS) an estimated £6 billion annually (Department of Health and Social Care, 2022). Recent reports estimate that the prevalence of overweight and obesity in adults in England has increased from 52.9 % to 64.3 % between 1993 and 2019, with obesity alone rising from 14.9 % to 28 % (Baker, 2023). According to body mass index (BMI) - calculated by dividing weight in kilograms by height in metres squared (m^2) - overweight is categorised as a BMI of 25 to 29 kg/m^2 and obesity is categorised as a BMI greater than 30 kg/m^2. Overweight and obesity are associated with cardiovascular complications and an increased risk of developing CVD, including coronary heart disease, heart failure and sudden cardiac death (Carbone et al., 2019). Obesity contributes to the development of hypertension, dyslipidaemia, diabetes, and insulin resistance (Akil and Ahmad, 2011; Barroso et al., 2017), and alterations in heart structure and function have been observed in those with excessive fat accumulation, as indicated by BMI (Szabo et al., 2023). Each of these risk factors independently contribute to heart and vasculature damage, further increasing the risk of CVD. Therefore, the potential physiological roles of carnosine

outlined in the narrative review may be beneficial in the cardiovascular system of individuals with overweight or obesity by targeting these CVD risk factors. For this reason, individuals with overweight or obesity were selected as the target sample for the *in vivo* intervention studies in the following research programme.

1.3.5 Non-excitable tissues

HCDs are also present in non-excitable tissues (e.g., liver and adipose tissue); therefore, a summary of HCD concentrations and the physiological roles of carnosine and beta-alanine in these tissues is provided.

1.3.5.1 Concentrations of HCDs in non-excitable tissues

Beta-alanine is endogenously synthesised in the liver from uracil and 3-hydroxypropionate catabolism (Matthews and Traut, 1987). The demand for beta-alanine required to elicit physiological roles in the body far exceeds this endogenous supply. An absence of HCDs (carnosine, anserine, and homocarnosine) has been reported in the liver of rabbits (Davey 1957) and rats (Aldini et al., 2004); however, this analysis may have been limited by the sensitivity of the equipment used in these studies. A recent profiling of HCD concentrations across multiple species detected HCDs in the liver, with carnosine present in both mouse and human liver, and both carnosine and anserine being present in rat liver (Van der Stede et al., 2023). Species differences were also observed in adipose tissue HCDs, with carnosine and anserine present in the adipose tissue of rodents, but only carnosine was present in human adipose tissue (Van der Stede et al., 2023). The human adipose tissue samples were collected from both visceral and subcutaneous depots; however, the authors did not specify which depots

the rodent adipose tissue was sampled from. It may be that the distribution of HCDs differs between adipose tissue depots.

1.3.5.2 Physiological roles of carnosine in non-excitable tissues

The physiological role(s) of HCDs in non-excitable tissues, such as liver, remain unknown (Van der Stede et al., 2023). A brief review of the literature suggests that carnosine may exert antioxidative properties in the liver. Supplementation with 250 mg/kg/day of carnosine for 1 month in aged rats decreased malondialdehyde, diene conjugate and protein carbonyl levels, and increased vitamin E and superoxide dismutase activity, suggesting a physiological role of carnosine in age-related oxidative stress (Aydin et al., 2010). Carnosine supplementation also reduced DOX-induced oxidative stress by reducing protein carbonyl abundance and superoxide dismutase activity, and decreasing histological tissue damage in rats (Kumral et al., 2016), as well as decreasing liver lipid peroxidation (Kim et al., 2011).

Additionally, knowledge regarding the role of carnosine and beta-alanine in brown adipose tissue (BAT) and white adipose tissue (WAT) remains limited. Carnosine has demonstrated effects on adipose tissue, including decreases in adipocyte size (Schaalan et al., 2018), increases in UCP1 expression (Schaalan et al., 2018) and attenuation of body weight gain in rodents exposed to an obesogenic diet (Al-Sawalha et al., 2019; Mong et al., 2011; Rašković et al., 2023; Schaalan et al., 2018). A meta-analysis reported no beneficial effect of beta-alanine supplementation on body composition in human populations, although the studies included were conducted on well-trained participants with low baseline body fat (Ashtary-Larky et al., 2022). This may highlight the potential

of carnosine to decrease lipid accumulation in an obesogenic environment. Therefore, further investigations are needed to explore the therapeutic potential of carnosine in these non-excitable tissues.

1.4 Thesis introduction

1.4.1 Rationale

This thesis will investigate the metabolism and potential physiological roles of the HCD carnosine and its constituent amino acid, beta-alanine, within cardiac cells and tissue.

Cardiac tissue, like skeletal muscle, is rich in HCDs, such as carnosine (O'Dowd et al., 1988). An understanding of carnosine and beta-alanine metabolism, including HCD synthesis, metabolite distribution and the enzymes and transporters involved in the heart, is lacking. In skeletal muscle, the precursors of carnosine (beta-alanine and L-histidine) are transported into the cells. Intracellular beta-alanine concentrations are lower than L-histidine concentrations, and thus, beta-alanine availability has been identified as the rate-limiting factor for the synthesis of carnosine in skeletal muscle (Harris et al., 2006). Due to similarities between skeletal muscle and cardiac tissue, and the abundance of taurine in the heart (Ito et al., 2008), it can be speculated that beta-alanine is transported into cardiomyocytes via the taurine transporter (TauT), although this assumption requires confirmation. Furthermore, the storage of carnosine and beta-alanine in cardiac tissue and cells upon exposure to exogenous sources remains unknown. It is uncertain whether cardiac cells can directly take up and store exogenous carnosine and beta-alanine and whether, as observed in skeletal muscle, beta-alanine acts as the rate-limiting factor for carnosine synthesis in the heart.

To address these gaps in the literature, this programme of work seeks to identify the expression of the enzymes and transporters involved in carnosine and beta-alanine metabolism in cardiomyocytes, determine whether cardiac cells can take up and store

carnosine and beta-alanine when exposed to exogenous sources and assess whether an increase in intracellular beta-alanine allows carnosine synthesis to occur. This will provide insights into the viability of carnosine and beta-alanine to aid the planning of future supplementation interventions. If the supplement cannot be taken up by the cells, and the relevant enzymes and transporters are not expressed, there would be no need for supplementation trials designed to investigate the direct effect of this on cardiac physiology.

Evidence from animal models suggests a promising therapeutic potential for carnosine in the heart, with possible differing roles within the cardiovascular system depending on the health status of the tissue. It is suggested that carnosine may improve calcium handling and muscle contractility in a healthy environment, while mitigating myocardial injury markers and histopathological changes in diseased environments. The direct application of these findings to humans, however, is currently lacking, necessitating further investigation to ascertain the therapeutic possibilities of carnosine and beta-alanine in the cardiovascular system in human participants. Many of the *in vivo* pre-clinical studies utilise acute cardiac injury models to investigate the beneficial effects of carnosine in a diseased environment, yet translating and replicating these findings in a human population is challenging as such events cannot be predicted and inducing them would be unethical. Most studies reported in the literature involve the supplementation of carnosine in animal models. Carnosine dipeptidase 1 (CN1), or serum carnosinase, catalyses the breakdown of carnosine in human serum; however, this enzyme is absent in non-primate mammals (Jackson et al., 1991). Therefore, oral beta-alanine supplementation may be a more effective method for increasing intracellular or

muscular carnosine levels in human participants, as ingested carnosine may be metabolised before reaching the tissue. In skeletal muscle, a higher dose of carnosine supplementation (13 g/day) is required to achieve an increase in muscle carnosine content similar to that shown with 6.4 g/day of beta-alanine supplementation (Harris et al., 2006). Using beta-alanine supplementation in animal models, rather than carnosine, may facilitate the translation of results to human models.

To address the challenges identified, using models of metabolic conditions associated with cardiovascular disease (CVD), such as prediabetes or overweight/obesity, may be more appropriate for beginning the *in vivo* investigation of the therapeutic potential of beta-alanine supplementation in diseased conditions in humans. Although, the use of animal models cannot be fully disregarded, as the direct analysis of cardiac tissue is essential to fully understand the therapeutic potential and metabolism of carnosine and beta-alanine; something that cannot be obtained from live human participants. Therefore, this programme of work seeks to utilise various experimental models, both rodent and human, to explore the physiological impacts of carnosine and beta-alanine in a metabolic condition associated with CVD. Understanding these impacts will provide evidence in favour, or against, the use of beta-alanine supplementation as a therapeutic strategy.

1.4.2 Aims

The overall aims of this research programme were to investigate the metabolism of carnosine and beta-alanine in the heart and to explore the physiological role(s) of beta-alanine supplementation on cardiac function and health in a metabolic disease model

associated was an increased risk of CVD. Several studies were employed in this work: *in vitro* cardiomyocytes were used to explore carnosine and beta-alanine metabolism, and *in vivo* murine and human models of metabolic disease conditions with an associated CVD risk were used to investigate the use of beta-alanine supplementation on cardiac function and health.

1.4.3 Objectives

The aims of this thesis were achieved through the following objectives:

1. To enhance the understanding of carnosine and beta-alanine metabolism within cardiomyocytes by identifying the carnosine and beta-alanine metabolism-related enzymes and transporters expressed by rat cardiomyocytes and explore the influence of exogenous carnosine and beta-alanine on these (**Chapter 2**).
2. To investigate whether exogenous carnosine and beta-alanine can be taken up and stored by cardiac cells and tissues and to explore whether HCD synthesis is affected by the cellular uptake of beta-alanine (**Chapter 2** and **Chapter 3**).
3. To explore the effects of 12 weeks of beta-alanine supplementation on cardiac structure and morphology in mice challenged with an obesogenic diet (**Chapter 3**).
4. To evaluate the feasibility and tolerability of three months of beta-alanine supplementation in humans with overweight and obesity using a randomised controlled trial approach (**Chapter 4**).
5. To explore the effects of three months of beta-alanine supplementation on echocardiographic, haemodynamic, and biochemical markers of cardiac function and health in humans with overweight and obesity (**Chapter 4**).

1.4.4 Hypotheses

The hypotheses for this research programme are:

1. Cardiomyocytes express the enzymes and transporters required for carnosine and beta-alanine metabolism.
2. Exogenous beta-alanine can be taken up by, and stored in, cardiomyocytes and subsequently synthesised into HCDs.
3. Beta-alanine supplementation mediates the negative effects of overweight and obesity on the cardiovascular system in *in vivo* models.

Chapter 2

Effects of carnosine and beta-alanine on amino acid and histidine-containing dipeptide content in rat cardiomyocyte cells

The data within this current chapter are supported by the identification and development of an appropriate *in vitro* cardiomyocyte cell model, as reported in **section 2.1.**

2.1 Optimisation of cardiomyocyte cell experimental model

This experimental chapter aimed to investigate the uptake and utilisation of carnosine and beta-alanine in cardiomyocyte cells to deepen the understanding of carnosine metabolism in the heart and to determine whether carnosine and beta-alanine can be taken up when cells are exposed to exogenous sources. This was approached from several perspectives, with the first being the methodological development to identify a relevant cell model for this investigation. Four different experimental models were explored to determine the optimal *in vitro* model.

2.1.1 Immortalised rodent cardiomyocyte cell lines

Initially, an immortalised rat cardiomyocyte cell line (H9c2) was identified, which colleagues at the university had previously used, providing a robust starting point. Immortalised cell lines have a high proliferation rate and can be utilised to model both physiological and pathological conditions. H9c2 cells present a promising model for studying heart disease as they elicit a hypertrophic response comparable to primary neonatal cardiomyocytes (Watkins et al., 2011), and for studying myocardial metabolic activity as they express multiple genes involved in oxidative metabolism at levels comparable to those observed in primary cardiomyocytes (Zordoky and El-Kadi, 2007). Additionally, H9c2 cells exhibit electrophysiological, morphological, and biochemical properties similar to those of primary cardiomyocytes (Hescheler et al., 1991). These

findings establish the H9c2 cell line as an alternative to isolated primary cardiomyocytes. The H9c2 cell line was chosen over the HL-1 cell line (an immortalised mouse cardiomyocyte cell line) as H9c2 cells exhibit greater similarity to primary cardiomyocytes in regard to energy metabolism, including cellular ATP, mitochondrial bioenergetics and mitochondrial morphology, when the two cell lines were compared (Kuznetsov et al., 2015).

2.1.2 Primary mouse cardiomyocytes

Primary cells are isolated directly from the tissue of interest and maintain the morphological and functional characteristics of cardiomyocytes. Therefore, a primary mouse cell culture model was attempted based on Ackers-Johnson et al. (2016); however, significant challenges were encountered in obtaining a sufficient quantity of cardiomyocytes for experimentation (**Figure 2.1**). This was likely due to the method used for mouse culling. The referenced protocol involves sacrifice by direct needle perfusion of the left ventricle in an anaesthetised mouse, whereas the approach used in this experiment involved hearts derived from mice culled via cervical dislocation. This may have affected the viability of the cardiomyocytes obtained. In addition, primary cell culture is difficult to maintain and has a limited lifespan, meaning that serial passaging cannot occur to the same extent as with immortalised cell lines, limiting the number of experiments that can be completed.



Figure 2.1. Cardiomyocyte isolated from a seven-day old mouse pup (28th June 2021).

2.1.3 Human cardiomyocyte immortalised cell line

The primary target of this body of work is human populations and, to this end, a human cell line model was identified that would work effectively for the planned investigations. The AC16 cell line, a novel immortalised human cardiomyocyte cell line derived from adult human ventricular cardiomyocytes (Davidson et al., 2005), was used; however, this model proved to be ineffective, primarily due to difficulties in inducing differentiation in these cells. The protocol detailed by Davidson et al. (2005), who established the proliferating AC16 cell line, and the manufacturer (Sigma-Aldrich, UK) was followed. The protocol describes the differentiation of the cells using differentiation media containing Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham [DMEM-F-12 (Gibco, Fisher Scientific, UK)] and 2 % horse serum (Thermo Fisher Scientific, UK) for two weeks. The methodology for the proliferation and differentiation of AC16 cells is briefly described in section **2.1.3.1**.

2.1.3.1 AC16 cell line proliferation and differentiation

AC16 cells were obtained from Sigma-Aldrich (UK) and cultured according to both Davidson et al. (2005) and the supplier's instructions. Cells were cultured in growth media comprised of DMEM/F12 supplemented with 12.5 % Embryomax foetal bovine serum [FBS (Sigma-Aldrich, UK)], 2 mM Embryomax L-glutamine (Sigma-Aldrich, UK), and 1 % penicillin/streptomycin (v/v) (Fisher Scientific, UK). Cells were grown until 90 % confluent, dissociated with trypsin-EDTA (Sigma-Aldrich, UK), and sub-cultured into fresh T-75 flasks. AC16 cells were plated with growth media and incubated for approximately 24 hours to allow cell attachment. Growth media was replaced with differentiation media comprised of DMEM/F12 supplemented with 2 % horse serum (Thermo Fisher Scientific, UK), 2 mM L-glutamine, 1 % penicillin/streptomycin (v/v), and 1 X ITS (insulin, transferrin, and selenium) liquid media supplement (Sigma-Aldrich, UK). Differentiation media was replaced every two days, and cells were differentiated for six days (until apoptosis occurred). All tissue culture was performed under sterile conditions in a Class I biological safety cabinet using sterile flasks and reagents. Cell incubation was carried out at 37 °C and 5 % CO_2. AC16 cells used in the experiments were at a maximum passage number of 8. The supplier recommends not exceeding passage 10 to ensure that there is no significant alteration of cell marker expression and functionality.

2.1.3.2 AC16 cell line differentiation gene expression experiment

During differentiation, apoptosis was observed on day six (**Figure 2.2**), and by day seven, all cells had detached from the wells.

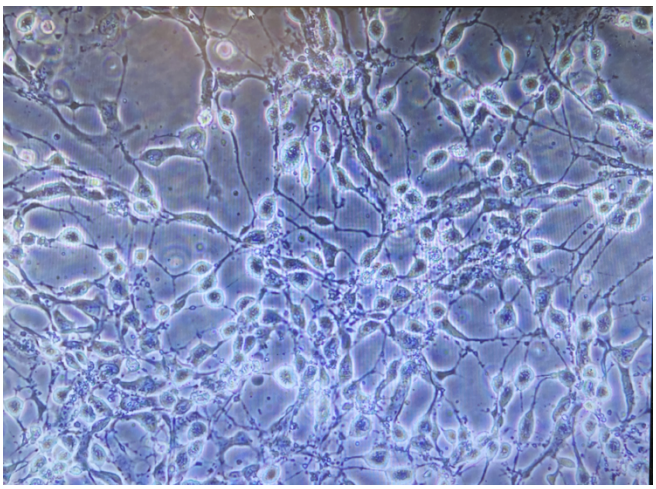


Figure 2.2. Apoptotic AC16 cells on day six of differentiation (passage seven).

According to Davidson et al. (2005), four specific genes respond to differentiation in AC16 cells: GATA binding protein 4 (*GATA4*), nuclear factor of activated T cells 4 (*NFATc4*), myocardin (*MYCD*), and bone morphogenetic protein-2 (*BMP2*). Real-time quantitative polymerase chain reaction (RT-qPCR) was performed on the AC16 cells; RT-qPCR is detailed in section **2.3.2**. Primer sequences are identified in **Table 2.1;** primers were designed and purchased from Thermo Fisher Scientific (UK). The expression of these genes was characterised on day zero (vehicle control), one, three, five, and six of differentiation, however, the results were extremely variable and did not align with the results reported in Davidson et al. (2005). Another laboratory in the United States has published work using differentiated AC16 cells, but there are contradictory findings between this work and the original AC16 paper. After email discussions with this laboratory, they stated that *BMP2* is absent in their differentiated AC16 cells, although according to Davidson et al. (2005), the *BMP2* gene should be activated when the cells are differentiated. This laboratory also experienced cell death after day 5 and used their cells within 48 hours of the differentiation process. Due to these difficulties and contradictions, the decision was made not to continue with the AC16 cell line.

Table 2.1. Forward and reverse primers used in AC16 differentiation gene expression analysis (Davidson et al., 2005)

Gene	Forward	Reverse
GATA4	CCCCAATCTCGATATGTTTG	GATTATGTCCCCGTGACTGT
NFATc4	AGACTCCAAGGTGGTGTTCA	TGATACCCTGGATAGGGACA
MYCD	CTTTTCCTGTCACACCCAAC	CTGCTTTACGGCATCTTCAT
BMP2	CCACCATGAAGAATCTTTGG	TGACCAACGTCTGAACAATG

2.1.4 Primary human cardiomyocyte cells

Primary human cardiomyocytes were available for purchase from Promocell (Germany) and Creative Bioarray (USA); however, the inventory of donors included only human cardiomyocyte samples from individuals with cardiomyopathy (undergoing a heart transplant). Any studies using these cells would likely create a false baseline and cause issues with the translation of findings. To ensure an appropriate model is selected, human cardiomyocytes from healthy individuals are required (i.e., individuals who have died from other causes and donated organs to science). Since this preliminary work (and the experiments) were completed, two studies have used primary human tissue to quantify the concentrations of HCDs in the heart. Carnosine and anserine have been identified as being present in cardiac tissue; however, this was observed in samples collected from patients undergoing open heart surgery (Van der Stede et al., 2023), and most donors in Gonçalves et al. (2023) had underlying cardiac conditions.

2.1.5 Conclusion

Given that the most viable cell model available was the H9c2 rat ventricular cardiomyocyte cell line, the next stage of the programme of work utilised this cell line. This is detailed in the remainder of this experimental chapter.

2.2 Introduction

Carnosine is a HCD synthesised from beta-alanine and L-histidine. Carnosine has potential physiological roles, including intramuscular pH buffering, regulation of calcium handling and sensitivity, antioxidant properties and metal ion chelation (for a comprehensive review, see Boldyrev et al., 2013). The dietary availability of carnosine and its constituent amino acids is a key determinant of tissue carnosine content (Harris et al., 2012). The endogenous synthesis of beta-alanine may occur via the catabolism of pyrimidines (e.g., uracil), the degradation of HCDs, aspartate metabolism during digestion, or as a by-product during the conversion of L-alanine to pyruvate (Hayaishi et al., 1961). Primarily, beta-alanine is endogenously produced via uracil degradation in the liver, where it is released into the bloodstream and then transported into tissues (Fritzson and Pihl, 1957). The concentration of beta-alanine in serum is undetectable under basal conditions when individuals have not been exposed to supplementary sources (Harris et al., 2006), suggesting that the demand for beta-alanine to be able to fulfil physiological roles far exceeds this endogenous supply (Boldyrev et al., 2013). Intramuscular carnosine content can be increased with exogenous carnosine or beta-alanine, either obtained through the diet or via supplementation. Beta-alanine is considered the rate-limiting precursor for carnosine synthesis in skeletal muscle (Harris et al., 2006). It is still unclear whether carnosine synthesis can occur in cardiac tissue with exogenous beta-alanine provision.

Several genes and proteins control the processes involved in the metabolic pathway of carnosine and beta-alanine; these are identified in **Table 2.2**.

Table 2.2. Carnosine-and beta-alanine metabolism related enzymes and transporters

	Gene	Function
Enzymes		
Carnosine Synthase 1	*CARNS1*	Carnosine synthesis
Carnosine Dipeptidase 1	*CNDP1*	Carnosine hydrolysis
Carnosine Dipeptidase 2	*CNDP2*	Carnosine hydrolysis
4-aminobutyrate-2-Oxoglutarate Transaminase	*GABA-T*	Beta-alanine transaminase
Alanine-glyoxylate Aminotransferase 2	*AGXT2*	Beta-alanine transaminase
Transporters		
Sodium and chloride dependent-taurine transporter	*SLC6A6/TauT*	Amino acid transporter
Proton-coupled amino acid transporter	*SLC36A1/PAT1*	Amino acid transporter
Peptide transporter 1	*SLC15A1/PEPT1*	Nonspecific peptide transporter
Peptide transporter 2	*SLC15A2/PEPT2*	Nonspecific peptide transporter
Peptide-histidine transporter 1	*SLC15A4/PHT1*	Nonspecific peptide transporter
Peptide-histidine transporter 2	*SLC153/PHT2*	Nonspecific peptide transporter

Research has begun to ascertain the expression of carnosine and beta-alanine metabolism-related genes within skeletal muscle (Everaert et al., 2013; Saunders et al., 2017a); however, knowledge is currently lacking regarding the synthesis and metabolism of carnosine and beta-alanine in the heart. Beta-alanine transport into muscle is primarily thought to occur via two transporters, TauT and PAT1, and synthesis with L-histidine in the muscle is regulated by CARNS1. Carnosine can be transported across the cellular membrane via nonspecific peptide transporters and is degraded into its constituent amino acids (L-histidine and beta-alanine) by Carnosine Dipeptidase 2

[CNDP2 (also known as tissue carnosinase)]. It may be hypothesised that the processes/pathways of carnosine and beta-alanine in cardiac tissue will utilise these enzymes and transporters. It has been reported that two transaminases (GABA-T and AGXT2) are involved in beta-alanine metabolism in cardiac tissue, catalysing the degradation of beta-alanine into MSA (Blancquaert et al., 2016). This may limit the beta-alanine available for carnosine synthesis and requires further investigation. The metabolism of exogenous carnosine and beta-alanine, utilising the enzymes and transporters described, is represented in **Figure 2.3**.

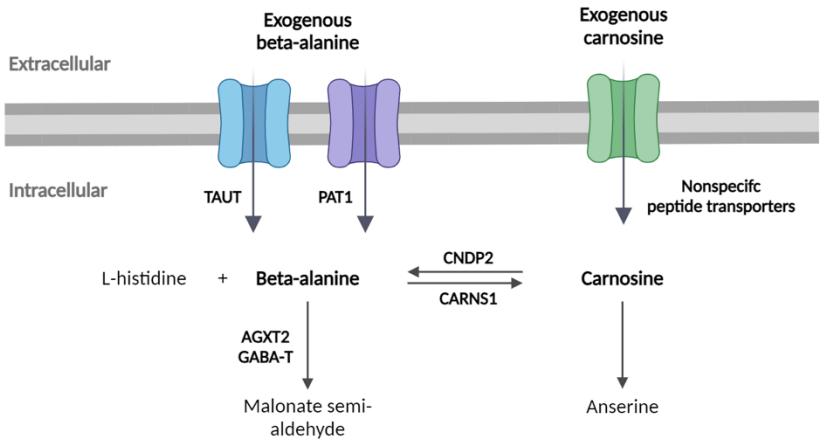


Figure 2.3. Simplified diagram depicting the metabolism of exogenous carnosine and beta-alanine. Created with Biorender.com.

Anserine is a methylated derivative of carnosine, consisting of beta-alanine and 3-methylhistidine. When compared to carnosine, it is more stable in blood plasma due to its resistance to degradation by carnosinase (Everaert et al., 2019). The synthesis of this dipeptide is dependent on the prior synthesis of carnosine (Gonçalves et al., 2021), as the formation of anserine involves transferring a methyl group from S-adenosylmethionine onto carnosine (McManus, 1962). This HCD is far less studied than

carnosine. An investigation into determining the concentrations of anserine in the heart, as well as the effect of exogenous carnosine and beta-alanine on anserine levels in cardiac tissue, is also required.

Investigation of carnosine and beta-alanine uptake into the cardiac cell is a critical determinant of exploring their viability for *in vivo* supplementation studies. Therefore, this study was designed to ensure that exogenously sourced carnosine and/or beta-alanine can accumulate in cardiac cells. This was unknown at the time the study was designed. The study aimed to identify the expression of carnosine and beta-alanine-related enzyme and transporter genes in H9c2 cardiomyocyte cells. Cells were then exposed to a variety of concentrations of carnosine or beta-alanine. The enzymes and transporters identified as being able to convert or traffic these two compounds were assessed by RT-qPCR, exploring their responses to the exogenous sources of carnosine and beta-alanine. The second objective of the study was to explore the influence of exogenous carnosine and beta-alanine on the intracellular concentration of associated amino acids (taurine, beta-alanine, and L-histidine) and HCDs (carnosine and anserine). As of yet, the intracellular abundance of these compounds in response to carnosine or beta-alanine are unrecorded.

2.3 Materials and methods

2.3.1 H9c2 cell culture

Rat ventricular cardiomyoblast-derived H9c2 cells were kindly donated by Dr John Dickenson [Nottingham Trent University (NTU)]. H9c2 cells are a subclone of the original clonal cell line derived from embryonic BD1X rat heart tissue (Kimes and Brandt, 1976) and have been established as an alternative to isolated primary cardiomyocytes (Kuznetsov et al., 2015).

2.3.1.1 Growth and maintenance

Cells were grown and maintained in T-75 tissue culture flasks in growth media composed of Dulbecco's Modified Eagle's Medium [DMEM (Lonza, UK)] supplemented with 10 % FBS (Gibco, Thermo Fisher Scientific, UK), 2 mM L-glutamine (Thermo Fisher Scientific, UK), and 1 % penicillin and streptomycin (v/v) (Thermo Fisher Scientific, UK). All tissue culture was performed under sterile conditions in a Class I biological safety cabinet using sterile flasks and reagents. Cell incubation was carried out at 37 °C and 5 % CO_2. The growth media (three replicates) was analysed on an amino acid analyser (protocol detailed in section **2.3.3**) to determine the concentrations of beta-alanine, L-histidine, carnosine, anserine, and taurine. The analysis showed only the presence of L-histidine (manufacturer concentration of 0.042 g/L) and taurine (from the FBS) in the growth media; beta-alanine, carnosine, and anserine were not detected.

2.3.1.2 Cell passaging

Cells were passaged at 70–80 % confluency, as observed by light microscopy. Growth media was aspirated, and cells were washed with 10 mL sterile phosphate-buffered saline (PBS) to ensure FBS was removed, as it can prevent trypsinisation from occurring. The PBS was aspirated, and cells were incubated in 5 mL of TrypLE solution (Thermo Fisher Scientific, UK) for 5 minutes at 37 °C until cell detachment occurred. Cell detachment was checked under the microscope. Cells were resuspended with an additional 5 mL of growth media to ensure complete cell detachment, and the solution was collected into a 15 mL Falcon tube. The Falcon tube was centrifuged at 1000 rpm for 5 minutes at room temperature. The supernatant was aspirated, and the cell pellet was resuspended in an appropriate volume of growth media before counting (section **2.3.1.3**) and/or sub-culturing into fresh flasks for further proliferation or plates for experiments. The growth media was changed, or cells were passaged, approximately twice a week, depending on proliferation rate and splitting ratio (typically 1:5). All experiments for gene expression analysis were performed using passages 11 to 23, and all experiments for amino acid analysis were performed using passages 14 to 26. No differences, including observable cell deterioration, changes in response to treatment, or changes in proliferation and differentiation, were noted between cell passage numbers by the investigator.

2.3.1.3 Cell counting and seeding

Cells were counted using a Luna II™ automated cell counter (Logos Biosystems, South Korea), where 10 μL of cell suspension was combined with 10 μL of 0.4 % Trypan Blue solution (Gibco, Fisher Scientific, UK). Of this combined solution, 10 μL was aliquoted

onto the Luna™ cell counter slide (Logos Biosystems, South Korea). Cells were seeded at various densities depending on the experimental requirements: 1.5 x 10^5 cells/well for gene expression and 1 x 10^6 cells/well for amino acid analysis. Once seeded, cells were cultured for approximately 24 hours in growth media to allow cell attachment. Following this, growth media was removed, and adherent cells were washed with PBS before being differentiated as described in section **2.3.1.4**.

2.3.1.4 Cell differentiation

Mono-nucleated cardiomyoblasts can be differentiated into a more cardiomyocyte-like phenotype when cultured in low serum concentration media containing *all-trans* retinoic acid (Branco et al., 2015; Daubney et al., 2015; Kimes and Brandt, 1976). The addition of *all-trans* retinoic acid enhances cardiac differentiation (Ménard et al., 1999). Based on the work by Daubney et al. (2015), differentiation was induced in the cells by replacing growth media with differentiation media, composed of DMEM supplemented with 1 % FBS, 2 mM glutamine, 1 % penicillin and streptomycin (v/v), and 10 nM *all-trans* retinoic acid (Sigma-Aldrich, UK). Cells were differentiated for 7 days, and differentiation media was changed every 48 hours. Cells were observed under light microscopy to confirm a cardiomyocyte-like phenotype by the formation of multinucleated elongated myotubes (Daubney et al., 2015). The differentiation media (three replicates) was analysed on the amino acid analyser (protocol according to section **2.3.3**) to determine the metabolite concentrations of beta-alanine, L-histidine, carnosine, anserine, and taurine. The analysis showed only the presence of L-histidine (manufacturer concentration of 0.042 g/L) in the differentiation media; beta-alanine, carnosine, anserine, and taurine were not detected.

2.3.1.5 Cryopreservation and thawing of cells

Cells were cryopreserved for long-term storage and to prevent high passage numbers. Cells were grown until 70-80 % confluent and prepared as described in section **2.3.1.2**. Cells were counted (section **2.3.1.3**) and then resuspended in growth media with 10 % Dimethyl Sulfoxide, a cryoprotectant, at 1 x 10^6 cells/cryovial. Cryovials were stored at -80 °C in a freezing container ["Mr Frosty" (Nalgene, UK)] to ensure cells were frozen at -1 °C /minute for 24 hours. Cryovials were then transferred to liquid nitrogen for long-term storage.

Cells were removed from storage and rapidly thawed at 37 °C in a bead bath. The vial of cells was removed from the bead bath before complete thawing had occurred and transferred into 10 mL of growth media. This solution was added to a T-75 tissue culture flask and incubated at 37 °C in a 5 % CO_2 atmosphere. Growth media was replaced after 24 hours to remove any dead cells, and the remaining adherent cells were maintained as described in sections **2.3.1.1** and **2.3.1.2**.

2.3.2 Gene expression experiment

H9c2 cells were seeded at approximately 1.5 x 10^5 cells/well in 6-well cell culture plates and incubated for approximately 24 hours in growth media to allow cell attachment. Growth media was removed, and adherent cells were washed with PBS before being differentiated as described in section **2.3.1.4**. On day seven, differentiation media was removed, adherent cells were washed with PBS, and treatment was then added. Cells were treated with 0.0 (vehicle control), 0.1, 0.5, 1.0, 5.0, and 10.0 mM of carnosine (Thermo Fisher Scientific, UK) or beta-alanine (Thermo Fisher Scientific, UK) in growth

media for 4 hours. Following treatment, ribonucleic acid (RNA) was isolated. Six biological replicates were completed for each concentration.

2.3.2.1 RNA isolation

Total RNA was isolated using TRI reagent solution (Invitrogen, Thermo Fisher Scientific, UK). This monophasic reagent preserves the integrity of RNA by using phenol to denature proteins and guanidine isothiocyanate to inhibit the RNA-degrading enzymes (ribonucleases). At the end of the treatment time, the media was aspirated, and cells were washed with PBS. One mL of TRI reagent was added to each well and pipetted up and down to ensure maximal cell lysis. The cell lysate in the TRI reagent was transferred to a 1.5 mL Eppendorf tube and stored at -80 °C until RNA isolation.

RNA isolation was performed using the phenol-chloroform method and alcohol-based precipitation. First, samples were warmed to room temperature. For each sample, 200 µL of chloroform per 1 mL of TRI reagent was added to each TRI reagent lysate and vortexed for 15 seconds. The mixture was then incubated for 3 minutes at room temperature to enable phase separation. The chloroform method separates RNA from deoxyribonucleic acid (DNA) and proteins into three distinct layers: the upper clear aqueous phase containing RNA, the white interphase containing DNA, and the pink organic phase containing protein. The sample was then centrifuged at 12,000 x *g* for 10 minutes at 4 °C, after which the upper aqueous layer was separated without disturbing the white interphase layer and transferred to a fresh 1.5 mL Eppendorf tube for further isolation. To this, 2 x the volume of ice-cold isopropanol was added and mixed by inverting the tube. This was then stored overnight at -20 °C to aid RNA precipitation.

The following day, the lysate was centrifuged at 12,000 x *g* for 10 minutes at 4°C to form the gel-like RNA pellet. The supernatant was carefully discarded (without disturbing the pellet) and the pellet was washed by resuspension in 750 µL of ice-cold 75 % ethanol. This was vortexed for a few seconds and then centrifuged at 7500 x *g* for 10 minutes at 4 °C. The supernatant was carefully discarded, and the ethanol washing procedure was repeated once more. After the second wash, the supernatant was completely removed, ensuring all ethanol was removed, as any remaining ethanol will inhibit the reverse transcription enzymes. If any ethanol remained, the tube was left open to air dry in a fume hood. The RNA pellet was resuspended in 20 µL of nuclease-free water (NFW). The pellet was dissolved in NFW by pipetting up and down and then immediately placed on ice.

2.3.2.2 RNA quantification

RNA yield (ng/µL) and quality were measured using a Nanodrop 2000C spectrophotometer (Thermo Fisher Scientific, UK), and RNA purity was determined by calculating the absorbance ratio between 260 and 280 nm. A ratio between 1.8 and 2.0 was deemed sufficient. 1 µL of NFW was used as the blank to remove background interference during sample measurement. 1 µL of each RNA sample was then used to assess RNA yield and quality. The sample stage was cleaned between each measurement.

2.3.2.3 Complementary DNA synthesis

Reverse transcription uses the reverse transcriptase enzyme for the synthesis of RNA into the more stable form of complementary DNA (cDNA). The cDNA can then be used directly as a template for polymerase chain reaction (PCR) amplification.

1 µg of each quantified RNA sample was diluted to a final volume of 10 µL with NFW and reverse transcribed using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, UK) to obtain cDNA. The components of this kit were kept on ice due to their temperature sensitivity. A reverse transcription master mix was prepared; for each sample, this consisted of 2 µL of RT buffer, 0.8 µL of dNTP, 2 µL of random primers, 1 µL of reverse transcriptase and 4.2 µL of NFW. 10 µL of this master mix was added to 10 µL of the diluted RNA sample, forming a total reaction volume of 20 µL. Reverse transcription was performed on a PCR thermal cycler (Applied Biosystems, Thermo Fisher Scientific, UK) with the following settings: 25 °C for 37 seconds, 37 °C for 120 minutes to allow reverse transcription, 85 °C for 5 seconds to inactivate the enzyme, followed by an infinite holding cycle of 4 °C until samples were removed. cDNA was then diluted into 20 µL NFW (total volume of 40 µL) and stored at -20 °C until use.

2.3.2.4 Real-time quantitative polymerase chain reaction

Gene expression and quantification were measured using RT-qPCR. The amplification of each gene is measured in each PCR cycle (40 cycles in total) using fluorescence. The cycle threshold (Ct) represents the number of amplifications (cycles) taken for this fluorescence to occur. Ct values are inversely related to the amount of target nucleic acid

in the sample: a lower Ct indicates a greater amount of gene expression, while a higher Ct indicates lower gene expression.

RT-qPCR was carried out on a QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, UK). FastGene® 2x IC Green Universal qPCR Mix (Geneflow, UK) was used for fluorescence. FastGene® IC Green is an intercalating dye that allows higher sensitivity to detect genes at a lower Ct value. The passive reference dye ROX was used as a loading control to normalise the fluorescence signals during the PCR run. 2 µL of 50 µM ROX Additive was added to 1 mL of the FastGene® 2x IC Green mix and protected from light to prevent bleaching of the DNA dye and to maintain fluorescent signal intensity. For each gene of interest, a qPCR reaction mix was made comprised of 10 µL of the FastGene® 2x IC Green Universal qPCR low ROX mix (Geneflow, UK), 0.2 µL of a 20 µM forward and reverse primer mix for the gene of interest and 8.8 µL of NFW. The qPCR reaction mix was protected from light. 19 µL of the qPCR reaction mix was then added to an Applied Biosystems™ MicroAmp™ Optical 96-Well Reaction Plate (Fisher Scientific, UK) with 1 µL of the diluted cDNA (a total volume of 20 µL in each well). A no template control was added to the plate for each gene of interest using 19 µL of the reaction mix with 1 µL of NFW. All samples and controls were plated in duplicate. An adhesive PCR plate seal (Fisher Scientific, UK) was added to the top of the plate before it was briefly centrifuged to ensure adequate mixing of the qPCR reaction mix and cDNA. All plate preparation, reagents, and samples were kept on ice.

The PCR was run according to the FastGene® 2x IC Green Universal qPCR Mix kit instructions, as described in **Table 2.3**. Primer sequences of the genes of interest were

identified in the literature and are presented in **Table 2.4**. Sequences were confirmed using NCBI BLAST analysis, and primers were then designed and purchased from Thermo Fisher Scientific (UK). The reference gene used was 18S. 18S was stable across all treatment conditions, with Ct values remaining within two Cts. The coefficient of variation for 18S was 0.07 % for beta-alanine treatments (including control) and 0.06 % for carnosine treatments (including control).

Table 2.3. RT-qPCR cycling protocol

Stage	Step	Temperature °C	Duration (seconds)	Cycles
Hold	1 *(Initial denaturation)*	95	120	1
PCR	1 *(Denaturation)*	95	5	40
	2 *(Annealing and elongation)*	60	30	
Melt curve	1	95	5	Continuous
	2	60	60	
	3 *(Dissociation)*	95	15	

Abbreviations: PCR, polymerase chain reaction.

Table 2.4. Forward and reverse primer sets used in the RT-qPCR analysis

Gene	Forward	Reverse	Reference
Carns1	GCGGCGTCAGCAAGAAGTT	CACCAAGCAGTCATCCCAGAA	Barca et al. (2019)
Cndp1	CCTAGAAGAATACCAGAAGAGC	GGGACTAGACGGATTGAAA	Barca et al. (2019)
Cndp2	TTCAAGGTGTACATGGGC	AAAGGTCAAGGTCACAGGA	Barca et al. (2019)
TauT	TGG-CCG-ACA-GCA-TTC-CA	GCC-TTC-TCT-AAG-GTG-CCT-TCC-T	Everaert et al. (2013)
Pat1	TGGTTGTACCAGTCGGTGAA	GGCCAGAACACATGTCACAC	Broberg et al. (2012)
Pept1	TGTGTTTGTCCTCGGCAGTGG	CTTTAGCCCAGTCCAGCCAGT	Barca et al. (2019)
Pept2	CATGAAATCTGTGCTCCAGG	AGGAGGCAGGAAAACAAAA	Barca et al. (2019)
Pht1	TGAAGGCCTTGGAGTCTTT	TTGGAAATACACTGTCCAGTAA	Barca et al. (2019)
Pht2	CCAGATGCAGTCCACCTA	AACATTGGCCAGCAGGAG	Barca et al. (2019)
Gaba-T	CCT-TCA-TGG-GTG-CTT-TCC-A	CAA-AGG-AAG-GGA-TGT-CAA-TCT-TG	Everaert et al. (2013)
Agxt2	GAT-AGG-CTG-CCA-ATC-AAC-AAT-GT	TGC-ACT-GGA-GAA-TCT-CGA-CAA	Blancquaert et al. (2016)

2.3.2.5 Interpretation and analyses

The Ct values across duplicates were averaged for all genes of interest and the 18S reference gene. Results were obtained using the comparative Ct method. The Δ-Ct values were calculated for each sample and gene of interest using:

$$\Delta\text{-Ct} = \text{Ct (gene of interest)} - \text{Ct (18S reference gene)}$$

To compare the relative fold change between treatment groups:

$$\Delta\Delta\text{Ct} = \Delta\text{-Ct (treated sample gene of interest)} - \Delta\text{-Ct (control sample gene of interest).}$$

The mRNA expression was calculated using Livak and Schmittgen (2001):

$$\text{mRNA expression} = 2^{-}\,\Delta\Delta\text{Ct}$$

2.3.3 Amino acid analysis (Biochrom 30)

The Biochrom 30 functions as a high-performance liquid chromatography ion exchange system (**Figure 2.4**).

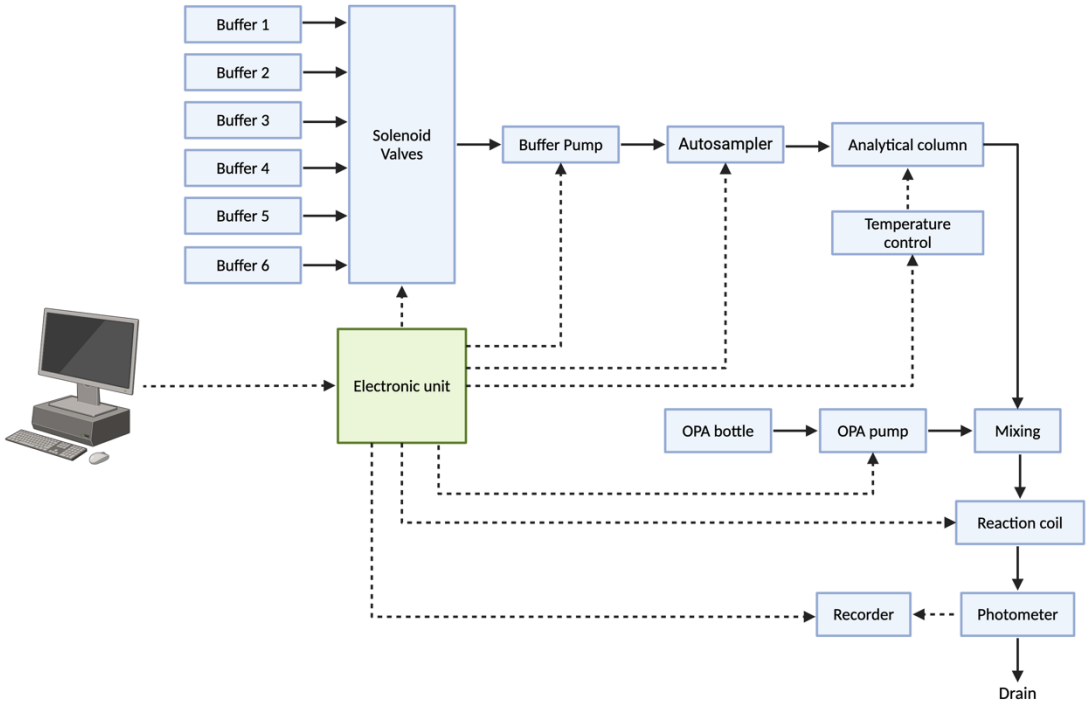


Figure 2.4. Biochrom 30 amino acid analyser adapted from the manufacturer manual. Created with Biorender.com. Abbreviations: OPA, o-phthaldialdehyde.

In this process, the sample is injected from an autosampler onto a cation exchange resin analytical column. This analytical column is negatively charged, whereas the amino acids, upon introduction from the autosampler at a low pH, are positively charged. Throughout the analysis, the temperature of the column is adjusted, and lithium buffers with varying pH and molarity are pumped through the column to separate the amino acids based on their net charge (determined by the pKa of their ionised group). When the iso-ionic point of an amino acid is reached, the ionic attraction is lost, allowing the elution of amino acids from the column for quantification. After each sample analysis, the system flushes

the column with a strong base buffer, followed by the equilibrating buffer, to restore it to its original pH.

2.3.3.1 Biochrom 30 sample preparation and analysis

H9c2 cells were seeded at approximately 1 x 10^6 cells/well in 10 cm cell culture dishes and cultured for approximately 24 hours in growth media to allow cell attachment. Growth media was removed, and adherent cells were washed with PBS before being differentiated (section **2.3.1.4**). On day seven, differentiation media was removed, adherent cells were washed with PBS, and cells were then treated with 0.0 (vehicle control), 0.1, 0.5, 1.0, 5.0, and 10.0 mM of carnosine or beta-alanine in growth media for 4, 24, or 72 hours. The media was not refreshed during the treatment. Six biological replicates were completed.

The protocol for amino acid analysis was adapted from Santos et al. (2021). Following treatment, the culture media was collected, and cells were lysed in 0.5 % Triton X-100 (Sigma-Aldrich, UK) in 0.2 mM sodium chloride. Cell lysates were used to examine intracellular concentrations, and culture media used to explore extracellular concentrations. All samples were added (1:1) to 5 % 5-sulphosalicylic acid deproteiniser (Sigma-Aldrich, UK) with an internal standard of 500 µM Norleucine (Fisher Scientific, UK). Norleucine is commonly used as an internal standard for acidic and neutral amino acid analysis (Harduf et al., 1977) because it can elute from the column without interfering with other amino acids, it is not a naturally occurring amino acid, and it remains stable with no degradation over time. Samples were incubated on ice for 30 minutes and then centrifuged at 10,000 rpm at 4 ^{o}C for 5 minutes. The supernatant was

collected, filtered through a 0.22 µm centrifugal filter tube (Corning, UK), and stored at -80 ^{o}C until analysis. Before analysis, the samples were thawed on ice, and 200 µL of the sample was transferred to an autosampler vial (Restek Corporation, USA).

Amino acid (taurine, beta-alanine, and L-histidine) and HCD (carnosine and anserine) concentrations in both cell lysates and culture media were determined using a Biochrom 30 (Biochrom Ltd, UK) with o-phthaldialdehyde [OPA (section **2.3.3.2**)] post-derivatisation. OPA was used for the post-derivatisation as it allows for a very low level of detection, improving sensitivity through fluorescence detection (Kühnreich and Holzgrabe, 2016). Before sample analysis, retention times for each amino acid and HCD of interest were determined by separately running 250 µM of each compound in lithium loading buffer to identify the time at which the peak occurs. The column was maintained at 50 ^{o}C, and the fluorescence detector was set at an excitation wavelength of 340 nm and an emission wavelength of 450 nm. A set of six lithium buffers of varying pH and ionic strength were used to separate the amino acids [**Table 2.5** (Biochrom Ltd, UK)]. The EZ Chrom Elite Software (Agilent Technologies, USA) was used to determine the peak area (**Figure 2.5**). Analytes were normalised to the internal standard of Norleucine. When amino acids and HCDs of interest could not be detected in the samples, this may have been due to either the absence of the metabolite or the concentration being below the machine's limit of detection.

Table 2.5. Biochrom 30 lithium citrate buffers

Product Code	Lithium Buffer	pH	Molarity (M)
80-2038-15	Lithium Buffer 1	2.80	0.2
80-2038-16	Lithium Buffer 2	3.00	0.3
80-2099-83	Lithium Buffer 3	3.15	0.5
80-2097-18	Lithium Buffer 4	3.50	0.9
80-2037-69	Lithium Buffer 5	3.55	1.65
80-2038-20	Lithium Regeneration 6		0.3
80-2038-10	Lithium Loading Buffer	2.20	0.2

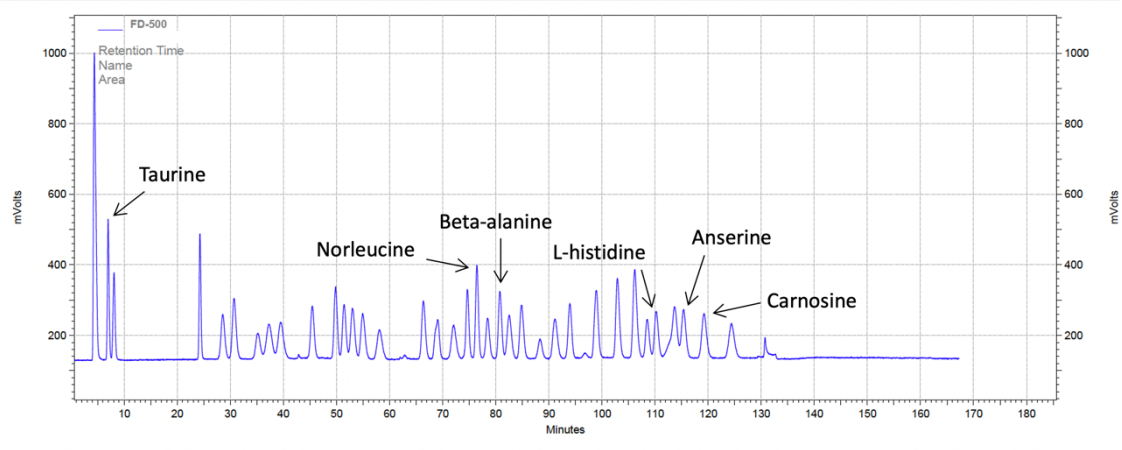


Figure 2.5. A chromatogram depicting the peaks observed on the Biochrom 30.

2.3.3.2 O-phthaldialdehyde preparation

The following protocol was used to prepare 2 L of OPA solution; approximately 50 mL of OPA is required per sample. 88 g of potassium hydroxide pellets (Fisher Scientific, UK) were dissolved in 500 mL of ultrapure water. 100 g of boric acid (Fisher Scientific, UK) was then added to the same beaker and sonicated at 30 °C until dissolved. In separate beakers, 2.1 g of the detergent Brij (Sigma-Aldrich, UK) was dissolved in 250 mL of ultrapure water, and 1.2 g of OPA crystals were dissolved in 15 mL of methanol. Both beakers were sonicated at 30 °C until the compounds had dissolved. The compounds

were filtered through a filter system with 0.22 μM filter paper (Merck Millipore, USA). Before use, the filter paper was flushed with a few drops of methanol using a Pasteur pipette. The filter paper was also changed between compounds. Filtered solutions were poured into a 2 L volumetric flask. 10 mL of 2-mercaptoethanol (Acros Organics, Thermo Fisher Scientific, UK) was added to the volumetric flask, and the resulting solution was topped up to 2 L with ultrapure water.

2.3.4 Statistical analysis

Analysis of variance (ANOVA) statistical components were used with GraphPad Software Inc. Prism version 9.2.0. A one-way ANOVA with Dunnett's multiple comparison post-test was used to compare the gene expression of each carnosine or beta-alanine concentration with the vehicle control. Data are presented as fold change, with statistical analysis being conducted on the Δ-Ct data. A one-way ANOVA with Dunnett's multiple comparison post-test was used to analyse the Biochrom 30 amino acid analyser data, comparing each carnosine or beta-alanine concentration with the vehicle control. Data are presented as mean ± standard deviation (SD) with statistical significance determined as * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

2.4 Results

2.4.1 Cardiomyocyte expression of carnosine and beta-alanine metabolism-related genes

The first experiment of this study sought to confirm the expression of carnosine and beta-alanine metabolism-related genes in cardiomyocytes. The H9c2 cardiomyocyte cells expressed the following genes of interest (**Table 2.6**): *Carns1 (*synthesis), *Cndp1* and *Cndp2* (hydrolysis)*, TauT* and *Pat1* (beta-alanine transporters)*, Pept1, Pept2, Pht1,* and *Pht2* (non-specific peptide transporters), and *Gaba-T* (beta-alanine transaminase). The beta-alanine transaminase *Agxt2* was not expressed in H9c2 cardiomyocyte cells.

Table 2.6. Gene expression of carnosine and beta-alanine-related enzymes and transporters in H9c2 cells

Function	Gene	Arbitrary unit
Carnosine synthesis	*Carns1*	0.00048 ± 0.00024
Carnosine hydrolysis	*Cndp1*	0.00286 ± 0.00293
	Cndp2	0.08374 ± 0.07057
Amino acid transporter	*TauT*	0.00287 ± 0.00239
	Pat1	0.00996 ± 0.00421
Non-specific peptide transporter	*PepT1*	0.00627 ± 0.00562
	Pept2	0.00009 ± 0.00004
	Pht1	0.06403 ± 0.05451
	Pht2	0.00070 ± 0.00046
Beta-alanine transaminase	*Gaba-T*	0.00004 ± 0.00003
	Agxt2	Not expressed

Data are presented as mean ± SD

2.4.2 Carnosine supplementation upon expression of carnosine and beta-alanine metabolism-related genes

Given that the cardiomyocyte cells expressed the enzymes and transporters related to carnosine and beta-alanine metabolism, the next stage of the experiment aimed to explore the effect of exogenous carnosine on the expression of these genes. The administration of exogenous carnosine for four hours did not affect the expression of any of the genes related to carnosine synthesis and hydrolysis (**Figure 2.6**), beta-alanine transporters (**Figure 2.7**), non-specific peptide transporters (**Figure 2.8**), or beta-alanine transamination (**Figure 2.6**).

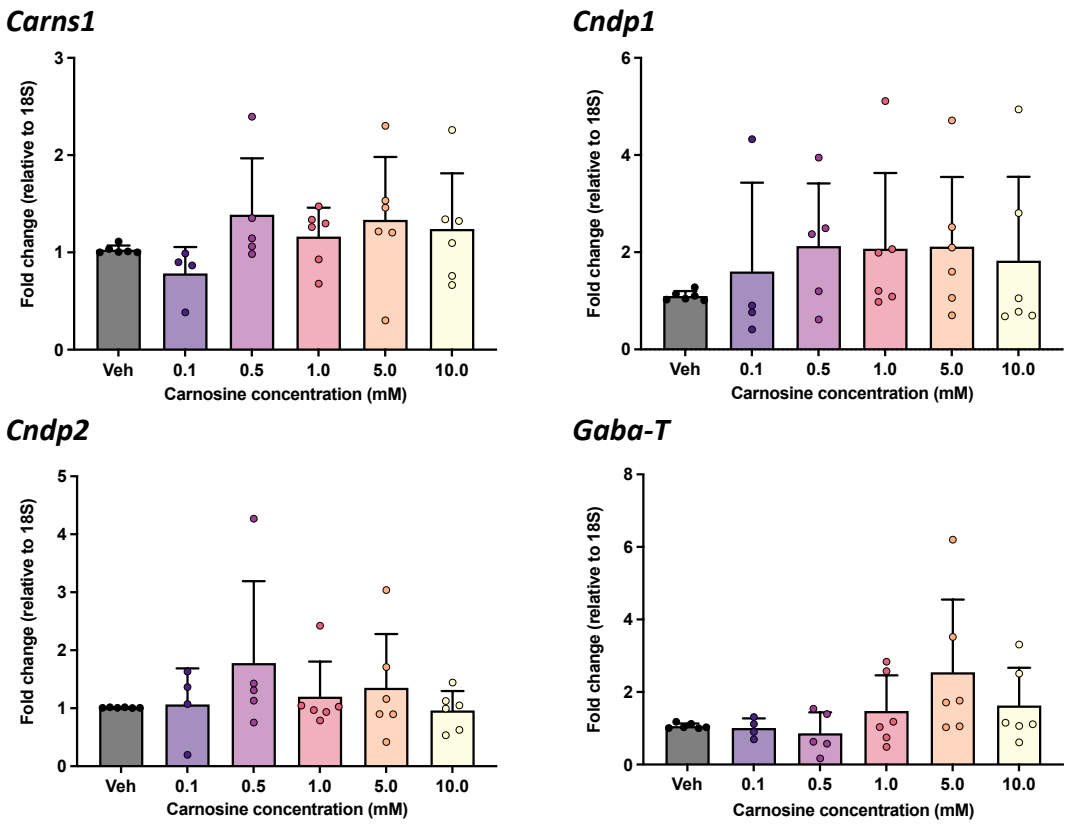


Figure 2.6. Gene expression of enzymes involved in carnosine synthesis and hydrolysis, and beta-alanine transamination following four hours of carnosine treatment, as assessed by RT-qPCR. Data are presented as mean with individual values ± SD. Statistical comparisons used Dunnett's post-test to compare treatment means to vehicle control mean. Abbreviations: Veh, vehicle control.

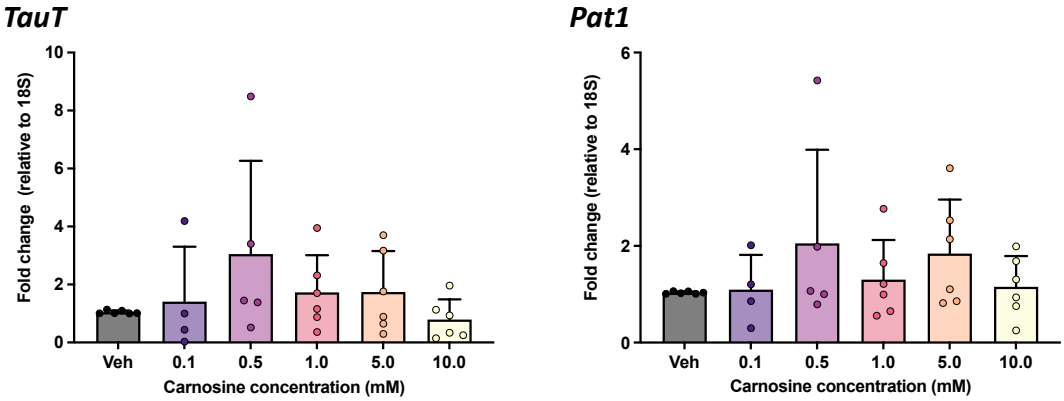


Figure 2.7. Gene expression of amino acid transporters following four hours of carnosine treatment, as assessed by RT-qPCR. Data are presented as mean with individual values ± SD. Statistical comparisons used Dunnett's post-test to compare treatment means to vehicle control mean. Abbreviations: Veh, vehicle control.

