The Influence of Exercise Training on the Profile of Circulating Metabolites and Small Extracellular Vesicles, and the Bioactivity of Human Plasma

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Declaration:

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of PhD is entirely my own work, and that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge breach any law of copyright, and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

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Dedication

For teaching me what it means to be strong, I would like to dedicate this thesis to my Mother, Fiona.

Acknowledgements

The study of exercise physiology has proven to be the great passion of my life. I would to acknowledge the people who have help to cultivate my passion for studying exercise science and helped me to complete the work contained within this thesis.

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Maybe one day I can pay you back by teaching you how to make really good figures.

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Ian Darragh

Abstract

Exercise factors are circulating signalling molecules that are responsive to acute exercise and exercise training. This thesis investigated (i) whether circulating metabolites, a source of exercise factors, change in response to short term exercise training, (ii) whether circulating metabolites and small extracellular vesicles (EVs; 'carriers' of exercise factors) are altered in an association with divergent histories of exercise training, and (iii) whether plasma extracted from men divergent exercise training histories influenced BT-549 cells.

Nine sessions of sprint interval training reduced the abundance of 11 fatty acids in circulation in blood samples taken at rest. A history of exercise training was associated with alterations in the abundance of 44 of metabolites. Under standardized measurement conditions the reliability of plasma metabolite concentrations varied largely at the level of individual metabolites with ~48% of metabolites displaying *good*-to-*excellent* reliability. A history of exercise training was not associated with an altered abundance of small EVs across multiple methods of small EV identification. BT-549 cells cultured with media supplemented with plasma derived from men with divergent exercise training histories had increased cell proliferation, greater anoikis, and lower extracellular matrix invasion compared to standard cell-culture media conditions.

This thesis provides insight into whether circulating metabolites are altered at rest in response to, or associated with, exercise training. This thesis does not support a paradigm wherein the 'exercise factor environment' at rest is largely different between healthy individuals with divergent training histories as assessed in terms of metabolite profile, small EV presence, or bioactive effects on BT-549 cells.

Peer-Reviewed Publications arising from this thesis

Literature review

Darragh, I.A.J., O'Driscoll, L., Egan, B., 2021. Exercise Training and Circulating Small Extracellular Vesicles: Appraisal of Methodological Approaches and Current Knowledge. Frontiers in Physiology 12, 1894. https://doi.org/10.3389/fphys.2021.738333

Original Research Article

Darragh, I.A.J., Aird, T.P., O'Sullivan, A., Egan, B., Carson, B.P., 2022. The resting serum metabolome in response to short-term sprint interval training. Eur J Appl Physiol. https://doi.org/10.1007/s00421-022-05115-x

Original Research Article

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Additional Peer-Reviewed Publications Independent of this thesis

Original Research Article

Mooney, T., Malone, S., Izri, E., Dowling, S., **Darragh, I.A.J.,** 2021. The running performance of elite U20 Gaelic football match-play. Sport Sci Health. https://doi.org/10.1007/s11332-021-00760-9

Original Research Article

Nugent, F.J., Flanagan, E.P., **Darragh, I.,** Daly, L., Warrington, G.D., 2022. The Effects of High-Repetition Strength Training on Performance in Competitive Endurance Athletes: A Systematic Review and Meta-Analysis. The Journal of Strength & Conditioning Research. https://doi.org/10.1519/JSC.0000000000004217

Conference Proceedings Arising from this Thesis

International Biochemistry of Exercise (Toronto, Canada, May 2022)

Poster Presentation

The resting serum metabolome in response to short term interval training

Brian P. Carson, **Ian A.J Darragh,** Tom P. Aird, Aifric O'Sullivan, Brendan Egan

Poster Presentation

The influence of exercise training history on the resting plasma metabolome in humans across two separate days

Ian. A.J. Darragh & Brendan Egan

All-Ireland Postgrad Conference in Sport Science, Phys Activity and Physical Education (Dublin, Ireland September 2022)

Oral Presentation

Within-subject variability and the influence of exercise training history on the resting plasma metabolome in male athletes

Ian. A.J. Darragh & Brendan Egan

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List of Abbreviations

Interleukin-6 (IL-6)

Extracellular Vesicle (EV)

L-ß-Aminoisobutyric acid (BAIBA)

International Society For Extracellular Vesicles (ISEV)

Maximal Oxygen Uptake (VO2max)

1 Repetition Maximum (1RM)

Maximum Power Output (W_{max})

Metabolic Control Analysis (MCA)

Principal Component Analysis (PCA)

Mass Spectrometry (MS)

Liquid Chromatography and Mass Spectrometry (LCMS)

Gas Chromatography and Mass Spectrometry (GCMS)

Partial-Least Squares Discriminant Analysis (PLSDA)

Intraluminal Vesicle (ILV)

Multivesicular Body (MVB)

Minimal Information for the Study of Extracellular Vesicles (MISEV)

Nanoparticle Tracking Analysis (NTA)

microRNA (miRNA)

Sprint Interval Training (SIT)

The Metabolomics Innovation Centre (TMIC)

Kaiser-Mayer-Olkin (KMO)

Analysis of Variance (ANOVA)

False Discovery Rate (FDR)

Fold Change (FC)

Fatty Acid Binding Protein (FABP)

3-hydroxyacyl-CoA dehydrogenase (βHAD)

Endurance Trained Participants (END)

Strength Trained Participants (STR)

Recreationally Active Control Participants (CON)

Intraclass Correlation Coefficient(s) (ICC)

Lecithin-Cholesterol Acyltransferase (LCAT)

High Density Lipoprotein (HDL)

Sodium Dodecyl Sulphate (SDS)

Size Exclusion Chromatography (SEC)

Polyvinylidene Difluoride Membrane (PVDF)

Bovine Serum Albumin (BSA)

Transmission Electron Microscopy (TEM)

Lysophosphatidylcholines (LPCs)

Phosphatidylcholines (PCs)

Sphingomyelins (SMs)

Fetal Bovine Serum (FBS)

Serum-Free Media (SFM)

Roswell Park Memorial Institute Medium - 1640 (RPMI)

Extracellular Matrix (ECM)

Minimal Essential Medium (MEM)

2-Hydroxyethyl Methacrylate (poly-HEMA)

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Chapter 1
Introduction

1.1. INTRODUCTION

In 1961 a series of experiments performed by M.S Goldstein observed that cross-transfusion of blood from exercised to non-exercised dogs elicited hypoglycaemia, even in circumstances where both donor and recipient dogs were de-pancreatized (i.e., hypoglycaemic effects could not be mediated by insulin secretion in either animal). This observation stimulated Goldstein to speculate about the presence of 'a hypoglycaemic factor of muscular work' that could provide an explanatory mechanism for the observation that muscle contraction stimulated increases in glucose uptake (Goldstein, 1961). This piece of work went largely unrecognised by Goldstein's peers, and indeed at the time of writing, the original article describing Goldstein's work has only ~100 citations. Interest in whether there were humoral factors elicited by muscular work that mediated 'beneficial' physiological effects remained scant until the mid-1990s when a series of experiments identified that the cytokine Interleukin-6 (IL-6) demonstrated was robustly increased in circulation in response to acute exercise, and infusion of IL-6 enhanced skeletal muscle glucose uptake (Pedersen and Febbraio, 2008). These observations regarding IL-6 acknowledged and revived interest in Goldstein's initial findings, as IL-6 was suggested to be Goldstein's 'muscular work factor' (Pedersen et al., 2004). Goldstein's term for the then-speculated factor being paraphrased into the more manageable term of 'exercise factor' (Pedersen et al., 2004).

Exercise factors have no unified definition, but have recently been defined as humoral molecules that are released in response to acute exercise and/or exercise training and demonstrate evidence of bioactivity(ies) (e.g. through eliciting altered signal transduction and/or gene expression) that correspond to identified 'health benefits' (Chow et al., 2022). However, within this thesis, I will consider an exercise factors in a more encompassing fashion and define them as *'any circulating molecule whose abundance robustly changes in response to acute exercise or exercise training and whose altered abundance can be linked with altered*

bioactivity(ies) that can be associated with exercise adaptations and/or health benefits'. This definition acknowledges that exercise-associated decreases in the abundance of certain (discussed subsequently in this Chapter, but also sees discussions on the metabolite homovanillic acid in Chapters 4 and Chapter 7 of this thesis) can also potentially elicit 'beneficial' bioactivities (i.e., an exercise-induced decrease in the circulating abundance of a molecule that otherwise inhibits 'beneficial' signalling).

All 'major' species of molecule (e.g. protein, nucleic acid and metabolites) show responsiveness to both acute exercise (Contrepois et al., 2020) and exercise training (Fernández-Sanjurjo et al., 2018; Hoffmann and Weigert, 2017; Sakaguchi et al., 2019) and therefore it is possible that many molecule types and individual molecules within types may act as exercise factors. In some circumstances, exercise factors have been delineated into different categories such as 'myokines' – a term which traditionally referred to protein exercise factors that originate specifically from skeletal muscle (Whitham and Febbraio, 2016) – but has recently been used to refer to any exercise factor that originates from skeletal muscle (Brooks et al., 2023; Chow et al., 2022). Exercise factors have also been referred to as 'exerkines' - a term originally applied only to RNA and protein exercise factors that are measured or identified in circumstances where tissue origin is ambiguous (e.g. from antecubital venous plasma) (Safdar et al., 2016), but also has recently been used to refer to any exercise factor that is measured or identified in circumstances where tissue origin is ambiguous (Chow et al., 2022). To avoid the challenges of dealing with an unsettled and developing terminology of exercise factor 'sub-types' (i.e. myokines and exerkines). This thesis will only employ use of the term 'exercise factor' as this term is that which is most closely aligned to the original (Goldstein, 1961; Pedersen et al., 2004) and is ambiguous enough to be encompassing of all contexts (i.e. acute or chronic exercise responses and molecules of different species and tissue origins).

1.2. EXERCISE FACTOR RESPONSES TO ACUTE EXERCISE AND EXERCISE TRAINING

The response of exercise factors to acute exercise is generally suggested to follow what could be considered a 'typical' hormonal response. In essence, the physiological responses that are required to initiate and sustain exercise (e.g. altered tissue metabolism, increased mechanical tension, changes in blood flow dynamics) (Hawley et al., 2014) act as the stimuli for the release of exercise factors, which then circulate and mediate local or distal effects through intracellular uptake and metabolism or receptor-mediated alterations in signal transduction that is related to metabolic effects (e.g., increased glucose uptake) and/or changes in gene expression (Severinsen and Pedersen, 2020). The majority of research concerning exercise factors to date has focused on acute exercise responses (Chow et al., 2022; Safdar et al., 2016; Severinsen and Pedersen, 2020), this likely in-part due to the degree of metabolic and mechanical perturbations elicited by exercise (Hawley et al., 2014) representing a logical stimulus for exercise factor release and the historic president that prototypical exercise factors such as IL-6 have been identified through studying acute exercise responses (Pedersen and Febbraio, 2008).

Considerably less is understood regarding the response of exercise factors to exercise training (i.e. alterations in the humoral presence of factors that are more attributable to repeated exercise *training* compared to individual acute exercise bouts). The most often reported response of exercise factors to exercise training has been described as 'post-training' changes in the abundance of exercise factors outside of the immediate post-exercise period (ideally >24hrs post exercise) (Fernández-Sanjurjo et al., 2018; Hoffmann and Weigert, 2017; Sakaguchi et al., 2019). Consequently, responses of exercise factors to exercise training have been acknowledged and encompassed into recent expansion(s) of the definitions of some exercise factor subtypes (myokines and exerkines) (Chow et al., 2022). However, it is not currently understood, and indeed is less intuitively apparent, what effects an altered presence

of exercise factors at rest in response to exercise training may be responsible for. Comparisons to exercise factors derived from acute exercise could suggest that chronic exercise factor responses represent a residual mechanism for conveying exercise 'health benefits' outside of the immediate proximity to acute exercise bouts or serve a mechanism for maintaining exercise adaptations. However, at the current time, this notion is purely speculative and shifts in the resting presence of exercise factors in response to chronic exercise training may equally represent a residual circulating artifact of the alterations to tissue metabolism induced by exercise training (Haskell, 1994).

1.3. METABOLITES AS EXERCISE FACTORS

Metabolites are low molecular weight (~1.5kDa) and mostly organic chemicals that are generally categorised as the reactants, intermediates or products of metabolic pathways (Dunn et al., 2011). While metabolites have only recently gained open recognition as a family of exercise factors (Brooks et al., 2023; Chow et al., 2022), it is notable that by definition, the first and currently most well characterised (in terms of release kinetics and bioactivity(ies)) molecule that is identified to behave as an exercise factor is the metabolite lactate (Brooks et al., 2023; Owles, 1930). Several hundred metabolites will change in circulation in response to acute exercise (Contrepois et al., 2020; Schranner et al., 2020) and some exercise responsive metabolites such as muscle-derived L-ß-Aminoisbutyric acid (BAIBA) have been associated with 'beneficial' metabolic effects, such as 'browning' of adipose tissue (Chow et al., 2022; Tanianskii et al., 2019)

Circulating metabolites are also responsive to exercise training with some studies reporting athletes with divergent exercise training backgrounds (Schranner et al., 2021) or different levels of fitness (Monnerat et al., 2020) present with different plasma metabolite profiles at rest. In response to exercise training, several metabolic processes can be altered at rest, such as lipid oxidation (Smorawiński et al., 2001) and muscle protein synthesis

(Figueiredo, 2019). Given the nature of how metabolites are produced (i.e. as the product of metabolic reactions) it is possible to speculate that changes to metabolic processes at rest (e.g., protein synthesis or fat oxidation) may create altered abundance(s) of tissue metabolites that may then permeate to circulation. However, it is currently unclear the extent to which changes in circulating metabolites at rest in association with exercise training are representative of a shift in the presence of metabolites as signalling molecules (i.e. an 'exercise factor' response) or simply as residual products of altered tissue metabolism (components of a so-called 'metabolic fingerprint') (Kosmides et al., 2013) or both.

1.4. EXTRACELLULAR VESICLES AS EXERCISE FACTOR CARRIERS

Extracellular vesicles (EVs) are a family of doubly membraned particles that are released by most eukaryotic cell types and are enriched with heterogenous cargoes (e.g. metabolites, nucleic acids and proteins) derived from their cell of origin (van Niel et al., 2018). EVs are continually present in all biofluids but demonstrate enhanced release in response to physiological stimuli and can elicit bioactivity through uptake and delivery of their molecular cargo to recipient cells (Yáñez-Mó et al., 2015). Interest in small EVs (<150nm) within the context of studying exercise factors has recently developed traction, primarily due to the observation that many circulating proteins as candidate exercise factors did not contain signal (secretory) peptide sequences, but were present in online EV expression databases (Safdar et al., 2016). Small EVs have been identified to enrich in circulation in response to acute exercise (Nederveen et al., 2021). This coupled with the observation that EVs carry 'bioactive' molecular cargoes have generated a degree of consensus that small EVs may represent a medium through which some or many exercise factors are 'packaged', transported and consequently exchanged between cells during exercise and the immediate post-exercise recovery period (Chow et al., 2022; Murphy et al., 2020; Nederveen et al., 2021; Safdar et al., 2016; Vechetti et al., 2020). The response of small EVs to exercise training is less clear. At

rest, the *cargo* of circulating small EVs appear altered in association with exercise training (i.e. a response that mimics that which has previously been reported for exercise factors that were not associated with EVs) (Nederveen et al., 2021). However, some studies have also reported that the *number* of circulating small EVs may also be increased at rest in response to exercise training (Bei et al., 2017; Ma et al., 2018). Therefore, it is currently unclear whether the response of small EVs to exercise training is represented exclusively by a shift in small EV cargo profile, or the abundance of small EV cargo and small EVs themselves.

1.5. HEALTH PROMOTING EFFECTS OF THE EXERCISE FACTOR MILIEU

Aside from demonstrating some kind of responsiveness to acute or exercise training, the second essential characteristic for validating a given candidate molecule as an exercise factor is that the molecule is demonstrated to exert some kind of physiological effect or response (Chow et al., 2022). Indeed, as exercise factors are generally considered within the context of contributing to the 'health promoting' effects of exercise (Booth et al., 2017; Whitham and Febbraio, 2016), the 'bioactivities' of exercise factors are also largely discussed within the context of also being health promoting (Carson, 2017; Chow et al., 2022; Hojman et al., 2018; Safdar et al., 2016; Whitham and Febbraio, 2016). Although, by current definition an exercise factor must merely demonstrate a capacity to exert some kind of physiological influence and linking the bioactivity of a candidate exercise factor to a health benefit *per se* is not necessary for validating candidacy as an exercise factor (Chow et al., 2022).

Studies employing omics technology have simultaneously identified several hundred factors that demonstrate concentration change(s) in response to acute exercise across multiple exercise factor species (metabolites, RNA and protein) (Catoire et al., 2014; Contrepois et al., 2020; Morville et al., 2020; Schranner et al., 2020; Wei et al., 2023) and several hundred proteins have also been identified in association with preparations of small EVs separated from acutely exercised plasma (Whitham et al., 2018). This evidences that the 'landscape' of

potential exercise factors is vast. Despite this possibility, the number of candidate exercise factors that have identified 'bioactivities' is comparably lower (e.g. <100) (Chow et al., 2022; Hoffmann and Weigert, 2017). Several exercise factors are also reported to have identified 'overlapping' physiological effects (e.g., the proteins Irisin and Angiopoietin-like protein 4 and the metabolite BAIBA are suggested to induce 'browning' of white adipose tissue), which suggests redundancies and/or complementarities across individual exercise factors in terms of effects (Chow et al., 2022).

An alternative approach to exploring the physiological relevance of exercise factors is to employ a more broad and ambiguous approach by exploring cellular or physiological effects of exercise-derived plasma or sera, which, in theory, would contain the entirety of the exercise factor milieu and is technically a method for mimicking the initial work of Goldstein (Goldstein, 1961). Surprisingly limited research investigating the effects of exercise-associated plasma or sera has been performed, with the majority of studies examining the influence of sera on the proliferation or activity of cancer-cell lines (Hojman et al., 2018; Metcalfe et al., 2021; Soares et al., 2021). In particular, there is limited evidence regarding the physiological effects of sera or plasma derived from exercise trained individuals or individuals with a history of exercise training at rest, despite many reports of the 'profile' of exercise factors changing rest in response to chronic exercise (Chow et al., 2022; Fernández-Sanjurjo et al., 2018; Hoffmann and Weigert, 2017; Sakaguchi et al., 2019).

1.6. THE PURPOSE OF THIS THESIS

The purpose of this thesis is to broadly examine how 'exercise factors' are altered at rest in association with exercise training. Using metabolites as the exercise factor 'family' of choice, this thesis examines the influence of exercise training or exercise training history on three broad elements of the 'exercise factor response'; Firstly, whether a short-term exercise training intervention divergent histories of exercise training alter the presence of metabolites

at rest and whether potential alterations in the presence or concentration of circulating metabolites is durable across separate days; secondly, whether the presence of small extracellular vesicles are altered in association with different histories of exercise training and whether the presence of small EVs is durable across separate days; thirdly, whether plasma extracted from individuals with different histories of exercise training at rest, which is inferred to represent a snapshot of the totality of the 'exercise factor environment', exerts differing effects on functional assays designed to mimic cancer hallmarks in a triple-negative breast cancer cell line.

Specific Aims of this thesis

Aim 1: Investigate whether metabolites shift in resting serum and plasma of 'exercise trained' individuals at rest.

Aim 2: Investigate whether the abundance of small EVs is altered in exercise trained individuals at rest using orthogonal methods that adhere to guidelines provided by the International Society for the Study of Extracellular Vesicles (ISEV).

Aim 3: Investigate whether plasma extracted from exercise trained individuals at rest elicits 'anti-cancer' effects in BT-549 triple-negative breast cancer cells.

Chapter 2
Literature Review

Please note: Portions of this literature review are adapted from the following published article. Darragh, I.A.J., O'Driscoll, L., Egan, B., 2021. Exercise Training and Circulating Small Extracellular Vesicles: Appraisal of Methodological Approaches and Current Knowledge. Frontiers in Physiology 12, 1894.

This article is available as **Appendix A**

2.1. EXERCISE FACTORS

2.1.1 Introduction

When individuals undertake repeated bouts of exercise (i.e. exercise training), acute molecular responses and chronic adaptive changes occur that result in functional changes at the levels of cells, tissues, organs and systems. These changes ultimately produce improvements in health status, and/or exercise capacity and performance, amongst other phenotypic changes. Currently, the totality of processes that regulate exercise adaptation are incompletely understood, and uncovering novel mechanisms that contribute to, or are characteristic of, this process represents a central topic of research for exercise physiologists (Egan and Sharples, 2023).. Acute exercise induces the enrichment in circulation of a vast array of factors including metabolites, several RNA species, and peptides/proteins (Contrepois et al., 2020). These may be derived from a variety of sources that include contracting skeletal muscle, among other prominent examples such as endothelial, cardiac, hepatic, and adipose tissues (Estébanez et al., 2020; Murphy et al., 2020). The physiological relevance of the enrichment of many of these factors is mostly unknown, but they may contribute to regulation of homeostasis and substrate metabolism during and after exercise (Murphy et al., 2020), and/or serve as the initiating signals for the adaptations that occur in response to repeated bouts of exercise (Hoffmann and Weigert, 2017). Indeed, the prevailing sentiment is that the activity of exercise factors convey 'health' benefits and have been speculated to provide preventative or remedial effects in numerous diseases, such as diabetes, cardiovascular disease and some cancers (Whitham and Febbraio, 2016).

While all 'major' molecular species have the potential to act as exercise factors, a particular focus of this thesis is the role of metabolites as exercise factors. Metabolites are low molecular weight (~1.5kDa) and mostly organic chemicals that are generally categorised as the reactants, intermediates or products of metabolic pathways (Dunn et al., 2011). As such,

metabolites represent a signal for ongoing molecular processes (e.g. protein-protein interactions) and/or the terminal consequences of metabolic pathways (Nicholson and Wilson, 2003). Metabolites are worthy to be considered a prominent aspect of the overall 'exercise factor response', as hundreds of metabolites can demonstrate a change in concentration shifts in response to acute exercise (Schranner et al., 2020) and select metabolites, namely lactate, possess strong and robust circulating responses to exercise with concomitant metabolic and signalling effects identified (Brooks et al., 2023). Circulating metabolites may also demonstrate responsiveness to exercise training evidence by individuals with a history of exercise training possessing altered 'profiles' of circulating metabolites compared to alternatively exercise trained individuals (Al-Khelaifi et al., 2018; Monnerat et al., 2020; Schranner et al., 2021). Here I define "profiles" as the detection of individual factors as present or absent, and/or changes in their estimated abundance(s) in circulation. The observation of differential profiles suggests that exercise training may induce changes to the circulating milieu that are somewhat durable, rather than only transient and present during, and soon after, exercise. In the case of circulating metabolites the idea of altered 'metabolite profiles' in response to exercise training can additionally suggest that metabolites may not only serve as 'bioactive' exercise factors, but may also produce detectable and characterizable 'metabolic fingerprints' of exercise training (section 2.2.2).

Extracellular vesicles are a family of lipid bilayer encapsulated molecules that have regulated released from all nucleated cell types and are measurable in most common biofluids (e.g. blood, sweat, urine) (van Niel et al., 2018). EVs contain heterogenous bioactive molecules or "cargo" (e.g., metabolites, nucleic acids, proteins), which are derived from their cell of origin, and are capable of being taken up into cells that are either proximal or distal to their site of release. These features have led to the assumption that the primary function of EVs is to serve as "messages" between cells (Yáñez-Mó et al., 2015).EVs undergo circulating

enrichment in response to acute exercise (reviewed by (Estébanez et al., 2020) and (Nederveen et al., 2021)) and this has produced speculation that EVs may represent a medium of transport for exercise factors (Murphy et al., 2020; Safdar et al., 2016). Under current models, it is speculated that the principal mechanism by which many exercise factors appear in circulation is packaged as cargo within released EVs (Estébanez et al., 2020). This is suggested to serve as a means of protecting certain factor types (e.g. RNA) from degradation, while also explaining how proteins devoid of secretory peptide sequences may still enrich in circulation in response to exercise (Safdar et al., 2016). Much of the current interest regarding the response of EVs to exercise has been focused on a subfraction of EVs in smaller size ranges (diameters of 50-150 nm), termed "small EVs" (Estébanez et al., 2020; Nederveen et al., 2021; Vechetti et al., 2020), with several studies having now investigated the effect of acute exercise and exercise training on the circulating profile (particle number, concentration/abundance, cargo and/or cargo density) of small EVs (section 2.3.4.)

When exercise factors are measured using analytical methods that enable simultaneous detection of many individual molecules in a single sample (i.e., 'omic' technologies), hundreds of candidate exercise factors from multiple molecular species (i.e. RNA, proteins, metabolites) can often be identified (Contrepois et al., 2020). This evidences that the landscape of exercise factors may present as an excitingly comprehensive and mixed milieu. This also creates an intimidating scenario in terms of searching for individual exercise factors for which determine potential 'beneficial' effects could be attributed to. An alternative perspective may be that it is more important to first explore whether there are robust bioactivities that can be attributed to plasma/sera derived from acutely exercised or exercise trained individuals (Hojman et al., 2018). The reason that exploring the effects of exercise-derived plasma/sera is important is that these blood fractions will, in theory, contain the 'entirety' of the exercise factor milieu and

therefore if bioactivity cannot firstly be attributable to exercised plasma/sera then the potential for 'health promoting' effects of exercise factors may consequently be overstated.

2.1.2. Overview of the response of exercise factors to acute exercise

For each subcategory of exercise factors (i.e. metabolites, nucleic acids, proteins), the number of individual molecules that are reported to change in response to acute exercise is often estimated to be hundreds (Fernández-Sanjurjo et al., 2018; Guseh et al., 2020; Sakaguchi et al., 2019). A small number of molecules may decrease in abundance in response to acute exercise, but it is generally considered that the majority of changes in exercise factors are in the form of increased circulating abundance(s) (Fernández-Sanjurjo et al., 2018; Murphy et al., 2020; Severinsen and Pedersen, 2020; Son et al., 2018). Some individual factors are well-described and understood in terms of kinetics of response to exercise and subsequent metabolic and/or molecular effects, such as the metabolite lactate (Brooks, 2018; Hall, 2010), and the myokine interleukin-6 (IL-6) (Pedersen and Febbraio, 2008). Mention of the latter molecule is of particular importance as an illustration of a prototypical exercise factor. During and soon after a bout of aerobic exercise, circulating IL-6 is robustly (and sometimes substantially) increased, is mostly derived from contracting skeletal muscle (Pedersen and Febbraio, 2008), and exerts relevant effects during (e.g. enhanced hepatic glucose output and adipose tissue lipolysis) and after (e.g. enhanced insulin sensitivity in skeletal muscle and enhancing pancreatic β-cell mass) exercise (Severinsen and Pedersen, 2020).

Collectively, these observations regarding IL-6 are seminal, as they established an intellectual foundation for the paradigm of how many exercise factors are now generally presumed to function. However, for the majority of exercise factors, limited information is available beyond (sometimes inconsistent) reports that indicate a change in circulating concentration(s) in response to acute exercise. The details regarding subcategories of exercise factors (e.g. potential bioactivities, variability within and between subcategories, and

variability in response to different types of exercise) are beyond the scope of this chapter, but are discussed elsewhere in relation to metabolites (Kelly et al., 2020; Sakaguchi et al., 2019), RNAs (Fernández-Sanjurjo et al., 2018; Sapp et al., 2017), and proteins (Eckel, 2019; Hoffmann and Weigert, 2017; Severinsen and Pedersen, 2020). As EVs are proposed as potential carriers of many of these exercise factors (Safdar et al., 2016), there is an implication that the circulating response of EVs to acute exercise should mimic the general response of exercise factors. In the case of an exercise-induced "increase", this change would manifest through a combination of an absolute increase in the circulating abundance of EVs, a change in cargo profile, and/or an increase in cargo density per EV.

2.1.3. Study design considerations for investigating the adaptive response to exercise training

Prior to considering whether exercise factors and/or small EVs are responsive to exercise training, it is salient to consider the various study design approaches that are employed to study exercise adaptation (figure 2.1). Describing the features, advantages and disadvantages of each of these designs is pertinent to this chapter, as acknowledgement of the limitations of study designs also informs the extent to which inferences can be made regarding measured changes in exercise factors and/or EVs associated with exercise training. There are three broad study designs that can be employed to examine adaptive changes in response to exercise training, namely pre-post intervention studies, cross-sectional studies, and longitudinal/prospective cohort studies.

2.1.4. Pre-post intervention study designs

Pre-post intervention study designs represent a direct approach for investigating change in physiological and performance phenotypes in response to exercise training. There are numerous ways that these designs can be implemented including approaches of randomized or non-randomized control trials of parallel groups, single group designs, and detailed n=1 case

studies, amongst others as detailed elsewhere (Hecksteden et al., 2018a). However, the common feature within this design category is the exposure of participants to a period of structured exercise training focussed on changing some aspect of health or fitness, and a subsequent comparison of physiological and/or performance outcomes. In human trials, this outcome is achieved generally by either a within-group pre-post comparison (single group design), or between-group comparison to a sedentary control group (parallel group design).

There are also well-established models of exercise training in rodents, which provide the advantage of a high degree of control over homogenous groups, and arguably can provide more detailed mechanistic insight into adaptations to exercise. The advantages provided by rodent studies include control over ambulatory activity, feeding times and dietary composition, environmental conditions, sleep/wake cycles, and compliance with training to extents that are often not possible in human studies. However, these experimental advantages must be tempered with the caution that innate differences (e.g. morphological and metabolic) between rodents and humans can make it difficult to reproduce some findings between species (Fuller and Thyfault, 2021). Additionally, within-subject designs in rodents are often impossible to conduct for invasive measures such as muscle and blood sampling. This is principally due to methodological limitations such as requiring the excision of whole skeletal muscles, or physiological limitations such as blood volumes required for downstream analyses. For example, rats have a total blood volume of ~10 to 25 on average depending on bodyweight (Lee and Blaufox, 1985), whereas mice have generally less than 2 (Riches et al., 1973). Considering the volume of plasma required for many assays, including for the analysis of small EVs, where multiple independent assays are required and thus large blood volumes are desirable (Théry et al., 2018), sampling at serial timepoints in an individual rodent is generally unfeasible.

Therefore, exercise training studies in rodents typically involve randomized groups selected from a homogenous inbred lineage either subjected to a period of forced daily exercise training (e.g. treadmill running or swim training), or allocated as a group of sedentary controls. Groups are subsequently compared after being euthanised at the same time-point corresponding to the end of exercise training intervention. An important point to note is that with such a design, any differences observed between groups are technically cross-sectional in nature i.e. differences between trained and untrained groups (section 2.1.5). Therefore, results should be described as characteristic of the trained state, rather than as training-induced changes in a phenotype or outcome variable.

In general, the primary advantage of training intervention studies in rodents or humans is that phenotypic changes can be attributed to certain characteristics of the exercise training stimulus controlled as independent variables such as the frequency, intensity, duration and type of exercise bouts. However, these interventions also suffer from notable limitations. For example, most training studies employ sedentary participants and relatively short intervention durations (e.g. several weeks to months) (Hecksteden et al., 2018a). However, even short (e.g. two weeks) interventions can result in substantial increases in aerobic fitness, and induce marked changes in transcripts and proteins in skeletal muscle (Egan et al., 2013; Perry et al., 2010). Sedentary individuals are the most responsive to the onset of an exercise training intervention, and therefore the changes that often occur in short interventions are unlikely to be reflective of what would continue to occur were the training intervention planned appropriately and continued in an extended fashion. Short-term training studies cannot be assumed to represent the same adaptive processes present in individuals who have extensive exercise training histories (e.g., years/decades), which are typically assessed in cross-sectional study designs (section 2.1.5). Nor can the time course for the changes in physiological

responses and/or performance outcomes that are observed in short-term training studies being interpreted as continuing in a linear manner.

A final consideration is that especially in studies of the molecular regulation of adaptation in humans, control groups are often absent and single group within-subject designs are employed. This type of design has implications regarding the extent that pre- to post-intervention differences can be accurately quantified because the absence of a control group does not allow analyses to account for factors such as the regression to the mean artefact, and/or random variability, ever-present and uncontrollable, in biological measurements between- and within-individuals (Atkinson and Batterham, 2015).

2.1.5. Cross-sectional studies

Cross-sectional studies encompass a form of observational research that broadly involves an isolated comparison of measurements representative of traits of interest between members of distinct population groups (Levin, 2006). For effects of exercise training, cross-sectional studies tend to involve recruiting and stratifying participants into exercise-trained and sedentary or untrained groups, based on standardized criteria of fitness (e.g. maximal oxygen uptake, VO_{2max}; maximal power output, W_{max}; one repetition maximum, 1RM), training history or competitive status (e.g. elite cyclist or powerlifter) and/or exercise performance (e.g. personal best running a set distance, or weight lifted in competition). Subsequently, differences in relevant resting measurements and/or physiological responses and performance outcomes are compared between groups. In these reports, group differences are then often inferred as the indications of the consequences of exercise training, with the reasoning that when other major confounders (e.g. age, sex, indices of health status) are controlled for, the largest determinant of difference between groups is the regular participation in specific type(s) of exercise training.

The advantage of this type of study design is that it is often less expensive, less of time burden, and relatively simple to perform in comparison to training interventions. This generally

allows the collection of larger sample sizes at lower cost, and sometimes the recruitment of a higher calibre of trained participant such as elite athletes. The more likely participation of elite athletes in cross-sectional studies is due to having lower time commitments and generally not interfering with athletes' training regimes. However, cross-sectional studies are limited mostly by the fact they only provide a "snapshot" at a specific moment in time, and therefore indicate the prevalence of traits or responses between exercise-trained or untrained groups at the time of measurement (Sedgwick, 2014). These studies are not capable of providing information on *how* a parameter of interest has changed over time to eventually qualify a participant for inclusion in the trained group. Additionally, some performance-associated traits such as VO_{2max} may be relatively-stable (Edgett et al., 2018), and thus can provide some reliable ecological insight from a single timepoint measure.

This case is potentially less for many discrete biological measurements (such as circulating factors), which may have an inherent within-individual biological variability, often day-to-day and that is often unknown, or unaccounted for. For example, both of the exercise factors IL-6 and Fibroblast growth factor 21 are reported to have both innate diurnal and inter-individual variations in resting circulating concentrations (Sothern et al., 1995; Yu et al., 2011). Under these designs, the standardisation of preparation for participants is paramount including, but not limited to, preceding days' dietary intake, preceding night's sleep, duration of fasting and morning ambulation for morning fasted samples, and time since last bout of exercise. Therefore, in cross-sectional studies where repeated experimental measures are not employed (e.g. duplicate or triplicate sampling of participants under similar resting or experimental conditions across several laboratory visits), the results of some measures may be less reliable, which could reduce inferential utility.

While in many cases between-group differences may most obviously be the consequence of differences produced by prolonged exercise training, quantifying the exact

contribution of training to individual phenotypes is not possible in cross-sectional studies. Importantly, there is often no consensus on minimum performance or physiological thresholds that can be used to delineate an individual as "exercise-trained". For example, one excellent approach is the physiology of road cyclists, wherein the categories of trained, well-trained, elite, and world class have been delineated based on training status, estimated by the weekly frequency of training sessions, the total volume of weekly training and the total year spent training; racing status estimated by the number of competition days per year and international ranking of the rider; and physiological capacity, estimated by common physiological parameters associated with performance (e.g. W_{max} and VO_{2max}) (Jeukendrup et al., 2000).

More recently, a general framework that can be applied to any athletic event has been proposed – within this approach individuals can be clustered into one of five 'tiers' (ranging from sedentary to 'world-class') with the qualifying criteria for each tier being delineated by factors such as training frequency, degree of athletic identity, performance results relative to the 'population average' of the event and the 'level' of competition the individual competes at (e.g. regional, national, international) (McKay et al., 2022). However, adoption of these frameworks for participant classification remains a rare exception as many studies opt to use different and sometimes arbitrary minimum thresholds for their trained participants. This lack of consistency can have the effects of making comparisons between studies more difficult and making the trained groups somewhat heterogenous and not divergent enough from the untrained group in order reveal meaningful between-group differences. For example, if a study categorises a participant as 'trained' if they demonstrate a VO_{2max} >60 kg^{-1} min^{-1}, this approach may subsequently produce a group with individuals with VO_{2max} values ranging 60-80 kg^{-1} min^{-1}. This group would represent a mixed population group of trained, well-trained and elite athletes of similar physiological phenotypes, but in fact, they would likely be heterogenous on parameters such as training volume, lactate threshold, and mechanical efficiency. Indeed,

obvious physiological differences between individuals within this example range have been reported, e.g. in citrate synthase activity, an indication of mitochondrial mass (Jacobs and Lundby, 2012).

Lastly, cross-sectional studies often request that participants do not engage in moderate-to-vigorous exercise for a fixed period of time (e.g. ~24 to 48 h) before coming to the laboratory, but it is often difficult to validate adherence to this recommendation, with the problem being that the residual effects of acute exercise may also be present in these samples i.e., the "last bout effect", which describes residual, but transient, physiological changes induced by acute exercise that extend beyond the cessation of exercise (e.g. for ~24-48 hours), but do not manifest as an adaptive response to long-term exercise training (section 2.1.7.).

2.1.6. Longitudinal and prospective cohort study designs

Like cross-sectional designs, longitudinal study designs are a form of observational research, with the principal difference being that participants are monitored over an extended period of time, and re-sampled at various intervals (Caruana et al., 2015). Some studies may also employ overlapping longitudinal and interventional designs, although these reports tend to be rare in exercise science (Hecksteden et al., 2018a). Longitudinal studies possess the principal advantages of cross-sectional studies (i.e. easier to measure larger sample sizes compared to a structured/supervised training intervention) and some of the limitations (heterogenous groups of participants, limited standardisation of participant preparation prior to measurements, arbitrary thresholds of group qualification). However, this design may provide additional insight into the reliability of measures and the stability of traits within- and between-groups. Such between-group comparisons would require recruitment of a control or comparator group with the design then being a prospective cohort study. Longitudinal and prospective cohort designs are often also applied to measure the physiological and performance development of well-trained and elite individual athletes and teams preparing for competitive

events (Gabbett, 2005; García-Pallarés et al., 2010; Jones, 1998). While the latter is not specifically a training intervention per se (as the researchers often have no direct control over the training of the participants), these types of observational design does provide insight into long-term development that can occur in conjunction with regular, intensive exercise training. However, these studies can be confounded by concurrent interventions employed by athletes (e.g. nutrition and recovery strategies), and fluctuations in training strategies across monthly or annual cycles that may not necessarily be tracked extensively by researchers. These confounders can make it more challenging to associate which specific elements of the exercise training process have the largest proportional influences on specific physiological or performance outcomes.

2.1.7. "Last bout effect": the importance of sample timing

An important consideration across all types of study purporting to measure adaptive changes with exercise training is the proximity of measurement of the outcome interest to the final exercise bout of the training intervention, or most recent exercise bout in the case of cross-sectional designs. This consideration is especially important for outcomes with short half-lives of response/decay, such as changes in circulating parameters, but less important for outcomes with longer half-lives of response/decay. An example of the latter is that one effect of prolonged resistance exercise training is an increase in muscle mass (Egan and Zierath, 2013), which can be said to be a chronic adaptation given that it persists for several days and weeks after training cessation before declining steadily over several months (Mujika and Padilla, 2000a, 2000b).

In contrast, exercise elicits obvious beneficial effects as improvements in glycaemic control and insulin sensitivity, but these are likely transient and related to the most recent bout of exercise. For example, a single bout of exercise modestly lowers blood glucose concentrations in the immediate post-exercise period in patients with type 2 diabetes (Minuk et al., 1981), but also improves whole-body insulin sensitivity for up to 48 h after exercise

cessation (Koopman et al., 2005; Mikines et al., 1988; Perseghin et al., 1996). The additive effect of repeated bouts of exercise i.e. training, over and above those that are seen in the aftermath of an acute bout of exercise in isolation is relatively modest (Perseghin et al., 1996). In cross-sectional terms, although well-trained athletes have markedly-enhanced insulin sensitivity compared to sedentary individuals (King et al., 1987), the habitual state of an athlete is between two individual exercise training bouts, thereby making it difficult to distinguish differences between acute responses and chronic effects of exercise. However, when well-trained or physically-active individuals cease training or reduce their daily activity, a decline in insulin sensitivity rapidly occurs towards sedentary levels at a time when declines in aerobic fitness or increases in adiposity are negligible (Heath et al., 1983; Krogh-Madsen et al., 2010). In turn, a single bout of exercise is sufficient to restore insulin sensitivity in these detrained individuals to their previously trained values (Heath et al., 1983), suggesting the effects of exercise for improved whole-body insulin sensitivity are mediated in large part by the acute effects of a single bout of exercise, rather than a generalized training effect i.e. the absence of a synergistic effect.

Therefore, the physiological responses to a single bout of exercise, i.e. acute responses to exercise, can extend to 24 to 48 h after the cessation of an individual exercise bout, and thus some convergence exists between the effects of an acute bout of exercise and those effects associated with adaptation to exercise training. This phenomenon was coined by William L. Haskell (Haskell, 1994) as the “last bout effect” and proposes that some metabolic effects and purported health benefits of exercise (e.g. lowering of blood pressure or circulating lipoprotein profile) are attributable to the biological consequences of the most recent bout of acute exercise, rather than to a true training adaptation.

The overall implication is that in studies where the post-intervention, or cross-sectional, blood samples are taken within close proximity (e.g. <48 h) to the final, or most recent, training

bout, respectively, it may be difficult to discriminate whether some outcomes represent a chronic adaptation to training, or an extended residual effect of the last exercise bout. This point is particularly salient when interpreting current literature on the effect of exercise training on the resting profile of exercise factors, metabolites and circulating small EVs given the research designs employed to date (discussed in sections: 2.2.7 – metabolites and 2.3.4. – small EVs)

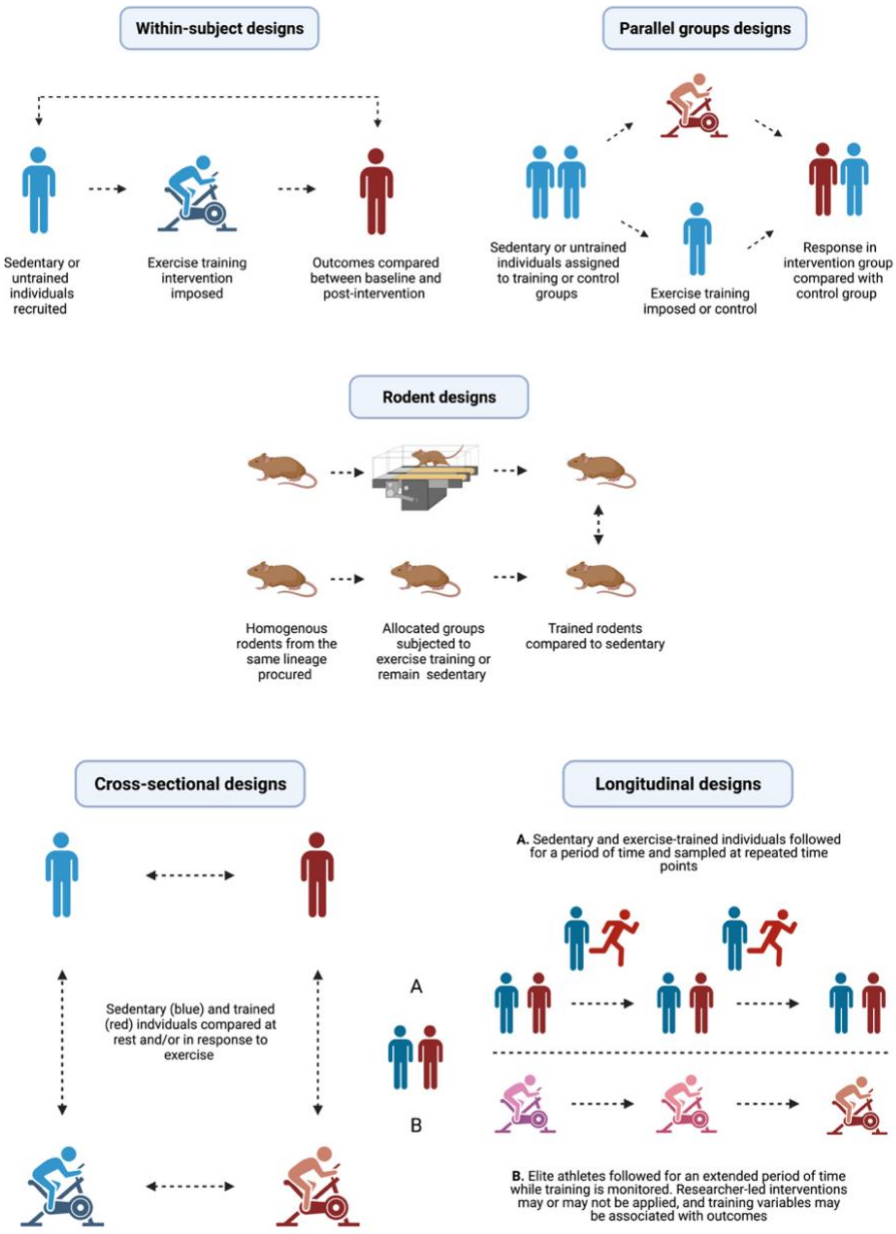

Figure. 2.1. Overview of study designs used to investigate the effect of exercise training on physiological and/or performance outcomes.

2.1.8. Does exercise training alter the resting profile of circulating exercise factors?

A pertinent question to our understanding of the biological importance and mechanistic consequence of exercise factors is whether these factors function as transient, beneficial responses exclusively related to the enrichment of factors induced by acute exercise, or whether exercise training induces more persistent changes to circulating exercise factors that are measurable distally from acute exercise. Clearly, exercise training alters the *resting* proteome (Ferreira et al., 2014; Holloway et al., 2009; Padrão et al., 2016), transcriptome (Pillon et al., 2019) and metabolome (Castro et al., 2019; Klein et al., 2020) of tissues such as skeletal (Holloway et al., 2009; Klein et al., 2020; Padrão et al., 2016; Pillon et al., 2019) and cardiac (Ferreira et al., 2014) muscle. Assuming that the internal biomolecular composition of a cell influences the host of factors it releases into circulation (Uhlén et al., 2019), exercise training-induced changes in the resting profile of exercise factors is a physiologically-plausible outcome. Conversely, to reiterate the example of IL-6, in healthy individuals exercise training does not appear to alter resting plasma concentrations of IL-6, nor the kinetics of a circulating response induced by acute exercise (Fischer et al., 2004). There are numerous other experimental studies that have investigated the influence of exercise training on the resting abundance or concentration of a small number of selected candidate miRNA and protein targets in circulation. Detailed discussion of these reports is beyond the scope of this chapter, but collectively the training-induced response is equivocal; some factors increase after a period of exercise training, while others decrease, with occasional inconsistencies in individual factors across studies. Again, variations in experimental design and sample timing as previously described must be considered, and interested readers are referred to reviews with appropriately collated tables of these studies for miRNA (Fernández-Sanjurjo et al., 2018) and protein (Son et al., 2018).

An alternative approach to measurement of hypothesis-driven candidate targets is unbiased, hypothesis-free omics approaches surveying the broadest possible profile of exercise factors, albeit exercise training studies paired with these approaches are limited to date. One example is in the response to a four month treadmill training intervention (20 min at 12-15 m/s per day, 6 d/week), which produced numerous differences at 24 h post-intervention in the resting plasma proteome of male Sprague-Dawley rats (54 proteins in higher abundance, 47 proteins in lower abundance) in trained compared to sedentary controls (Wei et al., 2018). Similarly, cross-sectional studies have reported differences in the resting plasma proteomes of moderately-trained male and female endurance athletes (16 proteins in higher abundance, 23 proteins in lower abundance) compared to sedentary controls (Santos-Parker et al., 2018) and several studies have identified differences in the resting serum/plasma metabolome of individuals who have been exposed to exercise training interventions or possess different histories of exercise training (section 2.2.6). Therefore, there is some indication that exercise training does result in alterations to resting concentrations of exercise factors in circulation.

2.2. METABOLITES AND METABOLOMICS

2.2.1. Metabolites as exercise factors

A class of exercise factor that is of particular relevance to this thesis are metabolites. Exercise can impose substantial increases in the demand for ATP and consequently markedly accelerates the rate of systemic metabolism; for example, during intense exercise, up to 100 fold changes in skeletal muscle ATP turnover rate (Gaitanos et al., 1993; Hargreaves and Spriet, 2020) with concomitant 20-30 fold increases in whole-body VO_2 (Jones and Poole, 2005) in humans. The result of the marked increases in metabolic rate elicited by exercise is a concomitant increase in a vast network of metabolic reactions (Hargreaves and Spriet, 2020) and systemic 'support' responses (such as redirection of blood flow and mobilization of circulating substrates) (Hawley et al., 2014). An additional consequence of the metabolic

demand elicited by exercise is a considerable increase in the abundance of many circulating metabolites (i.e. a shift in the circulating metabolome).. Circulating metabolites (such as various fatty acids, monocarboxylates and amino acids) are demonstrated to have the capacity to act as signalling moieties through various means (Baker and Rutter, 2023), such as targeted activation of various G-protein-coupled-receptors (Husted et al., 2017) or activation/inactivation of intracellular protein signalling complexes through allosteric binding (e.g., uptake of circulating leucine enhancing activation of the mTOR complex) (Baker and Rutter, 2023). Collectively, the capacity of metabolites to possess a circulating 'responsiveness' to acute exercise and a capacity to as signalling molecules creates a clear demonstration that metabolites have potential to function as exercise factors (Chow et al., 2022)

In fact, the latter point is already well substantiated, as arguably the most well-understood exercise factor is the monocarboxylate metabolite lactate. Lactate is produced through the reduction of pyruvate and represents the terminal point of glycolysis (Brooks, 2020) and is primarily believed to be produced as a means of increasing the amount of cytosolic NAD^+ thereby preventing a 'bottleneck' in aerobic metabolism (Brooks, 2020; Hargreaves and Spriet, 2020). When lactate production increases (e.g. during exercise) extracellular transport increases dramatically (evidenced by the fact that blood lactate concentrations can be measured as up to 20 fold of resting concentrations) with robust and well defined curvilinear kinetics that shift with the intensity of exercise (Ferguson et al., 2018). Circulating lactate functions as a substrate (through uptake in local or distant cells and re-oxidation to pyruvate/donation of H^+ or conversion to glucose at the liver) (Brooks, 2018). However, lactate also has identified signaling effects that can be mediated through direct receptor binding (e.g. HCAR1) and altered signal transduction mediated by lactate-induced shifts in redox status and epigenetic modifications through lactylation of lysine histone residues (potentially altering gene expression patterns) (Brooks et al., 2022). Collectively, this evidence regarding the exercise

responsiveness and functions of lactate demonstrate a clear role as an exercise factor, an idea that is now well-accepted (Brooks et al., 2023; Chow et al., 2022).

2.2.2. Metabolic fingerprinting

Metabolites represent a signal for ongoing molecular processes (e.g. protein-protein interactions) and/or the terminal consequences of metabolic pathways (Nicholson and Wilson, 2003). Consequently, identifying and quantifying numerous metabolites simultaneously is argued to be more representative of the "true" phenotype of a sample in an as close to a "real-time" sense as is currently possible (Belhaj et al., 2021). This is because, in comparison to protein and RNA – that generally have half-lives of several hours (for RNA) (Yang et al., 2003) to several days (for protein) (Chen et al., 2016), endogenous metabolites represent products of *ongoing* reactions and therefore individual metabolite concentration(s) will be more tightly linked to the rate of their upstream reaction at the time of sampling (Küken et al., 2019). Similar to equivalent terms for other levels of molecular organization (i.e. proteome and transcriptome), the term "metabolome" has been coined and in its most literal sense refers to the totality of metabolites that are present in a cell, tissue, organ or organism (Beecher, 2003; Fiehn, 2001). Although, this is term is routinely and acceptably used in a more pragmatic sense, such as for any circumstance where a comprehensive and systematic profiling of many metabolites has been performed on a sample (Nicholson and Lindon, 2008).

The ability to detect many metabolites simultaneously via the development of metabolomics technologies (section 2.2.3) has led to the development of a concept referred to as 'metabolic fingerprinting' which refers to idea that 'global' changes to metabolites (via concentration changes of numerous previously identified metabolites and/or a number of previously undetected metabolites surpassing detection limits) that are produced in response to different physiological stimuli (e.g. acute exercise or metabolic disease) occur in distinct patterns ('fingerprints') (Kosmides et al., 2013). For example acylcarnitines, which are

intermediary metabolites produced during lipid oxidation (McCoin et al., 2015; Reuter and Evans, 2012) and demonstrate elevated circulating concentrations during metabolic conditions such as insulin resistance (Mihalik et al., 2010) and also exercise (Hansen et al., 2015; Xu et al., 2016). Interestingly, the evidence for role of acylcarnitines as an independent factor that induce insulin resistance is limited and instead it has been suggested that increased circulating acylcarnitines are instead a reflective 'signal' of insulin resistance (i.e. an indication of metabolic inflexibility) (Schooneman et al., 2012). Conversely, increased circulating acylcarnitines during exercise may simply represent a signal of an increased rate of lipid oxidation (Xu et al., 2016).

The idea of determining whether 'metabolic fingerprints' can be detected in blood is potentially particularly valuable as at any given time, blood is interacting with many tissues in in a bidirectional manner i.e. in the case of metabolites, blood is constantly both delivering metabolites to and receiving metabolites from many tissues (Dunn et al., 2011). Subsequently, 'metabolic fingerprints' identified in blood may be representative of the systemic physiological state of an organism (Psychogios et al., 2011) and therefore could act as 'markers' for specific physiological (e.g. exercise) and pathological (e.g., disease) states. Indeed, 'metabolic fingerprinting' has been applied to plasma samples as a means of attempting to identify 'markers' of disease development or previously unidentified biological processes relevant to disease pathology for conditions such as cardiovascular disease (Barderas et al., 2011) and breast cancer (Oakman et al., 2011) as well as the detection of other physiological 'disruptions' such as jet lag (Minami et al., 2009).

The concept of 'metabolic fingerprinting' also serves well to highlight an issue with the identification of metabolites as exercise factors. Often, several hundred metabolites are identified to changes in response to acute exercise (Section 2.2.4). While lactate clearly exemplifies that metabolites certainly can act as exercise factors (Brooks et al., 2023), although

lactate could also be argued to simultaneously act a component of a 'metabolic fingerprint' of exercise intensity due to its relationship with anaerobic metabolism and exercise in the heavy and severe domains (Ferguson et al., 2018; Jamnick et al., 2020). Regardless, the example of acylcarnitines also suggests that not all 'exercise responsive' metabolites may function as exercise factors (in terms of having strict kinetics or directly exerting bioactive effects). Instead, some circulating metabolites may only represent components of a 'metabolic fingerprint' of exercise – in the sense that a change the amount of circulating acylcarnitines may only serve to inform that changes in tissue metabolism are occurring (Hansen et al., 2015). This latter argument highlights the complexity of changes to the circulating milieu in response to exercise and that a particular challenge with using metabolomics technology as a mean of identifying changes to exercise factors in response to either acute exercise or exercise training (Section 2.2.4 – acute exercise & 2.2.6 – exercise training) is determining which metabolites may have candidacy as exercise factors (based on bioactivity) and what metabolites may only play a role in constructing 'metabolic fingerprints'.

2.2.3. The history of metabolomics and methods of metabolomic analysis

The first non-reductionist approach attempts of studying multiple metabolites concurrently appeared in the late 1960s with the development of mathematical modelling techniques such as metabolic control analysis (MCA) and biochemical systems analysis (Nicholson and Lindon, 2008; Savageau et al., 1987). Mathematically, these methods are highly similar (Savageau et al., 1987) and the former holds popularity over the latter (Fell, 1992). For the sake of brevity, only the concept of MCA will be described, this being for historical context and subsequent comparison to more current techniques. Fundamentally, MCA involves establishing a number of variables (metabolites) and parameters (enzymes) that compose an isolated metabolic system. Historically, this technique involves a series of *in vitro* experiments that involve impairing the activity of each parameter (the enzyme) and determining the

resultant effect on the variable (the metabolite concentration) (Savageau et al., 1987). From these experiments, the proportional contribution of each enzyme to the concentration of each metabolite can be established and a *flux control coefficient*, which describes the general rate of flux through a metabolic system, and the proportional contribution of relevant enzymes to the overall flux of the system can both be estimated (Fell, 1992). There are two primary assumptions that must be satisfied to perform MCA – firstly that the reactions encompassing the studied system are wholly interconnected (i.e. all reactions are associated and share common metabolites and coenzymes – 'a metabolic pathway') and secondly that the system can be observed in a stable steady-state (i.e. the net rate of appearance of all metabolites in the system is equal to the net rate of degradation of all metabolites in the system). The most apparent limitation of MCA is that estimating a flux control coefficient requires a large amount of *in vitro* work to identify the relationship between each parameter and variable that are associated with a specific metabolic system. However, it is often for this exact reason that the development of MCA or similar techniques are signalled as important in the development of the field of metabolomics, as the laborious nature of these methods assisted in generating the demand for chemical assays that were capable of simultaneously identifying and quantifying a large number of metabolites and thus lay the foundation for modern metabolomics (Nicholson and Lindon, 2008). Techniques like MCA also arguably maintain merit, as unlike most common metabolomics analysis (described subsequently), MCA pays attention not only to the concentration of metabolites, but also directly considers the enzymes and individual reactions that influence the presence of metabolites in a system (Fell, 1992).

Arguably, the earliest published reports that could be considered as more directly ancestral to modern "metabolomic" studies appear in the late 1960s and early 1970s (Dunn et al., 2011), with a notable example being a brief communication provided by Linus Pauling and colleagues in 1971, where it was first demonstrated that ~250 or ~280 unique chemical

substances could be detected in samples of human urine and breath, respectively, using gas-liquid chromatography, even though the unique chemical identities or quantities of each substance were not discerned (Pauling et al., 1971). While this report is clearly limited, it is also seminal, in the respect that from a technical perspective, these scientist provided demonstration that the simultaneous detection of numerous chemical substances (i.e. metabolites) in biological samples was possible. However, it was not until the refinement of proton nuclear magnetic resonance spectroscopy (^{1}H-NMR) for the detection of small metabolites in the late 1980s that produced the first methodological approaches capable of simultaneously identifying and quantifying multiple metabolites (Nicholson and Lindon, 2008). This period also culminated with the first recommendations for the multivariate analysis of 'metabonomic' data through the use of statistical techniques that can be used to estimate the presence of latent variables, such as principal component analysis (PCA) by Jeremy Nicholson and colleagues in 1999 (Nicholson et al., 1999). This technique and other similar techniques remain common practice for the analysis of metabolomic data today (Worley and Powers, 2013).

The principle of NMR functions by exploiting the spin property of atoms that have uneven proton/neutron ratios (e.g. ^{1}H, C^{13}), when specific atoms with spin properties are excited at fixed radio wave frequencies (e.g. ~900Mv for hydrogen), atoms will intermittently transition between ground and excitation states, when this circumstance is induced, the energy resonance released by state transitions can be measured via a spectrophotometer and raw spectral data can be captured. When raw spectral data undergo a Fourier transformation, a series of peaks referred to as "chemical shifts" are produced. Here, the integral of each peak will be proportional to the number of protons present in an atom, whereas the leftward/rightward shift of each individual peak will be representative of the degree of "shielding" experienced by a specific proton, which itself is representative of the electron density of functional groups that

are in association with the excited proton(s). Through this approach, specific metabolites can be identified in samples, either by knowing the height and position of their spectral peaks *a priori,* or the identity of compounds can be inferred based on what can be discerned about the chemical properties of the excited atoms and their associated functional groups (Bharti and Roy, 2012). The principal advantages of ^{1}H-NMR is that is can be performed on "raw" biological samples. i.e. samples are not permanently altered by undergoing ^{1}H-NMR and can undergo multiple analysis (high reproducibility of findings) or be recovered and subsequently used for different assays. The second advantage of ^{1}H-NMR is that it is technically 'unbiased' in the sense that the assay parameters do not need to be specifically tuned to detect different types of metabolites (i.e. ^{1}H-NMR can be used in an 'exploratory' fashion) . However, ^{1}H-NMR inherently lacks sensitivity and is generally only capable of detecting 30-40 metabolites from a biological samples - detected metabolites also tend to be those that are larger and/or more abundant (e.g. glucose in blood). The reason for this insensitivity of ^{1}H-NMR is due to spectral overlap between metabolites, i.e. small and lowly abundant metabolites may have their spectral "peaks" hidden within the integrals of larger metabolites, particularly if the larger metabolites have wide spectral peaks (i.e. they cover a large area of the spectrophotometer reading) (Dunn et al., 2011).

With respect to current approaches to metabolomic analysis, use of ^{1}H-NMR remains common alongside several hyphenated combinations of chromatography and mass spectrometry (MS), which are a more sensitive alternative, being capable of identifying and quantifying up to several hundred metabolites simultaneously. The general principal of MS works by separating analytes into positive and negative ions, with individual isotopes becoming detectable by through the estimation of specific mass/charge ratios, these are converted to spectral peaks that can be used to identify and quantify/semi-quantify individual metabolites. Mass spectrometry is particularly effective for the detection of metabolites, as metabolites tend

to be singularly charged and thus more easy to identify in comparison to other molecules like proteins, which may have regional variations in ionic charge (Dunn et al., 2011). Generally, the number of metabolites identified by MS can be increased when coupled with various types of chromatography, each of which may vary technically, but will always essentially involve the prior separation of metabolites into stationary phases with each phase then analysed sequentially. The use of a chromatography step prior to the analysis of metabolites via MS reduces the magnitude of a phenomenon known as the "matrix effect", which broadly speaking, refers to the altered ionization of individual analytes due to interactions with the charges of other compounds being concurrently detected during MS; this effect can consequently result in the under/over estimation of the concentration of certain analytes (Zhou et al., 2017).

The most common combinations of chromatography and mass spectrometry that are used for metabolomic analysis are gas chromatography-mass spectrometry (GC-MS), capillary electrophoresis-mass spectrometry (CE-MS) and liquid chromatography-mass spectrometry (LC-MS) (Roca-Rivada et al., 2012) each of which have identified variations in terms of the advantages and disadvantages that are conveyed (Dunn et al., 2011). While MS based approaches technically do have the capacity to the identify spectral peaks that are representative of thousands of metabolites simultaneously in an unbiased (untargeted) fashion, the subsequent chemical identification (i.e. valid identification of metabolites) of what each measured spectral peak represents is highly challenging. This is primarily due to computational challenges with resolving many spectra and the incompleteness of reference databases for metabolite identification (Wishart, 2011). In addition, true quantification of metabolites identified using MS based approaches tend to require the addition of chemical analogues within specific reference ranges. This means that in circumstances where quantification is desired, metabolites of interest must be selected prior to analysis (Dunn et al., 2011; Gertsman and Barshop, 2018). Regardless, the consistent advantage of MS based techniques is that they are capable of

identifying a greater number of metabolites compared to ^{1}H-NMR, particularly with respect to metabolites that are in lower concentration ranges (e.g. nanomolar ranges). However, MS based techniques are also limited in the sense that it is challenging to produce good reproducibility between runs (it is difficult to compare samples that were not analysed simultaneously) (Roca-Rivada et al., 2012). In addition, the need for reference standards for metabolite quantification means the cost of MS based analysis can become unwieldly for many researchers in circumstances where it is desired to quantify many (i.e. hundreds) of metabolites.

While there has been great advancement in the tools that are capable of facilitating the simultaneous analysis of metabolites over the past two decades. Although, in contrast to earlier methods, like MCA, something is 'lost' using more popular techniques like MS based approaches and ^{1}H-NMR, as these techniques can only identify and quantify many metabolites meaning that shifts in metabolic reactions are inferred, as opposed to measured. Whereas, earlier techniques like MCA also involved the measuring of the reactions with which metabolites were produced from or used for, which provides an additional layer of detail to data that is directly informative to experimental questions (Savageau et al., 1987).

In practical terms, the number of metabolites that are capable of being identified by most popular modern forms of metabolic analysis' (i.e. ^{1}H-NMR and MS based approaches) generally range in the mid-to-low hundreds; in the case of the human metabolome this represents a minor fraction of what could be the full scope of metabolites that are encompassed therein (Wishart, 2011). For example, the serum metabolome alone has been estimated to contain up to potentially ~4000 distinct metabolites (Psychogios et al., 2011). Therefore, while many analysis may be referred to as "metabolomic" analysis (for example, within this thesis), it is important to comment in most circumstances, use of this term will be comparatively less accurate than in cases where the use of equivalent terms (e.g. genomic, proteomic) are applied for 'omic' analysis of other species of molecules (DNA, proteins). However, the term

metabolomics is still used throughout this thesis due to the fact that this is common parlance within the field (Nicholson and Lindon, 2008).

2.2.4. The response of the blood metabolome to acute exercise

The study of the response of circulating metabolome to acute exercise using popular metabolomic methods (e.g. ^{1}H-NMR and MS based approaches) is in relative infancy, but has been summarized recently in several recent systematic (Khoramipour et al., 2022; Schranner et al., 2020) and narrative (Belhaj et al., 2021; Heaney et al., 2019; Kelly et al., 2020; Sakaguchi et al., 2019) reviews.

The most quantitative overview regarding the response of the circulating metabolome to acute exercise has been provided by an article authored by Schranner and colleagues (Schranner et al., 2020). This systematic review is specifically concerned with changes in metabolite concentration (measured via mass spectrometry coupled with various types of chromatography or ^{1}H-NMR) in biofluids in response to acute exercise (defined in this case as metabolites that experience a concentration change from the point of exercise cessation to 24h post exercise). Authors identified 57 experiments (51 aerobic exercise, 6 resistance exercise) from 27 studies and included only metabolites that were reported to change in at least two studies. Across these experiments 196 metabolites were found to change (71 metabolites increased, 35 decreased, 90 mixed response) in biofluids (urine, serum, plasma or capillary blood) after acute exercise. The primary classes of metabolites responsive to acute exercise were amino acids/amino acid derivatives (53) and lipids (95), which are a representative signal of the shifts in skeletal muscle metabolism and substrate mobilization that are necessary for maintaining an exercising state (Schranner et al., 2020). From a physiological perspective, this observation is certainly not novel, as the increases in circulating lipids, amino acids or common amino acid derivatives (e.g. creatine) during exercise are long identified and relatively well understood (Hawley et al., 2014). Instead, the pertinent observation reported by Schranner and

colleagues is that what is observed with commonly applied metabolomic techniques (primarily ^{1}H-NMR and various forms of mass spectrometry coupled with a chromatography step) appears consistent with what is already understood about circulating metabolite responses to exercise from earlier work using traditional reductionist techniques (Schranner et al., 2020). Therefore, metabolomic methods may be indeed be capable of broadly mapping the landscape of the circulating metabolite response to exercise (i.e. may be capable of capturing 'metabolic fingerprints' of exercise or identifying metabolites that may be candidate exercise factors).

A comment made by some review articles is that the results of current experimental studies indicate that measured changes in the circulating metabolome in response to acute exercise are partially tuned to the mode of exercise engaged in – with aerobic exercise largely inducing changes in TCA intermediaries and lipids (serving as indexes of increased flux through the TCA cycle and oxidative phosphorylation activity) and resistance exercise largely inducing the concentration of anabolic hormones and 'anabolic' amino acid derivatives (e.g. creatine) (Khoramipour et al., 2022; Schranner et al., 2020). This narrative holds appeal, as it paints an exciting scenario where different 'modes' of acute exercise elicit clearly distinct and bespoke 'metabolic fingerprints' that are easily associated with the exercise 'mode' that produced them. However, it is worth commenting that this idea is not entirely substantiated and has the potential to be somewhat overstated. This is in-part due to the considerable bias most studies have had toward studying endurance over resistance exercise (Khoramipour et al., 2022; Schranner et al., 2020). Across studies investigating aerobic exercise there has been a consistent observation of increases in TCA intermediates, branched-chain amino acids (BCAAS) and Acylcarnitines across both intense and long duration bouts of aerobic exercise. For example, after an incremental test (with metabolites detected using untargeted LC-MS) (Contrepois et al., 2020) or marathon running using targeted LC-MS (Schader et al., 2020) or untargeted GC-MS (Shi et al., 2020). These observations would corroborate the idea that in

response to aerobic exercise, the 'metabolic fingerprint' is largely reflective of the metabolic activity associated with aerobic exercise (Khoramipour et al., 2022).

With respect to resistance exercise, there are six studies published (Schranner et al., 2020) of which only two use ecological models of resistance training and healthy participants (Berton et al., 2017; Yde et al., 2013). The first of these studies by Berton and colleagues asked 10 healthy male participants to complete 4 sets of 10 repetitions of two exercises at 70% of 1RM (bilateral leg press and leg extension). Plasma was extracted 5 minutes post exercise and analysed via ^{1}H-NMR. A total of 49 metabolites were detected and significant increases in the abundance of metabolites associated with glycolytic flux (pyruvate, lactate) and amino acids/amino acid breakdown (alanine, 2-oxoiocapoate) in serum (Berton et al., 2017) were observed. Yde and colleagues subjected 12 healthy males to 10 sets of 8 single leg extensions at 80% of 1RM. Analysing serum extracted immediately post exercise from these participants using ^{1}H-NMR demonstrated increases in an unspecific number of metabolites, but report observing an increase in the plasma content of amino acids and amino acid derivatives after resistance exercise (Yde et al., 2013). These studies collectively provide some suggestion that the response of the circulating metabolome to resistance exercise is somewhat reflective of the metabolism of resistance exercise (Khoramipour et al., 2022). However, these two studies both analyse metabolites via ^{1}H-NMR, which only detects a small number of highly abundant metabolites (section 2.2.3) and therefore it is difficult to discern how prominent the 'metabolic fingerprint' of resistance exercise may be against the backdrop of a larger metabolome.

Interestingly, a recent study by Morville and colleagues asked 10 healthy male participants to perform crossover bouts of either resistance (five sets of 10 repetitions at 90% of 10RM across five exercises) or aerobic (60 minutes of continuous cycling at a workload approximating 70% of VO_{2max}) exercise with plasma extracted 15 minutes post exercise and using a broad untargeted metabolomics approach (Ultrahigh performance LC-MS) that

detected a total of 836 metabolites. Interestingly of the 765 that were identified to be responsive to exercise (irrespective of mode), 615 metabolites were found to be altered similarly by both resistance and aerobic exercise, while only 51 and 93 metabolites were uniquely changed by aerobic and resistance exercise respectively. Similar to other studies (Contrepois et al., 2020; Schader et al., 2020; Shi et al., 2020), the metabolites that were unique to aerobic exercise were largely associated with lipid metabolism, while metabolites altered in response to resistance exercise were associated with glycolysis and amino acid catabolism (Morville et al., 2020). While this similarly demonstrates there are detectable and unique responses of the circulating metabolome to resistance or aerobic exercise respectively, the majority of the 'exercise responsive metabolome' is common across exercise modes. This suggests that resistance and aerobic exercise may not have divergent 'metabolic fingerprints' *per se* and instead may represent slight variations of a more generalised circulating metabolite response to exercise.

2.2.5. Exercise training induced alterations to tissue metabolism at rest: a skeletal muscle perspective

Prior to discussing whether there is evidence to suggest whether the circulating metabolome appears to also be altered by exercise training, it is valuable to discuss the extent to which exercise training may have the capacity to alter tissue metabolism at rest i.e. whether there is a prior physiological basis to suggest the circulating metabolome should be altered at rest in association with exercise training. Exercise training elicits durable systemic changes that benefit both performance and health. This is primarily achieved by stimulating remodelling of numerous tissues (e.g. skeletal and cardiac muscle, bone, vasculature, adipose) (Heinonen et al., 2014) that coordinate to enhance specific systemic metabolic capacities (e.g. fatigue resistance or maximal force production) (Egan and Zierath, 2013). Broadly, the process of exercise adaptation is thought to occur through activation of signal transduction induced by cellular 'stressors' associated with acute exercise (e.g. mechanical tension, metabolic flux,

altered redox status) leading to altered gene expression. The gradual accumulation of the products of 'exercise responsive' genes across repeated exercise bouts produce an eventual restructuring of relevant cells that underpin an 'exercise trained' phenotype; the nature of exercise adaptation requires that cells are remodelled in a way that produces changes that are helpful in enhancing an organisms capacity to withstand imposed stresses (e.g. mechanical loading or 'metabolic' stress) (Egan and Sharples, 2023; Egan and Zierath, 2013). This gives credence to the idea that exercise training may elicit changes to the metabolism of relevant tissues that are persistent outside of an exercising state (i.e. at rest); which has implications for the potential existence of 'metabolic fingerprints' associated with exercise training (section 2.2.2). The purpose of this section is to describe how physiological adaptations induced by exercise training may create a potential landscape where it is plausible that elements of systemic metabolism may become altered at rest. For brevity, this section will only discuss exercise adaptation within the context of skeletal muscle tissue. This is because skeletal muscle is the most studied organ within the context of exercise training; skeletal muscle also represents approximately 40% of body mass (Janssen et al., 2000; Lee et al., 2000) and is responsible for ~30% of resting energy expenditure (Zurlo et al., 1990). Therefore skeletal muscle is the tissue for which the greatest degree of information regarding exercise adaptation is available and also represents a major site of resting metabolism and thus functions as the most robust example of how exercise training induced alterations to tissue composition can influence metabolism at rest.

In the most general sense exercise adaptation within skeletal muscle can be typified by an altered abundance and organization of subcellular and local extracellular structures that are relevant for producing force or sustaining repeated contractions (Egan and Sharples, 2022; Egan and Zierath, 2013). However, in more categorical terms, adaptations of skeletal muscle in response to exercise can largely be identified by alterations in the abundance of relevant

proteins; changes in the organization and abundance of relevant cell organelles; the organization and abundance of stored intracellular substrates and remodelling of the local skeletal muscle environment. When specific examples are provided, it can also often be relatively clear how each of these factors can contribute to improving certain metabolic capacities of individual muscle fibres (and consequently whole muscle performance when summed across many muscle fibres). For example, in response to regular mechanical loading (e.g. the loads experienced during bouts of resistance exercise) muscle fibres respond by producing an increased number of myofibril proteins (i.e. creating an increased myofibril density within individual muscle fibres and an increased potential for force production) (Jorgenson et al., 2020; Wackerhage et al., 2019). The increased ATP turnover and shifts in myofiber redox status induced by increased metabolic flux (e.g. as experienced during more 'aerobic' style exercise sessions) can act as initiating signals for transcriptional programs regulating genes that encode for key substrate transporters (e.g. GLUT4 and CD36) (Egan and Zierath, 2013; Fritzen et al., 2020; Richter and Hargreaves, 2013) and metabolic enzymes (e.g. citrate synthase) (Egan and Zierath, 2013; Granata et al., 2018) that subsequently increases the abundance of these proteins thereby enhancing the potential for increased metabolic flux during exercise.

Exercise training induced changes in protein abundance and function also couple with changes to relevant cell organelles. For example, aerobic exercise is associated with expansion of the mitochondrial reticulum (through an increased mitochondrial volume) – that couples with an increased abundance of metabolic enzymes to enhance the capacity for glucose and fat oxidation (Granata et al., 2018). Resistance exercise is identified to induce an accretion of ribosomes (Figueiredo, 2019) and translocation/differentiation of satellite cells into myonuceli (Murach et al., 2021), which may contribute to changes in muscle mass through an increased potential for muscle protein synthesis via enhanced transcriptional and translational capacities

within individual muscle fibres. It is important to note that skeletal muscle contractile activity exists on a continuum ranging from high frequency, low force activity (e.g., 'aerobic exercise') and high force, low frequency activity (e.g, 'resistance exercise') (Egan and Zierath, 2013). Therefore, the latter example(s) (as well as other exercise adaptations discussed subsequently) of mitochondrial biogenesis or satellite cell translocation are not examples of adaptations that occur exclusively in response to aerobic or resistance exercise respectively, instead these represent examples of adaptations that manifest most profoundly when a specific type of contractile activity (e.g, 'aerobic exercise' or 'resistance exercise') is emphasized (Egan and Sharples, 2023).

In response to aerobic exercise training, it is long known that the resting abundance(s) of both glycogen (Bergström et al., 1967) and intramyocellular lipid (Morgan et al., 1969) are increased. While this firstly implies an increased capacity for metabolic flux during exercise (simply through a greater abundance of substrate locally available for utilization), in more recent years it has also been demonstrated that exercise training may also reorganise the subcellular location and morphology of theses substrates in a way that may have consequent implications for skeletal muscle metabolism. For example, untrained individuals tend have been reported to have no variations in the abundance of glycogen in each of the major subcellular glycogen fractions (the intermyofibrillar fraction, the intramyofibrillar fraction and the subsarcolemmal fraction) or between muscle fibre types (broadly categorised as either type-I or type-II only) (Nielsen et al., 2010). However, elite cross country skiers have been observed to contain greater total glycogen in type I fibres (compared to type-II) and also have variations in the distribution of glycogen between fibre types – with type-I fibres possessing 82% greater intramyofibrillar glycogen and 33% more subsarcolemmal glycogen compared to type-II fibres (Nielsen et al., 2011) (see (Nielsen and Ørtenblad, 2013) for more detailed discussion of this topic).

Interestingly, and somewhat in contrast to glycogen, exercise training induced increases in intramyocellular lipid appear to occur almost exclusively in the intermyofibrillar space, although similarly to glycogen, this appears to occur largely in type-I muscle fibres (Gemmink et al., 2020). In addition, exercise training may alter the composition of intramyocellular lipid droplets as distinct alterations in the morphology of lipid droplets in exercise trained muscle (compared to exercise untrained muscle) have been identified. Namely that the lipid droplets of exercise trained individuals are smaller and more numerous compared to exercise untrained individuals and tend to be more abundantly coated in lipid droplet regulatory proteins (e.g. PLIN5) that are associated with lipid droplet turnover and fatty acid oxidation (Gemmink et al., 2020; Seibert et al., 2020). This is also in contrast to glycogen, where exercise training induced changes may only occur in terms of the number of glycogen particles with no change to individual glycogen particle morphology (Jensen et al., 2021). Collectively this indicates that exercise training does not simply increase the storage of intracellular substrate store (at least within skeletal muscle), but can also re-organise the distribution and composition of substrates to an extent that may influence how they are metabolised.

The local muscle environment has also demonstrated evidence of remodelling in association with exercise training with the most prominent example being the enhanced angiogenesis and capillarisation of individual muscle fibres that is traditionally most associated with aerobic exercise training (Egginton, 2009; Prior et al., 2004). This adaptation is beneficial to the exercising skeletal muscle because it increases the opportunity for O_2 and circulating substrate extraction. Muscle fibre angiogenesis could also influence a circulating 'metabolic fingerprint', as a theoretically a greater capillary to fibre ratio may increase the potential for metabolite/release and clearance (i.e. a given metabolite has a greater or lesser degree of opportunity to appear in circulation in altered abundance due to a higher capacity for release or clearance). Other elements of the local skeletal muscle environment such as extracellular

matrix proteins like glycoproteins, collagens and proteoglycans also demonstrate evidence of remodelling in response to exercise training and may aid in improving performance through enhanced force transduction (Csapo et al., 2020; Kritikaki et al., 2021). However, in contrast to angiogenesis, less is currently known about the extent to which the extracellular matrix remodels in response to exercise training or the exact relevance of extracellular matrix adaptation to skeletal muscle performance and function.

Given the examples of how the local skeletal muscle environment can remodel in response to exercise training and the inference as to how this remodelling may influence changes in resting metabolism, it is pertinent to consider whether the resting skeletal muscle metabolome is indeed altered in response to periods of exercise training. This is essential to the concept of a circulating 'metabolic fingerprint' of exercise training, as a fundamental assumption of the concept of 'metabolic fingerprinting' is that a change in the circulating metabolome is reflective of a shift in tissue metabolism (Dunn et al., 2011). With respect to metabolomic studies, little evidence currently exists. There are four available studies (three in humans, one in rats) have that examined the response of the skeletal muscle metabolome to periods of resistance (Gehlert et al., 2022; Huffman et al., 2014; Szczerbinski et al., 2021) or Aerobic (Huffman et al., 2014; Starnes et al., 2017; Szczerbinski et al., 2021) exercise training However, of these four studies, three have taken their post-training muscle biopsies <24h post the final exercise training session (Gehlert et al., 2022; Huffman et al., 2014; Starnes et al., 2017) which renders inference on these papers difficult as the influence of exercise training cannot be decoupled from the residual effects of an acute exercise bout (Section 2.1.7).

The only study reported to take post-intervention muscle biopsies >24 post intervention is that of Szczerbinski and colleagues (Szczerbinski et al., 2021). In this work, normoglycemic (n = 11), prediabetic (n = 13) and diabetic (n = 8) males (age range for all participants between 43-53 years) participated in 3 sessions-per-week of mixed exercise training (45 minutes of

resistance training + 30 minutes of aerobic training) for 12 weeks with vastus lateralis muscle biopsy samples were extracted 48h before and after training. Biopsies were analysed using a targeted ultra-high performance LC coupled with tandem MS approach which identified 408 metabolites in muscle samples. Data were analysed using null hypothesis testing only and several metabolites which were not different between groups at baseline were found to be responsive to the exercise training intervention. These included 20 acylcarnitines (15 which increased in abundance after training, 5 which decreased in abundance), 2 biogenic amines (histamine and putrescene, which both increased after training) and 9 ceramides (all decreased in abundance after training) (Szczerbinski et al., 2021). This data is preliminary, largely in part due to the high degree of variability that is created by participants from three clinically different (normoglycemic, prediabetic and diabetic) groups.

However, additional data using more dynamic methods (i.e. measuring the rate of certain metabolic processes, instead of inferring rate changes through differences in metabolite concentration(s)) is available that provides some indication that the resting skeletal muscle metabolome may be measurably altered in response to exercise training. Cross-sectional studies comparing endurance athletes, strength athletes or sedentary control participants have observed that individuals with a history of endurance training present with lower resting respiratory-exchange-ratios (14-24%) (Jurasz et al., 2022; Smorawiński et al., 2001). This suggests that substrate utilisation is different in endurance athletes at rest and demonstrates that systemic metabolism can be observably altered at rest in association with certain types of exercise training.Three resistance training intervention studies (reviewed in (Figueiredo, 2019)) (8-10 weeks in length) have observed increases in resting mixed muscle (~40-100%) (Balagopal et al., 2001; Kim et al., 2005) and myofibrillar fractional synthesis rates (50-75%) (Kim et al., 2005; Wilkinson et al., 2008). Importantly, one of these studies performed unilateral aerobic or resistance exercise (on different legs, in the same participants) and only observed

increases in myofibrillar fractional synthesis rate in the resistance trained leg at rest (Wilkinson et al., 2008). The results from these cross sectional (Jurasz et al., 2022; Smorawiński et al., 2001) and training (Balagopal et al., 2001; Kim et al., 2005; Wilkinson et al., 2008) studies suggest that the rates of metabolic processes (e.g. fat oxidation/substrate utilisation and muscle protein synthesis) may shift in response to exercise training in a manner that is somewhat related to the mode of exercise training engaged in.

2.2.6. The response of the resting blood metabolome to exercise training

Compared to acute exercise, there has been considerably less investigation into whether exercise training alters profiles of the circulating metabolome at rest (i.e. whether there metabolites as exercise factors are altered at rest and/or there are 'metabolic fingerprints' of exercise training). In humans free of diabetes, to my knowledge seven studies have investigated the influence of an exercise training intervention on targeted (Schranner et al., 2021; Yan et al., 2009) or untargeted (Al-Khelaifi et al., 2018; Duft et al., 2017; Monnerat et al., 2020; Pechlivanis et al., 2013) profiles of the resting plasma (Kuehnbaum et al., 2015; Monnerat et al., 2020; Schranner et al., 2021) or serum (Al-Khelaifi et al., 2018; Duft et al., 2017; Pechlivanis et al., 2013; Yan et al., 2009) using either LC-MS (Al-Khelaifi et al., 2018; Monnerat et al., 2020; Schranner et al., 2021), GC-MS (Yan et al., 2009), Capillary-Electrophoresis MS (Kuehnbaum et al., 2015) or ^{1}H-NMR (Pechlivanis et al., 2013). These seven studies are summarised in table 2.1, however two studies do not report the proximity to exercise with which blood samples were extracted (Monnerat et al., 2020; Pechlivanis et al., 2013) and one study reports samples being extracted in the immediate post-exercise period (Al-Khelaifi et al., 2018). One study consists of both an 11 week training intervention where participants were sampled less than 24h after the most recent exercise bout and a baseline cross-sectional comparison with sedentary controls where trained participants were rested for three days prior to sampling (Yan et al., 2009). For brevity, the results of three of these studies (Al-

Khelaifi et al., 2018; Monnerat et al., 2020; Pechlivanis et al., 2013) are not discussed, as the residual influence of acute exercise bouts cannot be discounted (section 2.1.7) making the extent to which the results of these studies are reflective of an influence of exercise training are difficult to ascertain. For one study (Yan et al., 2009) only the results of the cross-sectional comparison are discussed, for the same reason.

The two relevant training studies investigate either 6 weeks of high intensity interval cycling exercise in obese (BMI > $25m^2$ - means not reported) women (n = 9) (Kuehnbaum et al., 2015) and 24 weeks of combined aerobic and resistance training (n = 22, training group n = 11, control group n = 11) in 'middle aged' (mean age = 48 $\pm$ 6.1 years) obese men (mean BMI 31 $\pm$ 1.4m^2) (Duft et al., 2017). Both of these studies employed a similar analytical approach, by first analysing the collective profile of all identified metabolites using a principle least-squares discriminant analysis (PLSDA), followed by univariate null-hypothesis testing. This style of analytical workflow employing a selection of a multivariate and univariate method(s) is a common approach for the analysis of metabolomics data (Blaise et al., 2021), and for that reason, a brief aside is required at this stage of the thesis in order to describe these approaches in more detail. The interpretation of data produced via PLSDA models is not intuitive without some explanation of how models are constructed therefore, prior to discussing the results of exercise training study a brief explanation of PLSDA is provided. PLSDA is a multivariate technique involves using the values of individual factors (metabolites) to create estimates of latent variables (a 'metabolome' or 'metabolic fingerprint' as examples) that explain the greatest proportion of variance; once latent variable values for each participants are calculated an algorithm is applied where the projection of each participant is rotated until the maximum separation between groups can be projected (Westerhuis et al., 2010). In more simple terms – PLSDA creates a representation of the 'metabolome' from each individual participant based on the values of each metabolite that is provided; An algorithm then seeks to create a

situation where, when estimates of participants 'metabolomes' are projected (in two or three dimensional space) the values of individual participants from the same group cluster as closely to each other as possible while the centroids of participant clusters (i.e. different groups) are maximally separated (Ruiz-Perez et al., 2020). It is *imperative* to highlight that PLSDA models *will always produce separation between groups* (as the PLSDA algorithm is constructed to maximise differences between groups) and therefore upon superficial visual inspection PLSDA models always create an appearance of a strong, positive finding. Therefore, to avoid misinterpretation, prior to models being inspected, parameters of model quality must be estimated an inspected; these are normally R^2 (the total proportion of variance explained by the model) and more importantly Q^2, which is an estimate of how well a given PLSDA model produces similar discrimination on unseen data (i.e. an estimate of how generalisable the predictions of the model may be) (Westerhuis et al., 2008). Presuming a model presents with indications of 'good' quality based on R^2 and Q^2 values (which themselves have subjective degrees of interpretation in terms of 'goodness') the 'weight' of the contribution each individual metabolite to the estimate of the 'metabolome' can be estimated (often using values that termed 'VIP' or 'loading' scores), these are the factors that are estimated to the contribute the most to each latent variable (i.e the 'metabolome') and therefore drive the discrimination between groups (Quintás et al., 2012).

In the case of the first training study performed by Kuehnbaum and colleagues, obese women performed 6 weeks of high intensity cycling exercise (10 x 60s cycling at 90% of heart-rate max). In this study, 41 metabolites were identified in plasma extracted from these women using capillary-electrophoresis MS of which three metabolites (the acylcarnitines C2 and C0 and the purine hypoxanthine) were associated with 'training responsiveness' (a variable that was not defined by the authors). Subsequently, these three metabolites were used to construct a PLSDA model with good quality parameters ($R^2 = 0.78$, $Q^2 = 0.62$). This would suggest that

abundance of these three metabolites are capable of delineating between individuals with high or low 'training responsiveness' to a reasonable degree, lending credence to the idea of a 'metabolic fingerprint' of exercise training. However, as 'training responsiveness' is an undefined variable in this study, it is difficult to interpret what this model actually represents. In addition, the plasma concentration of each of the three metabolites (C2, C0 and hypoxanthine) was not observed as significantly different between pre and post intervention resting samples via univariate testing (Kuehnbaum et al., 2015).

The second training intervention was performed by Duft and colleagues (Duft et al., 2017). In this study 22 middle aged men completed 24 weeks of combined aerobic and resistance training (n =11) or were left as sedentary control (n = 11). Serum samples were extracted prior to training and 72hr after the last training session. 41 metabolites were identified in serum samples using 1^{H}-NMR and a PLSDA model was constructed with good model parameters (R^2 = 0.98, Q^2 = 0.62) using all 41 metabolites. The metabolites with the top-20 VIP loading scores largely consisted of amino acids (10) and intermediates of the TCA cycle (e.g. carnitine, glucose, lactate, pyruvate). Only two metabolites were significantly different between the exercise training group and control group at rest post intervention (tyrosine and pyruvate, both in higher abundance in exercise training group) (Duft et al., 2017).