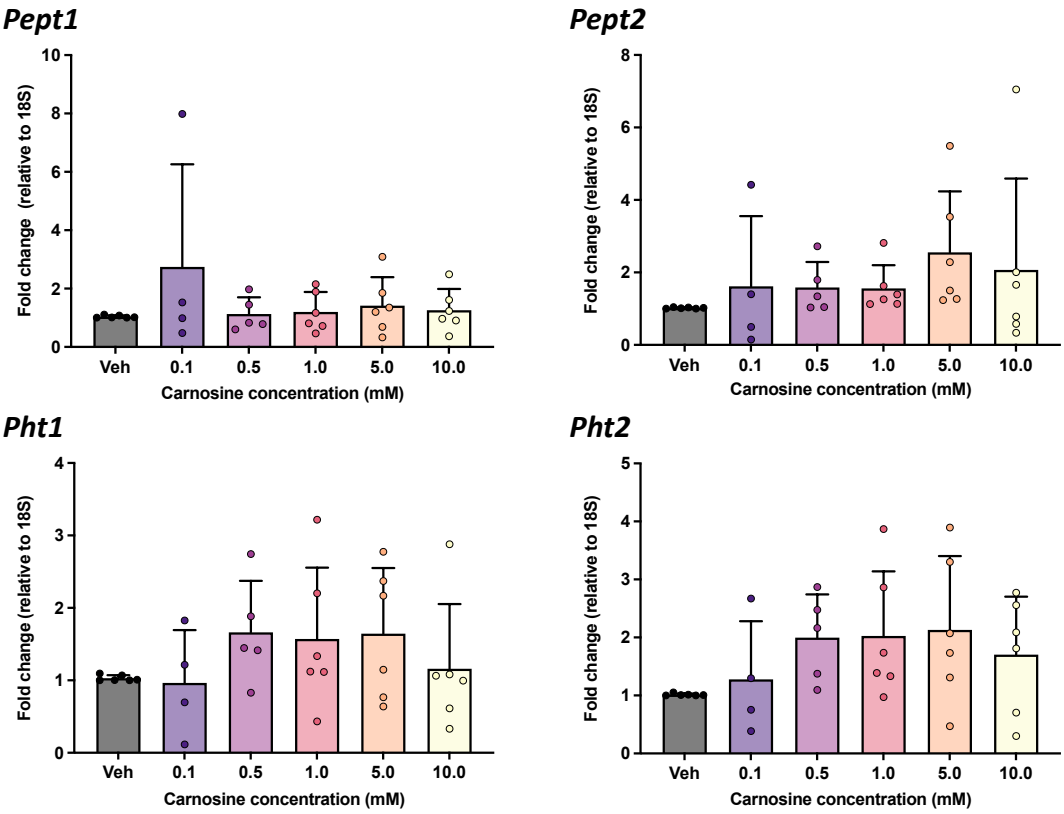


Figure 2.8. Gene expression of non-specific peptide transporters following four hours of carnosine treatment, as assessed by RT-qPCR. Data are presented as mean with individual values ± SD. Statistical comparisons used Dunnett's post-test to compare treatment means to vehicle control mean. Abbreviations: Veh, vehicle control.

2.4.3 Beta-alanine supplementation upon expression of carnosine and beta-alanine metabolism-related genes

The genes involved in carnosine and beta-alanine metabolism did not respond to exogenous carnosine; therefore, the cells were exposed to the rate-limiting precursor of carnosine synthesis, beta-alanine. Exogenous beta-alanine did not affect the expression of the genes related to carnosine synthesis and hydrolysis [*Carns1*, *Cndp1,* and *Cndp2* (**Figure 2.9**)], the beta-alanine transporter *Pat1* (**Figure 2.10**), non-specific peptide transporters *Pept1, Pept2,* and *Pht2* (**Figure 2.11**), or beta-alanine transaminase *Gaba-T* (**Figure 2.9**).

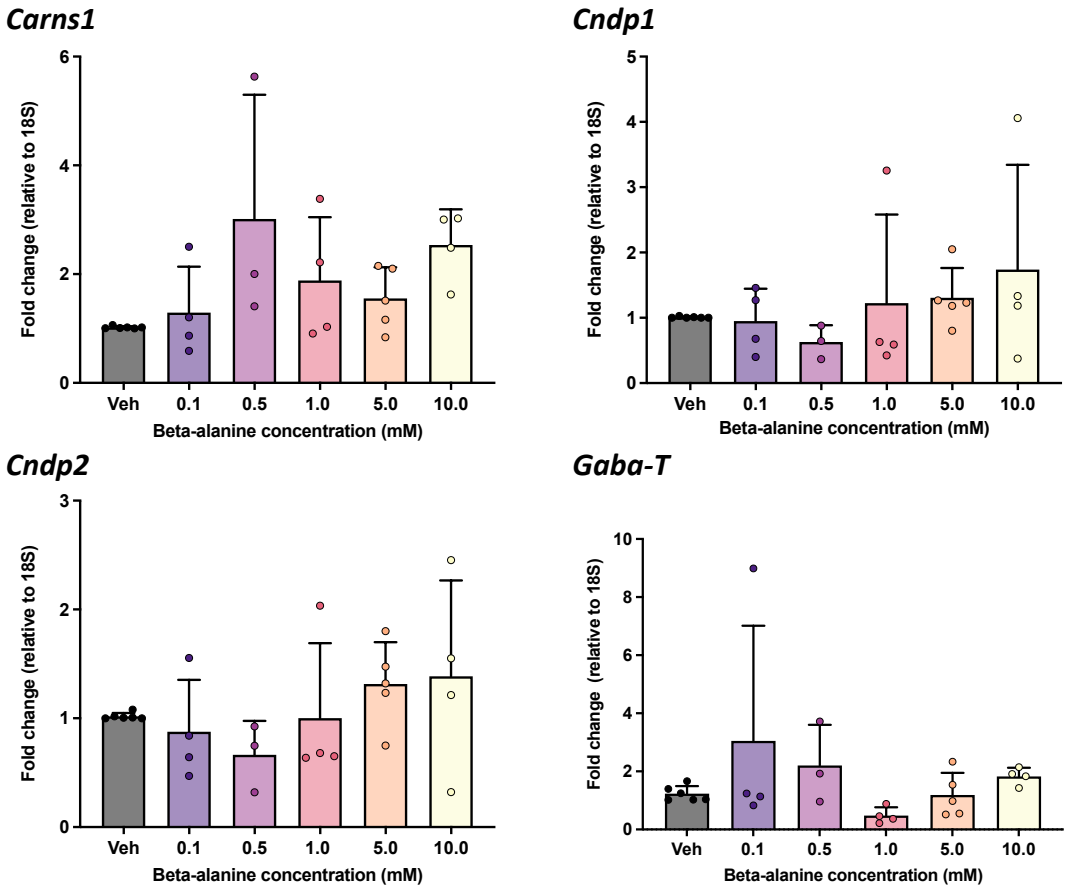


Figure 2.9. Gene expression of enzymes involved in carnosine synthesis and hydrolysis, and beta-alanine transamination following four hours of beta-alanine treatment, as assessed by RT-qPCR. Data are presented as mean with individual values ± SD. Statistical comparisons used Dunnett's post-test to compare treatment means to vehicle control mean. Abbreviations: Veh, vehicle control.

The administration of exogenous beta-alanine for four hours significantly decreased the expression of *TauT* at 0.5 mM [-62 % (p = 0.015)], 1.0 mM [-54 % (p = 0.021)], and 10.0mM [-59 % (p=0.028)], with no significance at 0.1 mM [-50 % (p = 0.102)] and 5.0 mM [-12 % (p=0.597)], when compared to the vehicle control (**Figure 2.10**).

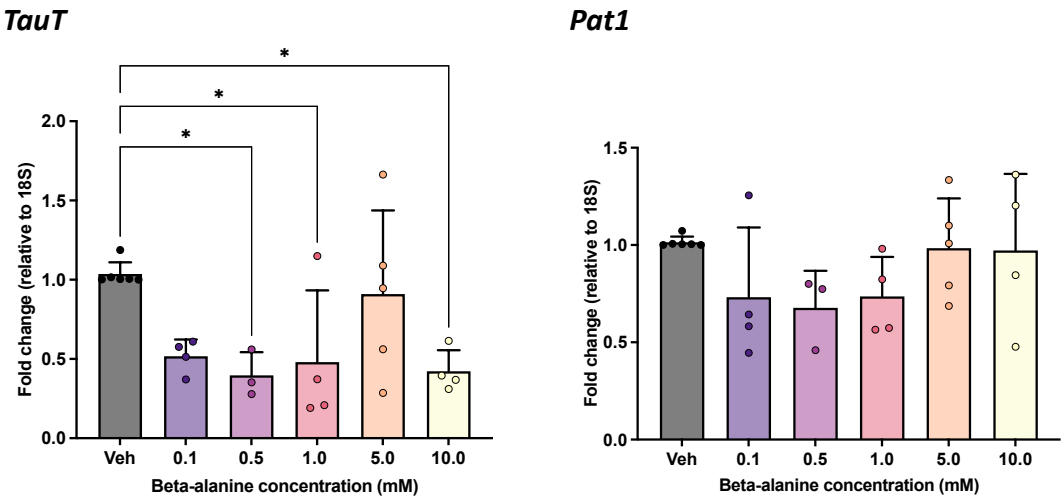


Figure 2.10. Gene expression of amino acid transporters following four hours of beta-alanine treatment, as assessed by RT-qPCR. Data are presented as mean with individual values ± SD. Statistical comparisons used Dunnett's post-test to compare treatment means to vehicle control mean. * p<0.05. Abbreviations: Veh, vehicle control.

Pht1 expression was significantly decreased at all beta-alanine concentrations [-35 to -66 % (p<0.05)], except at 5.0 mM [-48 % (p=0.066)] (**Figure 2.11**), when compared to the vehicle control.

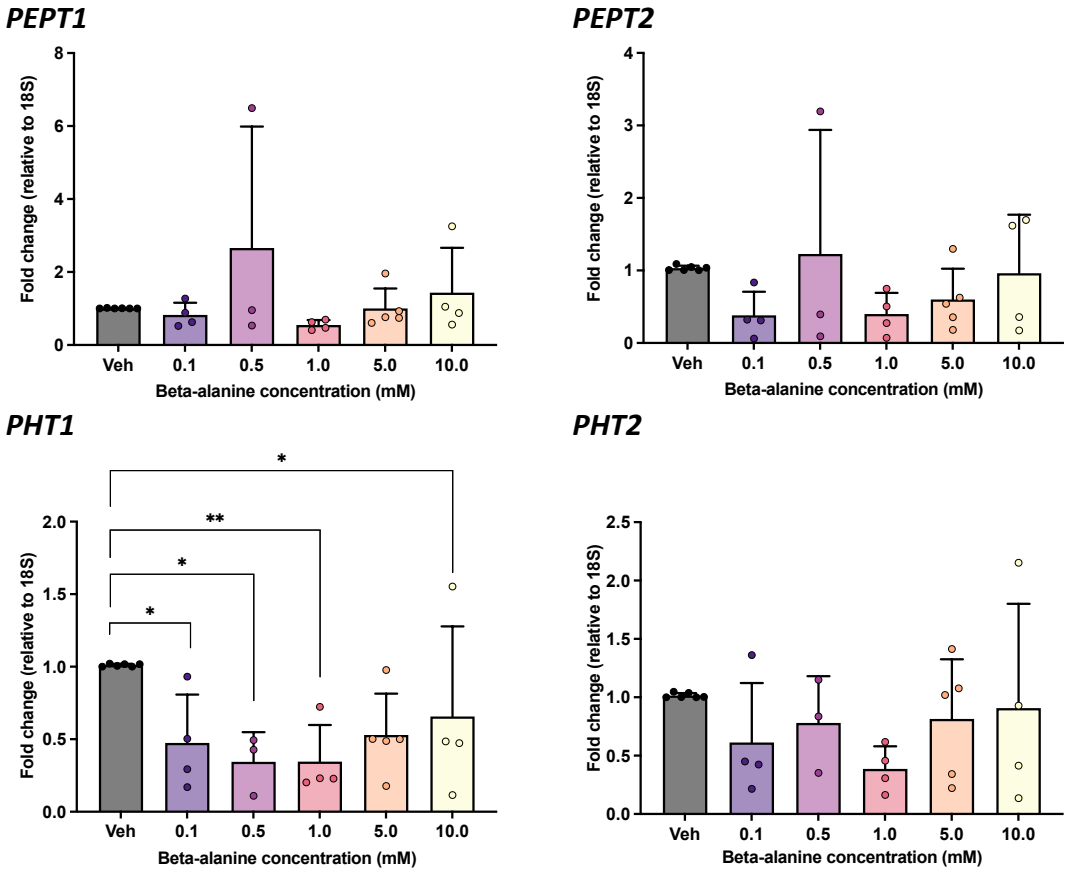


Figure 2.11. Gene expression of non-specific peptide transporters following four hours of beta-alanine treatment, as assessed by RT-qPCR. Data are presented as mean with individual values ± SD. Statistical comparisons used Dunnett's post-test to compare treatment means to vehicle control mean. * $p<0.05$, ** $p<0.01$. Abbreviations: Veh, vehicle control.

2.4.4 Carnosine supplementation on intracellular (cell lysates) histidine-containing dipeptide and associated amino acid concentrations

Given the lack of gene expression response to exogenous carnosine, the transport of carnosine into the cell through the quantification of carnosine and associated amino acid concentrations, both intracellularly and extracellularly, was investigated using the Biochrom 30 amino acid analyser.

2.4.4.1 Intracellular histidine-containing dipeptide concentrations

Intracellular carnosine was not detected in vehicle control samples or cells exposed to 0.1 mM of exogenous carnosine for 4, 24, and 72 hours. There was an increase in intracellular carnosine when H9c2 cells were exposed to 0.5, 1.0, 5.0, and 10.0 mM of carnosine for 4, 24, and 72 hours, with greater increases observed at higher concentrations of exogenous carnosine (**Figure 2.12**). Intracellular concentrations of anserine could not be detected in any samples across all exogenous carnosine concentrations and time points.

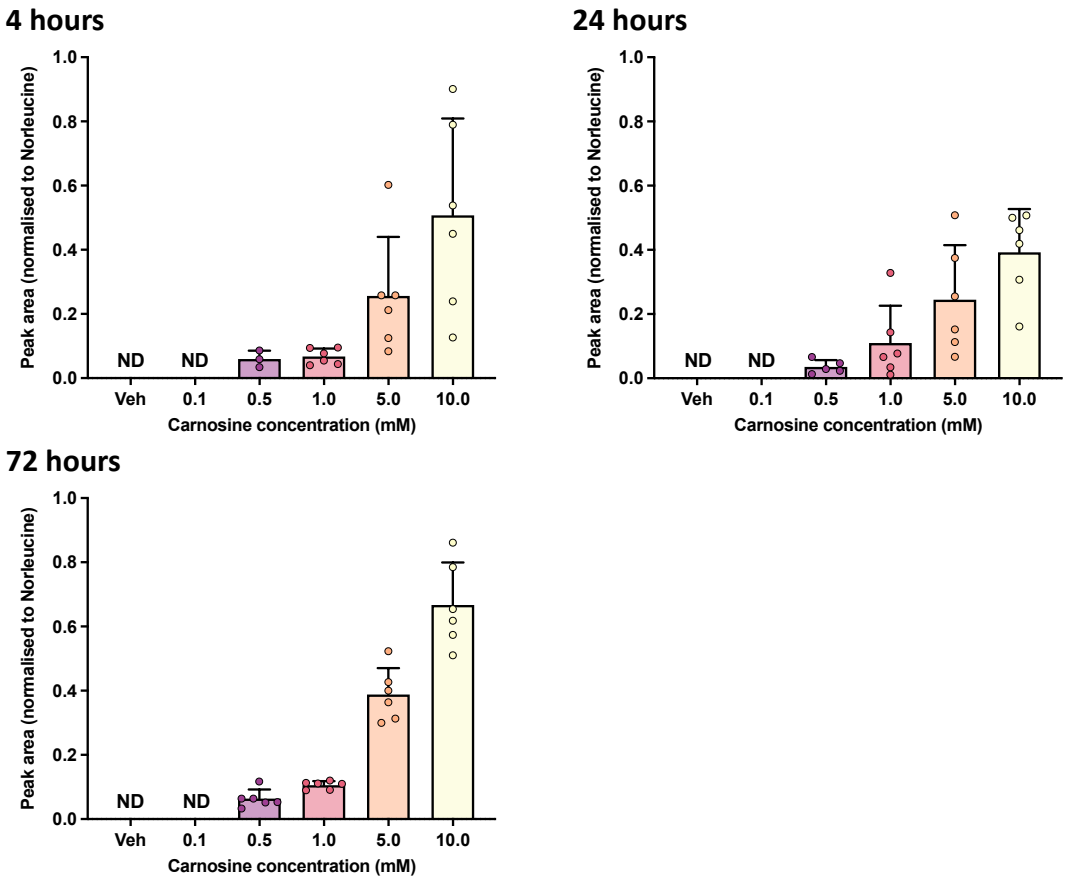


Figure 2.12. Intracellular carnosine concentration in H9c2 cells following exposure to exogenous carnosine for 4, 24, and 72 hours. Data are presented as mean with individual values ± SD. Abbreviations: ND, not detected; Veh, vehicle control.

2.4.4.2 Intracellular beta-alanine concentration

Following 4 and 24 hours of exogenous carnosine, intracellular concentrations of beta-alanine remained unchanged, with all concentrations not being significantly different from the vehicle control [p>0.05 (**Figure 2.13**)]. Following 72 hours of exogenous carnosine, the intracellular concentration of beta-alanine was significantly higher in cells exposed to 10.0 mM of exogenous carnosine compared to the vehicle control (p=0.001). The intracellular concentrations of beta-alanine for all other exogenous carnosine concentrations at the 72-hour time point were not significantly different from the vehicle control [p>0.05 (**Figure 2.13**)].

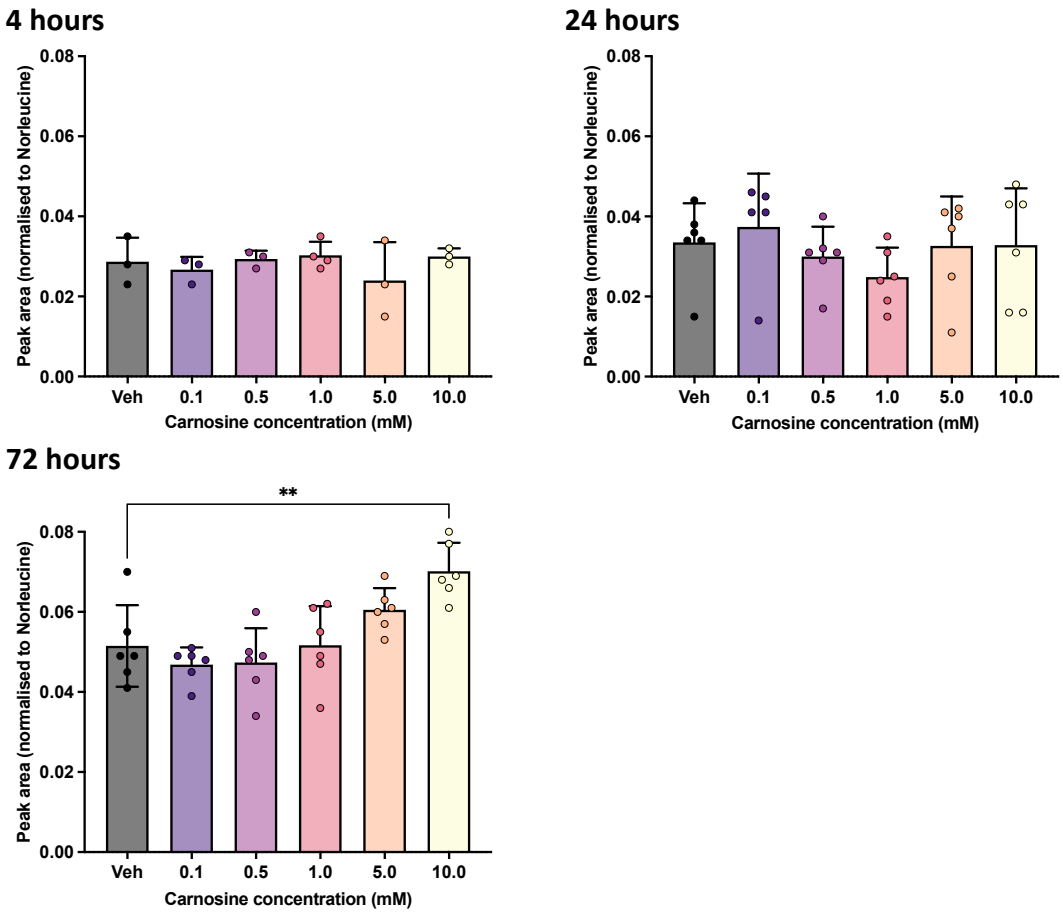


Figure 2.13. Intracellular beta-alanine concentration in H9c2 cells following exposure to exogenous carnosine for 4, 24, and 72 hours. Data are presented as mean with individual values ± SD. Statistical comparisons used Dunnett's post-test to compare treatment means to vehicle control mean. ** p<0.01. Abbreviations: Veh, vehicle control.

2.4.4.3 Intracellular L-histidine concentration

The intracellular concentration of L-histidine remained unchanged following exposure to increasing concentrations of exogenous carnosine for 4, 24, and 72 hours, with all concentrations not being significantly different from the vehicle control [p>0.05 (**Figure 2.14**)].

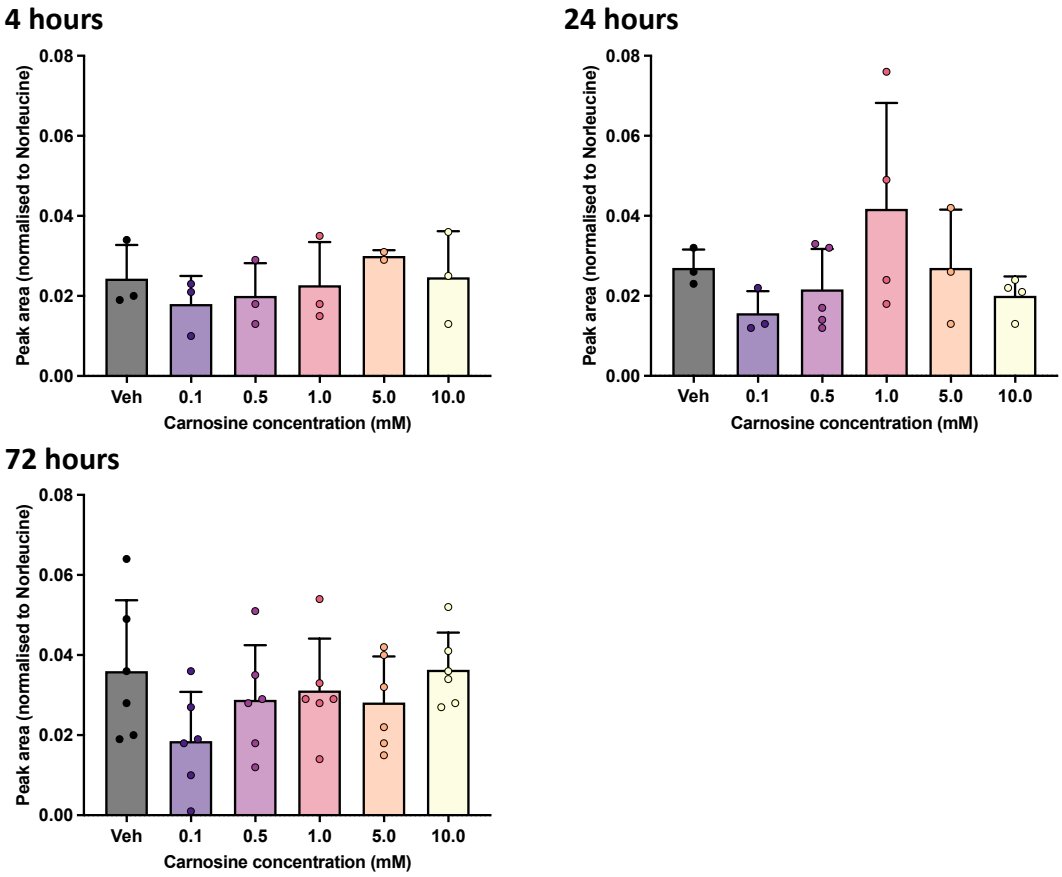


Figure 2.14. Intracellular L-histidine concentration in H9c2 cells following exposure to exogenous carnosine for 4, 24, and 72 hours. Statistical comparisons used Dunnett's post-test to compare treatment means to vehicle control mean. Data are presented as mean with individual values ± SD. Abbreviations: Veh, vehicle control.

2.4.4.4 Intracellular taurine concentration

The intracellular concentration of taurine remained unchanged following exposure to increasing concentrations of exogenous carnosine for 4, 24, and 72 hours, with all

concentrations not being significantly different from the vehicle control [p>0.05 (**Figure 2.15**)].

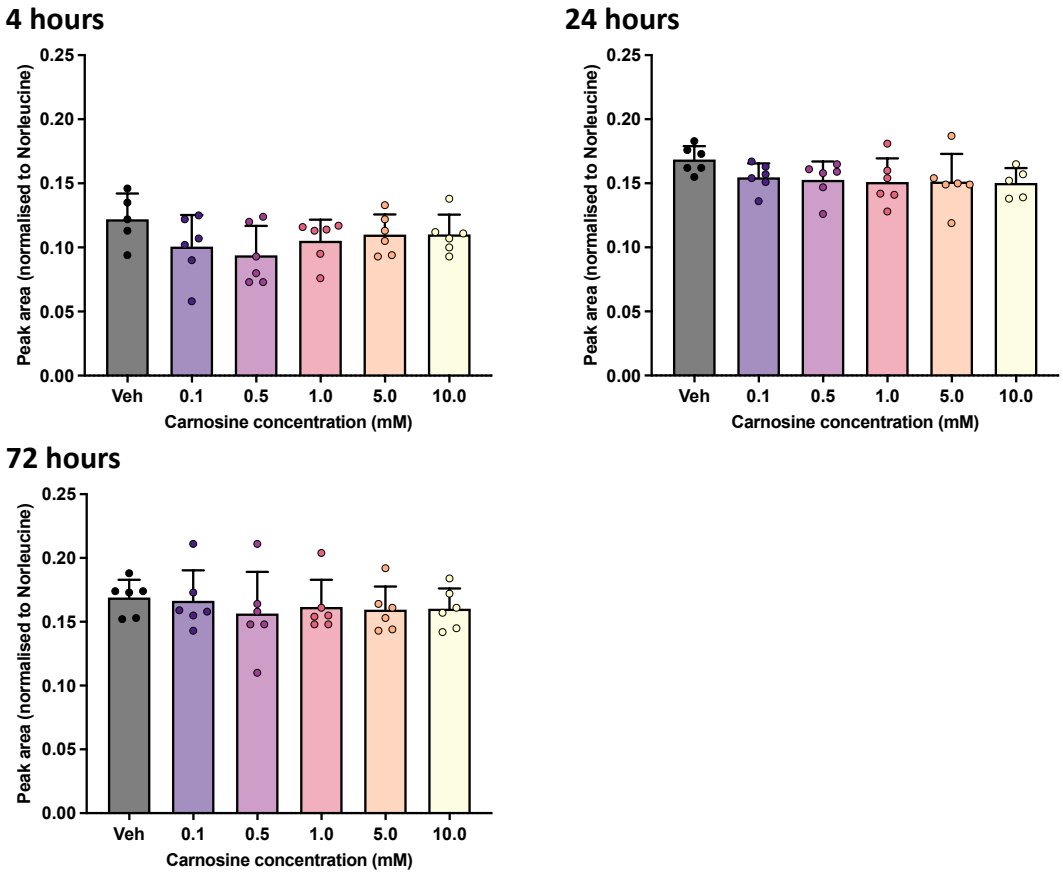


Figure 2.15. Intracellular taurine concentration in H9c2 cells following exposure to exogenous carnosine for 4, 24, and 72 hours. Statistical comparisons used Dunnett's post-test to compare treatment means to vehicle control mean. Data are presented as mean with individual values ± SD. Abbreviations: Veh, vehicle control.

2.4.5 Carnosine supplementation on extracellular (culture media) histidine-containing dipeptide and associated amino acid concentrations

2.4.5.1 Extracellular histidine-containing dipeptide concentrations

Extracellular concentrations of carnosine could not be detected in vehicle control samples. The levels of extracellular carnosine increased with the increase in exogenous carnosine concentration for all time points, confirming that the samples were exposed to the increasing concentrations in the media. The extracellular carnosine levels for the

5.0- and 10.0-mM exogenous carnosine concentrations were unquantifiable due to surpassing the upper limit of detection of the Biochrom 30 [>1000 mV (**Figure 2.16**)]. The extracellular concentrations of anserine could not be detected in any extracellular samples across all exogenous carnosine concentrations and time points.

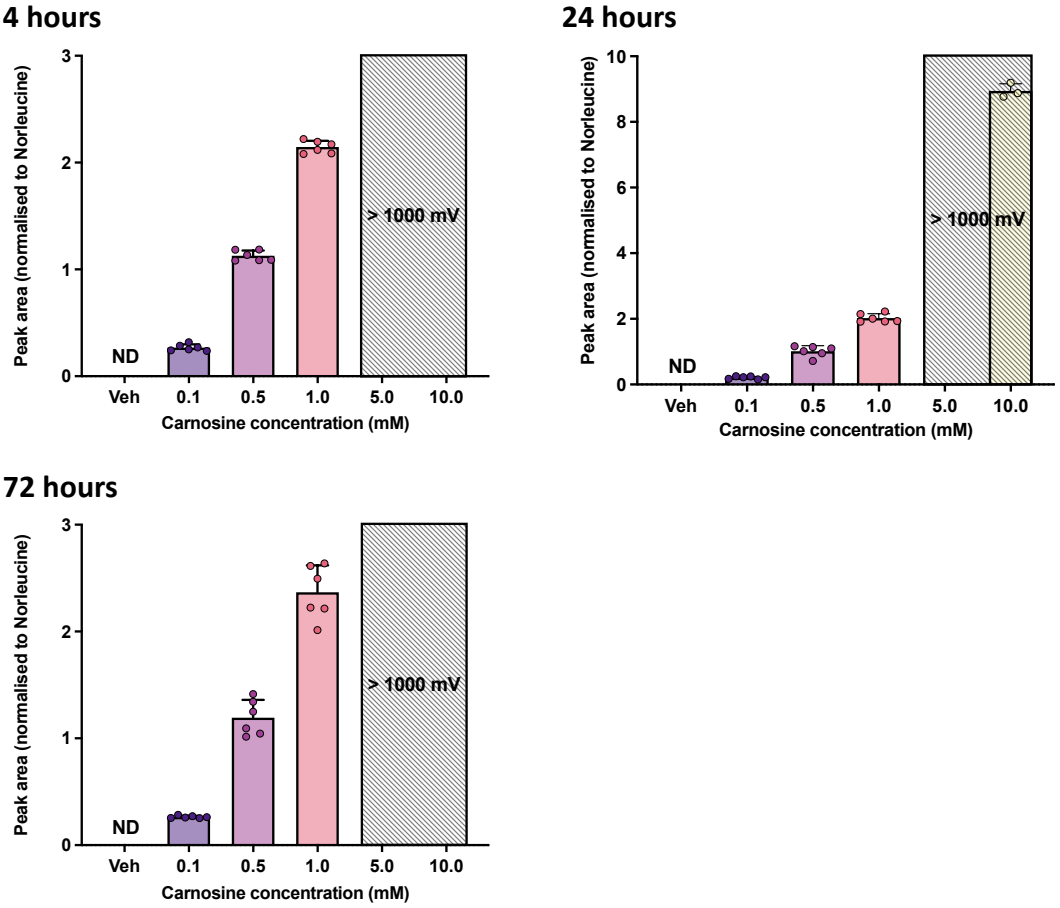


Figure 2.16. Extracellular carnosine concentration in H9c2 cells following exposure to exogenous carnosine for 4, 24, and 72 hours. Data are presented as mean with individual values ± SD. Abbreviations: Veh, vehicle control.

2.4.5.2 Extracellular beta-alanine concentration

Following 4, 24, and 72 hours of exogenous carnosine, the extracellular concentrations of beta-alanine were not detected in the vehicle control samples or in samples exposed to 0.1 mM exogenous carnosine. There was an increase in extracellular concentrations

of beta-alanine when cells were exposed to 0.5, 1.0, 5.0, and 10.0 mM of exogenous carnosine for all time points (**Figure 2.17**).

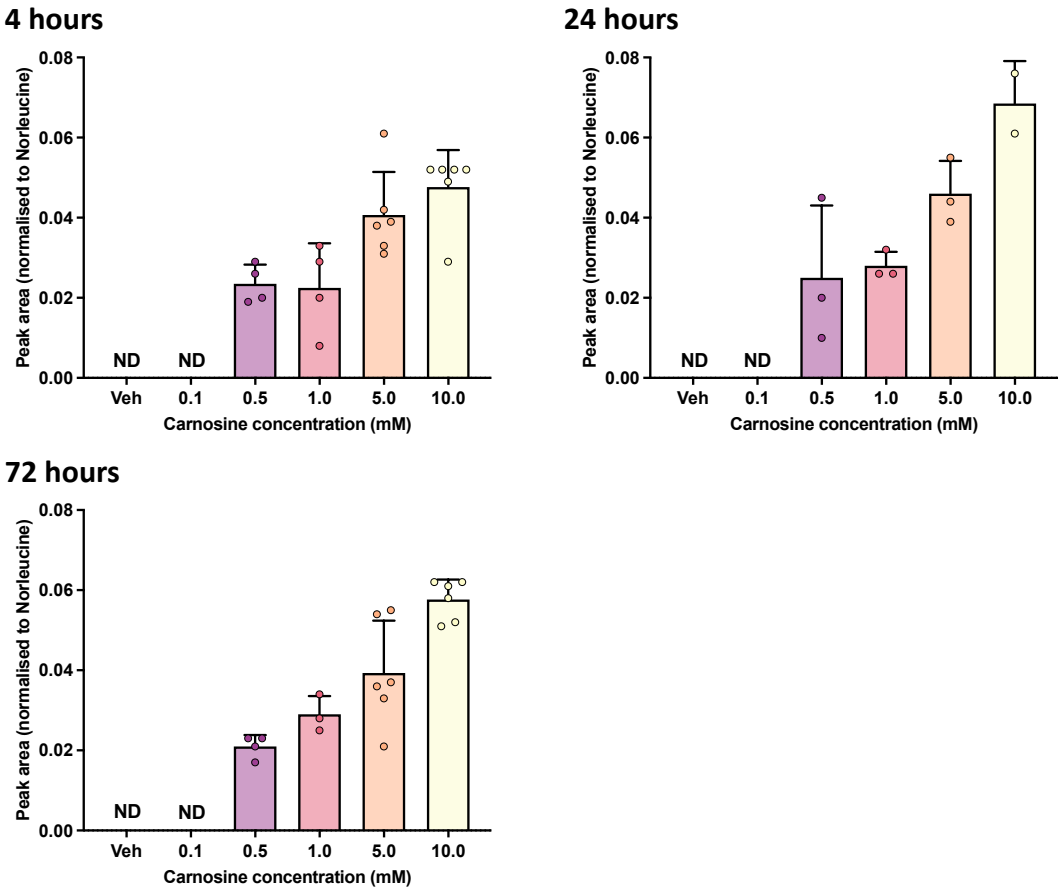


Figure 2.17. Extracellular beta-alanine concentration in H9c2 cells following exposure to exogenous carnosine for 4, 24, and 72 hours. Data are presented as mean with individual values ± SD. Abbreviations: ND, not detected; Veh, vehicle control.

2.4.5.3 Extracellular L-histidine concentration

The extracellular concentration of L-histidine remained unchanged compared to the vehicle control when the H9c2 cells were exposed to 0.1 to 5.0 mM of exogenous carnosine for 4, 24, and 72 hours [p>0.05 (**Figure 2.18**)]. The extracellular concentration of L-histidine was increased following exposure to 10.0 mM of exogenous carnosine for the 4-hour (p=0.020) and 24-hour (p=0.040) time points when compared to the vehicle control; this was not observed in the 72-hour time point [p=0.181 (**Figure 2.18**)].

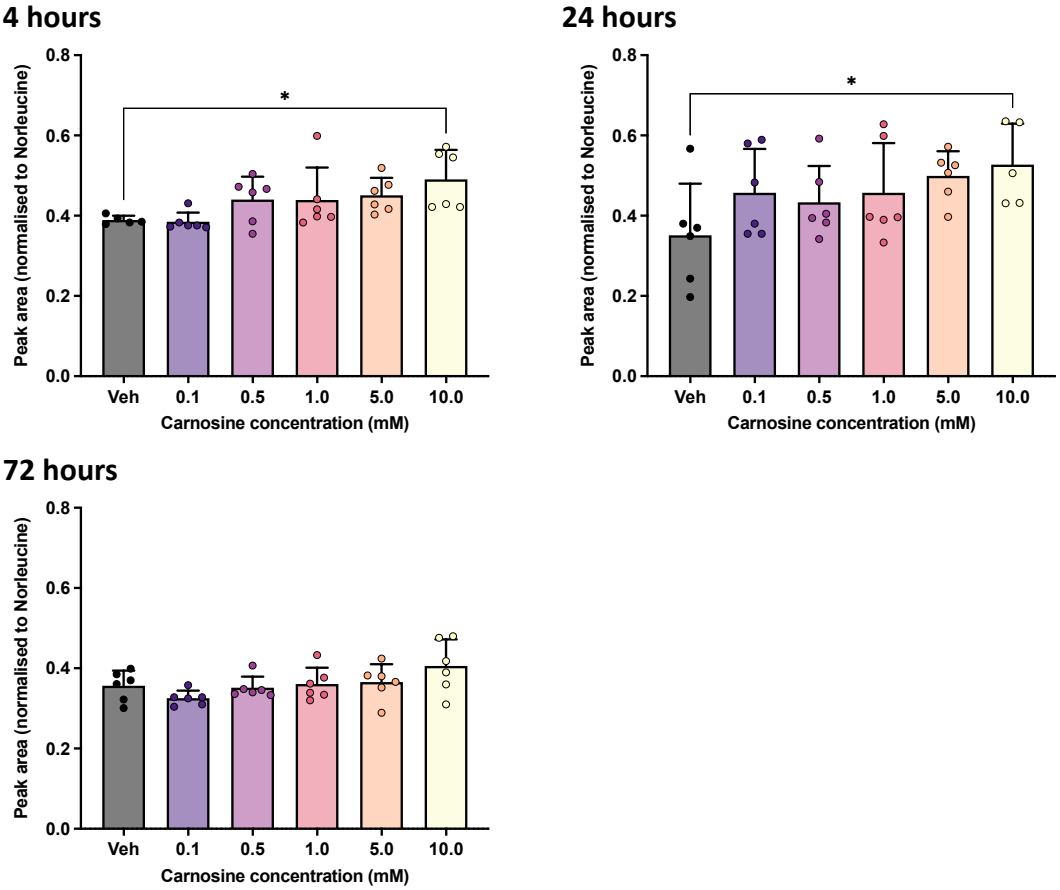


Figure 2.18. Extracellular L-histidine concentration in H9c2 cells following exposure to exogenous carnosine for 4, 24, and 72 hours. Data are presented as mean with individual values ± SD. Statistical comparisons used Dunnett's post-test to compare treatment means to vehicle control mean. * $p<0.05$. Abbreviations: Veh, vehicle control.

2.4.5.4 Extracellular taurine concentration

The extracellular concentration of taurine remained unchanged following exposure to increasing concentrations of exogenous carnosine for 4 and 24 hours, with all concentrations not being significantly different from the vehicle control [$p>0.05$ (**Figure 2.19**)]. The extracellular concentration of taurine could not be analysed for the 72-hour time point due to noise issues when attempting to quantify the peak area on the Biochrom 30.

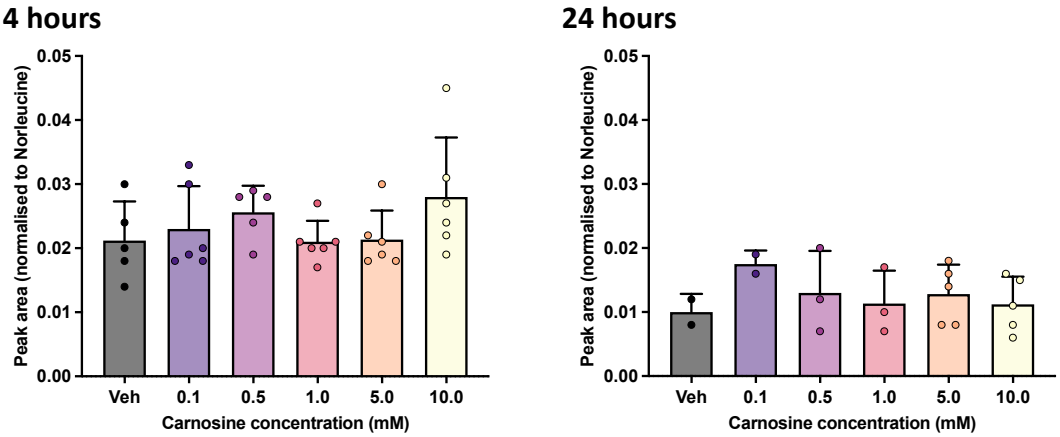


Figure 2.19. Extracellular taurine concentration in H9c2 cells following exposure to exogenous carnosine for 4 and 24 hours. Taurine could not be detected in the 72-hour samples. Data are presented as mean with individual values ± SD. Statistical comparisons used Dunnett's post-test to compare treatment means to vehicle control mean. Abbreviations: Veh, vehicle control.

2.4.6 Beta-alanine supplementation on intracellular (cell lysates) histidine-containing dipeptide and amino acid concentrations

The gene expression data showed an influence of exogenous beta-alanine on some of the transporters involved in carnosine and beta-alanine metabolism. Therefore, the transport and synthesis of exogenous beta-alanine were then investigated using the Biochrom 30 amino acid analyser.

2.4.6.1 Intracellular beta-alanine concentration

Following 4 and 24 hours of exogenous beta-alanine, the intracellular concentration of beta-alanine increased with all concentrations, except for 0.1 mM, when compared to the vehicle control [$p<0.05$ (**Figure 2.20**)]. Following 72 hours of exogenous beta-alanine, all exogenous concentrations of beta-alanine were significantly different from the vehicle control, including 0.1 mM of beta-alanine [$p<0.05$ (**Figure 2.20**)].

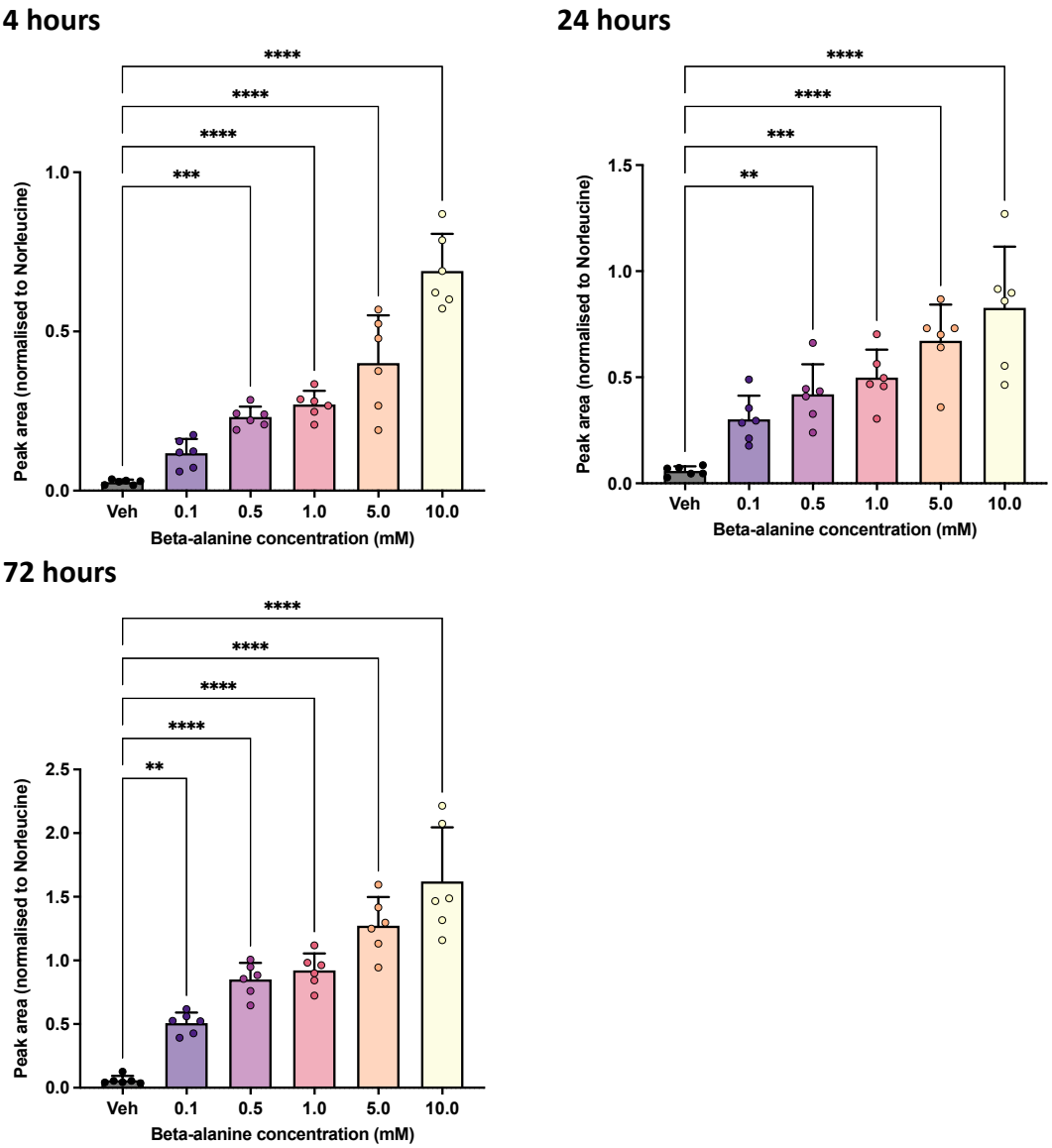


Figure 2.20. Intracellular beta-alanine concentration in H9c2 cells following exposure exogenous beta-alanine for 4, 24, and 72 hours. Data are presented as mean with individual values ± SD. Statistical comparisons used Dunnett's post-test to compare treatment means to vehicle control mean. ** p<0.01, *** p<0.001, **** p<0.0001. Abbreviations: Veh, vehicle control.

2.4.6.2 Intracellular L-histidine concentration

The intracellular concentration of L-histidine remained unchanged following exposure to increasing concentrations of exogenous beta-alanine for 4, 24, and 72 hours, with all concentrations not being significantly different from the vehicle control [p>0.05 (**Figure 2.21**)].

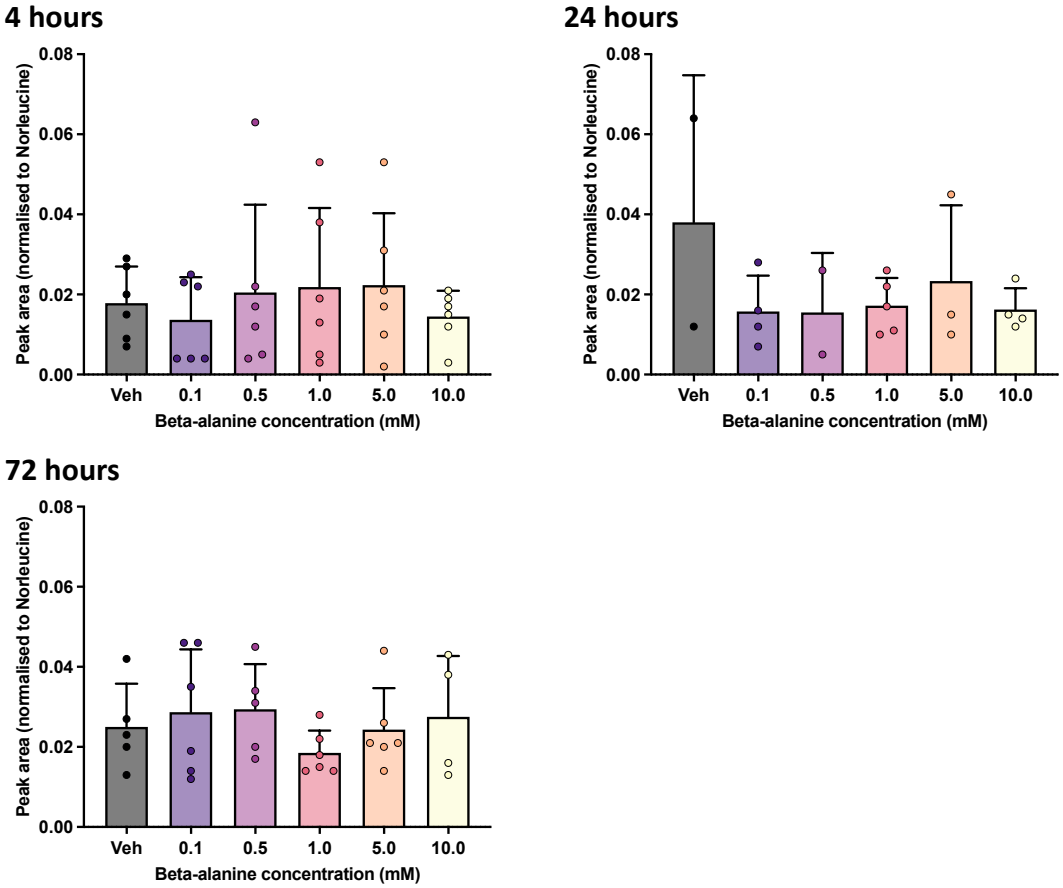


Figure 2.21. Intracellular L-histidine concentration in H9c2 cells following exposure to exogenous beta-alanine for 4, 24, and 72 hours. Data are presented as mean with individual values ± SD. Statistical comparisons used Dunnett's post-test to compare treatment means to vehicle control mean. Abbreviations: Veh, vehicle control.

2.4.6.3 Intracellular taurine concentration

The intracellular concentration of taurine decreased in H9c2 cells when exposed to increasing concentrations of exogenous beta-alanine for 4, 24, and 72 hours (**Figure 2.22**). Following 4 and 72 hours of exogenous beta-alanine, all concentrations, except for 0.1 mM, were significantly different from the vehicle control ($p>0.05$). Following 24 hours of exogenous beta-alanine, all concentrations were significantly different from the vehicle control, with intracellular taurine not being detected when H9c2 cells were exposed to 10.0 mM of exogenous beta-alanine.

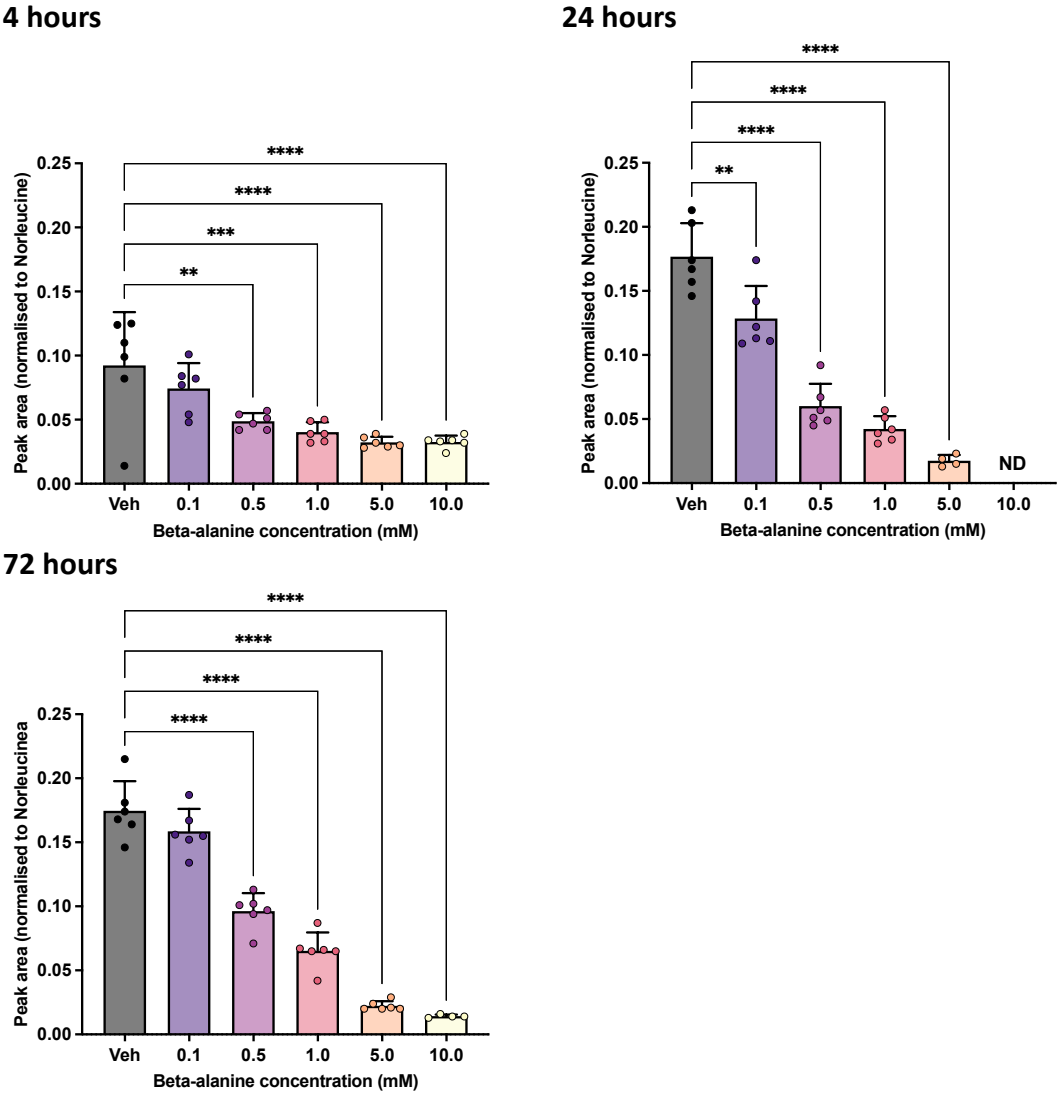


Figure 2.22. Intracellular taurine concentration in H9c2 cells following exposure to exogenous beta-alanine for 4, 24, and 72 hours. Data are presented as mean with individual values ± SD. Statistical comparisons used Dunnett's post-test to compare treatment means to vehicle control mean. ** p<0.01, *** p<0.001, **** p<0.0001. Abbreviations: ND, not detected; Veh, vehicle control.

2.4.6.4 Intracellular histidine-containing dipeptide concentrations

Intracellular concentrations of carnosine and anserine (HCDs) were undetectable across all exogenous beta-alanine concentrations and time points (4, 24, and 72 hours).

2.4.7 Beta-alanine supplementation on extracellular (culture media) histidine-containing dipeptide and amino acid concentrations

2.4.7.1 Extracellular beta-alanine concentration

Extracellular concentrations of beta-alanine could not be detected in vehicle control samples. The levels of extracellular beta-alanine increased with the increase in exogenous beta-alanine concentration for all time points, confirming that the samples were exposed to the increasing concentrations in the media (**Figure 2.23**). The extracellular concentrations could not be quantified for 5.0 and 10.0 mM of exogenous beta-alanine due to the peaks surpassing the upper limit of detection (> 1000 mV).

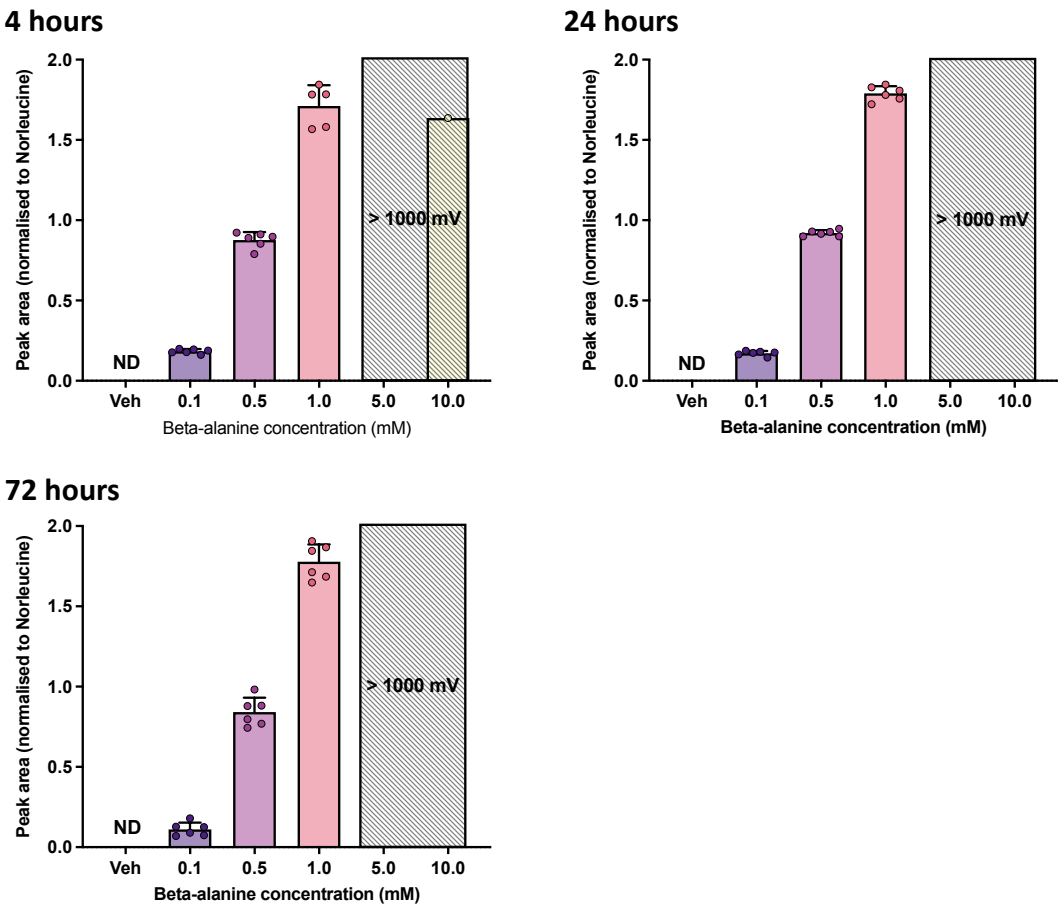


Figure 2.23. Extracellular beta-alanine concentration in H9c2 cells following exposure to exogenous beta-alanine for 4, 24, and 72 hours. Data are presented as mean with individual values ± SD. Abbreviations: ND, not detected; Veh, vehicle control.