In terms of cross-sectional studies, Yan and colleagues identified an unreported number of metabolites via GC-MS and built a PLSDA model comparing national level rowers (n = 16) and sedentary controls (n = 12) with R^2 and Q^2 values of 0.98 and 0.981 respectively. The 11 metabolites with the highest VIP scores were amino acids (n = 4), fatty acids (n = 4) and metabolites associated with carbohydrate metabolism (e.g lactate), and all of these metabolites were also significantly different between sedentary participants and rowers (6 metabolites in lower abundance in rowers, 5 metabolites in greater abundance) (Yan et al., 2009).

The final study investigating an association with exercise training and the profile of the resting metabolome is a cross-sectional study by Schranner and colleagues (Schranner et al., 2021). In this study, men from four exercise training backgrounds ('Natural' bodybuilding n = 4, Endurance training n = 6, sprint training n = 4 or sedentary 'controls' n = 4) had blood plasma extracted in a 'rested' state (at least 24h after the last exercise training bout). A targeted metabolomics approach using LC-MS identified 151 distinct metabolites (with 43 metabolite sums and ratios calculated *post-hoc*). Again, a PLSDA model was constructed, but this time with poorer parameters compared to other studies (R^2 = 0.48, Q^2 = 0.30). In this study, metabolites with the greatest VIP scores were largely related to metabolites associated with amino acid metabolism (e.g. isoleucine, leucine, the sum of BCAAs) and ß-oxidation (e.g. CPT-1 ratio and the kyneurine/tryptophan ratio). Only four metabolites demonstrated some significance between groups via univariate testing (serotonin, putrescine, C14:1 and C14) and none of these metabolites were in the top-ten VIP scores for the PLSDA model (Schranner et al., 2021).

Collectively, there is some consistency across all four of these studies (Duft et al., 2017; Kuehnbaum et al., 2015; Schranner et al., 2021; Yan et al., 2009) as each produced PLSDA models with reasonably estimates of model quality, and thematically metabolites associated with similar metabolic processes (e.g. fat oxidation and amino acid metabolism) appear to consistently produce the highest VIP scores. Coupled with the observation that both fat oxidation and protein synthesis can be altered with exercise training at rest (section 2.2.5), it is intuitively appealing to consider whether the 'shifts' in circulating metabolites related to amino acid and fat oxidation in response to exercise training represent a 'signal' of these processes (i.e. a metabolic fingerprint).

While this speculation is appealing, there are limitations to this inference. Firstly, two studies report identifying a low number of metabolites (41) (Duft et al., 2017; Kuehnbaum et

al., 2015) and one study (Yan et al., 2009) does not report the total number of metabolites detected at all. Added to this, two studies 'selectively' include metabolites in their PLSDA models – only including metabolites related to fat oxidation (Kuehnbaum et al., 2015) or amino acid and fat metabolism (Yan et al., 2009). While both of these studies (Kuehnbaum et al., 2015; Yan et al., 2009) produce PLSDA models with reasonable quality, these models are biased by researcher selection of metabolites and it is unclear what 'themes' would emerge if all metabolites identified in the studies were used to build PLSDA models. Schranner and colleagues includes 194 variables and produces results that is 'consistent' with other studies (Kuehnbaum et al., 2015; Yan et al., 2009) in terms of what 'types' of metabolites contribute most to a PLSDA model (i.e. metabolites related to fat oxidation and amino acid metabolism) (Schranner et al., 2021), yet this is only one study, and one with a low sample size (n = 19 total). Additionally, some interventional studies that have investigated whether the resting metabolome is altered at rest in response to exercise training have employed previously sedentary and/or obese participants (Duft et al., 2017; Kuehnbaum et al., 2015) and it is uncertain whether there would a similar response in leaner, fitter individuals. These studies often find limited (Duft et al., 2017; Schranner et al., 2021; Yan et al., 2009) or no significant (Kuehnbaum et al., 2015) differences in the abundance of circulating metabolites via univariate testing. An observation that is arguably contradictory to the contention that metabolites are a class of exercise factor that can be modified in circulation at rest. Although overall, it appears little is known regarding the influence of exercise training on the presence of the circulating metabolome at rest and it is currently difficult to draw clear inferences about how the circulating metabolome may respond to exercise training.

Study	Study Design	Participants	Exercise training protocol	Nutritional Control	Proximity From Exercise	Biofluid	Metabolomics Technique	Targeted/Untargeted	No. Metabolites Identified	Statistical Analysis Employed	Results
Schranner et al 2021.	Cross-Sectional	Untrained Controls (n = 4); Natural Bodybuilders (n = 4); Endurance Athletes (n = 6); Sprint Athletes (n = 5)	N/A	Standardised diet provided for 24hrs prior to sampling; 2000-2500kcal for controls, 3000-3500kcal for athletes. Macronutrient ratios not reported.	Participants requested to refrain from exercise for 24hrs prior to sampling. Exact data not reported.	Plasma	Mass Spectrometry with Liquid Chromatography	Targeted	151 (+ 43 calculated metabolite ratios/sums) - 194 features included in analysis	Partial least squared discriminate analysis & Univariate Hypothesis testing	PLSDA Produced the following model parameters: Q2Y = 0.304, R2X = 0.258 , R2Y = 0.488. Separation between pre-defined class structures driven by metabolites associated with Amino Acid metabolism, Hydroxysphingolipids and Phosphotidylcholines. 4 metabolites were significantly different between groups (1 metabolite different between control + others, 1 between endurance + others, 1 between bodybuilding + others, 1 between sprint + others)
Monnerat et al. 2020	Cross-Sectional	Amateur (VO2max = 61.0 , n = 5) and Elite (VO2max = 76.3 , n = 5) middle distance runners (5000-10000m)	N/A	None reported	Not reported	Plasma	Mass Spectrometry with Ultra-High performance Liquid Chromatography	Untargeted	169	Partial-Least Square Discriminant Analysis & Univariate Hypothesis testing	Two dimensional PLSDA model produced an estimated accuracy of 0.4 (R2 = 0.9, Q2 = 0.2). Under resting conditions, separation between pre-defined class structures driven by metabolites associated with Alpha Linoleic Acid Metabolism, Glutathione metabolism and Carnitine synthesis. No metabolites significantly different between groups under resting conditions
Pechlivanis et al. 2012	Training study	Previously untrained males (n = 14)	Participants were randoy assigned to one of two exercise training groups. In both exercise training groups participants performed 2-3 sets of 80m maximal sprints with either 10s or 60s rest. Training was performed 3x per week for 8 weeks	None reported	Not reported	Serum	1H-NMR	Untargeted	33	Partial-Least Square Discriminant Analysis & Univariate Hypothesis testing	Two dimensional PLSDA model produced an estimated accuracy of 0.4 (~R2Y = 0.9, ~R2X = 0.48 ~Q2 = 0.75). Under resting conditions, separation between pre-defined class structures were driven by methylguanidine, citrate, glucose, taurine, trimethylamine N-oxide, choline-containing compounds, histidine and acetoacetate/acetone

Al-Khelafi et al. 2018	Cross-Sectional	191 (171M, 20F) National level Athletes from various Team (Rugby Union, Baseball, Volleyball, Basketball) & Individual (Boxing, Sprint Canoe, Sprint Kayak, Motorcycle Racing, Tennis, Triathlon, Long Distance Running) sports. Sports were categorized as moderate (n = 44) or high (n = 144) power or moderate (n = 71) and high (n = 120) endurance	N/A	None reported	Either Immediately post competition (i.e. post exercise) or at an unspecified time out of competition.	Serum	Mass Spectrometry with Ultra-High performance Liquid Chromatography	Untargeted	743	Partial-Least Square Discriminant Analysis & Univariate Logistic Regression	Two dimensional PLSDA model produced an estimated accuracy of 0.4 . Separation between moderate and high endurance class structures (R2Y = 0.66, Q2Y = 0.45) were driven by metabolites associated with diacyl glycerol and gamma-glutamyl amino acids, steroids, GABA derivatives, and monohydroxy fatty acids; while separation between moderate and high power class structures (R2Y = 0.88, Q2Y = 0.52) were primarily driven by metabolites associated gamma glutamyl amino acids, sterols, phospholipids, lysolipids, and xanthine metabolites. 38 metabolites associated with diacylglycerol, gamma-glutamyl amino acid, eicosanoids, and monohydroxy fatty acid metabolism were associated with 'endurance' categorical; 33 metabolites primarily related to phospholipid, lysolipid, gamma-glutamyl amino acid and sterol metabolism pathways were associated with power categorical.
Yan et al. 2009	Intervention	28 male participants, 8 elite rowers, 8 novice rowers, 12 sedentary controls. Rowers were sampled prior to two weeks of intensive training and at the end of each training week. Controls were also sampled at each interval without exercise intervention.	11 sessions a week (30hrs of training a week). Rested cross sectional analysis with sedentary controls at baseline	All Participants ate the same meal in a canteen for the duration of the study (3 weeks) with the following energy intake and macronutrient content rowers' nutrient composition: protein, 130 g; carbohydrates, 700 g; fat, 90 g; 20% oligosaccharide beverages, 850 ;	Baseline sample taken 3 days post exercise (for rowers); post week 1 and week 2 training blood samples were taken the day after the	Serum	Time-of-Flight - Mass Spectrometry with Gas Chromatography	Targeted	Not reported	Partial-Least square Discriminant analysis and univariate hypothesis testing	A two dimensional PLSDA with the following parameters: R2Y = 0.92 , Q2Y = 0.92 with seperation between control participants and rowers driven by metabolites associated with Glucose Metabolism, Energy Metabolism, Oxidative Stress, lipid metabolism, Amino Acid Metabolism and an unidentified pathway. The following metabolites were in significantly greater

				energy, 4,100 kcal; control subjects' nutrient composition: protein, 90 g; carbohydrates, 490 g; fat, 63 g; 20% oligosaccharide beverages, 590 mL; energy, 2,850 kcal)	most recent training session						abundance in rowers at rest Alanine, Lactate, Cystine, Glutamic Acid, Valine and Glutamine. The following metabolites were in significatly lower abundance in rowers Linoleic Acid, Palmitic Acid, Oleic Acid, Citric Acid and B-D-Methylglucopyranoside
Keuhnbaum et al. 2015	Intervention	9 obese women (BMI 25-36) participated in 6 weeks of high intensity interval cycling training	Participants performed 10 x 60s cycling intervals at a workload estimated to elicit 90% of HRmax. Participants performed 1 minute of recovery cycling at 50w between each interval. Cadence was fixed between 80-100RPM. Participant completed 18 sessions (3x p/w for 6 weeks)	Overnight fasted	Baseline sample taken prior to the first session, post-training sample taken 1-2 days after the most recent session	Plasma	Multisegment Injection-Capillary electrophoresis-mass spectrometry	Untargeted identification with targeted QC	41	Partial Least-Squares discriminant analysis	A two dimensional PLSDA with the following parameters: R2 = 0.78, Q2 = 0.62 with separation between baseline and post training samples being primarily driven by 4 metabolites C2, Hypoxanthine, C0, GSH-Cys-SS. No metabolites were significantly different at baseline or post-training at rest.
Duft et al. 2017	Intervention	22 obese men (BMI = 31 kg/m2) were randomly assigned to a control group (n = 11) and exercise training group (n = 11) performed 24 weeks of combined aerobic and resistance training or continued their regular physical activity.	Participants in the exercise training group performed 3 sessions per week of combined endurance and resistance training for 24 weeks. Sessions consisted of 30 minutes resistance training - which involved 3 sets of 8-10RM using the following exercises (leg press, leg extension, leg curl, bench press, lateral pull down and arm curl) and 30 minutes aerobic training - which involved 30 minutes of walking or running at a speed eliciting a response of 50-85% estimated VO2peak	Overnight fasted	Baseline samples taken before first training session, post-intervention sample taken 72hrs post exercise	Serum	1H-NMR	Untargeted	44	Partial Least Squares Discriminate analysis & Univariate Hypothesis testing	A two dimensional PLSDA with the following parameters R2 = 0.98 , Q2 = 0.62 with separation between control participants exercise trained participants (post intervention) were primarily driven by Tyrosine, Histidine, 2-Oxoisocaproate, Pyruvate, Phenylalanine, Choline, Betaine, Carnitine, Creatinine, Ornithine, Valine, Alanine, Leucine, Glutamine, Asparagine, 2-Aminiobutyrate and Lactate; All of these metabolites were also significantly different in abundant in the exercise training group (18 metabolites increased in abundance, 2 metabolites decreased).

Table 2.1. Summary of studies that have investigated changes in fractions of the resting serum or plasma metabolome in response to or association with exercise training

2.3. SMALL EVs

2.3.1. Small extracellular vesicles as exercise factor carriers

Extracellular vesicle is a generic term used to describe any lipid bilayer-encapsulated particle that is naturally-released by cells and is incapable of independent replication (Théry et al., 2018). EVs are released by most eukaryotic cell types and are enriched with heterogenous cargoes (e.g. metabolites, RNA species, proteins) derived from their cell of origin. At rest, EVs are continually released and removed from circulation (Matsumoto et al., 2020), but can demonstrate enhanced release in response to physiological stimuli; for example, in response to hypoxia or shear stress in vascular endothelial cells (Hromada et al., 2017). Circulating EVs elicit bioactivity through uptake and delivery of their molecular cargo to recipient cells, often different from the tissue of origin, and are implicated in the regulation of physiological processes, such as coagulation and antigen presentation (Yáñez-Mó et al., 2015).

For exercise physiologists, interest in EVs developed greater traction with the observation that many circulating proteins as candidate exercise factors did not contain signal (secretory) peptide sequences, and yet were present in online EV expression databases (Safdar et al., 2016). Currently, rigorous investigations of EVs in the context of response to acute exercise are limited, but reports are often interpreted as indicating EVs respond in a manner that mirrors that of exercise factors more broadly. Specifically, that EVs are transiently-enriched in circulation during and after exercise, and elicit relevant bioactivity through the delivery of their cargo to recipient cells (Nederveen et al., 2021; Vechetti et al., 2020).

EVs are broadly separated into three subtypes, which are delineated by their mechanism of biogenesis, namely (i) exosomes, (ii) ectosomes (a.k.a. microparticles or microvesicles) and (iii) apoptotic bodies (van Niel et al., 2018). Exosomes represent released intraluminal vesicles (ILVs), which are derived from multi-vesicular bodies (MVBs) that originate with inward budding of the plasma membrane. Because of their mechanism of origin, exosomes are

expected to fall within the size range of MVB-associated ILVs i.e. ~50-150 nm (Kalluri and LeBleu, 2020; van Niel et al., 2018). Ectosomes are defined as EVs which originate via direct shedding from the plasma membrane. The exact mechanism of ectosome shedding in non-apoptotic cells is less well-described, but is suggested to involve altered membrane asymmetry via cytoskeleton remodelling mediated by Ca^{2+}-sensitive aminophospholipid translocases ("floppases" and "flippases"). Ectosomes have a broader reported size range of 50 nm to 1 μm (van Niel et al., 2018). Exosomes currently dominate the general interest in EVs in the domains of exercise physiology, metabolism and adaptation (Safdar and Tarnopolsky, 2018; Vechetti et al., 2020). As such, throughout this chapter, we will only consider reports which have endeavoured to separate and characterise EVs that are within the size-range of exosomes (i.e. 50-150 nm). However, as EVs with similar physical characteristics to exosomes can bud directly from the plasma membrane (and therefore are not exosomes) (van Niel et al., 2018) and technical challenges regarding the isolation of "pure" samples of individual EV subtypes (see below) hence we refer to these particles using the more collective term of "small EVs".

2.3.2. Separation and characterization of EVs

Pertinent to any discussion of EVs is acknowledgement that there are currently no routinely-used direct methods for the characterisation or quantification of EVs from biofluid samples. Instead, separation of biofluid fractions and multiple methods for identification and characterisation are required to identify both the presence and quantity of EVs in a sample. This has implications regarding the inferential value of individual experimental studies concerning small EVs in any context. To enable critical discussion of relevant exercise studies, a brief overview of how EVs are currently recommended to be identified and analysed by the International Society of Extracellular Vesicles (ISEV) via the MISEV2018 Guidelines is provided subsequently and summarised in table 2.2.

Most contemporary EV separation techniques involve separation of particles from a biofluid sample (e.g. blood, sweat, milk, saliva) based on either physical properties (such as size and density), specific expression of EV marker proteins, or a combination of both (reviewed by (Doyle and Wang, 2019)). Currently numerous methods are used to separate small EVs from biofluids, the most common of which are differential ultracentrifugation, density gradient separation, size exclusion chromatography, and immunoprecipitation (Cocozza et al., 2020). While these common techniques all may be used individually to separate small EVs, some may also be employed in combination for additive effects (Théry et al., 2018). However, the parameters by which these techniques work to separate particles tend to overlap across EV subtypes and various other molecules, thus it is often considered unavoidable that preparations of EVs will contain a heterogenous mix of particles e.g. several EV subtypes with similar physical or expression characteristics (Cocozza et al., 2020; Théry et al., 2018), and non-EV molecules such as argonaute proteins (Arroyo et al., 2011), lipoproteins (Jamaly et al., 2018) and exomeres. The latter are secreted, bioactive and non-membranous protein complexes with diameters of ~35 nm (Zhang et al., 2018, 2019). Accordingly, ISEV recommends referring to EVs within separated samples using clearly-defined operational terms based on identified physical and/or biochemical characteristics of particles within said samples. For example, instead of referring to EVs by the labels of specific subtypes, isolated EVs should be classified as “small” or “medium/large”, and fixed size ranges for each term be declared, which can be determined at researchers’ discretion. Alternatively, EVs can be termed based on the positive detection for specific markers (e.g. $CD63^+$ EVs), or the use of multiple defined parameters together such as “small $CD63^+$ EVs” (Théry et al., 2018).

Small EV characteristic	MISEV recommendation	Approaches	Analytic considerations
Quantity of EVs in a sample	A quantified estimate of both the source of EVs (e.g. extracted whole blood/plasma volumes) and EVs themselves should always be provided	-Particle quantification (e.g. NTA, flow cytometry) -Total protein quantification (e.g. SDS page) -Total Lipid quantification (e.g. SDS page)	-None of these methods exclusively quantify EVs -EV quantification is improved when methods are used in conjunction and to provide ratios indicative of purity e.g. protein/particle or lipid/particle
EV marker identification	- At least three positive protein markers associated with EVs; including at least one transmembrane protein and one cytosolic protein. -A Purity control - such as proteins that are identified as common contaminants (e.g. lipoproteins in plasma)	Traditional methods of protein identification (e.g. Western blot)	-"mixed" signal may be determined across positive markers and therefore it is important multiple markers are used -High contaminant signal may have implications for interpretation of some quantification methods (e.g NTA)
Characterization of single vesicles	-At least two different, but complimentary methods of vesicle visualization should ideally be employed	Microscopic based techniques (e.g. electron or atomic force microscopy) Single particle analysers (e.g NTA)	-Most Microscopy techniques are not interchangeable in terms of the information they provide -Different techniques may need to be employed depending on the EV size range of interest

Table 2.2. Summary of the minimal information for the study of extracellular vesicles guidelines.

Once small EVs have been separated from biofluid, it is then essential to validate the presence of small EVs and provide an estimate of small EV quantity, particularly in circumstances where enrichment is presumed to occur (e.g. exercise) (Safdar et al., 2016; Vechetti et al., 2020). Broadly, the MISEV2018 guidelines recommend use of three principal indicators to identify the extent of small EV presence in a biofluid sample (table 2.2). These are the quantification of particles in a small EV size range; the detection of marker proteins associated with small EV membranes and contaminants; and the visualization of small EVs derived from a sample (Théry et al., 2018).

The first indicator is the quantification of small EVs involving the estimation of the size range and concentration of particles within the sample. This enables determination of the distribution of particle diameters contained within a sample, and subsequent estimation of the concentration of particles that fall within the size range of small EVs (50-150 nm). Several methods are available for determining physical characteristics of these particles, but the most common technique employed in exercise studies is nano-particle tracking analysis (NTA). This

technique measures the Brownian motion of individual particles via detection of scattered light, and by employing the Stokes-Einstein equation to determine diffusion coefficients can estimate distributions of particle concentration and diameter (Doyle and Wang, 2019). Traditional or modified methods of flow cytometry are also sometimes employed for the quantification of EVs by particle count (Welsh et al., 2020). Additionally, estimating total quantity of protein or lipid is useful as a global indication for determining the relative abundance of specific small EV markers or common contaminants (e.g., lipoproteins). These are recommended to be estimated via standard approaches, e.g. BCA assay or global protein stain of SDS-PAGE, and can also be usefully combined to estimate sample characteristics like unit protein per particle (Théry et al., 2018). The second indicator required for small EV enrichment is the characterization of common markers of EV status, which are generally "validated" small EV surface marker proteins such as CD63 (van Niel et al., 2018). Identification and quantification of at least three markers is recommended, encompassing an EV-associated protein and/or cytosolic protein, and at least one negative protein marker. The third, and final, indicator is the visual characterization of single vesicles, which is most commonly accomplished by using transmission or scanning electron microscopy, but may also employ techniques that can visualise vesicle topography, such as atomic force microscopy (Doyle and Wang, 2019; Théry et al., 2018).

Importantly, when considering the presence or bioactive effects of small EV cargoes, endeavouring to achieve a reliable indication of high abundance of small EVs in the sample is important, because some non-EV molecules that co-exist in separated samples may also serve as carriers for factors that are proposed as bioactive cargo of small EVs, such as microRNA (miRNA) and protein (Arroyo et al., 2011; Zhang et al., 2019). Subsequently, to determine any roles or responses of small EVs to acute exercise or exercise training, it is essential that a rigorous characterisation of small EVs is made. The technical approach (e.g. the number and

types of methods) required to characterise the presence of EVs in a sample is extensive, and may be challenging to accomplish. However, when considering the question of whether small EVs represent an important "carrier" of exercise factors, the extent to which any given report demonstrates the presence/quantity of small EVs will have substantial influence on the inferential value of the reported results.

2.3.3. Changes in the small EV profile in response to acute exercise

When investigating the response to acute exercise, the profile of small EVs would then ideally be compared between pre-, during and/or post-exercise samples using a multi-methods approach based on ISEV guidelines (table 2.2). Namely, enrichment would be evident as greater particle concentration (e.g. a greater NTA or flow cytometry signal), and quantities of total protein and small EV protein markers would be measurable in post- compared to pre-treatment (e.g. exercise) samples, together with a consistent (and ideally low) indication of contamination within preparations of small EVs from both pre- and post-treatment (Théry et al., 2018). The confidence with which enrichment could be inferred would then be based not on the signal of a single indicator, but instead on the general signal across multiple independent methods (table 2.2).

Currently several studies have investigated elements of the response of circulating small EVs to acute aerobic (Bertoldi et al., 2018; Brahmer et al., 2019; Doncheva et al., 2022; D'Souza et al., 2018; Frühbeis et al., 2015; Gao et al., 2021; Guescini et al., 2015; Karvinen et al., 2020; Lovett et al., 2018; McIlvenna et al., 2023; Neuberger et al., 2021; Oliveira et al., 2018; Rigamonti et al., 2020; Warnier et al., 2022; Whitham et al., 2018; Yin et al., 2019; Zhang et al., 2021), resistance (Annibalini et al., 2019; Conkright et al., 2022; Just et al., 2020; Vechetti et al., 2021) or combined plyometric and downhill running (Lovett et al., 2018) exercise. Most of these studies have attempted to characterise circulating small EVs separated from plasma (Annibalini et al., 2019; Brahmer et al., 2019; Conkright et al., 2022; Doncheva

et al., 2022; D'Souza et al., 2018; Frühbeis et al., 2015; Gao et al., 2021; Guescini et al., 2015; Just et al., 2020; Lovett et al., 2018; McIlvenna et al., 2023; Neuberger et al., 2021; Rigamonti et al., 2020; Vechetti et al., 2021; Warnier et al., 2022; Whitham et al., 2018; Yin et al., 2019), although four studies have separated small EVs from serum (Bertoldi et al., 2018; Karvinen et al., 2020; Oliveira et al., 2018; Zhang et al., 2021).

An important point is that platelets are purported to be the largest contributors to the circulating EV pool (Yáñez-Mó et al., 2015). Therefore, results between studies that separate EVs from serum and plasma, which are rich in and depleted of platelets respectively, will have inherit differences in their EV profiles, particularly as platelets may contribute to the exercise-associated small EV pool (Brahmer et al., 2019; McIlvenna et al., 2023). This contribution is likely to be more apparent in preparations of small EVs separated from serum, and may influence differences in indications of small EV responses to exercise (e.g. enrichment) between studies.

In addition to differences in biofluid sources, these studies have not had consistent methodological approaches, which hampers broad conclusions on the nature of the response of circulating small EVs to acute exercise. For example, four studies have only examined changes in the concentration of specific miRNAs from plasma samples taken before and after aerobic exercise (D'Souza et al., 2018; Guescini et al., 2015; Karvinen et al., 2020; Yin et al., 2019). The inference was that these miRNAs represent small EV cargo. However, in each of these studies, miRNA concentrations (transcript abundance) were quantified in both pre- and post-exercise samples, but additional methods of small EV characterisation were only applied to post-exercise samples (D'Souza et al., 2018; Guescini et al., 2015; Karvinen et al., 2020) or not at all (Yin et al., 2019). Problematically, this type of technical approach provides no indication as to whether the abundance of small EVs was in fact changed by the exercise bout and cannot associate changes in the concentration of these miRNAs with a concomitant change

in the estimated abundance of small EVs. Given the heterogenous nature of preparations of small EVs, it is difficult to conclude that the data from these studies are definitively indicative of a change in small EV profile in response to acute exercise.

Thirteen studies have employed a single methods approach of small EV enrichment using either NTA (Annibalini et al., 2019; Just et al., 2020; Lovett et al., 2018; Rigamonti et al., 2020; Vechetti et al., 2021), flow cytometry (Bei et al., 2017; Conkright et al., 2022; Gao et al., 2021), western blot (Bertoldi et al., 2018; Doncheva et al., 2022; Neuberger et al., 2021; Zhang et al., 2021) or a microfluidic chip capture antibody based method of single vesicle analysis (McIlvenna et al., 2023) to estimate small EV enrichment in response to acute aerobic (Bei et al., 2017; Bertoldi et al., 2018; Doncheva et al., 2022; Gao et al., 2021; Neuberger et al., 2021; Rigamonti et al., 2020), resistance (Annibalini et al., 2019; Conkright et al., 2022; Just et al., 2020; Vechetti et al., 2021) or combined plyometric and downhill running (Lovett et al., 2018) exercise. Each of these studies, except two (Conkright et al., 2022; Lovett et al., 2018), observed an increase in their respective measures within preparations of small EVs taken after exercise, which potentially indicates an exercise-induced enrichment in small EVs. However, using only measures of particle count is problematic because these only provide an indication of the concentrations of particles with specific size characteristics, which in practice would have low specificity for individual subtypes of EVs. For example, the use of NTA alone can reduce the accuracy of results as this measure is highly sensitive to factors such as nutrition status (Brahmer et al., 2019; Jamaly et al., 2018; Mørk et al., 2016). While a single method analysis only identifying EV markers (e.g., exclusively using western blot) disallows any direct quantification of particles in preparations of small EV (i.e. the 'degree' of enrichment estimated is becomes subjective) and may be accidentally or intentionally selective for different sub-populations of small EVs (as the expression of most 'EV markers' is highly heterogenous across different EVs) (Kowal et al., 2016).

Five studies have employed multi-method approaches in response to acute aerobic exercise (Brahmer et al., 2019; Frühbeis et al., 2015; Oliveira et al., 2018; Warnier et al., 2022; Whitham et al., 2018). Two of these studies have seen increases in both NTA signal and protein concentration within preparations of small EVs derived from human plasma (Frühbeis et al., 2015; Whitham et al., 2018). One study employing a proteomics approach observed an exercise-induced increase in the abundance of 299 proteins (some of which were markers of small EVs such as CD81), which coincided with an increase in NTA signal within preparations of small EVs (Whitham et al., 2018). Another study estimated an increase in the small EV marker proteins FLOT1 and HSP70 via Western blot, which also coincided with an increase in NTA signal comparing pre- and post-exercise samples (Frühbeis et al., 2015).

Small EV preparations from Wistar rats exposed to acute low, moderate or high intensity exercise demonstrated greater concentration of small EV-sized particles, total protein and CD63 abundance compared to samples derived from a sedentary control group. Importantly, APOIV, a lipoprotein and indicator of contamination preparations of separated EVs, was present but had similar abundance across all conditions (Oliveira et al., 2018). Another study using a pre-post analysis of acute exercise in humans with minimal lipoprotein contamination used NTA and Western blot analysis of small EV markers (i.e. CD9, CD63, Syntenin, CD41b, Tsg101, CD81), or a novel multiplex array that enables concurrent detection of 41 surface EV proteins (Brahmer et al., 2019). These methods produced mixed results by observing no differences in small EV-sized particle concentration by NTA, but increases in multiple markers of small EVs via Western blot and multiplex assay approaches (Brahmer et al., 2019). A more recent study using NTA and western blot analysis of small EV markers (CD9, TSG101, ALIX, CD81, HSP60) subjected healthy and prediabetic individuals to 60minutes of cycling exercise (at a power output corresponding to 55% of VO_{2max}) only observed increases in small EV particle count (via NTA) after 30 minutes of exercise in healthy individuals during

normoxic exercise. In addition only three of the five small EV 'marker' proteins increased during exercise (CD81, HSP60 and TSG101) (Warnier et al., 2022).

In this section, I have focussed on appraising the approaches employed for determining whether small EV enrichment occurs, rather than focussing on the specifics of the exercise bouts, sample timing, or the specific changes in cargo or concentration during and after exercise. These specific details have been the subject of several recent reviews (Estébanez et al., 2020; Nederveen et al., 2021; Vechetti et al., 2020), and it is now often accepted that small EVs are enriched in circulation in response to acute exercise (Murphy et al., 2020; Safdar et al., 2016; Vechetti et al., 2020; Whitham and Febbraio, 2016), despite the methodological limitations of studies to date. Nevertheless, rigorously measuring small EV enrichment in response to exercise remains challenging to accomplish, as multi-method approaches are required and agreement across selected methods may not always be apparent (Brahmer et al., 2019; Warnier et al., 2022). However, the majority of studies to date have not provided sufficient characterization of small EVs to definitively conclude that enrichment has occurred. That said, of the limited number of studies where multi-method approaches have been applied, acute exercise arguably does induce an enrichment of small EVs in circulation. This is consistent with the general response of exercise factors, and therefore the proposed role of small EVs as a medium through which exercise factors are transported in circulation.

2.3.4. Does exercise training alter the resting profile of circulating small EVs?

There are currently fifteen available reports (across thirteen experimental studies) that have investigated the influence of exercise training on indicators of small EV abundance and/or cargo (summarised in table 2.3). Under the assumption that small EVs are carriers of exercise factors and that exercise factors experience training-induced alterations in resting concentrations in circulation, there are several scenarios wherein *increases* could be represented. The first is alterations in the cargoes associated with preparations of small EVs

i.e. the same approximate quantity of circulating small EVs, but with individual molecules presenting as novel cargo, or as cargo of greater abundance, in exercise-trained states (i.e. an increase in the "density" of a specific cargo per EV or novel molecules appearing in detectable abundances, as a result of exercise training). The second is an increase in the quantity of circulating small EVs i.e., a greater total abundance of cargoes in circulation, that is caused by an increase in the total amount of circulating small EVs in exercise-trained states (i.e., an exercise training induced increase in the concentration of circulating small EVs at rest). The third scenario would be some combination of these two scenarios (e.g., a greater amount of both small EV concentration and cargo abundance). The next subsections will describe the relevant studies in both rodents and humans and whether any of these scenarios are observed.

Results from rodent studies

Eleven studies have investigated the influence of exercise training on the small EV profile of rodents (Barcellos et al., 2020; Bei et al., 2017; Bertoldi et al., 2018; Castaño et al., 2020; Chaturvedi et al., 2015; Gao et al., 2021; Hou et al., 2019; Liu et al., 2022; Lou et al., 2022; Ma et al., 2018; Xiang et al., 2020). All of these studies, except one– which was interested in the expression of VEGF protein (Liu et al., 2022), were specifically interested in the miRNA cargo of small EVs, their differential expression and potential biological relevance, and employ an experimental design wherein animals subjected to aerobic exercise training are sampled after the cessation of training. Preparations of small EVs were then separated from plasma (Barcellos et al., 2020; Bei et al., 2017; Castaño et al., 2020; Gao et al., 2021; Liu et al., 2022; Lou et al., 2022, 2022; Ma et al., 2018; Xiang et al., 2020) or serum (Bertoldi et al., 2018; Chaturvedi et al., 2015; Hou et al., 2019), and compared to those of sedentary controls. Collectively, these studies have been inconsistent in their approaches towards separating and characterising small EVs, have tended to measure only a limited number of specific miRNA as

representative of small EV cargoes, and have reporting or methodological issues, which all combined, interferes with the inferential utility of these works.

For example, C57BL/6 mice subjected to four weeks of treadmill running at either a low (5 m/min) or moderate (10 m/min) speed, and sampled 24 h after the last exercise bout, had an increase in the resting abundance of both $CD34^+/VEGFR2^+$ small EVs and associated miR-126 cargo, in an intensity-dependent manner (Ma et al., 2018). These findings suggest that exercise training induces an increase in the resting concentration of some small EVs, namely those derived from endothelial progenitor cells based on their status as $CD34^+/VEGFR2^+$. However, in this study, the $CD34^+/VEGFR2^+$ small EVs were extracted from an initial separation of small EVs via differential ultracentrifugation using an additional procedure involving magnetic bead sorting. While a particle count assay (NTA) of these preparations estimated a ~2 to 2.5-fold increase in particles in trained compared to sedentary groups, no difference was visually-apparent in the abundance of either CD34 or VEGFR2 markers in associated Western blots, and these markers were not quantified. As NTA is a nonspecific method of small EV quantification, these results imply a disagreement between assays and call into question the inference of the increased resting concentration of small EVs in response to exercise training.

Additionally, Sprague-Dawley rats subjected to four weeks of swim exercise and sampled 24 h after the last exercise bout exhibited no change in NTA signal or the abundance of selected small EV markers (TSG101 and CD81) compared to controls i.e. the basal abundance of small EVs was unchanged by training (Hou et al., 2019). Interestingly however, 12 miRNA were observed to be differentially expressed (11 upregulated, 1 downregulated) in the small EV preparations from the exercise-trained rats, suggesting that, even in the absence of a change in the resting abundance of small EVs, the cargo of small EVs may change with exercise training (Hou et al., 2019). Additionally, Sprague-Dawley rats subjected to four weeks

of daily treadmill running (at 60% of predicted VO_{2max}) at sampled 24h after the last exercise bout demonstrated no difference in the abundance of small EVs via either NTA or subjective appraisal of western blot bands of CD9, CD63 and TSG101, but a 3x increase in VEGF protein observed in small EV preparations derived from exercise-trained rats (Liu et al., 2022). Similar results were also observed in C57BL/6 mice subjected to 60 minutes of treadmill running at 18m/min for 9 consecutive days and sampled 24h after the last exercise bout, where no difference in the abundance of small EVs via either NTA or subjective appraisal of western blot bands of CD81 and TSG110 was observed. However, a 3x increase in miR-122-5p observed in small EV preparations derived from exercise-trained mice was found (Lou et al., 2022).

Four other studies do report increases in signals indicative of particle enrichment and small EV marker abundance from small EV samples from exercise-trained rats (Barcellos et al., 2020; Bei et al., 2017) and mice (Chaturvedi et al., 2015; Gao et al., 2021). However, of these studies Barcellos et al. (Barcellos et al., 2020) sampled 1 h after the last exercise bout, Gao et al. (Gao et al., 2021) sampled immediately after the last exercise bout (personal communication), and Bei et al. (Bei et al., 2017) sampled on average 6 to 12 hours after the last exercise bout (personal communication), i.e. each study sampled in the immediate post-exercise period, when exercise-induced enrichment may still be present). I was unable to ascertain the time point of sampling from the work of Chaturvedi et al. (Chaturvedi et al., 2015) or Xiang et al. (Xiang et al., 2020). Regardless, in training studies where sampling is in close proximity to the end of the last exercise bout, it is difficult, or sometimes impossible, to parse out whether results are indicative of the effect of acute exercise, residual bout effects, a true training effect, or a combination of the three.

Other studies have waited 24 h before taking post-training samples (Hou et al., 2019; Ma et al., 2018), but residual influences of an acute exercise bout may still be apparent within these and shorter time periods (section 2.1.7). To my knowledge, one study has investigated the influence of exercise training on circulating small EV profiles in samples extracted >24 h after the last training bout (Castaño et al., 2020). Castano et al. subjected male C57BL/6 mice to five weeks of progressive high intensity treadmill running, and small EVs were isolated from plasma 48 h after the last training bout. No differences were observed in small EV abundance via an acetylcholinesterase assay, or subjective appraisal of Western blots of three small EV markers. Additionally, it should be noted, however, that acetylcholinesterase has been proven to not be a generic marker of EVs (Liao et al., 2019). Regardless, profiling of 378 miRNA transcripts by qPCR revealed seven differentially expressed miRNAs (6 miRNA with higher abundance, 1 miRNA with lower abundance) when comparing exercise-trained to sedentary groups (Castaño et al., 2020).

Collectively in these rodent studies, methodological limitations in terms of providing adequate indication of the presence of small EVs within samples separated from biofluids, and potential confounding by residual influences of the last exercise bout must be acknowledged, but some reports do indicate altered miRNA cargo within preparations of small EVs taken at rest after a period of exercise training (Castaño et al., 2020; Hou et al., 2019).

Results from human studies

To my knowledge, there are currently five reports describing the influence of prior exercise training on preparations of circulating small EVs derived from resting humans (Estébanez et al., 2021; Garai et al., 2021; Hou et al., 2019; Nair et al., 2020; Xhuti et al., 2023) (table 2.3), four of which separated small EVs from plasma (Estébanez et al., 2021; Hou et al., 2019; Nair et al., 2020; Xhuti et al., 2023) and one which has separated small EVs from serum (Garai et al., 2021). The first study reported 1.8-fold greater abundance of miR-342-5p at rest in preparations of small EVs derived from young (19 to 22 years) male rowers with at least one

year of training experience compared to sedentary controls (*n* =16 in each group). However, small EVs were not formally characterised in this study, despite a small EV separation protocol being applied to plasma samples, and miR-342-5p abundance measured as the only target of interest. The next study compared resting miRNA profiles (via RNA sequencing) of small EV samples derived from endurance-trained and sedentary older (~69 y) males, albeit with only *n*=5 in each group (Nair et al., 2020). Seven differentially expressed miRNAs were identified between trained and untrained individuals, with 4 increased and 3 decreased in preparations of small EVs derived from the trained individuals. However, this study provided minimal information regarding the characterisation of small EVs in samples by reporting only a single NTA result and making no comparisons between groups. Additionally, while the aerobic fitness of the participants was discordant (VO_{2max} of 34.4±1.1 and 21.7±1.2 mL kg^{-1} min^{-1} in trained and untrained groups respectively), and the trained participants clearly had superior fitness, the average VO_{2max} for these individuals is still approximately half of what is commonly-reported for well-trained athletes of younger age (Jeukendrup et al., 2000; Jones et al., 2021). Therefore it is unclear whether a greater magnitude of fitness at younger age would create additional or alternative differences in the profile of resting small EVs between either the trained or sedentary group in the aforementioned study (Nair et al., 2020). The third study was in male and female older adults (~73 y) that compared an 8 week resistance exercise training intervention (n=28) to a sedentary control group (n=10) (Estébanez et al., 2021). However, the analysis was limited to the presence of small EVs via quantification of total exosome protein, identification of six marker proteins of small EVs via Western blot, and one proposed miRNA cargo of small EVs (miR-146a-5p) (Estébanez et al., 2021). Of these, only the small EV marker CD63 exhibited a differential pattern between groups with the increase of ~7% in the training groups being less than the ~43% increase in the sedentary control group. Therefore, an attenuation of small EVs expressing CD63 may be a response to resistance exercise training,

but overall, the data from this study are also limited in their coverage. Another study involved 30 elderly (mean age 74.9 y) men participating in 36 sessions (3x per week for 12 weeks) of home-based banded resistance exercise (Xhuti et al., 2023). In this study, preparations of small EVs separated from pre and post intervention (72h post exercise) plasma samples were analysed using NTA, western blot of five small EV markers (CD63, ALIX, CD9, CD81,TSG101) and three contaminants (Albumin, ApoA1 and Calrecticulin). The presence of 14 miRNA was also screened for (using RT-PCR, 7 miRNA detected). No differences between were observed in the presence of small EVs between pre and post-training samples, although 4 miRNA were observed to have significantly higher abundance(s) in small EV preparations extracted post-intervention (miR-23a, miR-27a, mIR-146a, miR-92a) (Xhuti et al., 2023).

The final study consisted of two pilot experiments that investigated the potential influence of exercise training on the circulating profile of small EVs at rest (Garai et al., 2021). The first experiment consisted of healthy participants (n = 14, participant sex not reported) performing a three day-per-week concurrent (combined aerobic & resistance) training protocol for 6 months. Resting blood samples were taken at baseline and after the end of the training intervention, with each sample being reported as taken at least 24 hours after any vigorous exercise. In this experiment, no difference was determined in the number of particles within separated preparations of small EVs via NTA before and after exercise training, although 7 miRNA (2 increased, 5 decreased) were estimated to be differentially expressed via a nanostring array (Table 2.3). The second experiment reported in this study, is a cross sectional experiment whereby “lifelong” older (aged >60) athletes were identified via their responses to the international physical activity questionnaire (Craig et al., 2003) and their resting profile of small EVs was compared to the baseline samples of the individuals that completed the exercise intervention in the first experiment. Again no difference was estimated in the resting abundance of small EVs between the older athletes and younger individuals at baseline via NTA, however

three miRNA were estimated to be differentially expressed (all decreased) by nanostring array (table 2.3) (Garai et al., 2021). Although for this latter experiment, as the older participants were recruited solely based on their response to a lifestyle physical activity questionnaire, it is arguably difficult to discern the extent to which these (minor) differences in miRNA associated to separated preparations of small EVs may have been influenced by exercise training. Particularly as the older participants in this study, were on average 59 years older than the younger participants (Garai et al., 2021) and because the resting profile of small EVs may also be altered by the process of ageing (Noren Hooten, 2020).

2.3.5. Bioactivity of small EVs obtained from exercise trained and untrained individuals

Consideration of the bioactivity and/or beneficial physiological effects of small EVs may provide insight into whether training-induced changes in the resting profile of small EVs is a potential mediator of some of the benefits of exercise training, or perhaps methodological artifact. Six studies to my knowledge have performed *in vivo*/*ex vitro* experiments to investigate such effects (Bei et al., 2017; Castaño et al., 2020; Hou et al., 2019; Ma et al., 2018; Wang et al., 2020). However, I have not included discussion of two of these studies due to the post-training sample time point being <24 h after the last exercise training bout (Bei et al., 2017; Gao et al., 2021).

Of the remaining studies, two have investigated the bioactive effects of preparations of small EVs derived from exercise-trained mice exclusively in cell lines (Ma et al., 2018; Wang et al., 2020), and the other two have investigated potential *in vivo* effects through injection of small EVs derived from exercise-trained rodents into sedentary control animals (Bei et al., 2017; Castaño et al., 2020). In the first study (Ma et al., 2018), preparations containing $CD34^+$/$VEGFR2^+$ small EVs were co-incubated with cultured brain endothelial cells that were exposed to 18 h of 25 mM glucose and/or 6 h of hypoxia (1% O_2, 5% CO_2) as a model of endothelial cell injury. Co-culturing of cells with small EVs derived from exercise-trained or

sedentary C57BL/6 mice produced reductions in cell apoptosis of ~5, 10, and 20% for sedentary, low and moderate exercise-trained samples respectively. Cell migration and tube formation was increased similarly in a condition-dependent manner. These effects were speculated to be mediated by miR-126, a transcript increased in preparations derived from the exercise-trained mice. In these cells, preparations of small EVs enhanced the expression of the angiogenic protein VEGF, while anti-miR-126 decreased VEGF expression and removed the described effects of small EV co-incubation (Ma et al., 2018). These results suggest that exercise training enhanced the abundance of specific transcripts within preparations of small EVs, which in turn are capable of enhancing the expression of local factors associated with some exercise adaptations, such as angiogenesis in this case.