2.4.7.2 Extracellular L-histidine concentration

The extracellular concentration of L-histidine remained unchanged compared to vehicle control when the H9c2 cells were exposed to all exogenous beta-alanine concentrations for 4, 24, and 72 hours [p>0.05 (**Figure 2.24**)].

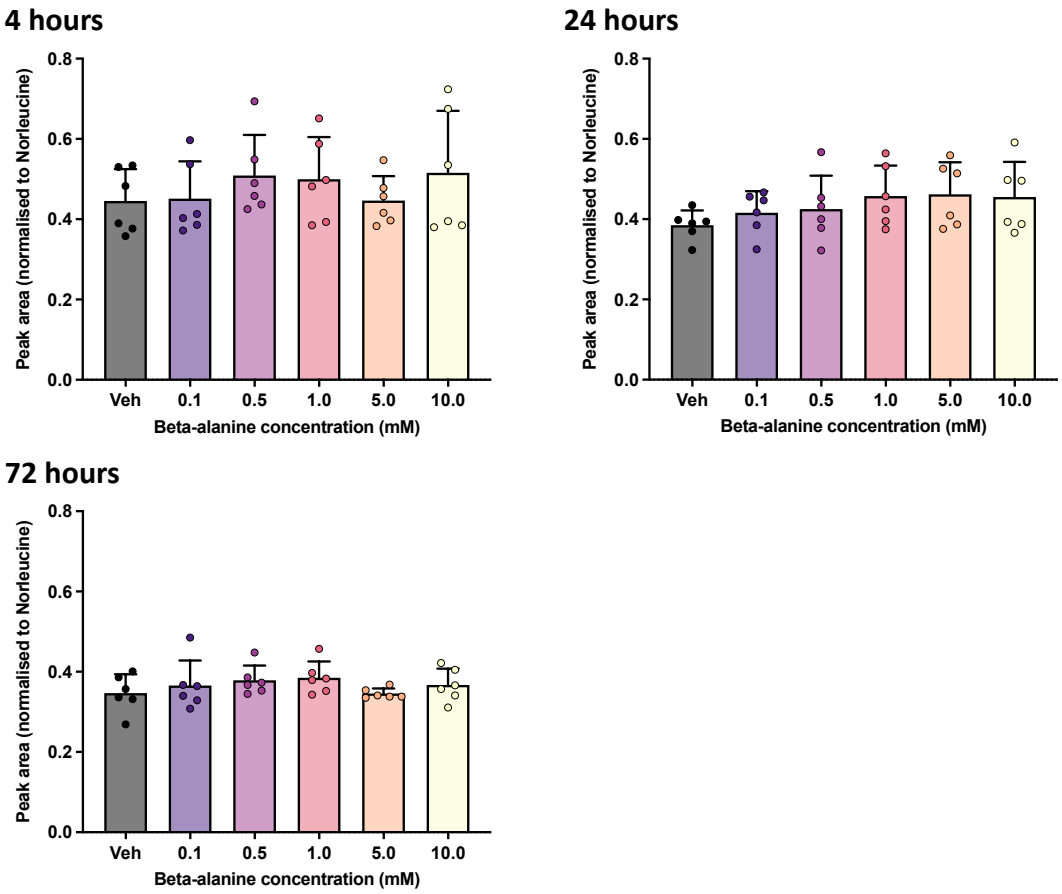


Figure 2.24. Extracellular L-histidine concentration in H9c2 cells following exposure to exogenous beta-alanine for 4, 24, and 72 hours. Data are presented as mean with individual values ± SD. Statistical comparisons used Dunnett's post-test to compare treatment means to vehicle control mean. Abbreviations: Veh, vehicle control.

2.4.7.3 Extracellular taurine concentration

Following 4 and 24 hours of exposure to exogenous beta-alanine, there was an increase in the extracellular taurine concentrations, with all exogenous beta-alanine concentrations, except for 0.1 mM, being significantly different from the vehicle control at the 4-hour time point (**Figure 2.25**). Extracellular taurine could not be detected in the

72-hour vehicle control sample; however following exposure to exogenous beta-alanine, the extracellular taurine concentration increased.

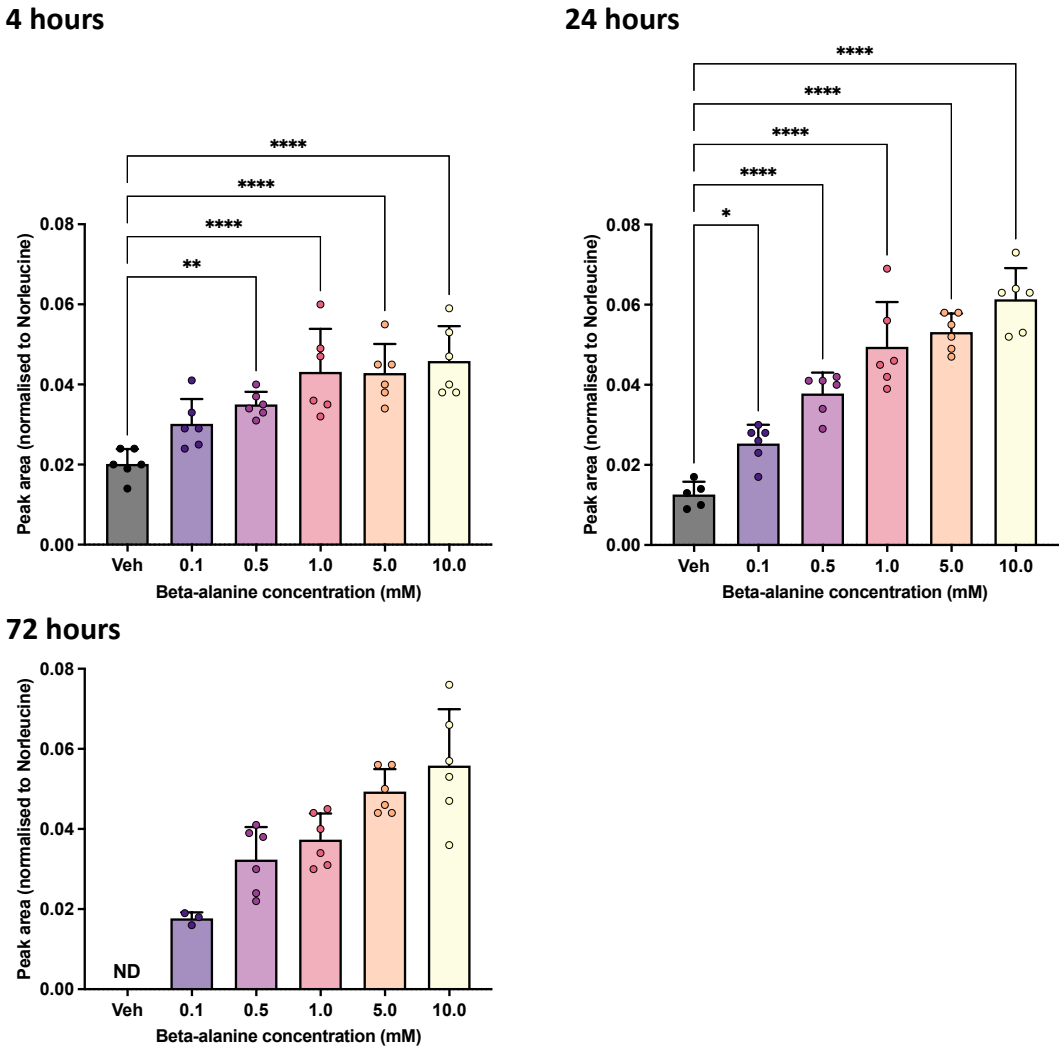


Figure 2.25. Extracellular taurine concentration in H9c2 cells following exposure to exogenous beta-alanine for 4, 24, and 72 hours. Data are presented as mean with individual values ± SD. Statistical comparisons used Dunnett's post-test to compare treatment means to vehicle control mean. * $p<0.05$, ** $p<0.01$, **** $p<0.0001$. Abbreviations: Veh, vehicle control.

2.4.7.4 Extracellular histidine-containing dipeptide concentrations

The extracellular concentrations of carnosine and anserine could not be detected in any culture media samples across all exogenous beta-alanine concentrations and time points.

2.5 Discussion

This experiment presents the expression of genes related to carnosine and beta-alanine metabolism in H9c2 cardiomyocyte cells and explores the uptake and storage of exogenous sources of carnosine and beta-alanine in these cells. The H9c2 cardiomyocytes expressed the genes required for carnosine and beta-alanine metabolism, suggesting that the enzymes and transporters are present for the synthesis and transport of these metabolites into the cell. The amino acid analysis showed the uptake and accumulation of exogenously sourced carnosine and beta-alanine in cardiomyocyte cells; however, the increase in intracellular beta-alanine in the cardiomyocytes did not lead to the synthesis of HCDs (i.e., carnosine and anserine).

2.5.1 Carnosine and beta-alanine metabolism-related genes in response to carnosine and beta-alanine

Rat cardiomyocytes express mRNA for most of the enzymes and transporters associated with carnosine and beta-alanine metabolism, which suggests that cardiomyocytes, at least rat ventricular cardiomyocytes, have the potential to generate the enzymes and transporters required for carnosine and beta-alanine uptake and synthesis. Firstly, the study showed the presence of the carnosine synthesis enzyme and the transporters that are required for the accumulation of carnosine and beta-alanine. This was further confirmed by the intracellular amino acid and HCD analyses, which demonstrated the cardiomyocytes' capacity to take up exogenous beta-alanine and carnosine. It was shown that *Carns1* was expressed by H9c2 cells, suggesting that the cells have the capacity to synthesise carnosine when exposed to exogenous beta-alanine. Despite this,

intracellular carnosine concentrations did not increase with the uptake and accumulation of exogenous beta-alanine.

Given that it is currently unknown to what extent cardiomyocytes would be exposed to carnosine and beta-alanine, H9c2 cells were exposed to increasing concentrations of carnosine and beta-alanine in a dose-dependent manner. No impact on cell viability or metabolic activity was observed at any of the carnosine or beta-alanine concentrations. Cells were regularly observed throughout the experiment; these observations of media colour (stable pH), cell adherence, and cell morphology indicated that the H9c2 cells did not exhibit signs of metabolic stress, detachment, or apoptosis during the experimental treatment. These concentrations were chosen because, as detailed in Creighton et al. (2022), the physiological concentrations of HCDs, including carnosine, in the heart are suggested to range between 0.1 and 10 mM. This is a plausible assumption, as other concentrations of cardiac amino acids fall within this range. Ideally, treatment concentrations would have been based on the Km of the enzyme or transporter for the relevant substrate. It is generally assumed that saturation is reached at 5 x Km, beyond which no further effect will be observed. Enzyme kinetic data have primarily been studied within the olfactory bulbs and skeletal muscle, so it is unclear what values are representative of cardiac tissue. The major issue with these values is the large variation and lack of consensus between studies, which complicates the decision-making process regarding which concentrations to use in experiments. Several studies have reported the enzyme affinity of beta-alanine for *Carns1*, including values of 1.0–1.5 mM in rat cerebellum, spinal cord, and olfactory bulbs (Ng and Marshall, 1978), 2.3 mM in rat brain (Skaper et al., 1973), 0.46 mM in homogenous recombinant mouse (Drozak et al., 2010),

0.09 mM in homogenous recombinant human (Drozak et al., 2010), and between 5 and 10 mM in mouse olfactory bulbs (Horinishi et al., 1978). Skaper et al. (1973) reported that optimal synthesis of carnosine was observed with concentrations of $\geq$ 6.0 mM of beta-alanine in rat brains.

The metabolites of interest were measured in the growth and differentiation media to determine what the cardiomyocytes were exposed to, independently of the exogenous treatment. Beta-alanine and carnosine were absent from both the growth and differentiation media, meaning that the cells were only exposed to these metabolites through the exogenous treatment. Since the demand for beta-alanine in a whole-body organism exceeds the endogenous supply from the liver, the heart is likely exposed to very minimal endogenous concentrations and relies on exogenous sources, as replicated in this study. The exposure to L-histidine and taurine through the growth and differentiation media can be considered physiologically relevant. These amino acids are present in human plasma and, therefore, the heart would naturally be exposed to them via circulation. Similarly, in this model, the cardiomyocytes are exposed to these free amino acids through the media, maintaining physiological relevance.

The reduced mRNA expression of the transporter *TauT* suggests potential competition between both beta-alanine and taurine for *TauT* and indicates that this solute carrier protein could play a role in the accumulation of beta-alanine in cardiomyocytes. This finding aligns with Saunders et al. (2017a), who showed downregulation of *TAUT* in skeletal muscle following 24 weeks of beta-alanine supplementation in physically active, healthy males. The downregulation of *TAUT* during exposure to exogenous beta-alanine

may limit beta-alanine uptake into the cell, thereby maintaining intracellular carnosine homeostasis (Saunders et al., 2017a). Everaert et al. (2013), however, showed an increase in *TauT* expression following 8 weeks of beta-alanine supplementation in mouse skeletal muscle. This discrepancy may be due to the mice being exposed to a supraphysiological dose when compared to humans. Regardless, the data presented in this experiment, alongside these two studies, suggest that the metabolism of beta-alanine supplementation may be more dependent on the transport of beta-alanine across the tissue and cell membrane, rather than the activity of *Carns1*. To confirm that exogenous beta-alanine predominantly uses *TauT* for uptake into cardiomyocytes, future research should aim to inhibit the transporter to identify the effect this has on subsequent beta-alanine uptake. Hypotaurine, a competitive *TauT* inhibitor, reduced beta-alanine uptake in skeletal muscle C2C12 cells, confirming that beta-alanine uptake transport in skeletal muscle is at least, in part, mediated by *TauT* (Santos et al., 2021). Additionally, it was demonstrated that *Carns1* was expressed in the H9c2 cells, suggesting that the cells have the ability to synthesise carnosine when exposed to exogenous beta-alanine. *Carns1* expression did not change with beta-alanine supplementation, again suggesting that the regulation of intracellular carnosine may not be dependent upon carnosine synthesis but instead may shift toward dependence on the transport of beta-alanine into the cells. ATP is a necessary co-factor for *Carns1*; literature has stated that H9c2 cells contain high cellular ATP levels (Kuznetsov et al., 2015) and are similar to primary cardiomyocytes.

2.5.2 Histidine-containing dipeptide and amino acid concentrations in response to carnosine and beta-alanine

Changes in gene expression do not necessarily translate into changes in protein expression and do not always represent the activity levels of the enzymes and transporters. Additionally, protein expression does not always equate to functional activity; a protein may be expressed (determined by techniques such as Western Blotting), but this does not necessarily mean that the protein is active, and, additionally, low levels of protein can still exhibit activity. Therefore, following the gene expression analysis, this study used amino acid analysis to determine the influence of the exogenous provision of carnosine and beta-alanine on intracellular concentrations of amino acids and HCD. This analysis helped determine whether the cells were directly taking up the supplements.

H9c2 cells expressed the transporters responsible for amino acid transport across the cell membrane, and exogenous beta-alanine was potentially able to utilise these to increase intracellular beta-alanine content. It was hypothesised that exogenous beta-alanine would synthesise with L-histidine to increase intracellular carnosine content. The results, however, showed no increase in intracellular carnosine concentration when cells were exposed to exogenous beta-alanine at all time points, indicating no synthesis occurred over the 72-hour timeframe. Both the cells and culture media contained L-histidine, suggesting that L-histidine was unlikely to be a rate-limiting factor for carnosine synthesis in this experiment. It has been suggested that when ample beta-alanine is provided and the transporter, *TauT*, becomes saturated, the activity of *Carns1* itself may

be the rate-limiting factor in carnosine synthesis (Bakardjiev and Bauer, 1994; Gonçalves et al., 2020; Harris et al., 2006). The enzyme affinity of beta-alanine for *TauT* is suggested to be 40 µM, albeit this value was measured in chick skeletal muscle (Bakardjiev and Bauer, 1994). *Carns1* has been described as a "sluggish" enzyme (Drozak et al., 2010) with a Kcat value (substrate molecules converted into product per second) of less than 1 $second^{-1}$, and its enzymatic activity has been suggested to be 10 times lower in myocardial tissue compared to skeletal muscle (Bulygina and Kramarenko, 1995). Therefore, the development of potential strategies to increase this enzymatic activity may be necessary (Gonçalves et al., 2020).

Beta-alanine supplementation is commonly used as a model of taurine deficiency (Allo et al., 1997; Harada et al., 1990; Jong et al., 2012; Pansani et al., 2012; Parildar et al., 2008), as taurine and beta-alanine share the sodium/chloride dependent transporter, *TauT*, and may compete for this in a time-dependent manner (Jong et al., 2010). Harris et al. (2006) showed an increase in plasma taurine levels when participants were supplemented with acute beta-alanine (10–40 mg/kg of body weight). Interestingly, in the current study, the intracellular concentration of taurine decreased with the provision of exogenous beta-alanine, while the extracellular concentration of taurine increased. This finding suggests that the decrease in intracellular taurine with exogenous beta-alanine may be due to beta-alanine limiting the uptake of taurine from the culture media, potentially due to competition between the amino acids for *TauT*. It is possible that beta-alanine supplementation not only limits taurine uptake but may also stimulate taurine export from the cells. The influx of beta-alanine may prompt the cells to release taurine through its transporter to maintain homeostasis (via a shuttle-exchange

mechanism) or interfere with taurine retention mechanisms, resulting in increased taurine in the media. This observation of reduced intracellular taurine alongside increased taurine concentration in the media during beta-alanine treatment could be explained by such mechanisms. The practical consequences of these interactions between beta-alanine and taurine are currently unknown and warrant further investigations.

Intracellular carnosine increased when H9c2 cells were exposed to concentrations above 0.5 mM of exogenous carnosine for 4, 24, and 72 hours, highlighting the ability of H9c2 cells to uptake and accumulate carnosine when exposed to higher concentrations. No changes were observed in the intracellular concentrations of beta-alanine and L-histidine with exogenous carnosine provision, indicating that even though there were relatively high expression levels of the *Cndp2* transcript in H9c2 cells, this carnosinase enzyme may have exhibited low activity. *Cndp2* functions optimally at a pH of 9.5 (Bellia et al., 2014) and the pH of cardiac muscle is not within this range (Vaughan-Jones et al., 2009), which may explain why there is no activity to hydrolyse carnosine into its constituent amino acids. When H9c2 cells were exposed to increasing concentrations of exogenous carnosine at and above 0.5 mM for 4, 24, and 72 hours, there was an increase in the extracellular concentration of beta-alanine. Given that both growth and differentiation media contain FBS, there may be the presence of *Cndp1*; *Cndp1*, serum carnosinase, is responsible for the hydrolysis of carnosine into its constituent amino acids. This increase in beta-alanine would typically be expected to be accompanied by an increase in extracellular L-histidine, although, this concentration remained unchanged at all exogenous carnosine concentrations and time points. The DMEM

already contains substantial levels of L-histidine compared to beta-alanine, where beta-alanine is undetectable in vehicle control samples. Therefore, minor increases in L-histidine concentration may have an insignificant impact on total concentrations, unlike beta-alanine where the non-detectable baseline makes even small variations noticeable in the overall concentration.

2.5.3 Anserine concentration in cardiomyocytes

Recent literature has identified anserine as a HCD present in cardiac tissue (Gonçalves et al., 2023; Van der Stede et al., 2023). Therefore, anserine was included in the amino acid analysis. This was only explored as an *n* of 3 for each concentration and timepoint, as it was included after initial experiments had begun. Intracellular anserine, a methylated analogue of carnosine, was not detected when H9c2 cells were supplemented with increasing concentrations of carnosine or beta-alanine for 4, 24, or 72 hours. The synthesis of anserine is dependent on the intracellular concentration of carnosine and the prior synthesis of this HCD (Gonçalves et al., 2021). Despite observing increases in intracellular carnosine concentration with the administration of exogenous carnosine, there were no subsequent detectable increases in intracellular anserine concentrations. Similar to the *Carns1* enzyme, the activity level of the anserine synthesis enzyme may be the rate-limiting factor. Therefore, enzyme activity assays for measuring the enzyme responsible for anserine synthesis would need to be developed and conducted. Additionally, the administration of exogenous beta-alanine did not increase intracellular anserine, likely because the accumulation of beta-alanine did not subsequently synthesise into carnosine and therefore could not lead to increased anserine concentrations.

2.5.4 Limitations

This study is not without limitations; for example, this study examined the relative expression of genes but not the associated proteins that they encode for or the activity levels of these enzymes and transporters. Future work needs to include *Carns1* activity assays to determine whether this enzyme is the rate-limiting factor for carnosine synthesis in cardiomyocytes when exogenous beta-alanine is provided. In this programme of work, a collaboration with Dr Guilherme Artioli was scheduled to occur in January 2023 to develop the methodology for a *Carns1* activity assay, adapting the methodology previously published by Nagai et al. (2003). I was due to start as a visiting researcher for two months at Manchester Metropolitan University on the 16th of January 2023. Due to medical reasons that occurred over the 2022 Christmas break for Dr Guilherme Artioli, this visit had to be postponed and ultimately cancelled due to the timeframe of the doctoral studies. This assay would have been used to address the questions emerging from this experiment, specifically whether *Carns1* enzyme activity could explain the lack of carnosine synthesis from exogenous beta-alanine in rat cardiomyocytes.

Additionally, this study focused on two HCDs, carnosine and anserine. Other HCDs that may be present in cardiac tissue include homocarnosine and N-acetylcarnosine. Previous literature has suggested that N-acetylcarnosine may be the predominant HCD in myocardial tissue (O'Dowd et al., 1988); however, Van der Stede et al. (2023) showed the absence of homocarnosine and N-acetylcarnosine in cardiac tissue sampled from mice, rats, and humans, indicating that carnosine and anserine are the HCDs present in the heart of these species. It needs to be highlighted that the human tissue in the

Van der Stede et al. (2023) study was sampled from a diseased model, and, therefore, different pathologies, along with associated metabolic changes, may influence HCD content. It was not possible to detect N-acetylcarnosine with the Biochrom 30 amino acid analyser as the acetylation of the carnosine dipeptide occurs on the amino group, meaning that the OPA cannot react with the n-acetylcarnosine. Lastly, a key limitation is that H9c2 cells are derived from rat ventricular cardiomyocytes, not human. Therefore, the translation of the results presented in this chapter needs to be approached with caution; however, as explained in the optimisation work (section **2.1**), H9c2 cells were the most appropriate model available for this study.

2.6 Conclusion

H9c2 rat ventricular cardiomyocytes express the carnosine and beta-alanine-related metabolism genes required for the synthesis and transport of exogenous carnosine and beta-alanine. This study demonstrated the uptake and accumulation of exogenously sourced carnosine and beta-alanine into H9c2 cardiomyocytes, indicating viability for *in vivo* supplementation studies. The activity of *Carns1*, the enzyme responsible for synthesising beta-alanine and L-histidine into carnosine, may be the rate-limiting factor for increasing the content of carnosine in H9c2 cardiomyocytes when the cells are exposed to ample exogenous beta-alanine; however, further investigation and method development are required to test this hypothesis.

Chapter 3

Effects of beta-alanine supplementation on carnosine-related metabolism and cardiac structure/morphology in mice challenged with a high-fat diet

3.1 Introduction

Obesity is a significant public health concern and continues to be the leading cause of premature death globally (The GBD 2015 Obesity Collaborators, 2017). The development of obesity is multifactorial, with several factors contributing to the complex aetiology, including environmental (diet and physical activity), genetic, social, psychological, and economic (Aronne et al., 2009). This study will focus on dietary factors contributing to obesity and the subsequent imbalance between energy intake and energy expenditure in mice. Metabolic stress and obesity, resulting from an excess energy intake in the form of dietary calories, are directly associated with an increased risk of CVD (Powell-Wiley et al., 2021). Excess body weight can alter heart structure and morphology, contributing to this risk (Carbone et al., 2019). CVD is the primary cause of mortality and morbidity among individuals with obesity (The GBD 2015 Obesity Collaborators, 2017), and the efforts to control this using nutritional (diet) and lifestyle (exercise) interventions remain a challenge. Therefore, effective and feasible practical interventions must be developed, focused on supporting individuals with obesity and improving their CVD outcomes (Powell-Wiley et al., 2021).

The use of animal models to research diet-induced obesity (DIO) allows for a thorough exploration of the mechanistic action of novel interventions, whilst maintaining strict, controlled conditions. DIO models, such as high-fat diet (HFD), were first introduced in 1959 (Mašek and Fábry, 1959). Subsequent studies have consistently demonstrated that HFD models of obesity in rodents cause increased body weight, WAT accumulation, and the induction of dyslipidaemia and insulin resistance (Buettner et al., 2006). Mice are frequently chosen for these models due to their genetic and thermoregulatory

similarities to humans (Peltonen and McKusick, 2001), and implementation of dietary challenges in mice induces an obese phenotype closely resembling human pathophysiology (De Moura E Dias et al., 2021), allowing them to be used to investigate obesity-related cardiac injury and dysfunction (Abel et al., 2008).

Beta-alanine, a non-essential, non-proteinogenic amino acid, synthesises with L-histidine to form the dipeptide carnosine and is considered the rate-limiting precursor in the endogenous synthesis of carnosine (Harris et al., 2006). This reversible reaction is regulated by the CARNS1 enzyme (for synthesis) and CNDP1 and CNDP2 (for the hydrolysis of carnosine into its constituent amino acids) enzymes (Bellia et al., 2014). Exogenous carnosine exhibits poor bioavailability in humans due to the presence of serum carnosinase, CNDP1, whereas rodents have limited presence of this enzyme in their serum (Jackson et al., 1991), as they lack the signal peptide in the CNDP1 gene required to secrete the enzyme (Boldyrev et al., 2013). Consequently, when supplemented to rodents, carnosine remains intact after ingestion, elevating plasma concentrations and thus preventing the translation of results into a human model, since humans are unable to process the HCD in the same way. Anserine, a methylated analogue of carnosine, is present in the cardiac tissue of both mice and humans (Van der Stede et al., 2023), and its synthesis is dependent upon the prior synthesis of carnosine, as evidenced by the absence of anserine in the tissues of CARNS1$^{-/-}$ rats, which are unable to synthesise carnosine (Gonçalves et al., 2021). Dietary supplementation of beta-alanine in mice has been shown to increase intramuscular levels of HCDs. Mice treated with 1.2 % beta-alanine in drinking water for 8 to 12 weeks showed increases in skeletal muscle concentrations of carnosine and anserine of 156 %

and 46 %, whereas a concentration of 0.6 % beta-alanine did not induce any significant changes (Everaert et al., 2013). Additionally, a beta-alanine concentration exceeding 3 % in drinking water has been suggested to reduce taurine concentration in murine cardiac tissue (Dolan et al., 2019). This conclusion is consistent with the findings outlined in **Chapter 2**, where it was observed that beta-alanine supplementation decreased intracellular taurine concentration in rat cardiomyocytes. These results also showed the expression of genes related to carnosine and beta-alanine metabolism in cardiomyocyte cells, and **Appendix 4** shows the expression of these genes in control and HFD mice. Taken together, these results suggest the potential for cardiac tissue to utilise beta-alanine in a similar way to skeletal muscle, as well as the potential importance of taurine in the physiological function of the heart (Schaffer et al., 2010). Therefore, a concentration of 1.2 % beta-alanine in drinking water (w/v) was selected for this experimental protocol. This dose of beta-alanine supplementation aligns with the doses used in published research designs where no adverse effects were reported or measured in those who consumed it (Dolan et al., 2019), and supplementing with beta-alanine rather than carnosine may improve the translation of results into humans.

Exogenous carnosine has shown improvements in rodent models of cardiac injury and histopathology, measured using colour staining (e.g., haematoxylin, eosin, Evans blue and triphenyltetrazolium chloride), including heart infarct size area ratio, myocardial atrophy, vascular degeneration, and interstitial edema (Dursun et al., 2011; Hou et al., 2020; Kumral et al., 2016; Zieba et al., 2003). These effects have been shown in animal studies that have predominantly explored the cardioprotective properties of carnosine when cardiac tissue is subjected to acute challenges such as cardiotoxicity reagents (i.e.,

adriamycin/DOX) and I/R injury (through occlusion). Obesity-related cardiac dysfunction may result from changes in haemodynamic factors, structure, function, and contractility (Abel et al., 2008; Ebong et al., 2014). Although these studies suggest carnosine's potential role in acute cardiac injury, the HCD may also be successful in utilising these properties for the treatment and management of cardiovascular pathologies associated with metabolic diseases, such as obesity (Stegen et al., 2015). Carnosine supplementation in obese rats reduced the elevated systolic blood pressure observed in untreated, obese controls (Aldini et al., 2011; Al-Sawalha et al., 2019), suggesting the potential benefits of carnosine in the cardiovascular system of an obese phenotype.

This study investigated the effects of beta-alanine supplementation on carnosine-related metabolism and cardiac structure and morphology in male mice challenged with a HFD for 12 successive weeks. This is an appropriate timeframe to establish the phenotypic and metabolic characteristics of obesity (Odom et al., 2022; Savetsky et al., 2015) and to induce histopathological cardiac changes (Lepczyński et al., 2021). The primary hypothesis was that 12 weeks of beta-alanine treatment, administered alongside the HFD, would improve morphological and histological cardiac markers in mice compared to HFD-fed mice treated with vehicle control drinking water. The data presented in this chapter will contribute to an understanding of the potential impacts of beta-alanine supplementation in the pathology of obesity.

Note: Macroscopic observations during culling and tissue collection, which were not part of the original hypothesis, led to further analysis of non-excitable tissues (liver, WAT, and BAT) and the influence of beta-alanine supplementation on fat infiltration.

3.2 Materials and methods

3.2.1 Animal care

Mice were housed per the 'Code of Practice for the Housing and Care of Animals Used in Scientific Procedures' and aligned with the United Kingdom Home Office recommendations and requirements. This study was designed to cause the minimum suffering or distress to the mice consistent with the scientific objectives and in accordance with the United Kingdom's (Scientific Procedure) Act 1986. The protocol was reviewed by Nottingham University Animal Welfare and Ethics Review Body. Mice were housed in the Bioscience Support Facility at NTU. Clinical observations were performed prior to dosing and daily thereafter. All observations were recorded.

3.2.1.1 Animal housing

Mice were group-housed (four per cage) in Tecniplast Sealsafe Plus cages containing corncob bedding, chew sticks, sizzle-nest material (Datesand Ltd., Cheshire, UK), and play tunnels, unless stated otherwise. The mice were kept under controlled conditions: 20-24 °C, humidity of 45-65 %, and ventilation of at least 15 air changes per hour. Mice were exposed to a uniform 12 hours of continuous artificial light in each 24-hour period (light/dark cycle: 07:00 lights on and 19:00 lights off). Light exposure and temperature can vary with respect to both rack location and cage position within the rack; therefore, cage placement in the rack was randomly changed three times a week. To minimise temperature variation, cages were housed in the same room, and each cage contained the same number of mice.

3.2.1.2 Pre-test procedures

Mice were subjected to an acclimatisation period of 11 days upon arrival at the facility, before the start of the experimental protocol, to allow familiarisation with the laboratory environment and research staff, as well as for the monitoring of their health status. During this acclimatisation period, mice were fed *ad libitum* with standard chow feed (Rodent 2018 Envigo Global Certified Diet, USA) and tap water. Each animal, selected at random, was uniquely identified by the presence or absence of an ear-punch mark (no earmark, left earmark, right earmark, or left and right earmarks).

3.2.2 Animal sacrifice and tissue collection

Mice were humanely sacrificed using Schedule 1 cervical dislocation, with death confirmed by the permanent cessation of circulation (cutting of carotid artery), at the end of the 12-week intervention. Residual blood was collected immediately after sacrifice from the thoracic cavity using a needleless syringe and put into an SST microtainer tube (Becton, Dickinson and Company, USA). The microtainer was centrifuged at 15,000 x *g* for 20 minutes at 4 °C, after which the sera were collected, aliquoted, and stored at -80 °C for future analyses. Tissues were collected and weighed (Fisherbrand™ precision balance, Fisher Scientific, UK) immediately following blood collection. The heart was split into four sections using a mouse heart matrice. The heart was cut using a 'leapfrog' method (**Figure 3.1**). The apex (section 1), section 3, and section 4 were placed into separate cryovials (Thermo Fisher Scientific, UK) and immediately snap-frozen in liquid nitrogen before being stored at -80 °C. Section 2 was placed into ice-cold PBS for histological analysis and processed according to section **3.2.7**. One lobe of the liver was placed into ice-cold PBS for histological analysis and

processed according to section **3.2.9**, with the remaining liver being placed into a 1 mL cryovial and snap-frozen in liquid nitrogen before storage at -80 °C. A section of WAT (gonadal) and BAT (interscapular) were placed into ice-cold 4 % paraformaldehyde (PFA) and processed according to section **3.2.8**, with the remaining tissues being placed in 1 mL cryovials and snap-frozen in liquid nitrogen before storage at -80 °C. The following tissues were collected and weighed but were not subjected to further analyses: kidneys, pancreas, brain, quadriceps, gastrocnemius, tibialis anterior, and soleus. Blood and tissue collection and processing were completed as quickly as possible to minimise the biological degradation of samples.

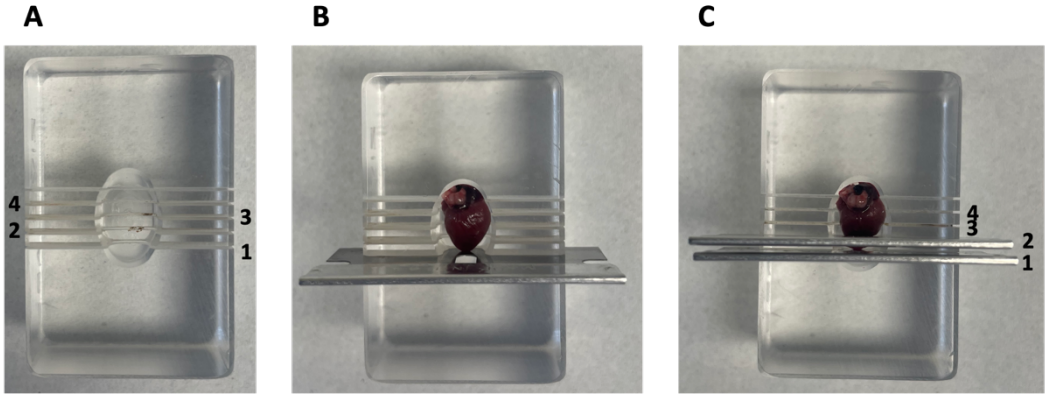


Figure 3.1. Heart matrice 'leapfrog' method. **A.** The heart matrice. **B.** The first blade was inserted at the end of the matrice (line 1), and the heart apex was aligned against it. **C.** The second blade was inserted at line 2 to initiate the first cut and remained in the matrice. The first blade was then removed and inserted into line 3 to make the next cut and left in place. The second blade was then removed and inserted into line 4 for the final cut. The sequence of sections, from the first cut to the final cut, went in the following order: apex, section 2, section 3, section 4. The blade was consistently inserted into the matrice at a 45-degree angle.

3.2.3 Study design

6-week-old male C57BL/6J mice (*n* = 32) were obtained from Charles River (UK) and housed as described in section **3.2.1**. After acclimatisation, cages were randomly

assigned to 1 of 4 experimental groups, each receiving a specific diet and treatment for 12 successive weeks. Group CHOW VC (*n* = 8) received standard chow and vehicle control tap water; Group CHOW BA (*n* = 8) received standard chow and 1.2 % beta-alanine in tap water (w/v); Group HFD VC (*n* = 8) received 60 % HFD (custom diet TD.06414, Teklad, USA) and vehicle control tap water; Group HFD BA (*n* = 8) received 60 % HFD and 1.2 % beta-alanine in tap water. Treatments are further described in section **3.2.3.1**. Mice were split into 2 cohorts of 16 mice due to the maximum capacity of the Promethion Core. The second cohort of mice arrived at the facility and started and ended their intervention one week after the first cohort of mice. Each cohort consisted of four cages, with one cage assigned to each experimental group.

3.2.3.1 Diet and drinking water

Mice had *ad libitum* access to their assigned diet and drinking water. **Table 3.1** shows the composition of the standard chow and HFD. Food weight and water volume were recorded three times a week. The mean weekly food and drink intake per cage were calculated, and cumulative food and drink intake were analysed. Food and drink were refreshed two to three times a week. Drinking water stock was prepared according to **Table 3.2** and was restocked at least once a week.

3.2.4 Body weight

Body weight was monitored and measured three times a week. This was completed on non-consecutive weekdays (Monday, Wednesday, and Friday).

Table 3.1. Macronutrient composition of the standard chow diet and the 60% HFD

Nutrients	Standard chow	60 % HFD
Protein (%)	24.0 (18.4)	18.3 (23.5)
Fat (%)	18.0 (6.0)	60.3* (34.3)
Carbohydrate (%)	58.0 (44.2)	21.4 (27.3)
Metabolisable energy (kcal/g)	3.1	5.1

Percentage information presented as kcal from (by weight). *Approximate fatty acid profile (% of total fat): 36 % saturated, 41 % monounsaturated, 23 % polyunsaturated. Abbreviations: HFD, high-fat diet.

Table 3.2. Drinking water treatments

Treatments	Dose	Preparation
Beta-alanine (BA)	12 g/L (1.2 %).	12 g of beta-alanine powder (Carnosyn, Natural Alternatives International, USA) was dissolved in 1 L of tap water. Stock was protected from light and stored at 4 °C.
Vehicle control (VC)	n/a	1 L of tap water. Stock was protected from light and stored at 4 °C.

3.2.5 Promethion Core (including indirect calorimetry)

The 16-channel Promethion Core, a metabolic and behavioural phenotyping system, was used during the final week [**Figures 4.2 and 4.3** (Sable Systems International, USA)]. This system was used to assess metabolic (indirect calorimetry) and behavioural adaptations in response to the dietary interventions, as well as any changes in health status via the monitoring of individual food and water intake and activity. Indirect calorimetry is a non-invasive technique used to determine energy expenditure and fuel utilisation by the continuous measurement of gaseous oxygen consumption ($\dot{V}O_2$) and carbon dioxide

production ($\dot{V}CO_2$) over a period of time. The respiratory exchange ratio (RER), calculated by the ratio of $\dot{V}CO_2$ and $\dot{V}O_2$, indicates the primary substrate utilised for energy (an RER value of 0.7 indicates exclusive utilisation of lipids, while an RER value of 1.0 indicates exclusive utilisation of carbohydrates). Total energy expenditure (TEE) consists of three main components: resting energy expenditure (REE), the thermic effect of feeding, and the thermic effect of activity. The modified Weir equation relates metabolic rate to $\dot{V}O_2$ and $\dot{V}CO_2$ and is calculated using the following formula:

$$\text{Energy expenditure} = [3.941\ (\dot{V}O_2) + 1.106\ (\dot{V}CO_2)] \times 1440$$

Mice were placed in the Promethion Core with a cage mate and part of their group cage bedding for 48 hours to acclimatise to the new environment (Soto et al., 2019), after which they were individually housed in the system for 120 hours. During this latter period, no data were collected for the first 24 hours to allow the mice to acclimatise to the isolation. Data were collected for the subsequent 96 hours, with the final 48 hours being plotted. Cages contained only corncob bedding; chew sticks, sizzle-nest, and play tunnels were not included. The fixed running wheel accessory in the Promethion Core cage was rendered inactive throughout the experimental protocol to prevent any changes in body composition through an increase in activity. Mice had *ab libitum* access to food and water. Sufficient food and water were provided only at the start of group acclimatisation and isolation to prevent interference with the mice during data collection. Cages were maintained at 22 °C, with an internal ventilation fan speed of 50 %**.** Food and drink hopper masses and photoelectric beam interruptions versus time stamps were measured continuously, and respiratory gases were measured every 2.5

minutes for 15 seconds. This enabled food and water intake, energy expenditure, $\dot{V}O_2$, $\dot{V}CO_2$, RER, and locomotor and ambulatory activity to be calculated. Data were analysed using CalR [version 1.3 (Mina et al., 2018)].



Figure 3.2. 16-channel Promethion Core system in the Bioscience Support Facility.

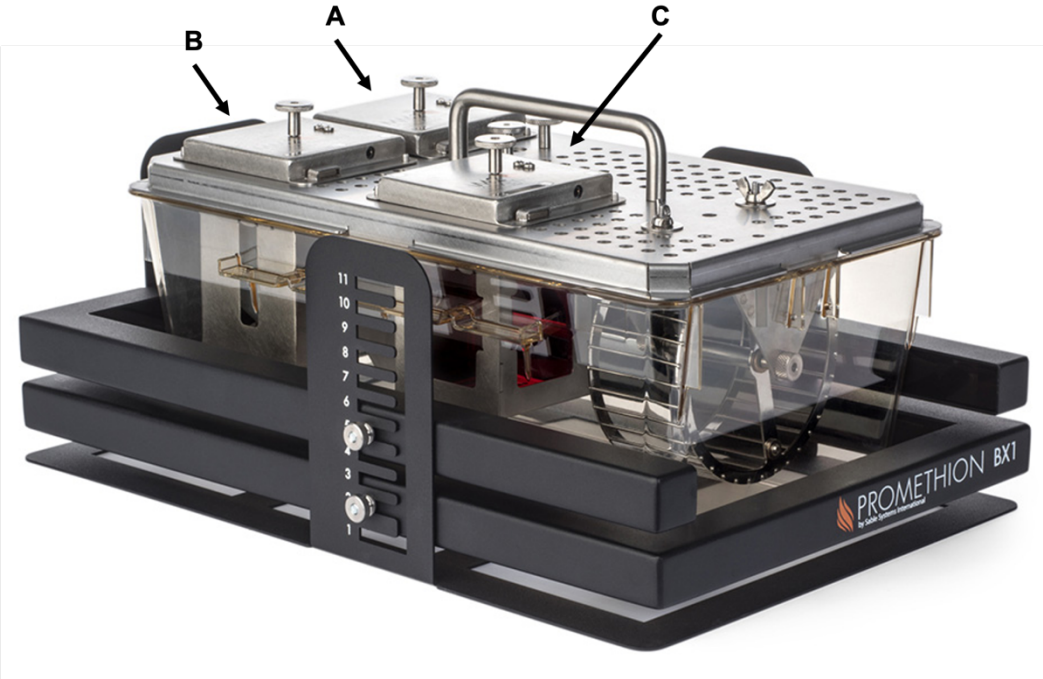


Figure 3.3. Individual Promethion Core cage includes ceiling-mounted food hopper **(A)** and water spigot **(B)** attached to a MM2 load cell and a ceiling-mounted sleeping hut **(C)**. A specialised grill was added to the food hopper for the high-fat diet. Image taken from Sable International.

3.2.6 Amino acid analysis

3.2.6.1 Biochrom 30 sample preparation and analysis

The protocol for amino acid analysis of tissue was adapted from Coburn et al. (2016). The tissues subjected to this analysis were the heart (section 3, as detailed in **Figure 3.1**), liver, WAT, and BAT. Sample preparation was completed on ice unless stated otherwise. Approximately 20 mg of frozen tissue was pulverised cryogenically using a pestle and mortar and transferred into a 2 mL Eppendorf tube containing a bulk bead. The sample was then homogenised by adding 120 µL of 10 % 5-Sulfosalicylic acid in lithium loading buffer (w/v) (Biochrom Ltd, UK) and placing the sample in a TissueLyser LT (QIAGEN, Germany) for 1 minute. The samples were then centrifuged at 13,400 x *g* at 4 °C for 10 minutes. The supernatant was collected and transferred into a fresh Eppendorf tube, and 120 µL of the internal standard Norleucine (500µM) in lithium loading buffer was

added to the supernatant before being centrifuged at 13,400 x *g* at 4 °C for 10 minutes. The supernatant was collected and filtered through a 0.22 µm centrifugal filter tube, with 200 µL of the filtered sample being transferred to an autosampler vial, ready for analysis.

The concentrations of amino acids (taurine, beta-alanine, and L-histidine) and HCDs (carnosine and anserine) were determined using a Biochrom 30 (Biochrom Ltd, UK) with OPA post-derivatisation as detailed in section **2.3.3.1**. Analytes were normalised to the internal standard Norleucine and the weight (mg) of tissue used.

3.2.6.2 O-phthaldialdehyde preparation

OPA solution was prepared as detailed in section **2.3.3.2**.

3.2.7 Cardiac histology

3.2.7.1 Tissue preparation

Immediately upon excision, hearts were cut using a mouse heart matrice (**Figure 3.1**), and section 2 was used for histological analysis (as described in section **3.2.2**). This section of the heart was placed into ice-cold PBS and kept on ice until tissue processing later that day.

3.2.7.2 Tissue processing

A metal container containing isopentane (Fisher Scientific, UK) was placed in a polystyrene box with liquid nitrogen and allowed to equilibrate for approximately 10 minutes. Equilibrium was achieved when the isopentane turned milky white in colour and the inside of the metal container began to freeze.

The tissue was removed from the PBS and dried. A drop of optimal cutting temperature compound [OCT (VMR International, US)] was placed on a piece of cork, the tissue was then placed onto this and fully covered with another drop of OCT. Bubbles in the OCT were avoided. The cork was held in the isopentane for three minutes. The cork was removed from the isopentane, wrapped in foil, and temporarily placed on dry ice whilst other samples were processed. Samples were then stored at -80 °C until sectioning (section **3.2.7.3**). The isopentane was continuously stirred throughout to prevent it from freezing solid.

3.2.7.3 Tissue sectioning

Tissues were placed into a cryostat (Leica Biosystems, UK) at -20 °C degrees and sections were cut at 10 µm. Sections were mounted onto Superfrost™Plus Adhesion microscope slides (Epredia, USA) and stored at -80 °C until staining.

3.2.7.4 Haematoxylin and eosin staining

Cardiac tissue was stained with Harris' haematoxylin (Epredia™ Shandon™, Fisher Scientific, UK) and aqueous eosin [H & E stain (Epredia™ Shandon™, Fisher Scientific, UK)]. The haematoxylin stains nuclei blue/purple, and eosin stains the basic protein components in the cytoplasm and the connective tissue fibres in varying shades of pink.

Microscope slides were removed from the -80 °C freezer and allowed to air dry for 20 minutes. Staining was completed at room temperature. The slides were immersed in 100 % acetone for one minute and then rinsed in deionised water. The slides were immersed in haematoxylin for two minutes before being washed in Scott's water (100 µL of 10 M

sodium hydroxide in 800 mL of deionised water) and then rinsed in tap water. The slides were then immersed in eosin for 10 seconds before being washed in tap water. The slides were then immersed in 70 % ethanol for 30 seconds, followed by immersion twice in 100 % ethanol for 1 minute at a time. Lastly, the slides were immersed twice in xylene for one minute at a time, with the slides being left in the second xylene until mounting. The slides were fixed with a cover slip (Menzel Gläser, Germany) using Eukitt® Quick-hardening mounting media (Sigma-Aldrich, USA), and air bubbles were removed before the slides were left overnight in a fume hood to dry.

3.2.7.5 Van Gieson's staining

Histochemical characterisation of collagen in cardiac tissue was performed using Van Gieson's staining. This stain allows the differentiation of collagen from other connective tissues (i.e., muscle). The picric acid and acid fuchsin allow the collagen to be stained red/pink and the surrounding connective tissues are stained yellow.

Staining was completed at room temperature. Microscope slides were removed from the -80 °C freezer and allowed to air dry for 20 minutes. The slides were immersed in 100 % acetone for one minute and then rinsed twice in deionised water for two minutes at a time. The slides were then immersed in pre-filtered Van Gieson's stain [picro-acid fuchsin (Clin-Tech Ltd, UK)] for 5 minutes before being dipped in 100 % ethanol approximately 10 times. The slides were then immersed twice in 100 % ethanol for two minutes at a time, before being immersed three times in xylene for five minutes at a time. Slides were fixed with a cover slip using mounting media, ensuring no air bubbles, before being left overnight in a fume hood to dry.

3.2.8 White and brown adipose tissue histology

3.2.8.1 Tissue preparation

Immediately upon excision, tissues were placed in a 7 mL bijoux tube containing 4 % PFA for 24 hours at 4 °C. The samples were then washed in PBS and transferred into a fresh bijoux tube containing 70 % ethanol, where they were stored at 4 °C until embedding.

3.2.8.2 Paraffin wax embedding

Fixed samples were loaded into individual, labelled histology Swingsette™ tissue cassettes (Simport Scientific inc, Quebec, Canada). Tissues were processed for paraffin embedding overnight using an enclosed, automated tissue processor [**Table 3.3** (Excelsior AS, Epredia, Thermo Fisher Scientific, UK)]. Tissues were embedded into paraffin wax blocks using the Epredia Histostar Embedding Center (Thermo Fisher Scientific, UK). Tissue blocks were stored at room temperature until sectioning.

3.2.8.3 Tissue sectioning

Paraffin blocks were inverted on ice prior to sectioning to facilitate the process. 5 µm sections of WAT and BAT were cut using a rotary microtome (Leica Biosystems, UK) with a cool-cut block attachment. Sections were immediately placed into a digital tissue section bath set to 50 °C (CellPath, UK) containing distilled water and mounted onto glass microscope slides (Fisherbrand™, Fisher Scientific, UK). Slides were then placed on a digital slide-drying hot plate set to 48 °C (CellPath, UK) for approximately 2 hours to facilitate adherence of sections to the slides.

Table 3.3. Stepwise procedure of tissue processing for histology using the Excelsior AS

Step	Reagent		Temperature (°C)	Time (minutes)	Drain Time (seconds)
1	Dehydration (Ethanol)	75 %	30	60	30
2		90 %	30	60	30
3		95 %	30	60	30
4		100 %	30	60	30
5		100 %	30	60	30
6		100 %	30	60	60
7	Clearing (Xylene)		30	60	30
8			30	60	30
9			30	60	120
10	Infiltration (Wax)		62	80	120
11			62	80	120
12			62	80	120

The vacuum was on for each step.

3.2.8.4 Adipocyte staining

Staining was performed at room temperature. Mounted WAT and BAT sections were stained with H & E to identify individual adipocytes. Sections were dewaxed in xylene for five minutes, three times, then rehydrated in 100 % ethanol twice for two minutes at a time to remove the xylene. The sections were washed in deionised water twice for two minutes at a time to replace the ethanol with water, increasing the compatibility for water-soluble dyes. Following this, the sections were immersed in haematoxylin for five minutes before being washed in Scott's tap water for two minutes. Sections were briefly dipped twice in acid alcohol (0.5 % hydrochloric acid in 70 % ethanol) before being washed in Scott's tap water for two minutes. The sections were then immersed in eosin for 5 minutes and briefly dipped into 100 % ethanol 10 times to clear the eosin. Lastly,

sections were placed in 100 % industrial methylated spirit (IMS) twice for two minutes at a time before being immersed in xylene three times for five minutes at a time to clear any remaining IMS. Once stained, sections were fixed with a cover slip using mounting media before being left overnight in a fume hood to dry.

3.2.9 Liver histology

A lobe of the liver was placed into ice-cold PBS (section **3.2.2**) and processed and sectioned according to sections **3.2.7.2** to **3.2.7.4**. Histological examination of livers using H & E staining revealed a potential issue with tissue processing and staining. A representative image has been provided in **Appendix 2** to illustrate the observed issues.

3.2.10 Histology imaging

Stained heart, WAT, and BAT sections were imaged using a light microscope (Leica Thunder Imaging System) with 10 X and 40 X magnification, and the micrographs were processed using Leica Application Suite X (LasX, Leica). WAT adipocyte diameters were analysed on LasX by randomly selecting 0.1 mm^2 areas and measuring the maximum diameter of all intact adipocytes in that area (adipocytes crossing the border were excluded); a total of 188 to 350 adipocytes were measured per section. Adipocytes were categorised based on size: small (< 50 μm), medium (50 to 69 μm), large (70 to 89 μm), and very large (>90 μm) (Verboven et al., 2018).

3.2.11 Liver triglyceride assay

Liver tissue triglyceride was quantified using a commercial triglyceride assay kit (ab65336, Abcam, UK) and was completed according to the manufacturer's instructions. This assay protocol converts triglycerides to free fatty acids and glycerol. The glycerol is subsequently oxidised to generate a colourimetric reaction that can be read spectrophotometrically at 570 nm. All reagents were included in the kit, except for NP-40 Surfact-Amps™ Detergent Solution (Thermo Fisher Scientific, UK), which was used to keep lipids in solution and does not create a background. All reagents were equilibrated to room temperature and gently mixed before use unless stated otherwise.

3.2.11.1 Standard preparation

A 0.2 mM triglyceride standard was prepared fresh using the Triglyceride Standard (1 mM) reagent, by diluting 100 µL of the 1 mM standard in 400 µL of Assay Buffer V/Triglyceride Assay Buffer. The standard curve dilutions were prepared as described in **Table 3.4**. The standards were plated in a clear 96-well plate (flat-bottom) in duplicate, along with a background control, with a final volume of 50 µL in each well.

3.2.11.2 Tissue preparation

Frozen liver tissue (approximately 100 mg) was pulverised into a fine powder using a pestle and mortar on liquid nitrogen. The weight of each tissue powder was recorded for later calculations in section **3.2.11.4**. The tissue powder was resuspended in 1 mL of 5 % NP-40 (in deionised water) buffer, and samples were slowly heated at 95 ˚C in a Grant QBT2 heat block for 5 minutes, ensuring that the NP-40 solution was visibly cloudy before being removed. Following this, the samples were cooled to room temperature

before the heating step was repeated to ensure maximum solubilisation of all triglycerides (the tissue had dissolved). Samples were then returned to room temperature and centrifuged for two minutes at maximum speed to remove any insoluble material. The supernatant was collected and diluted 10-fold with deionised water. The samples were plated in the 96-well plate in duplicate.

Table 3.4. Standard preparation for liver triglyceride assay

Standard (nmol/well)	0.2 mM triglyceride standard (µL)	Assay buffer V/triglyceride assay buffer (µL)
0	0	150
2	30	120
4	60	90
6	90	60
8	120	30
10	150	0

3.2.11.3 Assay procedure

The cholesterol esterase/lipase buffer was reconstituted in 220 µL of Assay Buffer V/Triglyceride Assay Buffer and kept on ice. 2 µL of this solution was added to all wells containing the standards, background control, and samples. The plate was incubated at room temperature for 20 minutes on a Stuart SSM1 mini-orbital shaker (Cole-Parmer, USA) to allow the conversion of triglycerides into fatty acids and glycerol. During this time, a triglyceride reaction master mix was prepared containing 46 µL of Assay Buffer V/Triglyceride Assay Buffer, 2 µL of OxiRed Probe/Triglyceride Probe, and 2 µL of Enzyme Mix VI/Triglyceride Enzyme Mix. The probe was warmed on a heat block at 37 ˚C for 5 minutes, and the enzyme mix was reconstituted in 220 µL of Assay Buffer V/Triglyceride

Assay Buffer and kept on ice before being added to the master mix. Following the 20-minute incubation, 50 µL of this triglyceride reaction master mix was added to each well containing the standards, background controls, and samples. The plate was briefly mixed on the orbital shaker before being protected from light (wrapped in foil) and incubated at room temperature for 60 minutes. Following this, the plate was read on an Infinite® 200 PRO plate reader (Tecan, Switzerland) at an optical density of 570 nm.

3.2.11.4 Determination of triglyceride concentration

Duplicates were averaged for each standard, background control, and sample, and the averaged background control sample (standard 0 nmol) was subtracted from all standard and sample averages to correct the absorbance. Corrected absorbance values of standards were plotted, and the best-fit curve was drawn to calculate the trend-line equation. The concentration of triglycerides in each sample was calculated using the following formula, where **B** is the amount of triglyceride in the sample calculated from the standard curve (nmol), and **V** is the sample volume added to the wells (50 µL). These values were then normalised to tissue weight.

$$\text{Concentration of triglycerides} = B/V$$

3.2.12 Statistical analysis

A power calculation to determine the sample size required for meaningful effects was conducted using the guidelines provided by the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3R). Due to the lack of preliminary data for this study, the estimated effect size was obtained from Everaert et al. (2013)

due to similar experiments/models being used. The power calculation was performed using estimated effect sizes and based on a significance level of 0.05, a desired power of 0.90, and a two-sided hypothesis test. This power analysis indicated that a sample size of seven was required. To account for attrition (spontaneous deaths during the experiment), a final sample size of eight mice per group was selected. Statistical analysis was performed using GraphPad Prism version 9.2.0. Indirect calorimetry measures were analysed using CalR [version 1.3 (Mina et al., 2018)]. Data are presented as mean ± SD unless otherwise stated. Two-way ANOVAs plus Tukey's post-test were used to examine differences between treatment groups, with one factor of diet (with two levels: CHOW and HFD) and one factor of supplementation (with two levels: VC and BA). Two-way ANOVAs plus Tukey's were used for the Promethion Core data when considering the light cycle (inactive phase) and dark cycle (active phase). Statistical comparisons are presented between CHOW VC vs. CHOW BA, CHOW VC vs. HFD VC, CHOW BA vs. HFD BA and HFD VC vs. HFD BA. Indirect calorimetry measures were also analysed using linear regression and analysis of covariance (ANCOVA); body weight was used as the covariant to determine if the effect of body weight on the metabolic variables was statistically significant. When amino acid/HCD concentrations could not be detected in VC groups in the Biochrom 30 analysis, *t*-tests were conducted between CHOW BA and HFD BA. Statistical significance was considered to be $p<0.05$ and was reported as: * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

3.3 Results

3.3.1 Body and tissue weight

3.3.1.1 Body weight gain

Body weight at the start of each week of the intervention and the total change in body weight are shown in **Figure 3.4**. There was a significant time-by-group interaction on body weight over the 12 weeks of the intervention ($F(33,308) = 24.48$, $p<0.0001$), with a main effect of time ($p<0.0001$) and group ($p<0.001$). There were no post-test differences in body weight between groups at baseline ($p>0.05$). Beta-alanine supplementation attenuated the body weight gain in HFD mice, with this difference starting to occur at week seven of the intervention ($p=0.022$). Throughout the 12-week intervention, there was no difference in body weight between CHOW VC and CHOW BA mice ($p>0.05$). HFD VC exhibited a significantly higher body weight compared to CHOW VC from weeks 4 to 12, and HFD BA mice were significantly heavier than CHOW BA mice from weeks 7 to 12. The total percentage change in body weight from week 1 to week 12 was calculated and showed a significant diet-by-supplementation interaction ($F(1,28) = 9.239$, $p=0.005$), with a main effect of diet ($p<0.0001$) and supplementation ($p=0.001$). The HFD showed a greater increase in total body weight over the 12-week intervention when compared to CHOW mice, with beta-alanine supplementation attenuating this increase. The weekly percentage change in body weight is presented in **Table 3.5**.

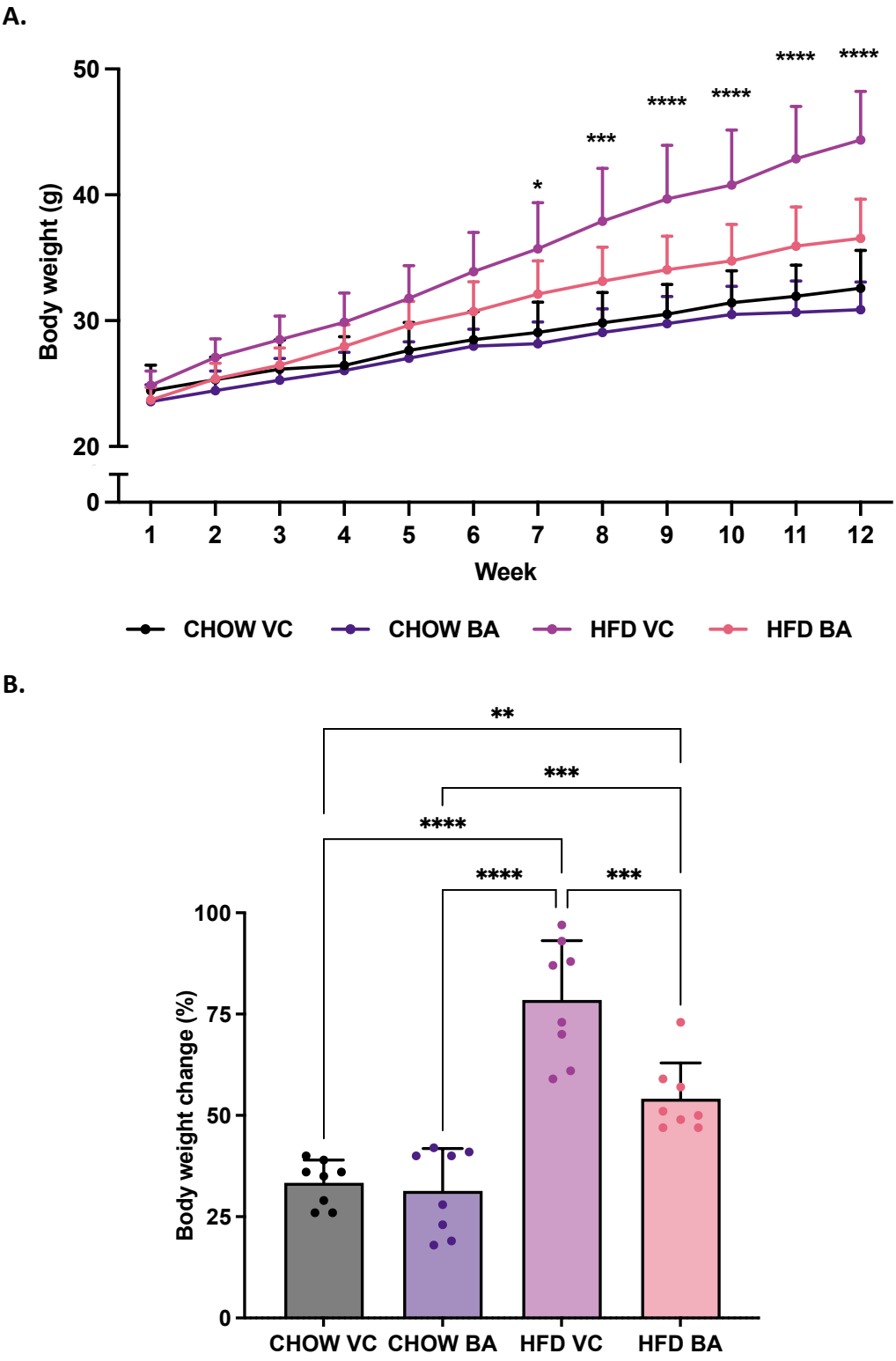


Figure 3.4. Body weight over the 12-week intervention**. A.** Body weight on day 1 of each week of the 12-week intervention. Comparisons are only shown between HFD VC and HFD BA. **B.** Total change in body weight from week 1 to week 12 of the intervention. Line graph data are presented as mean ± SD. Bar chart data are presented as mean ± SD with individual values. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001. Abbreviations: BA, beta-alanine; HFD, high-fat diet; VC, vehicle control.

Table 3.5. Weekly body weight percentage change over the 12-week intervention.

	CHOW VC	CHOW BA	HFD VC	HFD BA
Week 1 to Week 2	3.6 ± 2.2	3.8 ± 2.0	8.9 ± 2.7	7.1 ± 2.2
Week 2 to Week 3	3.2 ± 2.4	3.4 ± 3.1	5.2 ± 2.2	4.3 ± 2.2
Week 3 to Week 4	1.2 ± 1.6	3.1 ± 2.0	4.8 ± 2.8	5.6 ± 2.0
Week 4 to Week 5	4.5 ± 1.6	3.8 ± 1.9	6.4 ± 2.4	6.0 ± 1.4
Week 5 to Week 6	3.2 ± 2.0	3.6 ± 2.4	6.7 ± 1.3	3.6 ± 2.1
Week 6 to Week 7	2.0 ± 1.4	0.6 ± 2.4	5.2 ± 1.4	4.5 ± 1.1
Week 7 to Week 8	2.7 ± 1.6	3.2 ± 1.3	6.1 ± 2.7	3.2 ± 1.6
Week 8 to Week 9	2.3 ± 1.4	2.4 ± 1.3	4.7 ± 2.7	2.8 ± 1.0
Week 9 to Week 10	3.0 ± 2.0	2.4 ± 2.2	2.8 ± 2.5	2.1 ± 1.5
Week 10 to Week 11	1.6 ± 1.8	0.5 ± 1.2	5.2 ± 1.9	3.3 ± 1.7
Week 11 to Week 12	1.9 ± 2.0	0.8 ± 1.7	3.6 ± 1.7	1.8 ± 1.9

Data presented as mean ± SD. Abbreviations: BA, beta-alanine; HFD, high-fat diet; VC, vehicle control.

3.3.1.2 Tissue weights

Tissue weights for each group following the 12-week intervention are shown in **Figure 3.5 to Figure 3.8.**

There was no significant diet-by-supplementation interaction for heart tissue weight ($F(1,27) = 0.00130$, $p=0.972$), with neither diet ($p=0.430$) nor supplementation ($p=0.711$) influencing heart tissue weight (**Figure 3.5**).

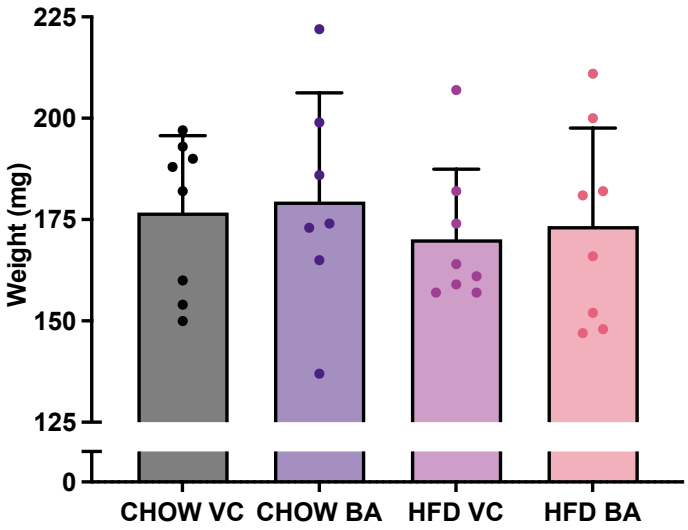


Figure 3.5. Heart tissue weight following the 12-week intervention. Data are presented as mean with individual values ± SD. Abbreviations: BA, beta-alanine; HFD, high-fat diet; VC, vehicle control.

Tissue weights of WAT and BAT are shown in **Figure 3.6**. There was a significant diet-by-supplementation interaction for WAT weight ($F(1,28) = 8.764$, $p=0.006$), with main effects for diet ($p<0.0001$) and supplementation ($p=0.0001$). HFD increased the weight of WAT, with a higher weight being shown in WAT of HFD VC mice compared to CHOW mice ($p<0.0001$). Beta-alanine supplementation reduced the weight of WAT in mice challenged with a HFD (HFD VC vs. HFD BA, $p<0.0001$), however, this was not reduced to CHOW levels. WAT was heavier in HFD BA mice when compared to CHOW BA ($p=0.002$). There was no difference in WAT weight between CHOW VC and CHOW BA mice ($p=0.695$). There was a non-significant diet-by-supplementation interaction for BAT weight ($F(1,27) = 2.198$, $p=0.150$); however, there was a main effect for diet ($p<0.0001$) and supplementation ($p=0.012$). The HFD increased the weight of BAT, with a higher weight being shown in BAT of HFD VC mice compared to CHOW VC ($p<0.001$). Beta-alanine supplementation reduced the weight of BAT in mice challenged with a HFD (HFD VC vs. HFD BA, $p=0.035$). The weight of BAT in HFD BA mice was similar to that of CHOW

mice (CHOW BA vs. HFD BA, p=0.080). There was no difference in BAT weight between CHOW VC and CHOW BA mice (p=0.823).

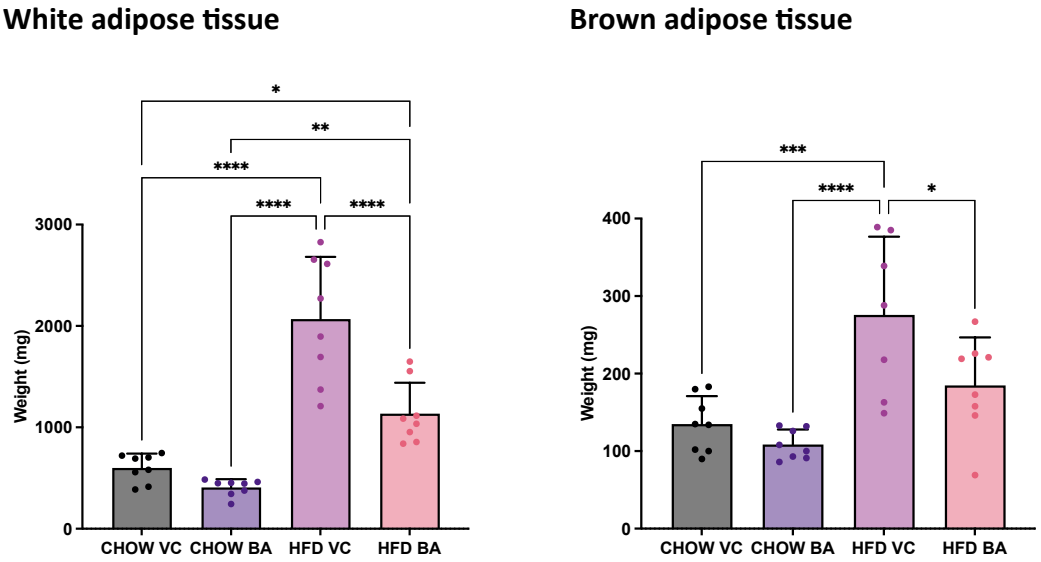


Figure 3.6. White adipose tissue and brown adipose tissue weights following the 12-week intervention. Data are presented as mean with individual values ± SD. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001. Abbreviations: BA, beta-alanine; HFD, high-fat diet; VC, vehicle control.

Tissue weights of solid organs (liver, pancreas, and kidneys) and brain are shown in **Figure 3.7.** There was a significant diet-by-supplementation interaction for pancreas weight (F (1,28) = 4.727, p=0.038), with main effects for diet (p=0.001) and supplementation (p=0.032). Pancreas weight was increased in HFD VC compared to CHOW VC (p=0.002). The elevation in pancreatic weight with HFD was attenuated with beta-alanine supplementation. There was a significant difference between HFD VC and HFD BA mice (p=0.020), with pancreatic weight in HFD BA mice being similar to that of CHOW mice (CHOW BA vs. HFD BA, p=0.716). There were no differences in liver, kidney, or brain tissue weights between groups; neither HFD nor beta-alanine supplementation influenced these organs. There was a non-significant diet-by-supplementation interaction for liver weight (F (1,27) = 0.2620, p=0.613), with no main effect for diet

(p=0.770) nor supplementation (p=0.839). There was a non-significant diet-by-supplementation interaction for kidney weight (F (1,28) = 0.438, p=0.514), with no main effect for diet (p=0.544) nor supplementation (p=0.606). There was a non-significant diet-by-supplementation interaction for brain weight (F (1,27) = 3.090, p=0.090), with no main effect for diet (p=0.437) nor supplementation (p=0.859).

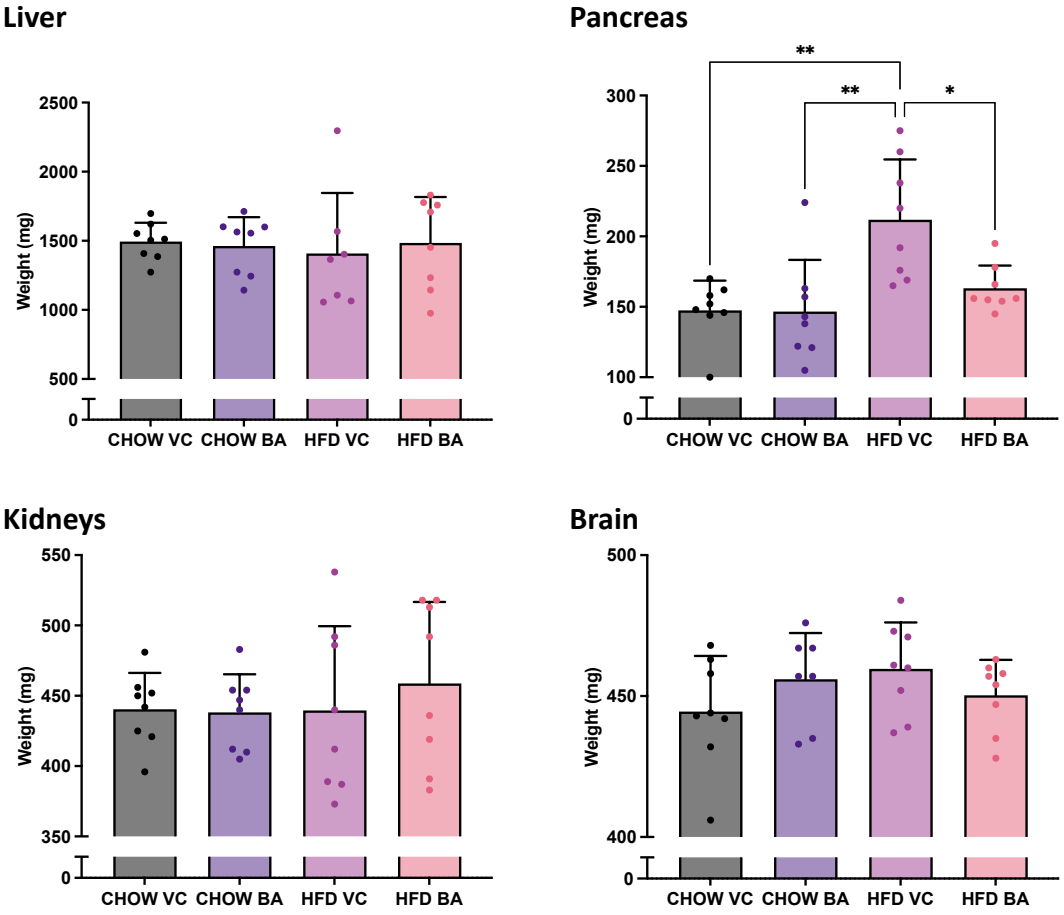


Figure 3.7. Solid organ and brain tissue weights following the 12-week intervention. Data are presented as mean with individual values ± SD. * p<0.05, ** p<0.01. Abbreviations: BA, beta-alanine; HFD, high-fat diet; VC, vehicle control.

No differences in tissue weights of the quadriceps, gastrocnemius, tibialis anterior, and soleus were observed between groups, with neither HFD nor beta-alanine supplementation influencing these skeletal muscle weights (**Figure 3.8**). There was a

non-significant diet-by-supplementation interaction for quadricep weight (F (1,27) = 0.06267, p=0.804), with no main effect for diet (p=0.357) nor supplementation (p=0.067). There was a non-significant diet-by-supplementation interaction for gastrocnemius weight (F (1,28) = 1.258, p=0.272), with no main effect for diet (p=0.475) nor supplementation (p=0.415). There was a non-significant diet-by-supplementation interaction for tibialis anterior weight (F (1,28) = 0.00947, p=0.923), with no main effect for diet (p=0.236) nor supplementation (p=0.794). There was a non-significant diet-by-supplementation interaction for soleus weight (F (1,27) = 2.700, p=0.112), with no main effect for diet (p=0.491) nor supplementation (p=0.593).

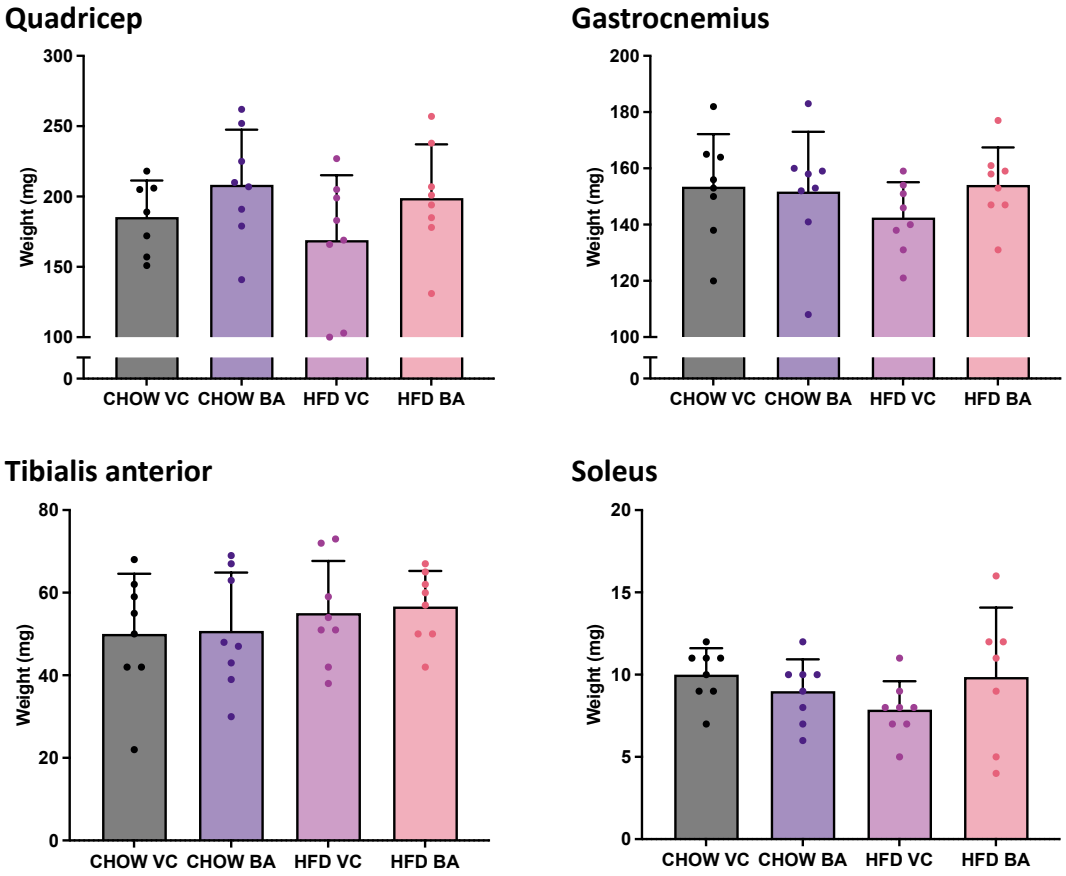


Figure 3.8. Skeletal muscle tissue weights following the 12-week intervention. Data are presented as mean with individual values ± SD. Abbreviations: BA, beta-alanine; HFD, high-fat diet; VC, vehicle control.

3.3.2 Food and water intake

3.3.2.1 Group-housing cages

There were no apparent differences in total water intake between the group-housing cages during the first 11 weeks of the 12-week intervention (**Table 3.6**). Neither HFD nor beta-alanine supplementation affected the water consumption of male mice. There was a decrease in total food intake in the HFD cages; however, the total calories consumed were higher in the HFD cages. Beta-alanine supplementation did not appear to affect food consumption in both CHOW and HFD cages (**Table 3.6**). These data were collected when mice were grouped housed, with each cage (and average) consisting of four mice.

Table 3.6. Water and food, including calorie, intake per mouse per day in the first 11 weeks of the intervention

	CHOW VC	CHOW BA	HFD VC	HFD BA
Cohort 1				
Drinking water (mL)	4.0	4.0	3.1	3.6
Food intake (g)	3.3	3.2	2.7	2.6
Food intake (kcal)	10.23	9.92	13.77	13.26
Cohort 2				
Drinking water (mL)	3.6	3.3	3.6	3.7
Food intake (g)	3.0	3.0	2.8	2.7
Food intake (kcal)	9.3	9.3	14.28	13.77

Calculated as: (total cage intake over the 11-weeks divided by the number of days recorded) divided by number of mice per cage. Abbreviations: BA, beta-alanine; HFD, high-fat diet; VC, vehicle control.

3.3.2.2 Individual cages (Promethion Core)

Assessment of individual food consumption (**Figure 3.9**) showed that HFD-fed mice consumed less food compared to CHOW mice. Beta-alanine supplementation did not

affect food intake; there were no differences between VC and BA mice in both CHOW and HFD groups. All groups exhibited an increase in food intake from the light to dark cycles.

Assessment of individual water consumption (**Figure 3.10**) showed a reduction in water intake in HFD VC mice compared to HFD BA and CHOW VC mice. There were no differences in water intake between HFD BA and CHOW BA mice or between CHOW VC and CHOW BA mice. All groups exhibited an increase in water intake from the light to dark cycles

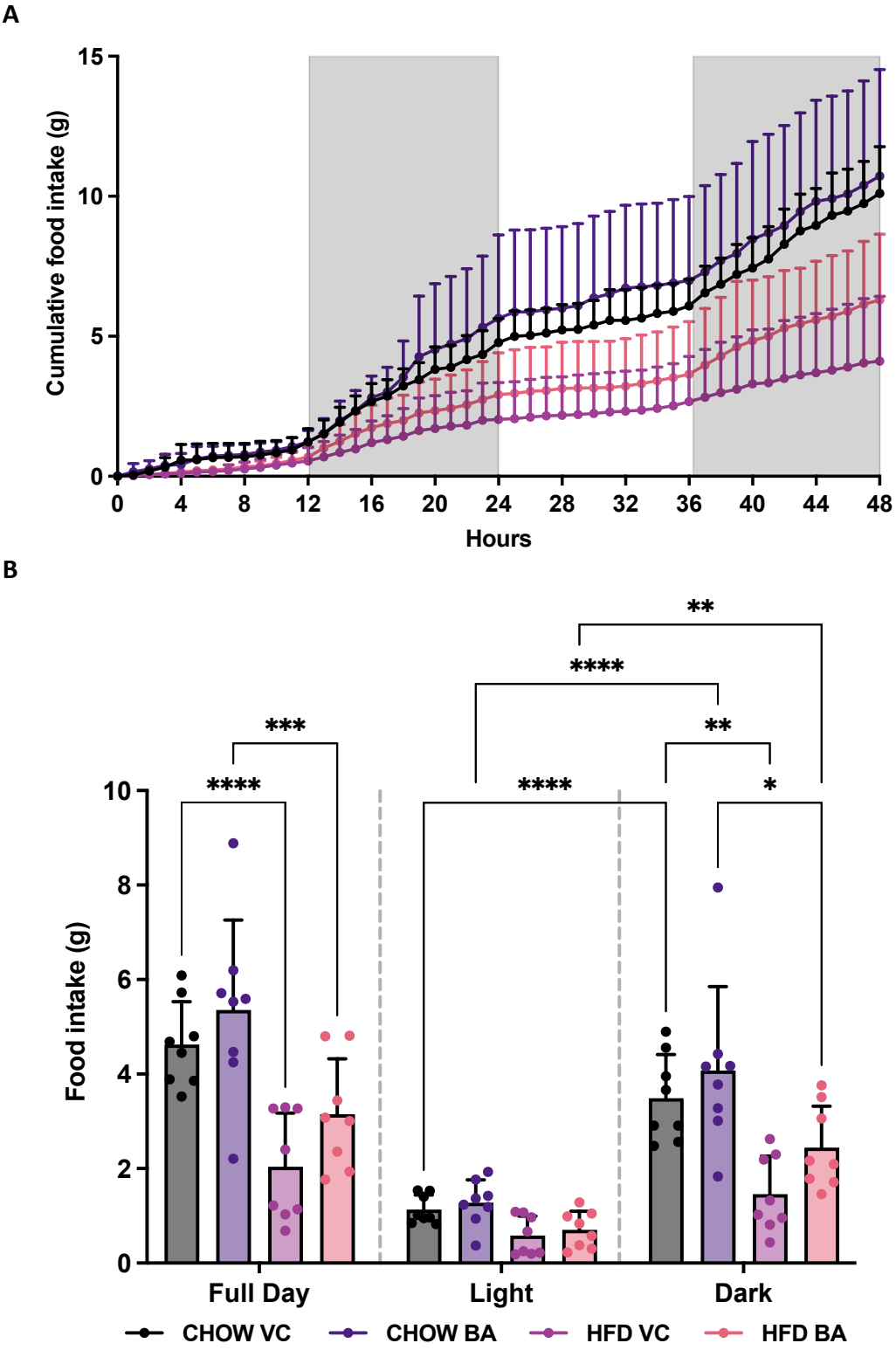


Figure 3.9. Food intake during 48 hours of Promethion Core analysis. **A.** Cumulative food intake. **B.** Mean food intake during light and dark cycles. Line graph data are presented as mean ± SD. Bar chart data are presented as mean ± SD with individual values ± SD. Comparisons are displayed between CHOW VC vs. CHOW BA, CHOW VC vs. HFD VC, CHOW BA vs. HFD BA and HFD VC vs. HFD BA. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. Abbreviations: BA, beta-alanine; HFD, high-fat diet; VC, vehicle control.

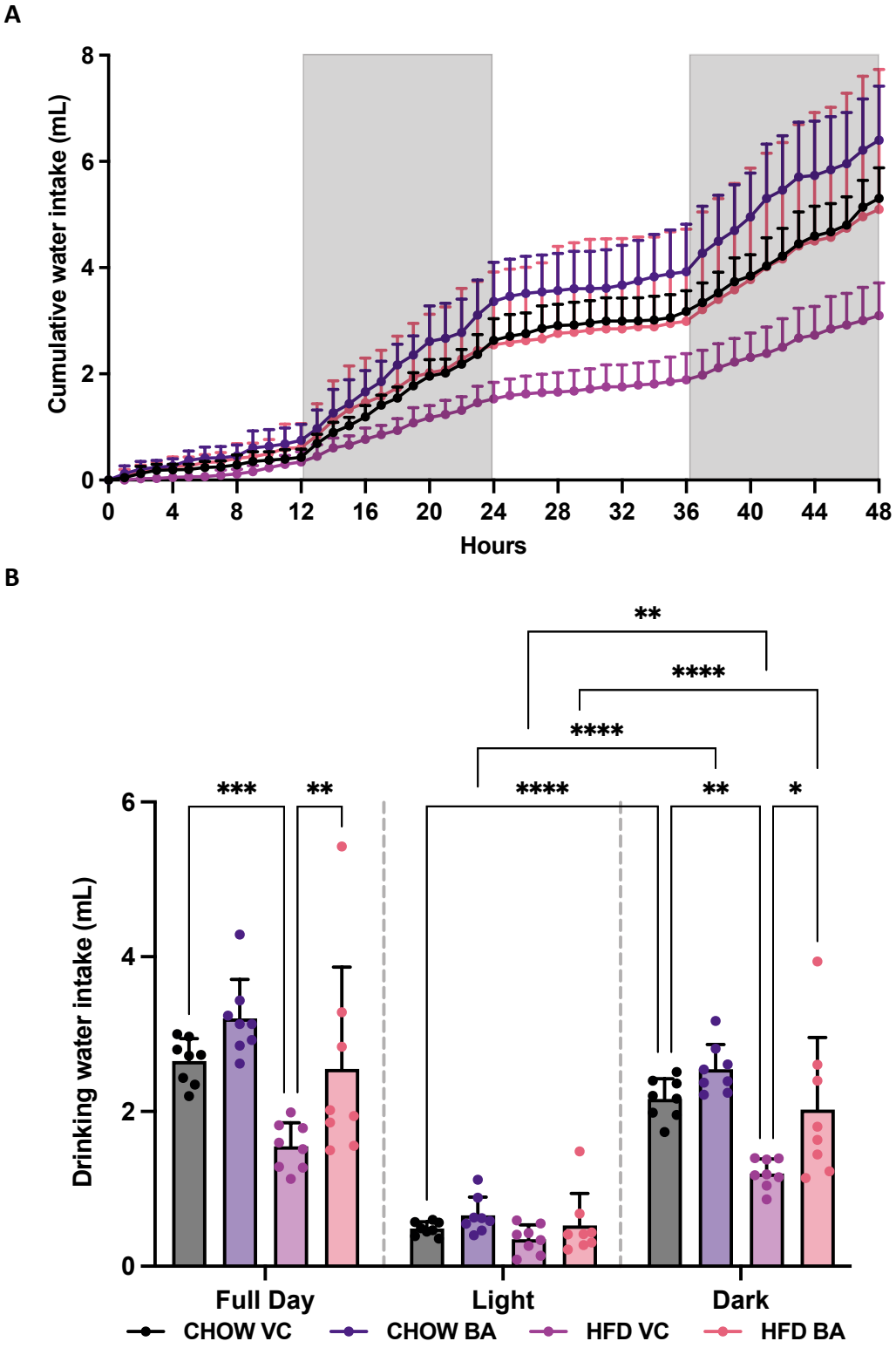


Figure 3.10. Water intake during 48 hours of Promethion Core analysis. **A.** Cumulative drinking water intake. **B.** Mean drinking water intake during light and dark cycles. Line graph data are presented as mean ± SD. Bar chart data are presented as mean with individual values ± SD. Comparisons are displayed between CHOW VC vs. CHOW BA, CHOW VC vs. HFD VC, CHOW BA vs. HFD BA and HFD VC vs. HFD BA. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. Abbreviations: BA, beta-alanine; HFD, high-fat diet; VC, vehicle control.

3.3.3 Indirect calorimetry

3.3.3.1 Respiratory exchange ratio

Assessment of RER (**Figure 3.11**), using the Promethion Core, showed that HFD VC and HFD BA mice exhibited a lower RER compared to CHOW VC and CHOW BA mice. Beta-alanine supplementation did not influence RER, with there being no differences between HFD VC and HFD BA mice, nor between CHOW VC and CHOW BA mice. HFD VC mice were, however, the only group to show no change in RER between light and dark cycles ($p=0.245$); all other groups, including HFD BA, exhibited an increase in RER from light to dark cycles.

Assessment of the two constituent parts of RER, $\dot{V}O_2$ (**Figure 3.12**) and $\dot{V}CO_2$ (**Figure 3.13**), using the Promethion Core, showed that HFD treatment increased $\dot{V}O_2$ in both VC and BA mice in the dark cycle, compared to CHOW mice. HFD VC exhibited a higher $\dot{V}O_2$ in the light cycle compared to CHOW VC mice ($p<0.001$); there was no difference between CHOW BA and HFD BA mice in the light cycle ($p=0.094$). Beta-alanine supplementation did not affect $\dot{V}O_2$ in CHOW mice. $\dot{V}CO_2$ was increased in all groups from the light to dark cycles; however, neither diet nor BA supplementation influenced $\dot{V}CO_2$ in either cycle.

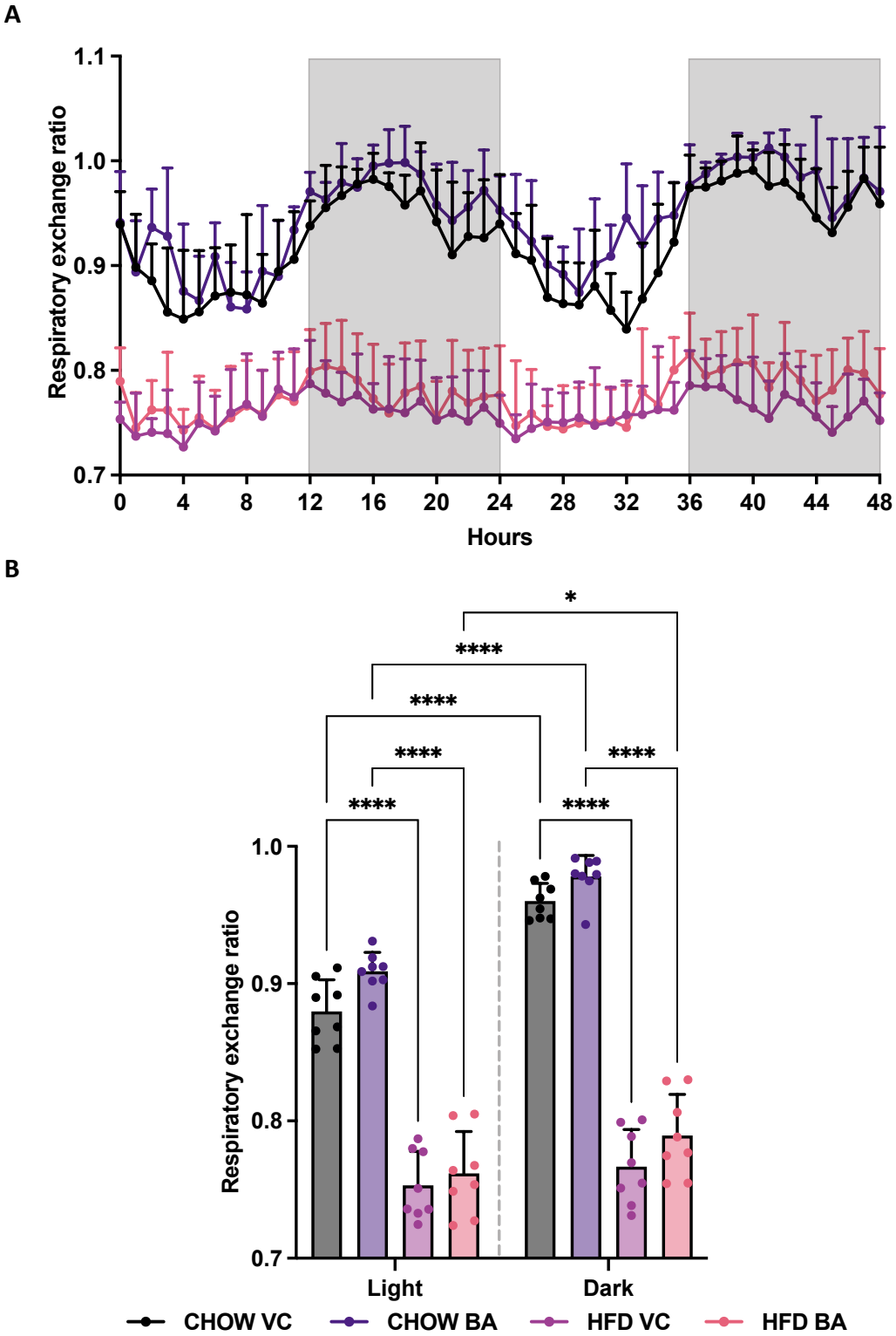


Figure 3.11. Respiratory exchange ratio (RER) following the 12-week intervention. **A.** Mean hourly RER. **B.** Mean RER during light and dark cycles. Line graph data are presented as mean ± SD. Bar chart data are presented as mean with individual values ± SD. Comparisons are displayed between CHOW VC vs. CHOW BA, CHOW VC vs. HFD VC, CHOW BA vs. HFD BA and HFD VC vs. HFD BA. * $p<0.05$, **** $p<0.0001$. Abbreviations: BA, beta-alanine; HFD, high-fat diet; VC, vehicle control.

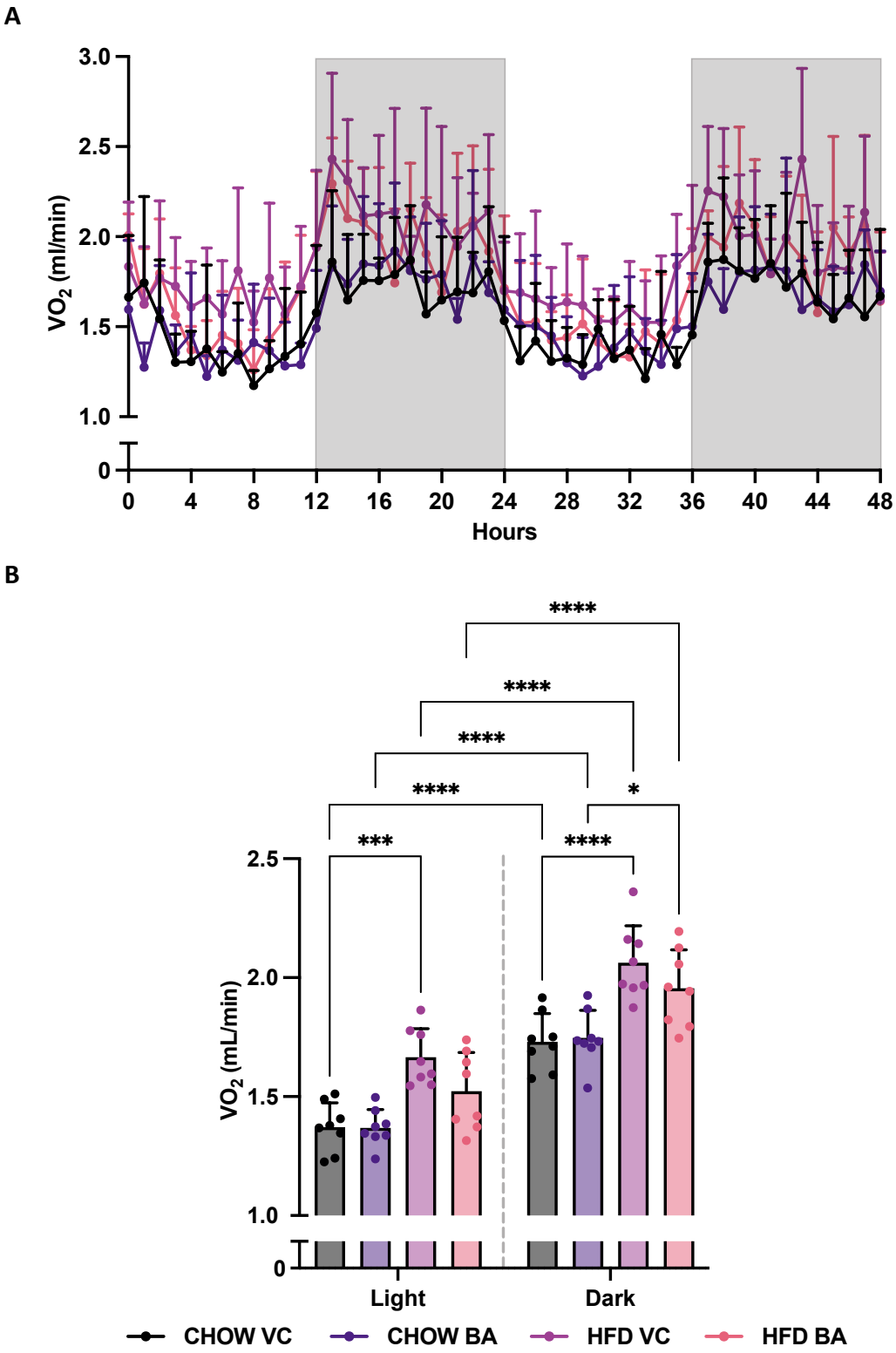


Figure 3.12. Oxygen consumption ($\dot{V}O_2$) following the 12-week intervention. **A.** Mean hourly $\dot{V}O_2$. **B:** Mean $\dot{V}O_2$ during light and dark cycles. Line graph data are presented as mean ± SD. Bar chart data are presented as mean with individual values ± SD. Comparisons are displayed between CHOW VC vs. CHOW BA, CHOW VC vs. HFD VC, CHOW BA vs. HFD BA and HFD VC vs. HFD BA. * $p<0.05$, ** $p<0.01$, **** $p<0.0001$. Abbreviations: BA, beta-alanine; HFD, high-fat diet; VC, vehicle control.

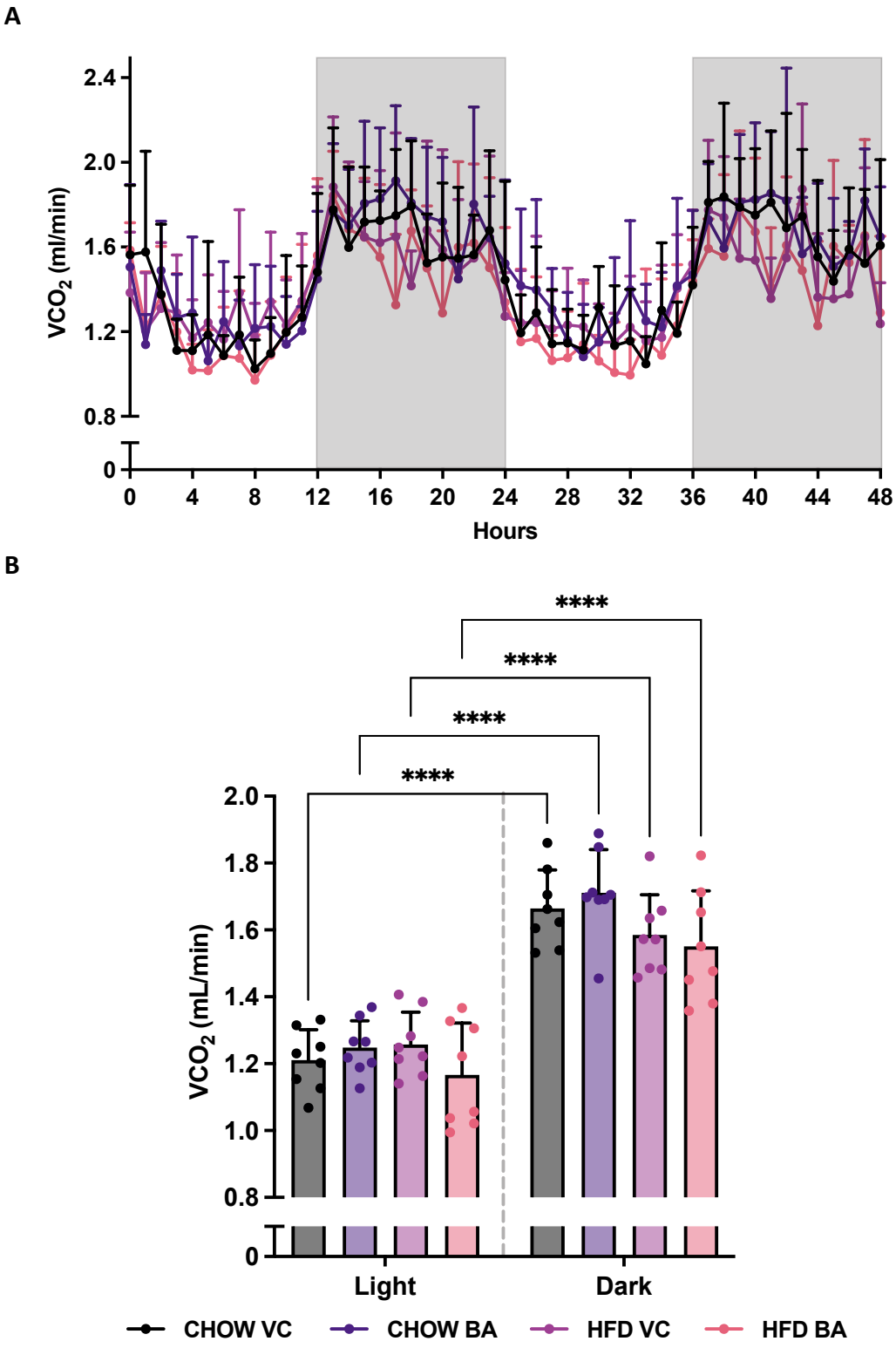


Figure 3.13. Carbon dioxide production ($\dot{V}CO_2$) following the 12-week intervention. **A.** Mean hourly $\dot{V}CO_2$. **B.** Mean $\dot{V}CO_2$ during light and dark cycles. Line graph data are presented as mean ± SD. Bar chart data are presented as mean with individual values ± SD. Comparisons are displayed between CHOW VC vs. CHOW BA, CHOW VC vs. HFD VC, CHOW BA vs. HFD BA and HFD VC vs. HFD BA. **** $p<0.0001$. Abbreviations: BA, beta-alanine; HFD, high-fat diet; VC, vehicle control.

3.3.3.2 Energy expenditure

Assessment of energy expenditure (**Figure 3.14**), using the Promethion Core, showed that beta-alanine supplementation attenuated the increase in energy expenditure observed in HFD-fed mice. HFD VC mice had a higher energy expenditure during both the light (p=0.002) and dark (p=0.002) cycles compared to CHOW VC. There was no difference between HFD BA and CHOW BA in either the light (p=0.380) or dark (p=0.196) cycles. Beta-alanine supplementation did not affect energy expenditure in CHOW mice during either the light (p>0.999) or dark (p=0.983) cycles when compared to CHOW VC mice. Energy expenditure increased in all groups from the light to dark cycles.

Linear regression and subsequent ANCOVA to explore the influence of body weight on energy expenditure showed no significant difference in slope angle between all groups (**Figure 3.15**). This indicates no significant effect of body weight on energy expenditure during both light (p=0.569; CHOW VC, r^2=0.5144; CHOW BA, r^2=0.1212; HFD VC, r^2=0.0409; HFD BA, r^2=0.2946) and dark cycles (p=0.514; CHOW VC, r^2=0.4269; CHOW BA, r^2=0.2604; HFD VC, r^2=0.0002; HFD BA, r^2=0.1878).

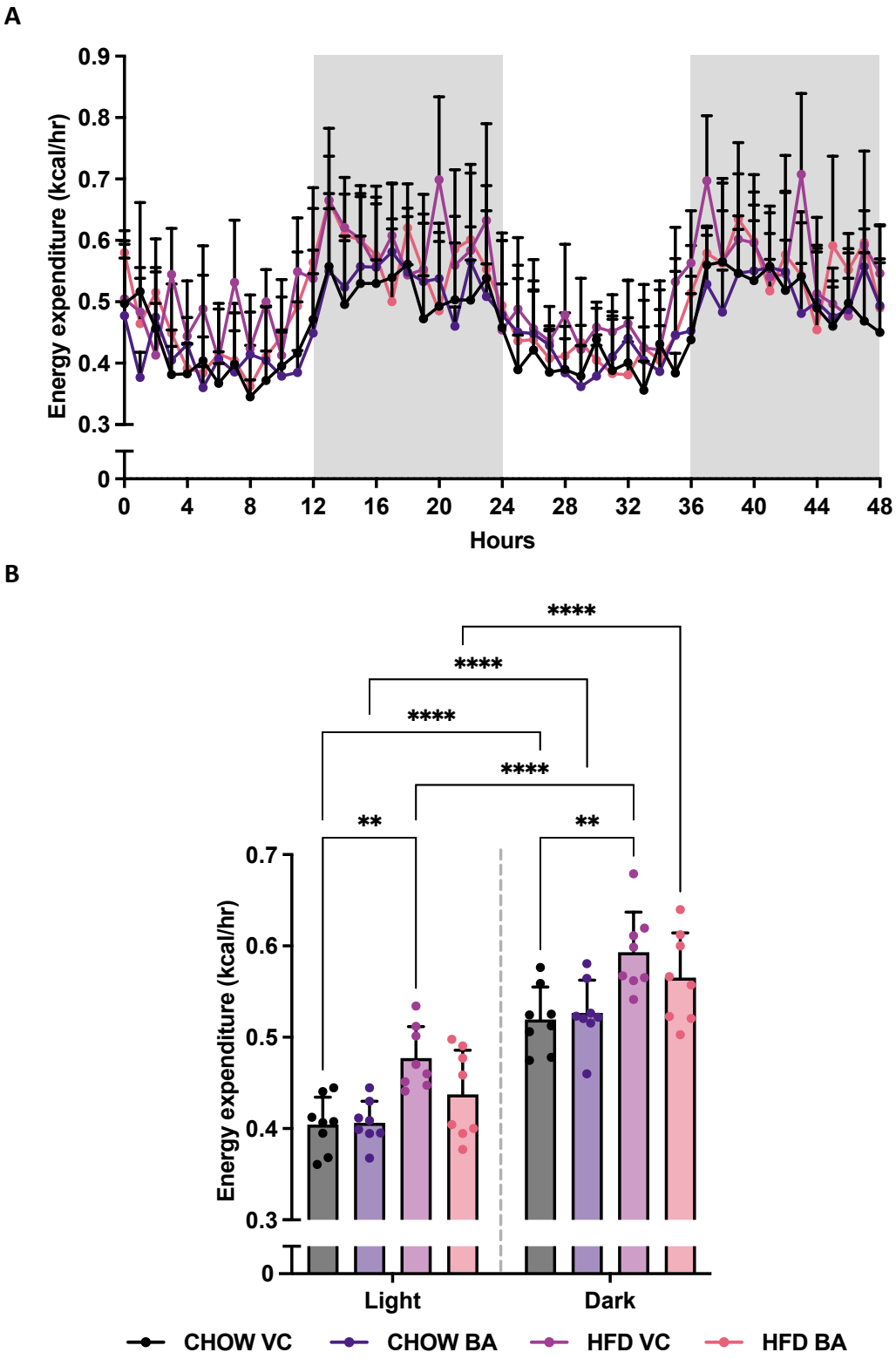


Figure 3.14. Energy expenditure following the 12-week intervention. **A.** Mean hourly energy expenditure. **B.** Mean energy expenditure during light and dark cycles. Line graph data are presented as mean ± SD. Bar chart data are presented as mean with individual values ± SD. Comparisons are displayed between CHOW VC vs. CHOW BA, CHOW VC vs. HFD VC, CHOW BA vs. HFD BA and HFD VC vs. HFD BA. ** $p<0.01$, **** $p<0.0001$. Abbreviations: BA, beta-alanine; HFD, high-fat diet; VC, vehicle control.

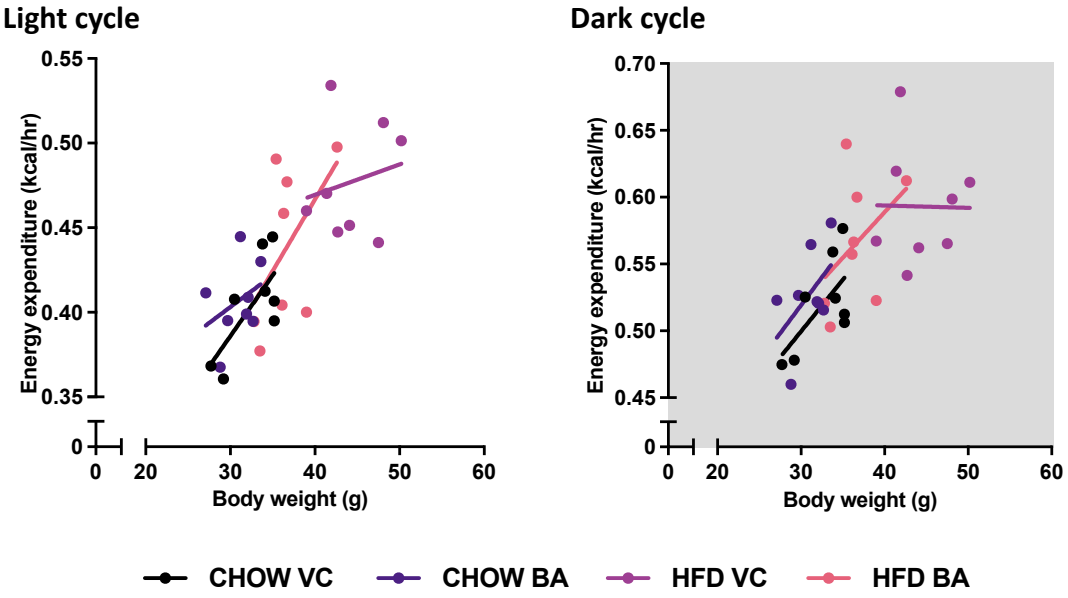


Figure 3.15. Linear regression to determine the influence of body weight on energy expenditure following the 12-week intervention during light and dark cycles. Abbreviations: BA, beta-alanine; HFD, high-fat diet; VC, vehicle control.

3.3.3.3 Locomotor and ambulatory activity

Assessment of locomotor and ambulatory activity (**Figure 3.16** and **Figure 3.17**), using the Promethion Core, showed that beta-alanine supplementation attenuated the reduction in activity observed with HFD. HFD VC mice had significantly lower pedestrian meters during the dark cycle when compared to CHOW VC ($p=0.009$) and HFD BA ($p=0.018$) mice. There was no difference in the pedestrian meters accumulated by HFD BA and CHOW BA mice during the dark cycle ($p=0.979$). All groups exhibited expected nocturnal behaviour, with pedestrian meters being lower in the light cycle compared to the dark cycle. All groups exhibited an increase in percentage of time spent walking and an increase in the percentage of time spent still and asleep during the dark cycle compared to the light cycle (**Figure 3.17**). Heat maps have been generated for each mouse and are presented in **Appendix 3**.

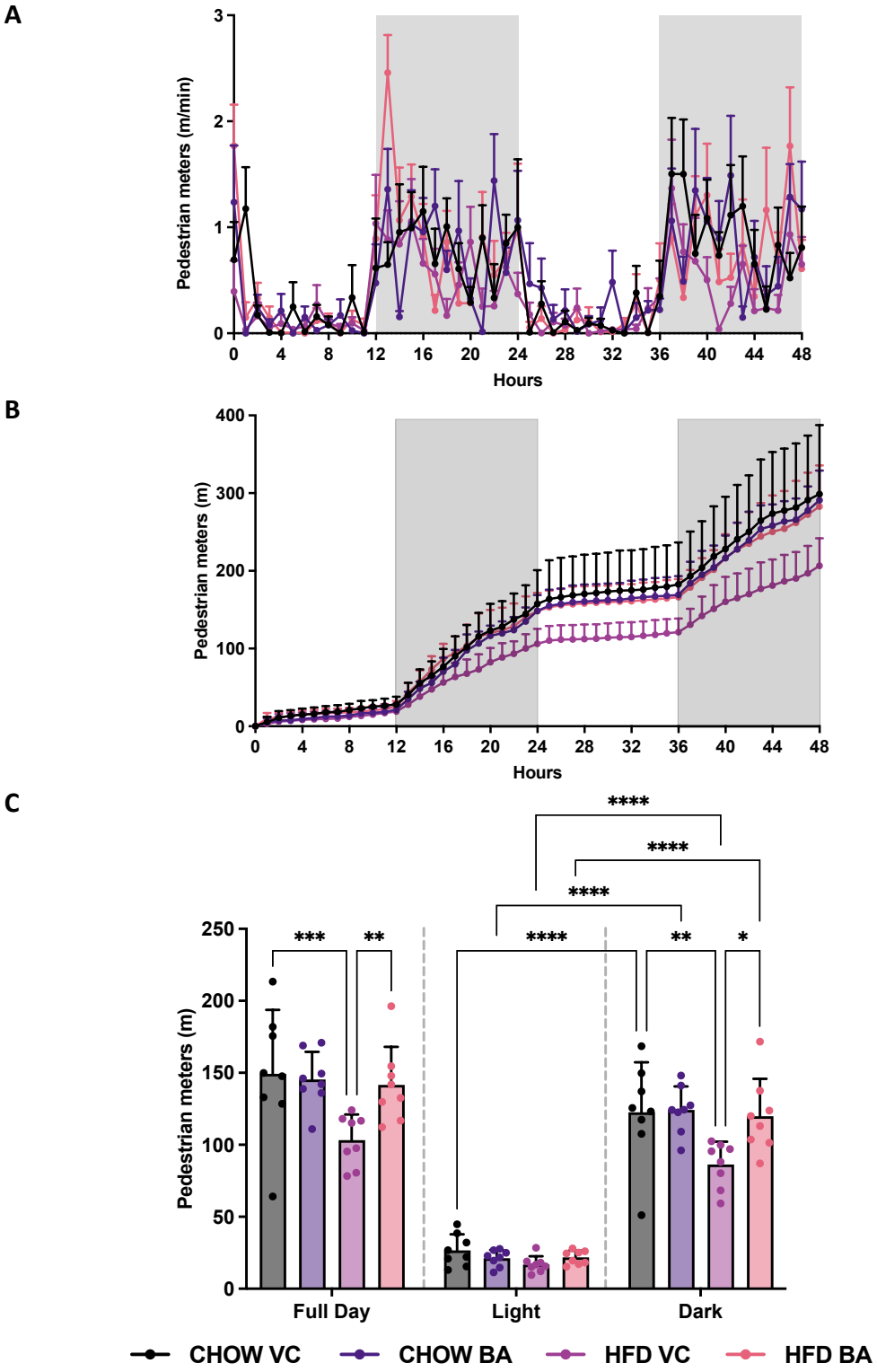


Figure 3.16. Mean pedestrian meters following the 12-week intervention. **A.** Mean hourly pedestrian meters. **B.** Cumulative pedestrian meters. **C.** Mean pedestrian meters across a full day and light and dark cycles. Line graph data are presented as mean ± SD. Bar chart data are presented as mean with individual values ± SD. Comparisons are displayed between CHOW VC vs. CHOW BA, CHOW VC vs. HFD VC, CHOW BA vs. HFD BA and HFD VC vs. HFD BA. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001. Abbreviations: BA, beta-alanine; HFD, high-fat diet; VC, vehicle control.