Similar findings were reported in an *in vivo* model of cardiac injury induced in Sprague-Dawley rats (Hou et al., 2019). Sedentary rats received a direct intramyocardial injection of small EVs derived from exercise-trained rats two days prior to a surgically-induced myocardial infarct. Rats who had received injections of preparations of small EVs derived from exercise-trained counterparts demonstrated ~40% lower infarct size 24 h post-surgery, and enhanced cardiac function through a lower reduction in ejection fraction and fractional shortening 4 weeks post-surgery, compared those injected with small EVs from sedentary controls. These results were similarly associated with the presence of a single miRNA in small EV samples (miR-342-5p), which was measured to reduce the abundance of some apoptotic signalling proteins (Caspase-9 and Jnk2), and potentiate proliferative Akt signalling, in cultured cardiomyocytes exposed to hypoxic damage (Hou et al., 2019). In follow-up work to Ma et al., Wang et al. again used preparations containing $CD34^{+}$/$VEGFR2^{+}$ small EVs derived from plasma from exercise-trained mice (60 min/d at 10 m/min, 5 d/week for four weeks). Co-incubation of these EV preparations with neuronal N2a cells subjected to hypoxia-reoxygenation injury resulted in protection against injury (increased cell viability, decreased

apoptosis), and restored neurite length. These results coincided with greater secretion of brain-derived neurotrophic factor from these cells, and all effects were partially attenuated when inhibition of miR-126 or Akt-PI3K signalling was employed (Wang et al., 2020).

The work in each of these three studies should be noted as models that have potentiated recovery from injury in damaged cells or tissues (Hou et al., 2019; Ma et al., 2018; Wang et al., 2020) and therefore, these bioactivities do not necessarily translate into potentiating the function of healthy cells, or provide insight into mediating an exercise adaptation or training response in tissues. However, recent work by Castano et al. has attempted to address these questions. C57BL/6 mice were exposed to four weeks of high intensity interval training via treadmill running after which small EVs were separated from plasma collected 48 h after the last exercise training bout. These preparations, as well as preparations derived from sedentary control mice, were then injected intravenously daily for four weeks into separate groups of sedentary mice. At the end of the four week administration period, mice treated with exercise-trained small EVs demonstrated lower body mass (~6%) and epididymal fat mass, lower circulating triglycerides (~15%), improved lipid tolerance, and improved insulin sensitivity and glucose tolerance (glucose AUC ~35% lower), compared to the control mice (Castaño et al., 2020). Analysis of differentially-expressed miRNA between preparations of small EVs derived from exercise-trained or sedentary mice (discussed in Section 2.3.4 and Table 2.3) also yielded a thematic association with the regulation of the transcription factor forkhead box O1 (*FoxO1*), which in part regulates hepatic gluconeogenesis (Puigserver et al., 2003). Preparations of small EVs derived from the exercise-trained mice reduced hepatic *FoxO1* mRNA expression, as well as transcripts of several gluconeogenic proteins associated with *FoxO1* signalling. Thus, extended administration of small EV samples derived from exercise-trained mice was proposed to enhance glucose tolerance through small EV-mediated delivery of miRNAs that attenuated hepatic gluconeogenesis (Castaño et al., 2020).

In summary, small EVs derived from resting blood samples of exercise-trained rodents appear to exert bioactive, and potentially beneficial, effects in several tissues compared to sedentary counterparts. These results suggest that exercise-trained small EV samples may offer therapeutic potential in specific use cases, or may play a role in exercise adaptation, and/or homeostatic maintenance. However, more research is required to determine aspects such as the consistency of specific metabolic effects, the type and time course of exercise training required to induce reliable changes in resting small EV profiles in humans, the duration for which these changes are sustained after training cessation, and whether it is the entire small EV sample or specific cargo within those samples that are the strongest determinants of observed bioactivity

Article	Study design	Sampling	Intervention	Small EV identification	Small EV presence between groups	Cargo measured	Differences in cargo
Training Intervention reports							
Bertoldi et al. 2018	Male Wistar rats (*n* = 4-6 per group) from three different age groups (3 months old, 6 months old and 21 months old) were subjected to 2 weeks of treadmill running	18hrs after last exercise bout	7 days running per week for two weeks at 60% of VO_{2max}	Small EVs separated using a commercial assay kit(miRcury) quantified via acetylcholine esterase assay and CD63 via western blot	CD63 concentrations higher (~5%) in all trained groups. Esterase activity was lower (~30%) in exercise trained in 6 month and 21 month groups	Aβ 1-42 via western blot	No differences between in Aβ 1-42 groups or conditions.
Ma et al. 2018	Eight to ten week old male C57BL/6 mice were left sedentary or subjected to four weeks of low or moderate treadmill running (*n* = 4-6 mice per group)	24 hours after last exercise bout	60 minutes of either low (5m·min) or moderate (10m·min) intensity treadmill running 5 days per week	Small EVs were separated from plasma via differential ultracentrifugation; and CD34/VEGFR2 magnetic bead sorting. Particles quantified via NTA and small EV markers (CD63, TSG101, CD34, VEGFR2) identified via western blot	$CD34^+$/$VEGFR2^+$ particles greater in low (↑ 2x) and moderate (↑ 4x) compared to sedentary (↑ 2x) in moderate compared to low	miR-126, identified and quantified via RT-PCR	**Low versus sedentary** miR-126 (↑ 1.5x) **Moderate versus sedentary** miR-126 (↑ 2.3x) **Moderate versus low** miR-126 (↑ 1.5x)
Hou et al. 2019	Six week old male Sprague-Dawley rats completed a four week swim training intervention. A minimum of 6 rats used per assay, total sample size not reported.	24 hours after last exercise bout	One week of progressive training and three weeks of two sessions of 90 minutes per day, five days per week.	Small EVs were separated from plasma via differential ultracentrifugation. Particles within small EV samples were quantified via nanoparticle tracking analysis, small EV "markers" CD81 and TSG101, identified by western blot and quantified with densitometry	No differences between groups	14 differentially expressed miRNA estimated between exercise trained and sedentary control rats via qRT-PCR	**Trained vs Sedentary** miR-1-3p (↑ 7.6x) miR-3571 (↑ 6.06x) miR-342-5p (↑ 4.33x) miR-208a-3p (↑ 3.09x) miR-184 (↑ 3.00); miR-122-5p (↑2.57x) miR-486 (↑ 2.36x) miR-3591 (↑ 2.27x) miR-206-3p (↑ 2.18x) miR-196b-5p (↑ 2.16) miR-760-3p (↑ 2.07x) miR-99a-5p (↑ 2.05x) miR-191a-5p (↓ 0.38x) miR-494-3p (↓ 0.44x)
Castaño et al. 2020	15-week old male C57BL/6J mice performed a five week high intensity treadmill running protocol or left as sedentary control. Multiple groups employed throughout minimum of *n* = 5 mice per group	48 hours after last exercise bout	15 sets of two minutes of running at 80% of maximal running speed. Speed increased by 2m.min every week. Days run per week not reported.	Small EVs separated from plasma using differential ultracentrifugation. Small EVs quantified via NTA and identified via TEM. Small EV "markers" CD63, CD9 and HSP70 identified via western blot	Only small EV markers (CD63, CD9 and HSP70) identified in both groups via western blot. Results not quantified	378 miRNA were screened for via RT-PCR, 7 differentially expressed	**Trained vs Sedentary** miR-133b-3p (↑ 11.03x) miR-205-5p (↑ 10.33x) miR-206-3p (↑ 9.56x) miR-133a-3p (↑ 9.46x) miR-19b-3p (↑ 2.73x) miR-30d-5p (↑ 2.24x) let-7g-5p (↓ 7.27x)

de Mendonça M,et al. 2020	C57BL/6J mice fed a high fat (*n* = 30) or control diet (*n* = 30) for 4 weeks and then subsequently subdivided further into exercising obese (*n* = 15) or sedentary control groups (*n* = 15) for each diet, for a further 8 weeks	72 hours after last exercise bout	60 minutes of running at 50% of peak speed, 5 days per week for 8 weeks	Small EVs separated commercial assay (EXO-quick) and identified via western blot of four markers (CD9, CD63, ALIX, TSG101) and one negative marker (GRP94), quantified via NTA and visualised via TEM	Only one marker (GRP94) was identified in all groups and not quantified	6 miRNA via qPCR	**Trained obese and versus sedentary obese** miR-22-3p (↓ ~2x) miR-29c (↓ ~2x) miR-122 (↓ ~20x) miR-192 (↓ ~3x)
Estébanez et al. 2021	38 individuals (16 males, 22 females), aged 70-85, self-reported as being free from chronic disease and medication use. Separated into a training (*n* = 28, 15 males, 13 females) and control (*n* = 10, 1 male, 9 females) groups	5-6 days after last exercise bout	3 sets of 12-8-12 reps of whole body progressive resistance training 2 sessions per week for 8 weeks	Small EVs separated from plasma using differential ultracentrifugation. Six small EV markers identified via western blot (CD9, CD14, CD63, CD81, FLOT-1, VDAC1) and quantified via optical densitometry	Percentage change in optical density of CD63 was significantly lower (36%) in the exercise trained group	miR-146-5p via qPCR	No difference in miR-146-5p between trained and sedentary groups
Garai et al. 2021	14 young males (mean age 23), self-reported as sedentary, but free from chronic metabolic disease participated in a concurrent (aerobic and resistance) training program	At least 24 hours after the last exercise bout	Resistance training performed at 85% of heart-rate maximum (exercises not reported) followed immediately by aerobic training (running and walking) at 65% of heart-rate maximum, three times per week for 6 months. Durations of exercise sessions not reported.	Small EVs seperated from pooled serum samples using Total Exosome Isolation reagent and traditional centrifugation. Unlabelled and labelled (CD63, CD81) preparations of small EVs quantified via NTA and visualised via TEM	No differences between groups	54 miRNA identified via nanostring multiplex array, 7 differentially expressed	**Post intervention versus baseline** miR-21-5p (↑ 1.5x) miR-451a (↑ 1.5x) miR-130a-3p (↓ 1.4x) miR-15b-5p (↓ 1.3x) miR-199b-3p (↓ 1.1x) miR-223-3p (↓ 1.01x)
Lou et al., 2022	Male C57BL/6J mice were exercised for 9 days consecutively and then euthanised or left as a sedentary control	24hrs after the last exercise bout	Mice ran at 18m/min on a treadmill with no incline for 60 minutes daily, for a period of nine days	Small EVs separated from plasma using differential ultracentrifugation. Small EV particle count was estimated using NTA, visualised using TEM and markers TSG101, CD81 and APOA1 were identified (but not quantified) using western blot	No differences between groups	miR-122-5p quantified between groups via RT-PCR	**Trained versus sedentary** miR-122-5p (3x ↑)
Liu et al., 2022	Sprague Dawley Rats were exercised daily for 4 weeks or assigned to sedentary control	24hrs after the last exercise bout	Rats ran for 20 minutes daily at a speed corresponding to 60% of their estimated VO_{2max}	Small EVs were separated from plasma using differential ultracentrifugation. Small EV particle count was estimated using NTA. Small EV markers CD9, CD63 and TSG101 were identified (but not quantified) using western blot. Small EVs visualised using TEM	No differences between groups	VEGF protein identified and quantified via Western blot	**Trained versus sedentary** VEGF (3x ↑)
Xhuti et al., 2023	Older adults (*n* = 30) performed 12 weeks of home-based resistance exercise using bands	72hrs prior to the first exercise session and 72hrs after the	Participants did 36 sessions of banded resistance exercise (3x sessions per week for 12 weeks). Participants performed	Small EVs were separated plasma using size exclusion chromatography concentrated with differential	No differences between groups	Small EVs were screen for 14 miRNA using RT-PCR.	**Post versus pre intervention** 4 miRNA were observed to be increased post exercise training (miR-23a ↑ 2x, miR-27a ↑

		last exercise bout	a series of self-directed banded resistance exercises (namely biceps curl, triceps extension, lateral raise, seated row, bench press, abdominal crunch, calf raise, chair squat, knee extension, knee flexion, hip flexion, and dorsi flexion) for 3 sets of 10-15 repetitions per exercise.	ultracentrifugation. Small EV particle count was estimated using NTA. Small EV markers (CD63, ALIX, CD9, CD81,TSG101) and contaminants (Albumin, ApoA1 and Calrecticulin) were identified via western blot and quantified using densitometry. Small EVs were visualised using TEM			0.5x, mIR-146a ↑ 0.5x, miR-92a ↑ 0.25x); 3 miRNA were not significantly different between pre/post training (miR-1, miR-133a, miR-199a) and 7 were not identified (miR-296, miR-141, miR-27b, miR-130, miR-181, miR-199, miR-34a)
Cross-sectional reports							
Hou et al. 2019	20 year old soldiers who were members of a rowing team (n =16) or sedentary (n = 16) provided resting blood samples.	24 hours after last exercise bout	Subjects provided resting blood samples	Small EVs were separated with differential ultracentrifugation. Particles within small EV samples were quantified via NTA	No differences between groups	miR-342-5p measured via qRT-PCR	**Trained versus sedentary** miR-342-5p (↑ 5x)
Nair et al. 2020	older men (> 65 years) were stratified into "exercise trained"(n = 5) or "sedentary" (n = 5) groups, based activity history	Overnight fasted proximity to last exercise bout not reported	Subjects provided resting blood samples	Small EVs separated from plasma using a commercial assay kit (Exoquick). Small EVs identified using NTA	No differences between groups	Whole-transcriptome RNA-seq applied to small EV samples	**Trained versus sedentary** miR-874-3p (↑ ~1.2x) miR-339-5p (↑ ~ 1.8x) miR-383-5p (↑ ~2x) miR-206 (↓ ~1.5x) miR-148a-3p (↓ ~ 1.2x) miR-486-5p (↓ ~ 1.1x) let7b-5p (↓ ~ 1.1x)
Garai et al. 2021	Older (average age 62) men who self-reported as exercise trained (n = 11) provided resting blood samples	Self-reported to have refrained from exercise for at least 24 hours	Subjects provided resting blood samples	Small EVs separated from pooled serum samples using Total Exosome Isolation reagent and traditional centrifugation. Unlabelled and labelled (CD63, CD81) preparations of small EVs quantified via NTA and visualised via TEM	No differences between groups	54 miRNA identified via nanostring multiplex array, 3 differentially expressed	**Trained older adult versus sedentary youth** miR-199b-3p (↓ 1.3x) miR-23a-3p (↓ 1.07x) miR-451a (↓ 1.11x)

Table 2.3. Studies in rodents or humans that have investigated the effect of exercise training on the profile of circulating small EVs either by examining the response to exercise training interventions or by performing cross-sectional comparisons of trained and untrained individuals

2.3.6. Summary

Appraisal of existing studies across rodents and humans regarding the influence of exercise training on the abundance of small EVs and their proposed miRNA cargoes leads me to conclude that the characterisation of small EVs within samples is generally inadequate by way of employing insufficient methodological approaches. Therefore, while some data indicate that the resting concentration of small EVs may change with exercise training, which has been commented by others (Brahmer et al., 2020; Nederveen et al., 2021), arguably the methods employed do not produce sufficient information to confidently state this to be the case.

Aside from the difficulty in accurately characterising small EVs and their cargo, it is pertinent to also consider physiological and teleological aspects of the proposition that resting profiles would indeed be changed by exercise training. Circulating concentrations of small EV are a dynamic balance, such that the concentration measured in a resting sample is indicative of the processes of small EV release and small EV uptake, and yet small EVs may have a relatively short half-life of ~7 minutes (Matsumoto et al., 2020). Thus, for enriched concentrations of small EVs to be observed in a resting sample distal to acute exercise, exercise training would have to induce augmented small EV release, attenuated small EV uptake, or both, at rest. To rigorously determine whether either or both occur would require determining which mechanisms govern systemic release and clearance of small EVs, and how exercise training either augments or attenuates these processes. Distinct from assessing small EV abundance, there is greater consistency across studies indicating that exercise training influences the miRNA profile of preparations of small EVs from resting samples. As exercise training can alter the transcriptomes (Pillon et al., 2019) and proteomes (Ferreira et al., 2014; Holloway et al., 2009; Padrão et al., 2016) of contractile tissues, a change in cargo profile is plausible independent of change in small EV abundance, because local changes to the biomolecular environment of a cell could alter the cargo that is released, even in the absence of an altered

rate of EV release. However, it is important to reiterate that without adequate characterisation of small EVs, the extent to which these differential miRNA profiles associate specifically to small EVs (even when small EV separations have been undertaken) is unknown and there remains the question as to why a persistent, as opposed to acute, change in resting profile of small EVs would occur with exercise training.

2.4. THE BIOACTIVITY OF PLASMA/SERA EXTRACTED IN ASSOCIATION WITH ACUTE EXERCISE OR EXERCISE TRAINING

2.4.1. The bioactivity of the exercise factor milieu: a case for investigating the influence of exercise-associated plasma and sera

One of the most appealing aspects regarding the concept of an 'exercise factor' pertains to the idea that the consequence of exercise factor function is to exert bioactivities that are beneficial to health (Carson, 2017; Chow et al., 2022; Severinsen and Pedersen, 2020). It is overwhelmingly clear that regular exercise participation conveys both preventive and remedial benefits in the case of the majority of chronic non-communicable metabolic diseases (Booth et al., 2017). The idea that some, or many, of the benefits of exercise are mediated by exercise factors is attractive, as this would imply that studying the characteristics of exercise factors could reveal a fertile landscape for novel and potentially translatable therapeutic agents (or targets) that can relieve symptomology by inducing 'beneficial' metabolic effects that normally occur during or after exercise (Whitham and Febbraio, 2016). There is evidence from specific exercise factors that give credence to this idea. For example, acute exercise exposure is well identified to enhance insulin sensitivity for ~24h (Sylow et al., 2021). Acute exercise also robustly increases the concentration of the circulating exercise factor IL-6 (sometimes up to 100x), which has, in this context, also been associated with enhanced insulin sensitivity (Pedersen and Febbraio, 2008). Acute infusion(s) of IL-6 (i.e. attempting to 'mimic' the IL-6 response to exercise) have been observed to increase glucose infusion rate during a hyperinsulinemic/euglycemic clamp in healthy and diabetic individuals (Pedersen and

Febbraio, 2007). Collectively, these findings were the inspiration for development of new chimeric protein known as IC7-Fc, which shares domains from IL-6 and the Ciliary Neutrophic Factor protein. Regular administration of IC7-Fc has been shown to enhance glucose tolerance in mice and has absent immunogenicity in macaques, a characteristic which technically makes this compound eligible for consideration to be approved for Phase I clinical trials (Findeisen et al., 2019). Consequently, IC7-fc is the first therapeutic compound that has been devised by knowledge gained from studying the response of an exercise factor and relevantly is a demonstration that there is the potential for translatable utility to be ascribed to the activity of some exercise factors.

Beyond IL-6 there is currently some degree of 'beneficial' activity ascribed to ~30 exercise factors, albeit to varying degrees, potential bioactivities of exercise factors range from increases in lipolysis, browning of adipose tissue, maintaining muscle mass and stimulating angiogenesis (Chow et al., 2022; Severinsen and Pedersen, 2020). The effects of exercise factors have largely been studied using an approach where a factor is identified as having some responsiveness to exercise (e.g. a circulating enrichment) in initial experiment(s) and then the activity of said factor is assessed via interrogation of signal transduction cascades and/or physiological effects through the administration of recombinant factor preparations using *in vitro* cell culture or *in vivo* animal models (Chow et al., 2022). Several reports have also demonstrated that small EVs separated from exercise-associated plasma also can promote beneficial effects. For example, small EVs derived from exercise trained mice promote recovery from hypoxia injury in endothelial (Ma et al., 2018) and neuronal (Wang et al., 2020) cells. While daily injection of small EVs derived from exercise trained rodents elicited reductions in body mass (~6%) and epididymal fat mass, lower circulating triglycerides (~15%), improved lipid tolerance, and improved insulin sensitivity and glucose tolerance (glucose AUC ~35% lower), compared to the control mice (Castaño et al., 2020).

Collectively, these findings work to suggest that both exercise factors and small EVs (in a context where they are presumably acting as carriers *of* exercise factors) can indeed elicit 'health promoting' effects and may serve to in-part mediate some of the health benefits of exercise (Chow et al., 2022; Safdar et al., 2016). 'Novel' candidate exercise factors are now routinely identified using 'omics' style methods, which will often simultaneously identify hundreds of factors in a set of samples (Contrepois et al., 2020). The potential bioactivity of factors are then often inferred through ontological 'clustering' of factors into themes or through screening identified factors and looking for individual molecules with already established bioactivities. An alternative approach for validating the extent to which exercise factors may mediate 'health benefits' is by examining the effects of exercise-derived plasma or sera. Investigating the bioactivity of plasma or sera within an exercise factor context is salient as, in theory, these blood fractions represent the totality of the 'exercise factor environment' and therefore in a physiological context, any 'health promoting' effects mediated by exercise factors should also be measurable with the treatment of plasma/sera (Hojman et al., 2018). The final section of this literature review will briefly outline the experimental models that can be employed to study the effects of exercise-associated plasma/sera and will briefly discuss what 'health promoting' effects of exercise-associated plasma/sera are currently identified.

2.4.2. Models developed for studying exercise-associated plasma or sera

Prior to discussing what is currently known about identified bioactivities that may be attributable to exercise-associated plasma/sera, it is useful to first outline experimental models that can be used to attempt to study the 'isolated' physiological effects of plasma or sera. Principally, these experimental models can be separated into two distinct approaches, namely cell culture experiments using media supplemented with exercise-associated plasma/sera or *in vivo* administration experiments where rodents or humans are injected or infused with plasma

derived from acutely exercised or exercise-trained individuals. A brief overview of each type of experimental model is outlined briefly in the subsections below.

Cell culture models involving media supplemented with human plasma/sera

Supplementing cell culture media with plasma or sera represents a simple model for making inferences regarding the potential influences of circulating changes induced by different homeostatic perturbances (e.g., food/supplement intake, disease, or exercise). The general process for models using supplemented media involves culturing cells in serum-free media (SFM) that is supplemented with plasma/sera extracted from humans in varying physiological states (e.g., acutely exercised) and measuring responses through changes in signal transduction, gene product abundance or assays designed to measure parameters of cell function (e.g., proliferation, growth/migration) (Allen et al., 2023).

The practical advantage of studying media supplemented with plasma/sera is that once an experimental protocol is established (e.g. certain assays, cell lines and/or media preparation strategies) it becomes straightforward to examine the influence of plasma/sera that has been extracted in different physiological states. For example, media supplemented with human plasma/sera from patients with metabolic diseases (e.g. liver disease) as a means of studying the influence of circulating factors on disease pathology (Allen et al., 2022) or individuals who have consumed specific meals, diets or supplements as a means of identifying how varying nutritional status can influence the activity of certain cell types (Cogan et al., 2019; Lees et al., 2021; Patel et al., 2019). These two 'different' types of supplemented media can investigated on the same type of cell (e.g. skeletal muscle cells) using similar assays (e.g. growth assays or measurements of protein turnover) to study different physiological scenarios e.g. in this example, how liver disease influences muscle cell atrophy (Allen et al., 2022) or how circulating aminoacidemia influences muscle cell growth (Patel et al., 2019). While this approach is innovative and relatively straightforward, it carries disadvantages that are largely translational in nature. These include issues with the concentration of factors in plasma/sera.

For example, even though media supplemented with human plasma/sera is more 'physiological' than alternatives (e.g. Foetal Bovine Serum) in terms of the abundance of specific nutrients and growth factors (Allen et al., 2023), the isolated environment in which cells are cultured may create a scenario where the amount or concentration of plasma/sera cells are exposed to is still beyond what a given cell may experience *in vivo* e.g. (the ratio of a given factor/cell is still supraphysiological). Therefore certain effects or influences may present in an exaggerated fashion that fails to be replicated with translation to more physiological experimental models. This limitation is also compound by the absence of elements of the physiological environment in cell culture models (i.e. interstitial barriers) that regulate the extent to which plasma/sera or circulating factors can make direct contact (i.e. directly influence) cells *in vivo*.

In vivo models involving the infusion or injection of plasma/sera

There also exist some approaches for studying the exchange of circulating factors between organisms *in vivo*, primarily through using models of parabiosis, which technically refers to the physiological union of two individuals (e.g. conjoined twins), but is more often used to describe an experimental model where animals with different phenotypes (e.g. diseased and non-diseased) are surgically joined and the influence of each respective phenotype on the other is measured through various means (Finerty, 1952). Variations of this experimental model originated in the 1890s and utilised regularly in the mid-20th century across various diseases and conditions (Eggel and Wyss-Coray, 2014; Finerty, 1952) until losing vogue until 2005 when certain regenerative capacities (e.g., rejuvenation of satellite cells) of older mice were found to be restored through parabiosis with younger counterparts (Conboy et al., 2005). Interestingly, it was through a model of parabiosis established by M.S Goldstein in 1961 where blood transfusion from exercised to non-exercised and non-exercised diabetic mongrel dogs elicited decreases in blood glucose concentration that lead to the initial speculation of the presence of an 'exercise factor' (Goldstein, 1961). However, parabiosis models are highly

extreme as the recipient organism (e.g. diseased or unexercised animal) is essentially fully and chronically exposed to the circulating environment of the 'donor' organism, which in the case of exercise factor studies, must be continually 'exercising' (Goldstein, 1961). Thus, parabiosis can only provide 'proof-of-concept' level insight into the *in vivo* benefits of exercise factors and is not feasible for models that consider more translationally relevant 'chronic' administration experiments that may mimic traditional pharmacologic intervention (as described below).

There is limited examples of more translatable models of *in vivo* administration of exercise-associated plasma to unexercised organisms. Studies using this kind of model are almost exclusively concerned with whether the transfer of plasma from exercise trained rodents conveys benefits to the *cognitive* function of sedentary rodents (De Miguel et al., 2021; Horowitz et al., 2020), but have served as inspiration for an ongoing Phase II clinical trial exploring the benefits of exercise-derived plasma on tasks of cognition and recall in patients with early-stage Alzheimer's disease (Tari et al., 2022). The results of these studies (De Miguel et al., 2021; Horowitz et al., 2020) are discussed in section 2.4.3. The design of these studies are explained here in more abstract terms as a means of assessing the parameters of the experimental model. Principally, these studies both involve administering plasma from mice that were granted free access to participant in voluntary wheel running for either 28 days (Horowitz et al., 2020) or 6 weeks (De Miguel et al., 2021) to age-matched sedentary controls. Exercise-derived plasma was administered in an intermittent fashion (~every 3rd day) for a period of 27 (Horowitz et al., 2020) or 28 days (De Miguel et al., 2021).

Therefore, the basic design of these studies is to grant the provision of exercise factors (via exercise-derived plasma) to diseased individuals in a manner that mimics a traditional pharmacological intervention (a fixed dose received at regular intervals while symptomology is monitored). Potential advantages of this study design is that individuals may be exposed to

an ‘exercise factor response’ that they themselves are incapable of producing endogenously (e.g. the plasma of elite level athletes could be transferred to sedentary older adults) and if found to be effective, studying the benefits of exercise-derived plasma is clinically translatable prior to refinement. In other words, plasma can be administered as a therapeutic agent prior to discovery of what specific exercise factors may be mediating beneficial effects. Potential disadvantages of this model are firstly related to the ‘dose’ of plasma that may be required. Typically studies have administered ‘doses’ of plasma that are ≤10% of expected plasma volume of the recipient individual (De Miguel et al., 2021; Horowitz et al., 2020; Tari et al., 2022), which may represent only a fraction of the ‘exercise factor’ response experienced by the individual that the plasma was derived from and could be insufficient to elicit beneficial effects in the recipient individual. Secondly, a disadvantage may be the regularity with which plasma may need to be administered i.e. if the benefits of exercise-derived plasma are largely transient e.g. ~24h, impractically regular dosing strategies may be required to maintain beneficial effects. Therefore, while there is collectively limited examples of *in vivo* experimental designs that have been employed to explore the administration of exercise-derived plasma, straightforward and potentially translatable models do exist and are likely to be explored more thoroughly in the future.

2.4.3. The potential bioactivity of plasma/sera derived from acutely exercised individuals

Results from cell culture studies

There has been a reasonable degree of investigation into the influence of plasma/sera derived from acutely exercised individuals. Currently, the majority of research has focused on the influence of cell culture media supplemented with exercise-derived sera on the growth and proliferation of various types of cancer cells (Metcalfe et al., 2021; Orange et al., 2020; Soares et al., 2021), although some studies have also investigated the influence of serum derived from

acutely exercised humans on the activity on C2C12 myoblasts (Nguyen et al., 2014); Adipocytes (Flores-Opazo et al., 2019); and CD4+ T-cells (Palmowski et al., 2021). The bias towards studying media supplemented with sera over plasma largely due to reaction(s) between common cell culture media types and latent clotting factors present in plasma that produces a 'clumping' of media upon mixing (Calatzis et al., 2001). While the influence of clotting factors in plasma can be attenuated via heparinisation or heat inactivation, these interventions are inconvenient and reduce the physiological relevance of plasma, therefore the use of sera (which has already clotted) to condition media is often preferred by researchers (Allen et al., 2023).

There are currently two meta-analyses that have investigated the influence of acute exercise-derived sera specifically on the proliferation of cells derived from prostate, breast, lung and colon cancer cell lines and have similarly reported that culture media supplemented (5-10%) with serum extracted from acutely exercised human(s) reduces cell proliferation (standardized mean difference ~-1.5) (Orange et al., 2020; Soares et al., 2021). However, due to the proximity of their publication, the studies employed by these meta-analysis overlap completely, so agreement of results is expected. Although one of these meta-analyses did report a sub-analysis examining whether isolating studies that extracted serum from individuals who had performed high (exercise performed > 80% VO_{2peak}) or moderate intensity (< 80% of VO_{2peak}) exercise protocols altered the overall effect size of analysis, observing that excluding moderate intensity exercise studies tended to increase the overall effect size (implying serum derived from individuals who had performed high intensity exercise was more effective at reducing cell proliferation) (Soares et al., 2021).

While there are numerous reports of the influence of media supplemented with exercise-derived serum on cancer cell proliferation as a broad outcome measure, there is limited additional evidence regarding additional responses of cancer cells in terms of metabolism or aspects cell function. Media enriched with 10% pooled serum extracted from

acutely exercised mice induced a 50% reduction in proliferation of MCF-7 cells with a concomitant increase in total caspase activity suggesting that influence of exercise-derived serum is enhanced apoptosis (Hojman et al., 2011). However, despite reductions in cell proliferation of ~10% increased apoptosis was not observed in LoVo or CaCo-2 cells cultured with media supplemented with 10% serum derived from acutely exercised men who were in remission from colon or rectal cancer (Devin et al., 2019). Therefore, while the influence of exercise-derived serum on the proliferation of cancer cells is arguably robust (Orange et al., 2020; Soares et al., 2021), it is unclear whether this is related to serum induced increases to apoptosis or reductions to growth signalling (Metcalfe et al., 2021) or whether exercise-derived serum influences other aspects of cancer cell activity *in vitro*.

Information is considerably more limited regarding the influence of exercise-derived serum on cells that are not derived from tumours . In C2C12 cells, media supplemented with 5% serum extracted from healthy children immediately after 60 minutes of cycling exercise (2 x 30 minute bouts at 55% of peak power output) has been demonstrated to enhance proliferation of myoblasts (~30-50%, compared to serum extracted at rest) over 48h (Nguyen et al., 2014). While media supplemented with 10% serum extracted from healthy males after 60 minutes of cycling at 70% of VO_{2max} increased GLUT4 protein content of primary human adipocytes by ~12% (compared to adipocytes cultured with resting plasma) (Flores-Opazo et al., 2019). Interestingly, media supplemented with 50% serum extracted from men who had performed 30 minutes of treadmill running (25 minutes at 70% VO_{2max}, 5 minutes at 95% VO_{2max}) had no effect on the viability, gene expression or subset differentiation of CD4+ T cells compared to media supplemented with serum extracted from a resting control group (Palmowski et al., 2021).

Collectively, the influence of serum extracted from acutely exercised individuals is mixed, with some evidence suggesting variations in responses that may depend on cell-type

(e.g. decreased proliferation across cancer cell-lines, with increased proliferation in muscle cell lines) and some results that could be argued as reflective of 'exercise adaptations' (e.g. increased GLUT4 expression) (Egan and Zierath, 2013; Flores-Opazo et al., 2019). All of these observations would align with the idea that exercise factors may convey a diverse variety on 'health benefits' that may vary with different tissue types (Chow et al., 2022). However, the available research is largely preliminary, and experimentally the extent to which many experimental variables (e.g., sera concentrations, incubation time, participant disease status, exercise/mode/duration/intensity) influence assay results is not well-established. Therefore it is difficult to attribute confidence to the extent to which sera extracted from acutely exercised individuals influences the activity of different cell-types.

Results from in vivo studies

Currently there is one *in vivo* study examining the influence of administration of sera/plasma extracted immediately from acutely exercised individuals to sedentary animals or humans. One study has investigated *in vivo* tumour formation induced by subcutaneous injection of LNCaP cells that had previously been cultured (for 72h) in media supplemented with 5% human serum extracted at rest or 2h post 60 minutes of cycling exercise (at 55% peak power output) in SCID mice over a period of 42 days. Mice injected with LNCaP cells that had been cultured in media supplemented with post-exercise serum demonstrated no tumour incidence after 14 days and ~30% lower tumour volume 34 days post LNCaP cell injection (Rundqvist et al., 2013).

In addition, there are two other studies where plasma was likely to be extracted in the early post-exercise period (De Miguel et al., 2021; Horowitz et al., 2020). In both of these studies, C57BL/6 mice were housed for a period of for a period of 42 (Horowitz et al., 2020) or 28 days (De Miguel et al., 2021) and granted unlimited access to participate in voluntary wheel running. Mice were then sacrificed and plasma is reported as being extracted at the end

of the last night cycle (6:00am-9:00am) (De Miguel et al., 2021) or the day after each exercise period ended (i.e. the morning of the 43rd day) (Horowitz et al., 2020). C57BL/6 mice who are provided with the opportunity to participate in wheel running will preferentially run at the beginning of the dark cycle (~18:00pm), but will continue to intermittently participate in wheel running until the end of the dark cycle (~6:00am) (Bains et al., 2018; Harri et al., 1999). Therefore, while the reporting of these studies (De Miguel et al., 2021; Horowitz et al., 2020) make it difficult to determine the exact proximity to exercise with which plasma was extracted, it is likely to be <24h post exercise. In the case of both studies, plasma extracted from 'exercised' mice was then pooled and administered to a sedentary cohort of aged (18 month old) mice for a period of either 27 (200µl of plasma administered via orbital injection every 3rd day) (De Miguel et al., 2021) or 28 (100µl of plasma administered to into an unreported injection site, 4 times over administration period) days (Horowitz et al., 2020). Parameters of neurogenesis and cognitive function assessed after the plasma administration period and in the case of both studies, indications of enhanced neurogenesis (determined through fluorescence intensity of immunolabelled neurons) and cognitive performance (determined through memory test scores) were greater in aged mice who had received treatment of plasma extracted from mice who had access to voluntary wheel running, in comparison to plasma extracted from young, sedentary mice (De Miguel et al., 2021; Horowitz et al., 2020). Collectively, these two studies provide some indication the some of the 'neuroprotective' effects (Booth et al., 2017) elicited by exercise can be conveyed externally through the administration of exercise-derived plasma. However, study results are limited by poor control over the exercise condition (by choosing voluntary wheel running as an intervention), sample extraction (not stating the exact proximity to exercise with which plasma was extracted) and the confounding influence of 'exercise training' (mice had ~ 1 month – 6 weeks of regularly wheel running exposure). These results are sufficient to suggest that more rigorous exploration of the 'beneficial' effects of *in*

vivo administration of exercise-derived plasma are warranted. Indeed, these studies have served as justification for an ongoing phase II clinical trial in humans, where plasma derived from young and 'fit' (aged 18-40 y, VO_{2max} > 55 $m{\cdot}kg^{-1}{\cdot}min^{-1}$) males are administered to human adults with early-stage Alzheimer's disease intermittently (two, four week administration periods) over the course of a year, with cognitive test score performance being monitored for up to 5 years post intervention (Tari et al., 2022).

2.4.4. The potential bioactivity of plasma/sera derived from exercise trained individuals at rest

In addition to examining whether sera extracted from acutely exercised individual demonstrates indication of 'beneficial' bioactivities, it is then also worth examining whether sera/plasma extracted from exercise trained individuals at rest may also demonstrate 'beneficial' effects. Effects may arguably be expected if the presence of exercise factors are altered at rest in exercise trained individuals (section 2.1.8). Similar to studies focusing on sera derived from acutely exercised individuals, the majority of studies examining the *in vitro* bioactivity of plasma/sera extracted from exercise trained individuals have been performed using cancer cells (Metcalfe et al., 2021). This section will only cover *in vitro* models as no *in vivo* studies investigating administration of sera/plasma derived from exercise trained individuals at rest to sedentary counterparts have currently been published.

A series of early studies (all using blood samples derived from the same cohort) observed that supplemented media enriched with 10% serum extracted 24h post exercise from men who regularly engaged in a community based exercise program (consisting of swimming, walking and light calisthenics exercises) induced 25-30% reductions in proliferation (Barnard et al., 2007, 2003; Leung et al., 2004; Tymchuk et al., 2002, 2001) and 2-30x increases in apoptosis (Barnard et al., 2007, 2003; Leung et al., 2004) compared supplemented media from obese controls in prostate cancer derived LNCaP and PC-3 cells (Barnard et al., 2007, 2003;

Tymchuk et al., 2002). In particular, one of these studies observed that the decrease in proliferation of LNCaP cells induced by culture with serum-derived from exercise trained participants was associated with an increase in p53 (2x) and decrease (~50%) in PCNA protein (Leung et al., 2004). This observation would imply that the principal mechanism by which exercise trained serum influenced LNCaP cells was apoptosis (Leung et al., 2004; Paunesku et al., 2001), particularly as serum derived from exercise trained participants did not alter proliferation of p53 negative LN-56 cells (Leung et al., 2004).

Arguably, these results contrast with studies investigating serum extracted immediately after acute exercise bouts, where culture with serum supplemented media reduces proliferation but has failed to induce increases in apoptosis (Devin et al., 2019; Rundqvist et al., 2013), leading to the suggestion that acutely exercise serum primarily reduces proliferation via reduction(s) in growth signalling (Metcalfe et al., 2021). However, all studies that demonstrate reduced cell viability or apoptosis from 'exercise trained' sera use samples derived from the same cohort of participants and do not report how recently after exercise cessation that serum samples were extracted (Barnard et al., 2007, 2003; Leung et al., 2004; Tymchuk et al., 2002, 2001). Therefore, the enhanced apoptosis observed in media supplemented with serum from exercise trained participants in these reports may be related to the residual presence of exercise factors released during a recent (i.e. <24h) exercise bout (Metcalfe et al., 2021). Additionally, media supplemented with 5 (Rundqvist et al., 2013) or 10% (Devin et al., 2019) serum extracted immediately post exercise did not increase measures of apoptosis in CaCo-2 and LoVo cells (Devin et al., 2019) or LNCaP cells (Rundqvist et al., 2013), suggesting that serum extracted from acute exercise does not increase apoptosis of cancer cells.

A key element of study design regarding the unified cohort used in studies that have demonstrated enhanced apoptosis in prostate cancer cells is that all observations of 'enhanced' cell death are reported as in comparison to sera extracted from obese (~31 kg/m^2, compared to

26kg/m^2 in the exercise trained group) sedentary 'controls' (Barnard et al., 2007, 2003; Leung et al., 2004; Tymchuk et al., 2002, 2001). Media supplemented with 5 or 10% sera extracted from obese compared to lean Zucker rats has been demonstrated to enhance proliferation of LNCaP cells by ~15% (Lamarre et al., 2007) with similar results (15-25% increased proliferation) observed in other prostate cancer cell lines (22RV1, DU145 and PacMetUT1) that are cultured in media containing 5% sera from obese compared to lean human males (Sherman et al., 2020). Therefore, the results of 'decreased' apoptosis and cell viability observed in response to media supplemented with serum from exercise trained individuals (Barnard et al., 2007, 2003; Leung et al., 2004; Tymchuk et al., 2002, 2001) are actually being reported relative to a control condition (obesity) that potentiates cell viability (instead of acting as a 'baseline') and therefore an 'anti-cancer' inference cannot be made from the results of these studies. Although, recently, media supplemented with 10% plasma extracted from community-dwelling older adults (aged 73.38 ± 11.28 y; body mass index 27.8 ± 4.9 kg/m^2) at rest (48h after last exercise sessions) after 16 sessions of combined aerobic and resistance training (across 8 weeks) has been observed to reduce proliferation of PC3 cells by ~5% compared to cells supplemented with pre-intervention plasma (Peres et al., 2022). This observation suggests that exercise-trained plasma extracted at rest may still convey some beneficial effects, although in the case of the aforementioned study (Peres et al., 2022) the magnitude of this effect appears small to the point where the extent to which results are translationally relevant (i.e. into *in vivo* studies) is not clear.

Additional reports regarding the influence of media supplemented with plasma/sera from exercise trained individuals at rest on cells that are not tumour derived are considerably limited. One study observed that media supplemented with 5% post-intervention serum extracted from obese (BMI = 30kg/m^2) women who participated in 12 weeks of home-based circuit style training at rest (72h post last exercise bout) elicited very limited (~10%)

enhancements in wound healing (compared to cells cultured with pre-intervention serum) of human coronary artery endothelial cells whose regenerative capacity was impaired with the chemotherapeutic agent 5-fluruouracil (Mclaughlin et al., 2023). Lastly, media supplemented with 10% resting (extracted at least 24h post exercise) serum extracted from elderly (aged 72 ± 11 y) heart-failure patients after 3 weeks of combined aerobic and calisthenics training (30 minutes sessions 2x daily, 6 days per week, for 3 weeks, 36 sessions total) enhanced the colony number (35% increase compared to pre-intervention serum) and adhesion activity (30% increase compared to pre-intervention serum) of Colony Forming Unit Endothelial cells extracted from the buffy coat of young, healthy volunteers (Gatta et al., 2012).

Collectively, there is limited evidence that indicates that plasma/sera extracted at rest from individuals who have experienced exercise training demonstrates meaningful 'beneficial' effects on cell culture preparations. Some studies have provided minor indication(s) of 'beneficial' effects (Gatta et al., 2012; Mclaughlin et al., 2023; Peres et al., 2022), these all are reported as within-group 'improvements' in assay results using sera derived from clinical populations (diseased older adult patients). Therefore, it is difficult to ascertain whether different results produced by comparing post- versus pre-intervention sera are representative of 'health promoting' effects conveyed by shifts in the profile of exercise factors, or are 'disease reduction' effects potentially conveyed by reductions in circulating factors that progress disease e.g. inflammatory cytokines. Further research involving younger, disease-free individuals is required to gain a clearer insight into whether exercise training elicits changes to plasma/sera that may exert apparent effects on cultured cells.

2.5. CONCLUDING REMARKS

The purpose of this chapter was to outline what is known regarding the 'exercise factor' response to acute exercise and exercise training, both in terms of 'exercise factors' from a traditional perspective (i.e. as independent circulating molecules) and as the cargo of

circulating small EVs. This chapter placed particular emphasis on the role circulating metabolites may play as exercise factors and while it is clear that some metabolites (e.g. lactate) do function as prototypical exercise factors, some metabolites whose circulating abundance(s) change in association with exercise may only serve to act as a representative signal of change in tissue metabolism (either at rest, or during exercise). There is some indication that there are 'metabolic fingerprints' of both acute exercise and exercise training, but with respect to exercise training in particular, studies tend to poorly control for the residual influence of acute exercise bouts, use small sample sizes, and arguably employ inappropriate statistical models. Both exercise factors and small EVs appear to share similar kinetic responses to acute exercise and the profile of both exercise factors and small EV cargoes appear to be altered with exercise training across different molecular species (i.e. protein, RNA and metabolites). While the acute responses of small EVs appears consistent in terms of abundance (demonstrating an enrichment in response to acute exercise), the response of small EVs to exercise training has mixed responses with some evidence of an increased abundance of small EVs present at rest in association with exercise training, and some studies indicating no change in abundance of small EVs. However many studies use individual, rather than orthogonal, methods of small EV identification, which is largely insufficient for making robust inferences about the abundance of small EVs present in a sample.

Lastly, this chapter aimed to assess the hypothesis that many or most exercise factors demonstrate evidence of 'health-promoting' bioactive effects. Instead of focusing on individual exercise factors, this chapter sought to interrogate whether there is evidence that the totality of the exercise factor milieu (i.e. plasma/sera extracted from acutely exercised or exercise trained individuals at rest) demonstrates reliable evidence of bioactivity. Collectively there appears to be some robust evidence that sera derived from acutely exercised individuals reduces the proliferation of many types of cancer cells *in vitro* with some preliminary *in vivo* reports also

producing suggestion that exercise-associated plasma may provide beneficial effects to the cognitive function of aged rodents, and potentially possess anti-cancer effects. There is currently limited evidence demonstrating any beneficial effects of plasma/sera extracted from exercise-trained individuals at rest, but most studies performed demonstrate weak methodological design in terms of poorly controlling for the most recent exercise session and through the reporting results against inappropriate control measures (e.g. obese participants), making the influence of exercise trained sera/plasma difficult to discern.

Subsequently, the endeavour of this thesis is to explore the response of metabolites and small EVs to exercise training using samples extracted from exercise trained individuals at rest and under controlled measurement conditions. Lastly, this thesis endeavours to investigate whether plasma extracted from exercise-trained individuals exerts 'health promoting' bioactivity.