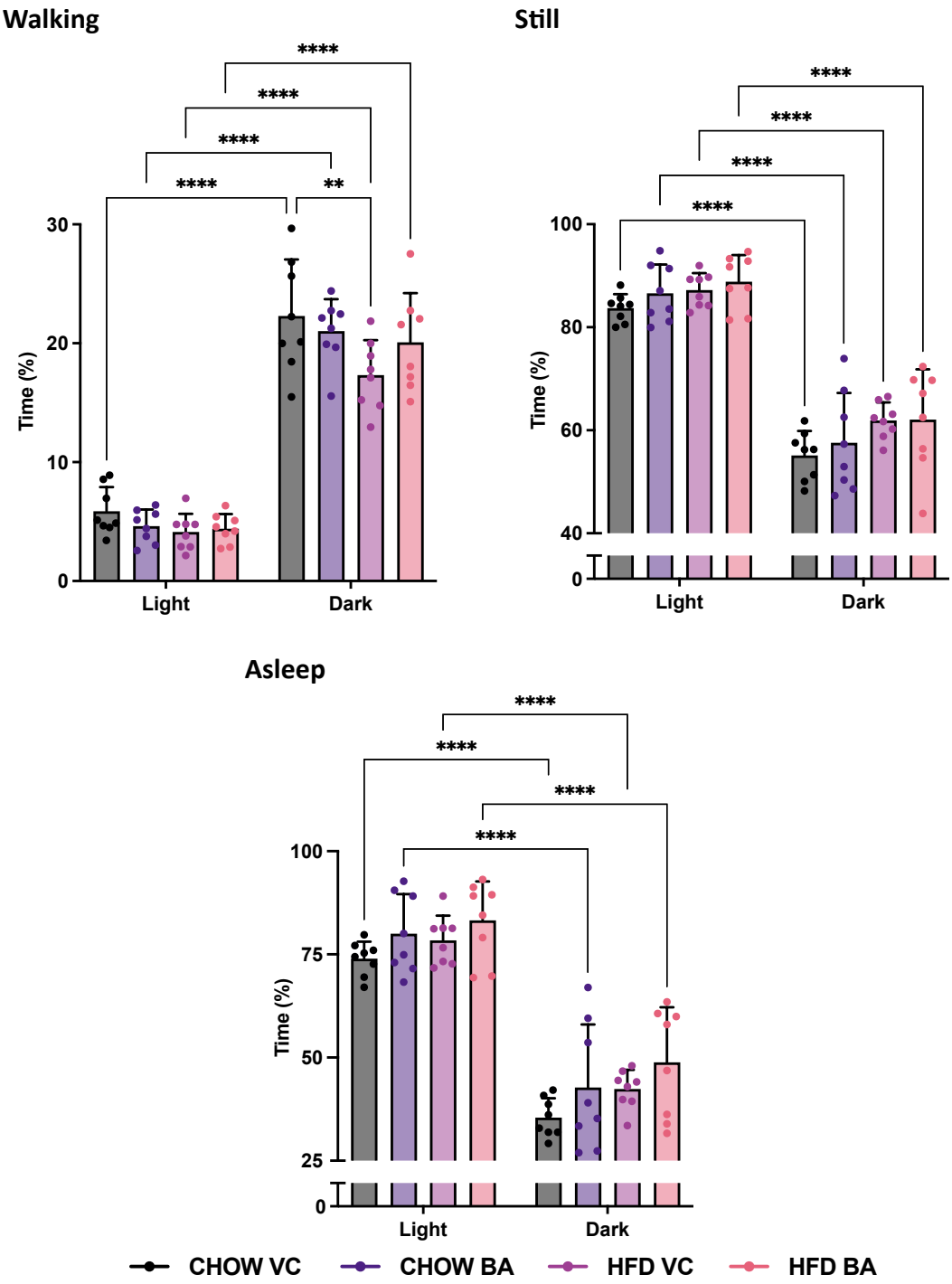


Figure 3.17. Mean percent of time spent completing each activity following the 12-week intervention during light and dark cycles. Data are presented as mean with individual values ± SD. Comparisons are displayed between CHOW VC vs. CHOW BA, CHOW VC vs. HFD VC, CHOW BA vs. HFD BA and HFD VC vs. HFD BA. ** p<0.01, **** p<0.0001. Abbreviations: BA, beta-alanine; HFD, high-fat diet; VC, vehicle control.

3.3.4 Cardiac tissue

3.3.4.1 Amino acid and HCD concentrations

Assessment of amino acid and HCD concentrations in cardiac tissue is shown in **Figure 3.18**. Beta-alanine supplementation enabled the detection of beta-alanine levels in the hearts of CHOW mice. A two-way main effects ANOVA showed no significant main effect of diet (p=0.0731) or supplementation (p=0.2349). There was no difference in cardiac beta-alanine concentration between CHOW BA and HFD BA mice (p=0.239). Beta-alanine was not detected in the hearts of CHOW VC mice or in two of the HFD VC mice.

There was no significant diet-by-supplementation interaction for L-histidine concentration ($F(1,11) = 2.675$, p=0.130), with no main effect of diet (p=0.125) or supplementation (p=0.131). Taurine quantification was unattainable in all groups due to the levels in the cardiac tissue exceeding the upper limit of quantification of the Biochrom 30 amino acid analyser (> 1000 mV).

Cardiac anserine and carnosine levels were detected with beta-alanine supplementation in both CHOW and HFD groups, with neither carnosine nor anserine being detected in VC mice. Unpaired *t*-tests showed there were no significant differences in anserine (p=0.788) or carnosine (p=0.054) concentrations between CHOW BA and HFD BA mice, indicating no influence of the HFD on intracellular levels of the HCDs.

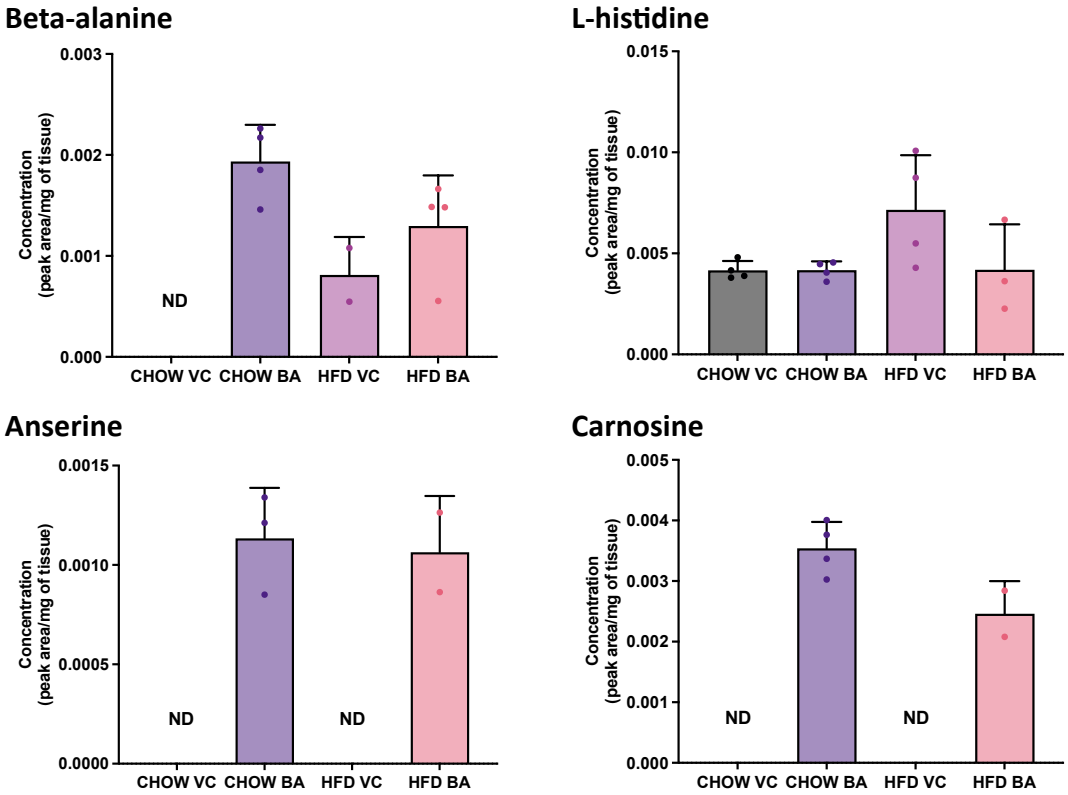


Figure 3.18. Amino acid and histidine-containing dipeptide concentrations in heart following the 12-week intervention. Taurine was above the upper limit of quantification level of the Biochrom 30 amino acid analyser in the heart. Data are presented as mean with individual values ± SD. Abbreviations: BA, beta-alanine; HFD, high-fat diet; ND, not detected; VC, vehicle control.

3.3.4.2 Morphology and collagen staining

Histological examinations of cardiac tissue morphology and collagen showed no differences between CHOW and HFD mice (*n* = 4), nor were there any differences with beta-alanine supplementation (**Figure 3.19** and **Figure 3.20**). All groups showed normal tissue morphology.

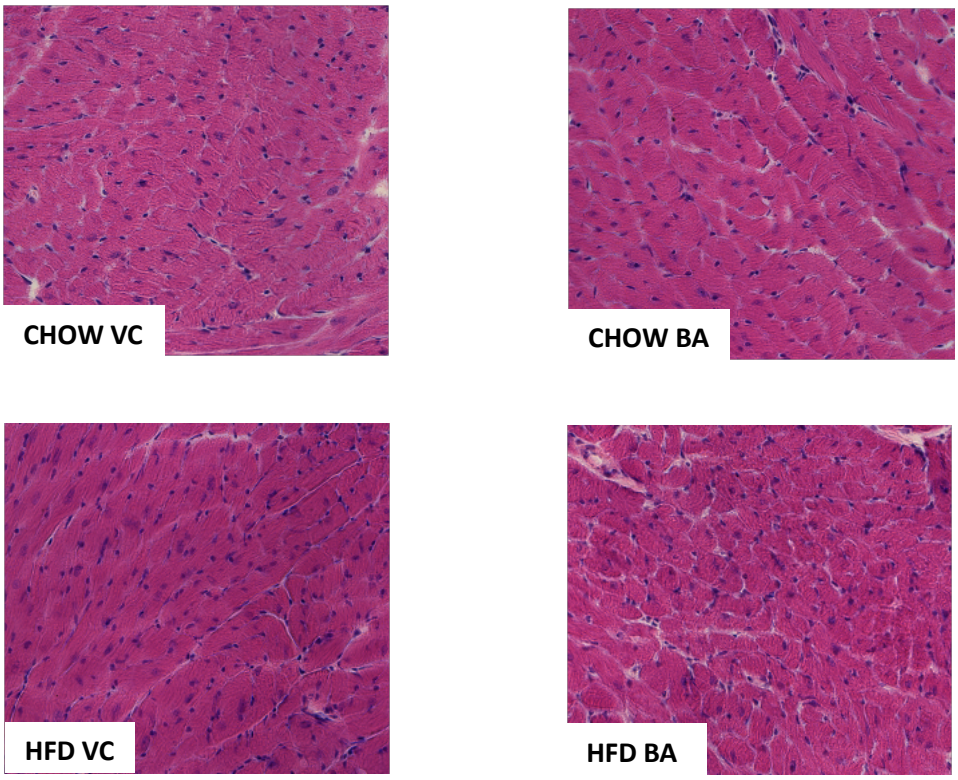


Figure 3.19. Representative histological slides of haematoxylin and eosin staining in cardiac tissue for all treatment groups (40 X magnification). Abbreviations: BA, beta-alanine; HFD, high-fat diet; VC, vehicle control.

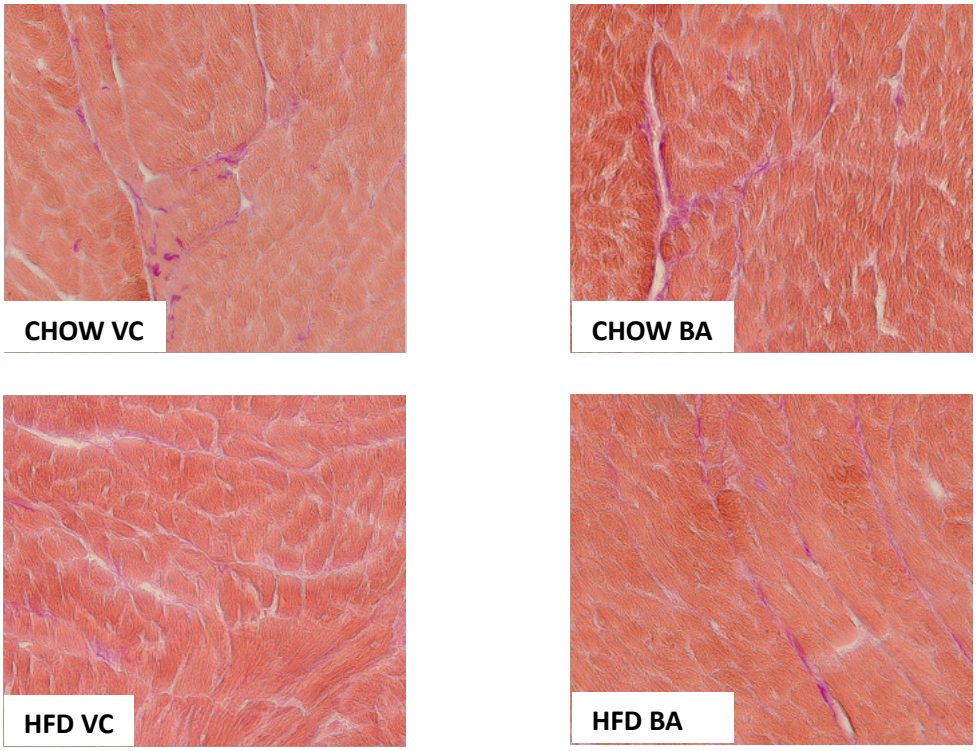


Figure 3.20. Representative histological slides of Van Gieson's staining in cardiac tissue for all treatment groups (40 X magnification). Abbreviations: BA, beta-alanine; HFD, high-fat diet; VC, vehicle control.

3.3.5 Liver

3.3.5.1 Amino acid and HCD concentrations

Assessment of amino acid and HCD concentrations in liver (**Figure 3.21**) showed that beta-alanine concentration appeared higher in mice supplemented with beta-alanine; however, this was not significant between groups. There was no significant diet-by-supplementation interaction for liver beta-alanine concentration ($F(1,12) = 1.1047$, $p=0.326$), with no main effect of diet ($p=0.440$) but a main effect for supplementation ($p=0.018$). There was no significant diet-by-supplementation interaction for liver L-histidine concentration ($F(1,11) = 0.01209$, $p=0.914$), with no effect for diet ($p=0.071$) nor supplementation ($p=0.537$). There was no significant diet-by-supplementation interaction for liver carnosine concentration ($F(1,10) = 4.110$, $p=0.070$), with no main

effect of diet (p=0.170) nor supplementation (p=0.788). Anserine could not be detected in the livers of all groups. Taurine quantification was unattainable for liver samples as concentrations exceeded the upper limit of quantification of the Biochrom 30 amino acid analyser (> 1000 mV).

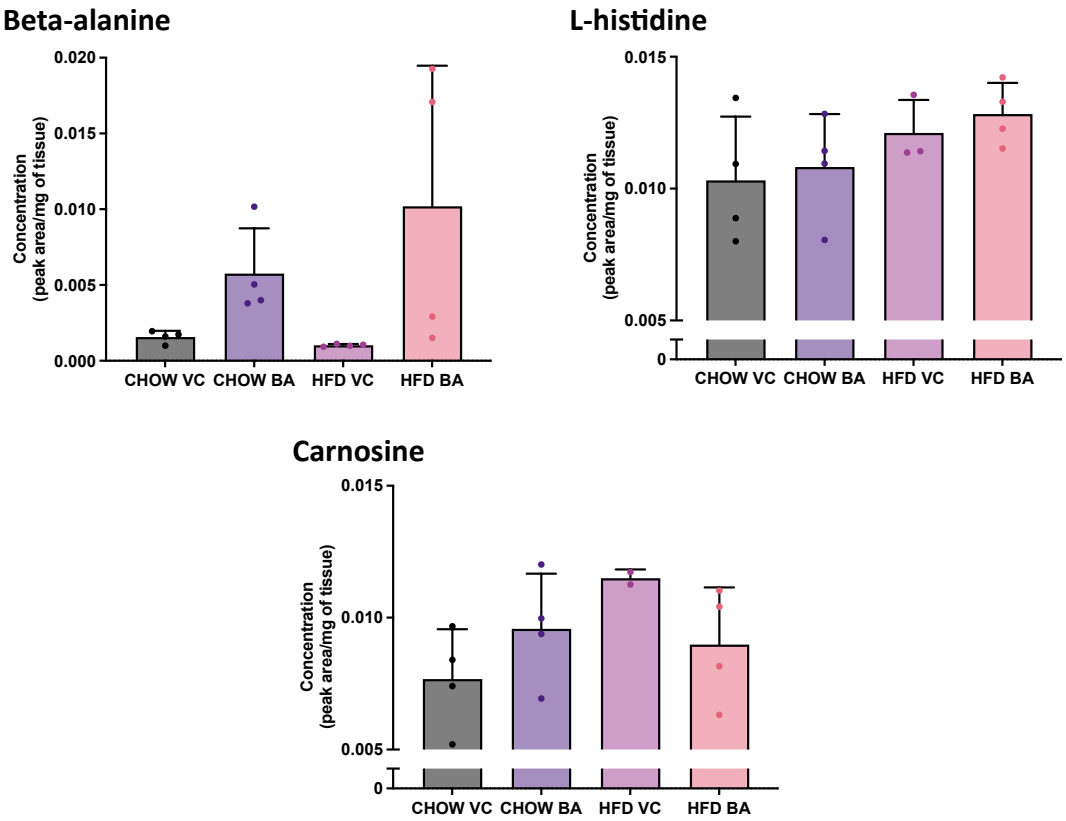


Figure 3.21. Amino acid and histidine-containing dipeptide concentrations in liver following the 12-week intervention. Taurine was above the limit of quantification level and anserine could not be detected. Data are presented as mean with individual values ± SD. Abbreviations: BA, beta-alanine; HFD, high-fat diet; VC, vehicle control.

3.3.5.2 Liver triglyceride concentrations

Following culling and collection of the tissues, macroscopic observations indicated differences in fat deposition in the liver; HFD VC mice had marbling of the liver, indicative of a higher lipid content. Subsequent assessment of liver triglyceride content (**Figure 3.22**) showed that the HFD VC mice had a higher concentration of liver triglyceride

compared to all other groups. There was a significant diet-by-supplementation interaction for liver triglyceride concentration (F (1,28) = 8.307, p=0.008), with a main effect for diet (p<0.0001) but not for supplementation (p=0.094). Beta-alanine supplementation decreased liver triglyceride concentration in mice challenged with a HFD (p=0.015). There was no difference between HFD BA and CHOW BA groups (p=0.550) or between CHOW VC and CHOW BA (p=0.848).

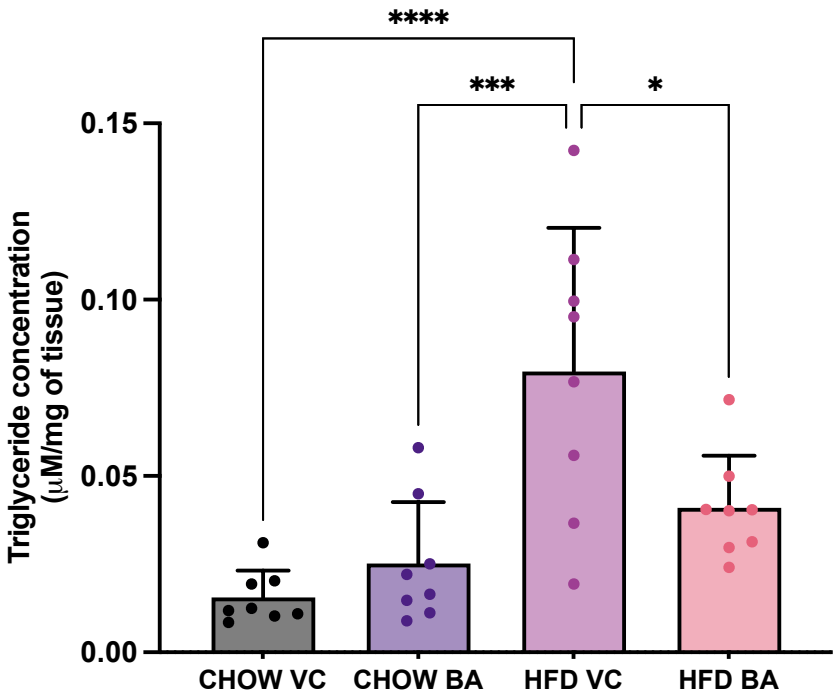


Figure 3.22. Liver triglyceride content following the 12-week intervention. Data are presented as mean with individual values ± SD. * p<0.05, *** p<0.001, **** p<0.0001. Abbreviations: BA, beta-alanine; HFD, high-fat diet; VC, vehicle control.

3.3.6 White adipose tissue

3.3.6.1 Amino acid and HCD concentrations

Assessment of amino acid and HCD concentrations in WAT (**Figure 3.23**) showed that beta-alanine supplementation increased WAT beta-alanine concentration in both CHOW and HFD mice. An unpaired *t*-test showed no significant difference between CHOW BA and HFD BA mice (p=0.628), indicating that diet did not influence this effect. Beta-alanine concentrations could not be detected in WAT of CHOW and HFD VC mice. There was no significant diet-by-supplementation interaction for taurine concentration (F (1,8) = 1.060, p=0.333), with no main effect of diet (p=0.073) nor supplementation (p=0.759). L-histidine, anserine, and carnosine could not be detected in WAT of any group.

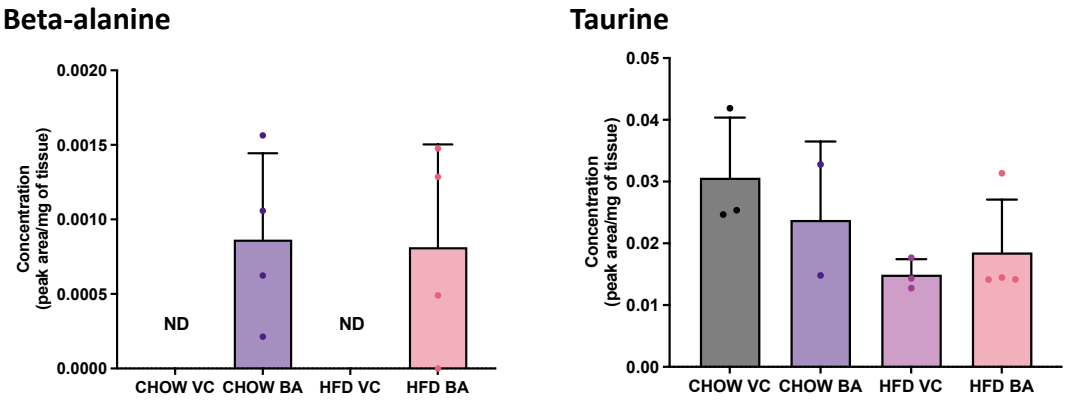


Figure 3.23. Beta-alanine and taurine concentrations in white adipose tissue following the 12-week intervention. L-histidine, anserine, and carnosine could not be detected in WAT. Data are presented as mean with individual values ± SD. Abbreviations: BA, beta-alanine; HFD, high-fat diet; ND, not detected; VC, vehicle control.

3.3.6.2 Adipocyte morphology

H & E staining of WAT (*n* = 4) showed that adipocytes were larger in the WAT of HFD mice compared to CHOW mice (**Figure 3.24**). Beta-alanine supplementation did not affect

adipocyte size, with no observable differences between CHOW VC and CHOW BA mice, or between HFD VC and HFD BA mice.

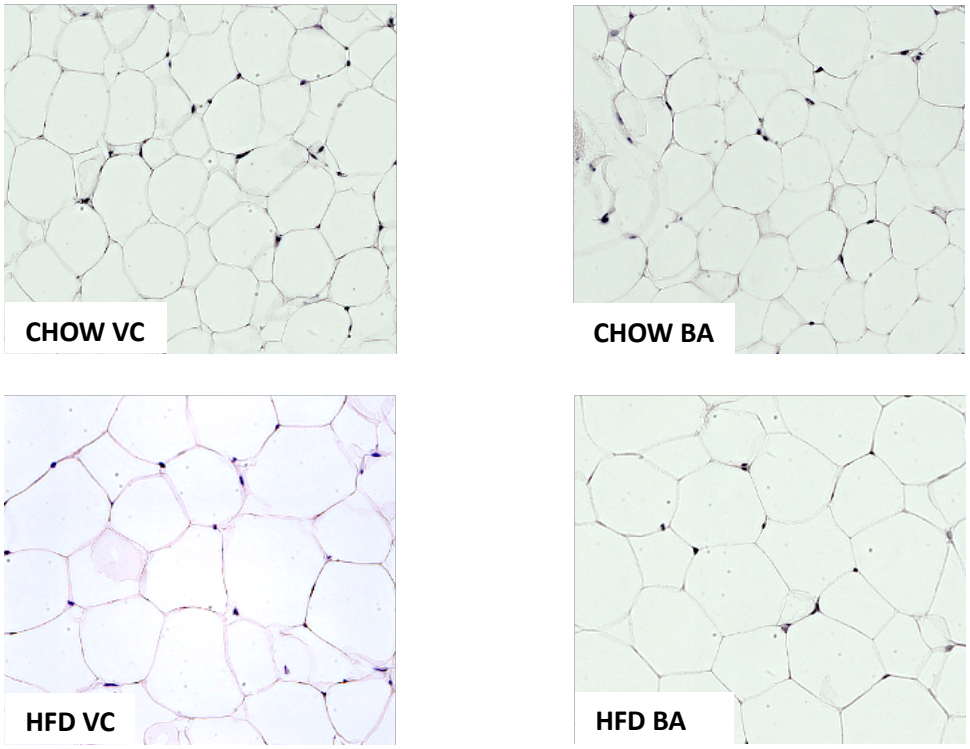


Figure 3.24. Representative histological slides of haematoxylin and eosin staining in white adipose tissue for all treatment groups (40 X magnification) showing increased adipocyte size with a high-fat diet. Abbreviations: BA, beta-alanine; HFD, high-fat diet; VC, vehicle control.

Further assessment of adipocyte diameter, as described in section **3.2.10**, showed that HFD mice had a higher proportion of very large adipocytes (diameter > 90 µm) and a smaller proportion of medium adipocytes (diameter 50–69 µm) compared to CHOW mice (**Figure 3.25**). This resulted in a higher mean adipocyte size in HFD mice compared to CHOW mice. The administration of beta-alanine did not influence the adipocyte size proportions in either CHOW or HFD mice. There was a non-significant interaction for mean adipocyte diameter ($F(1,12) = 1.000$, $p=0.337$), with a main effect of diet ($p=0.0001$) but no main effect of supplementation ($p=0.337$).

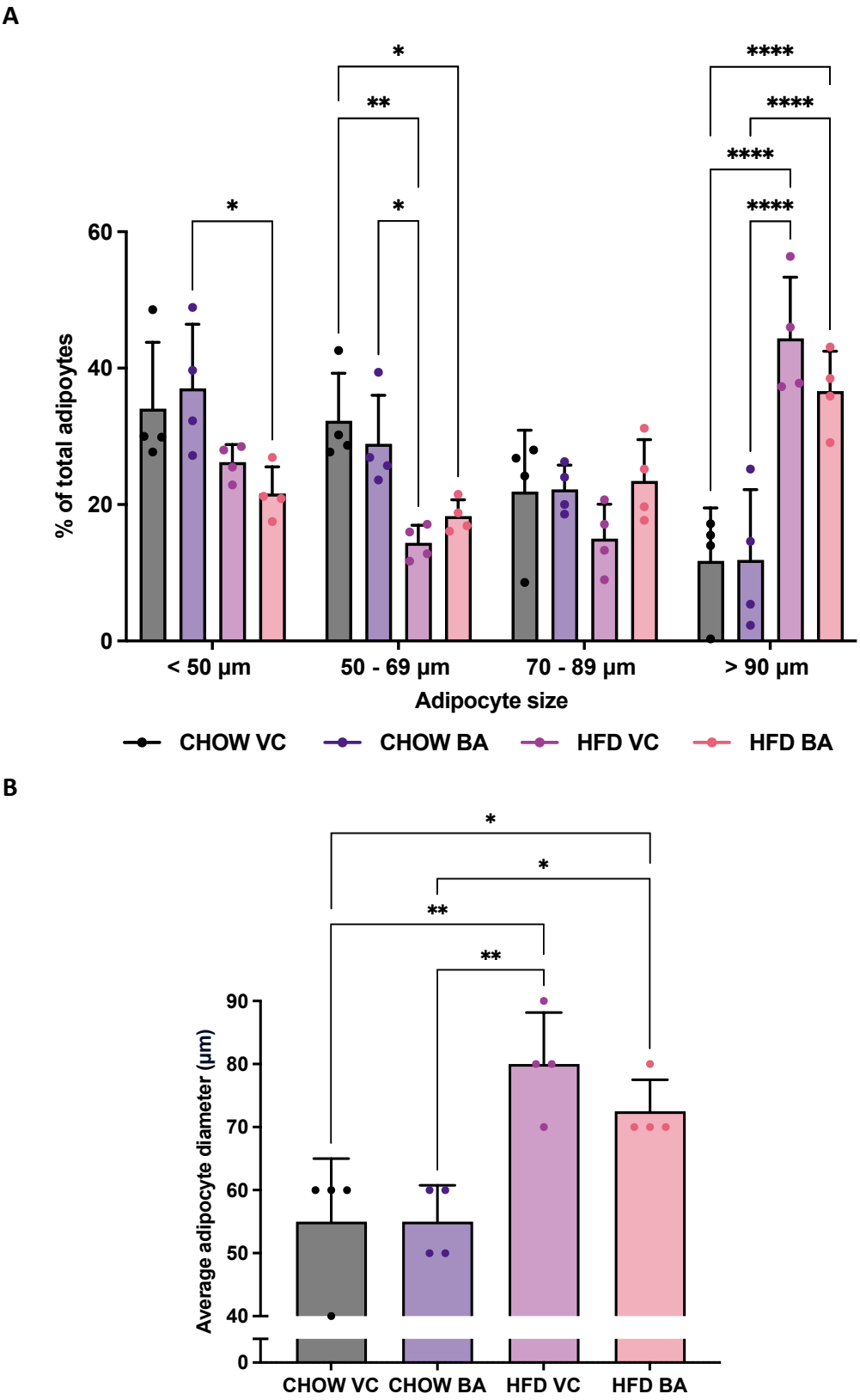


Figure 3.25. Adipocyte size in WAT. **A.** The distribution of adipocyte size in WAT. The frequency of small (< 50 µm), medium (50–69 µm), large (70–89 µm) and very large (> 90 µm) adipocytes in groups. **B.** The average diameter of adipocytes in WAT. Data are presented as mean with individual values ± SD. * p<0.05, ** p<0.01, **** p<0.0001. Abbreviations: BA, beta-alanine; HFD, high-fat diet; VC, vehicle control.

3.3.7 Brown adipose tissue

3.3.7.1 Amino acid and HCD concentrations

Assessment of amino acid and HCD concentrations in BAT (**Figure 3.26**) showed that beta-alanine supplementation increased beta-alanine concentrations in BAT of both CHOW and HFD mice. An unpaired *t*-test showed no difference in beta-alanine concentration between CHOW BA and HFD BA mice (p=0.406), indicating no influence of diet on beta-alanine uptake into BAT. Beta-alanine could not be detected in BAT of CHOW VC and HFD VC mice. L-histidine, anserine, and carnosine could not be detected in BAT of any group. Taurine quantification was unattainable for all BAT samples as concentrations exceeded the upper limit of quantification of the Biochrom 30 amino acid analyser (> 1000 mV).

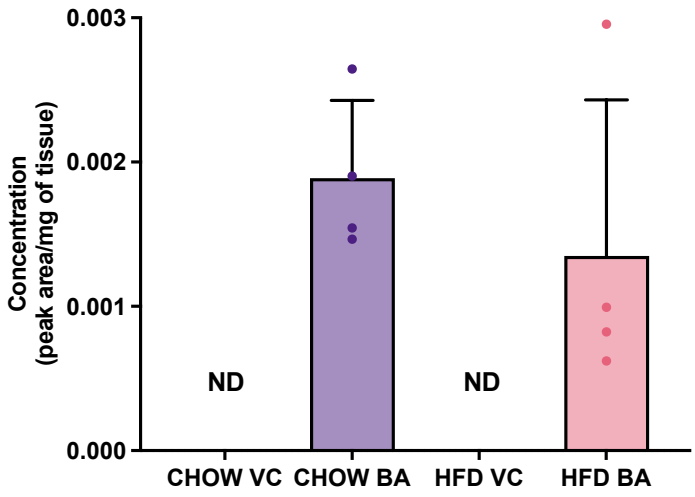


Figure 3.26. Beta-alanine concentration in brown adipose tissue following the 12-week intervention. Taurine was above the limit of quantification level, and L-histidine, anserine, and carnosine could not be detected in BAT. Data are presented as mean with individual values ± SD. Abbreviations: BA, beta-alanine; HFD, high-fat diet; ND; not detected; VC, vehicle control.

3.3.7.2 Adipocyte morphology

H & E staining of BAT (*n* = 2) showed an increase in BAT adipocyte size in HFD VC mice. The administration of beta-alanine attenuated this increase in HFD mice, resulting in smaller adipocytes with a relatively uniform size that appeared similar to the BAT of CHOW mice (**Figure 3.27**).

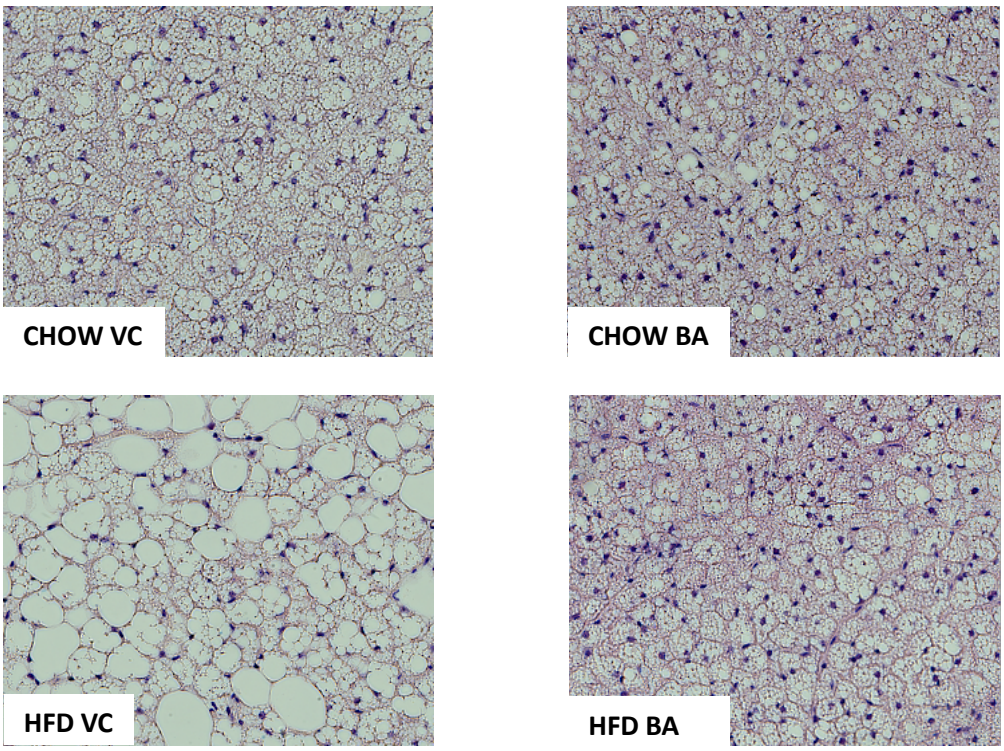


Figure 3.27. Representative histological slides of haematoxylin and eosin staining in brown adipose tissue for all treatment groups (40 X magnification), showing beta-alanine attenuated the increase in BAT lipid content seen with HFD. Abbreviations: BA, beta-alanine; HFD, high-fat diet; VC, vehicle control.

3.4 Discussion

Beta-alanine supplementation increased cardiac carnosine and anserine concentrations, suggesting that the increase in cardiac beta-alanine concentration, seen with beta-alanine supplementation, facilitated the synthesis of HCDs. There were no morphological or histological changes in the heart with the consumption of a HFD for 12 weeks, and, perhaps unsurprisingly, as a result, beta-alanine supplementation did not influence these outcomes. Macroscopic observations highlighted a potential role for beta-alanine supplementation in attenuating body and tissue weight gain when ingested alongside a HFD.

3.4.1 Diet-induced obesity phenotype

C57BL/6J male mice were selected for this study as both the sex (Casimiro et al., 2021) and strain (De Moura E Dias et al., 2021) exhibit heightened susceptibility to diet-induced increases in body weight and fat accumulation. The administration of a 60 % HFD over 12 weeks effectively induced an obesogenic phenotype in this experiment. The criteria for the successful implementation of an obesogenic phenotype in mice are stated to be a > 15 % increase in body weight between groups (Svensson et al., 1996) and the accumulation of WAT (Matias et al., 2018), both of which were observed in this experiment. This study showed a significant increase in body weight in HFD VC mice compared to CHOW mice four weeks into the HFD intervention. This finding is consistent with the literature, where an increase in body weight with the consumption of a HFD becomes apparent at this time point (Wang and Liao, 2012).

3.4.2 Adverse effects of beta-alanine supplementation in mice

No adverse effects of beta-alanine supplementation were noted in this study. Everaert et al. (2013) showed that mice treated with control drinking water consumed more than those supplemented with 1.2 % beta-alanine water (-35 % vs. control). Herein, there were no such disparities in either drinking or eating behaviours between groups during the first 11 weeks of the intervention when total energy intake was calculated for each cage. In the Promethion Core, individual mouse analysis showed that HFD mice consumed less food than CHOW mice; however, there was no difference between the HFD VC and HFD BA mice. This may be explained by either the specialised grill on the food hopper of the Promethion Core, food was observed as becoming stuck and, therefore, investigators had to cut it up into smaller pieces, or that the mice do not need to eat as much as the HFD contains more calories.

Behavioural habits remained consistent across groups, with similar still and sleeping behaviour in both light and dark cycles. Additionally, there were no occurrences of spontaneous deaths throughout this study. Beta-alanine supplementation causes a tingling/prickling sensation of the skin (paraesthesia) in some human participants (Harris et al., 2006). Liu et al. (2012) showed that beta-alanine induces itch-associated behaviour in mice via the G-protein coupled receptor, MrgprD. The mice were injected with 40 mg/mL of beta-alanine (Liu et al., 2012); however, this was higher than the dose of beta-alanine used in this experiment (12 mg/mL in drinking water). Additionally, in Liu et al. (2012), mice were injected with a bolus dose, whereas the mice in this experiment consumed the beta-alanine supplement through drinking water, which would have been administered in regular, smaller doses, throughout the day. Both the lower dose and

intermittent administration of the supplement may suggest that the peak of beta-alanine in the blood will not be as high, reducing any potential adverse effects or itch-associated behaviour. No changes in behaviour or condition of the fur/skin were noted by investigators in this study, suggesting that the mice were not likely to have been negatively affected by the beta-alanine supplementation, although this cannot be directly confirmed.

A dose of > 3 % beta-alanine has been suggested to reduce cardiac taurine concentration (Dolan et al., 2019), with decreases in taurine likely to be associated with poor overall health (Singh et al., 2023). Given the limited understanding of complications that may be associated with taurine depletion in the heart, mice were supplemented with 1.2 % of beta-alanine (w/v) dissolved in drinking water to mitigate any potential impact of the supplementation on taurine concentration. The amino acid analysis could not determine whether 1.2 % beta-alanine decreased cardiac taurine concentration, as the concentrations were above the upper limit of detection of the machine and could not be quantified.

3.4.3 Cardiac tissue

3.4.3.1 Cardiac tissue amino acid and HCD concentrations

The first part of the study aimed to confirm that beta-alanine supplementation could be taken up and utilised in an *in vivo* model (mouse cardiac tissue). Beta-alanine supplementation increased beta-alanine, carnosine, and anserine concentrations in the mouse heart. The increase in the HCDs with beta-alanine supplementation suggests that beta-alanine accumulated in the heart and was able to synthesise with L-histidine, which

is already abundant in heart tissue, to form carnosine, and this synthesis of carnosine contributed to the increase in anserine. The synthesis of HCDs may be dependent on the supplementation duration. In **Chapter 2**, the accumulation of intracellular beta-alanine with beta-alanine supplementation did not subsequently increase intracellular HCDs. In Zhao et al. (2020), the administration of beta-alanine (20 mg/mL) in the drinking water of mice for 7 days increased the intracellular levels of carnosine in the heart compared to untreated controls (mean ± SD: 0.31 ± 0.05 nmol/mg of protein vs. 2.26 ± 0.08 nmol/mg of protein) but did not change cardiac levels of anserine (mean ± SD: 0.0754 ± 0.008 nmol/mg of protein vs. 0.068 ± 0.001 nmol/mg of protein). In this experiment, beta-alanine was administered for 12 weeks, and anserine was shown to be increased in the hearts of mice supplemented with beta-alanine during this time. Therefore, 7 days may not be sufficient for the subsequent synthesis of anserine, whereas the longer duration of 12 weeks in the current experiment enabled anserine synthesis to occur.

The HFD implemented in this study did not affect the uptake and increase of beta-alanine and HCDs in the heart of beta-alanine-supplemented mice, suggesting that obesity does not affect accumulation and synthesis. Lower levels of carnosine have been observed in hypertrophic and failing mouse hearts (Sansbury et al., 2014) and in diabetic rat hearts (Liu et al., 2016); however, due to the concentrations of beta-alanine and HCD being undetectable or absent in the majority of VC mice, it cannot be confirmed whether the implementation of a HFD would lower endogenous cardiac HCD concentrations in mice not supplemented with beta-alanine. Stegen et al. (2015) showed that HFD did not alter carnosine, anserine, or total HCD content in skeletal muscle; however, a decrease in beta-alanine content has been observed in the skeletal muscle of HFD mice (Han et al.,

2023). In cardiac tissue, no association has been shown between anserine content and body mass in human participants (Gonçalves et al., 2023), suggesting that an increase in body mass does not influence endogenous HCD concentrations. This highlights the uncertainty regarding the effect of HFD on intramuscular concentrations of beta-alanine and HCDs in the literature.

3.4.3.2 Cardiac tissue morphology and histology

It has been suggested that beta-alanine and carnosine supplementation can improve myocardial injury markers and morphological/histological parameters in a diseased heart model (Creighton et al., 2022). Beta-alanine supplementation has reduced the infarct size from I/R injury in rats treated with 3 % (Allo et al., 1997) and 100 mM (Hou et al., 2020) beta-alanine. A similar effect has been shown with carnosine supplementation, with decreased histological damage induced by DOX in rats (Kumral et al., 2016) and rabbits (Zieba et al., 2003). Therefore, this study aimed to investigate the effect of beta-alanine supplementation on cardiac morphology and histology in mice challenged with a HFD. Whilst existing literature showed that the consumption of a 12-week HFD induces myocardial morphological changes, observed using H & E and Mallory's trichrome staining (Lepczyński et al., 2021), such alterations were not evident in the current experiment using similar histological analyses. The absence of physical myocardial injury, observed through histological analyses, may have negated the capacity and necessity for improvement with beta-alanine supplementation. In a study published after the completion of this experiment, mice fed a high-fat, high-sucrose diet for 16 weeks exhibited a decrease in histological myocardial fibrosis, observed with Masson's trichrome staining, when supplemented with 80 mM carnosine from week 7

of the diet (Berdaweel et al., 2024). The longer exposure to the HFD or the addition of sucrose in the diet may explain why histological changes were observed in the hearts of these mice (Berdaweel et al., 2024), compared to the current experiment.

The effect of HFD and beta-alanine supplementation on cardiac function measures (i.e., haemodynamic or ECG) could not be investigated in this study due to the conditions of the project license, which do not include direct physical myocardial injury or cardiac functional measurements in the experimental design. Mice fed a HFD for five weeks exhibited early left ventricular dysfunction and more pronounced myocardial interstitial fibrosis, as well as changes in cardiac myocyte size and left ventricular mass (Ternacle et al., 2017). Additionally, eight weeks of HFD showed impairments in left ventricular EF (Carbone et al., 2015). Berdaweel et al. (2024) did not, however, show any changes in CO, SV, or EF in mice fed a high-fat, high-sucrose diet for 16 weeks, indicating that the administration of carnosine had no beneficial effects on these measures. RNA-Seq was completed in this experiment to gain insight into underlying molecular mechanisms and pathways related to cardiac processes, including functional pathways, that could be influenced by both HFD and beta-alanine supplementation to direct future research. RNA-Seq data has not been included in this thesis, as the data was received in November 2023 (after funding had finished). An increase in cardiac HCDs in a cardiospecific α–myosin heavy chain transgenic overexpression of CARNS1 mouse model enriched the expression of enzymes involved in glycolysis, fatty acid oxidation, and the citric acid cycle, which may influence cardiac metabolism. Additionally, it enriched the cardiac muscle contraction pathway, including MYL1, MYL2, MYL3, MHC7, troponin, and calsequestrin (Yan et al., 2022). These transcriptomic and proteomic changes may contribute to

upregulating the contractile and calcium-binding function of the CARNS1 transgenic heart, however, the CARNS1 overexpression cardiospecific transgenic model increases myocardial HCD levels supra-physiologically (carnosine: 30 to 40-fold increase, anserine: 10 to 12-fold increase) compared to beta-alanine supplementation (carnosine: 7-fold increase, anserine: no change) (Zhao et al., 2020). It would be relevant to explore, through our RNA-Seq analysis, whether similar improvements can be observed with dietary increases in cardiac HCDs (i.e., via prolonged beta-alanine supplementation).

3.4.4 Indirect calorimetry

Metabolic flexibility is the capacity to respond to environmental changes (e.g., activity levels) in metabolic demand, enabling organisms to transition between the utilisation of fats and carbohydrates for energy (Duivenvoorde et al., 2015; Goodpaster and Sparks, 2017). Since mice are nocturnal and crepuscular, there is an increase in metabolic demand from the light cycle to the dark cycle, as shown by the Promethion Core data. Obesity is associated with metabolic inflexibility (Goodpaster and Sparks, 2017), which is line with the findings from the HFD VC mice in this study, where they showed no difference in RER between light and dark cycles. The HFD BA mice, however, exhibited an increase in RER from the light to dark cycle, as also observed in the CHOW mice. This suggests that beta-alanine may restore metabolic flexibility in HFD mice, allowing them to be less restricted to lipid utilisation for energy and potentially enabling them to adapt to varying metabolic demands. HFD mice primarily utilise lipid oxidation for energy production, leading to an RER closer to 0.7, whilst CHOW mice primarily utilise carbohydrate oxidation for energy, exhibiting an RER closer to 1.0. Diurnal RER was decreased with the HFD in both VC and BA groups. This alteration of substrate utilisation

is expected with the consumption of this diet. RER, the ratio between $\dot{V}CO_2$ and $\dot{V}O_2$, was lowered in HFD mice compared to CHOW mice because $\dot{V}O_2$ increased while $\dot{V}CO_2$ remained constant. There was no difference in RER values between HFD VC and HFD BA, indicating that beta-alanine does not affect substrate utilisation in obese mice.

3.4.5 Non-excitable tissue amino acid and histidine-containing dipeptide concentrations

3.4.5.1 Liver

Beta-alanine and carnosine were detected in the liver of all groups, including VC mice; this is expected as beta-alanine is endogenously produced in the liver via uracil and 3-hydroxypropionate catabolism (Matthews and Traut, 1987). The other HCD, anserine, was not detected in the liver, which aligns with Van der Stede et al. (2023), who observed carnosine as the only HCD present in mouse liver. Previous literature has shown a decrease in taurine in the liver of mice supplemented with 3 % beta-alanine (Lee and Kim, 2007); taurine concentrations could not be quantified in this study, however, the dose used (1.2%) was less than 3 %.

3.4.5.2 Adipose tissue

Beta-alanine was not detected in WAT or BAT of VC mice and was only detected in WAT and BAT of mice supplemented with beta-alanine, suggesting that beta-alanine can be successfully taken up and accumulated in these adipose tissues. The increase in beta-alanine concentration in WAT did not influence intracellular taurine concentrations, with similar levels observed across all groups. Van der Stede et al. (2023) showed that

carnosine and anserine were the HCDs present in mouse adipose tissue; however, carnosine and anserine were not detected in WAT and BAT in this study. This may be explained by the absence of L-histidine, which was not detected in WAT and BAT samples from all groups, potentially rendering L-histidine as the rate-limiting factor for HCD synthesis in these tissues when ample beta-alanine is provided through supplementation. Low levels of L-histidine have previously been observed in mouse WAT and BAT (Zhou et al., 2021). The discrepancies in concentrations between studies may be explained by amino acid and HCD concentrations being below the limit of detection or due to different methodologies being used for quantification (Boldyrev et al., 2013).

3.4.6 Body weight and tissue fat infiltration

Ingestion of beta-alanine in HFD mice attenuated the increase in body weight gain. This is consistent with other studies that have demonstrated decreases in body weight gain in HFD rodents supplemented with carnosine (Al-Sawalha et al., 2019; Mong et al., 2011; Rašković et al., 2023; Schaalan et al., 2018; Yang et al., 2014). The analysis of dietary food consumption (group and individual cage data) revealed comparable food and energy intake among the HFD groups. Therefore, the observed difference in weight gain cannot be attributed to a reduction in energy intake in the HFD BA mice. HFD VC mice exhibited an increase in energy expenditure when compared to all treatment groups. Energy expenditure is the sum of basal metabolic rate (energy expended whilst at rest), the thermic effect of feeding (energy required for the digestion, absorption, and storage of nutrients), and the thermic effect of activity (energy expended during physical activity). The increase in energy expenditure in HFD VC mice cannot be explained by the activity levels of the mice, as the mice were less motile (pedestrian meters) compared to all

other groups. Additionally, HFD mice were consuming a diet predominantly containing fat; therefore, the increase in energy expenditure cannot be explained by the thermic effect of feeding, as carbohydrates and protein will elicit a larger thermic effect of feeding, increasing energy expenditure when compared to the consumption of fat (carbohydrate: 6–8 %; protein: 25–30 %; fat: 2–3 %). Therefore, the elevation in energy expenditure may be explained by HFD-induced thermogenesis or the higher body weight in HFD VC mice (Licholai et al., 2018; Rothwell and Stock, 1982). Previous literature has shown a positive association between body weight and energy expenditure (Corrigan et al., 2020). This may be explained by the kinetic energy law: the movement of a larger mass necessitates more energy, and by extension, larger animals require increased energy for both resting metabolism and activity under standard conditions (Kleiber, 1932; White and Seymour, 2005). Further analysis of the relationship between energy expenditure and body weight in this study using linear regression did not show a significant difference between groups, potentially suggesting it is the HFD that is causing the elevation in energy expenditure rather than the body weight. Additionally, the prevention of weight gain with the HFD in the beta-alanine-supplemented groups cannot be explained by a change in RER. Weight loss can occur from shifting from utilising lipid oxidation to glucose oxidation due to an increase in the metabolic economy (higher yield of ATP per mole of oxygen) from glucose when compared to fat. There was no difference in RER or $\dot{V}O_2$ between the HFD VC and HFD BA groups.

An obesogenic diet will lead to the deposition of fat into multiple organs and tissues (Mathur et al., 2007), resulting in an increase in their weight, including the liver (Tsuru et al., 2020), BAT (Shimizu et al., 2014), and pancreas (Mathur et al., 2007). Previous

studies have demonstrated a reduction in liver weight and fat accumulation in rodent obesity models treated with carnosine (Aydın et al., 2017; Mong et al., 2011; Yang et al., 2014). There was no change in liver weight between the groups in this study; however, the increase in liver triglyceride concentration with the consumption of a HFD was attenuated with the administration of beta-alanine, suggesting less fat infiltration into the liver in beta-alanine-supplemented HFD mice. The pancreatic weight was increased in HFD VC mice; this may be due to pancreatic steatosis, where an increase in fat intake leads to pancreatic inflammation and increased fat infiltration (Zhao et al., 2015). This increase in pancreatic weight was attenuated in HFD BA mice, suggesting the supplement decreases the HFD pancreatic response by reducing inflammation and/or fat infiltration in the tissue; however, this needs to be confirmed using histological analysis. Histological analysis showed an increase in lipid droplet size in BAT of HFD VC mice, suggesting that increased fat infiltration is responsible for the increase in BAT weight in this group. The administration of beta-alanine decreased the lipid droplet sizes in BAT to levels similar to those seen in CHOW mice, suggesting that beta-alanine supplementation also affects the infiltration of fat into BAT.

The observed reduction in body weight, fat accumulation, liver triglyceride concentration, and pancreatic weight with beta-alanine supplementation in HFD mice, compared to HFD VC mice, could imply a potential effect on lipid metabolism and nutrient absorption. Pancreatic lipases play a crucial role in dietary fat digestion by hydrolysing the triglycerides into fatty acids and monoglycerides, aiding their absorption and storage in the adipose tissue (Birari and Bhutani, 2007; De Pradhan et al., 2017; Emimmal and Sankar, 2019). Excess fat deposition leads to the development of obesity.

If pancreatic lipases are inhibited, the digestion of triglycerides is impeded, reducing the absorption and storage of dietary fat (Birari and Bhutani, 2007). Orlistat, an FDA-approved treatment for weight loss in the UK, specifically targets pancreatic lipases, inhibiting fat digestion and absorption to reduce energy intake through gastrointestinal mechanisms (Birari and Bhutani, 2007). A primary side effect of this treatment is gastrointestinal issues, such as oily stools and spotting, indicating that the system is excreting ingested fats. To investigate whether beta-alanine supplementation may be utilising this pathway, future studies should include the collection of faeces to assess digestive efficiency [the fraction of calories consumed that are absorbed by the gut versus lost to stool (Reho et al., 2022; Soto et al., 2019)], as well as examination of the gastrointestinal system and lipase activity assays. It is important to note that this theory is conjecture and requires further investigation to determine the mechanisms behind the decrease in lipid metabolism. Mong et al. (2011) did not observe an effect of carnosine supplementation on faecal lipid concentration; however, this may be a direct effect of beta-alanine and may highlight differences between the roles beta-alanine and carnosine play.

If beta-alanine is utilising these mechanisms, it may explain why beta-alanine supplementation decreases the weight of the WAT gonadal pad in HFD mice. Adipocyte hypertrophy (increase in adipocyte cell size) and hyperplasia (increase in the number of adipocyte cells) contribute to the expansion of WAT during the consumption of a HFD. Histological analysis of WAT showed no difference in adipocyte size between HFD VC and HFD BA mice, suggesting that beta-alanine supplementation does not affect the storage of triglycerides in existing adipocytes. Hyperplasia involves the proliferation of

adipocytes to store additional triglycerides during the development of obesity (Faust et al., 1978), triggered when adipocytes have reached a 'maximum capacity'. Hyperplasia is enhanced during the consumption of a HFD (Jo et al., 2009), particularly in C57BL/6 mice (Faust et al., 1978; Jo et al., 2009). Therefore, the decrease in WAT with beta-alanine supplementation may be due to there being fewer triglycerides to store, reducing the need for hyperplasia.

Obesity has been shown to promote the activation of adipose tissue inflammation. The accumulation of WAT induces oxidative and metabolic stress, recruiting pro-inflammatory cytokines (Hotamisligil, 2017). During histological analysis, it was observed that there appeared to be non-adipose cells, possibly inflammatory, in WAT of HFD VC mice, suggesting pronounced inflammatory infiltration in HFD VC sections that was not observed in HFD BA WAT (**Appendix 2**). As this was beyond the scope of the research hypothesis, further research is required to investigate the direct role of beta-alanine in the inflammatory pathogenesis of obesity.