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Chapter 3
The resting serum metabolome in response to short-term sprint interval training

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The full text version of this article is available as **Appendix B**

Prior to reading this chapter please note: All supplementary materials referred to in this chapter and the raw metabolomics (count) data analysed are available for secure download from the open science framework at the following link: https://osf.io/xj7ag/

3.1. ABSTRACT

Purpose: To investigate the response of a targeted fraction of (168 metabolites) of the resting serum metabolome to 9 sessions of Sprint Interval Training (SIT). **Methods:** 34 recreationally-active males provided resting blood samples before (baseline) and 48 to 72 h after (post) a short-term (9 session) cycle ergometer-based SIT intervention. A targeted analysis of 168 metabolites was performed on serum using liquid chromatography mass spectrometry (LC-MS). 160 distinct metabolites were identified and combined with four calculated metabolite sums and three calculated metabolite ratios creating a panel of 167 individual factors. Data were analysed using principal component analysis and univariate testing of all factors classified into 5 metabolite subgroups. **Results:** SIT improved anaerobic capacity measured by average power output during a Wingate test ($p<0.01$; mean difference = 38W, 95% confidence interval [26,51]) and aerobic capacity measured by average power output in a 20 minute cycling test ($p<0.01$; 17W [12,23]). Limited separation was discernible in the targeted serum metabolome between baseline and post-intervention when projected on the first and second principal component(s). However, univariate testing identified 11 fatty acids that had lower concentrations (false discovery rate <0.05) in post-intervention samples. **Conclusions:** These findings demonstrate that this short-term SIT intervention had limited effect on the serum metabolome at rest, but a subfraction of fatty acids are potentially sensitive to short-term exercise training.

3.2. INTRODUCTION

Sprint interval training (SIT) is a term used to describe a method of exercise training composed of individual exercise bouts that are each characterized by sets of brief (i.e. < 30s), highly intensive activity periods and extended inter-set recovery periods (i.e. > 2 minutes) (MacInnis and Gibala, 2017). When discussed within the context of exercise training for the general population, SIT is often promoted as an effective and time-efficient approach (Gibala et al., 2012) to enhance physiological indices of aerobic fitness (e.g. VO_{2max}) (Gist et al., 2014) and/or health (e.g. blood pressure) (Gibala et al., 2012). Notably, even short-term SIT interventions (6 sessions performed over two weeks) have demonstrated increased time to task failure during continuous exercise trials with coincident increases in markers of physiological remodelling to exercise training, such as skeletal muscle maximal citrate synthase activity (Burgomaster et al., 2005). These observations imply that adaptations elicited by SIT are detectable in the early stages of a training intervention, and makes short-term SIT a useful tool for studying systemic exercise adaptation.

Metabolomic analysis refers to the comprehensive systematic profiling of metabolites in a biological sample (Nicholson and Lindon, 2008). Specific or collective metabolite concentrations represent the outcomes of metabolic reactions and therefore metabolomic analysis arguably provides the most informative insight into the general metabolic state of tissues at the time of sampling (Belhaj et al., 2021). In this regard, blood represents a tissue of particular interest. This is partially due to the mixed composition of the circulating metabolome i.e. the metabolite content of blood consists of contributions from many tissues, and thus may provide insight into metabolic shifts that are representative of the physiologic state of an organism as a whole; the study of blood is also practically appealing due to the ease with which samples can be obtained compared to other tissue types (Dunn et al., 2011), for example muscle biopsies – which require more invasive and technically challenging procedures to extract and tend to produce greater patient discomfort compared to blood samples (Ekblom, 2017).

Interestingly, changes to the circulating metabolome at rest can manifest as a consequence of exercise training (Sakaguchi et al., 2019). For example, eight weeks of sprint training (repeats of 80m running sprints) induced changes in the ^{1}H-NMR metabolomic profile of serum of moderately trained men at rest that were primarily related to changes in circulating TCA intermediates, glucose and choline-containing molecules (Pechlivanis et al., 2013). Cross-sectional studies employing LC-MS have also demonstrated that exercise-trained individuals with different training backgrounds (bodybuilders or endurance athletes or untrained controls) each present with distinct serum metabolomic profiles at rest, with differences between groups in being driven by differences in metabolites related to Amino Acid & Hydroxysphingolipid and Phosphotidylcholine metabolism (Schranner et al., 2021); and that competitive middle distance runners (5000-10000m) with a greater VO_{2max} present with divergent profiles of the plasma metabolome at rest compared to athletes with a lower VO_{2max} from the same competitive discipline, with differences between athlete groups primarily related to differences in metabolites associated with Alpha Linoleic & Glutathione metabolism and Carnitine synthesis (Monnerat et al., 2020).

Collectively this provides indication that exercise training may exert some influence on the blood metabolome at rest, but it is currently unclear how the resting blood metabolome responds to short-term exercise training. Here, we present a secondary analysis on a subset of data derived from a larger trial, that has also been detailed in a previous publication (Aird et al., 2021), the aim of the current analysis is to provide insight into whether a short-term intervention of SIT (9 sessions performed over 3 weeks) induces changes to the serum metabolome at rest using a targeted analysis of a collective 167 individual metabolites and calculated metabolite sum/ratios.

3.3. METHODS

3.3.1. Participants

Recreationally active males (n = 34; age = 25.14 ± 4.20y), height = 1.82 ± 0.08m, body mass = 82.97 ± 9.87 kg, BMI = 25.57 ± 2.26 kg/m^2, baseline VO_{2max} = 42.26 ± 4.85 mL kg^{-1} min^{-1}, data presented as mean ± standard deviation, also available in Table S3.1) were recruited for this study and signed written informed consent forms prior to undergoing any experimental procedures. All procedures performed in this study received ethical approval by the Faculty of Education and Health Sciences research ethics committee at the University of Limerick (Ireland) (Ethics No: 2016_18_11_ EHS).

3.3.2. Exercise training intervention and performance measures

All participants successfully completed 9 sessions of SIT. Each session consisted of a fixed number of "Wingate"-style sprint intervals, where participants were asked to pedal with maximal effort on bicycle ergometer (Monark, 894E, Sweden) against a resistance equivalent to 7.5% of pre-intervention body mass for 30 seconds, before resting passively on the ergometer saddle for 4 minutes between each interval. At the beginning of each session, participants warmed up with 5 minutes of unloaded cycling at a cadence of 60-70RPM and each session ended with a 3 minute cool down of unloaded cycling at the same pedalling rate. The volume of each session was increased incrementally, whereby in sessions 1-3 participants performed 4 intervals, 5 intervals in sessions 4-6, and 6 intervals in sessions 7-9. Sessions were separated by 48-72 hours (i.e. typically performed on a Monday, Wednesday and Friday) and participants were asked not to perform any vigorous physical activity outside of the prescribed training sessions, which was confirmed weekly with participants at the first training session of each week. For performance outcome measures, participants performed a single maximal effort 30 second Wingate test to measure anaerobic capacity, and a 20 minute test to measure aerobic capacity. During the 20 minute test, which was performed on a different mechanically braked

cycling ergometer (SRM, Germany) cadence was fixed at 85 RPM while power adjusted in a variable fashion in accordance with participant's effort. Prior to the first assessment (the Wingate test) participants completed a standardised 5 minute warm-up on a Monark cycle ergometer that consisted of unloaded cycling at 60-70 rpm. These tests were performed in this order, 60 minutes apart, at both baseline, and 48 to 72 hours after the final SIT session, respectively. Prior to beginning the 9 session SIT intervention, participants completed a 7-day weighed food diary which was entered to online software (Nutritics. Version 5.031). Participants were asked to maintain their habitual nutrition throughout the SIT intervention and prior to each training and lab testing session, participants performed a 24 h dietary recall with researchers, which was compared to initial 7-day weighed food diary to ensure habitual diet was maintained (Table S3.1). Additionally, participants refrained from alcohol and caffeine intake for 24 h and 12 h, respectively before baseline and post-intervention blood samples which were obtained in the fasted state.

These data are derived from a larger trial where participants were additionally randomized into one of four intervention groups whereby each training session was performed under different nutrition conditions, namely: fasted (n = 8), carbohydrate-fed (n = 9), whey protein concentrate-fed (n = 8), or whey protein hydrolysate-fed (n = 9). During this intervention, participants received a pre-prepared supplement drink 45 minutes before each exercise training session only; specific details of this intervention are available elsewhere (Aird et al., 2021). No significant difference in performance outcomes or evidence of differences in the profile of baseline/post-intervention serum metabolomes between the nutrition intervention groups were observed (Table S3.1). Therefore, for the present analysis, participants were pooled into a single collective group to increase statistical power.

3.3.3. Blood sample acquisition and storage

For blood extraction, a 9 mL blood sample was taken from an antecubital vein in an overnight fasted state at baseline and post intervention (48-72 hours after the final exercise bout) into Serum Monovette® vacutainer tubes (Sarstedt, Munich, Germany). All blood samples were extracted at a consistent time of day (between 7.30-8:30am). To facilitate clotting, extracted blood samples were left to stand for 30 minutes at room temperature, samples were then centrifuged for 10 minutes at 2000g (20°C). Serum sample aliquots were stored at -80ºC before being sent to an analytical facility for the subsequently described metabolomics analysis.

3.3.4. Serum metabolomics

All serum metabolomics analyses were performed by The Metabolomics Innovation Centre (TMIC, Calgary, Canada) using custom assays employing targeted methods. Each assay involved a combination of direct injection mass spectrometry with reverse phase LC-MS/MS using an ABSciex 4000 Qtrap® tandem mass spectrometry instrument (Applied Biosystems/MDS Analytical Technologies, Foster City, CA, USA) coupled with an Agilent 1260 series UHPLC system (Agilent Technologies, Palo Alto, CA, USA). These instruments were used to quantify a targeted selection of up to 168 metabolites and can provide identification and quantification of numerous metabolite species (specifically, amino acids, acylcarnitines, biogenic amines & derivatives, uremic toxins, glycerophospholipids, sphingolipids and sugars, TMIC prime assay) and free fatty acids. Assays involved the derivatization and extraction of analytes, and the selective mass-spectrometric detection using multiple reaction monitoring pairs and metabolites that are subsequently quantified using Isotope-labelled internal standards and other internal standards. This assay is an adapted method used for targeted analysis of metabolites in urine (López-Hernández et al., 2021) that have been detailed elsewhere (Zheng et al., 2020). Upon identification and quantification,

metabolites were subsequently classified into four distinct metabolite groups (Amino Acids, Peptides and Analogues; Fatty Acids and Fatty Acid conjugates; Acylcarnitines; and Glycerophospolipids & Phosphopshpingolipids) using the "ChemOnt" taxonomic classification technique (Feunang et al., 2016). Fourteen of our detected metabolites belonged to taxonomy groups for which <3 metabolites were measured; these metabolites were gathered into a single group labelled "Others". We also calculated four metabolite sums (branched chain amino acids, gluconeogenic amino acids, essential amino acids, total acylcarnitine) and three metabolite ratios (acylcarnitine/carnitine, C2/C0 and kynurenine/tryptophan ratios respectively), as this can reduce data variation and provide insight into whether metabolic processes may be altered when certain clusters of metabolites with similar bioactivities have changed in a collected fashion (Petersen et al., 2012). Eight metabolites in our targeted panel were below the limit of detection (histamine, cis-hydroxyproline, dopamine, carnosine, nitro-tyrosine, diacetyl-spermine, tyramine, phosphocreatine), which collectively left the total number of variables generated for analysis (individual metabolites and metabolite sum/ratios) at 167 (168 potentially identifiable metabolites on the targeted assay panel, minus eight undetected metabolites, plus seven calculated metabolite sums/ratios for a total of 167 variables prepared for analysis, Table S3.2).

3.3.5. Principal component analysis

Prior to dimension reduction a Kaiser-Mayer-Olkin (KMO) test of sampling adequacy and Bartlett's test of sphericity were performed on data to ensure they met the minimum standards for PCA. Bartlett's test of sphericity produced a significant result (X^2 (166) = 95506, $p < 0.01$), while a Kaiser-Mayer-Olkin test of mean sampling adequacy produced a result of 0.5, which is a low, but acceptable value (Kaiser and Rice, 1974). Subsequently, a multilevel PCA was performed on all metabolites and metabolite sums and ratios using an orthogonal rotation and with factors mean zeroed and scaled for unit variance. Multilevel PCA refers to a

modified analysis applicable to repeated-measures design and involves decomposition of within-subject variation after the values for each selected variable (in this case each individual metabolite, metabolite sum or ratio) are collectively decomposed into linear variables (termed principal components) each of which explain an independent (orthogonal) and descending (i.e. PC1 explains the largest individual amount of variance, PC2 the second largest amount of variance) of total data variance. This approach has been demonstrated to increase model accuracy for paired-sample data (Liquet et al., 2012). All PCA were performed using mixOmics (version 6.14.1), a curated analysis package designed for exploratory analysis/feature selection within the environment of the statistical programming language R (Rohart et al., 2017). The number of principal components used was seven, which was selected based on appraisal of the "elbow" of a scree plot estimating the point where the proportion of variance explained by each principal component becomes approximately level (Table S3.3).

3.3.6. Mixed ANOVA of performance outcomes and univariate analysis of metabolites

As this was a secondary analysis of data from a combined exercise and nutrient intervention, I first performed a mixed analysis of variance (time * nutrient intervention) (ANOVA) to determine whether there were performance differences between nutrient intervention groups at either baseline or post-intervention. Univariate testing was also performed on metabolite data, where a series of pairwise t-tests with a Benjamini-Hochberg correction to adjust for multiple comparisons was applied to each of the five categorical metabolite groups (Amino Acids, Peptides and Analogues; Fatty Acids and Fatty Acid Conjugates; Acylcarnitines; Glycerophospolipids & Phosphopshpingolipids and Others) in isolation (Table S3.4). To determine common metabolic processes associated with altered metabolites, significant results underwent pathway enrichment analysis using a free online web-platform (MetaboAnalyst) (Xia et al., 2009). All statistical tests were considered to have produced a significant result if a p-value < 0.05 (or FDR <0.05 for pairwise analysis of

metabolites) was calculated. ANOVA results are reported with a partial-eta squared standardized estimate of effect size (η_p^2).

3.4. RESULTS

3.4.1. Performance results

The results of 9 sessions of SIT on performance outcome measures are presented in Figure 3.1. A significant main effect of time was observed for average Wingate power ($F = 41.88$, $p < 0.01$, $\eta_p^2 = 0.58$, baseline = 640 ± 74 W, post-intervention = 678 ± 75 W, mean difference = 38W, 95% confidence interval= [26,51]) and average power during the 20 minute test ($F = 41.29$, $p < 0.01$, $\eta_p^2 = 0.57$, baseline = 182 ± 30W, post-intervention = 199 ± 30 W, mean difference = 17 W, 95% confidence interval = [12,23]).

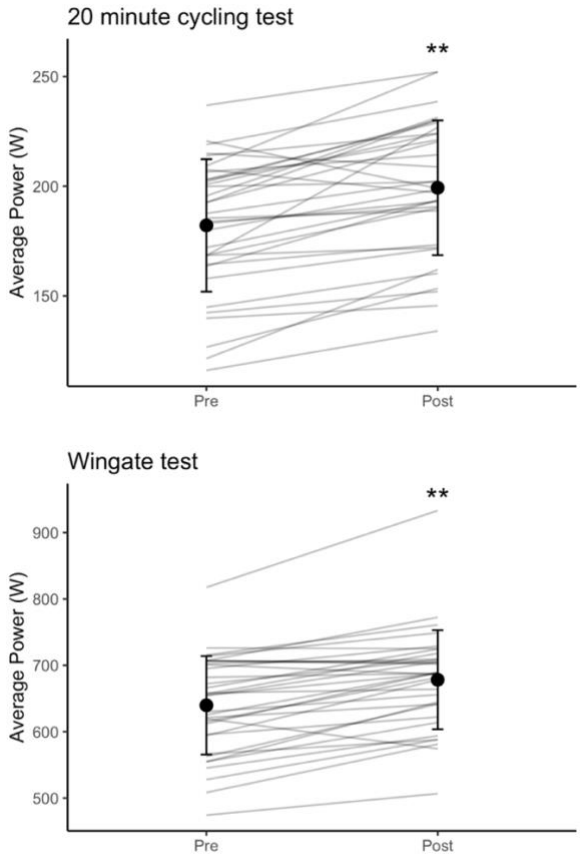

Figure 3.1. Performance changes in response to three weeks of sprint interval training. Filled dots indicate collapsed group means, error bars represent standard deviations. Filled lines indicate individual participants. * Indicates a significant main effect for intervention ($p < 0.05$) ** ($p < 0.01$).

3.4.2. Principal component analysis of the serum metabolome

The results of a Multilevel PCA to investigate whether SIT intervention influenced any changes in the serum metabolome are reported in Figure 3.2; There was no clear spatial separation between baseline and post-intervention samples, suggesting that 9 sessions of SIT did not influence the overall profile of this targeted analysis of the serum metabolome at rest.

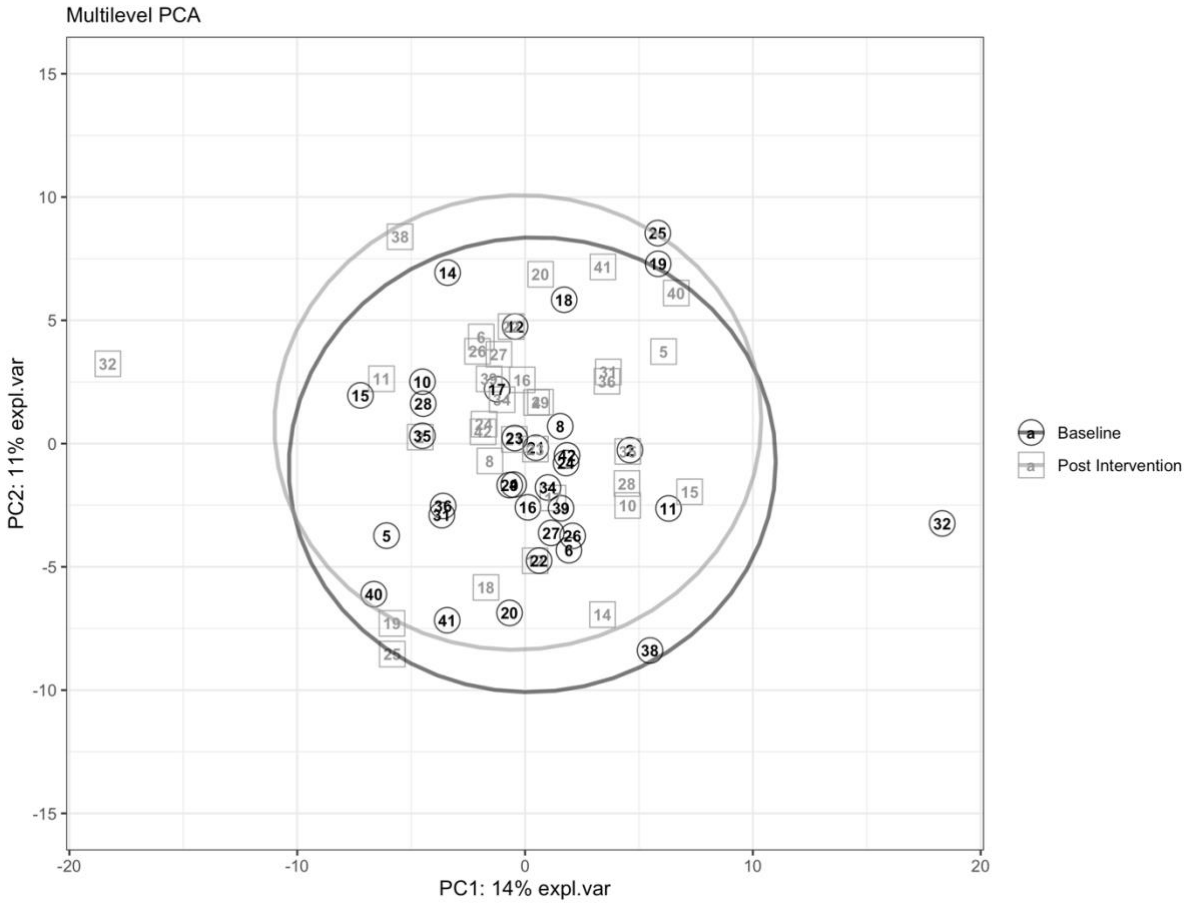

Figure 3.2. Principal Component Analysis of resting plasma metabolomes for all participants. Small panes represent simple analysis of participant's baseline ($R^2X = 0.58$) (A.) and post-intervention ($R^2X = 0.56$) (B.) metabolomes in isolation. Large pane (C.) displays results of a multilevel analysis containing both participants' baseline and post intervention samples. Individual numbers represent individual participants ($R^2X = 0.52$). Circles represent a baseline metabolome samples, while squares represent post intervention metabolome samples; individual numbers represent participant IDs.

3.4.3. Univariate analysis comparing baseline and post intervention circulating metabolites

The results of univariate analysis of all metabolites within each categorical metabolite group comparing serum metabolites, metabolite sums and metabolite ratios at baseline and post-intervention are presented in Table S3.4. The following metabolites, all belonging to the Fatty Acids and Fatty Acid Conjugates group, had significantly lower circulating concentrations: Oleic acid (Fold Change (FC) = 0.73, p.adj= 0.001); cis-8, 11, 14 Eic osatrienoic acid (FC = 0.81, p.adj= 0.002); Alpha Linolenic acid (FC = 082, p.adj= 0.003), Mystiric acid (FC = 0.73, p.adj = 0.004); Margaric acid (FC = 0.82, p.adj = 0.004); Gamma Linolenic acid (FC = 0.93, p.adj = 0.012); Linoleic acid (FC = 0.84, p.adj = 0.012); Stearic acid (FC = 0.90, p.adj = 0.016); Pentadecylic acid (FC = 0.93, p.adj = 0.023); Palmitic acid (FC = 0.90, p.adj = 0.023) and Palmitoleic acid (FC = 0.82, p.adj = 0.027) (Figure 3.3). When the 11 total fatty acids with significantly lower concentrations were included in a subsequent enrichment analysis, one significant pathway – biosynthesis of unsaturated fatty acids (7/36 hits, p.adj <0.01) was identified.

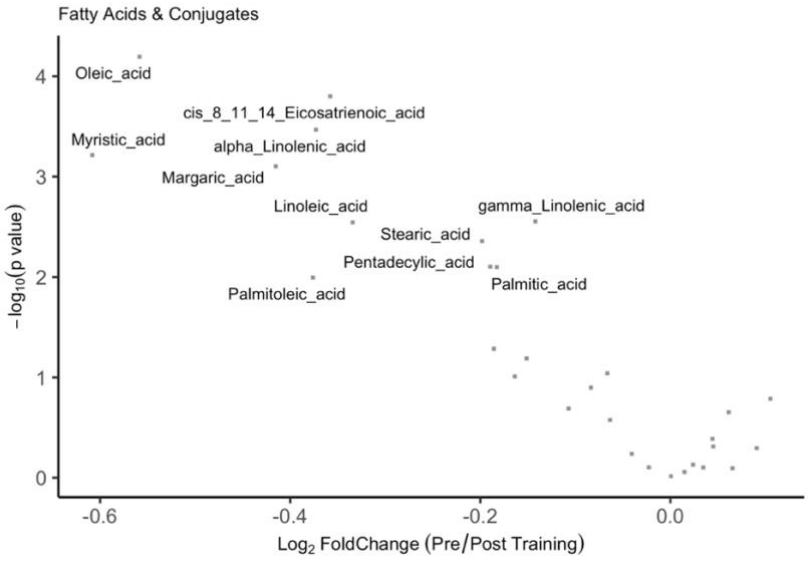

Figure 3.3. Volcano plot of Fatty Acids, named metabolites are those which were found to be differentially (adjusted p value < 0.05) abundant between baseline and post-intervention samples.

3.5. DISCUSSION

The aim of this study was to investigate whether a short-term intervention of SIT induces changes in the serum metabolome of recreationally-active males at rest using a targeted analysis of 167 metabolites and metabolite sums/ratios. Nine sessions of SIT improved the average power output of exercise tests indicative of anaerobic and aerobic capacity, with increases of 6% and 17% for the Wingate and the 20 minute test, respectively. These results align with previous studies that have demonstrated this specific SIT protocol (multiple sets of 30s all-out sprints, separated by 4 minutes recovery) can enhance measures of exercise and performance capacity (e.g. average Wingate power outputs, cycling time-trial performance(s)) (Hazell et al., 2010; Little et al., 2010) and physiological indices of aerobic capacity (e.g. VO_{2max} and skeletal muscle maximal citrate synthase activity) over short-term intervention periods (i.e. 6 training sessions) (Burgomaster et al., 2005; Hazell et al., 2010; Little et al., 2010).

After establishing the sufficiency of 9 sessions of SIT to increase performance, it was then investigated whether there was a coinciding change in the resting serum metabolome was evident. An unsupervised dimension reduction technique adjusted for paired samples (multilevel PCA) was applied to the metabolomic profile of resting serum samples collected before the beginning of the SIT intervention and 48-72 hours after the final performance test. PC1 and PC2 explained a low proportion of variance (14% and 11% respectively) and no apparent separation of baseline and post-intervention metabolomes was discernible at the group level (Figure 3.2). This implies the resting serum metabolome as measured by this targeted analysis as a whole was not altered by 9 sessions of SIT. The only other study to date of the effects of an exercise training intervention on the resting serum metabolome was almost three-times longer than the current study (24 exercise sessions, compared to 9) (Pechlivanis et al., 2013). In that study, participants were exposed to repeated 80 m running sprints, three times

per week for 8 weeks with significant separation observed between the baseline and post-intervention resting serum metabolome profiles using PLSDA. Separation between groups was observed to primarily be driven by metabolites classified as amino acids, monocarboxylates and some lipids (Pechlivanis et al., 2013). However, in addition to being a longer training intervention, this study also used ^{1}H-NMR to generate metabolomic profiles. ^{1}H-NMR is less sensitive than LC-MS and biases towards metabolites that are present in higher abundances (Gika et al., 2019). Therefore, while ^{1}H-NMR detects fewer metabolites compared to LC-MS, metabolites that are detected display lower variability, and larger mean differences in the concentration of metabolites may be more readily detected using this technique (Emwas, 2015).

Two other studies using cross-sectional designs have investigated the effect of exercise training on the resting serum metabolome using PLSDA on metabolites identified via LC-MS, and have demonstrated differences in the profile of the resting circulating metabolome that may be related to exercise training history and ability levels (Monnerat et al., 2020; Schranner et al., 2021). Firstly, in trained middle distance runners (Monnerat et al., 2020) group separation in the profile of the plasma metabolome between athletes with a low (61 $mL{\cdot}kg^{-1}{\cdot}min^{-1}$) or high (76 $mL{\cdot}kg^{-1}{\cdot}min^{-1}$) VO_{2max} at rest, was driven primarily by metabolites associated with alpha linolenic-acid metabolism, glutathione metabolism and carnitine metabolism (Monnerat et al., 2020). In another study by Schranner and colleagues, group separations in the resting serum metabolome of athletes from different sports and training backgrounds (sprinters, bodybuilders, endurance athletes and sedentary controls) was observed to primarily be driven by metabolites associated with amino acid metabolism, various phospholipids and metabolites associated with fatty acid oxidation. Additionally, distinct metabolites or metabolite sum/ratios that were influential to PC1 and PC2 the PLSDA model produced in this study were also found to be in altered abundance between groups; for example – compared to sedentary and endurance participants, bodybuilders had lower concentration of metabolites associated with

amino acid metabolism (e.g. Isoleucine, Leucine, the sum of BCAAs). While markers of ß-oxidation (e.g. CPT-1 ratio and the Kyneurine/Tryptophan ratio) were significantly higher in endurance athletes (compared to bodybuilders and controls) (Schranner et al., 2021). However, cross-sectional studies are only capable of capturing "snapshots" of parameters that can be used to classify an individual's training status (e.g. VO_{2max}) (Darragh et al., 2021; Jeukendrup et al., 2000; McKay et al., 2022). Therefore, while cross-sectional studies can associate changes in the circulating metabolome at rest with a history of exercise training, these designs cannot directly quantify the isolated contribution of exercise training to parameters in resting blood samples at the time of sampling (Darragh et al., 2021). Cross-sectional studies investigating the resting blood metabolome may also be confounded by the contribution of additional factors that can coincide with a high level of exercise training, but are not directly induced by exercise adaptation itself e.g. dietary habits (Burke et al., 1991).

Next, pairwise testing was performed to establish the effect of SIT on the abundance of individual metabolites. When pairwise testing was performed on the full set of identified metabolites within each of the five metabolite subgroups (Table S3.4) a total of 11 metabolites were identified to have lower concentrations (FDR <0.05) after SIT intervention, all of which belonged to the Fatty Acids & Conjugates group (Figure 3.3. Oleic acid, cis-8, 11, 14 Eicosatrienoic acid, Alpha linolenic acid, Mystiric acid, and Margaric acid, Gamma linolenic acid, Linoleic acid, Stearic acid, Pentadecylic acid, Palmitic acid and Palmitoleic acid). Pathway analysis of these 11 fatty acids identified one significant pathway: biosynthesis of unsaturated fatty acids, which could suggest that SIT induced an alteration of this metabolic process that subsequently became evident in the resting serum metabolome. Additionally, in Rats, aerobic training (90 min per day treadmill running, 6x per week 11 week) (Scorpio et al., 1984) or 8 weeks of ab libitum wheel running (Petridou et al., 2005) has been reported to induce reduced rates of fatty acid biosynthesis in hepatic (Petridou et al., 2005; Scorpio et al., 1984) ,

skeletal muscle and adipose tissues (Petridou et al., 2005). Lower hepatic rates of appearance of some of our identified fatty acids (palmitic acid) have been reported in response to 18 sessions of continuous exercise training (20 minute ergometer cycling sessions at power outputs estimated to elicit VO_2 responses of 60-80% of VO_{2max}) in sedentary men (Shojaee-Moradie et al., 2007). However, these studies involve intervention durations of eight (Petridou et al., 2005), eleven (Scorpio et al., 1984) and six (Shojaee-Moradie et al., 2007) weeks, respectively. To our knowledge, no study to date has investigated whether these processes are attenuated in response to short-term exercise training. However, exercise training can also increase the content of transport proteins involved in the metabolism of medium/long chain fatty acids such as CD36 and fatty-acid binding protein (FABP) on both sarcolemmal (Perry et al., 2008) and mitochondrial (Perry et al., 2008; Talanian et al., 2010) membranes by approximately 16-20% in human skeletal muscle. This could potentially increase the capacity for the oxidation of medium/long chain fatty acids at rest, which is an alternative suggestion as to why decreased concentration of certain fatty acids at rest were observed in the current study. Previously no change has been reported in the content of either CD36 or FABP in response to six-sessions of SIT (Burgomaster et al., 2007) or in the activity of 3-hydroxyacyl-CoA dehydrogenase (βHAD, a proxy of total β-oxidation capacity) (Burgomaster et al., 2005). However, increases in these markers of fatty acid transport and oxidation have been reported in response to short-term training interventions employing other modes of exercise (namely; High intensity interval training consisting of 7 sessions of 10 x 4 minute cycling intervals performed at 90% of VO_{2peak} performed every other day for 14 days) (Talanian et al., 2010). Additionally, 15-20% increases in skeletal muscle βHAD activity were previously reported to have occurred in response to the 9 session SIT intervention that comprises the current study (Aird et al., 2021). Therefore, while the current study produced an ontological suggestion that short term SIT is associated with a reduced biosynthesis of fatty acids, it is unclear whether

this association was influenced by a decreased synthesis of fatty acids *per* se or an increased oxidation of medium/long chain fatty acids at rest. Monounsaturated fatty acids have previously been suggested to decrease in response to exercise training, although the concentration of polyunsaturated fatty acids may increase (Nikolaidis and Mougios, 2004). The results of the current study partially agrees with these observations, given that the metabolite with the largest fold change decrease was Oleic acid (27%), which is the most abundant circulating monounsaturated fatty acid (Mashek and Wu, 2015). Although contrary to this, the current study also observed significant decreases in several polyunsaturated fatty-acids (e.g. Linoleic acid, Gamma linoleic acid, cis-8-11-4 Eicosatrienoic acid). Primary myotubes extracted from healthy men (40-62 years old) post 12 weeks of combined strength and endurance training (interval cycling training and whole-body resistance exercise) have been shown to increase Oleic acid uptake (~30%) and oxidation (~46%) (Lund et al., 2017) and 4 weeks of single leg extensor exercise training (60-120 minutes) has been shown to increase Oleic acid incorporation to skeletal muscle membrane phospholipids (~10%) also in healthy males (Helge et al., 2001). Together, these data suggest an altered "profile" of fatty acid oxidation as a result exercise training that may potentially result in lower circulating concentrations of select fatty acids.

3.6. LIMITATIONS

The current study is limited by our use of a targeted approach for the identification of 168 (160 identified above the limit of detection) metabolites and 7 added metabolite sum ratios (167 analysed variables total). This approach identifies only a subfraction of the global serum metabolome, which has in some estimates been speculated to contain upwards of several thousand metabolites (Psychogios et al., 2011). Additionally, the current study may limited by the number of exercise training sessions employed, it is possible that larger changes in the resting serum metabolome may have been detected by an intervention of a similar length (3

weeks) – but containing a greater number of training sessions (i.e. > 9 training sessions). The current study may also be considered limited by the absence of a control group, whose inclusion would have enabled us to identify a causal relationship between the reduced concentrations of fatty acids identified via univariate analysis and short-term SIT. The current study is potentially limited by standardisation through the use of food diaries and dietary recall rather than prescribed nutrition pre and post-intervention.

3.7. CONCLUSION

In conclusion, despite improving the anaerobic and aerobic capacity of recreationally-active males, SIT did not induce changes in the serum metabolome at rest when analysed by PCA. However, univariate analysis did reveal decreases in the concentrations of some fatty acids in response to SIT. Therefore, changes in this metabolite sub-fraction could potentially represent an early shift in the resting serum metabolome. Further research regarding the influence of short-term exercise training interventions on physiological adaptations combined with metabolomic profiling are required to determine whether changes in circulating metabolites are reflective of an early adaptation to exercise training.

Chapter 4

Within-Subject Variability and the Influence of Exercise Training History on the Resting Plasma Metabolome in Men

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The full text version of this article is available as **Appendix C**

Prior to reading this chapter please note: All supplementary materials referred to in this chapter and the raw metabolomics (count) data analysed are available for secure download from the open science framework at the following link: https://osf.io/v8w6y/

4.1. ABSTRACT

This study investigated within-subject variability in the circulating metabolome under controlled conditions, and whether divergent exercise training backgrounds were associated with alterations in the circulating metabolome in resting samples. Thirty-seven men comprising of endurance athletes (END; body mass, 71.0±6.8 kg; fat-free mass index, 16.9±1.1 kg/m^2), strength athletes (STR; 94.5±8.8 kg; 23.0±1.8 kg/m^2), and recreationally active controls (CON; 77.6±7.7 kg; 18.1±1.0 kg/m^2) provided blood samples after an overnight fast on two separate occasions. A targeted profile of 166 individual metabolites and metabolite features was performed using liquid chromatography and mass spectrometry on plasma samples, and analysed with intraclass correlation coefficients (ICC), a multilevel principal component analysis, and univariate t-tests adjusted for multiple comparisons. The median ICC was 0.49, with 46 metabolites displaying *good* reliability, and 31 metabolites displaying *excellent* reliability. No difference in the abundance of any individual metabolite was identified within groups when compared between visits, but a combined total of 44 metabolites were significantly different (false discovery rate <0.05) between groups (END vs. CON, 42 metabolites; STR vs. CON, 10 metabolites; END vs. STR, 5 metabolites). Under similar measurement conditions, the reliability of resting plasma metabolite concentrations varies largely at the level of individual metabolites with ~48% of metabolites displaying *good*-to-*excellent* reliability. A history of exercise training was associated with alterations in the abundance of ~28% of metabolites in the targeted profile employed in this study.

4.2. INTRODUCTION

Exercise training elicits a broad range of physiological adaptations in many tissues and organ systems (Egan and Zierath, 2013; Hawley et al., 2014). When a specific type of exercise

training is emphasised, divergent exercise training phenotypes can manifest (e.g. endurance-trained or strength-trained athletes) (Coffey and Hawley, 2017; Egan and Zierath, 2013), which include specific anthropometric and performance traits that can differ markedly in different athletic populations (Degens, 2019).

Metabolites are low molecular weight (mostly organic) chemicals that are usually the reactants, intermediates, or products of metabolic pathways (Dunn et al., 2011; Nicholson and Lindon, 2008). The metabolome represents the collective output of metabolic reactions, and is arguably the most accurate representation of the phenotype of a sample at the time of measurement (Belhaj et al., 2021; Patti et al., 2012). The circulating metabolome is of interest as it represents metabolite contributions from all tissues, and is an integrated snapshot of systemic metabolism (Dunn et al., 2011). Considering the extent and diversity with which exercise training can induce physiological remodelling, there is emerging interest in whether exercise training history or exercise training interventions alter the profile of the circulating metabolome (Khoramipour et al., 2022). Profiling of the circulating metabolome in a resting state using a cross-sectional design between groups with divergent histories of exercise training can infer the influence of exercise training while attenuating the confounding residual effects of recent exercise training sessions (Darragh et al., 2021).

To date, two cross-sectional studies have investigated the relationship between exercise training and the circulating metabolome at rest (Monnerat et al., 2020; Schranner et al., 2021), both of which suggest alterations associated with divergent exercise training history and/or performance characteristics. However, both studies comprised of only small sample sizes and the extent of control of dietary intake and the last exercise training session was unclear. Moreover, the studies acquired samples at only a single time-point. When interpretating the durability of alterations to the circulating metabolome related to exercise training, these methodological issues are salient given the dynamic nature of the circulating metabolome and

the evident within-subject variability in human samples (Agueusop et al., 2020; Breier et al., 2014; Floegel et al., 2011; Yin et al., 2022).

Therefore, using a targeted profile of metabolites measured in plasma samples taken at rest, the present study firstly investigated the within-subject variability in the circulating metabolome while controlling for time of day of sampling, recent dietary intake, time since last meal, and time since last exercise training session. Secondly, we investigated whether exercise training history was associated with alterations in the circulating metabolome by comparing samples from recreationally-active controls and two groups of exercise-trained individuals with divergent training histories and performance characteristics.

4.3. METHODS

4.3.1. Participants

Men (n=38) who were endurance-trained (END; *n* = 13), strength-trained (STR; *n* = 13), and recreationally active controls (CON; *n* = 12) were recruited for this study (Table 1 & Supplementary Table S4.1). To qualify for the respective training group, participants self-reported being able to meet the following criteria: END, at least two of the following running performances, 5000 m <20 min, 10000 m <40 min or 16000 m <64 min; and STR, a one repetition maximum for at least two of the following, squat ≥200 kg, bench press ≥140 kg, deadlift ≥220 kg. Three recent performances were recorded (Supplementary Table S4.1), and these were verified via social media accounts (e.g. Instagram), training logs (e.g. Garmin Connect, Strava) and/or public databases (e.g. www.worldathletics.org, www.openpowerlifting.org). Based on the recently-proposed Participant Classification Framework (McKay et al., 2022), END was comprised of n=8 Tier 3/Highly-trained athletes and n=5 Tier 4/Elite athletes, whereas STR consisted of n=9 Tier 3/Highly-trained athletes and n=4 Tier 4/Elite athletes. The CON participants were Tier 1/Recreationally Active, but did not participate in intensive or sport-specific training.

4.3.2. Study design

This study received ethical approval from the Research Ethics Committee of Dublin City University (DCUREC/2021/079) in accordance with the Declaration of Helsinki, and each participant provided written informed consent prior to participation. Participants arrived at the laboratory on two separate occasions, during each of these visits participants were in an overnight fasted state and had not exercised for at least 24 h beforehand (Table 4.1). Height was measured using a stadiometer (model 213, SECA, Germany), and body mass and body composition (fat mass, %body fat and fat-free mass) were assessed by bioimpedance spectroscopy (SOZO®, ImpediMed, Australia) (Esco et al., 2019). Participants lay supine for 10 min while answering questions to verbally confirm compliance with all pre-visit preparations including the timing of their most recent meal and exercise training session. A venous blood sample was then taken from a superficial forearm vein.

4.3.3. Dietary control

Participants were provided with standardised meals (GourmetFuel™, Dublin, Ireland) for consumption on the day prior to each visit, which were delivered by a member of the research team. The meals provided 30 kcal/kg body mass with a macronutrient ratio of 50/25/25 for carbohydrate, protein and fat, respectively. Participants were permitted to consume caffeine during the day before each visit in doses that contained negligible additional nutrients (i.e. tea or coffee without milk), but were asked to consume only water on the morning of each visit. Participants were also asked to abstain from alcohol consumption in the 24 h preceding each visit.

4.3.4. Blood sampling, processing, and storage

Whole venous blood was drawn by inserting a 21G butterfly needle (Greiner, Bio-One, Austria) into an antecubital vein. The initial 4 mL of blood was drawn into a generic vacutainer and discarded. Subsequently ~50 mL of whole blood was drawn into six 9 mL blood collection tubes coated with ACD-A anticoagulant (Greiner, Bio-One, Austria). Blood samples were immediately placed on ice and centrifuged at 1500*g* for 15 min at 4ºC. Directly after centrifugation, plasma samples were separated into aliquots, and stored at -80ºC. Samples were collected and stored between August and December 2021, and were analysed in batch in May 2022.

4.3.5. Plasma metabolomics

Plasma metabolomics analyses were performed by TMIC (Calgary, Canada) using a targeted assay capable of detecting up to 172 metabolites and employing LC-MS. Further details of this method are detailed in Supplementary Methods, and have been described elsewhere (Zheng et al., 2020). One hundred and fifty-nine (159) metabolites were detected by LC-MS and classified into five distinct metabolite groups (Amino Acids, Peptides and Analogues; Fatty Acids and Fatty Acid Conjugates; Acylcarnitines; Glycerophosphocholines & Phosphosphingolipids; and Other) using the "ChemOnt" taxonomic classification technique (Feunang et al., 2016) (Supplementary Table S4.2). In addition, four metabolite sums (branched chain amino acids, gluconeogenic amino acids, essential amino acids, total acylcarnitine) and three metabolite ratios (acylcarnitine/carnitine, C2/C0, and kynurenine/tryptophan) were calculated (Petersen et al., 2012), resulting in a total of 166 metabolite features included in the final analysis.

4.3.6. Principal Component Analysis

Prior to dimension reduction a Kaiser-Mayer-Olkin test of sampling adequacy and Bartlett's test of sphericity were performed on data to ensure they met the minimum standards

for PCA. Bartlett's test of sphericity produced a significant result ($X^2(165)=103410$; $P<0.01$), while a KMO test of mean sampling adequacy produced a result of 0.5, which is a low, but acceptable value (Kaiser and Rice, 1974). A multilevel PCA using an orthogonal rotation with features mean zeroed and scaled for unit variance was then performed on the full set of 166 metabolites, metabolite sums and ratios using mixOmics (version 6.14.1), a package designed for use within the statistical programming language R (Rohart et al., 2017). Multilevel PCA refers to a modified analysis applicable to repeated-measures design that involves decomposition of within-subject variation prior to dimension reduction. This approach has been demonstrated to increase model accuracy for paired-sample data (Liquet et al., 2012).

4.3.7. Statistical analysis and calculation of Intraclass Correlation Coefficients

Date are reported as mean ± SD unless otherwise stated. All analyses were performed using the base version of the programming language R (4.2.1) or the 'Rstatix' package (0.7.0). For variables in which a difference in outcomes between visits was possibly expected (body composition, timing of each sample, time since last meal and exercise session), data were analysed using a two-way (Visit * Group) mixed ANOVA. No interaction effect was observed for any variable, but given our *a priori* interest in between-group differences, in the presence of a significant main effect for Group, *post-hoc* pairwise comparisons were performed using the Bonferroni correction. In circumstances where Visit was not a relevant independent variable (e.g. height, age, number of days between visit 1 and visit 2), data were analysed using a one-way ANOVA with *post-hoc* pairwise comparisons again performed using the Bonferroni correction. All ANOVAs are reported with a relevant estimate of standardized effect size (η_p^2).

Univariate testing of metabolite data was performed using paired t-tests with the Benjamini-Hochberg method to control for the FDR of multiple comparisons. This approach was first applied to the full set of identified metabolite data (166 features) within each group. When no significant differences were observed between visits for each group, Visit 1 and Visit

2 data were averaged for participants in each group, and the same series of pairwise tests were performed to compare groups (i.e. END vs. CON; STR vs, END; END vs. STR). ICC for each individual metabolite was calculated using the ICC2 method (Shrout and Fleiss, 1979). The reliability of circulating metabolites was interpreted using arbitrary thresholds (<0.4, '*poor*'; 0.4 to 0.5, '*fair*'; >0.5 to 0.75, '*good*'; and >0.75 '*excellent*') that were established for analysis of psychometric test scores (Cicchetti, 1994), but have been employed to describe the reliability of circulating metabolites (Agueusop et al., 2020; Floegel et al., 2011; Li-Gao et al., 2019). All statistical tests rejected the null hypothesis at an alpha level of <0.05, or an FDR <0.05 for univariate tests.