These macroscopic observations of liver, WAT, and BAT led to further investigation through RNA-Seq. RNA-Seq was not completed on the pancreas due to further research being required to select the most appropriate pancreatic cell type to study; this could not be completed within the time constraints of this project. The pancreas is a heterogeneous tissue, comprised of multiple endocrine (in the islets of Langerhans) and exocrine (acinar and duct tissue) cell types. Single-cell technologies for RNA-Seq would allow the profiling of gene expression levels in individual cells; however, the cells

targeted in this analysis would need to be carefully selected. The RNA-Seq analyses for the liver, WAT, and BAT will be processed for publication.

As mentioned previously, similar RER values observed in both the HFD VC and HFD BA groups, which suggests that gastrointestinal metabolism may not be the primary mechanism responsible for the positive effects of beta-alanine on body weight and tissue fat infiltration; the fat continues to enter the gastrointestinal system and undergoes metabolism in both groups. Notably, body weight was significantly lower in HFD BA mice, compared to HFD VC mice, at week seven. This attenuation in body weight gain in HFD BA may be explained by the increase in pedestrian meters, reflecting enhanced activity levels. Beta-alanine supplementation has been shown to improve exercise capacity (Hobson et al., 2012; Saunders et al., 2017b). The HFD BA mice may have been able to sustain physical activity (i.e., walking) for longer durations than the HFD VC mice by utilising carnosine's ergogenic effect as an intramuscular hydrogen ion buffer (Hill et al., 2007). Since muscle acidosis contributes to the onset of fatigue during physical activity, beta-alanine supplementation in this study may have increased intramuscular carnosine concentrations, thereby increasing intracellular buffering capacity, delaying the onset of fatigue, and allowing for prolonged activity. While beta-alanine supplementation appeared to have improved the exercise capacity of the HFD mice, no additional benefit of beta-alanine supplementation in CHOW mice was observed when comparing their activity levels to CHOW VC.

HFD BA mice were more active than HFC VC mice, showing higher accumulated pedestrian meters in the Promethion Core; however, it is not possible to determine

whether this increased activity is directly responsible for the reduced weight gain, or if the attenuation of weight gain allowed them to be more active. The HFD BA mice did not, however, exhibit an increase in energy expenditure when compared to HFD VC mice, which would be expected with an increase in activity levels. Future research should consider measuring skeletal muscle carnosine concentration and serially monitoring the activity of the mice throughout the experimental timeline, rather than only during the final week, as was done in this study. This limitation arose because the mice could not remain in the Promethion Core system for the full duration of the study due to project license constraints and severity limits, including single-housing restrictions.

3.4.7 Limitations

The main limitation of this study is the translatability of the findings from mice to humans. It is important to acknowledge the difference in beta-alanine dose between animal and human participants (Dolan et al., 2019), as well as the difficulty in selecting the optimal dose. Recommendations suggest that equivalent mouse dosing is 12.3 times the human dose (Nair and Jacob, 2016); however, this is based on systemic methods of administration (injections). Retrospective calculations for this experiment, based on the average drinking water intake for each mouse in the beta-alanine group, show that the dose provided in this study was nearly equivalent to the maximum dose used in the literature for human populations [6.4 g/day (Trexler et al., 2015)]. The calculation is detailed below:

1. For a 60 kg human, a dose of 6400 mg/day (6.4 g/day) equates to 106 mg/kg/day.
2. The equivalent mouse dose is 1303.8 mg/kg/day (106 mg/kg/day x 12.3).
3. For an average 30 g mouse, 40 mg/day is required (1303.8 mg/kg/day x 0.03 kg).

4. The amount of beta-alanine required in each mL of water to achieve 40 mg/day, based on the mouse drinking 3.7 mL/day, is 10.8 mg/mL (40 mg/day divided by 3.7 mL/day).
5. For 100 mL stock, 1080 mg is required (10.8 mg/mL x 100 for 100 mL), which equates to 1.08 % beta-alanine.

A Western diet is considered to be composed of 45% kcal from fat and 30% kcal from sugar; therefore, a 45 % HFD with sucrose-supplemented water may be considered more relevant to the human population (Speakman, 2019). This experiment implemented a 60 % HFD as it was a more economical method (in terms of time and resources) to reliably maximise the development of obesity at a faster rate compared to a 45 % HFD. The findings from this study need to be validated in an *in vivo* human model.

Functional myocardial measurements, such as blood pressure and echocardiography, would have strengthened the study; however, these could not be conducted under the project license conditions. The assessment of cardiac mitochondrial function was attempted with the first cohort of mice; however, potentially due to the length of time between culling and mitochondrial isolation, the mitochondria were not viable. Additionally, the experiment should be repeated in female mice to address both sexes' behavioural and metabolic responses to the diet and beta-alanine supplementation.

3.4.8 Summary

This study provides valuable insights into the potential role of beta-alanine supplementation on weight gain and tissue fat infiltration- observations not part of the

original hypothesis- highlighting the need for additional research to address the emerging mechanistic actions identified and suggested, including lipase activity assays and faecal analysis. The mice were supplemented with beta-alanine whilst consuming a HFD; obesity had not been induced first. This approach allowed the preventive role of beta-alanine supplementation during the development of a metabolic disease to be explored. Future research needs to investigate the restorative function of beta-alanine by supplementing mice after an obesogenic phenotype has been established. This would allow the translation of findings into individuals with obesity, who are likely to implement interventions once obesity has developed. It is important to note that addressing obesity typically involves a multifaceted approach, including, diet, physical activity, and lifestyle modifications. Therefore, the study of a combination of beta-alanine supplementation with diet and physical activity interventions in obesity is also of particular importance.

3.5 Conclusion

Beta-alanine supplementation was taken up into mouse cardiac tissue and synthesised into carnosine and, subsequently, anserine. 12 weeks of a HFD did not induce any morphological or histological changes in the heart, preventing any potential beneficial effects of beta-alanine supplementation and HCDs on histopathology from being observed. Beta-alanine supplementation had an ameliorative effect on body weight gain, WAT accumulation, and pancreatic weight in HFD mice, potentially indicating an effect on fat infiltration. Further research is required to understand the mechanisms beta-alanine is utilising in body weight and tissue fat accumulation when exposed to a HFD.

Chapter 4

Beta-alanine supplementation on cardiac function and health in humans with overweight and obesity: a randomised, controlled feasibility study

4.1 Introduction

Overweight and obesity are characterised by excessive abdominal fat accumulation and chronic, systemic, low-grade inflammation (Calder et al., 2011) and are associated with metabolic diseases such as hypertension, dyslipidaemia, and CVD. CVD is the leading cause of morbidity and mortality worldwide, with it being responsible for approximately two-thirds of deaths related to high BMI (The GBD 2015 Obesity Collaborators, 2017). Lifestyle modifications, including physical activity and diet changes, are important for the management and prevention of overweight and obesity, yet they can be challenging to implement, long-term adherence is difficult to sustain and effectiveness varies between individuals (Fappa et al., 2008). Therefore, there is a need for the development of safe, cost-effective, and sustainable strategies. These should be aimed at preventing disease progression, preserving cardiovascular structural and functional outcomes, and improving cardiometabolic health in individuals with overweight and obesity.

Emerging *in vivo* evidence from animal models suggests that HCDs, such as carnosine, may have implications for cardiovascular function and health by exhibiting effects on calcium handling and EC coupling (Gonçalves et al., 2021), and haemodynamic parameters (Aldini et al., 2011; Niijima et al., 2002). Human studies evaluating the effects of carnosine on cardiovascular function and health in clinical populations are limited. Supplementation of 0.5 g/day of carnosine for six months did not improve markers of cardiac function measured using transthoracic echocardiography (TTE) in patients with chronic heart failure (Lombardi et al., 2015). Similarly, 12-weeks supplementation of 2 g/day of carnosine in adults with overweight or obesity showed no changes in blood lipid markers, blood pressure, or high-sensitivity C-reactive protein [hs-CRP (De Courten et

al., 2016)]. A notable limitation of these existing studies is that the low dose of carnosine used is likely to have only modest effects on tissue carnosine concentrations, which may limit the potential impact of the HCD.

Oral carnosine supplementation is inefficient at increasing intramuscular carnosine content in humans because the HCD is hydrolysed into its constituent amino acids (beta-alanine and L-histidine) by tissue carnosinase in the small intestine. Any remaining intact carnosine enters the circulation where the peptide bonds are degraded by serum carnosinase (Perry et al., 1968). Beta-alanine has been identified as the rate-limiting precursor for carnosine synthesis (Harris et al., 2006). While beta-alanine supplementation has been shown to increase skeletal muscle carnosine content in humans (Hill et al., 2007), its impact on cardiac tissue remains uncertain. In the *in* vivo pre-clinical study, the findings of which are presented in **Chapter 3,** 12 weeks of 1.2 % beta-alanine supplementation increased carnosine concentration in the cardiac tissue of mice. Furthermore, beta-alanine is a more cost-effective option than carnosine, addressing the economic requirements for feasible lifestyle interventions.

A meta-analytical assessment of human trials evaluating beta-alanine and carnosine supplementation on cardiometabolic outcomes (Matthews et al., 2021) showed a high risk of bias in the existing literature, including the study by De Courten et al. (2016). A Cochrane risk of bias 2.0 assessment highlighted concerns regarding participant randomisation and biases in the measurement, selection, and reporting of outcomes (Matthews et al., 2021). These studies (de Courten et al., 2016) also exhibited baseline imbalances that were not adjusted for in statistical analyses, increasing the risk of chance

bias. The study by Lombardi et al. (2015) also presents a risk of bias due to it adopting an open-label approach, lacking a clear randomisation process, and not reporting adherence, acceptability, or side-effect outcomes. Supplementation of beta-alanine has not yet been trialled in a human clinical population with an associated CVD comorbidity, such as overweight and obesity. Although beta-alanine supplementation is considered safe in healthy populations (Dolan et al., 2019), research needs to explore the side effects, adherence, and therapeutic potential of beta-alanine supplementation in lower-risk clinical populations, such as individuals with overweight and obesity, before extending investigations to higher-risk groups (e.g., peripheral arterial disease or chronic heart failure patients). Therefore, the current study aimed to address these shortcomings within the study design and results.

The principal research objective of this study was to assess the feasibility (whether this study can be completed) and tolerability (including attrition rates, blinding effectiveness, and side effects) of three months of beta-alanine supplementation in adults with overweight or obesity. The secondary research objective was to investigate the potential impact of beta-alanine supplementation on cardiovascular function and health, exploring its influence on haemodynamic, echocardiographic, and biochemical markers. These research objectives were selected to determine the relevant methodology, estimates of tolerability, and outcome variability to aid the planning of future, full-randomised controlled trials to assess the efficacy of beta-alanine supplementation as a therapeutic strategy.

Note: This study is a subset of a larger study completed in collaboration with Dr Joseph J. Matthews (a fellow PhD candidate at NTU, supervised by Professor Craig Sale, Professor Kirsty Elliott-Sale, Dr Mark Turner, and Dr Livia Rocha Dos Santos). The exploratory outcomes of the study also include cardiometabolic measurements, which are included and discussed in Joseph's thesis. The publication of this study will include all measurements; however, not all preregistered outcomes were explored due to the absence of change in other markers and change in study population. This larger study has been accepted for publication in Obesity (23/10/24), with an open-access dataset (zenodo.org/records/14165012).

4.2 Materials and methods

4.2.1 Ethical approval

The study was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice. Ethical approval was granted by the NTU and the NHS Health Research Authority [HRA (REC reference: 21/NW/0280)] ethics committees. The study was preregistered on www.clinicaltrials.gov (Study ID: NCT05329610). All testing and data collection were carried out at NTU.

4.2.2 Recruitment

The study was conducted between March 2022 and July 2023 and comprised of two phases of participant recruitment. In the first phase of recruitment, the inclusion criteria required participants to have a glycated haemoglobin (HbA1c) value between 42 and 48 mmol/mol (6.0 to 6.4 %). The study population was defined as adults with prediabetes. Recruitment primarily occurred through primary care services in the Nottinghamshire area. Five local General Practice's (GPs) screened their electronic patient databases to identify eligible individuals meeting the following criteria: aged 18 to 75 years, BMI $\geq$ 25 to < 40 kg/m^2 and HbA1c 42 to 48 mmol/mol (6.0 to 6.4 %). Eligible individuals received a standardised letter containing the Participant Information Sheet (**Appendix 5**). Secondary recruitment strategies targeted both the local and NTU communities. This involved circulating recruitment posters on social media platforms [Nottinghamshire resident Facebook groups, X (formerly Twitter), and the Nextdoor app], within local community groups (Nottingham U3a and libraries), around NTU Clifton campus, and in GP waiting areas. A study advertisement was placed in the local newspaper (Nottingham

Post, with a reported readership of 24,754). Enrolled participants were provided with study posters to aid recruitment through word of mouth (participant referral). Prospective participants were instructed to contact the research team if interested in participating.

In July 2022, due to challenges with recruitment, the study protocol was amended; the eligibility criteria were widened by removing the requirement for a baseline HbA1c value of 42 to 48 mmol/mol (6.0 to 6.4 %). This amendment was necessary to ensure the study could be completed within the doctoral studies timeframe. The NHS HRA ethics amendment was given a favourable opinion on 1st September 2022. As a result, the study population was redefined as being adults with overweight or obesity. Those who initially visited the laboratory in the first recruitment phase but were deemed ineligible due to an HbA1c < 42 mmol/mol (< 6.0 %) were re-invited via email. In the second recruitment phase, four GPs screened their patient databases, including two GPs who newly joined the recruitment process and two who re-contacted previously screened individuals. The screening used the same criteria mentioned above (including an HbA1c value in the prediabetes range). The updated recruitment poster was redistributed in the same locations as previously described. Additionally, the study was advertised on university-wide communication platforms, including NTU's internal networks Yammer and All-company, as well as on the institution's website.

4.2.3 Experimental design

The study used a triple-blinded, randomised, placebo-controlled, parallel-group design. The supplements were manufactured, supplied, and blinded by Natural Alternatives

International (Carlsbad, CA, USA). Natural Alternatives International had no role in study design, methods, or analyses. Blinding was maintained for all participants, data collectors, and outcome assessors. Supplement allocation was disclosed by the manufacturer only after data analyses were completed. A schematic representation of the study design is provided in **Figure 4.1**. Testing for visits one and three took place in the morning after an overnight fast. Participants were not required to fast for visit two. For all visits, participants were instructed to refrain from vigorous exercise and alcohol consumption for 24 hours and to avoid caffeine for 12 hours before each visit. This was confirmed verbally upon arrival to the laboratory.

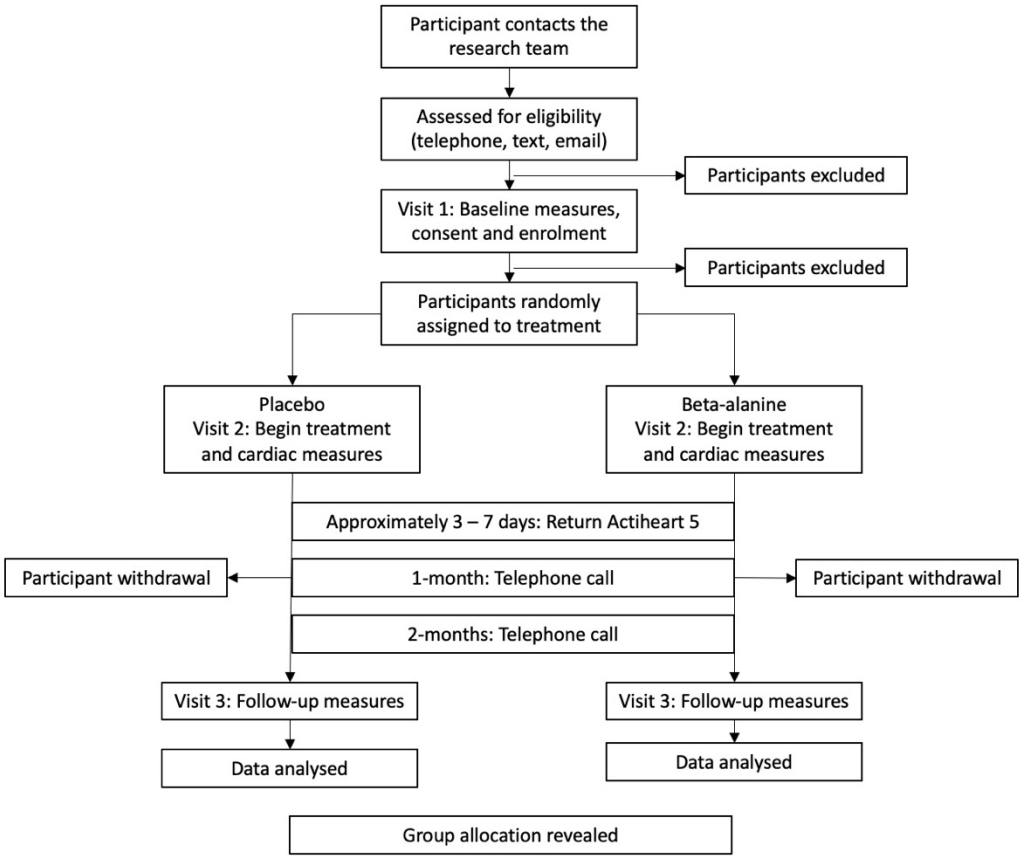


Figure 4.1. Overview of the study design.

4.2.3.1 Initial screening

Participants were assessed for eligibility via telephone or email prior to visit one. BMI was measured during visit one to confirm eligibility. The exclusion criteria included: weight loss or gain of $\geq$ 5 kg in the prior six months; current participation in another clinical research trial; substance abuse, presence of an eating disorder or purging behaviour; known mental health illness requiring active treatment; known cognitive impairment; inability to understand conversational English; the presence of type-1 *diabetes mellitus* or type-2 *diabetes mellitus*; use of weight loss or glucose-lowering drugs (e.g., orlistat, thyroxine, metformin, insulin, glucagon-like-peptide-1 analogues), long-term corticosteroids or other drugs which may impact on the measurement of study outcomes; use of carnosine or beta-alanine supplements in the prior six months; currently breastfeeding, pregnant or consideration of pregnancy; or known comorbidities which may impact on study aims (e.g., cancer, heart failure, or chronic kidney disease) or measurement of study outcomes (e.g., sickle cell anaemia or previously known haemoglobinopathy).

4.2.3.2 Visit one: Baseline measures, consent, and enrolment

Participants were provided with verbal and written participant information before completing a general health history questionnaire (**Appendix 6**) and providing written informed consent (**Appendix 7**). Participants were then re-assessed for eligibility. **Table 4.1** lists the experimental procedures completed during visit one, further detailed in section **4.2.7**.

4.2.3.3 Visit two: Begin treatment and cardiac measures

Table 4.1 lists the experimental procedures completed during visit two, further detailed in section **4.2.7**.

4.2.3.4 Visit three: Follow-up measures

Table 4.1 lists the experimental procedures completed during visit three, further detailed in section **4.2.7**. During this visit, participants were asked if they had made any conscious changes to their diet, lifestyle, or medication during the intervention.

4.2.3.5 Monthly telephone calls

Participants received a telephone call one and two months into the intervention, where the number of tablets taken or missed was noted (adherence). Participants completed the modified General Assessment of Side Effects (GASE) questionnaire (**Appendix 8**).

Table 4.1. Experimental procedures completed at each laboratory visit

Visit 1 (baseline)	Visit 2	Visit 3 (follow-up)
Height	CNAP	Body weight
Body weight	TTE	Waist circumference
Waist circumference	GASE questionnaire	Hand-grip strength
Hand-grip strength	Actiheart 5	GASE questionnaire
GASE questionnaire	Collect/begin treatment	24-hour dietary recall
24-hour dietary recall		Blood and urine samples
Blood and urine samples		CNAP
		TTE
		Actiheart 5

Abbreviations: CNAP, continuous non-invasive blood pressure; GASE, General Assessment of Side Effects; TTE, transthoracic echocardiography.

4.2.3.6 Sample size

General guidelines suggest that 12 participants per arm are required to estimate a parameter for feasibility trials (Julious, 2005). Therefore, the target enrolment for this study was 15 participants per arm (30 participants total), to account for an assumed attrition rate of 20 % (due to participant withdrawal).

4.2.3.7 Minimisation procedure

A 1:1 group allocation was performed using a minimisation procedure; sex, age, BMI, and HbA1c, all recorded at visit one (baseline), were used as prognostic factors to minimise imbalances. A member of the research team, not involved with participant recruitment or data collection, performed the minimisation and allocated participants to receive either the placebo or beta-alanine intervention.

4.2.4 Supplementation

Participants received either sustained-release beta-alanine (SR CarnoSyn® beta-alanine tablet, Natural Alternatives International, Carlsbad, CA, USA) or a taste- and appearance-matched tapioca starch placebo (Natural Alternatives International, Carlsbad, CA, USA) in tablet form at a dose of 4.8 g/day for three months. Despite altered plasma pharmacokinetics, sustained-release beta-alanine is highly efficacious for muscle carnosine synthesis (Stellingwerf et al., 2011; Varanoske et al., 2019). If participants had taken all the tablets provided, this would provide a 60 to 80 % increase in muscle carnosine content (Harris et al., 2006). This dose is considered safe, with no associated adverse events or implications on clinical markers of renal, hepatic, and muscle function (Dolan et al., 2019). Participants were required to take the supplement in 4 doses of 2 x

600 mg tablets daily. Participants were instructed to consume each dose alongside their main meals (i.e., breakfast, lunch, and dinner) and before bed; however, depending on the participant's routine, the supplement did not have to be taken with food.

The first dose of the intervention was administered in the laboratory during visit two after cardiac measurements were completed. Participants were supervised for 30 minutes to monitor side effects. The most widely known side effect of beta-alanine is paraesthesia, which has been described as a “pins and needles” or “tingling” sensation occurring within 20 minutes of consumption and lasting up to one hour. It has been reported to affect the ears, forehead, scalp, upper trunk (including the arms and back of hands), and the base of the spine and buttocks (Harris et al., 2006). This study aimed to ameliorate these side effects by using the sustained-release formula of beta-alanine, which results in a lower peak plasma concentration of beta-alanine compared to an equivalent dose of free beta-alanine (Décombaz et al., 2012).

4.2.5 Primary outcome measure

4.2.5.1 Adherence

Adherence, the probability that a randomised participant received the assigned intervention, was recorded at three time points (one month, two months, and follow-up) from supplement diaries and tablet counts (adherence = actual supplement ingested (g)/ expected supplement ingested (g)). To promote adherence, participants were provided with a weekly pill organiser box and received weekly automated text messages to remind them to organise and take their supplements (FireText Communications Ltd). Participants were also provided with an information sheet on how to take their

supplement (**Appendix 9**) and a diary to record each dose (**Appendix 10**). Participants reported supplement intake during the monthly telephone calls (section **4.2.3.5**) and were instructed to return their supplement diary along with any remaining tablets at follow-up.

4.2.6 Secondary outcome measures

4.2.6.1 Attrition rate

Attrition rate was measured as the probability that a randomised participant completed both baseline and follow-up measures.

4.2.6.2 Side effects

The presence and severity of side effects were recorded using a modified version of the GASE questionnaire [**Appendix 8** (Rief et al., 2009)]. Participants completed the GASE questionnaire on five different occasions: at visit 1 (baseline), visit 2 (30 minutes after ingestion of the first dose), the 1-month telephone call, the 2-month telephone call, and at visit 3 (follow-up). The GASE questionnaire results were analysed by separating the scores into paraesthesia side effects and other side effects, with the severity scores summed for each category.

4.2.6.3 Blinding

Blinding to the intervention was evaluated at follow-up using a -1, 0, +1 scale. Participants were asked whether they thought they had been assigned to the placebo group (-1), the beta-alanine group (+1), or did not know (0).

4.2.7 Exploratory participant-centred outcome measures

4.2.7.1 Anthropometrics

Height was measured to the nearest 0.1 cm using a stadiometer (Seca, Hamburg, Germany) with participants' shoes removed. The stretch stature method was used, with the head positioned in the Frankfort horizontal plane. Body weight was measured to the nearest 0.1 kg with participants wearing minimal clothing. BMI was calculated as weight (kg) divided by height squared (m^2). Waist circumference was measured to the nearest 0.1 cm using a flexible, non-elastic tape (HaB Direct, Warwick, UK) at the narrowest point of the abdomen, between the lower costal border and the top of the iliac crest. Participants were instructed to take a deep inhalation followed by a gentle exhalation, with the waist circumference measurement being taken at the end of the exhalation.

4.2.7.2 Handgrip strength

Handgrip strength was measured using a Jamar hydraulic handgrip dynamometer, following the Southampton protocol (Roberts et al., 2011). Participants sat in a standard chair with forearms resting on the arms of the chair, and wrists positioned just over the end of the chair arm. The position of the dynamometer handle was adjusted according to participant's hand size and comfort; this position was noted at baseline and replicated at follow-up. The researcher supported the base of the dynamometer on the palm of their hand during the procedure to negate the effect of gravity on peak strength. Participants were instructed to squeeze the dynamometer handle and were told to stop when the needle stopped rising. Grip strength was recorded to the nearest 1 kg. Each hand was tested in triplicate, and the highest of the six measurements was used in statistical analyses.

4.2.7.3 Dietary intake

Participants specified their habitual dietary preference at baseline [i.e., mixed (omnivorous), vegetarian, or vegan]. Since beta-alanine can be acquired through the consumption of foods such as meat, poultry, and fish, habitual dietary decisions can influence intramuscular concentrations of beta-alanine and carnosine (Everaert et al., 2011; Harris et al., 2007). Dietary intake was assessed using a 24-hour recall at both baseline (the 24 hours preceding baseline) and follow-up (the 24 hours preceding follow-up). Participants were asked to replicate, as closely as possible, the diet they followed in the 24 hours before baseline for the 24 hours preceding the follow-up. Although no analysis was conducted on this data for the thesis, it was used to ensure consistency between visits.

4.2.7.4 Blood sampling and analysis

Fasting venous blood samples (20 mL) were obtained from the antecubital fossa using a 21G needle (Becton, Dickinson and Company, USA) at baseline and follow-up. Participants remained in a semi-reclined, resting position throughout the procedure. Samples were collected sequentially into pre-coated collection tubes (Becton, Dickinson and Company, USA) in the following order: lithium heparin (green tube), SST™II (yellow tube), K2/EDTA (purple tube), and sodium fluoride/potassium oxalate (grey tube). Each tube was inverted 6 to 10 times. A 10 μL aliquot of whole blood was immediately taken from the K2/EDTA tube, before centrifugation, for the immediate analysis of HbA1c using a Quo-Lab® HbA1c Analyzer (EKF Diagnostics, Germany) for the minimisation allocation. The SST™II tube was incubated at room temperature for 30 minutes to allow for clotting

before centrifugation. All tubes were centrifuged at 2000 x *g* at 4 °C for 10 minutes. The resulting plasma and sera were then aliquoted and frozen at -80 °C for future analysis.

Plasma concentrations of hs-CRP, alkaline phosphatase (ALP), alanine transaminase (ALT), aspartate aminotransferase (AST), creatine kinase (CK), gamma-glutamyl transpeptidase (GGT), lactate dehydrogenase (LDH), albumin, total bilirubin, total protein, and urea, as well as serum concentrations of total cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL), triglycerides, and creatinine were measured spectrophotometrically using a Pentra c400 clinical chemistry analyser and associated ABX Pentra assays [**Table 4.2** (Horiba ABX S.A, France)]. All samples from each participant were analysed in the same run to eliminate inter-assay variation, with each sample measured in duplicate. Controls and calibrators were run prior to each assay. The results of these blood markers are also presented in Joseph J Matthews' thesis.

4.2.7.5 Urine sampling and analysis

Urine samples were collected at baseline and follow-up. Participants were required to have voided in the morning before attending the laboratory. Participants were instructed to collect a mid-stream urine sample of approximately 30 mL. The urine sample was decanted into 2 x 15 mL Falcon tubes and centrifuged at 400 x *g* at 4 °C for 10 minutes. The supernatant was sterile filtered (0.2 µm) and aliquoted into individual Eppendorf tubes before being frozen at -80 °C for future analysis. The results of the urine markers are presented in Joseph J Matthews' thesis.

Table 4.2. Penta c400 assay parameters

Assay	Assay range	Limit of detection	Limit of quantitation	Calculated linear assay (R^2)
CVD and lipid biomarkers				
Total Cholesterol (mmol/L)	0.10 – 15	0.10		0.993
LDL (mmol/L)	0.04 – 9.5	0.04		0.9963
HDL (mmol/L)	0.05 – 4.5	0.02	0.05	0.992
Triglyceride (mmol/L)	0.08 – 67.2	0.08		0.9994
CRP high sensitivity (mg/L)	0.12 – 100		0.12	0.996
CK (U/L)	8 – 4500	8		0.9930
LDH (U/L)	10 – 3600	10		0.991
Liver and kidney function				
ALP (U/L)	6 – 6000	6		0.998
ALT (U/L)	4 – 1800	4		0.9964
AST (U/L)	4 – 1800	4		0.9966
Creatinine (µmol/L)	12.2 – 4800		12.2	0.999
GGT (U/L)	4 – 3000	4		0.9984
Albumin (g/dL)	0.46 – 11.2	0.02		0.9889
Total bilirubin (µmol/L)	2.4 – 1350	1.49	2.4	0.995
Total protein (g/L)	6.0 – 200	1.4	6.0	0.9796
Urea (mmol/L)	0.31 – 250	0.31		0.996

Taken from (accessed 27th November 2023):
https://toolkits.horiba-abx.com/documentation/navigation.php?relDir=clinical_chemistry%2F01_reagent_notices%2F03_pentra_c400
Abbreviations: ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate aminotransferase; CK, creatine kinase; CRP, c-reactive protein; CVD, cardiovascular disease; GGT, gamma-glutamyl transpeptidase; HDL, high-density lipoprotein; LDH, lactate dehydrogenase; LDL, low-density lipoprotein.

4.2.7.6 Continuous non-invasive blood pressure

Non-invasive continuous haemodynamic measurements were recorded using a CNAP® (continuous non-invasive blood pressure) Monitor (CNSystems, Graz; Austria). The CNAP® Monitor allows for the measurement of blood pressure continuously in real-time, using plus contour analysis to determine haemodynamic parameters non-invasively. The system provides outputs for continuous finger blood pressure (systolic, diastolic, mean arterial pressure, and heart rate) and advanced haemodynamics [systemic vascular resistance (SVR) and systemic vascular resistance index (SVRI)].

The CNAP® monitoring system consists of a reusable double-finger cuff, the cuff controller, a non-invasive blood pressure (NBP) cuff, and the CNAP® monitor. Infrared light sensors detect blood flow in the double-finger cuff. The continuous adjustment of the inflation and deflation of the finger cuffs maintains a constant blood flow and pulsation by exerting pressure on the artery. The pressure required to maintain constant blood flow corresponds to the real arterial blood pressure. Continuous blood pressure is automatically calibrated using the oscillometric blood pressure values obtained at the brachial artery with the NBP arm cuff, providing absolute arterial blood pressure values rather than finger arterial pressure. The monitor displays the recorded arterial pressure waveform and calculates the corresponding haemodynamic values.

An appropriately sized NBP cuff (adult or large adult) was positioned on the upper part of the left arm, with the tubing placed directly above the brachial artery. The finger cuff size (small, medium, or large) was determined using the graphic on the CNAP controller. If a participant's finger fell between two sizes, the larger cuff was selected. Both the NBP

cuff and finger cuff sizes were noted at baseline to ensure consistent set-up at follow-up. The double-finger cuff was then positioned on the proximal joints of the index and middle fingers of the left hand. The finger cuffs were connected to the cuff controller, and the cuff controller was fastened around the participant's forearm using the Velcro fixation strap, ensuring it remained on the upper side of the participant's arm. For support and comfort, a box and rolled-up towel were placed under the participant's hand and arm. This also helped to keep the tubing that connects the double-finger cuff to the cuff controller as flat as possible (**Figure 4.2**).

Participants' biometric data (age, height, weight, and sex) were required for automatic calibration. Participants remained in a supine position, in the dark and with no noise throughout the 20-minute measurement. NBP calibration was performed at the beginning and then again 10-minutes into the procedure. The alarm system on the monitor was disabled to ensure the participant remained in complete silence. The 5 minutes of continuous data collected immediately after the 10-minute NBP calibration were used for statistical analyses.



Figure 4.2. CNAP monitor set up. Image taken from the manufacturer manual.

4.2.7.7 Resting transthoracic echocardiography

Resting TTE is a non-invasive method using ultrasound to provide real-time images of the beating heart and vessels for the quantitative assessment of cardiac function and anatomy (Lang et al., 2015). The images in this study were obtained according to the American Society of Echocardiography and the European Association of Cardiovascular Imaging (Lang et al., 2015). A systematic approach using 2-dimensional (2D) and movement-mode (M-mode) echocardiography was used for the comprehensive assessment of the heart at baseline and follow-up.

TTE measurements were recorded and analysed by an experienced sonographer using a commercial ultrasound system (Vivid e95 cardiac ultrasound, GE Healthcare) with a 4 MHz cardiac transducer. The sonographer was blinded to the condition of the trial. Participants were required to lie in the left lateral decubitus position for the duration of the examination, with comfort breaks provided as needed. Two windows of the heart

were analysed, avoiding bones/ribs: parasternal (adjacent to the sternum, on the left side of the heart) and apical (heart apex). The TTE scans took approximately 30 minutes to complete, depending on the participant's echogenicity (image quality). Echogenicity can vary between individuals due to differences in myocardium anatomy, acoustic windows, body composition, and disease state (Lang et al., 2015). Images were recorded during apnoea at end-expiration, and a minimum of three cardiac cycles were stored digitally to account for inter-beat variation. Offline analysis was performed by the same experienced sonographer using commercially available software (Echo-pac version 204.x, GE Medical Systems, Norway), with measurements averaged over a minimum of three consecutive cardiac cycles. Measurements were taken in triplicate, with the mean values being calculated and reported.

A 2D view was used for the evaluation of ventricular structure, and M-mode imaging was used to analyse the structural movement of the myocardium during the cardiac cycle. A 2D parasternal long axis orientation was acquired by placing the transducer probe on the $3^{rd}/4^{th}$ intercostal space to the left of the sternum, with the probe marker positioned towards the right shoulder. From this placement, the left ventricle minor axis dimensions were quantified from the M-mode echocardiography, facilitating the offline analysis of interventricular septal end diastole and systole (IVSd and IVSs), posterior wall thickness end diastole and systole (PWd and PWs), and left ventricular internal diameter end diastole and systole (LVIDd and LVIDs). During M-mode, end-diastolic measurements were taken when the left ventricular cavity was at its largest, and end-systolic measurements were taken when the left ventricular cavity was at its smallest.

2D echocardiography was used to assess left ventricular volume, with the apical 4-chamber (AP4CH) and 2-chamber (AP2CH) views being used to trace the left ventricular end-systolic volume (ESV) and end-diastolic volume (EDV). The transducer probe was positioned at the left 5th intercostal space (midclavicular) for the AP4CH view, with the transducer then being rotated 60° counterclockwise to capture the AP2CH view. SV was calculated using EDV and ESV, and CO was subsequently calculated from this. Stroke volume index (SVI) and cardiac index (CI) were calculated by normalising SV and CO to body surface area [BSA (Mostellar, 1987)]. The Simpson's biplane method, recommended by the American Society for Echocardiography for left-ventricle volume quantification (Lang et al., 2015), was used for left ventricular EF analysis. The assessment of left ventricular systolic function was calculated from the 2D volume data. Left ventricular EF was calculated using the following equation:

$$EF\ (\%) = (EDV - ESV) / EDV$$

4.2.7.8 Actiheart 5

The Actiheart 5 is a wearable, combined single-lead device for synchronous recordings of heart rate and activity. The monitor consists of an triaxial accelerometer to detect and quantify free-living physical activity and movement, and ECG sensors for continuous heart rate monitoring and analysis of cardiac inter-beat interval (IBI) variability.

Participants were fitted with a sample ECG electrode (Dormo, Pulse Medical, UK) at visit 1 and advised to wear it for 24 hours to ensure there was no significant discomfort from skin irritation or reaction. Participants were fitted with an Actiheart 5 (CamNtech Ltd,

Cambridge, UK) at the end of visit two and visit three according to the manufacturer's instructions. The participant's skin was cleaned with soap and water, and skin prep pads (Medigraphics Ltd, UK) were then used to remove the non-conductive skin layer to help reduce noise levels. If needed, participants were shaved prior to skin preparation. One ECG electrode was attached to the centre of the chest, below the apex of the sternum, and the Actiheart device with the wire connected was attached to this. The second ECG electrode was attached to the Actiheart clip at the end of the wire and placed into position on the same lateral level (**Figure 4.3**). This position is recommended to reduce noise from movement artefacts. For female participants, if needed, electrodes were placed approximately 10 cm higher. After the Actiheart was attached, a signal test was completed using the commercial software [Actiheart Software (version 5.1.24) 64 bit, CamNtech Ltd] to ensure appropriate placement and an adequate signal. The Actiheart was then set up for full waveform analysis (with IBI recordings), with data sampled at 256 Hz: 50 Hz (ECG:ACC). Participants were instructed to wear the device for 72 consecutive hours and document any times the device was removed [e.g., for water activities, when it became unattached (**Appendix 11**)]. Data were downloaded from the device and analysed offline using the Actiheart Software.



Figure 4.3. Actiheart 5 electrode placement. Image taken from CamNtech Ltd.

4.2.7.8.1 Daily energy expenditure analysis

The Daily Energy Expenditure window in the commercial software generated an overall summary of day-to-day energy expenditure. The Actiheart 5 system uses the recorded heart rate and activity levels, and requires participant age, weight, height, and sleeping heart rate to calculate activity energy expenditure (AEE). Sleeping heart rate was set in the Advanced Energy Expenditure window using the average heart rate over the recorded nights. The branched model, Group Cal JAP2007, was applied for AEE analysis; this is a group calibration of heart rate and activity (Brage et al., 2004), recommended for work conducted on adults where a step test calibration cannot be performed. REE was estimated using the Schofield equation (Schofield, 1985), which measures the basal metabolic rate adjusted for body weight. TEE was calculated as the sum of REE, AEE, and dietary-induced thermogenesis (DIT). DIT was estimated as 10 % of TEE, and physical activity level (PAL) was calculated by dividing TEE by REE. The average of two complete days of data was used in the statistical analysis, except for one participant where only one day of data was analysed at baseline and follow-up due to skin reaction. Participants

were excluded from analysis if their data required auto-filling (indicated by the software) or if they removed the Actiheart for sleeping.

4.2.7.8.2 Inter-beat interval analysis

Post-test analyses were completed at epoch 1, and activity counts, heart rate (bpm), average IBI, minimum IBI in the epoch, maximum IBI in the epoch, standard deviation of the IBI data (SD), and the root mean square of the successive differences of IBIs (RMSSD) were obtained. The low-frequency [LF (0.04–0.15 Hz)] and high-frequency [HF (0.15–0.4 Hz)] components were derived from the Fast Fourier Transform and were then used to calculate the ratio of the LF component to the HF component (LF/HF).

Each full day of data was analysed and averaged, with further analysis conducted on the sleeping and waking hours. The duration of missing data points was calculated for full day, sleeping hours, and waking hours data. To be included in the full-day and waking hour analyses, at least 10 hours of valid waking data was required over a 24-hour period (Simpson et al., 2022). Sleep onset and sleep termination times were determined as described in Collings et al. (2015) (**Figure 4.4**). Sleep onset was defined as the start of a prolonged absence of activity, coupled with a gradual decrease in heart rate, while sleep termination was identified by the recommencement of activity following an extended duration of minimal or no activity, accompanied by a sudden increase in heart rate. Sleeping and waking data were excluded when a participant's sleep onset and termination could not be determined. If participants only wore the device during waking hours, their data were removed from the full-day and sleeping hours analyses. The

software automatically excluded IBI points if more than 50 % of the time period was missing, with the corresponding activity and heart rate values being manually excluded.

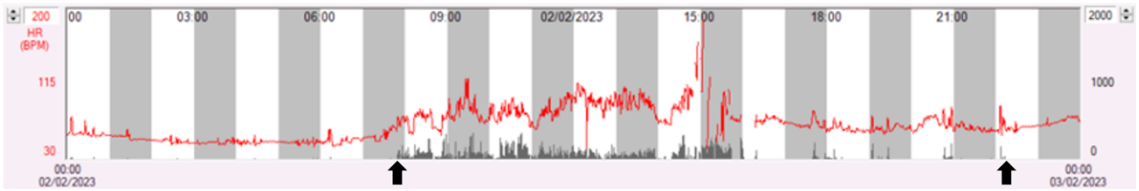


Figure 4.4. An example of sleep onset and sleep termination identification in the Actiheart 5 software. The black arrows indicate the start and end of sleep time. Red lines indicate heart rate and black lines indicate activity levels.

4.2.8 Statistical analysis

All analyses were performed using GraphPad Prism 10. Data are reported as mean ± SD unless stated otherwise. A one-way ANOVA was used to examine between-group differences for supplementation duration and supplement total intake. A two-way ANOVA was completed on all physiological measures, with one factor of treatment (with two levels: placebo or beta-alanine) and one factor of time (with two levels: baseline and follow-up); time was selected as the repeated measure. For significant main effects or interactions, a Bonferroni post-test was used. The GASE questionnaire results are reported as descriptive statistics (median aggregated score). For the Actiheart 5 analyses, full-day and waking hours measures were analysed using linear regression and ANCOVA; activity count was used as the covariant to determine if the effect of activity on the IBI variables was statistically significant. The analyses for publication, as outlined in the preregistration, will use a Bayesian approach.

4.3 Results

4.3.1 Participant recruitment

Participant inclusion at each stage of the study is illustrated in **Figure 4.5**.

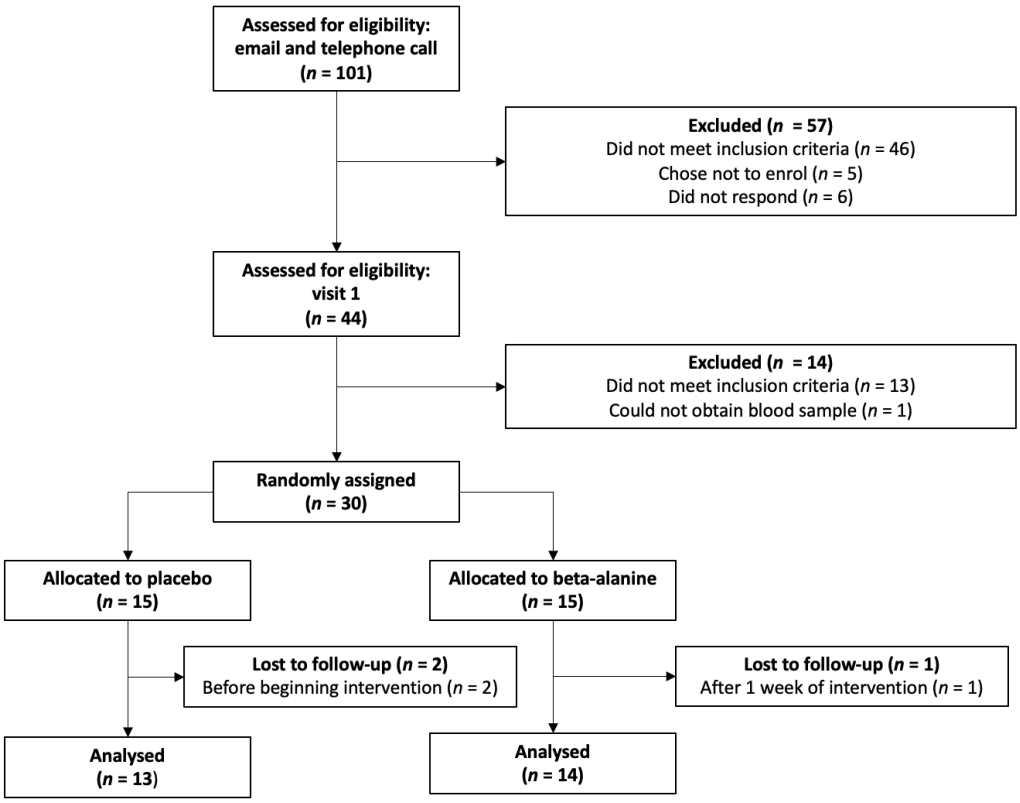


Figure 4.5. CONSORT flow diagram.

The first recruitment phase occurred between March 2022 and September 2022, during which 5 GPs distributed 839 recruitment letters. The research team deemed the recruitment rates unfeasible within the doctoral studies timeframe, prompting a revision of the eligibility criteria. The second recruitment phase occurred between September 2022 and March 2023, during which 4 GPs distributed 475 recruitment letters (121 of which were sent to individuals already contacted during the first recruitment phase). The resulting numbers of individuals screened and enrolled in both phases are presented in

Table 4.3. The recruitment approach involving GP letters resulted in the highest number of enrolled participants.

Table 4.3. Individuals screened and enrolled from each recruitment strategy

Strategy	Participants screened	Participants enrolled
First recruitment phase*		
GP letters	41	4
Community groups	5	2
Unknown	1	0
Second recruitment phase**		
GP letters	26	15
Community groups	3	1
Flyer location not stated	2	0
Social media	4	1
Participant referral	3	3
Nottingham Trent University	11	4
Unknown	5	0

* inclusion criteria included HbA1c value in prediabetes range. ** removal of HbA1c value in prediabetes range from inclusion criteria.

4.3.2 Participant characteristics

A total of 27 participants completed the intervention, with 13 participants (8 male and 5 female) allocated to the placebo group and 14 participants (7 male and 7 female) allocated to the beta-alanine group. Participant characteristics are summarised in **Table 4.4** and **Figure 4.6**, with the groups being well-matched across the minimisation prognostic factors. Results, according to BMI, categorised four participants as overweight and nine participants as obese in the placebo group, and seven participants as

overweight and seven participants as obese in the beta-alanine group. One participant was categorised as vegetarian in each group.

Further analysis was completed to calculate the metabolic syndrome severity risk for each participant at baseline and follow-up. This was calculated using the BMI or waist circumference, fasting glucose, triglycerides, HDL, and systolic blood pressure outcomes (Gurka et al., 2014). The score was adjusted for age (adult equation), ethnicity, and sex to allow for an individualised estimation of risk. The equation by Gurka et al. (2014) does not include an Asian ethnicity option. Both *z*-scores and percentiles based on BMI and waist circumference are reported in **Table 4.4** and **Figure 4.6**. Four participants were excluded from this analysis: three from the beta-alanine group [no systolic blood pressure measurement (1) and Asian ethnicity (2)], and one from the placebo group (Asian ethnicity). Based on the metabolic severity risk score, most participants were classified as having a higher risk of metabolic syndrome. There were no differences between baseline and follow-up or between treatment groups. Some individuals did show a decrease in their metabolic syndrome severity risk score between baseline and follow-up; however, this was observed in both treatment groups.

Table 4.4. Participant characteristics at baseline and follow-up

	Placebo		Beta-alanine		ANOVA		
	Baseline	**Follow-up**	**Baseline**	**Follow-up**	**Treatment**	**Time**	**T x T**
Age (years)	59 ± 10		57 ± 11				
Height (m)	1.687 ± 0.127		1.702 ± 0.083				
Weight (kg)	90.4 ± 15.3	90.8 ± 15.8	89. 2 ± 14.9	89.2 ± 14.4	0.815	0.578	0.627
BMI (kg/m^2)	31.6 ± 3.0	31.7 ± 3.2	30.6 ± 2.9	30.6 ± 2.9	0.385	0.603	0.639
Waist circumference (m)	1.03 ± 0.14	1.03 ± 0.13	1.03 ± 0.13	1.01 ± 0.11	0.884	0.398	0.132
Handgrip strength (kg)	37 ± 15	35 ± 13	39 ± 15	38 ± 16	0.744	0.090	0.612
HbA1c (mmol/mol)	40.2 ± 4.6	40.5 ± 6.4	39.5 ± 4.0	40.0 ± 4.1	0.749	0.526	0.901
HbA1c (%)	5.8 ± 0.4	5.9 ± 0.6	5.8 ± 0.4	5.8 ± 0.4	0.765	0.463	0.861
MetS *z*-score (BMI)	0.54 ± 0.51	0.57 ± 0.58	0.34 ± 0.41	0.39 ± 0.58	0.369	0.503	0.887
MetS percentile (BMI) (%)	68.63 ± 16.45	68.93 ± 16.66	62.40 ± 14.06	62.99 ± 18.93	0.379	0.800	0.936
MetS *z*-score (waist circumference)	0.46 ± 0.57	0.51 ± 0.63	0.32 ± 0.43	0.35 ± 0.59	0.529	0.575	0.919
MetS percentile (waist circumference) (%)	65.74 ± 18.74	66.44 ± 18.90	61.79 ± 15.88	62.06 ± 20.05	0.582	0.808	0.915

Metabolic syndrome was calculated using Gurka et al. (2014). Data are presented as mean ± SD. Abbreviations: ANOVA, analysis of variance; BMI, body mass index; HbA1c, glycated haemoglobin; MetS, metabolic syndrome.

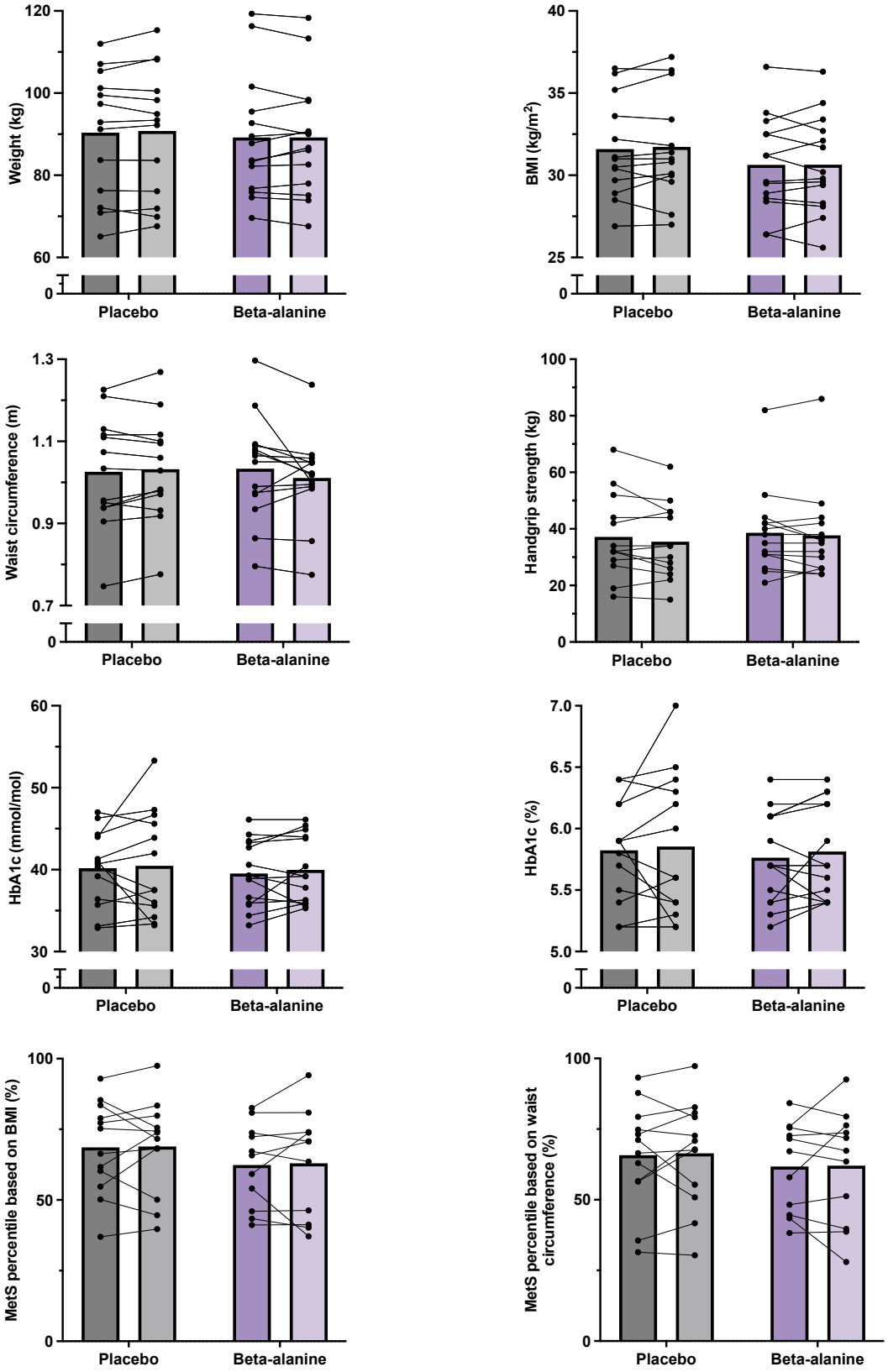


Figure 4.6. Participant characteristics at baseline (dark bar) and follow-up (light bar) of the three-month intervention. Data are presented as mean and individual values with before-after lines. Statistical data are presented in Table 4.4. Abbreviations: BMI, body mass index; HbA1c, glycated haemoglobin; MetS, metabolic syndrome.

4.3.3 Primary and secondary outcomes

The primary and secondary criteria evaluated the feasibility and tolerability of the study. These are reported as continuous outcomes.

4.3.3.1 Adherence

Participants were supplemented for 87 ± 4 days in the placebo group and 87 ± 8 days in the beta-alanine group, with no differences between groups (p=0.733). Adherence, as estimated from the mean return tablet count each month, was not statistically different between the placebo and beta-alanine groups (main effect of treatment; p=0.787) and remained consistent throughout the three-month supplementation period (main effect of time; p=0.127) (**Figure 4.7**). There was no treatment-by-time interaction for supplement adherence (p=0.864). Participants had a total supplement intake of 380.9 ± 36.7 g in the placebo group and 386.4 ± 48.3 g in the beta-alanine group, with no differences between groups (p=0.746).

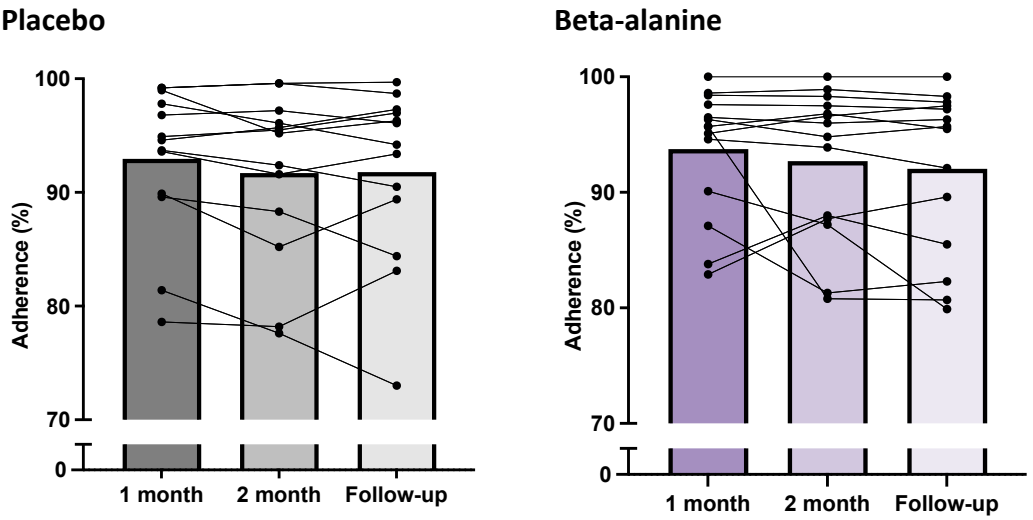


Figure 4.7. Supplement adherence (%) across the three-month intervention. Data presented as mean with individual values and before-after lines.

4.3.3.2 Attrition rate

Attrition was lower in the beta-alanine group (7 %) than in the placebo group (13 %). The two dropouts from the placebo group occurred prior to receiving the intervention, with the reasons cited as no response to the research team and venepuncture issues. The one dropout from the beta-alanine group occurred after one week of commencing supplementation.

4.3.3.3 Side effects

There were no adverse events during the intervention, with the participant-reported side effects showing comparable side effects between the placebo and beta-alanine groups that remained below baseline values (**Table 4.5**). Participant-reported paraesthesia was higher in the beta-alanine group compared to the placebo group; however, the highest prevalence was at baseline and remained below this value throughout the intervention.

Table 4.5. Participant reported side effects throughout the intervention

	Placebo	Beta-alanine
Paraesthesia		
Baseline	0 (0 – 1) [8%]	0 (0 – 4) [43%]
30 minutes post first dose	0 (0) [0%]	0 (0 – 1) [7%)
1-month telephone call	0 (0 – 1) [8%]	0 (0) [0%]
2-month telephone call	0 (0) [0%]	0 (0 – 4) [21%]
Follow-up	0 (0 – 2) [8%]	0 (0 – 3) [7%]
Other side effects		
Baseline	4 (0 – 11)	5 (0 – 16)
30 minutes post first dose	0 (0 – 6)	2 (0 – 10)
1-month telephone call	2 (0 – 13)	2 (0 – 11)
2-month telephone call	3 (0 – 19)	5 (0 – 12)
Follow-up	3 (0 – 21)	3 (0 – 10)

Data are presented as median aggregated score (range) and paraesthesia prevalence [%].

4.3.3.4 Blinding

Blinding scores were similar across the placebo and beta-alanine groups, with most participants in both groups scoring as 'placebo' or 'don't know' (**Table 4.6**).

Table 4.6. Blinding responses to the intervention at follow-up

Blinding Responses	Placebo	Beta-alanine
Placebo	5	8
Beta-alanine	1	2
Don't know	7	4
Blinding score index (-1 to +1)	**-0.31**	**-0.43**

4.3.4 Exploratory participant-centred outcomes

4.3.4.1 Lipid and CVD-related blood markers

There were no differences between the placebo and beta-alanine groups for all lipid and CVD-related blood markers, with no change between baseline and follow-up in either group (**Table 4.7** and **Figure 4.8** and **Figure 4.9**).