4.4. RESULTS

4.4.1. Reliability of metabolite concentrations between visits

The majority of the 159 detected metabolites displayed *fair* or better reliability between visits as evidenced by 98 metabolites (~62%) having ICCs ≥0.4, with 46 metabolites (~29%) displaying *good* reliability (ICCs >0.5), and 31 metabolites (~19%) displaying *excellent* reliability (ICCs >0.75) (Figure 4.1A; Supplementary Table S4.3). ICCs varied between metabolite groups with Glycerophosphocholines & Phosphosphingolipids displaying the greatest reliability (83% of metabolites with an ICC >0.5) and Acylcarnitines displaying the lowest reliability (30% of metabolites with an ICC >0.5) (Figure 4.1B).

Table 4.1. Participant characteristics and details of control measures for visits 1 and 2

Variable	END (n = 13)	STR (n = 13)	CON (n = 12)	Interaction F	Interaction p	Interaction η_p^2	Group F	Group p	Group η_p^2	Visit F	Visit p	Visit η_p^2
Age (years)	30 ± 6	25 ± 5	26 ± 2				3.285	0.05	0.166			
Height (m)	1.82 ± 0.06	1.80 ± 0.07	1.78 ± 0.07				0.876	0.426	0.052			
Body mass (kg)	71 ± 7 ##	94 ± 9	78 ± 8 ##	1.634	0.211	0.093	27.259	<0.001	0.63	0.124	0.727	0.004
FFM Index (kg/m^2)	17 ± 1 ##	23 ± 2	18 ± 1 $##	2.149	0.133	0.118	65.943	<0.001	0.805	0.671	0.419	0.021
Recent 5 km time (min:s)	16:30 ± 1:22											
Recent bench press (kg)		148 ± 30										
Recent back squat (kg)		228 ± 30										
Recent deadlift (kg)		253 ± 38										
Time training per week (hours)	9 ± 10	9 ± 2	4 ± 1 $$##				51.992	<0.001	0.594			
Average time between visits (days)	12 ± 10	9 ± 6	9 ± 5				0.02	0.98	0.001			
Maximum time between visits (days)	42	26	21									
Minimum time between visits (days)	7	7	6									
Time since most recent meal visit 1 (hours)	12 ± 2	12 ± 2	12 ± 1	0.939	0.401	0.052	1.132	0.334	0.062	0.711	0.405	0.02
Time since most recent meal visit 2 (hours)	12 ± 1	12 ± 2	13 ± 1	0.939	0.401	0.052	1.132	0.334	0.062	0.711	0.405	0.02
Time since most recent exercise visit 1 (hours)	46 ± 23	66 ± 66	75 ± 84	1.945	0.159	0.103	0.974	0.388	0.054	0.376	0.544	0.052
Time since most recent exercise visit 2 (hours)	44 ± 12	42 ± 17	88 ± 132	1.945	0.159	0.103	0.974	0.388	0.054	0.376	0.544	0.052
Average time of sampling visit 1	08:32	09:16	08:44	1.559	0.226	0.089	1.322	0.281	0.076	3.672	0.064	0.103
Average time of sampling visit 2	08:17	09:18	08:32	1.559	0.226	0.089	1.322	0.281	0.076	3.672	0.064	0.103
Maximum difference between visit 1 and visit 2 time of sampling (minutes)	54	23	54	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Minimum difference between visit 1 and visit 2 time of sampling (minutes)	1	1	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Notes: # represents a significant difference from STR (# p <0.05, ## p <0.01); $ represents a significant difference from END ($ p < 0.05, $$ p < 0.01

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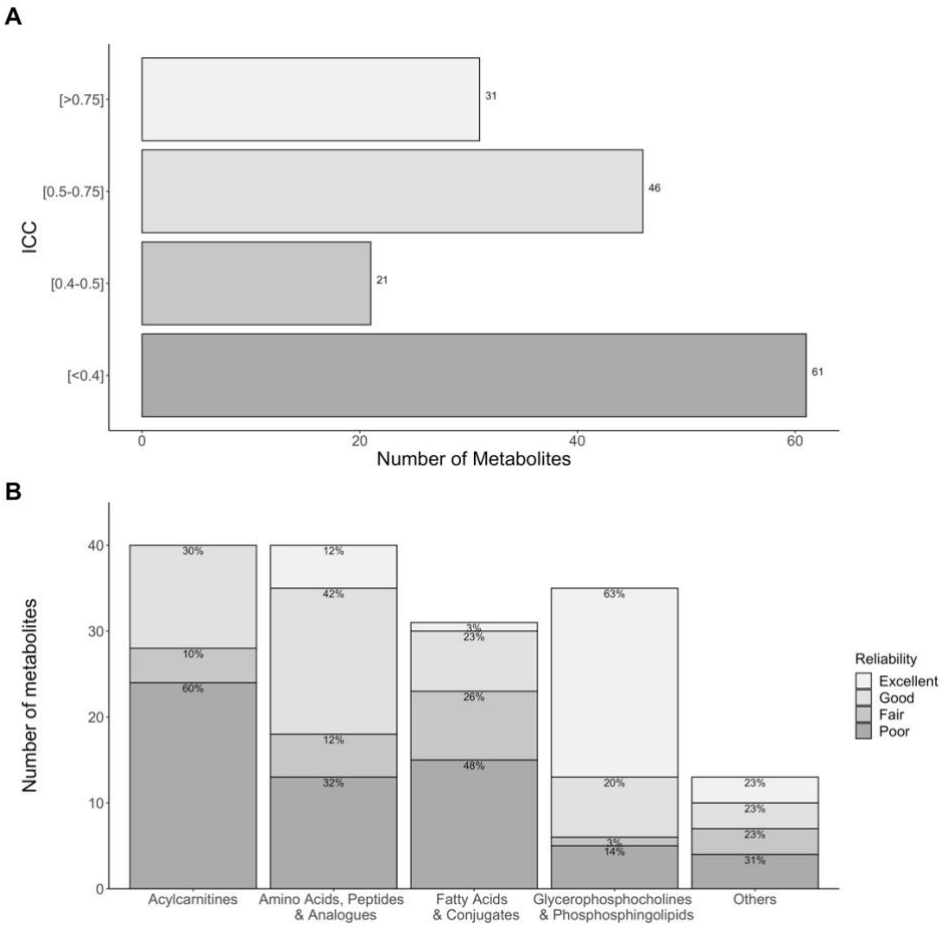


Figure 4.1. The intraclass correlation coefficients (ICC) of detected metabolites. **A.** This distribution of all metabolites by ICC interval. **B.** The number of metabolites in each group (y-axis) with the percentage of individual metabolites in each specific ICC interval

4.4.3. Within-subject and between group analyses

Multilevel PCA was performed to investigate whether clear spatial separations were apparent in within-subject, or between-groups analyses. This model produced no apparent clustering within groups or separation between groups, and different visits within participants tended to cluster closely implying the profile of a participant's metabolome was consistent between visits (Figure 4.2; Supplementary Table S4.4).

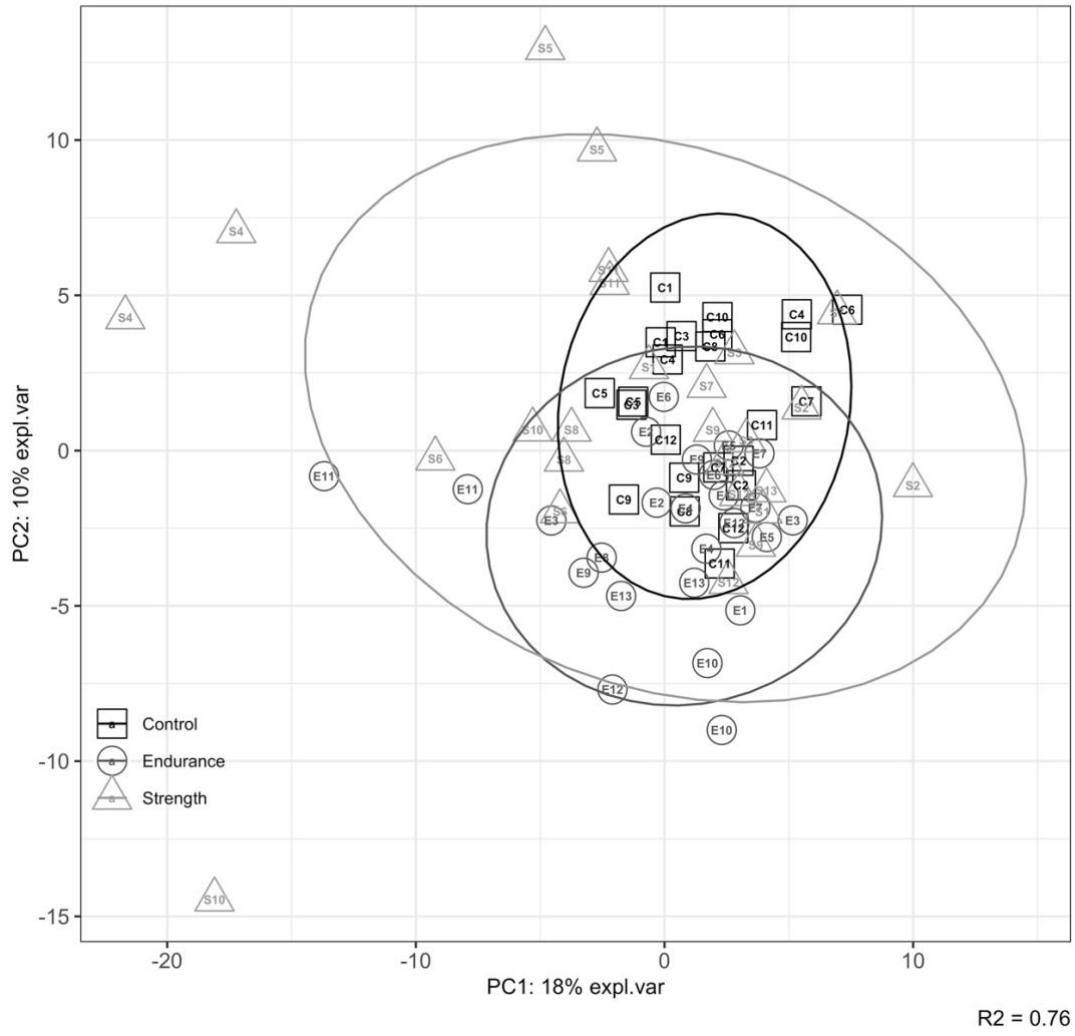


Figure 4.2. A multilevel principal component analysis (PCA) of the collapsed profile of targeted metabolomes from the participants for both visits; Circles represent Endurance athletes, triangles represent Strength athletes, and squares represent Control participants. PC1/PC2 refers to the first and second principal components i.e. latent variable structures that explain the largest and second largest unique proportion of total model variance, respectively.

The results of univariate within-subject analyses demonstrated that no metabolites displayed significantly different abundances between visits within any group (Figure 4.3). Univariate tests comparing the resting concentration of each metabolite between groups (END vs. CON; STR vs, CON; END vs. STR) revealed a combined total of 44 metabolites with differences between groups (FDR <0.05; Figure 4.4; Supplementary Table S4.5). Boxplots for each of these 44 metabolites are presented in Supplementary Figure 4.1. The largest number of differentially-abundant metabolites (42) was between END and CON, with 16 metabolites demonstrating lower abundance and 26 metabolites demonstrating higher abundance in END (Figure 4.4A). Of the 16 metabolites with lower abundance, 4 metabolites were in the Acylcarnitine group, 4 metabolites were in the Amino Acid, Peptide and Analogue group, 6 metabolites were in the Fatty Acid and Fatty Acid Conjugate group, 1 metabolite was in the Glycerophosphocholines & Phosphosphingolipids, and 1 metabolite was in the Others groups (Supplementary Table S4.5). Of the 26 metabolites with higher abundance, 6 metabolites were in the Acylcarnitine group, 8 metabolites were in the Fatty Acid and Fatty Acid Conjugate group, and 12 metabolites were in the Glycerophosphocholines & Phosphosphingolipids group (Supplementary Table S4.5).

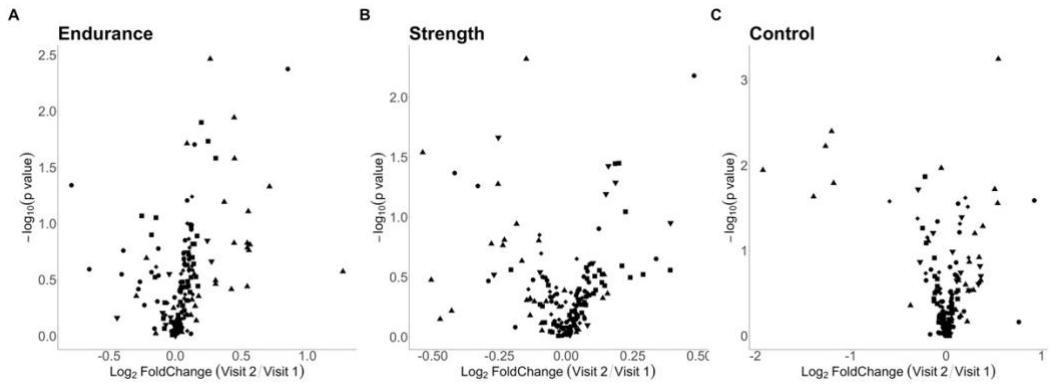


Figure 4.3. Univariate analysis of metabolites within each group. **A.** Endurance athletes, **B.** Strength athletes, **C.** Recreationally-active Controls. Shapes represent metabolites in specific metabolite groups with the following coding; squares = Acylcarnitines; circles = Amino Acids, Peptides & Analogues; triangles = Fatty Acids & Conjugates; diamond = Glycerophosphocholines & Phosphosphingolipids; inverted triangle = Others.

Comparing STR to CON (Figure 4.4B), 10 metabolites were differentially-abundant, with 5 metabolites demonstrating higher abundance (4 metabolites in the Fatty Acid and Fatty Acid Conjugate group, and 1 metabolite in the Others group), and 5 metabolites displaying lower abundance (all in the Fatty Acid and Fatty Acid Conjugate group) in STR (Supplementary Table S4.5). Comparing END to STR (Figure 4.4C), 5 metabolites were differentially-abundant with 2 metabolites displaying lower abundance (1 in the Amino Acid, Peptide and Analogue group, and 1 in the Others group) and 3 metabolites displaying higher abundance (all in the Glycerophosphocholines & Phosphosphingolipids group) in END (Supplementary Table S4.5).

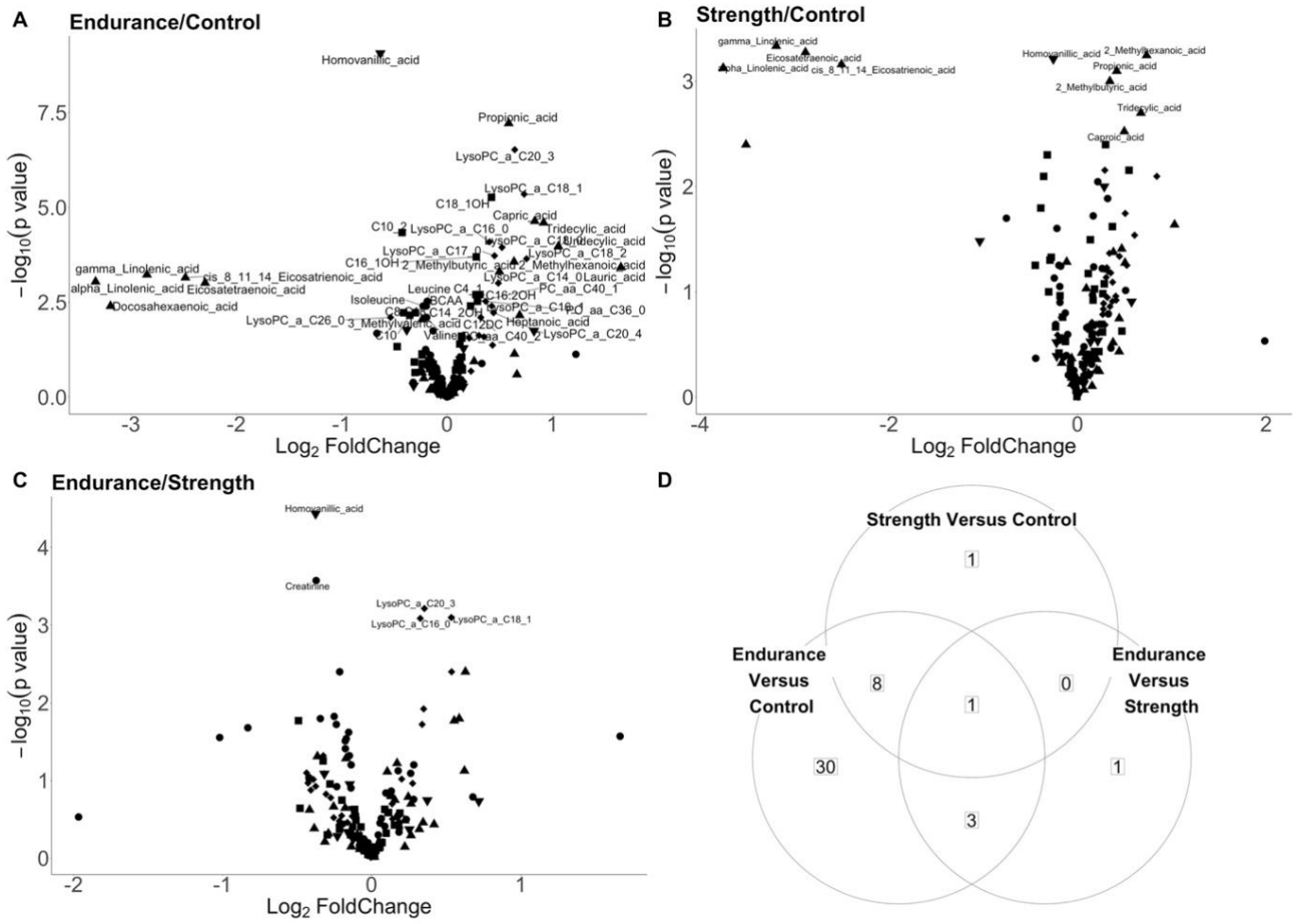


Figure 4.4. Univariate analysis of metabolites between each groups. **A.** Endurance athletes versus Control, **B.** Strength athletes versus Control, **C.** Endurance athletes versus Strength athletes. Annotated metabolites are differentially-abundant (FDR < 0.05). **D.** A Venn diagram showing the overlap of differentially expressed metabolites between individual group analysis. Shapes represent metabolites in specific metabolite groups with the following coding; squares = Acylcarnitines; circles = Amino Acids, Peptides & Analogues; triangles = Fatty Acids & Conjugates; diamond = Glycerophosphocholines & Phosphosphingolipids; inverted triangle = Others.

4.5. DISCUSSION

The present study employed a targeted profile of the circulating metabolome in resting plasma samples, firstly to investigate reliability when measured on two separate days. Under controlled conditions, namely time of day of sampling, recent dietary intake, time since last meal, and time since last exercise training session, the reliability of plasma metabolite concentrations varied largely at the level of individual metabolites. Specifically, ~48% of metabolites displaying *good*-to-*excellent* reliability in resting samples, whereas the remaining ~52% of metabolites displayed *fair*-to-*poor* reliability. Secondly, investigating whether divergent histories of exercise training were associated with alterations in the circulating metabolome revealed that the abundance of ~28% (44/159) of the metabolites detected in the targeted metabolite profile were altered in plasma between END or STR participants compared to CON.

Using ICC as an assessment of reliability, the median ICC across metabolites was 0.49, and ~62% of metabolites displayed an ICC ≥0.4. These data suggest that the majority of metabolites displayed *fair* or better reliability between visits, with ~48% displaying *good* to *excellent* reliability. However, the median ICC observed in the present study is lower than previously reported in other studies in blood samples taken from overnight fasted humans on multiple days, including both untargeted (median ICC of 0.65 for 1438 metabolites identified in serum, Ageusop et al., 2020; median ICC of 0.66 for 148 metabolites identified in plasma, Li-Gao et al., 2019), and targeted (median ICC of 0.57 for 163 metabolites identified in serum,

Floegel et al., 2011; median ICC of 0.62 for 138 metabolites identified in plasma, Yin et al., 2022,).

When analysed by metabolite group in the present study, the Glycerophosphocholines & Phosphosphingolipids group displayed the greatest reliability (median ICC of 0.78, with 63% of metabolites displaying ICCs >0.75, and only 14% displaying a ICCs <0.4), and the Acylcarnitine group displaying the poorest reliability (median ICC of 0.28, with 60% of metabolites displaying ICCs <0.4 and no metabolites displaying an ICC >0.75). In similar studies, phosphocholines and sphingolipids have likewise displayed *good*-to-*excellent* reliability (median ICCs ~0.6 to ~0.8) (Agueusop et al., 2020; Floegel et al., 2011; Yin et al., 2022), but our observation of *poor* reliability of the Acylcarnitine group is in contrast to previous studies in overnight fasted humans (median ICCs ~0.6 to ~0.9) (Agueusop et al., 2020; Breier et al., 2014; Floegel et al., 2011; Yin et al., 2022). The reliability of Acylcarnitines may vary in a manner that is dependent on the length of the Acylcarnitine chain with short-medium Acylcarnitines displaying higher ICCs compared to longer chains (Breier et al., 2014; Floegel et al., 2011). A difference may also exist between plasma and serum as evidenced by Acylcarnitines identified in serum having higher ICCs than those in plasma (Breier et al., 2014). The present study analysed a mixed panel of 42 Acylcarnitines ranging from C1 to C18, and measured metabolite concentrations in plasma, not serum, and at the level of specific Acylcarnitines, some of the reliability data are similar to other studies (Floegel et al., 2011; Yin et al., 2022). For example, C4, which we observed to have an ICC of 0.70, has previously been reported to have *excellent* reliability with an ICC of 0.79 in plasma (Yin et al., 2022), and 0.81 in serum (Floegel et al., 2011). Possible explanations for the lower reliability observed in the Acylcarnitines group in the present study may be related to the collection of plasma rather than serum, and to using a general classification of Acylcarnitines rather than classifying Acylcarnitines into separate categories of short or long, respectively. Overall these findings

suggest, in agreement with others (Agueusop et al., 2020; Breier et al., 2014; Floegel et al., 2011), that the reliability of circulating metabolite concentrations can vary considerably within metabolite groups, even when measured under controlled conditions. Establishing the reliability of individual metabolites and specific metabolite groups is important within analyses that aim to investigate differences between groups or time points by providing an indication of whether potential differences in abundance are likely to be robust.

For the analysis of between-group differences, multilevel PCA revealed no separation between participant groups, implying that the decomposed metabolomic profile was not different between groups. However, both visits from individual participants tended to cluster closely together, suggesting that the metabolomes from individual participants projected similarly between each visit, which agrees with the reliability data described above. The observation of no clear separation between groups using a multivariate method is in contrast to that reported by two other cross-sectional studies investigating the influence of exercise training history and performance characteristics on the circulating metabolome (Monnerat et al., 2020; Schranner et al., 2021). The first study compared elite long distance runners with "low" (<65 $mL \cdot kg^{-1} \cdot min^{-1}$; n=7) or 'high" (>75 $mL \cdot kg^{-1} \cdot min^{-1}$; n=7) values for maximal oxygen consumption (Monnerat et al., 2020), whereas the second study compared a control group (n=4) and endurance (n=6), sprint (n=5), and bodybuilding (n=4) athletes (Schranner et al., 2021). While both studies report clear separations between their athlete groups (Monnerat et al., 2020; Schranner et al., 2021), both studies also employed a PLSDA modelling approach in contrast to the PCA approach employed in the present study. In contrast to PCA, PLSDA is a 'supervised' method, wherein the model algorithm attempts to produce linear components that maximise the separation between pre-defined class structures (e.g. exercise training groups) (Ruiz-Perez et al., 2020). This technical difference between methods is important because it means that PLSDA models will produce inter-class separation, even in circumstances

where no true class structure exists in the data (Ruiz-Perez et al., 2020). Therefore, the utility of a PLSDA model is based on a quality assessment of the model parameters (Szymańska et al., 2012). While neither model from the previous exercise training studies are reported as producing model overfitting (i.e. the training model over predicting the cumulative variance produced by the full dataset) (Monnerat et al., 2020; Schranner et al., 2021), both models did have evidence of underfitting (Q^2 values greater than R^2 of half of the full datasets across multiple permutations), which implies poor predictive power. In addition, the model produced by Schranner and colleagues explained a low cumulative variance (R^2=0.5) (Schranner et al., 2021). Therefore, the contrasting results between the present study and these prior studies (Monnerat et al., 2020; Schranner et al., 2021) in terms of broad between-group differences may largely be due to differences in the selection of model algorithm(s).

When pairwise univariate testing was performed on our data, no individual metabolite differed between visits within each group, again implying that metabolites detected in the targeted metabolite panel were consistent between visits. However, univariate comparisons between groups, i.e. END vs. CON; STR vs CON; END vs. STR, identified a combined total of 44 metabolites with differential abundance between groups. The largest number of metabolites with differential abundance was between END and CON (42 metabolites), with a smaller number different between STR and CON (10 metabolites), and END and STR (5 metabolites). The mechanisms that explain these between-group differences remain to be elucidated, and discussion at present is speculative. For example, increased abundance of numerous lysophospholipids was observed between END and both other groups (10 lysphospholipids higher vs. CON; 3 lysophosholipids higher vs. STR). In plasma, lysophosopholipids can reflect the activity of lecithin-cholesterol acyltransferase (LCAT), an enzyme that is abundant in plasma and serves to facilitate the transport of cholesterol esters between high density lipoprotein (HDL) particles and hepatic tissue (Glomset, 1968; Tan et al.,

2020). Aerobic exercise training can increase the presence of circulating HDL (Kodama et al., 2007). Additionally, cross-sectional studies involving athletes with endurance training backgrounds have noted increased LCAT activity at rest (Gupta et al., 1993; Tsopanakis et al., 1988) and this activity is significant in the context of reverse cholesterol transport (Leaf, 2003). Therefore, one speculation would be that the increased abundance of lysophospholipids observed in END may indeed be related to this adaptation to aerobic exercise training.

Other notable results include the observation that of all metabolites identified to be differentially-abundant, nine (2-Methylbutyric acid; 2-Methylhexanonic acid; Alpha linolenic acid; cis,8,11,14 Eicosatrineoic acid; Gamma linolenic acid; Homovanllic acid; Propionic acid, Tridecyclic acid) were common to both athlete groups compared to CON in addition to these differences being directionally the same in both END and STR. Interestingly, one metabolite - Homovanillic acid, an endpoint of dopamine metabolism and marker of metabolic stress (Amin et al., 1992) - was also observed to be lower in END and STR compared to CON, but lower again in END vs. STR. Ultimately, elucidating the importance of these differentially-abundant metabolites will require further work, as would establishing whether these differences are definitively a consequence of exercise training given that a cross-sectional design can only establish association, rather than causation.

In athletes or exercise contexts the present study includes the largest sample size captured to date and is the first study to include reliability data for individual metabolites in an exercise context. This study is not without limitations, exercise training history and current performance status of participants was established largely through self-reported methods, and thus, objective measures of the physical fitness of participants (e.g. VO_{2max}) are lacking. Additionally, while our sample size is indeed the largest currently reported, some of our model parameters (e.g., the KMO score) are suggestive of us having low statistical power for multivariate modelling, a fact that may influence the results of the PCA. An important

limitation that precludes the broader applicability of the results is that the groups comprised of only male participants, which is important given the influence of endogenous and exogenous sex hormones on metabolism, and that the responses of several genetic, metabolic, and physiological parameters to exercise differs between males and females (Ansdell et al., 2020; D'Eon et al., 2002; Fu et al., 2009; Landen et al., 2019; Maher et al., 2010). Lastly, we employed a targeted metabolomic approach, and therefore our findings are restricted to only this subfraction of the circulating metabolome.

In conclusion, the present study found that when sampled under controlled measurement conditions, the resting plasma concentration of 166 metabolites and calculated metabolite sums and ratios did not differ overall between two visits, yet reliability was variable as evidenced by a large range in the average ICC within and between specific metabolite groups. Additionally, divergent histories of exercise training were associated with alterations in the circulating metabolome at rest, but future work will be required to determine the importance of these differences, and whether these differences are definitively a consequence of adaptations to exercise training.

Chapter 5
The Separation and Identification of Small EVs between men with Divergent Exercise Training Histories

Prior to reading this chapter please note: All supplementary materials referred to in this chapter available for secure download from the open science framework at the following link: https://osf.io/y5ras/

5.1. ABSTRACT

Small EVs are (<150nm) membrane encapsulated particles that carry bioactive cargoes, are released by all cell types and present in all human biofluids. EVs are shown to be responsive to acute exercise and are speculated to be carriers of so-called exercise factors. The current study sought to investigate whether CON, END and STR participants presented with an altered abundance of small EVs at rest and whether the abundance of small EVs was consistent within each group across two measurement days. 38 men (END n = 13, STR n = 13, CON n = 12) arrived to the lab on two separate occasions in a rested, overnight fasted state, having not exercised for ~40hrs and consuming identical 24hr nutrition. Whole plasma was collected and small EVs were separated using size exclusion chromatography and identified in accordance with the MISEV guidelines. No indication of any differences in the abundance of small EVs between groups or between visits within groups were detected across multiple methods of small EV identification (NTA, Amnis flow cytometry, Western blot of specific EV markers and contaminants). Targeted metabolomics of small EVs identified 96 distinct metabolites that were associated with the structure and function of small EVs and had consistent concentration(s) between groups. The results of the current study suggest that the abundance and metabolomic profile of small EVs derived from men with different exercise training histories are similar at rest.

5.2. INTRODUCTION

EVs are a family of membrane encapsulated vesicles that are secreted by all eukaryotic cell types and are present in all major biofluids. EVs are typified by their carrying of a variety of bioactive molecular cargo (e.g. nucleic acids, peptides, metabolites) that can be delivered to distally or proximally located recipient cells and exert bioactive effects (van Niel et al., 2018; Yáñez-Mó et al., 2015). EVs tend to exist in highly heterogenous populations that are operationally labelled based on size characteristics (i.e. small EVs – diameters ranging from 50-150nm, medium/large EVs diameters ranging from >150-1000nm) (Théry et al., 2018). This operational labelling is necessary, as there are currently no direct methods available for the isolation of EVs, nor is there uniform agreement of a 'gold standard' approach amongst common techniques employed to separate small EVs from popularly studied biofluids (e.g. plasma/serum, urine and cell-culture media) (Gardiner et al., 2016; Théry et al., 2018). Consequently, the extent to which small EVs can be isolated is highly variable and a rigorous multi-methods approach is necessary to confidently identify the presence of EVs in separated preparations (Théry et al., 2018).

Small EVs have become a particular topic of interest for exercise physiologists, with multiple reviews concerning interactions between small EVs and exercise published in recent years (Darragh et al., 2021; Denham and Spencer, 2020; Estébanez et al., 2020; Fuller et al., 2020; McIlvenna and Whitham, 2022; Nederveen et al., 2021; Safdar et al., 2016; Vechetti, 2019; Vechetti et al., 2020). This interest in small EVs was catalysed by an initial report that provided indication that small EVs may enrich in circulation in response to acute exercise (Frühbeis et al., 2015), an observation that stimulated speculation that small EVs may serve as a medium of transportation for circulating exercise factors (e.g. 'myokines' and/or 'exerkines') and could contribute to exercise metabolism and/or signalling for exercise adaptation through the delivery of bioactive cargo(es) (Safdar et al., 2016; Vechetti, 2019). To date, much of the

current research concerning small EVs has focused on acute exercise and it does indeed appear that small EVs enrich (in terms of small EV abundance with an additional shift in small EV 'cargo') during exercise (see Estébanez et al., 2020; Nederveen et al., 2021 for comprehensive reviews). However, a small number of studies have also focused on the influence of *exercise training* on the presence of small EVs (summarised in Nederveen et al., 2021) and some of these studies have reported that in response to exercise training, the abundance of circulating small EVs may also increase at rest (Barcellos et al., 2020; Bei et al., 2017; Chaturvedi et al., 2015; Estébanez et al., 2021; Gao et al., 2021; Ma et al., 2018). This observation has been (albeit tentatively) speculated as a novel response to exercise training (Ma et al., 2018; Nederveen et al., 2021). However, all of the studies that demonstrate an indication of increased presence of small EVs at rest in association with exercise training are limited either by employing single methods to identify the presence of circulating small EVs and/or by imposing poor control over the confounding influence of recent exercise bouts (i.e. the last bout of the exercise training program) (Nederveen et al., 2021). Given these limitations, the legitimacy of the idea that circulating small EVs may be increased at rest in association with exercise training warrants investigation.

The purpose of the current study was to identify whether the presence of circulating small EVs was altered in CON, END and STR participants at rest using orthogonal methods of small EV identification and characterisation while also controlling for confounding factors that may influence signals associated with small EV abundance (recent dietary intake and proximity to acute exercise).

5.3. METHODS

5.3.1. Participants and study design

Men (n=38) who were endurance-trained (END; *n* = 13), strength-trained (STR; *n* = 13), and recreationally active controls (CON; *n* = 12) were recruited for this study (Table 4.1). Based on the recently-proposed Participant Classification Framework (McKay et al., 2022), END was comprised of n=8 Tier 3/Highly-trained athletes and n=5 Tier 4/Elite athletes, whereas STR was comprised consisted of n=9 Tier 3/Highly-trained athletes and n=4 Tier 4/Elite athletes. The CON participants were Tier 1/Recreationally Active, but did not participate in intensive or sport-specific training. This study received ethical approval from the Research Ethics Committee of Dublin City University (DCUREC/2021/079) in accordance with the Declaration of Helsinki, and each participant provided written informed consent prior to participation.

Participants arrived to the laboratory on two separate occasions under controlled conditions. Participants were provided with standardised meals (30 kcal/kg as 50/25/25 for carbohydrate, protein and fat; GourmetFuel™, Dublin, Ireland) for consumption on the day prior to each visit, were asked to abstain from alcohol consumption for at least 24 h, arrived after an overnight fast and only having consumed water that morning, and were asked to abstain from exercise for at least 24 h before the visit (Table 4.1). Participants lay supine for 10 min before a venous blood sample was then taken from a superficial forearm vein.

5.3.2. Blood sampling, processing, and storage

Whole venous blood was drawn by inserting a 21G butterfly needle (Greiner, Bio-One, Austria) into an antecubital vein. The initial 4 mL of blood was drawn into a generic vacutainer and discarded. Subsequently ~50 mL of whole blood was drawn into six 9 mL blood collection tubes coated with ACD-A anticoagulant (Greiner, Bio-One, Austria). Blood samples were immediately placed on ice and centrifuged at 1500*g* for 15 min at 4ºC. Directly after

centrifugation, plasma samples were separated into aliquots, and stored at -80ºC. Samples were collected and stored between August and December 2021.

5.3.3. Separation of small EVs using Size-Exclusion Chromatography (SEC)

Whole plasma aliquots (2mL) were defrosted from -80°C on ice at room temperature. To remove large debris, defrosted samples were re-centrifuged at 2500g for 10 minutes at 4°C and then injected into a through a 33mm syringe with a 44µm polyether sulfone filter into a fresh 1.5mL Eppendorf. This initial filtration generally resulted in a loss of 400-800µl of plasma. Subsequently, 1mL of filtered plasma was added to a commercially available agarose-resin based size-exclusion chromatography column calibrated for this volume of sample (qEV1-35nm GEN2 smart column, Izon Science Europe, Lyon, France) designed for the specific collection of EVs in smaller size ranges (i.e. 35-350nm). Samples were collected by an automatic fraction collection device (Izon AFC V1, Izon Science Europe, Lyon, France) into 12 fractions of 700ul elutes. For a given sample, fractions 7-11 were then pooled together (as per the manufacturers recommendation and validated experimentally – figure S5.1). Columns were used a maximum of 9 times (as per the manufacturers recommendation); used columns were stored at 4°C with fresh buffer and columns were completely used within a week of their first use. After SEC was performed, samples were concentrated via 20-30 minutes of 4000g centrifugation at 4°C using a 10kDa protein concentrator PES10MWCO (Cat. #:88513, Sigma, St Louis, USA) to 150µl and aliquoted into fractions of 'small EV prep' that were stored at -80°C. Aliquots of small EV prep were never defrosted more than once. Small EV separation and analysis all took place between April and November of 2022, therefore all experimental performed on small EV preps were performed within 6 months of separation. The separation and identification procedure described above and subsequently has been submitted to, and assessed by, the EV-Track database (ID: EV230013) (Van Deun et al., 2017). This study design

received a score of 75%, which is above the average reported for the calendar year this work was performed (2022, 40%).

5.3.4. Nanoparticle Tracking Analysis

NTA was performed using NanoSight NS300 system (Malvern Technologies, Malvern, UK), configured with a 488 mm later and a high sensitivity CMOS camera to determine the size of particles in separated small EV preparations and provide an additional estimate of small EV concentration. Concentrated preparations of small EVs were pooled from elute fractions 7-11 and concentrated to 150µl as previously described. Sample were diluted 1:500 in PBS that had been filtered once through a 25µm polyether sulfone filter (specifically, 2µl of EV prep diluted in 998µl of filtered PBS). Samples were analysed under constant flow conditions (flow rate = 50) at 25 °C, camera level was set to 12 and screen gain set to 2. Five × 1 min videos were captured for each sample. Data was exported with a detection threshold of 10 and analysed manually in R.

5.3.5. Quantification of small EVs using Amnis imagestream

Small EV preparations (separated from plasma as described above) from each participant and visit were screened for the presence of CD63 (ExBio, Cat. #: 1A-552-T100, Vestec, Czech Republic). The CD63 antibody was centrifuged at 16,000g for 15 mins at 4°C to remove antibody aggregates. 10µl of Small EV prep was incubated with 10µl of Green Cell Mask^{tm} (1:1 dilution, Thermofisher, Cat# C37608, Waltham, USA) for 30 minutes on a thermomixer (37°C, 300RPM). Afterwards, the Cell Mask/Small EV combination was incubated with 4µl of CD63 antibody (1:5 dilution) in the dark for 60 minutes (at room temperature). The antibody/small EV prep was then diluted in 280µl of filtered PBS and this solution was centrifuged (1000g at 4°C) in a protein concentrator for one-minute intervals until it reached a concentrate of 100µl. The concentrated fraction was then placed in a new Eppendorf and acquired within 2hrs on ImageStream X MK II imaging flow cytometer at 60X magnification and low flow rate. PBS and unstained controls were run in parallel. All samples were screened for 5 mins. Once screened, 4% of NP-40 (EMD Millipore, Cat. #: 492016, Burlington, USA)

was added at 1:1 ratio with the sample and was re-screened, as the NP-40 control. Data analysis was performed using IDEAS software v6.2, generating summary statistics of the number of objects identified per mL, as well as the number of $CD63^+$ objects identified per mL.

5.3.6. Protein quantification of EV preparations

Single 50µl aliquots of EV prep were defrosted on ice and mixed with 50µl of lysis buffer sodium dodecyl sulphate (SDS) lysis buffer (250 nM Tris–HCl, pH 7.4; 2.5% SDS) and 4µl of proteinase inhibitor (1X proteinase inhibitor (Roche, Cat. #: 05892970001, Basel, Switzerland); lysed EV preps were then exposed to three bouts of five seconds of vortex, samples were rested on ice for ten minutes between each vortex bout, samples were then centrifuged at 13200 rpm for ten minutes at 4°C. The concentration of samples was then estimated via a Bradford colorimetric assay (Biorad, California, USA) in a 96 well polystyrene well that was read on a FLUOstar OPTIMA plate reader (BMG Labtech, Ortenberg, Germany) with an excitation wavelength of 570nm. Here, the absorbance of samples was compared that of a standard curve of known protein concentrations, sample protein concentrations were then predicted via linear regression.

5.3.7. Immunoblotting of EV markers and contaminants

Calibrated volumes of lysed EV prep estimated to contain 10µg of protein or a whole-cell lysate control (derived from the $Hs578Ts(i)_8$ triple-negative breast cancer cell line) were resolved via SDS page (on a 10% bis-acrylamide resolving gel) via electrophoresis. Proteins were then transferred onto polyvinylidene difluoride membrane (PVDF) that was blocked with 10mL of a blocking buffer solution consisting of PBS-tween and 5% dissolved Bovine serum albumin (BSA) (Sigma-Aldrich, Cat. #: A9413, St Louis, USA) for one hour. Membranes then underwent 3 x 5 minute washes in PBS-tween and were subsequently incubated with primary antibodies anti-CD63 (1:500;Abcam;Cat.#:ab68418, Cambridge, United Kingdom); anti-Albumin (1:2000;Abcam;Cat.#:ab190806, Cambridge United Kingdom) and anti-flotilin-1 (1:1000, Abcam;Cat#ab133497, Cambridge, United Kingdom) overnight under constant

rocking at 4°C. The following morning, membranes were washed 3 times and incubated with appropriate HRP-linked secondary antibodies (Rabbit for all primary antibodies) under constant rocking for one hour at room temperature. Membranes were once again washed three times, after which proteins were visualized by Super Signal® West Pico chemiluminescence substrate (Thermofisher, Cat. #: 34080, Waltham, USA) or Super Signal® West Femto maximum sensitivity substrate (Thermofisher, Cat. #: 3409s, Waltham, US). Proteins were detected using a chemidoc exposure system (BioRad laboratories). Images were exported and saved as .tif files at 600dpi.

5.3.8. Visualisation of small EVs using Transmission Electron Microscopy (TEM)

20µL of small EV preparation was placed onto parafilm (Sigma-Aldrich, Cat. #: P7793) and covered by a single-side formvar carbon-coated nickel grid (Ted Pella Inc, Cat. #: 01813) with the coated side facing the EV suspension droplet. The grid was incubated for 60min at room temperature and washed in 30µL of PBS (x3 times) on parafilm. Absorbent paper was used to remove excess PBS from the washing steps. A droplet of paraformaldehyde (2%) was placed on separate parafilm with the grid placed on top and then fixed for 10min. PBS washing steps were repeated and the grid was then contrasted in 2% uranyl acetate (BDH, Cat. #: 230550). Representative? images of both visits from one participant from CON, END and STR participants were taken and are presented at 2990x and 5970x magnification. All images were taken using the JEOL JEM-2100 transmission electron microscope at 120kV.

5.3.9. Targeted Metabolomics of EV preparations

Samples from each participant were pooled into 20µl fractions (10µl of small EV preparation from visit 1 was pooled with 10µl from visit 2) and a targeted metabolomic employing a mix of LCMS and Flow Injection Analysis and Mass Spectrometry was performed. For metabolite extraction Small EV preparations were dried under a stream of nitrogen and resuspended in 25 µl ice-cold Ethanol/PBS (85:15, v/v). Samples were then subjected to three cycles of 3-minute sonication in an ice bath followed by a 30 second snap-

freeze in liquid nitrogen. Afterwards, samples were centrifuged at 24,000×g for 5 min at 2 °C, and the metabolite-enriched supernatant was collected for metabolomic analysis. Metabolites were identified and quantified using the Biocrates AbsoluteID® p180 assay (Biocrates Life Sciences, Innsbruck, Austria), which is a targeted panel capable of identifying up to 188 metabolites. Metabolomic analysis was performed using a SCIEX QTRAP 6500+ mass spectrometer coupled to SCIEX ExionLC™ Series with UHPLC capability. This approach has previously been employed on preparations of human derived circulating small EVs and is more extensively detailed in a recent publication by Howard and Colleagues (Howard et al., 2022). Briefly, amino acids (n = 21) and biogenic amines (n = 21) were targeted in liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis using isotopically labelled internal standards and 7-point calibration curves. Acylcarnitines (n = 40), lysophosphatidylcholines (n = 14, LPCs), phosphatidylcholines (n = 76, PCs), sphingomyelins (n = 15, SMs), and hexose were measured semi-quantitatively by using 14 internal standards in flow injection analysis–tandem mass spectrometry analysis. 96 distinct metabolites (Table S5.1) were detected and classified appropriately into the following groups Glycerophosphocholines (n = 79) – whose direct parents were phosphatidylcholines (n = 70) and 1-acyl-sn-glycero-3-phosphocholines (n = 9); & Phosphosphingolipids (n = 14) using the ChemOnt method (Feunang et al., 2016). For analytical purposes, three metabolites (Serine, Spermine and Spermidine) were subjectively collected into a single sub-group (called Amino acids, Amines & Carboximidic acids) as each of these metabolites were the only identified members of their ChemOnt metabolite class.