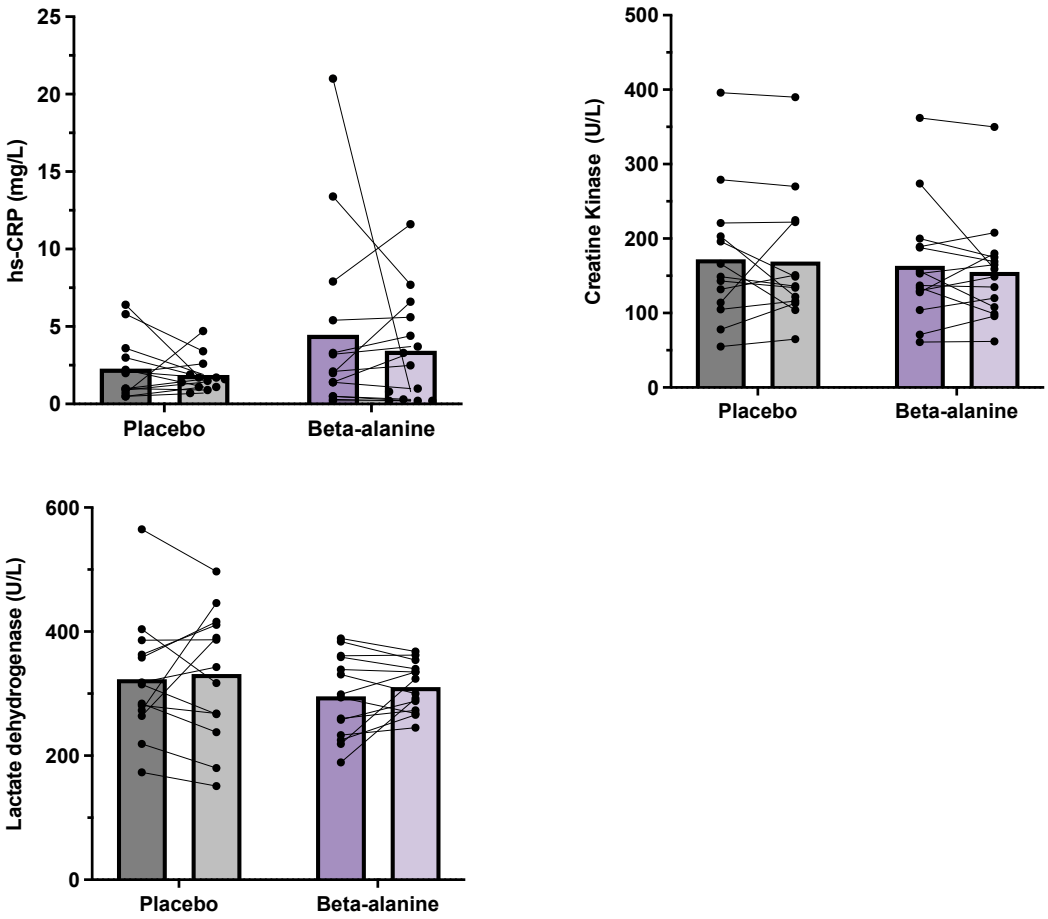


Figure 4.8. Serum high-sensitivity C-reactive protein (hs-CRP), creatine kinase and lactate dehydrogenase (LDH), measured using a Pentra c400, at baseline (dark bar) and follow-up (light bar) of the three-month intervention. Data are presented as mean and individual values with before-after lines. Statistical data are presented in Table 4.7.

Table 4.7. Lipid and CVD-related biochemical markers, measured using a Pentra c400, at baseline and follow-up (also presented in another PhD thesis: Joseph J Matthews)

	Placebo		Beta-alanine		ANOVA		
	Baseline	**Follow-up**	**Baseline**	**Follow-up**	**Treatment**	**Time**	**T x T**
Total cholesterol (mmol/L)	5.0 ± 1.5	5.0 ± 1.6	5.3 ± 1.0	5.1 ± 1.2	0.663	0.330	0.415
LDL (mmol/L)	3.0 ± 1.1	3.0 ± 1.2	3.1 ± 0.9	3.0 ± 1.1	0.846	0.384	0.654
HDL (mmol/L)	1.3 ± 0.4	1.3 ± 0.4	1.4 ± 0.3	1.3 ± 0.3	0.550	0.483	0.281
Triglycerides (mmol/L)	1.4 ± 0.5	1.4 ± 0.5	1.3 ± 0.4	1.4 ± 0.6	0.191	0.158	0.104
LDL/HDL ratio	2.5 ± 0.8	2.4 ± 0.9	2.5 ± 0.8	2.4 ± 1.0	0.904	0.441	0.826
Total cholesterol/HDL ratio	4.1 ± 1.0	4.1 ± 1.1	4.1 ± 0.9	4.0 ± 1.2	0.937	0.523	0.748
Hs-CRP (mg/L)	2.3 ± 2.0	1.9 ± 1.1	4.5 ± 6.0	3.4 ± 3.5	0.107	0.415	0.726
CK (U/L)	172 ± 91	169 ± 87	163 ± 79	155 ± 69	0.715	0.520	0.774
LDH (U/L)	323 ± 98	332 ± 105	296 ± 66	311 ± 40	0.406	0.338	0.782

Data are presented as mean ± SD. Abbreviations: CK, creatine kinase; HDL, high-density lipoproteins; hs-CRP, c-reactive protein high sensitivity; LDH, lactate dehydrogenase; LDL, low-density lipoproteins.

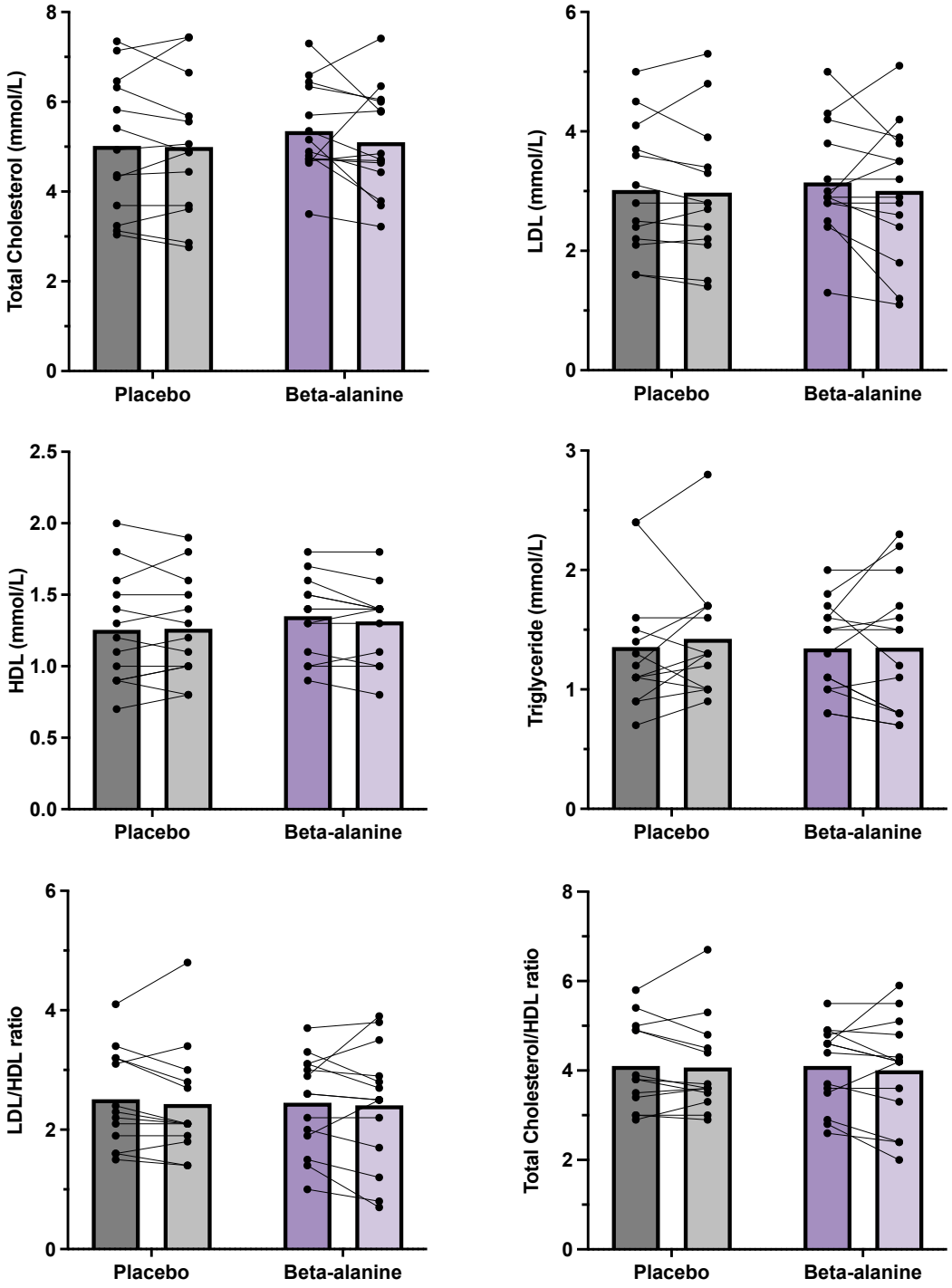


Figure 4.9. Blood lipid markers, measured using a Pentra c400, at baseline (black) and follow-up (purple) of the three-month intervention. Data are presented as mean and individual values with before-after lines. Statistical data are presented in Table 4.7. Abbreviations: HDL, high-density lipoproteins; LDL, low-density lipoproteins.

4.3.4.2 Liver and kidney function blood markers

Blood albumin concentrations had a significant main effect of time (p=0.002) but had no significant main effect of treatment (p=0.214) or treatment-by-time interaction (p=0.621). Post-test analysis showed a decrease in albumin in the beta-alanine group between baseline and follow-up (p=0.017). Blood urea concentrations showed a significant treatment-by-time interaction (F (1,25) = 4.310, p=0.048) but there were no significant main effects of time (p=0.939) or treatment (p=0.337). Post-test analysis showed no significant difference for blood urea concentration between treatment groups at baseline (p=0.203) or follow-up (p=>0.999), or within treatment group differences (beta-alanine, p=0.324; placebo, p=0.295). There were no differences between placebo and beta-alanine groups for all other liver and kidney function blood markers (ALP, ALT, AST, creatinine, GGT, total bilirubin, and total protein), with no change between baseline and follow-up in both treatments (**Table 4.8**).

Table 4.8. Liver and kidney biochemical markers, measured using a Pentra c400, at baseline and follow-up (also presented in another PhD thesis: Joseph J Matthews)

	Placebo		Beta-alanine		ANOVA		
	Baseline	**Follow-up**	**Baseline**	**Follow-up**	**Treatment**	**Time**	**T x T**
ALP (U/L)	81 ± 28	81 ± 27	82 ± 30	82 ± 31	0.939	0.803	0.803
ALT (U/L)	33 ± 16	33 ± 14	29 ± 12	31 ± 18	0.571	0.745	0.804
AST (U/L)	32 ± 8	31 ± 5	30 ± 5	31 ± 10	0.687	0.971	0.734
Creatinine (μmol/L)	97 ± 24	94 ± 22	92 ± 17	93 ± 19	0.696	0.207	0.146
GGT (U/L)	23 ± 16	28 ± 19	26 ± 17	28 ± 22	0.838	0.196	0.457
Albumin (g/L)	42.4 ± 2.0	41.4 ± 1.9	43.6 ± 2.6	42.3 ± 2.4*	0.214	**0.002**	0.621
Total bilirubin (μmol/L)	11.4 ± 4.3	11.7 ± 5.5	13.0 ± 6.0	13.1 ± 6.6	0.484	0.744	0.905
Total protein (g/L)	69.5 ± 2.9	69.2 ± 4.2	70.9 ± 5.2	70.3 ± 5.0	0.427	0.518	0.768
Urea (mmol/L)	5.1 ± 1.4	4.7 ± 1.1	4.3 ± 1.3	4.6 ± 1.4	0.337	0.939	**0.048**

Data are presented as mean ± SD. * significant within factor (time) differences. * $p<0.05$. Abbreviations: ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transpeptidase.

4.3.4.3 Continuous non-invasive blood pressure and haemodynamics

There were no differences between the placebo and beta-alanine groups for the continuous non-invasive blood pressure and haemodynamic parameters (systolic, mean arterial, diastolic, SVR, and SVRI), with no change between baseline and follow-up in both treatments (**Table 4.9** and **Figure 4.10**). There was a significant main effect of time on heart rate (p=0.041) but no significant main effect of treatment (p=0.733) or treatment-by-time interaction on heart rate (F (1, 24) = 0.2403, p=0.134). Post-test analysis showed a decrease in heart rate in the beta-alanine group between baseline and follow-up (p=0.030), with no change between baseline and follow-up in the placebo group (p>0.999). For all CNAP parameters, the beta-alanine group had an *n* of 13 due to technical issues with 1 participant's recording in visit 2.

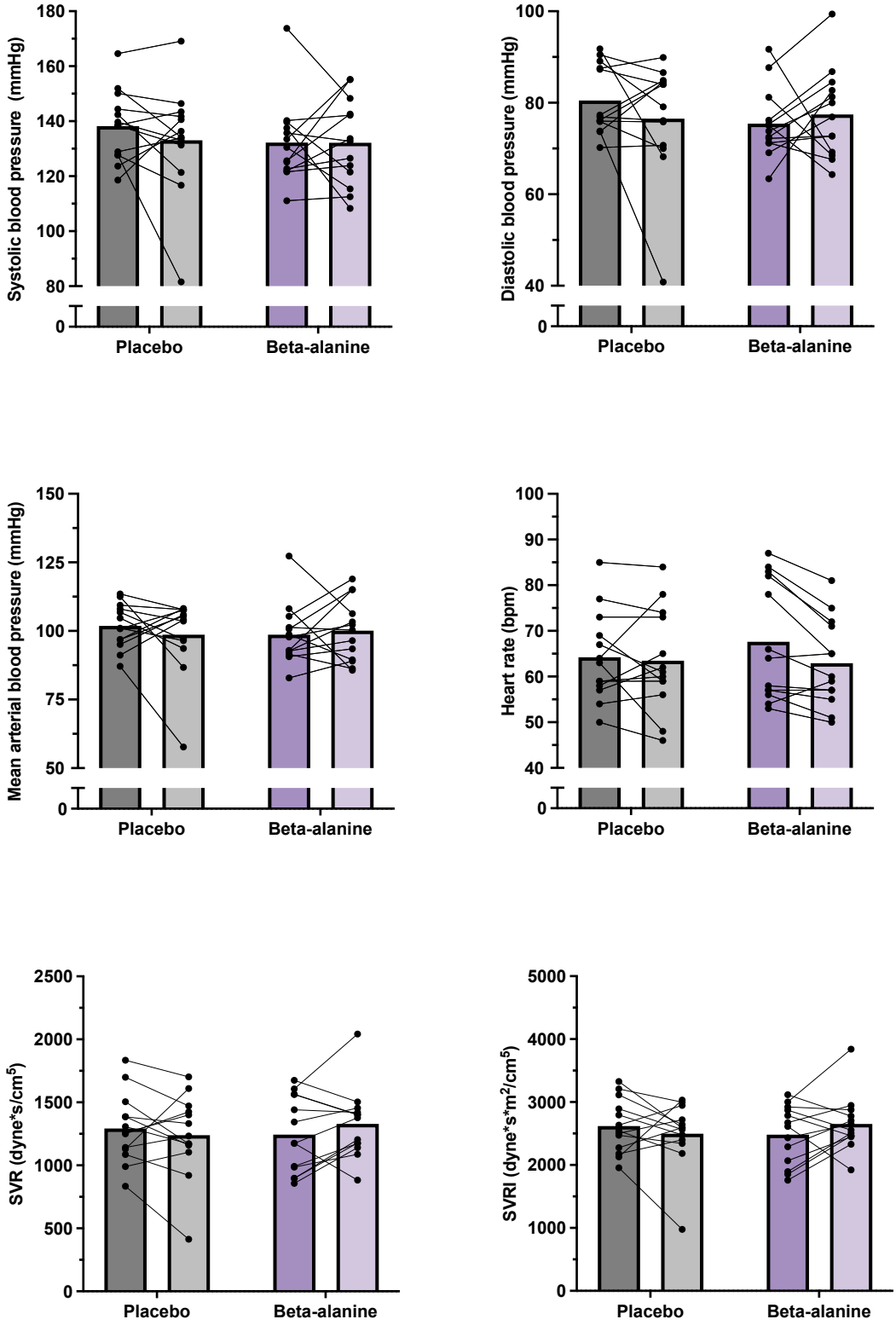


Figure 4.10. Markers of continuous finger blood pressure, measured using a CNAP monitor, at baseline (dark bar) and follow-up (light bar) of the three-month intervention. Data are presented as mean and individual values with before-after lines. Statistical data are presented in Table 4.9. Abbreviations: SVR, systemic vascular resistance; SVRI, systemic vascular resistance index.

Table 4.9. Haemodynamic parameters, measured using a CNAP monitor, at baseline and follow-up

	Placebo		Beta-alanine		ANOVA		
	Baseline	**Follow-up**	**Baseline**	**Follow-up**	**Treatment**	**Time**	**T x T**
Continuous finger BP							
Systolic (mmHg)	138.2 ± 12.9	133.1 ± 20.0	132.2 ± 15.1	132.1 ± 15.8	0.523	0.456	0.469
Diastolic (mmHg)	80.5 ± 7.5	76.5 ± 12.8	75.4 ± 7.6	77.4 ± 9.7	0.819	0.743	0.381
Mean arterial (mmHg)	101.8 ± 8.2	98.6 ± 14.0	98.7 ± 10.9	100.1 ± 11.3	0.490	0.683	0.218
Heart rate (bpm)	64 ± 10	63 ± 11	68 ± 13	63 ± 10*	0.733	**0.041**	0.134
Advanced haemodynamics							
SVR (dyne*s/cm^5)	1293.1 ± 276.5	1239.7 ± 328.1	1224.2 ± 302.9	1328.3 ± 278.7	0.854	0.734	0.151
SVRI (dyne*s*m^2/cm^5)	2617.3 ± 431.5	2497.3 ± 522.4	2483.2 ± 469.0	2652.4 ± 442.1	0.947	0.799	0.143

Data are presented as mean ± SD. * significant within factor (time) differences. * $p<0.05$. Abbreviations: BP, blood pressure; SVR, systemic vascular resistance; SVRI, systemic vascular resistance index.

4.3.4.4 Resting transthoracic echocardiography

There were no differences between the placebo and beta-alanine groups for all TTE structural parameters [IVSd, LVIDd, LVPWd, LVIDs, left ventricular outflow tract (LVOT), and right ventricular outflow tract (RVOT)], with no change between baseline and follow-up in both treatments (**Table 4.10** and **Figure 4.11**). Due to echogenicity and imaging issues, data analyses include an *n* of 10 for the placebo group and *n* of 12 for the beta-alanine group.

There were no differences between the placebo and beta-alanine groups for the TTE functional parameters (ESV, EDV, LV EF, SV, CO, SVI, and CI), with no change between baseline and follow-up in both treatments (**Table 4.11** and **Figure 4.12**). There was a significant main effect of time on heart rate (p=0.021) but no significant main effect of treatment (p=0.621) or treatment-by-time interaction on heart rate (F (1, 22) = 0.001, p = 0.979). Post-test analysis showed no difference between baseline and follow-up for the placebo (p=0.180) or beta-alanine group (p=0.192). Due to echogenicity and imaging issues, data analyses include an *n* of 12 for both groups.

Table 4.10. Cardiac structure parameters, measured using TTE, at baseline and follow-up

	Placebo		Beta-alanine		ANOVA		
	Baseline	**Follow-up**	**Baseline**	**Follow-up**	**Treatment**	**Time**	**T x T**
IVSd (cm)	1.0 ± 0.2	1.0 ± 0.1	0.9 ± 0.2	0.8 ± 0.2	0.255	0.121	0.393
LVIDd (cm)	5.0 ± 0.4	5.2 ± 0.6	4.9 ± 0.5	4.9 ± 0.6	0.312	0.335	0.479
LVPWd (cm)	1.0 ± 0.1	1.1 ± 0.2	1.0 ± 0.1	0.9 ± 0.2	0.287	0.789	0.190
LVIDs (cm)	3.4 ± 0.7	3.4 ± 0.6	3.4 ± 0.4	3.3 ± 0.5	0.916	0.367	0.489
LVOT (cm)	2.9 ± 0.3	2.9 ± 0.3	2.9 ± 0.2	2.9 ± 0.2	0.920	0.176	0.881
RVOT (cm)	3.1 ± 0.2	3.1 ± 0.3	3.0 ± 0.2	3.0 ± 0.3	0.474	0.907	0.478

Data are presented as mean ± SD. Abbreviations: IVSd, interventricular septal end diastole; LVIDd, left ventricular internal diameter end diastole; LVIDs, left ventricular internal diameter end systole; LVOT, left ventricular outflow tract; LVPWd, left ventricular posterior wall end diastole; RVOT, right ventricular outflow tract.

Table 4.11. Cardiac function parameters, measured using TTE, at baseline and follow-up

	Placebo		Beta-alanine		ANOVA		
	Baseline	**Follow-up**	**Baseline**	**Follow-up**	**Treatment**	**Time**	**T x T**
Heart rate (bpm)	66 ± 14	62 ± 12	64 ± 11	60 ± 8	0.621	**0.021**	0.979
ESV (mL)	50 ± 17	51 ± 17	50 ± 13	53 ± 15	0.888	0.424	0.625
ESV/BSA (mL/m^2)	24 ± 7	24 ± 6	24 ± 6	26 ± 8	0.626	0.504	0.550
EDV (mL)	116 ± 33	121 ± 32	120 ± 30	125 ± 25	0.750	0.173	0.956
EDV/BSA (mL/m^2)	55 ± 12	57 ± 11	58 ± 13	61 ± 11	0.458	0.176	0.859
EF (%)	57 ± 7	58 ± 8	59 ± 4	58 ± 7	0.694	0.921	0.622
Stroke volume (mL)	66 ± 21	68 ± 21	70 ± 19	72 ± 14	0.598	0.310	0.947
SVI (mL/m^2)	31 ± 8	32 ± 8	34 ± 9	35 ± 6	0.318	0.345	0.989
Cardiac output (L/min)	4.1± 1.0	4.1 ± 1.3	4.4 ± 1.0	4.0 ± 0.6	0.789	0.419	0.277
CI (L/min/m^2)	2.0 ± 0.4	2.0 ± 0.5	2.2 ± 0.5	2.0 ± 0.3	0.516	0.358	0.358

Data are presented as mean ± SD. Abbreviations: BSA, body surface area; CI, cardiac index; EDV, end diastolic volume; EF, ejection fraction; ESV, end systolic volume; SVI, stroke volume index.

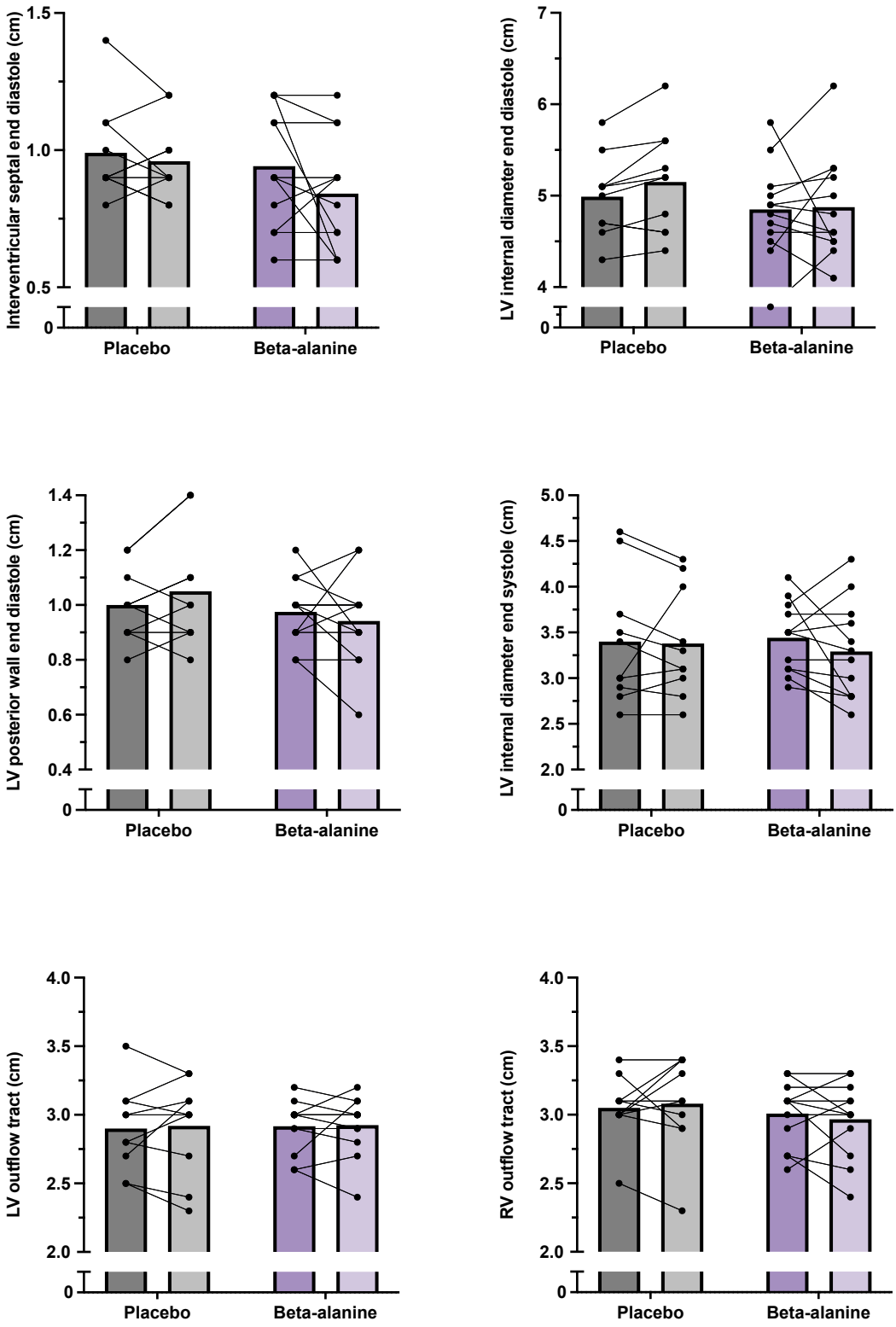


Figure 4.11. Cardiac structure parameters, measured using TTE, at baseline (dark bar) and follow-up (light bar) of the three-month intervention. Data are presented as mean and individual values with before-after lines. Statistical data are presented in Table 4.10. Abbreviations: LV, left ventricular; RV, right ventricular.

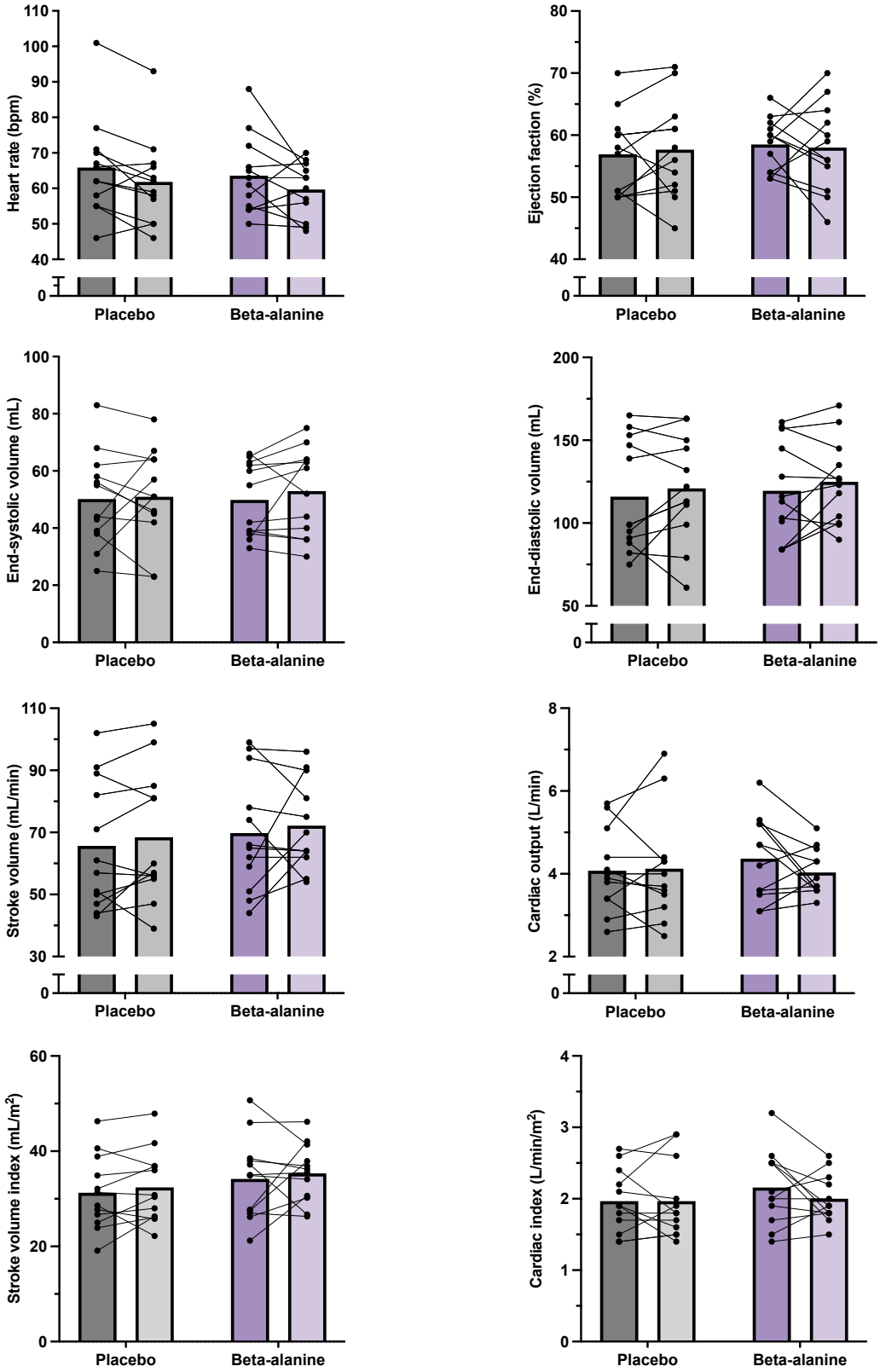


Figure 4.12. Cardiac function parameters, measured using TTE, at baseline (dark bar) and follow-up (light bar) of the three-month intervention. Data are presented as mean and individual values with before-after lines. Statistical data are presented in Table 4.11.

4.3.4.5 Actiheart 5

4.3.4.5.1 Energy expenditure

There were no differences between the placebo and beta-alanine groups for all estimated energy expenditure parameters (REE, AEE, DIT, TEE, and PAL), with no change between baseline and follow-up in both treatments (**Table 4.12** and **Figure 4.13**). Five participants were excluded from this analysis due to analysis issues (*n* = 2), reactions to the Actiheart electrodes (*n* = 1), and device removals at night (*n* = 2).

Table 4.12. Estimated energy expenditure parameters, measured using Actiheart 5, at baseline and follow-up

	Placebo		Beta-alanine		ANOVA		
	Baseline	**Follow-up**	**Baseline**	**Follow-up**	**Treatment**	**Time**	**T x T**
REE (kcal)	1617 ± 287	1620 ± 288	1715 ± 279	1712 ± 275	0.433	0.803	0.333
AEE (kcal)	710 ± 417	764 ± 386	706 ± 440	818 ± 614	0.890	0.419	0.776
DIT (kcal)	259 ± 58	265 ± 52	269 ± 67	281 ± 82	0.608	0.424	0.801
TEE (kcal)	2585 ± 578	2649 ± 521	2690 ± 667	2811 ± 824	0.609	0.425	0.803
PAL	1.61 ± 0.30	1.65 ± 0.28	1.57 ± 0.25	1.65 ± 0.34	0.831	0.373	0.796

Data are presented as mean ± SD. Abbreviations: AEE, activity energy expenditure; DIT, dietary-induced thermogenesis; PAL, physical activity level; REE, resting energy expenditure; TEE, total energy expenditure.

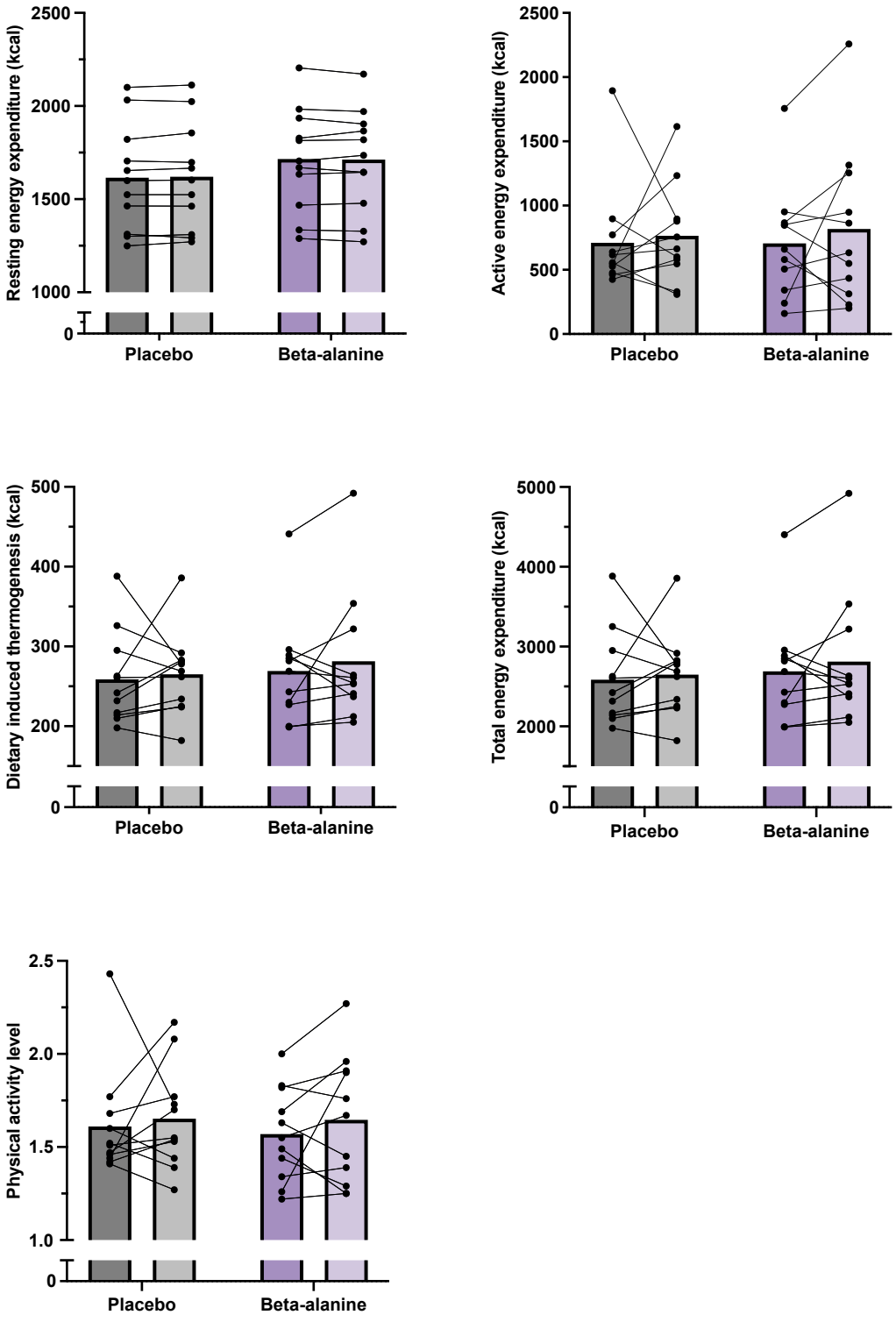


Figure 4.13. Estimated energy expenditure parameters, measured using Actiheart 5, at baseline (dark bar) and follow-up (light bar) of the three-month intervention. Data are presented as mean and individual values with before-after lines. Statistical data are presented in Table 4.12.

4.3.4.5.2 Full-day IBI analyses

There were no differences between the placebo and beta-alanine groups for all IBI analyses during full-day recordings (heart rate, average IBI, minimum IBI, maximum IBI, SD, RMSSD, LF, HF, and LF/HF), with no change between baseline and follow-up in both treatments (**Table 4.13**). Linear regression and subsequent ANCOVA for activity count determined no significant differences in slope angle between the different time points and treatment groups, indicating no significant effect of activity count on full-day IBI parameters. Eight participants' data were completely removed due to analysis issues (*n* = 5), participant reaction to the Actiheart electrodes (*n* = 1), and device removals at night (*n* = 2).

4.3.4.5.3 Waking hours IBI data analyses

There were no differences between the placebo and beta-alanine groups for IBI analyses during waking hours (heart rate, average IBI, minimum IBI, maximum IBI, SD, RMSSD, LF, and HF), with no change between baseline and follow-up in both treatments (**Table 4.14**). Linear regression and subsequent ANCOVA for activity count determined no significant differences in slope angle between the different time points and treatment groups, revealing no significant effect of activity count on full-day IBI parameters. There was a significant treatment-by-time interaction for LF/HF ($F(1, 16) = 5.062$, $p=0.039$), but there was no main effect of time ($p=0.711$) or treatment ($p=0.719$). Post-test analysis showed no differences between groups (baseline, $p=0.763$; follow-up, $p>0.999$) or within groups (placebo, $p=0.454$; beta-alanine, $p=0.133$). Nine participants' data were removed

due to analysis issues (*n* = 5), reaction to the Actiheart electrodes (*n* = 1), and being unable to identify sleeping and waking hours (*n* = 3).

4.3.4.5.4 Sleeping hours IBI data analyses

There were no differences between the placebo and beta-alanine groups for all IBI analyses during sleeping hours recordings (heart rate, average IBI, minimum IBI, maximum IBI, SD, RMSSD, LF, HF, and LF/HF), with no change between baseline and follow-up in both treatments (**Table 4.15**). Linear regression and subsequent ANCOVA for activity count determined no significant differences in slope angle between the different time points and treatment groups, indicating no significant effect of activity count on sleeping hours IBI parameters. 11 participants' data were completely removed due to analysis issues (*n* = 5), reaction to the Actiheart electrodes (*n* = 1), being unable to identify sleeping and waking hours (*n* = 3), and device removals at night (*n* = 2).

Table 4.13. Inter-beat interval parameters during full day, measured using Actiheart 5, at baseline and follow-up

	Placebo		Beta-alanine		ANOVA		
	Baseline	Follow-up	Baseline	Follow-up	Treatment	Time	T x T
Activity (counts)	27 ± 8	31 ± 18	28 ± 15	28 ± 10	0.787	0.534	0.623
Heart rate (bpm)	73 ± 10	72 ± 12	73 ± 9	73 ± 10	0.902	0.777	0.857
Average IBI (ms)	886 ± 120	873 ± 132	857 ± 93	859 ± 103	0.827	0.683	0.846
Minimum IBI (ms)	750 ± 119	762 ± 102	755 ± 79	753 ± 87	0.961	0.593	0.418
Maximum IBI (ms)	997 ± 125	1002 ± 180	979 ± 95	986 ± 124	0.772	0.746	0.949
SD (ms)	45.15 ± 12.07	48.97 ± 21.06	47.78 ± 9.38	49.79 ± 14.06	0.771	0.394	0.790
RMSSD (ms)	38.01 ± 17.44	40.93 ± 27.95	33.83 ± 11.7	36.11 ± 15.61	0.554	0.563	0.943
LF (ms^2)	898 ± 631	1558 ± 2116	1094 ± 387	1204 ± 624	0.842	0.278	0.435
HF (ms^2)	427 ± 292	696 ± 1167	408 ± 308	469 ± 406	0.606	0.367	0.567
LF/HF ratio	6.8 ± 3.6	6.6 ± 3.3	5.4 ± 2.0	5.4 ± 1.9	0.305	0.622	0.736

Data are presented as mean ± SD. Abbreviations: HF, high-frequency; IBI, inter-beat interval; LF, low-frequency; SD, standard deviation; RMSSD, root mean square of the successive differences of IBIs.

Table 4.14. Inter-beat interval parameters during waking hours, measured using Actiheart 5, at baseline and follow-up

	Placebo		Beta-alanine		ANOVA		
	Baseline	**Follow-up**	**Baseline**	**Follow-up**	**Treatment**	**Time**	**T x T**
Activity (counts)	51 ± 31	53 ± 33	49 ± 26	49 ± 13	0.797	0.883	0.790
Heart rate (bpm)	79 ± 11	78 ± 13	81 ± 8	82 ± 9	0.630	0.915	0.572
Average IBI (ms)	788 ± 112	804 ± 138	768 ± 74	761 ± 85	0.530	0.744	0.467
Minimum IBI (ms)	685 ± 115	707 ± 116	665 ± 70	662 ± 71	0.481	0.477	0.343
Maximum IBI (ms)	910 ± 121	929 ± 190	888 ± 80	884 ± 111	0.582	0.757	0.612
SD (ms)	40.51 ± 11.34	44.64 ± 23.77	45.55 ± 9.37	45.78 ± 14.77	0.643	0.595	0.635
RMSSD (ms)	31.53 ± 16.38	35.96 ± 30.43	32.00 ± 10.63	30.19 ± 11.91	0.736	0.808	0.565
LF (ms^2)	772 ± 678	1538 ± 2596	1113 ± 409	1197 ± 610	>0.999	0.372	0.472
HF (ms^2)	296 ± 240	648 ± 1362	330 ± 207	344 ± 203	0.622	0.439	0.472
LF/HF ratio	8.5 ± 4.8	7.3 ± 3.7	6.9 ± 2.6	7.7 ± 3.7	0.719	0.711	**0.039**

Data are presented as mean ± SD. Abbreviations: HF, high-frequency; IBI, inter-beat interval; LF, low-frequency; SD, standard deviation; RMSSD, root mean square of the successive differences of IBIs.

Table 4.15. Inter-beat interval parameters during sleeping hours, measured using Actiheart 5, at baseline and follow-up

	Placebo		Beta-alanine		ANOVA		
	Baseline	**Follow-up**	**Baseline**	**Follow-up**	**Treatment**	**Time**	**T x T**
Activity (counts)	1 ± 1	1 ± 1	1 ± 0	1 ± 1	0.765	0.692	0.692
Heart rate (bpm)	62 ± 9	63 ± 9	62 ± 5	61 ± 6	0.765	0.812	0.340
Average IBI (ms)	1000 ± 145	985 ± 137	990 ± 74	1011 ± 99	0.896	0.877	0.312
Minimum IBI (ms)	863 ± 138	853 ± 94	872 ± 69	885 ± 83	0.681	0.915	0.470
Maximum IBI (ms)	1135 ± 153	1111 ± 160	1114 ± 86	1136 ± 129	0.977	0.957	0.329
SD (ms)	53.37 ± 18.81	53.53 ± 17.44	56.51 ± 14.62	58.40 ± 17.92	0.619	0.792	0.825
RMSSD (ms)	47.62 ± 26.15	45.03 ± 22.10	38.78 ± 13.46	41.42 ± 17.55	0.522	0.995	0.585
LF (ms^2)	1169 ± 983	1137 ± 763	1684 ± 1678	1325 ± 794	0.440	0.555	0.622
HF (ms^2)	696 ± 645	559 ± 434	581 ± 628	495 ± 347	0.696	0.455	0.863
LF/HF ratio	4.8 ± 2.6	5.5 ± 2.8	5.4 ± 2.5	5.1 ± 2.4	0.962	0.494	0.156

Data are presented as mean ± SD. Abbreviations: HF, high-frequency; IBI, inter-beat interval; LF, low-frequency; SD, standard deviation; RMSSD, root mean square of the successive differences of IBIs.

4.4 Discussion

This study presents the feasibility and tolerability of beta-alanine supplementation in adults with overweight or obesity, and further explores the effect this intervention may have on markers of cardiovascular function and health. The high adherence, low attrition rates, and low participant-reported side effects, including paraesthesia, suggest that 4.8 g/day of beta-alanine is well-tolerated in adults with overweight or obesity. There was no influence of beta-alanine supplementation on haemodynamic, echocardiographic, and biochemical markers of cardiovascular function and health.

4.4.1 Feasibility and tolerability

The primary and secondary outcome measures of the study aimed to assess feasibility and tolerability by evaluating participant recruitment, retention, adherence, blinding, and side effects experienced during the intervention.

4.4.1.1 Adherence

Adherence to the intervention was high across the placebo and beta-alanine groups, with consistent intake observed throughout the three-month supplementation period. Both the placebo and beta-alanine-supplemented groups demonstrated a high level of supplement compliance, with over 90 % of the supplement doses being successfully consumed. To gain further insights into trial adherence and the acceptability of the supplement, qualitative measurements would have been a valuable addition to this study. Future research could consider conducting exit interviews, with questions related to the strategies used to enhance adherence (including preferences for researcher-

provided strategies), opinions on the supplementation dosage, and future adherence (willingness to continue taking the supplement regularly).

4.4.1.2 Recruitment and retention

The low response and enrolment rates with the initial eligibility criteria and the first phase of recruitment prompted a change in the inclusion criteria. This change led to the recruitment of the desired number of participants within the timeframe of the doctoral studies. Despite implementing a multi-faceted recruitment strategy, only six eligible individuals were enrolled in the trial within the first seven months of the first phase of recruitment. The successful recruitment and retention of participants in randomised, controlled trials is challenging (McDonald et al., 2006). A review of 73 publicly funded trials in the UK revealed that in 63 % of trials early recruitment was slower than expected, with fewer eligible participants than anticipated being a key contributing factor (McDonald et al., 2006).

During the first recruitment phase, an additional 13 participants attended the laboratory with HbA1c values from their last GP blood test falling in the prediabetes range; however, all these participants had an HbA1c value in the normal clinical reference range at visit 1. Several participants noted that these values may have been outdated due to not having seen their GP for at least 1 to 2 years, a consequence of COVID-19 restrictions. Participant retention and treatment compliance are also important indicators of trial success (McDonald et al., 2006). Upon recruitment into the trial, there was a high likelihood that participants would complete the intervention. A total of 30 participants were recruited into the trial, with observed attrition rates of 13 % and 7 % for placebo

and beta-alanine groups; this remained below the assumed 20 % dropout rate. Therefore, this suggests that while there were challenges in recruiting individuals to the study within the university research setting, once recruited, participants were able to be retained in the intervention.

Clinical trials often underrepresent individuals from low socioeconomic areas (Heiat et al., 2002). Socioeconomic diversity is crucial for conducting rigorous research with generalisable and impactful results (Emery et al., 2023). Individuals from a diverse range of demographics were recruited in this study; the recruitment strategies used targeted various areas within Nottinghamshire (including Clifton, West Bridgford, Chilwell, Sherwood, and Ilkeston), covering a range of socioeconomic demographics. The socioeconomic backgrounds of the 27 participants who completed the intervention were assessed using the Index of Multiple Deprivation (accessed via https://imd-by-postcode.opendatacommunities.org/imd/2019); this is an official measurement of small area deprivation (Lloyd et al., 2023) using the participant's postcode (home address). It is not an individual measure of the participant. The socioeconomic distribution of the participants showed that 26 % were from low socioeconomic areas (bottom 30 %), 26 % from middle socioeconomic areas (40^{th} to 70^{th} percentile), and 41 % from high socioeconomic areas (top 30 %); 7 % of postcodes were unmatched in the analysis. Whilst there is a slight overrepresentation from the high socioeconomic areas, the inclusion of participants from each socioeconomic area grouping suggests a reasonable level of diversity.

4.4.1.3 Side effects and blinding

Paraesthesia is a commonly reported side effect of beta-alanine supplementation. A meta-analytical study of 101 beta-alanine trials showed that 18.6 % of participants supplemented with beta-alanine reported experiencing paraesthesia (Dolan et al., 2019); however, this was not observed in the current study. Participant-reported general and paraesthesia side effects, assessed using the GASE questionnaire, showed no adverse effects of beta-alanine supplementation in adults with overweight or obesity; values remained below baseline and were comparable across the placebo and beta-alanine groups. The intensity and incidence of paraesthesia are determined by the extent and time to reach peak blood concentration of beta-alanine. The sustained-release formula and/or smaller individual doses used in the current study extend the absorption of beta-alanine, thereby lowering and extending the peak concentration of beta-alanine in the blood, and minimising the paraesthesia sensation (Décombaz et al., 2012). If individuals had experienced paraesthesia (a known side effect), it may have led to unintentional unblinding of group allocation. Blinding index scores were similar across the placebo and beta-alanine groups; scores indicated that participants were appropriately blinded to the intervention. It is important to establish successful blinding to beta-alanine supplementation, providing a basis for future study designs.

Beta-alanine supplementation is considered safe in healthy individuals regarding liver and kidney biochemical markers (Dolan et al., 2019; Harris et al., 2006). The current study showed no influence of placebo or beta-alanine supplementation on biochemical markers of liver and kidney function, further suggesting that beta-alanine is safe and well-tolerated in adults with overweight and obesity. Although a decrease in plasma

albumin concentration was observed in the beta-alanine-supplemented group, the decrease was small (-1.3 g/L), and the value remained within the NHS clinical reference ranges (35–50 g/L). While lower concentrations of albumin can suggest liver dysfunction or inflammation, this is unlikely to be the case in the current study. A meta-analytical study showed an increase in plasma ALT concentration, a transaminase enzyme associated with liver dysfunction, with beta-alanine supplementation (Dolan et al., 2019). The observed effect was small, remaining within clinical reference ranges, and may be attributed to an increase in the activity of transamination in response to elevated beta-alanine availability, rather than liver damage. The current study did not show any change in ALT with beta-alanine supplementation.

4.4.2 Cardiovascular-related exploratory outcomes

There was no influence of beta-alanine supplementation on lipid and CVD-related blood profiles in adults with overweight or obesity. Previous human studies have shown a decrease in fasting levels of triglycerides with the administration of carnosine at 1 g/day for 12 weeks in individuals with type 2 diabetes (Houjeghani et al., 2018) and diabetes nephropathy (Elbarbary et al., 2018). The decrease in total cholesterol and elevation in HDL shown in Elbarbary et al. (2018) was not reported in Houjeghani et al. (2018), even though the same intervention and a similar patient phenotype were used. The inconsistency in these outcomes is also demonstrated in other studies focused on individuals with overweight and obesity (De Courten et al., 2016) and prediabetes and type 2 diabetes (Saadati et al., 2023). As mentioned in section **4.1**, the high/unclear risk of bias in already published studies may cause false positive or negative effects by overestimating or underestimating the intervention effect. The more rigorous a study is,

the closer it is likely to be to the true effect of the intervention. To minimise the risk of bias, this study provided clear information on randomisation and allocation concealment and assessed and reported participant adherence and blinding to the intervention. Additionally, group assignments were revealed after outcome assessment (unblinded analysis). These improvements in study design and methodology may have reduced the effects when compared to other studies.

Additionally, the current study utilised multiple non-invasive techniques to directly explore cardiac function, including heart rate variability monitoring, haemodynamics, and echocardiography to quantify structural and functional outcomes. Beta-alanine supplementation did not influence any of these cardiovascular outcomes in adults with overweight and obesity. Individuals with overweight and obesity are at an increased risk of CVD and cardiovascular dysfunction. The changes in body composition associated with overweight and obesity can cause alterations in haemodynamics, ventricular function, cardiac morphology, and metabolic profile (Lavie et al., 2013; Poirier et al., 2006). While some individuals did exhibit biochemical and cardiovascular outcomes above clinical ranges, other participants had baseline values within normal ranges. This may explain the absence of change with beta-alanine supplementation. In human clinical trials, carnosine supplementation did not change blood pressure parameters in individuals with obesity (De Courten et al., 2016), diabetic nephropathy (Elbarbary et al., 2018), or prediabetes and type 2 diabetes (Houjeghani et al., 2018; Saadati et al., 2023). This may be because the participants in these studies were normotensive at baseline, with blood pressure and heart rate measurements within clinical reference ranges (De Courten et al., 2016; Elbarbary et al., 2018; Houjeghani et al., 2018; Saadati et al., 2023),

and some participants were reported as receiving prescribed medication for hypertension (Houjeghani et al., 2018). Most of the participants in the current study can be categorised as pre-hypertensive (systolic, 120–139 mmHg; diastolic, 80–90 mmHg) or hypertensive (systolic, > 140 mmHg; diastolic, > 90 mmHg). The promising effects of carnosine on hypertensive properties have been shown in pre-clinical *in vivo* models, with vasodilatory effects observed (section **1.2.4.4**); however, despite this, three months of beta-alanine supplementation did not result in improvements in any of the haemodynamic parameters measured using the CNAP and TTE.

Prescribed medications may negate the influence of beta-alanine supplementation on functional and structural cardiovascular outcomes. 69 % of participants in the placebo group and 43 % of participants in the beta-alanine group were regularly prescribed medications related to hypertension and CVD pathologies (e.g., cholesterol, lipids, and blood clots), including alpha-blockers (doxazosin), angiotensin-converting enzyme inhibitors (ramipril), angiotensin receptor blockers (losartan), anti-platelet agents (clopidogrel), aspirin, beta-blockers (atenolol, bisoprolol fumarate), calcium channel blockers (amlodipine), diuretics (indapamide), and statins (atorvastatin). It would be unethical to include participants with uncontrolled health conditions. The participant information sheet for the current study declared that if researchers found anything of clinical importance (e.g., high blood pressure), the participant would be advised to arrange an appointment with their GP.

The cardiovascular results in the current study may also be explained by the pre-existing defence mechanisms of the individuals or the mechanisms already provided by any

prescribed medication. As mentioned in **section 1.2**, it may be that the effects of beta-alanine and carnosine are more prominent in tissue with higher levels of dysfunction, whereby supplementation restores function when the endogenous defence mechanisms are inadequate, rather than improving existing function. This is demonstrated in pre-clinical *in vivo* animal cardiovascular studies, which use acute cardiovascular injury models, such as I/R or cardiotoxicity, to induce severe cardiac dysfunction when investigating carnosine and beta-alanine. In such high pathological states, beta-alanine and carnosine demonstrate beneficial effects. The pathology of the individuals included in this study may not have been sufficiently dysfunctional to elicit the restorative function of carnosine and beta-alanine. It is important to note that cardiometabolic and cardiovascular outcomes should be interpreted in line with the small sample size and feasibility study design.

4.4.3 Limitations

It is plausible that individuals with overweight or obesity may exhibit a profile considered too low-risk to fully utilise the therapeutic potential of beta-alanine supplementation on cardiovascular and cardiometabolic outcomes. Individuals were categorised as overweight or obese based on BMI measurements; however, BMI only considers total body fat. The distribution of body fat may need to be considered, as the obesity phenotype differs not only in the amount of excess body fat but also in its regional distribution, aspects not accounted for by BMI. Waist circumference may have been a more robust method, as it specifically measures abdominal obesity, providing insight into fat distribution. Analysis of waist circumference data in this study showed that all

participants, except for one female in the placebo group, are categorised as ‘high risk’ or ‘very high risk’.

Due to ethical implications, cardiac tissue could not be taken from a live human cohort. Results presented in **Chapter 2** and **Chapter 3** showed an increase in intracellular concentrations of beta-alanine in cardiomyocyte cells and mouse hearts when exposed to exogenous beta-alanine; however, the translation of these results into the human population needs to be approached with caution. Investigating the effect of exogenous beta-alanine supplementation on cardiac concentrations of beta-alanine and HCDs in human participants requires the development of a novel method, potentially utilising techniques such as magnetic resonance imaging or radiopharmaceutical tracer methods, to non-invasively detect and quantify the metabolism of beta-alanine in cardiac tissue. Urinary and serum/plasma carnosine, beta-alanine, and L-histidine can be quantified to indicate what is available to all tissues, although this only provides time-specific information on circulatory and excretion data and does not measure the rate of uptake into each tissue or the synthesis of the dipeptide therein. While these measurements were included in trial preregistration, they could not be completed within the timeframe of the doctoral studies.

4.5 Conclusion

Three months of sustained-release beta-alanine, at a dose of 4.8 g/day, is well-tolerated and does not cause adverse events in adults with overweight or obesity. Short-term supplementation of beta-alanine in adults with overweight and obesity was not associated with improvements in haemodynamic, echocardiographic, or biochemical markers of cardiac function and health. Longer duration studies and/or studies involving patients with higher BMI or established CVD may be needed to potentially demonstrate the therapeutic benefits of beta-alanine supplementation. Nevertheless, caution needs to be exercised, as higher-risk or higher-BMI individuals will likely be accompanied by increased prescription medications and existing pathologies, potentially requiring more extreme interventions to reduce pathology.

Chapter 5: General discussion and conclusion

5.1 Overview of main findings and research impact

This research programme aimed to investigate the metabolism of carnosine and beta-alanine in the heart and the physiological role(s) of beta-alanine supplementation on cardiac function and health in a disease model with an associated risk of CVD. To achieve these aims, the following objectives were investigated: 1) identify the carnosine and beta-alanine metabolism-related enzymes and transporters expressed by rat cardiomyocytes and explore the influence of exogenous carnosine and beta-alanine on these; 2) investigate whether exogenous carnosine and beta-alanine can be taken up and stored by cardiac cells/tissues and explore whether HCD synthesis is affected by the cellular uptake of beta-alanine; 3) explore the effects of 12 weeks of beta-alanine supplementation on cardiac structure and morphology in mice challenged with an obesogenic diet; 4) evaluate the feasibility and tolerability of three months of beta-alanine supplementation in humans with overweight and obesity using a randomised controlled trial approach; 5) explore the effects of three months of beta-alanine supplementation on echocardiographic, haemodynamic, and biochemical markers of cardiac function and health in humans with overweight and obesity. This thesis adds new contributions to the research knowledge on beta-alanine supplementation in metabolic disease, investigating its use on cardiac function and health in overweight and obesity.

5.1.1 Experimental models

This research programme investigated the uptake and therapeutic potential of beta-alanine supplementation in the cardiovascular system, with a focus on pathologies induced by metabolic disease (i.e., overweight and obesity). This work used a

translational research approach, from *in vitro* molecular experiments to whole-body system interactions using *in vivo* pre-clinical (murine) and clinical (human) models. The data from the cell, animal, and human experiments have contributed to understanding the mechanisms and processes involved in the heart when exposed to exogenous beta-alanine.

Table 5.1 identifies the main advantages and disadvantages of each of the three experimental models implemented: H9c2 immortalised cell line, mouse, and human. The primary endpoint of research into a nutritional intervention is a human model; however, the implementation of all three models allows for a thorough investigation of its therapeutic potential. The *in vivo* mouse model enabled the direct investigation of pathways and mechanisms in the heart, something that cannot be examined in a living human model. Additionally, the study of obesity and energy metabolism in whole-body models cannot be successfully or reliably replaced with computer-based or cell-culture models (Speakman et al., 2007).

Table 5.1. The advantages and disadvantages of the experimental models used in this programme of work

Experimental Model	Advantages	Disadvantages
Immortalised cell line (H9c2)	Fast rate of replication (infinite proliferation) Cost-effective No ethical considerations Controlled environmental conditions Provides insights into mechanisms and pathways	Cannot replicate the complex whole-body interactions in an organism Simplified representation of physiological mechanisms Grown in artificial environments Limited long-term study
Mouse (*in vivo*)	Isogenic Safety profile of pharmaceutical products Genetic similarity to humans Replicate human diseases Reproduce quickly Shorter timeframe for disease progression Controlled conditions (environment, food, activity) Compliance Directly access organs and tissue	Limited translation and applicability to humans Expensive Drug doses cannot be scaled to humans easily Physiological differences (nocturnal, feeding patterns)
Human (*in vivo*)	Direct applicability Provide informed consent Communication of any side effects or perceived difficulties completing the intervention	Genetically diverse (variability in responses to treatments) Recruitment challenges Limited control over participants and non-compliance Cannot directly access cardiac tissue in living participants

5.1.2 Experimental results

5.1.2.1 Carnosine and beta-alanine-related metabolism

The confirmation of carnosine and beta-alanine incorporation into the target cell or tissue is essential. If the supplement cannot be taken up, it will not be able to induce direct physiological effects, and consequently, any interventional study would be deemed unnecessary. Therefore, the first and second objectives of this research programme were to deepen the understanding of carnosine and beta-alanine metabolism in the heart, particularly in response to exogenous sources. The initial investigation (**Chapter 2**) focused on the uptake and accumulation of exogenous carnosine and beta-alanine in cardiomyocytes. Rat ventricular cardiomyocytes expressed the enzyme and transporter genes associated with carnosine and beta-alanine metabolism and subsequently showed an increase in intracellular carnosine and beta-alanine when exposed to exogenous sources, indicating successful uptake and storage of the supplements. Despite this, however, there was no increase in intracellular carnosine levels with beta-alanine supplementation, suggesting accumulated beta-alanine did not subsequently synthesise into carnosine.

In a whole-body organism, mice showed an increase in cardiac concentrations of beta-alanine, carnosine, and anserine after 12 weeks of 1.2 % beta-alanine supplementation (**Chapter 3**), suggesting that beta-alanine could be taken up and accumulated in mouse hearts and further synthesised into HCDs. The time the cells were exposed to beta-alanine may explain the discrepancy between the cell and mouse findings. The enzyme responsible for synthesising beta-alanine and L-histidine into carnosine, *Carns1*, has

been described as “sluggish” (Drozak et al., 2010), indicating that a longer duration may be required for synthesis to occur.

Beta-alanine supplementation is often used as a model of taurine deficiency. Lower levels of taurine are associated with adverse health effects such as obesity, hypertension, and liver disease (Singh et al., 2023). In rat ventricular cardiomyocytes, beta-alanine supplementation decreased intracellular taurine concentrations. This result was accompanied by an increase in extracellular taurine concentrations (in the culture media), suggesting competition between beta-alanine and taurine and indicating a possible role of beta-alanine in modulating taurine exposure (**Chapter 2**). The effect of beta-alanine supplementation on taurine concentrations in mouse hearts was inconclusive due to values exceeding the limit of quantification of the amino acid analyser (**Chapter 3**). The concentrations of beta-alanine used in the *in vivo* experiments were within the doses suggested by Dolan et al. (2019) to avoid a decrease in cardiac taurine concentrations, which, if exceeded, could cause adverse effects. The clinical signs and macroscopic observations in the pre-clinical study (**Chapter 3**), along with the side effects questionnaires completed by participants in the clinical trial (**Chapter 4**), did not indicate any concerns for those supplemented with beta-alanine.

5.1.2.2 Therapeutic potential of beta-alanine supplementation

With the knowledge gained from the previous metabolism results and the promising evidence that beta-alanine can be taken up by cardiac cells and tissue, this programme of work progressed to intervention experiments. The therapeutic potential of carnosine in various pathological conditions has been explored over the last 30 years. As of April

2022, there were 32 ongoing clinical studies investigating the use of carnosine, L-carnosine, beta-alanine, or carnosine-rich foods for treating various diseases (Caruso, 2022). Before this research programme, the literature lacked studies concerning the implementation of beta-alanine as a potential treatment for cardiovascular implications associated with metabolic disease. Most *in vivo* animal studies have induced cardiovascular dysfunction through acute injury models, although this brings into question the translation to humans given the inability to predict acute cardiac events and the difficulty in ensuring adequate pre-treatment before this occurrence. Consequently, it was necessary to explore the use of beta-alanine and carnosine in metabolic diseases with associated CVD risks (i.e., prediabetes and overweight and obesity), and thus this area became the focus for two further experiments.

This work did not find significant evidence, within the experimental conditions employed, to support the use of beta-alanine supplementation for improving cardiac function and health in a model of metabolic disease (overweight/obesity). The results in **Chapter 3** showed no effect of beta-alanine supplementation on cardiac morphology or histology in mice challenged with a HFD. The implementation of a HFD in mice has been shown to cause histological and morphological alterations in the myocardium within 12 weeks, as observed with tissue colour staining (Lepczyński et al., 2021). Previous literature have reported beneficial effects of carnosine on histological and morphological parameters of the heart (Allo et al., 1997; Hou et al., 2020; Kumral et al., 2016; Zieba et al., 2003). In this study, however, no morphological or histological changes were observed in the hearts of control HFD mice compared to control chow-fed mice. Therefore, since there were no histological and morphological changes in the heart with

a HFD, there was limited opportunity for beta-alanine supplementation and synthesised HCDs to provide any potential benefits or improvements.

The second nutritional intervention in this research programme involved supplementing human adults with overweight and obesity with 4.8 g/day of beta-alanine for three months (**Chapter 4**). Initially, the experiment aimed to recruit adults with prediabetes; however, identifying, screening, and enrolling this population proved challenging. Therefore, due to the timeframe of this research programme, the eligibility criteria were amended to focus on adults with overweight and obesity. The results showed no effect of beta-alanine supplementation on cardiac function or health in adults with overweight and obesity, as assessed by haemodynamic and echocardiographic measurements and cardiovascular-related biochemical markers. It is possible that the disease state of the participants was not advanced enough to elicit the potential beneficial effects of beta-alanine supplementation. For beta-alanine supplementation to promote any beneficial roles on cardiac function and health, a more pronounced level of cardiovascular dysfunction may be required. Another possible explanation is that 43 % of participants in the beta-alanine group were prescribed medication related to the cardiovascular system (e.g., hypertension and cholesterol), which may have masked the potential effects of the supplement.

Interestingly, and somewhat unexpectedly, HFD-induced body and tissue weight gains were attenuated in beta-alanine-treated mice, suggesting its potential use as a therapeutic agent for other obesity-related conditions. This finding warrants significant further investigation. Unlike these results, where mice exhibited a decrease in body

weight with beta-alanine supplementation when challenged with a HFD, there was no change in body composition among the human participants, as assessed by body weight, BMI, and waist circumference. The human participants already had an established overweight or obese phenotype prior to supplementation, whereas the mice developed this phenotype alongside beta-alanine supplementation. This difference suggests that beta-alanine may exert protective effects by preventing the development of obesity, rather than having a curative effect once obesity has already been developed.

It is difficult to control lifestyle factors in human participants, such as diet and exercise, and 56 % of participants across both treatment groups were regularly prescribed medication related to cardiovascular co-morbidities. In contrast, these factors can be controlled in mice. While, the extrapolation of murine data to humans needs to be approached with caution, this cross-species analysis is valuable, as pre-clinical experiments allow direct effects of the supplementation on cardiac tissue to be analysed, which cannot be performed in human participants. The dose and supplementation period in the mouse experiment (12 weeks) was comparable to that in the human clinical trial (3 months). Most murine studies in the literature have used carnosine supplementation, which limits translation into humans since rodents lack the carnosinase enzyme responsible for the hydrolysis of carnosine into its constituent amino acids. Therefore, the effects of carnosine supplementation shown in rodent models may partly result from elevated plasma carnosine concentration. The murine study in this programme of work used beta-alanine supplementation to improve data translation between species.

5.1.2.3 Key findings and research impact

The key findings from this programme of work, corresponding to the objectives and the impact of the research, are summarised in **Table 5.2**.

Table 5.2. Key findings and impact of the research

Objective	Key findings	Research impact
1	Rat ventricular cardiomyocytes expressed the genes for the enzymes and transporters involved in carnosine and beta-alanine related metabolism. Beta-alanine supplementation decreased the expression of *Tau-T.*	Rat ventricular cardiomyocytes express the genes required to facilitate the uptake and synthesis of carnosine and beta-alanine and indicate the potential of the cells to be able take up the supplement. There may be competition between taurine and beta-alanine in cardiomyocytes; the implications of this need to be further researched and considered in supplementation trials.
2	Carnosine supplementation can be taken up and stored by rat cardiomyocytes. Beta-alanine supplementation can be taken and stored by rat cardiomyocytes and murine cardiac tissue. The increase in beta-alanine levels increased HCD concentrations in murine heart tissue but not in rat cardiomyocytes.	Carnosine and beta-alanine can be taken up by cardiac cells and tissue. The difference in HCD synthesis between the *in vitro* and *in vivo* studies may indicate that longer durations of supplementation are required for beta-alanine to be synthesised into carnosine and then anserine. This provides evidence that the supplement can be taken up by the heart, therefore providing support for future intervention trials to be conducted looking at the direct effect on cardiac physiology.

3	12 weeks of beta-alanine supplementation demonstrated no beneficial effect on cardiac structure and morphology in mice challenged with an obesogenic diet. The high-fat diet did not demonstrate cardiac dysfunction in structure or morphology.	This study was the first to investigate the effects of beta-alanine supplementation on cardiac structure and morphology in mice challenged with an obesogenic diet. Functional cardiac measurements, such as echocardiography and blood pressure, need to be assessed to fully investigate the effect of beta-alanine supplementation on the heart in this experimental model.
4	Three months of beta-alanine was well-tolerated and adhered to in humans with overweight and obesity; evidenced by high adherence, low attrition rates, and low participant-reported side effects, which were comparable between both groups.	This was the first study to provide information regarding longitudinal tolerability and adherence to the intervention. This study provides information on recruitment and enrolment of clinical populations (prediabetes and overweight/obesity) using a multi-faceted recruitment approach.
5	There was no clear evidence that the use of beta-alanine supplementation affected echocardiographic, haemodynamic, or biochemical markers in humans with overweight or obesity.	This study was the first to investigate the use of beta-alanine supplementation on cardiac function and health in an overweight/obese population. Within the scope of the experimental measures, this study provides evidence that beta-alanine supplementation may not need to be recommended to humans with overweight or obesity to improve their cardiac function or health.

5.2 Limitations

The limitations of each experimental model implemented in this body of work have been identified in **Table 5.1**. This research programme began in October 2020, coinciding with the COVID-19 pandemic lockdowns and restrictions. This resulted in restricted access to the laboratories and substantial delays in the delivery of consumables throughout the first year of the PhD. Additionally, recruitment for individuals with prediabetes was affected. It became apparent that many interested individuals had not attended a recent GP appointment or blood test due to COVID-19, rendering their HbA1c values outdated. The feasibility of conducting human trial research within a restricted timeframe may require reconsideration of the research setting and personnel. A full research team in a clinical setting or a multicentre approach may be required, as the clinical study in this programme of work had only two investigators predominantly completing the recruitment and testing, and recruitment rates were deemed unfeasible in the doctoral timeframe with the initial inclusion criteria, noting also the further complications of COVID-19 mentioned above.

5.3 Future directions

Further research is essential to deepen our understanding of the findings and limitations from the studies in this thesis. The future directions specific to each study are briefly outlined in the corresponding chapters. The results from Chapter 2 highlight the need for *in vitro* research to focus on the potential trafficking of taurine and beta-alanine in and out of cardiomyocytes. This includes exploring how these systems are regulated, the transport mechanisms involved in taurine export from the cells, and how a decrease in intracellular taurine may impact cardiomyocyte function when exogenous beta-alanine is introduced. Incorporating a taurine transporter inhibitor, such as hypotaurine, could help elucidate the mechanisms by which taurine and beta-alanine are taken up by cardiomyocytes. Additionally, recent studies have begun investigating the intracellular concentrations of HCDs in heart tissue across species, including mice, rats, and humans (Gonçalves et al., 2023; Van der Stede et al., 2023); however, a key limitation of these studies is the use of heart tissue from diseased phenotypes. Therefore, future research needs to confirm the intracellular concentrations of HCDs in healthy heart tissue and assess whether a diseased phenotype affects HCD concentrations.

Considering the potential beneficial effects of beta-alanine supplementation identified in Chapter 3, an in-depth examination of the role beta-alanine plays in lipid metabolism is warranted. The pre-clinical *in vivo* experiment supplemented mice during the development of obesity, whereas the clinical trial supplemented humans with already developed overweight and obesity. To further align the mouse study with the human trial, the next stage of pre-clinical research would be to investigate the use of beta-alanine supplementation after the establishment of an obese phenotype. A future study

design would involve feeding mice a HFD for 12 weeks to develop the obese phenotype, followed by an extended period of beta-alanine supplementation while continuing exposure to a HFD. Follow-up studies could also incorporate additional lifestyle interventions, as would be typical for human individuals seeking to reverse the negative effects of obesity. These might include dietary changes (switching to a standard diet after obesity is established) and/or increased physical activity (providing mice with an exercise regime). Furthermore, the majority of *in vivo* rodent experiments have focused on using carnosine supplementation and its effects on cardiovascular function and health, particularly using models of acute cardiovascular injury to assess its preventative and restorative benefits. To aid the translation of these findings to human populations, future studies should consider the use of beta-alanine supplementation in models of acute cardiovascular injury.

A study examining the effects of beta-alanine supplementation in human participants consuming a high-fat, hypercaloric diet would begin to determine if the beneficial effects of beta-alanine supplementation on the reduction in body and tissue weight gains observed in mice in Chapter 3 can be replicated in humans. This study design could involve investigating the effects of beta-alanine supplementation in human participants who are consuming a HFD for four weeks- a timeframe after which the negative effects of overfeeding/HFD are generally considered to be reversible- to assess whether this protective effect against the development of obesity can be demonstrated in human populations.

To further investigate the effects of beta-alanine supplementation on cardiovascular and cardiometabolic health in humans, a longer supplementation period with serial measurements over the intervention period may be beneficial. Future research should also explore whether beta-alanine supplementation can be used alongside other medical treatments. Several participants in the study presented in Chapter 4 were on prescribed antihypertensive and lipid-lowering medications, which may negate the effect of supplementation on cardiovascular-related outcomes. It remains unclear whether beta-alanine supplementation would be effective in populations with more advanced cardiovascular conditions, such as coronary artery disease, atherosclerosis, and peripheral artery disease.

Future studies to assess the effect of beta-alanine supplementation on cardiovascular function, in both healthy and diseased participants, could incorporate a stress test (e.g., exercise) at baseline and follow-up. This stress test would assess the potential benefits on cardiovascular function when the cardiovascular system is at rest, under increased physical demands, and during the recovery period, focusing on whether the heart can adapt and respond more effectively. This would provide a more comprehensive evaluation of cardiovascular function compared to assessments solely measured at rest. Smith-Ryan et al. (2014) showed an increase in submaximal heart rate training thresholds with 28 days of beta-alanine supplementation (6.4 g/day). The study suggests this increase may be the result of an improvement in cardiac contractility; however, these mechanistic factors were not directly measured. Similar measures used in this programme of work, such as the CNAP monitor and TTE, can be conducted during an exercise test to allow for an overall assessment of heart contractility and function. This

study would also begin to investigate the real-world effectiveness of beta-alanine supplementation, where the cardiovascular system is constantly exposed to physical demands, especially in populations with compromised cardiovascular health.

5.4 Final conclusion

The H9c2 cells (**Chapter 2**) expressed the enzymes and transporters required for carnosine and beta-alanine metabolism. The cardiomyocyte (**Chapter 2**) and mouse (**Chapter 3**) experiments highlight the ability of cardiac cells and tissue to uptake and accumulate exogenous beta-alanine. These increases in beta-alanine in the mouse heart were able to increase the synthesis of carnosine, and subsequently, anserine. Further investigation into the administration of beta-alanine supplementation in mice consuming a HFD highlighted the therapeutic potential of beta-alanine in a metabolic stress environment and suggested a role for beta-alanine in fat infiltration into organs and tissues. Due to the absence of a dysfunctional cardiovascular system, the histological role of beta-alanine in diseased cardiac tissue could not be established in mice. The administration of beta-alanine supplementation in human adults with overweight or obesity did not influence cardiovascular structure or function, nor cardiovascular-related biochemical markers. This study highlighted the difficulties of completing clinical studies in a university setting and the breadth of recruitment required to achieve the desired enrolment numbers. Regardless, the study emphasised the strong compliance of individuals once enrolled and the safety of supplementing this population with beta-alanine, as reported adverse effects remained below baseline levels. Perhaps the most pertinent point of the *in vivo* studies is that beta-alanine may have potential use as a prophylactic therapeutic agent for those with a propensity to develop obesity and the concomitant morbidities associated with the disease, rather than as a curative agent; however, this requires further investigation.

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Appendices

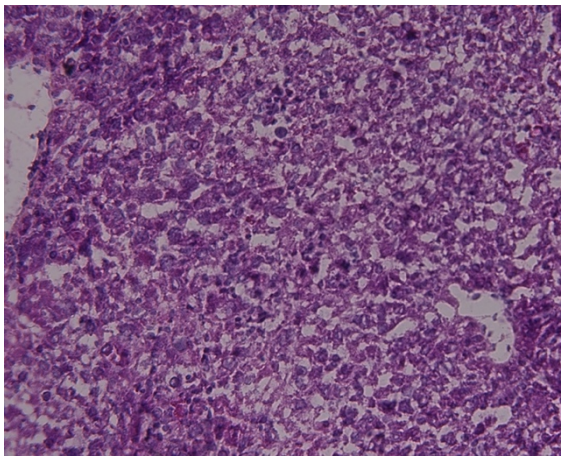
Appendix 1. Composition of 60% high-fat diet (Chapter 3).

Nutrients	**g/Kg**
Casein	265.0
L-Cystine	4.0
Maltodextrin	160.0
Sucrose	90.0
Lard	310.0
Soybean oil	30.0
Cellulose	65.5
Mineral Mix, AIN-93G-VX (94046)	48.0
Calcium Phosphate, dibasic	3.4
Vitamin Mix, AIN-93-VX (94047)	21.0
Choline Bitartrate	3.0
Blue Food Colour	0.1

Appendix 2. Mouse liver and white adipose tissue histology (Chapter 3)

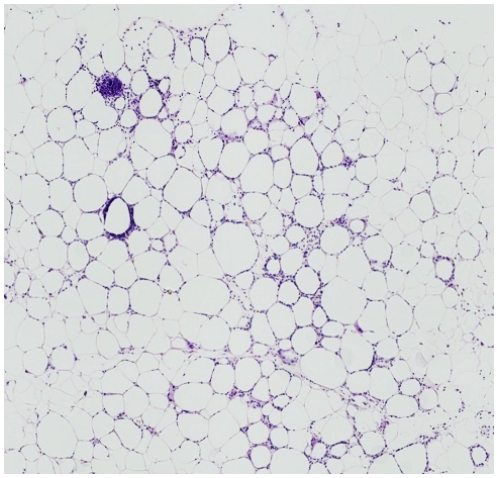
Liver

Histological examination of the liver using H & E staining highlights potential issues with tissue processing (isopentane method described in section **3.2.7.2**) and staining (method described in section **3.2.7.4**). A representative image has been provided to illustrate the observed issues. Alternative fixation techniques to enhance the tissue's histological quality are required in future research.



White adipose tissue

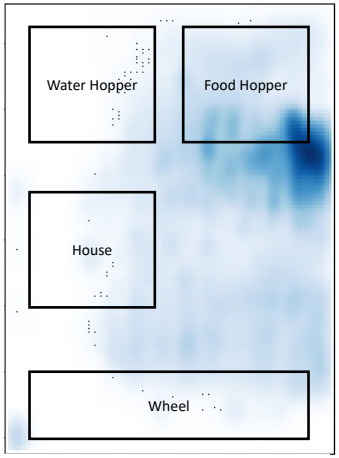
Histological examination of WAT showed a potential inflammatory response in HFD VC (multiple nuclei). A representative histological slide of H & E-stained WAT for HFD VC mice has been provided to illustrate the potential inflammatory response.



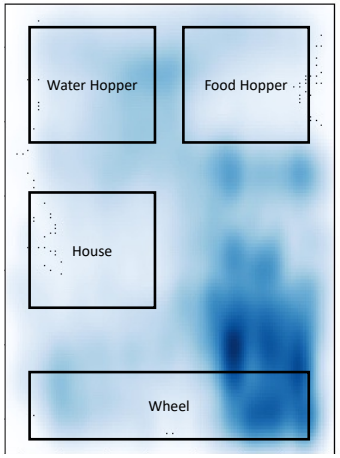
Appendix 3. Heat maps for each mouse generated from the Promethion Core (Chapter 3)

HFD VC heat maps

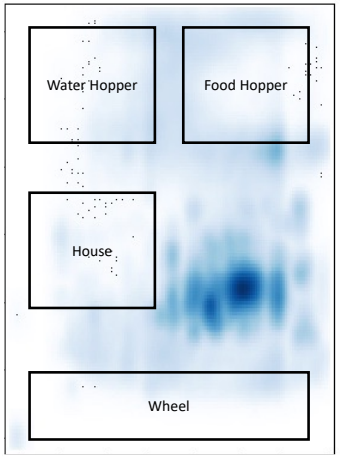
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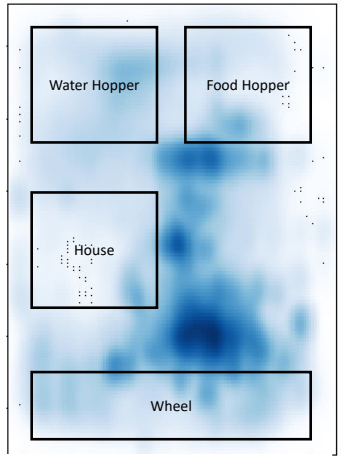
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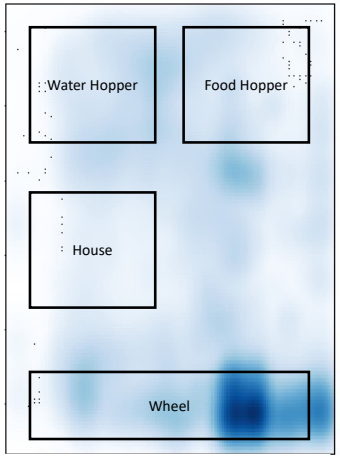
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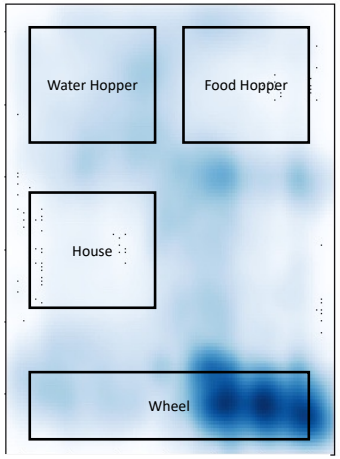
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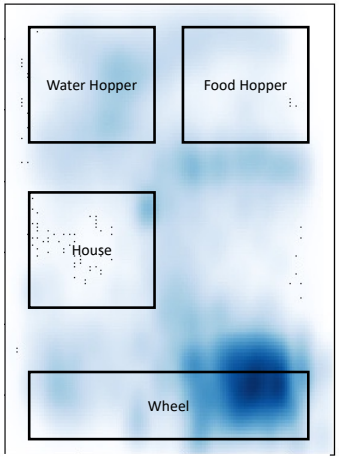
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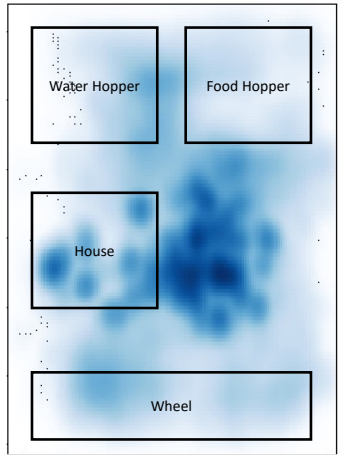
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M19



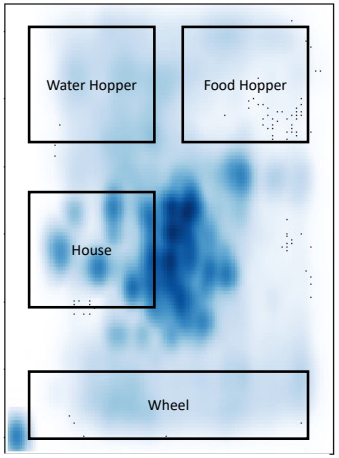
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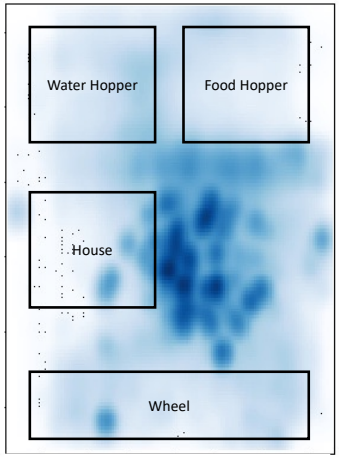
Appendix 3. Heat maps for each mouse generated from the Promethion Core (Chapter 3)

HFD BA heat maps

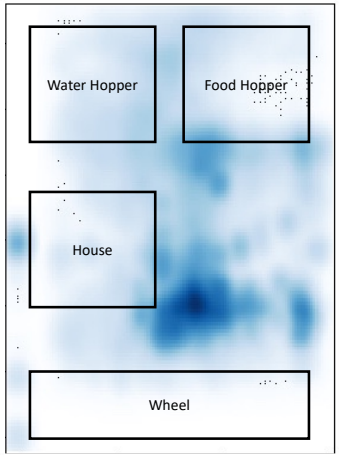
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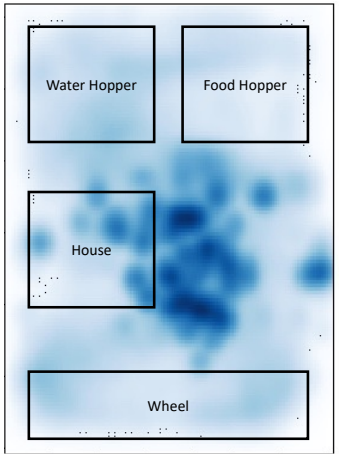
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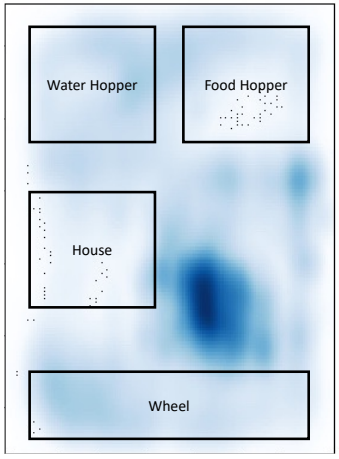
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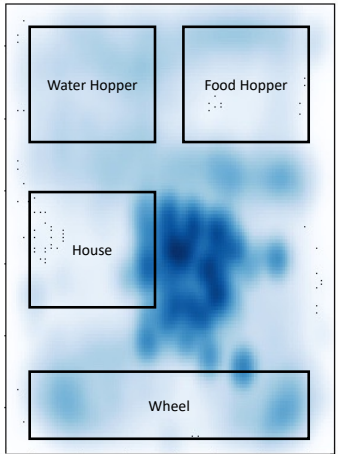
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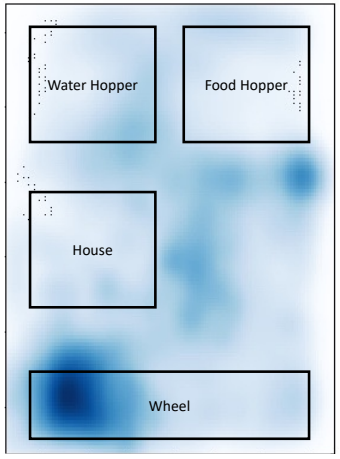
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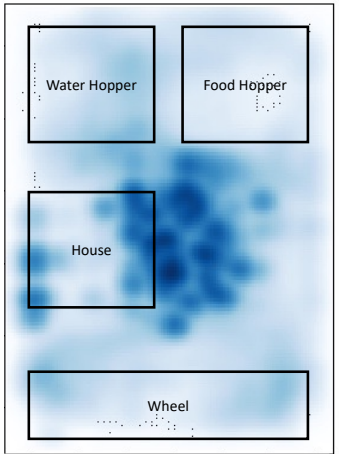
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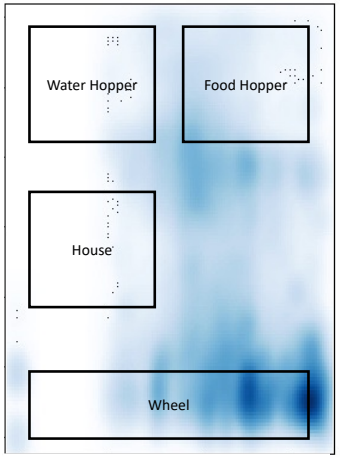
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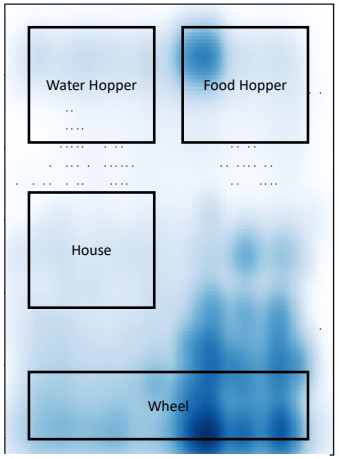
Appendix 3. Heat maps for each mouse generated from the Promethion Core (Chapter 3)

CHOW VC heat maps

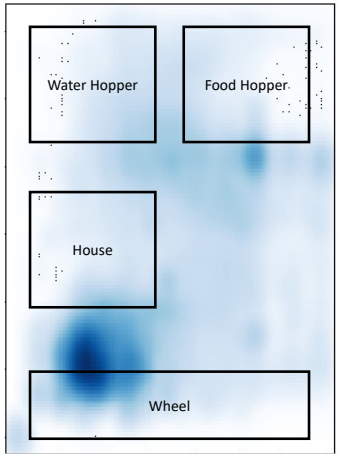
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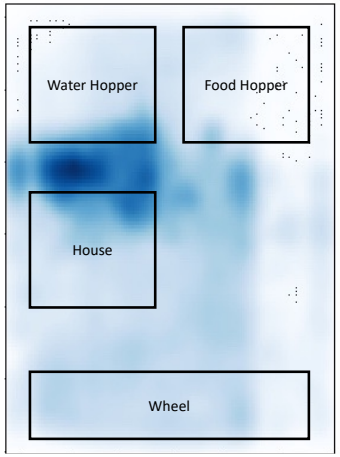
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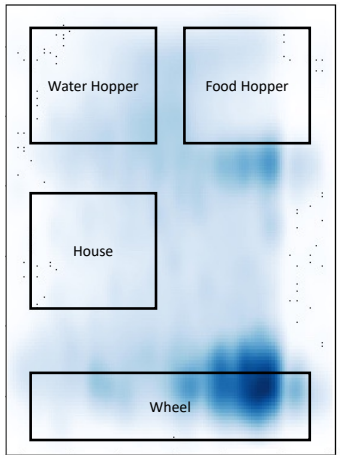
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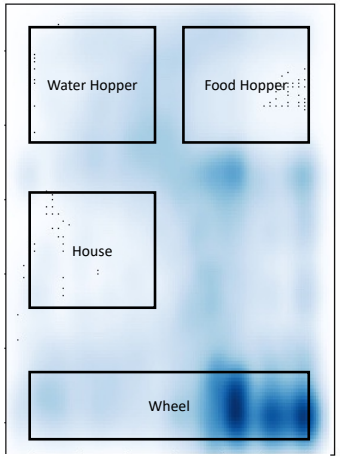
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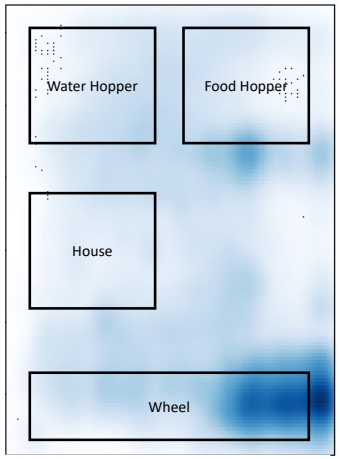
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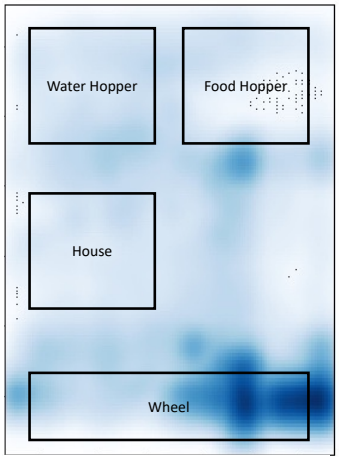
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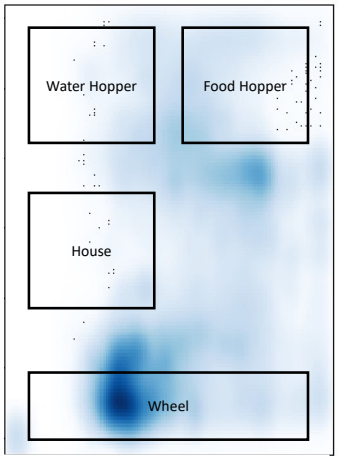
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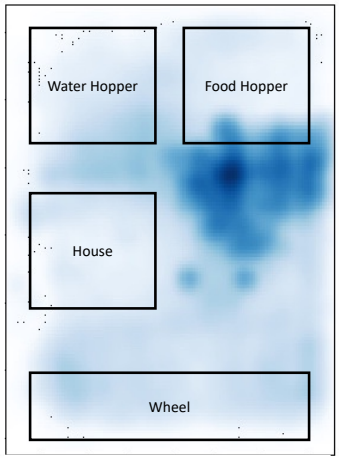
Appendix 3. Heat maps for each mouse generated from the Promethion Core (Chapter 3)

CHOW BA heat maps

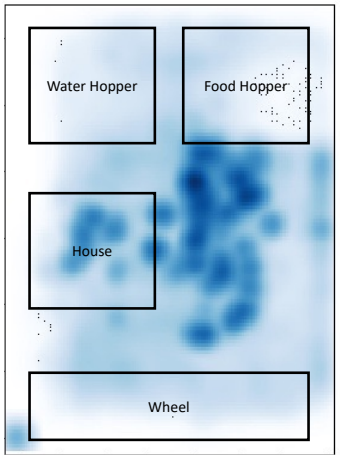
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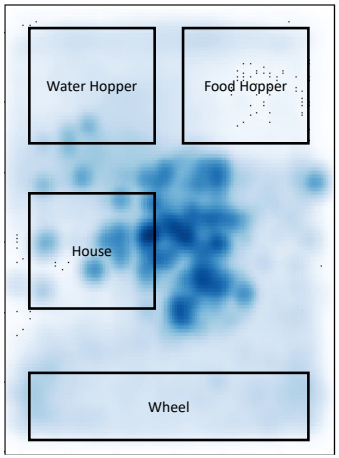
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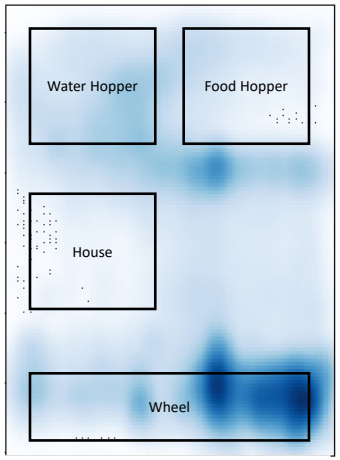
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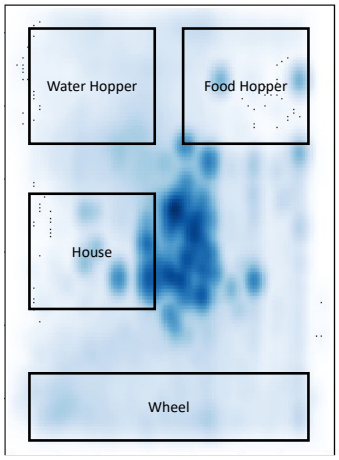
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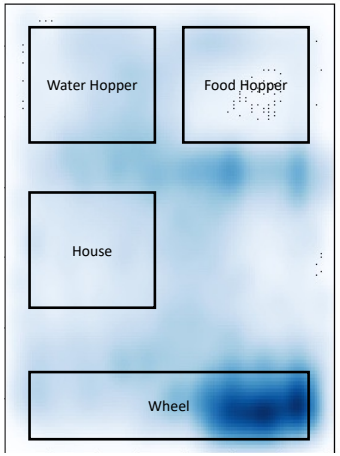
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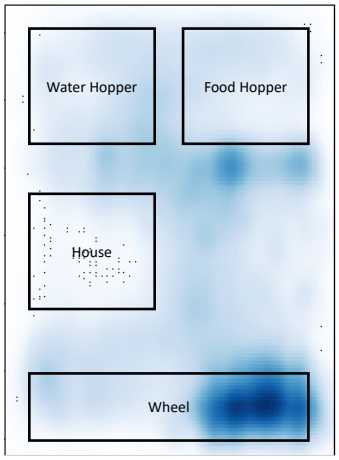
M30



M31



M32



Appendix 4. Carnosine and beta-alanine-related metabolism gene expression in mouse heart

To confirm the expression of carnosine and beta-alanine-related metabolism genes in an *in vivo* mouse model, and to aid the translation of the H9c2 cell data to a whole-body system, the hearts of control and HFD mice were subjected to RT-qPCR analysis before the design of the study reported in **Chapter 3**. Hearts from three groups of male and female mice (control and 12 weeks of 42% HFD or 60% HFD) were kindly donated by Dr Richard Hulse and Dr Lydia Hardowar (Nottingham Trent University). Mice were housed per the 'Code of Practice for the Housing and Care of Animals Used in Scientific Procedures' and aligned with the United Kingdom Home Office recommendations and requirements. Experiments were conducted in accordance with United Kingdom's (Scientific Procedure) Act 1986. Experimental animals were sacrificed by cervical dislocation and death was confirmed by cutting of the jugular vein. All tissues were snapped frozen in liquid nitrogen and stored at -80 ^{o}C until use.

Briefly, a section of the heart tissue was placed into an Eppendorf tube containing a pre-cooled bulk bead. The sample was homogenised for 5 minutes in 1 mL TRI reagent using the TissueLyser LT (QIAGEN, Germany). Total RNA was extracted using the phenol-chloroform method and gene expression analysis was conducted according to section **2.3.2**. Primers (Everaert et al., 2013; Blancquaert et al., 2016) and the arbitrary values of each gene are presented below. The control and HFD mice expressed all genes related to carnosine and beta-alanine metabolism except for *Pept1*, which could not be detected. *Agxt2* was detected at very small amounts (average Ct of 36 in the controls and 35 in the HFD groups).

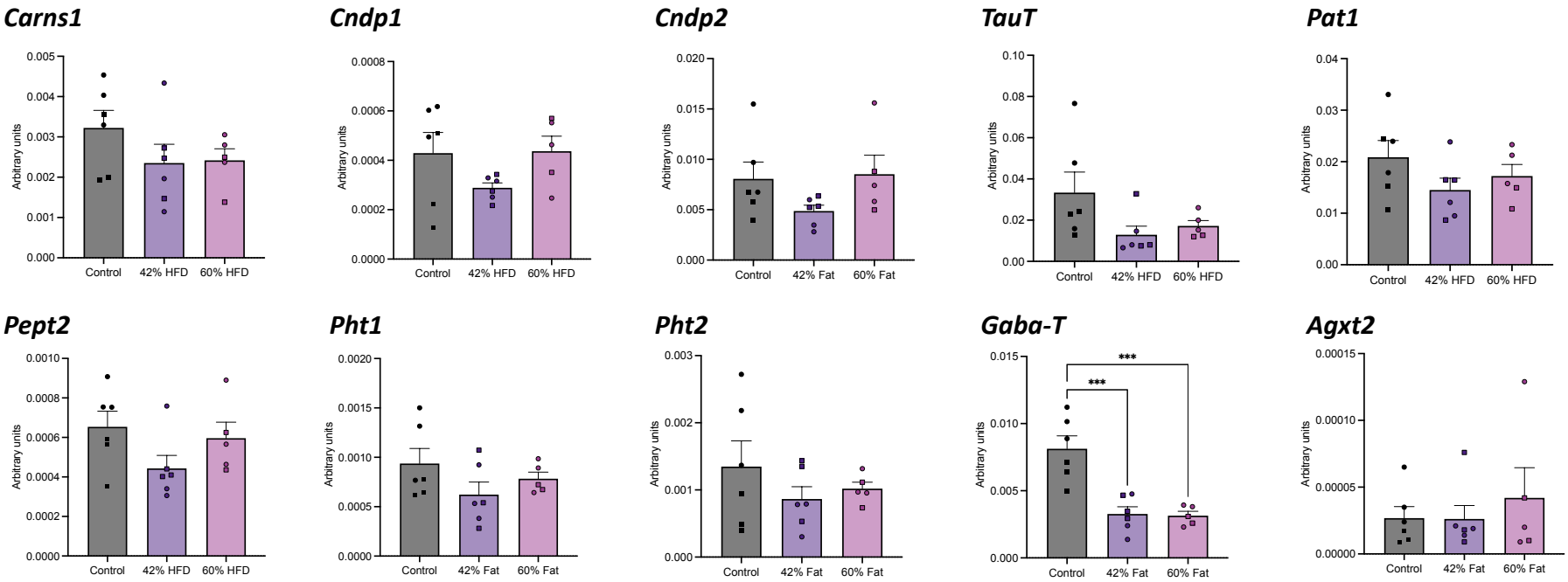
Appendix 4. Carnosine and beta-alanine-related metabolism gene expression in mouse heart

Herein, mouse cardiac tissue in control and HFD groups express most of the mRNA of the enzymes and transporters required for carnosine and beta-alanine metabolism. Therefore, mouse cardiac tissue has the potential to effectively utilise exogenous beta-alanine.

Forward and reverse primer sets used in mouse heart RT-qPCR analysis

Gene	**Forward**	**Reverse**
Carns1	GCGGCGTCAGCAAGAAGTT	CACCAAGCAGTCATCCCAGAA
Cndp1	CCTAGAAGAATACCAGAAGAGC	GGGACTAGACGGATTGAAA
Cndp2	TTCAAGGTGTACATGGGC	AAAGGTCAAGGTCACAGGA
TauT	TGG-CCG-ACA-GCA-TTC-CA	GCC-TTC-TCT-AAG-GTG-CCT-TCC-T
Pat1	TGGTTGTACCAGTCGGTGAA	GGCCAGAACACATGTCACAC
Pept1	TGTGTTTGTCCTCGGCAGTGG	CTTTAGCCCAGTCCAGCCAGT
Pept2	CATGAAATCTGTGCTCCAGG	AGGAGGCAGGAAAACAAAA
Pht1	TGAAGGCCTTGGAGTCTTT	TTGGAAATACACTGTCCAGTAA
Pht2	CCAGATGCAGTCCACCTA	AACATTGGCCAGCAGGAG
Gaba-T	CCT-TCA-TGG-GTG-CTT-TCC-A	CAA-AGG-AAG-GGA-TGT-CAA-TCT-TG
Agxt2	GAT-AGG-CTG-CCA-ATC-AAC-AAT-GT	TGC-ACT-GGA-GAA-TCT-CGA-CAA

Appendix 4. Carnosine and beta-alanine-related metabolism gene expression in mouse heart



Gene expression of beta-alanine and carnosine-related metabolism as assessed by RT-qPCR. Data are presented as mean with individual values ± SEM. Squares, male; circles, female. * statistically significant between groups * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

Appendix 5. Participant letter and information sheet: page one (Chapter 4)

Mr Joseph J Matthews
Erasmus Darwin Building
Nottingham Trent University
Clifton Campus
Clifton Lane
NG11 8NS
joseph.matthews2016@my.ntu.ac.uk

To whom it may concern,

I am writing on behalf of our Research Group at Nottingham Trent University. We are collaborating on a new study with researchers from Aston University, investigating the effects of a dietary supplement on health outcomes in adults based in Birmingham and Nottingham. The supplement, called beta-alanine, is naturally occurring and has the potential to improve cardiometabolic health markers..

This study has received the relevant approvals, and we have partnered with the NHS and local GPs, who are searching their patient databases for people who might be suitable to take part in our study. Your local GP or a member of your local healthcare team identified that you have a body mass index (BMI) in the range of overweight or obesity This means that you might be eligible to take part in our study and you have therefore received this letter.

Please note that no personal data has been passed from your GP to the Universities. Instead, this is a standardised letter that your local Practice is sending on our behalf. We will not have access to your name or contact details unless you choose to contact us about the study.

We have enclosed a Participant Information Sheet, which explains the purpose of the study, and what it involves should you choose to take part. Please do not hesitate to contact us if you would like to take part in the study, or if you have any further questions about the research. Please note you are under no obligation to take part, nor do you need to respond to this letter if you do not wish to.

Kind regards,

Mr Joseph J Matthews
Musculoskeletal Physiology Research Group
Nottingham Trent University

Appendix 6. Health history questionnaire (Chapter 4)

Health History Questionnaire

Participant Name: ..... **Date:**.....

Please complete this brief questionnaire to confirm fitness to participate:

1. Can you understand conversational English? Yes ☐ No ☐

2. **At present,** do you have any health problem for which you are:

(a) on medication, prescribed or otherwise Yes ☐ No ☐

(b) attending your general practitioner Yes ☐ No ☐

(c) on a hospital waiting list Yes ☐ No ☐

3. **In the past two years,** have you had any illness which require you to:

(a) consult your GP Yes ☐ No ☐

(b) attend a hospital outpatient department Yes ☐ No ☐

(c) be admitted to hospital Yes ☐ No ☐

4. **Have you ever** had any of the following?

(a) Convulsions/epilepsy Yes ☐ No ☐

(b) Asthma Yes ☐ No ☐

(c) Eczema Yes ☐ No ☐

(d) Diabetes Yes ☐ No ☐

(e) A blood disorder (*e.g.*, sickle cell anaemia, haemoglobinopathy) Yes ☐ No ☐

(f) Head injury Yes ☐ No ☐

(g) Digestive problems Yes ☐ No ☐

(h) Heart problems (*e.g.*, heart failure) Yes ☐ No ☐

Appendix 6. Health history questionnaire (Chapter 4)

(i) Problems with bones or joints Yes ☐ No ☐

(j) Disturbance of balance / coordination Yes ☐ No ☐

(k) Numbness in hands or feet Yes ☐ No ☐

(l) Disturbance of vision Yes ☐ No ☐

(m) Ear / hearing problems Yes ☐ No ☐

(n) Thyroid problems Yes ☐ No ☐

(o) Kidney or liver problems (*e.g.,* chronic kidney disease) Yes ☐ No ☐

(p) Allergy to nuts, alcohol etc. Yes ☐ No ☐

(q) Any problems affecting your nose e.g. recurrent nose bleeds Yes ☐ No ☐

(r) Mental health illness Yes ☐ No ☐

4. Has any, otherwise healthy, member of your family under the age of 50 died suddenly during or soon after exercise? Yes ☐ No ☐

Blood Sampling

5. Are there any reasons why blood sampling may be difficult? Yes ☐ No ☐

6. Have you had a blood sample taken previously? Yes ☐ No ☐

Covid-19

7. Have you had a high temperature, persistent cough, cold, flu, any flu like symptoms, or lost your sense of taste and smell in the last month? Yes ☐ No ☐

8. Have you had covid-19? Yes ☐ No ☐

(a) If yes, how was this confirmed? .....

(b) Were you hospitalised? Yes ☐ No ☐

(c) Detail the duration of the illness and severity of symptoms etc.

.....

.....

.....

Other (eligibility criteria)

1. Weight loss or gain exceeding 5kg in the last 6 months? Yes ☐ No ☐
2. Are you currently participating in another clinical research trial? Yes ☐ No ☐
3. Taken beta-alanine or carnosine supplements in the last 6 months? Yes ☐ No ☐
4. Taking any other medications or over-the-counter products? Yes ☐ No ☐
5. Are you pregnant, trying to become pregnant or breastfeeding? Yes ☐ No ☐

If YES to any question, please describe briefly if you wish (e.g. to confirm problem was/is short-lived, insignificant, or well controlled.) (*continue overleaf, if necessary*).

.....

.....

.....

.....

Appendix 7. Informed consent form (Chapter 4)

NOTTINGHAM TRENT UNIVERSITY

Aston University BIRMINGHAM UK

IRAS Project ID: 279587, REC Number: 21/NW/0280

Participant Statement of Consent to Participate in the Investigation Titled:

***β*-Alanine Supplementation in Adults with Overweight or Obesity: A Randomised Controlled Feasibility Trial**

Name of Chief Investigator: Mr Joseph J Matthews

Please **INITIAL** box

1. I confirm that I have read the Participant Information Sheet dated 21/07/2022 (version 3) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐

3. It has also been explained to me by a member of the Research Team that the risks and side effects that may result from my participation are as follows: parathesia (mild itching, skin tingling, or pins and needles), which are sometimes felt in the face, neck, or back of the hands. Not everybody will get these, and the effects are usually mild and last for around 30 minutes after taking the supplement. ☐

4. I understand that my personal and pseudonymised data collected during the study may be looked at by individuals from the Research Team at Nottingham Trent University and Aston University, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my data where it is relevant to the study. ☐

5. I agree to provide blood and urine samples as part of my involvement in this study, as described in the Participant Information Sheet dated 21/07/2022 (version 3), and I understand I will not gain any direct personal or financial benefit from them. ☐

6. I understand that after the study, anonymised (deidentified) research data will be publicly available and may be used for purposes not related to this study. However, it will not be possible to identify me from these data. ☐

When completed: 1 for participant; 1 for researcher site file (original).

Appendix 7. Informed consent form (Chapter 4)

NOTTINGHAM TRENT UNIVERSITY

Aston University BIRMINGHAM UK

7. I agree to my General Practitioner being informed of my participation in the study. ☐

8. I agree for my anonymised samples to be used in future research, here or abroad, which has ethical approval. **Note: if you wish to "opt-out" of this, please put an "X" in the box instead of your initials. Your samples will still be used for the current research but will not be used in future research.** ☐

9. I confirm that I have completed the health questionnaire and know of no reason, medical or otherwise that would prevent me from partaking in this research. ☐

10. It has been explained to me that there may be additional risks arising from the current COVID-19 pandemic. I agree to abide by the special measures which have been verbally explained to me for this study together with such Government Guidelines that are prevailing at the time. ☐

11. I, _____. agree to take part in the above study. ☐

_____	_____	_____
Name of Participant	Date	Signature

_____	_____	_____
Name of Person Taking Consent (Researcher)	Date	Signature

When completed: 1 for participant; 1 for researcher site file (original).

Appendix 8. Modified GASE questionnaire (Chapter 4)

Modified GASE-P: Generic Assessment of Side Effects
Patient Version

Please rate whether you have experienced one of the following complaints during the last seven days by using the scale below. Please indicate by circling "NO" or "YES" whether you believe that these symptoms are relate to the trial supplement.

0 = Complaint not present
1 = Mild: complaint causes mild distress or discomfort, but no impairment in daily functioning
2 = Moderate: complain causes moderate distress or discomfort or at least some impairment in daily functioning
3 = Severe: complaint causes severe distress and discomfort, severe impairment in daily functioning or acute danger to health

Please try to choose an answer even if you are not completely sure.

I have had the following complaints:

Complaint	Severity not present	mild	moderate	severe	Is this complaint related to the trial supplement?	
Paraesthesia: Tingling, Numbness or Prickly Sensation						
Scalp	0	1	2	3	NO	YES
Forehead	0	1	2	3	NO	YES
Ears	0	1	2	3	NO	YES
Shoulders	0	1	2	3	NO	YES
Upper chest/back	0	1	2	3	NO	YES
Upper arms	0	1	2	3	NO	YES
Forearms	0	1	2	3	NO	YES
Hands (palm)	0	1	2	3	NO	YES
Hands (dorsal)	0	1	2	3	NO	YES
Other (please state)	0	1	2	3	NO	YES
Headache	0	1	2	3	NO	YES
Hair loss	0	1	2	3	NO	YES
Dry mouth	0	1	2	3	NO	YES
Dizziness	0	1	2	3	NO	YES
Chest pain	0	1	2	3	NO	YES
Palpitations, *irregular heartbeat*	0	1	2	3	NO	YES
Breathing problems	0	1	2	3	NO	YES
Low blood pressure, *or other circulation problems*	0	1	2	3	NO	YES
Abdominal pain	0	1	2	3	NO	YES

Appendix 8. Modified GASE questionnaire (Chapter 4)

Nausea	0	1	2	3	NO	YES
Vomiting	0	1	2	3	NO	YES
Constipation	0	1	2	3	NO	YES
Diarrhoea	0	1	2	3	NO	YES
Reduced appetite	0	1	2	3	NO	YES
Increased appetite	0	1	2	3	NO	YES
Difficulty Urinating	0	1	2	3	NO	YES
Problems with sexual performance or sex organs	0	1	2	3	NO	YES
Females: painful or irregular menstruation	0	1	2	3	NO	YES
Skin rash or itching	0	1	2	3	NO	YES
Tendency to develop bruises	0	1	2	3	NO	YES
Fever, *increased temperature*	0	1	2	3	NO	YES
Abnormal sweating	0	1	2	3	NO	YES
Hot flashes	0	1	2	3	NO	YES
Convulsions or seizures	0	1	2	3	NO	YES
Fatigue, *loss of energy*	0	1	2	3	NO	YES
Tremor	0	1	2	3	NO	YES
Insomnia, *sleeping problems*	0	1	2	3	NO	YES
Nightmares, *abnormal dreams*	0	1	2	3	NO	YES
Back pain	0	1	2	3	NO	YES
Muscle pain	0	1	2	3	NO	YES
Joint pain	0	1	2	3	NO	YES
Agitation	0	1	2	3	NO	YES
Irritability, *nervousness*	0	1	2	3	NO	YES
Depressed mood	0	1	2	3	NO	YES
Thoughts about suicide	0	1	2	3	NO	YES
Anxiety, *fearfulness*	0	1	2	3	NO	YES
Further symptoms (please name these symptoms)	0	1	2	3	NO	YES

Appendix 9. "How to take your supplement" instructions (Chapter 4)

How to take your supplement

Example daily intake

- *Breakfast: take 2 tablets*
- *Lunch: take 2 tablets*
- *Dinner: take 2 tablets*
- *Before bed: take 2 tablets*

If you miss a dose, <u>DO NOT</u> make up for it by increasing your next dose.

Record your intake in the supplement diary.

Each dose of 2 tablets provides 1.2 grams of beta-alanine or tapioca starch (placebo).

The total daily intake will be 4.8 grams of beta-alanine or tapioca starch (placebo).

In the event of an emergency, you should call 999.

If you have any questions or concerns, you can contact the Research Team by:

- *Text: 07908678944 (our study phone number)*
- *Email: joseph.matthews2016@my.ntu.ac.uk*

Appendix 10. Supplement diary: page one (Chapter 4)

Supplement Diary					
Instructions: *Keep this record somewhere visible, like on your kitchen workshop, and use it as a tick sheet to record which doses you take and which ones you have missed.*					
Week Commencing	*Day*	*Breakfast*	*Lunch*	*Dinner*	*Before Bed*
	Monday	✓✓	✓✓	✓✓	✓✓
EXAMPLE	*Tuesday*	✓✓		✓✓	
	Wednesday	✓✓	✓✓	✓✓	✓✓
	Thursday		✓✓		✓✓
	Friday	✓✓	✓✓		✓✓
	Saturday		✓✓		✓✓
	Sunday	✓✓		✓✓	✓✓
	Total number of missed doses this week: 8				
Week Commencing	*Day*	*Breakfast*	*Lunch*	*Dinner*	*Before Bed*
	Monday				
	Tuesday				
	Wednesday				
	Thursday				
	Friday				
	Saturday				
	Sunday				
	Total number of missed doses this week:				
Week Commencing	*Day*	*Breakfast*	*Lunch*	*Dinner*	*Before Bed*
	Monday				
	Tuesday				
	Wednesday				
	Thursday				
	Friday				
	Saturday				
	Sunday				
	Total number of missed doses this week:				
Week Commencing	*Day*	*Breakfast*	*Lunch*	*Dinner*	*Before Bed*
	Monday				
	Tuesday				
	Wednesday				
	Thursday				
	Friday				
	Saturday				
	Sunday				
	Total number of missed doses this week:				

Appendix 11. Actiheart 5 diary (Chapter 4)

Actiheart Diary

Instructions: *Keep this record somewhere visible and use it as a diary to record the times you take the Actiheart off and put it back on (i.e., when having a shower/bath or when sleeping)*

You will wear the Actiheart for 3 days.

From ***to***

Date	*Time Removed*	*Time Reattached*	*Reason*