5.3.10. Analysis of EV Exocarta and Vesiclepedia databases

Metabolites from identified two established databases of EV markers and cargo, Exocarta (Keerthikumar et al., 2016) and Vesiclepedia (Pathan et al., 2019) were screened for available metabolomics/lipidomics data that were generated on EVs separated from blood

(plasma or serum) derived from human participants. Relevant data were downloaded and categorised in similar fashion to metabolites identified in the current study (Table S5.2) the proportion of different metabolite types and the number of overlapping individual metabolites were then compared.

5.3.11. Statistical Analysis

All data that contained both a within-groups and between groups independent variable (Nanoparticle Data, Flow-Cytometry Data, Immunoblot/Protein data) were analysed using mixed ANOVA (Group * Visit). In circumstances where visit was not a relevant independent variable (Height, age, number of days between visit 1 and visit 2 and Metabolomics data), data were analysed using a one-way ANOVA with *post-hoc* pairwise comparisons again performed using the Bonferroni correction. All ANOVAs are reported with a relevant estimate of standardized effect size (η_p^2). The profile of Metabolomics data between groups were also examined using a PCA and logistic regression analysis. Prior to dimension reduction into latent variables (Principal components 1 and 2, respectively) a Kaiser-Mayer-Olkin test of sampling adequacy and Bartlett's test of sphericity were performed on data to ensure they met the minimum standards for PCA. Bartlett's test of sphericity produced a significant result ($X^2(95)=$ 19885; $P<0.01$), while a Kaiser-Mayer-Olkin test of mean sampling adequacy produced an average result of 0.9 which is considered 'marvellous' (Kaiser and Rice, 1974). A PCA using an 'oblimin' rotation (to account for the high degree of correlation between metabolites) was performed, with features mean zeroed and scaled for unit variance. PCA was performed using the 'parameters' package in R, located within the 'easystats' ecosystem (Lüdecke et al., 2020). After dimension reduction, a multinomial logistic regression model was constructed using the 'nnet' R package (Beck, 2018) to investigate whether an individual's PC1 score (the latent variable composed of all metabolites that explained the highest proportion of total variance) was capable of predicting whether participants belonged to either the CON, END or STR

groups. Data were randomly partitioned into a 'training' set (consisting of 70% of the data) and a 'test' set (consisting of the remaining 20%). Models were evaluated based on the ability of the trained model to predict the test data and using Wald's test.

5.4. RESULTS

5.4.1.NTA and Amnis imagestream of small EV preparations

The results of NTA and Amnis imagestream flow cytometry of small EV preps are displayed in Figure 5.1. The diameter of particles were measured to be in the typical small EV size range by NTA for all groups (average diameter range = 129-138 nm, Figure 5.1A) no differences were observed between groups ($p = 0.68$, $F = 0.40$, $\eta_p^2 = 0.02$) or between visits within groups ($p = 0.63$, $F = 0.25$, $\eta_p^2 = 0.01$). For concentration of particles measured by NTA (particles/mL, Figure 5.1A & B.). no significant differences were observed between groups ($p = 0.21$, $F = 1.63$, $\eta_p^2 = 0.09$) or between visits within groups ($p = 0.40.$, $F = 0.73, 0.40.$ $\eta_p^2 = 0.02$). For the Amnis imagestream, no difference between groups or between visits within groups for any of the following variables; Particles/mL (Group - $p = 0.24$, $F = 1.5$, $\eta_p^2 = 0.08$, Visit – $p = 0.41$, $\eta_p^2 = 0.02$, Figure 5.1C); $CD63^+$ particles/mL (Group - $p = 0.46$, $F = 0.80$, $\eta_p^2 = 0.05$, Visit – $p = 0.24$, $\eta_p^2 = 0.04$, Figure 5.1D); %$CD63^+$ particles/mL (Group - $p = 0.10.$, $F = 2.50.$, $\eta_p^2 = 0.13$, Visit – $F = 0.09$, $p = 0.81$, $\eta_p^2 = 0.01$, Figure 5.1E).

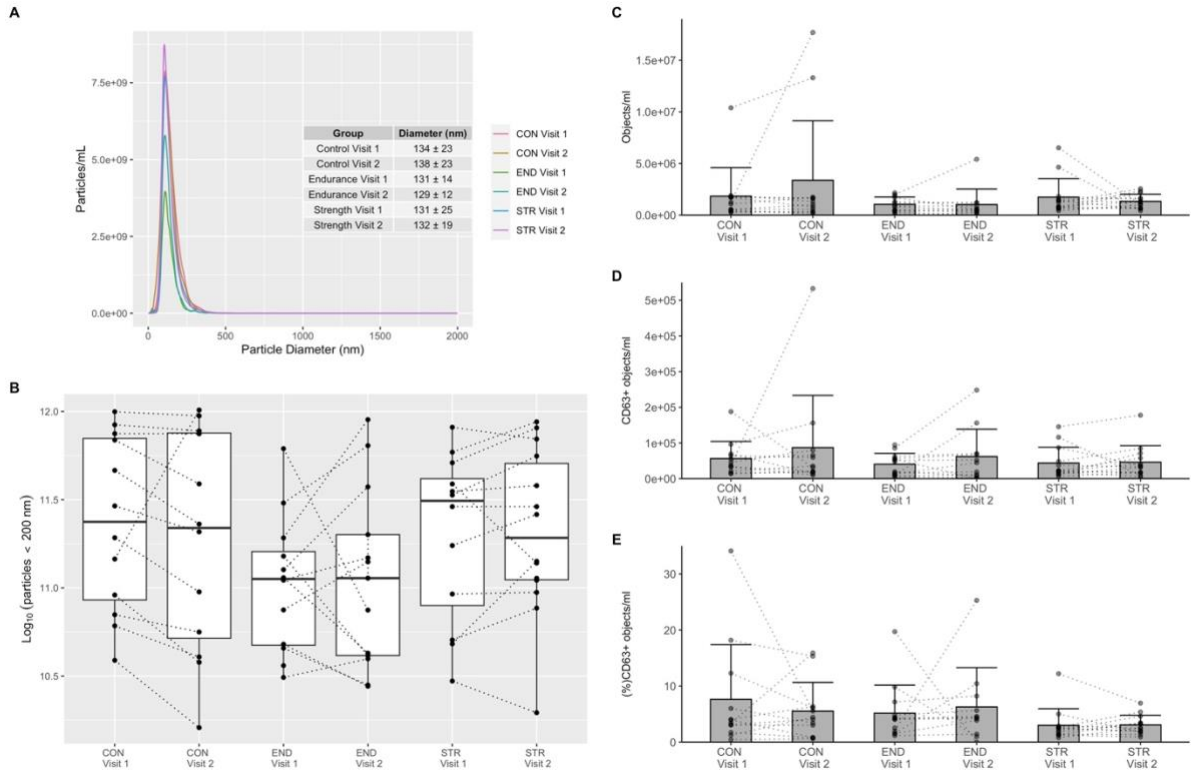


Figure 5.1. Particle Analysis of Small EV Preparations. **A.** Size distribution and concentration of particle in small EV preparations determined via NTA. **B.** Log10 of total particles identified via NTA. **C.** Number of objects/mL detected via AMNIS flow cytometry. **D.** Number of CD63+ objects/mL detected via AMNIS flow cytometry. **E.** Relative percentage of total objects per/mL detected via AMNIS flow cytometry that were CD63 positive. All analysis n = 75, two visits from 12 recreationally active controls, 13 endurance athletes (1 athlete only visit) and 13 strength athletes. For C, D, & E column height represents means and error bars representing standard deviation(s).

5.4.2. Immunoblotting of CD63, FLOT1 and Albumin

No difference in the amount of total protein present in small EV preparations was observed between groups ($p = 0.96$, $F = 0.04$, $\eta_p^2 = 0.01$) or between visits within groups (Figure S5.2) ($p = 0.83$, $F = 0.05$, $\eta_p^2 = 0.004$). The quantification of immunoblot stains of small EV markers (CD63, FLOT1) and albumin are presented in Figure 2 with representative images (Figure 2D). No difference between groups or between visits within groups for small EV markers CD63 (Group - $p = 0.90$, $F = 0.11$, $\eta_p^2 = 0.02$, Visit $p = 0.83$, $F = 0.05$, $\eta_p^2 = 0.004$, Figure 5.2A) and FLOT1 (Group – $p = 0.72$, $F = 0.34$, $\eta_p^2 = 0.05$, Visit – $p = 0.82$, $F = 0.05$, η_p^2 – 0.004, Figure 5.2B). The presence of albumin contamination was detected in all samples that

were tested (Figure 5.2. C), no significant differences were observed in the abundance of albumin between groups ($p = 0.26$, $F = 1.53$, $\eta_p^2 = 0.20$) or between visits within group ($p = 0.55$, $F = 0.38$, $\eta_p^2 = 0.03$).

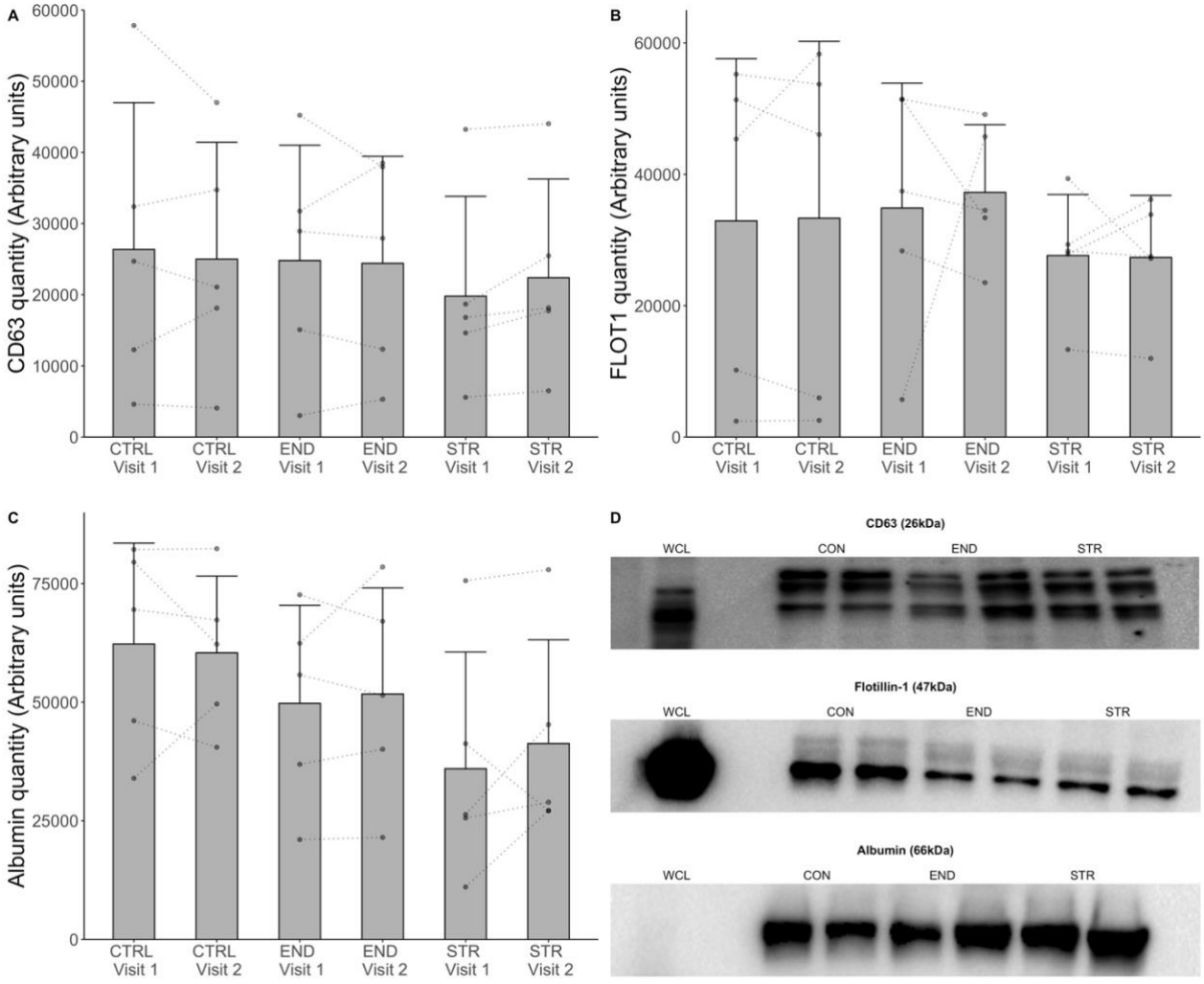


Figure 5.2. Identification of small EV marker proteins and contaminants via western blot. **A.** Densitometry of tetraspanin/EV marker CD63. **B.** Densitometry of small EV internal marker Flotilin-1. **C.** Densitometry of Albumin Contamination. **D.** Representative images of all three markers. All analysis are n = 5 replicates with column height representing means and error bars representing standard deviation(s), each replicate used different individual participants. WCL = Whole-Cell Lysate

5.4.3. Transmission Electron Microscopy of small EV preparations

TEM analysis demonstrated that samples derived from all three groups contained vesicular shaped objects in the shape of small EVs. These vesicles were predominantly found to be in the small EV size range (e.g., ≤ 150nm). Although, in some circumstances, larger

vesicles were visible. Distinct clustering or 'clumping' of some vesicles was also apparent. The appearance and subjective 'amount' of vesicles was largely similarly between groups (Figure 5.3).

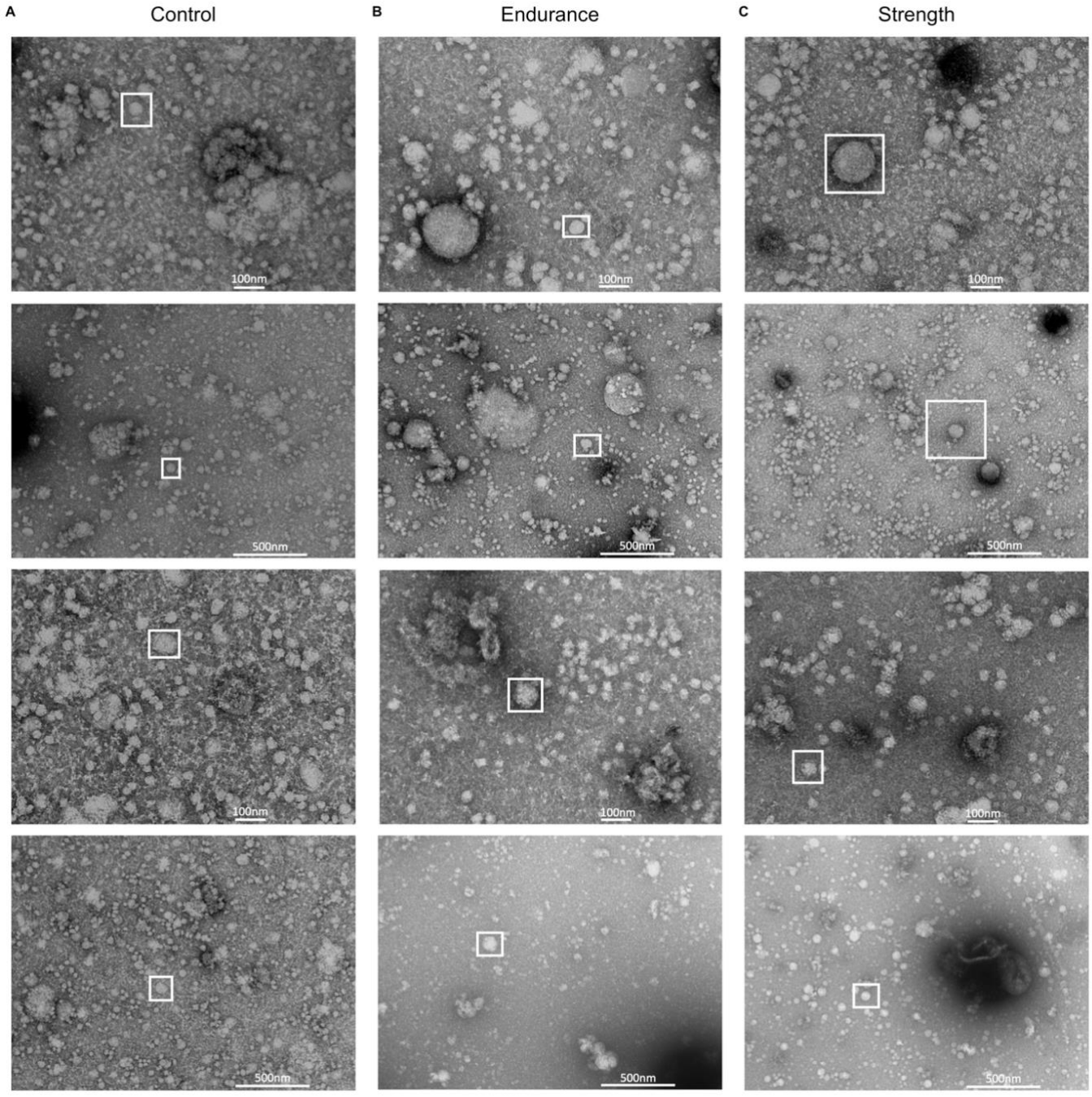
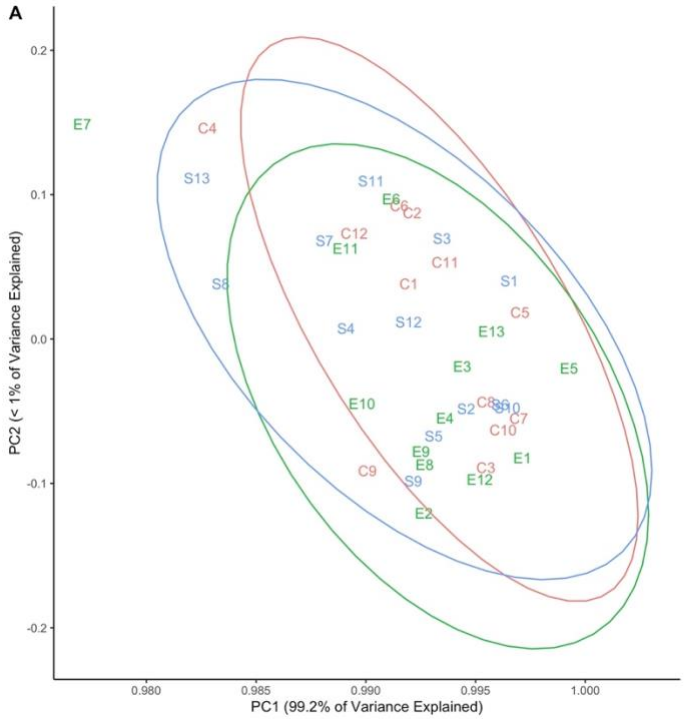


Figure 5.3. Visualisation of Small EVs via transmission electron microscopy. **A.** 4 representative images from a control participant. **B.** 4 representative images from an Endurance Participant. **C.** 4 representative images from a Strength participant. The top two panels represent images taken of small EV prep separated from plasma derived at each individual participants first visit to the lab, the lower two images represent small EV preps visualised from the second lab visit.

5.4.4. Targeted Metabolomic analysis of small EV preparations

From 188 metabolites in the targeted panel, 96 metabolites were identified in small EV preparations. These were largely classified as Glycerophosphocholines (n = 79), Phosphospingolipids (n = 13) and three independent metabolites (Serine, Spermine and Spermadine) (Table S5.3). PCA of identified metabolites explained a large proportion of variance (PC1 explained ~99% of variance), however when participants were projected no distinct clustering or separation of participant groups were apparent, implying the metabolome profile of small EV preparations from each participant were largely similar (Figure 5.4). A multinomial logistic regression model designed to predict each participants group membership using their PC1 score demonstrated poor predictive power (~20% accuracy, AIC = 91.40). Wald's test also did not produce significant p-values for the ability to predict END (p = 0.86) or STR (p = 0.49) group membership relative to the CON participants. A one-way ANOVA did observe a significant main effects between groups for 7 metabolites; PC aa C36:0 (p = 0.04 , F = 1.7 , η_p^2 = 1.7); PC ae C36:5 (p = 0.02 , F = 4.4 , η_p^2 = 0.2); PC ae C38:3 (p = 0.04 , F =3.6 , η_p^2 = 1.7); PC ae C40:4 (p = 0.04 , F = 3.6 , η_p^2 = 0.17); SM(OH) C24:1 (p = 0.04, F = 3.65 , η_p^2 = 0.17); Spermine (p = 0.02, F = 4.35 , η_p^2 = 0.21); Spermidine (p = 0.01 , F = 5.25 , η_p^2 = 0.231). However, no significant differences were observed between groups when post-hoc testing with a Bonferroni correction was applied (Table S5.3).

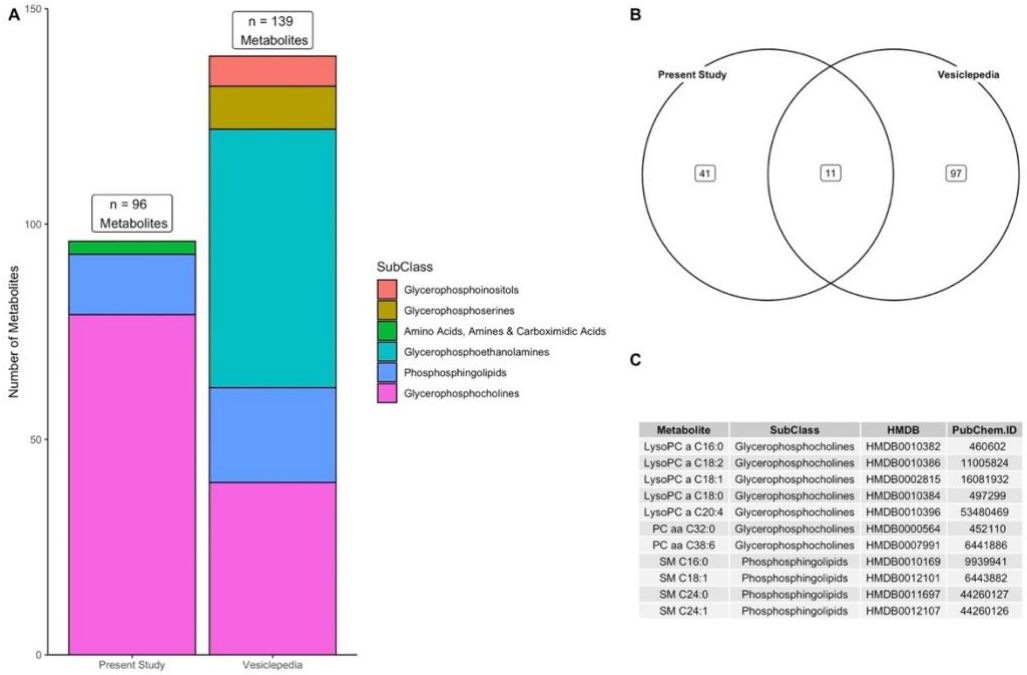


Metabolite	PC1 Loading Score	HMDB Identifier
Serine	-0.3563575	HMDB0000187
Spermidine	-0.3234598	HMDB0001257
Spermine	-0.3209216	HMDB0002172
LysoPC a C16:0	-0.2728077	HMDB0010382
LysoPC a C17:0	-0.2706535	HMDB0012108
LysoPC a C18:0	-0.2705628	HMDB0010384
LysoPC a C18:1	-0.2592601	HMDB0002815
LysoPC a C18:2	-0.2473516	HMDB0010386
LysoPC a C20:3	-0.2396120	HMDB0010394
LysoPC a C20:4	-0.2319627	HMDB0010396

Figure 5.4. The results of principal component analysis (PCA) on the 96 identified metabolites. **A.** The projection of participants PC1 and PC2 scores into two dimensional space. **B.** The Metabolites with the top ten largest loading scores, ranked in descending order. PC1 represents a single latent variable that explains the greatest proportion of variance (99.2%) while PC2 represents a latent variable that explains the second greatest proportion of variance (~0.8%) of variance. Ellipses represent 95% confidence intervals.

5.4.5. Analysis of EV databases

Data from two EV databases Exocarta (286 studies, 1116 metabolite/lipid entries) (Keerthikumar et al., 2016) and Vesiclepedia (1254 studies, 639 metabolite/lipid entries) (Pathan et al., 2019) were downloaded. Screening of these databases identified two studies (Losito et al., 2013; Weerheim et al., 2002) that examined the metabolite profile of circulating human EVs. These studies were both only available on the Vesiclepedia database. Across both studies (Losito et al., 2013; Weerheim et al., 2002) 161 distinct metabolites were identified (Table S5.2) that were mostly categorically similar to the current study (Figure 5.5. A). However, only 11 individual metabolites were identified in both the current study and on those downloaded from the Vesiclepedia database (Figure 5.5. C)



Metabolite	SubClass	HMDB	PubChem.ID
LysoPC a C16:0	Glycerophosphocholines	HMDB0010382	460602
LysoPC a C18:2	Glycerophosphocholines	HMDB0010386	11005824
LysoPC a C18:1	Glycerophosphocholines	HMDB0002815	16081932
LysoPC a C18:0	Glycerophosphocholines	HMDB0010384	497299
LysoPC a C20:4	Glycerophosphocholines	HMDB0010396	53480469
PC aa C32:0	Glycerophosphocholines	HMDB0000564	452110
PC aa C38:6	Glycerophosphocholines	HMDB0007991	6441886
SM C16:0	Phosphosphingolipids	HMDB0010169	9939941
SM C18:1	Phosphosphingolipids	HMDB0012101	6443882
SM C24:0	Phosphosphingolipids	HMDB0011697	44260127
SM C24:1	Phosphosphingolipids	HMDB0012107	44260126

Figure 5.5. A comparison between metabolites downloaded from the vesiclepedia database and those identified in the current study. **A.** The distribution of metabolites by metabolite SubClass. **B.** A venn diagram with the exact overlap of metabolites between vesiclepedia and the current study. **C.** A list of the identical metabolites identified between vesiclepedia and the current study.

5.5. DISCUSSION

The purpose of the current study was to investigate whether a history of exercise training influences the abundance of circulating small EVs at rest. CON, END and STR participants had blood samples extracted on two separate occasions with similar measurement circumstances (proximity from most recent exercise bout, 24hr nutrition and time of day). Preparation of small EVs that were separated from blood plasma using size-exclusion chromatography and small EVs were identified using orthogonal methods in line with the MISEV guidelines (Théry et al., 2018). These guidelines recommend that a faithful identification of EVs requires, at minimum, an indication of the abundance of EVs present in a sample (e.g. a particle count); an indication of the presence of EV 'markers' (both internal and transmembrane/external EV proteins); visualisation of single and/or multiple vesicles; and an indication to the degree of contamination produced by the method of small EV separation (as creating 'pure' samples of EV is considered practically impossible) (Théry et al., 2018).

Using a common method of EV separation (SEC) (Cocozza et al., 2020; Gardiner et al., 2016; Monguió-Tortajada et al., 2019) and following the aforementioned MISEV guidelines (Théry et al., 2018) as a template, it was observed that SEC separated out preparations of particles that followed the approximated size distribution of small EVs (i.e. 50-~150nm) (van Niel et al., 2018); were positive for both transmembrane (CD63) and internal (Flotilin-1) 'EV marker' proteins (Yáñez-Mó et al., 2015); and possessed a distinct vesicular shape when visualised using TEM. Notably, no significant differences (either between or within groups) were observed for quantitative (NTA &and flow cytometry) or semi-quantitative (densitometry of western blot images of EV markers) indices of EV presence and abundance. Collectively, these results would indicate that, in a resting state, exercise training may not influence the presence of circulating small EVs and that the abundance of circulating small EVs may be similar when blood samples are acquired under consistent measurement conditions.

Some indication of sample contamination was observed via the presence of albumin demonstrating that SEC did not produce 'pure' preparations of small EVs. However, no difference was observed in presence of albumin between groups or visits. It is noteworthy to mention that all established methods of EV separation are largely considered incapable of producing samples that are entirely devoid of any contamination (Théry et al., 2018). This latter point that has recently been demonstrated empirically in work produced by the O'Driscoll group, where many common methods of EV separation (namely; Differential Ultracentrifugation, SEC, Polyethylene-Glycol precipitation, Nickel-Based Isolation and several commercial EV separation kits) produce preparations of EVs that contain albumin contamination (to varying degrees) when used to separate EVs from human sera (McNamee et al., 2022). The observation that the presence of circulating small EVs at rest may not be altered by exercise training is in contrast with two previous studies, that employ the same study design and are produced by the same research group (Ma et al., 2018; Wang et al., 2020); These two

studies have both observed increased abundance of particles in the small EV size range (detected using NTA) in 'EV' preparations separated (using differential ultracentrifugation) from the plasma of exercise trained C57BL/6J mice (subjected to 4 weeks of low (5min/min) or moderate (10m/min) treadmill running for 60 min per day, 5 day per week) compared to a sedentary control groups; However, in the case of both studies, plasma samples were acquired 24hr after the final exercise bout (Ma et al., 2018; Wang et al., 2020), a time point at which residual influences of previous acute exercise bouts has been reported in preparations of small EVs (Lovett et al., 2018). In addition only one of these studies reports an additional method of small EV identification (beyond using NTA alone) and observed no difference in the presence of the 'EV marker proteins' CD63, CD34 or TSG101 via western blot (Ma et al., 2018). More recently, 12 weeks of home-based resistance exercise (progressive band-based resistance exercise 3 days per week) did not alter the abundance of five EV marker proteins (ALIX, CD63, TSG101, CD9, CD81) or the size/number of particles in the small EV size range, via NTA, in plasma samples derived from older adults (age, 75±57 y, total sample size unclear, reported n ranges from 9-38 depending on assay) at rest (~96hrs post most recent exercise bout) (Xhuti et al., 2023).

Therefore, while it has been speculated that exercise training influences the abundance of circulating EVs at rest (Nederveen et al., 2021), within any single study, this has never been demonstrated in a manner that is robust (i.e. across more than a single method of EV characterisation) and has currently never been indicated in preparations of circulating small EVs derived from human plasma. Therefore, the observation of the current study that the presence of small EVs may not be altered in men with a history of exercise training at rest is plausible and instead suggests that the abundance of circulating small EVs in exercise trained individuals is not altered at rest.

To gain some insight into the metabolite profile of human small EVs, a targeted metabolomics assay was applied to pooled samples of each participant's visit 1 and visit 2 small EV preparations. 96 metabolites were identified and analysed using PCA. No clustering or separation between CON, END and STR participants was apparent, implying the targeted metabolomic profile of groups were similar. A multinomial logistic regression model that aimed to predict whether an individual's exercise training history could be predicted based on their PC1 score demonstrated poor predictive power (only correctly guessing group membership in 20% of observations provided by the 'test' partition of the dataset) and produced non-significant values on Wald's test.

Having noted that the profile of metabolites between groups were similar, it was then considered whether identified metabolites had previously been associated with small EVs. Most metabolites identified in small EV preparations were Glycerophosphocholines (of which n =70 were phosphatidylcholines and n= 9 were 1-acyl-sn-glycero-3-phosphocholines) and phosphosphingolipids (n = 14) a finding that was thematically consistent with metabolites that have previously been deposited to the EV database Vesiclepedia (Figure 5.5). Both phosphatidylcholines and certain phosphosphingolipids (namely sphingomyelins – which all of our identified phosphosphingolipids were identified as – table S5.1) have previously been reported as enriched in the membranes of small EVs (relative to their cell of origin) (Laulagnier et al., 2004; Llorente et al., 2013; Subra et al., 2010). The physiochemical properties conveyed by membrane enrichment of phosphatidylcholines, sphingomyelins, as well as cholesterols (which were not included in the p180 metabolomics assay) have been speculated as necessary for providing EVs with the buoyancy required to maintain a robust structure in rapid extracellular fluid environments (e.g. blood plasma) (Record et al., 2014).

Three additional metabolites that were not lipid derived (Serine, Spermine and Spermidine) were also identified in small EV preparations. In addition, these three metabolites

also contributed the most to our latent PC1 variable by possessing the first, second and third largest loading scores respectively (Figure 5.4. B). Interestingly, little is known regarding the relevance of Serine, Spermine or Spermidine to the function or structure of EVs. While the presence of phosphatidylserine in EV membranes is well established as essential for recognition and uptake of EVs into recipient cells (Buzás et al., 2018). The relevance of free serine for EVs biogenesis and/or EV function is not immediately clear, but serine has previously been detected in preparations of small EVs derived from human and feline plasma (Howard et al., 2022), human serum (Luo et al., 2018) and urine (Puhka et al., 2017). Serine is a substrate by enzymes such as serine palmitoyl transferase, an enzyme essential for the production ceramide (Hanada, 2003), which is the backbone of all sphingomyelins and essential for producing the membrane curvature of endosomes (i.e. is necessary for the production of exosomes, a subtype of small EV) (Castro et al., 2014; Trajkovic et al., 2008). Inhibiting cellular ceramide production using the sphingomyelinase inhibitor GW4869 has also been reported to reduce the release of fluorescently tagged small EVs from Oli-neu cells by up to 80% (Trajkovic et al., 2008). There is also limited evidence regarding the relevance of spermine and its precursor spermidine to EVs. Spermidine synthase catalyses the conversion reaction of spermidine to spermine (Wu et al., 2007) and has previously been identified in preparations of human EVs separated from urine (Chen et al., 2012; Øverbye et al., 2015; Wang et al., 2012). Spermidine synthase and has also been identified to interact with myosin Va (Dolce et al., 2019), a motor protein that is involved in the transport of large intracellular vesicles that has also been identified to facilitate exocytosis and the extracellular release of vesicles (Rudolf et al., 2011), although the direct extent to which spermidine synthase contributes to EV release has not yet been identified or quantified.

There are several limitations to the current study. Firstly, only a single method of small EV separation was applied to whole plasma samples, this reduces the generalizability of our

results as it is unclear whether results would be similar using a different method of EV separation (e.g. differential ultracentrifugation) (Cocozza et al., 2020). The current study also only employed a generic selection of small EV markers, therefore our study does not provide indication as to whether the proportional amount of different subpopulations of small EVs is different between CON,END and STR participants (Kowal et al., 2016; Théry et al., 2018). The current study employed a targeted metabolomics approach, which was limited to 187 metabolites, therefore it is uncertain as to whether a wider coverage of the metabolome would have revealed differences between groups. The metabolomics analysis in the current study also involved the lysing of EV membranes via sonification, therefore, while many metabolites could be thematically associated with small EV membranes, it cannot directly be inferred whether these metabolites were actually associated to EV membranes prior or were internalised within EVs prior to sonification. Lastly, there is uncertainty with respect to the direct extent that identified metabolites can be solely attributed to EVs, as small EV preparations also contained albumin contamination. Although, albumin primarily transports fatty acids (not measured by the p180 targeted assay) (Rudolf et al., 2011) and a recent study performing lipidomic comparisons between album depleted/albumin enriched plasma fractions has observed that the abundance of glycerophospholipids and sphingolipids decrease (by ~ 0.5 - 2x) in albumin enriched fractions of plasma (Casulleras et al., 2022) suggesting that glycerophospholipids and sphingolipids may be less associated with the albumin fraction of plasma.

In conclusion, the presence and metabolomic profile of small EVs derived from the plasma of individuals with different exercise training histories were not different at rest. These findings suggest that the presence of circulating small EVs results in healthy individuals at rest, irrespective of their exercise training status. However, further research is required to explore whether the distinct bioactive cargo(s) (e.g. RNA, protein and additional metabolites) carried within EVs are altered in individuals with different exercise training histories.

Chapter 6
Functional assays in BT-549 cells reflective of cancer hallmarks are similar when cultured with media supplemented with plasma extracted from men regardless of exercise training history

6.1. ABSTRACT

Media supplemented with serum extracted from acutely exercise men has been shown to have 'anti-cancer' effects on prostate and breast cancer cell lines. The purpose of the current study was to examine whether media supplemented with resting (~40hrs post exercise) plasma samples extracted from CON, END and STR participants influenced the results of four functional assays designed to mimic cancer hallmarks (Proliferation, Migration, Anoikis Resistance and Extracellular Matrix Invasion) in BT-549 cells. Data were analysed using mixed linear models with post-hoc t-tests performed on significant main effects. BT-549 cells cultured with plasma supplemented media had increased cell proliferation ($p < 0.01$ for all groups, ~20-50% increase) that was not dependent on group or plasma concentration. Cell proliferation was lower between 48hr and 72hrs for all groups ($p < 0.01$ for all groups, ~20% lower). Media supplemented with 10% human plasma promoted greater migration ($p < 0.05$ for all human plasma groups – 15-20% difference, $p < 0.01$ for 1% FBS condition ~40% difference) at 48hrs. BT-549 cells cultured with 10% human plasma had lower extracellular matrix invasion ($p < 0.01$ for all human plasma groups, 10-20%) at 24hrs and 48hrs. Greater anoikis was observed for cells that were cultured in media that was 15% human plasma ($p < 0.05$ for all human plasma groups, anoikis, 15-20% greater than SFM control) at 24hrs. Resting plasma extracted from healthy males demonstrates 'nourishing' effects for proliferation and migration assays and deleterious effects observed for invasion and anoikis resistance assays that are not influenced by exercise training history. Previously reported 'anti-cancer effects' of serum supplemented media may be due to acute exercise effects, as opposed to exercise training.

6.2. INTRODUCTION

Often referred to as an individual disease, the term ‘cancer’ technically describes a group of over 100 different diseases (Siegel et al., 2020; Weinberg, 1996) that share a common pathology described by an initial localised development, growth and proliferation of ‘abnormal’ cells followed by an eventual expansion to new tissues (Weinberg, 1996). There is phenotypic variability across cancer subtypes in terms of cell-type origin, rate of occurrence, aggressiveness and responsiveness to treatment (Siegel et al., 2020). However, cancer has hallmark characteristics (namely – sustained proliferative signalling; evasion of growth suppression; extracellular invasion and metastasis, replicative immortality; altered angiogenesis; and a resistance to cell death) that manifest to varying degrees across all cancer cells (Hanahan and Weinberg, 2011, 2000).

A high habitual physical activity level has been associated with decreased risk for developing (relative risk reduction ~10-20%) and experiencing mortality from (relative risk reduction ~40-50%) several breast, prostate and colon cancer(s) (Brown et al., 2012; Mctiernan et al., 2019). There is currently limited understanding regarding the means by which physical activity (and consequently structured exercise) conveys remedial benefits in cancer patients or ‘anti carcinogenic’ effects to cancer-free individuals. Commonly speculated mechanisms include increased tumour ‘vulnerability’ mediated by alterations to tumour metabolism induced by acute exercise and enhanced immune cell recognition of tumour cells which may be caused by an exercise-induced increase in circulating ‘exercise factors’ (e.g. IL-6) (Hojman et al., 2018; Metcalfe et al., 2021; Pedersen et al., 2016).

Given the epidemiological relationship between high physical activity and reductions in cancer development or progression (Mctiernan et al., 2019) and the potential for exercise training to alter the presence of many ‘exercise factors’ at rest (Chow et al., 2022). It has also been considered whether anti-cancer effects may be present in human serum/plasma extracted

from exercise trained individuals outside of the immediate post-exercise period (~24hrs post exercise) (Hojman et al., 2018; Metcalfe et al., 2021). There are currently a limited number studies that have cultured cancer cells with media supplemented with serum from exercise trained individuals at rest (see – Metcalfe et al., 2021 for review). A series of early studies (all using blood samples derived from the same cohort) observed that media enriched with 10% serum extracted 24hrs post exercise from men who regularly engaged in a community based exercise program induced 25-30% reductions in growth (Barnard et al., 2007, 2003; Leung et al., 2004; Tymchuk et al., 2002) and 2-25x increases in apoptosis (Barnard et al., 2007, 2003; Leung et al., 2004) compared media enriched with serum extracted from obese controls in prostate cancer derived LNCap and PC-3 cells (Barnard et al., 2007, 2003; Tymchuk et al., 2002).

Two recent studies extracting resting samples >24hrs post exercise have found no effect of supplemented media containing 10% serum extracted from colorectal cancer survivors who had participated in 12 sessions of high intensity interval exercise on the growth or apoptosis of CaCo-2 and LoVo cells (Devin et al., 2019); or media supplemented with 5% serum extracted healthy men who had been subjected to 36 sessions of high intensity interval cycling on the growth of MDA-MB-231 and LNCaP cells (Baldelli et al., 2021). The reason for the discrepancy between these studies is unclear (Baldelli et al., 2021; Barnard et al., 2007, 2003; Devin et al., 2019; Tymchuk et al., 2002), but may be related to factors such as the diseased nature of some participants (Barnard et al., 2007, 2007; Devin et al., 2019; Tymchuk et al., 2002) and the number of functional characteristics of cancer cells that were investigated (proliferation and apoptosis only) (Baldelli et al., 2021; Barnard et al., 2007, 2003; Devin et al., 2019; Tymchuk et al., 2002). All of these studies (Baldelli et al., 2021; Barnard et al., 2007, 2003; Devin et al., 2019; Tymchuk et al., 2002) have supplemented media with blood serum and not plasma (Metcalfe et al., 2021; Soares et al., 2021). This is principally due to the

phenomenon that blood plasma treated with citrate-based anticoagulants (which are highly common) produces coagulation when mixed with cell culture media (Calatzis et al., 2001). There are strategies which can ameliorate the coagulation stimulating effects of cell culture media on human plasma (namely - heparinisation of plasma or heat-inactivation of clotting factors). However said approaches may reduce then reduce the physiological relevance of plasma supplemented media and therefore sera remains preferentially studied (Allen et al., 2023).

Therefore, the first aim of the current study were to establish a method of conditioning cell-culture media with human plasma while avoiding induced coagulation and additional methods of plasma treatment. The second aim of the current study was to investigate whether media supplemented with plasma from healthy men with divergent exercise training histories influenced the results of four functional assays designed to mimic cancer hallmarks (proliferation, migration, extracellular matrix invasion and cell-death resistance) in the BT-549 triple-negative breast cancer derived cell-line (Lasfargues et al., 1978).

6.3. METHODS

6.3.1. Participants and study design

Men (n=38) who were endurance-trained (END; *n* = 13), strength-trained (STR; *n* = 13), and recreationally active controls (CON; *n* = 12) were recruited for this study. Based on the recently-proposed Participant Classification Framework (McKay et al., 2022), END was comprised of n=8 Tier 3/Highly-trained athletes and n=5 Tier 4/Elite athletes, whereas STR was comprised consisted of n=9 Tier 3/Highly-trained athletes and n=4 Tier 4/Elite athletes. The performance characteristics of these participants are described in Chapter 4. Briefly, participants arrived to lab having consumed a standardised meals (30 kcal/kg as 50/25/25 for carbohydrate, protein and fat; GourmetFuel™, Dublin, Ireland) in the 24hrs prior to arrival,

were asked to abstain from alcohol consumption for at least 24 h, arrived after an overnight fast and only having consumed water that morning, and were asked to abstain from exercise for at least 24 h (CON = 81 ± 108hrs; END = 45 ± 18hrs; STR = 53 ± 48hrs since last exercise, mean ± SD). Participants lay supine for 10 min before whole venous blood was drawn by inserting a 21G butterfly needle (Greiner, Bio-One, Austria) into an antecubital vein. The initial 4 mL of blood was drawn into a generic vacutainer and discarded. Subsequently ~50 mL of whole blood was drawn into six 9 mL blood collection tubes coated with ACD-A anticoagulant (Greiner, Bio-One, Austria). Blood samples were immediately placed on ice and centrifuged at 1500*g* for 15 min at 4ºC. Directly after centrifugation, plasma samples were separated into aliquots, and stored at -80ºC. Plasma samples were only defrosted once before being discarded.

6.3.2. Culturing of BT-549 cells

BT-549 cells were awoken from cryopreservation and cultured in T75 flasks containing Roswell Park Memorial Institute Medium - 1640 (RPMI) (Moore et al., 1967) containing 10% Fetal-Bovine-Serum (FBS), 5% L-Glutamate and 1% PenStrep. Cells were maintained in an incubator with fixed temperature of 37°C and 5% CO_2 and containing CuSO4. Cells were grown to ~90% confluence and then either seeded or split. Cells were maintained for no more than 10 passages.

6.3.3. Sodium Phosphatase Cell Viability (Proliferation) Assay

BT-549 cells were seeded in 96-well plate at a concentration of 6 x 10^3cells per 100µl, to allow cells to fix to the plate, cells were cultured in their original media for 24hrs. After which wells were treated with 200µl of one of the following 11 conditions – FBS-free RPMI (containing only 5% glutamate); FBS-containing RPMI (10% FBS, 5% glutamate); and RPMI (containing 5% glutamate) enriched with either 10, 15 or 20% human plasma from CON, END and STR participants. Each condition was applied to wells in quintuplicate and to determine whether there was an influence of incubation period replicates of cells were also incubated with

these respective experimental condition(s) for periods of 24, 48 and 72hrs, respectively. On the day of each individual cytotoxicity assay, media was discarded from all wells and cells were washed twice with 100µl of room temperature phosphate-buffered saline (PBS, Sigma-Aldrich, Cat. #: P8537). Subsequently, 100µl solution of phosphatase substrate buffer consisting of 10mM p-nitrophenol phosphate diluted in a 1M sodium acetate buffer (4.1g of sodium acetate (Sigma Aldrich, Cat. #: S5636)) diluted in 500 mL of dH_2O and 500µl of triton X (Sigma-Aldrich, Cat. #: T8787), pH = 5.5, solution made by adding 0.27g of) was added to each experimental well. 96 well plates were wrapped in foil and incubated with phosphatase substrate buffer for 1hr (at 37°C and 5% CO_2). Post-incubation, the phosphatase reaction was stopped by adding 50µl of 1mM NaOH (VWR chemicals, Cat. #: 27963.101), absorbance was then read at 405nm using the FlouStar Optima microplate reader (BMG Labtech, serial #: 413-2103). Cell Viability was estimated by plotting absorbance values of each condition as relative to the value obtained for the standard cell culture condition (RPMI containing 10% FBS , 5% L-Glutamate and 1% PenStrep).

6.3.4. Cell Migration Assay

BT-549 cells were seeded in a 24 well plate at a concentration of 6 x 10^4 per well in 500µl of 10% FBS containing RPMI media for 24hrs at 37°C and 5% CO_2. The following day, cells were investigated visually to ensure proper formation of a cell monolayer. Each well was then 'scratched' by dragging a P200 pipette tip from the top to the bottom of the well in a single straight line. To remove floating dead cells, FBS-containing media was discarded and each well was washed twice with 500µl of media containing only RPMI and 5% Glutamate. Cells were then treated with fresh RPMI media that was modified to create the following five conditions; 'serum-free' – containing only RPMI, 5% Glutamate and 1% PenStrep; 'FBS' which consisted 'serum-free' RPMI that also contained 1% FBS; and three human conditions which consisted of 'serum-free' RPMI and also contained 10% of human plasma from a CON, END or STR

participant, respectively. After treatment, each well was immediately visualised using a 10x objective lens using an Olympus IX81 inverted microscope, this visualisation constituted '0hrs' and at this time, a suitable region of the 'wound' was identified in each well and marked using a permanent marker. Cells were returned to an incubator and visualised again at this exact region at 24hrs and 48hrs post scratch. Cell migration was quantified in imageJ using a bespoke plugin designed for wound-healing assays (Suarez-Arnedo et al., 2020).

6.3.5. Invasion Assay

Extracellular matrix (ECM) (Sigma-Aldrich, Cat. #: E1270) diluted in serum-free RPMI at a concentration of 1mg/mL was thawed at 4°C. 200µl of ECM was added to polyester transwell inserts with 8µm pore sizes (Falcon, Cat. #: 353097) and left to incubate overnight (37°C and 5% CO_2). The following day, BT-549 cells were seeded to each insert at a concentration of 8 x 10^4 cells in 200µl of 10% FBS containing RPMI. Cells were incubated in inserts for 24hrs at 37°C and 5% CO_2, afterwards FBS containing media was removed from the inserts, which were washed twice with 200µl of serum-free RPMI and then inserts were treated with 200µl of one of the following five conditions; serum-free RPMI; 1% FBS containing RPMI; and 10% containing human plasma RPMI from CON, END or STR participants, respectively. 500µl of RPMI containing 10% FBS was also added to the well surrounding each insert to promote cell invasion through the ECM. Cells were then incubated for 24 or 48hrs respectively at 37°C and 5% CO_2, Post-incubation, inserts were washed with a PBS soaked Q-tip and incubated for 10 minutes with 0.1% crystal violet (diluted in DiH2O) on a rocker at room temperature. Post incubation – inserts were washed again by being gently submerged in bath of PBS, inserts were dried passively on paper and then visualised and imaged using a 10x objective lens using an Olympus IX81 inverted microscope to confirm ECM invasion. To quantify the amount of invading cells, inserts were placed in 10% acetic acid (Sigma, Cat. #: 338826) for 10 minutes on a rocker, to elute the crystal violet. 100ul of elute from each

supplemented well was added to a 96 well plate and the absorbance was read at 595nm using a FluorStar Optima microplate reader. Cell invasion was quantified as being relative the result of the SFM treated control condition which was assumed *a-priori* to promote the greatest amount of invasion.

6.3.6 Anoikis Resistance Assay

To prevent cell adhesion, a 24-well plate was coated 200µl of 12 mg/mL poly (2-hydroxyethyl methacrylate) [poly-HEMA] (Sigma-Aldrich, Cat. #: P3932) diluted in 95% ethanol or a control condition of just 95% ethanol and left in a fume hud overnight. This process was repeated twice, after wells were seeded with BT-549 cells at a concentration of 6×10^4 per well in 500µl of 10% FBS containing RPMI. After seeding, wells were separated into five separate conditions by increasing the total amount of media in each well to a concentration of 600µl through the addition of 100µl of either; Serum-Free RPMI (containing only 5% L-Glutamate, SFM condition); 10% FBS; or 100µl of human plasma taken from either CON, END or STR participants. Cells cultured in the 95% ethanol control (i.e. could adhere to the plate) were cultured in 600µl of 10% FBS. Cells were incubated in suspension for either 24 or 48hrs, after wells were incubated with 60µl of almarBlue dye for 3.5hrs. Cell Survival was determined by reading plate(s) at an absorbance of 570nm on FluoStar Optima microplate reader. Cell survival was quantified as relative to the 95% ethanol control (assumed to be 100% cell survival).

6.3.7. Statistical Analysis

Data were analysed using in R using mixed linear models assembled with the 'Rstatix' package. A mixed model using the following formula for the initial proliferation experiment examined the main effect terms for Group Membership (i.e. CON,END,STR or SFM/FBS controls), Percent of Human Plasma dilution (10,15,20%), Incubation Duration (24,48 or 72hrs) and the interaction of these three main effects (Group * Plasma Dilution * Incubation

Duration). Data from the remaining functional assay experiments (Cell Migration, Anoikis Resistance and Extracellular Matrix Invasion) were analysed with models that examined main effect terms for Group Membership and Incubation Duration (24hr, 48hr) and their interaction (Group * Incubation Duration). On significant main effects, post-hoc differences were examined using a series of pairwise T-tests. Results were considered statistically significant if they rejected an α level of < 0.05.

6.4. RESULTS

6.4.1. Cell Viability assay

The results of Sodium Phosphatase Cell Viability Assay are presented in Figure 6.1. Significant main effects were observed for Group ($F = 4.92$, $p < 0.01$, $\eta_p^2 = 0.08$) and Incubation Duration ($F = 13.2$, $p < 0.01$, $\eta_p^2 = 0.18$) but no main effect was observed for the dilution percentage of human plasma ($F = 1.21$, $p < 0.31$, $\eta_p^2 = 0.01$) and no Group * Incubation interaction ($F = 0.136$, $p = 0.97$, $\eta_p^2 = 0.005$) or Group * Plasma Dilution percentage was observed ($F = 0.123$, $p = 0.97$, $\eta_p^2 = 0.004$). Post-Hoc tests for Group indicated that dilution of RPMI with plasma from CON ($p < 0.01$, mean difference = 24hr - 41%; 48hr; 72hr – 25%), END ($p < 0.01$, mean difference = 24hr – 16%; 48hr – 41%; 72hr – 25%) and STR ($p < 0.05$, mean difference = 24hr – 7%; 48hr – 33%; 72hr – 10%) had increased viability compared to SFM. The STR ($p < 0.01$, mean difference = 24hr – 16% ; 48hr – 5%; 72hr – 33%) and SFM groups ($p < 0.01$, mean difference = 24hr – 23% ; 48hr – 40% ; 72hr – 43%) were also observed to have lower viability compared to the 10% FBS control. Post-Hoc tests on the main effect of Incubation indicated that all human plasma groups had lower viability at 72hrs compared to 48hrs ($p < 0.01$, mean difference = CON - 25%; END – 24%; STR = 28%). The SFM group also had lower viability at 48hrs ($p = 0.01$, 16% difference) and 72hrs ($p < 0.01$, 20% difference) compared to 24hrs.

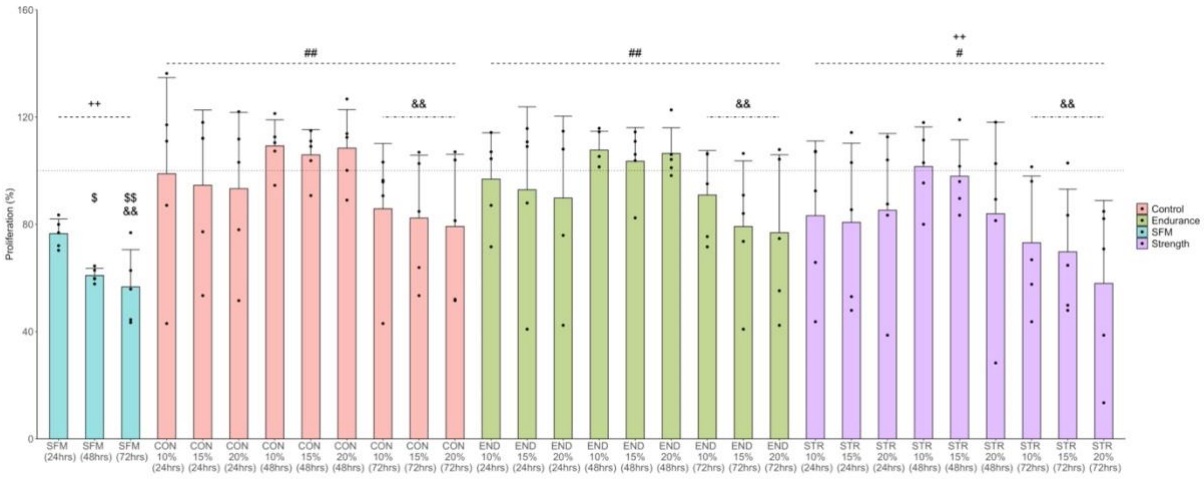


Figure 6.1. The results of a Sodium Phosphatase Cell Proliferation Assay comparing cell culture media diluted with 3 concentrations of human plasma (10,15,20%) from CON, END and STR participants across 24, 48 & 72hrs respectively. Bar height represents group mean and error bars represent standard deviation. & indicates a significant difference from 48hrs within groups, $ indicates a significant difference from 24hrs within groups, # indicates significantly different from SFM group, + indicates significantly different from FBS control (100% line) (all $p < 0.05$). A single presentation of a symbol represents $p < 0.05$, a double presentation of any symbol (e.g. ##) indicates $p < 0.01$.

6.4.2. Cell Migration Assay

The results of the Cell Migration Scratch Assay are reported in Figure 6.2. For the percent of cell migration, significant main effects were observed for Group ($F = 4.05$, $p < 0.01$, $\eta_p^2 = 0.18$) and Duration ($F = 91.35$, $p < 0.01$, $\eta_p^2 = 0.55$). No Group * Duration interaction effect was observed ($F = 0.97$, $p < 0.42$, $\eta_p^2 = 0.05$). Post-hoc tests on the main effect of Group observed a significant difference between all groups and the SFM condition at 48hrs only (FBS - $p < 0.01$, mean difference = 22%; CON - $p = 0.01$, mean difference = 13%; END – $p < 0.01$, mean difference = 17%; STR – $p = 0.03$, mean difference = 8%). Post-hoc tests on the main effect of Duration observed a significant difference between 24hrs and 48hrs ($p < 0.01$ mean difference = SFM – 15%; FBS – 30%; CON – 25%; END – 27%; STR – 24%). Post-hoc tests on the main effect of Duration observed a significant difference between 0hrs and 24hrs ($p < 0.01$, mean difference = SFM – 37%; FBS – 52%; CON – 42%; END – 44%; STR – 35%) and 24hrs and 48hrs ($p < 0.01$, mean difference = SFM – 25%; FBS - 79%; CON – 56% ; END – 64% ; STR – 47%).

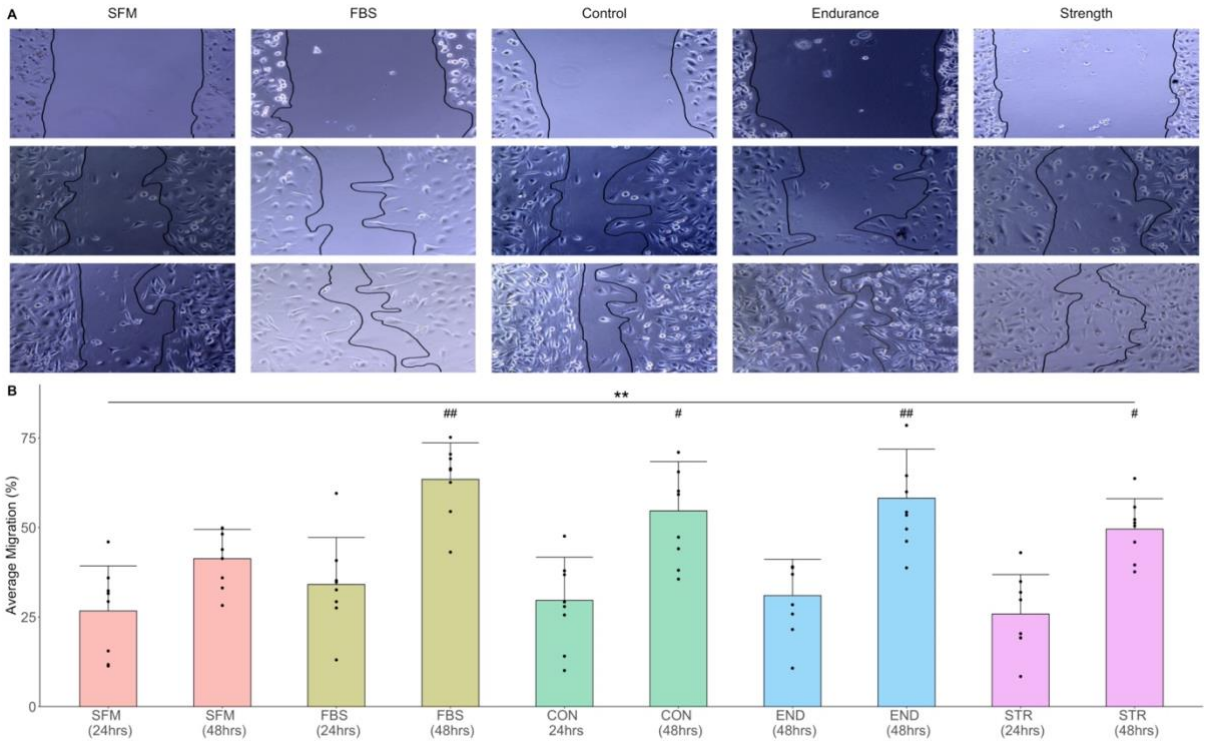


Figure 6.2. The results of a scratch assay promoting cell migration – cells were scratched and then incubated with SFM, 1% FBS containing RPMI or RPMI containing 10% of plasma from CON, END or STR participants. Images were taken at 0hrs, 24hrs & 48hrs respectively. Bar height represents group mean and error bars represent standard deviation. **A.** Representative images from each condition. **B.** The average cell migration (change score from 0hrs as a percentage) at 24hrs and 48hrs. # indicates a significant difference from SFM condition at 48hrs. * indicates a significant difference between 24 and 48hrs within groups. A single presentation of a symbol represents $p < 0.05$, a double presentation of any symbol (e.g. ##) indicates $p < 0.01$.

6.4.3. Extracellular Matrix Invasion Assay

The results of the Extracellular Matrix Invasion Assay are displayed in Figure 6.3. A main effect was observed for Group ($F = 7.94$, $p < 0.01$, $\eta_p^2 = 0.27$). No significant main effect was observed for Incubation ($F = 3.10$, $p = 0.08$, $\eta_p^2 = 0.03$). Post-Hoc testing on the significant main effect for group observed that the CON and STR groups all displayed significantly lower invasion than the SFM group at 24hrs (CON = $p < 0.01$, mean difference = 14%; STR $p = 0.01$, mean difference = 10%,). CON, END and STR groups all had significantly lower invasion compared to the SFM control at 48hrs ($p < 0.01$ for all groups, CON mean difference = 18%; END mean difference = 20%; STR mean difference = 16%). No significant difference was

observed between the 1% FBS group and SFM at either timepoint and no significant differences between human plasma conditions were observed ($p > 0.05$).

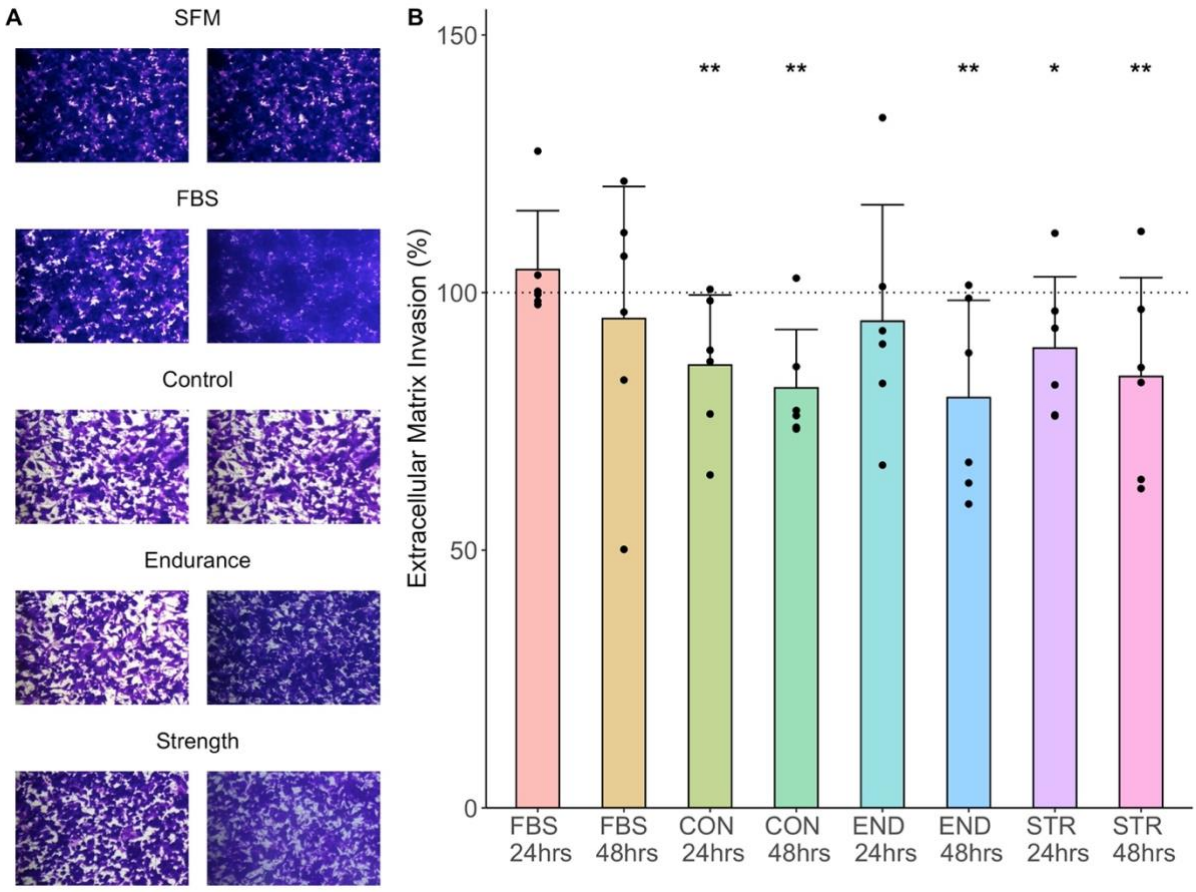


Figure 6.3. The results of extracellular matrix invasion assay. Bar height represents group mean and error bars represent standard deviation. Bar height represents group mean and error bars represent standard deviation. **A.** representative images from each condition – left hand panel represents 24hrs, right hand side panel represents 48hrs. **B.** Results of four biological replicates of colorimetric quantification of matrix invasion. ** represents a significant difference from SFM condition which is visualised as the 100% horizontal line.

6.4.4. Anoikis Resistance Assay

The results of Anoikis resistance assay are presented in Figure 6.4. A main effect was observed for Group ($F = 5.2$, $p < 0.01$, $\eta_p^2 = 0.34$) and Duration ($F = 24.04$, $p < 0.01$, $\eta_p^2 = 0.36$). No significant Group * Duration interaction was observed ($F = 2.18$, $p = 0.08$, $\eta_p^2 = 0.18$). Post-hoc tests on the main effect of Group demonstrated significantly greater anoikis for all human plasma conditions compared to SFM ($p < 0.01$ for all groups, mean difference – CON = 17%; END = 17%; STR = 19%) and FBS ($p < 0.01$ for all groups, mean difference –

CON = 14%; END = 14%; STR = 16%) at 24hrs only. The amount of anoikis increased significantly between 24hrs and 48hrs for the SFM ($p = 0.01$, mean difference = 20%), FBS ($p < 0.01$, mean difference = 16%) and CON groups ($p = 0.04$, mean difference = 10%).

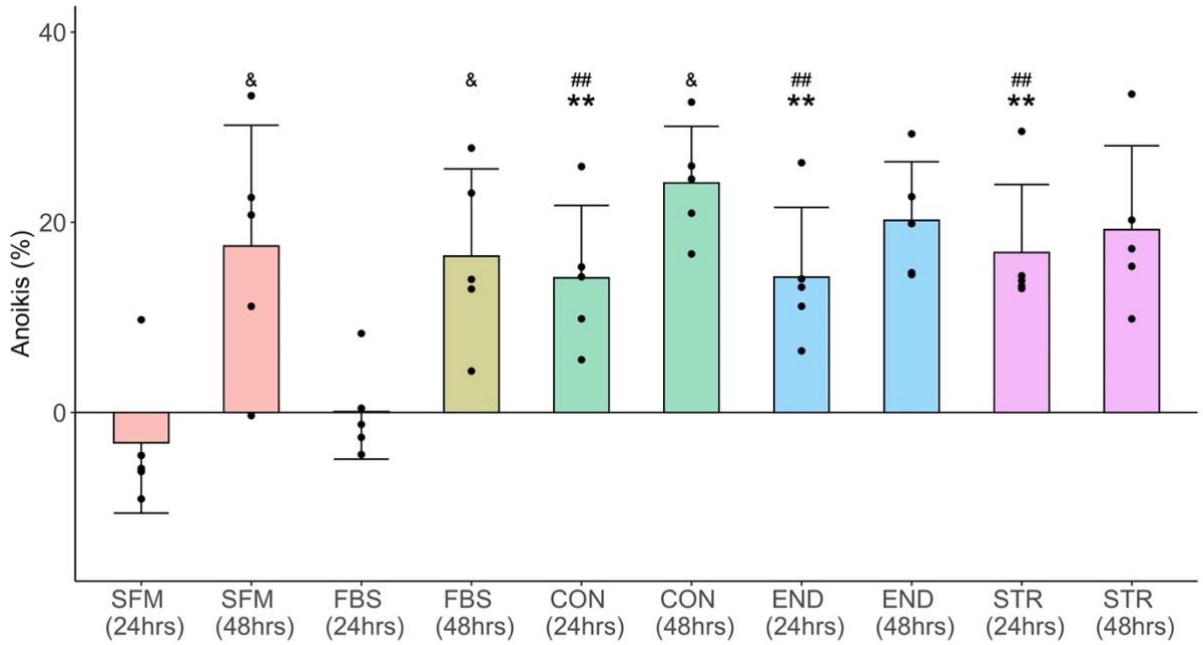


Figure 6.4. The results of incubation with media containing 15% plasma from CON, END and STR groups or 15% additional SFM or 10% FBS RPMI colorimetric Anoikis Resistance assay. Bar height represents group mean and error bars represent standard deviation. * represents a difference from SFM condition, # represents a difference from the 10% FBS condition. & represents a difference between 24hr and 48hrs duration within a given group. A single presentation of a symbol represents $p < 0.05$, a double presentation of any symbol (e.g. ##) indicates $p < 0.01$.

6.5. DISCUSSION

The collective purpose of the current study was to examine whether culturing BT-549 cells with media supplemented with plasma derived from healthy men who had a history of either Endurance Exercise Training, Strength Exercise Training or Recreational Activity (controls) influenced results of four functional assays designed to mimic hallmarks of cancer cell activity (proliferation, migration, cell-death resistance and extracellular matrix invasion). Plasma supplemented media was successfully produced and for each of the four functional assays, effects of culture with human plasma were observed. Although no robust indication(s) that any effects mediated by human plasma varied between CON, END or STR participants.

6.5.1. The successful establishment of plasma supplemented cell-culture media

The first aim of the current study was to successfully supplement cell culture media with human plasma (i.e. without clot formation). A successful approach for supplementing media with human plasma across varying plasma concentration(s) (10-20%) was found by mixture with RPMI media (Moore et al., 1967). This is not a novel achievement, but conditioning media with human plasma is challenging, but worthwhile due to the greater physiological relevance of plasma compared to sera (See Allen et al., 2023 and explanation below).

The issue with conditioning culture media with human plasma is related to fact that citrate (a popular anticoagulant), does not directly inactivate clotting factors. Instead, citrate inhibits clotting by chelating calcium, which is an essential substrate for fibrin formation (Oudemans-van Straaten et al., 2006). This means clotting factors are 'dormant' (instead of inactivated) in plasma produced with citrate-based anticoagulants and can therefore be reactivated with the re-introduction of sufficient calcium. Calcium is present in many common cell culture media types such as Minimal Essential Medium (MEM) (Eagle, 1959) and RPMI (Moore et al., 1967). Thus, mixture of plasma and cell culture media can stimulate clotting when insufficient citrate remains present to chelate the calcium present in media. The current study was initially attempted using Hs578T cells that are cultured in DMEM (which contains 200mg/L calcium chloride) (Eagle, 1959). This caused substantial clotting and rendered any attempts at experimentation impossible. It has previously been reported that reducing the calcium concentration of solutions mixed with plasma can reduce or prevent plasma clot formation (i.e. suggesting that the ratio of unchelated citrate/free calcium is what will determine whether plasma will clot or not when mixed with cell culture media) (Calatzis et al., 2001). Using this information, the parameters of the experiment were altered to instead use BT-549 cells as these are cultured with RPMI-1640 (which contains 100mg/L, Calcium Nitrate) (Moore

et al., 1967). With this transition, clotting was successfully eradicated, and we observed no incidences of clotting in any experimental replicates. This is notable, simply because we were able to successfully condition RPMI media with human plasma (with concentrations ranging from 5-50% human plasma in pilot work) without additional intervention(s) that are often recommended when attempting to condition cell culture media with human plasma (namely - heparinisation, recalcification of plasma prior to conditioning media or heat inactivation of clotting factors) (Ayache et al., 2006). It is desirable to avoid these approaches as each may alter the physiological relevance of plasma samples and thus influence experimental results (Allen et al., 2023; Ayache et al., 2006).

6.5.2. Investigate the influence of plasma supplemented media on proliferation, migration, anoikis-resistance and invasion assays designed to mimic cancer hallmarks in BT-549 cells

After successfully supplementing RPMI media with human plasma, it was then sought to determine whether incubating BT-549 cells with media containing different concentrations (10%,15%,20%) of human plasma for varying durations (24hr, 48hr and 72hr) would affect BT-549 cell proliferation. Cell proliferation was determined using an acid phosphatase assay that is based on the cellular conversion p-nitrophenol phosphate to p-nitrophenol – the quantity of which can be detected by reading absorbance at 405nm; this assay correlates strongly with cell number (determined via hemocytometer counts) and is sensitive enough to detect as few as 1000 cells per well (Yang et al., 1996). Human plasma concentrations and incubation durations were informed by what has previously been reported to induce measurable effects of proliferation prostate and breast cancer cell lines (conditioning media with 5-10% of human sera and 24-72hr incubations) (Metcalfe et al., 2021; Soares et al., 2021). For this assay, cell proliferation increased in all human plasma conditions (CON, END, STR) and incubation periods relative to a SFM control with the increases ranging from ~20-50% in a manner that depended on the incubation duration and not plasma concentration. The greatest increase in

cell viability being found at 48hrs for all groups. Within each individual group (SFM, CON, END, STR) – cell viability decreased between 48hrs and 72hs of incubation by ~15-20% implying this incubation period may be too long for maximum results on this assay. Collectively, these results demonstrate that plasma from healthy human males at rest promotes cell proliferation in BT549 cells in comparison to SFM and that exercise training status has no influence on this proliferative effect.

Initially, these results may be considered in contrast with earlier work demonstrating that LNCaP cells incubated for 48hrs with media supplemented with serum derived from men who participate in a community exercise program (i.e. are purportedly 'exercise trained') had ~ 40-60% lower cell proliferation compared to obese sedentary controls determined via cell-counting on a hemocytometer (Tymchuk et al., 2002, 2001) or various colorimetric assays assessing proliferation (Barnard et al., 2007, 2003; Leung et al., 2004). However, all five of these studies employ samples from the same cohort of participants and do not report how recently post-exercise serum samples were extracted (Barnard et al., 2007, 2003; Leung et al., 2004; Tymchuk et al., 2002, 2001). This is important to note, as it has previously been demonstrated that media supplemented with 5% serum extracted from healthy men 24hrs post an acute exercise bout significantly decreases absolute cell counts of both LNCaP and MDA-MB-231 (determined via hemocytometry count) by 12-24% (depending on whether the serum was extracted 0hrs, 12hrs or 24hrs post exercise); whereas media supplemented with serum samples taken 72hrs post exercise in the same participants had no influence on cell count number, even after 9 weeks of high intensity exercise training (Baldelli et al., 2021). Therefore, the decreased proliferation of LNCaP cells previously reported by other 'resting' studies (Barnard et al., 2007, 2003; Leung et al., 2004; Tymchuk et al., 2002, 2001), may not be an effect of exercise 'training' *per se*, as the residual influence of an acute exercise bout cannot be entirely discounted. Furthermore, a notable observation regarding examples where the 'resting'

samples from exercise trained participants have produced decreased cell proliferation is that the (sedentary) control participants in these studies tend to produce relative (%) proliferation values of~100-110% of an FBS control over a 48hr incubation (Barnard et al., 2007, 2003; Tymchuk et al., 2002, 2001). These values are similar to the 48hr incubation results from all groups incubated with media supplemented with resting human plasma in the current study (Figure 6.1.) and could suggest the results of increased proliferation previously attributed to the sedentary lifestyle control participants (Metcalfe et al., 2021) – is in fact more related to the fact that control participants were not in an acutely (i.e. 24hr post) exercised state (Barnard et al., 2007, 2003; Leung et al., 2004; Tymchuk et al., 2002, 2001).

To assess BT-549 cell migration, a scratch assay was performed with images taken at 0,24hrs and 48hrs (Figure 6.2). For all conditions, significant migration of BT-549 cells was observed between 24-48hrs. Greater cell migration was observed in the 1% FBS control condition and 10% CON, END and STR human plasma supplemented media conditions compared to the SFM control at 48hrs, with no differences observed between human plasma conditions or between any groups at 24hrs. In comparison to various proliferation assays, considerably less information exists regarding the influence of media supplemented with human plasma/serum on cell migration. Media supplemented with 1% of commercially acquired pooled human sera produces similar migration in both HeLa and SiHa cervical cancer cell lines compared to a 1% FBS condition (Heger et al., 2018). This study did not report the results of a SFM control condition, although the observation that media supplemented with human sera promoted similar migration to a 1% FBS control is consistent with the current study and may further suggest that media supplemented with resting human plasma conveys nourishing or beneficial effects to cancer cells *in vitro*.

With respect to the ECM invasion assay, media supplemented with 10% plasma from CON and STR groups produced lower ECM invasion (ranging from 10-20%) at both 24hrs and

48hrs. Significantly lower invasion was only observed for the END group at 48hrs (20% lower invasion). Similarly to other assays, no significant differences were observed between CON, END and STR conditions, implying that the reduction in invasion elicited by human plasma supplemented media was also not influenced by the exercise training history of the participants. There is limited available work examining whether human sera/plasma supplemented media influences ECM invasion across cancer cell-lines. One study has previously examined whether media supplemented with 10% (commercially purchased) pooled human serum altered the ECM invasion of HeLa and SiHa cervical cancer cells. These researchers observed that human sera increased invasion by 40% and 20% respectively compared to a 10% FBS control (Heger et al., 2018). The difference in these results may be explained by differences in the establishment of the ECM invasion assay between the current study and that of Heger and Colleagues. In the current study, 10% human plasma was added to the ECM containing-transwell 24hrs after cells were seeded, after which 10% FBS containing media was placed around the transwell to encourage cells to migrate through the ECM. In contrast, Heger and colleagues seeded all cells in SFM media and instead investigated whether adding 10% human supplemented serum *around the transwell* would alter invasion in comparison to surrounding the transwell with 10% FBS (Heger et al., 2018). The current study opted to add plasma directly to the cells (i.e. *within*, instead of around, the transwell), as the purpose of the current study was more concerned with whether *culture* of BT-549 cells with media supplemented with human plasma would alter BT-549 invasion through the ECM. In contrast, Heger and colleagues were more concerned in seeing whether human sera acted as a stronger signal for cell migration compared to 10% FBS (Heger et al., 2018). However, this does mean that within the current study, it is difficult to infer whether incubation with media supplemented with 10% human plasma reduced the competence of BT-549 cells to the point where they had a lower capacity for ECM invasion (i.e. had an 'anti-cancer' effect) – or was simply sufficiently

nourishing enough (as could arguably be evidenced by the results of the proliferation assays performed in the current study) for BT-549 cells to reduce the 'motivation' for cells to migrate through the ECM. Further and more detailed experimentation is required to determine which situation is more likely to be the case with respect this assay.

Anoikis is a form of programmed cell death that is initiated when cells become detached or inappropriately attached to their relevant extracellular matrix, resistance to anoikis is essential for tumour metastasis (Taddei et al., 2012). BT-549 cells are derived from the epithelial cells of a metastatic ductal tumour and therefore possess anoikis resistance (Lasfargues et al., 1978). The current study observed that suspended BT-549 cells that had their culture media supplemented with 15% human plasma had significantly higher rates of anoikis (i.e., *reduced anoikis resistance*) of ~14-19% compared to cells treated with additional SFM or 10% FBS, with no difference observed between CON, STR or END conditions. However, this effect of human plasma was only observed with 24hrs incubation and no significant differences in anoikis were observed between human plasma and control conditions at 48hrs. Therefore, media supplemented with human plasma may have only accelerated an inevitable induction of some anoikis in BT-549 cells. It has previously been reported that anoikis will occur in control conditions of BT-549 cells, with activation of cleaved caspase 3, 8 and 9 anoikis values of 8-12% has been observed in suspended BT-549 cells after 12.5hrs incubation in 2% FBS RPMI media (Zheng et al., 2020). The delay in anoikis in an FBS control condition in the current study may be due to the greater amount of FBS used by the current study (10%), particularly as the parameter of the SFM control was the *addition* of 100µl of SFM to wells already containing media that was enriched with 10% FBS. It is unclear why media supplemented with human plasma would cause an earlier induction of anoikis, particularly when the results of the cell proliferation and migration assays (which suggested a 'nourishing' effect of human plasma) are considered. No study has previously investigated the influence of media supplemented with

human sera/plasma on anoikis resistance. However, three studies have reported that incubation with media supplemented with 10% human serum for 48hrs induces apoptosis in LNCaP cells (Barnard et al., 2007, 2003; Leung et al., 2004) with increased p53 protein expression also measured in one circumstance (Leung et al., 2004). In contrast, 72hrs incubation with media supplemented with 10% resting and post-exercise serum from men has also been reported not to induce apoptosis in CaCo2 or LoVO cells, although the participants in that study were colorectal cancer survivors (Devin et al., 2019). Therefore, there is uncertainty with respect to how robust the influence of supplemented media enriched with healthy human plasma on anoikis and other cell-death pathways is, or by what mechanisms human plasma may induce potential acceleration to cell-death pathways in cancer cell lines.

6.5.3. Summary Remarks

The purpose of the current study was to investigate whether media supplemented with human plasma from CON, END and STR participants influenced four functional assays designed to be reflective of cancer hallmarks. Across all four assays no difference between CON, END or STR groups was observed, suggesting that exercise training history may not be a factor that alters the effects of human plasma supplemented media when plasma samples are extracted at rest, at least when participants are otherwise healthy. However, the extent to which inference can be made with respect to how exercise training history influences the effects of human plasma supplemented media are limited in the current study by the absence of acute exercise control conditions, as the influence of sera/plasma from exercised individuals (whether they have a history of exercise training or not) appear robust (Metcalfe et al., 2021; Soares et al., 2021) and it is plausible that media supplemented with exercised plasma extracted from individuals with an extended history of exercise training could convey altered effects to exercised plasma from sedentary or recreationally active controls. While no divergent effects between CON, END and STR groups were observed, ‘generic’ effects of media supplemented

with human plasma were observed across all functional four assays. In some circumstances, these produced results that were clearly beneficial to BT-549 cells (in the case of the proliferation and migration assays); However, for the anoikis and ECM invasion assays, results were less clear regarding whether media supplemented with human plasma had negligible, beneficial, or deleterious effects to BT-549 cells. These mixed results also highlight further limitations of the current study, as more detailed experimentation including factors like the expression of relevant proteins (e.g., program mediated cell death related proteins for the anoikis assay) may have provided greater clarity into confounding results. Lastly, the current study is limited by the selection of only using one cell line, which reduces how generalised any inferences regarding the influence of media supplemented with healthy human plasma to other types of cancer cells. In conclusion, the current study does provide indication that media supplemented with human plasma can exert influence on BT-549 cells, but further research is required to more clearly determined the directionality of said influence and whether it contains physiological relevance in more integrated settings.

Chapter 7
General Discussion

7.1. MAIN RESEARCH FINDINGS

The purpose of this thesis was to perform a broad exploration of how a subset of circulating exercise factors are altered at rest in association with exercise training by investigating several issues that underpin a potential 'exercise factor' response to exercise training. Using metabolites as a selected exercise factor 'type', this thesis specifically examined: whether a short-term exercise training intervention or a divergent exercise training background influenced of the presence of targeted panels of metabolites in sera/plasma and preparations of small EVs at rest (Chapters 3, 4 and 5 respectively); whether a targeted profile of plasma metabolites and the abundance of small EVs at rest were durable among individuals with divergent exercise training histories at rest (Chapters 4 & 5); and whether media conditioned with plasma derived from individuals with divergent exercise training histories at rest influenced the performance of BT-549 cells on functional assays that are designed to be reflective of cancer hallmarks (Chapter 6).

Each specific study and its respective key findings are briefly summarised below:

Study 1

Nine sessions of SIT training elicited no changes in a targeted profile of the serum metabolome when estimated via multilevel PCA, but with univariate analysis, 11 fatty acids were identified to have significantly lower concentrations (FDR < 0.05) in post-training serum samples. These fatty acids were ontologically associated with altered fatty acid biosynthesis.

Study 2

Under standardized measurement conditions (24hr dietary intake, proximity from the most recent exercise bout, time of day) the reliability of plasma metabolite concentrations varied largely at the level of individual metabolites with ~48% of metabolites displaying *good*-

to-*excellent* reliability. A history of exercise training was associated with alterations in the abundance of ~28% (n = 44) of metabolites in the targeted profile employed.

Study 3

No indication of any differences in the abundance of small EVs between groups or between visits within groups were detected across multiple methods of small EV identification (Nanoparticle Tracking Analysis, amnis flow cytometry, Western blot of specific EV markers and contaminants). Targeted metabolomics of small EVs identified 96 distinct metabolites that were associated with the structure and function of small EVs and had consistent concentration(s) between groups.

Study 4

BT-549 cells cultured with media conditioned with plasma derived from men with divergent exercise training histories had increased cell proliferation (~20-50% increase compared to SFM), greater migration (15-20% greater migration compared to SFM), greater Anoikis 15-20%) and lower extracellular matrix invasion (10-20%) at 24hrs and 48hrs. Therefore, resting plasma extracted from healthy males arguably demonstrated 'nourishing' effects for proliferation and migration assays and deleterious effects observed for Anoikis resistance and invasion assays, but these effects are not influenced by exercise training history.

7.2. ESTABLISHING EXPERIMENTAL CONTROLS THAT INCREASE CONFIDENCE IN HOW ATTRIBUTABLE RESULTS ARE TO EXERCISE TRAINING

When studying the idea that exercise factors respond to exercise training at rest, there are numerous confounding factors that are important to consider in order to ensure that any observation of differentially abundant exercise factors can be confidently attributed to a physiological response to *exercise training.* Examples of confounding influences that could be particularly relevant when studying exercise factor responses to exercise training include physiological influences such as the residual effects of the most recent acute exercise bout

(section 2.1.7) (Chow et al., 2022; Haskell, 1994); variability in sample acquisition (e.g. blood draw repetition), storage and transport (duration between acquisition and storage, duration stored for, duration a sample is thawed before analysis) (Lippi et al., 2005; Mazzocca et al., 2012; Yin et al., 2015) procedures; and analytical variability (e.g., 'batch' differences in 'omics' style analysis or assay variability using more traditional techniques like ELISAs or Western Blot) (Han and Li, 2022). As a means of endeavouring to ensure that the studies contained within this thesis possessed sufficient rigour in terms of experimental control to legitimately study an exercise factor response to exercise training, this thesis employed three experimental design features (namely - controlling for proximity from most recent exercise bout; 24hr dietary control; and acquiring samples under consistent measurement conditions across two separate days) that are noteworthy to acknowledge prior to the discussion of results.

Controlling for the 'residual influence' of acute exercise bouts by having a sample acquisition that is at least >24hrs post-exercise is acknowledged as important for studies that are aiming to investigate the influence of exercise training (Haskell, 1994; Hecksteden et al., 2018b, 2018a), particularly in the case of exercise factors (Chow et al., 2022). However, it is also common for experimental studies investigating the response of exercise factors (or small EVs as exercise factor carriers) to exercise training to regularly either not control for or not report controlling for the proximity of sample acquisition from the most recent exercise bout (see tables 2.1. and 2.3. for examples). In both chapter 3 and chapter 4, participants were asked to refrain from exercise for periods >24hrs prior to any blood sample extraction. However, chapter 3 is limited by the fact that exact estimates of when the last exercise bout was performed was not strictly recorded (participants were simply asked to return to the lab 2-3 days after their last training bout). In the case of chapter 4, participants were strictly reminded two mornings before each lab visit to cease exercise after the morning of that day and at each laboratory visit the exact time of each participants most recent bout was recorded. The exact recording of

approximately when participants most recently exercised is not conventional, but is simple to do and highly informative for the assessment of studies that are concerned with the influence of exercise training on exercise factors and is a strength of this thesis. Although, in the case of the studies contained within chapter 3 and chapter 4 it is not possible to determine the extent to which participants adhered to abstaining from exercise. However, the emphasis of the importance of abstinence from exercise and reminders that were sent to participants would have acted as strong encouragement for participants to adhere to the study requirements.

Dietary control is of particular importance for studies that are concerned with metabolites as exercise factors, as acute variations in dietary intake can produce different metabolomic 'signatures' in plasma and urine (Guasch-Ferré et al., 2018). Therefore, when studying metabolites as exercise factors, control of recent dietary intake increases confidence that differences are more attributable to exercise training (Belhaj et al., 2021), instead of dietary habits, which may vary between individuals with different exercise training backgrounds (Burke et al., 1991). Dietary control is also useful for studies that examine small EVs, as common EV contaminants (i.e. lipoproteins) are sensitive to dietary lipid intake and therefore can influence preparations of small EVs (Mørk et al., 2018, 2016). In both chapter 3 and chapter 4 attempts at dietary control were administered. In chapter 3 participants completed a week long food diary and then were asked to maintain a consistent day of habitual eating the day before each blood sample was set to be acquired, which was then assessed by 24hr recall. While use of dietary recall is better than no measurement, 24hr dietary recall also generally underestimates energy intake (Rutishauser, 2005). However in chapter 4, 24hr nutrition was controlled for via the provision of a set of professionally prepared meals that had consistent relative energy content (30kcal/kg bodyweight) and macronutrient ratios (50% carbohydrate, 20% protein, 20% fat), which is more robust than dietary intake and may 'homogenize' the influence of recent dietary intake across participants (as participants were consuming the same

exact meals, just in smaller or larger portion sizes based on their individual body mass). In both chapter 3 and chapter 4, participants had samples extracted in an overnight fasted state, and in the case of chapter 4 the time since participants consumed their last meal was recorded.

In the case of the samples extracted in chapter 4 – which were subsequently used for chapter 5 and chapter 6, participants were asked to repeat the same procedures (in terms of cessation from exercise and dietary intake) on two separate occasions (generally a week apart for most participants). This repetition of identical experimental procedures enabled investigation into potential differences in the abundance of metabolites or the presence of small EVs, remained durable across different measurement days where the fitness of the participants was unlikely to be dramatically change (i.e. a week) and enables some degree of inference as to whether differences between individuals with divergent exercise training histories were potentially durable (i.e. attributable to data variability caused by exercise training, instead of other factors) (Hecksteden et al., 2018b). While collectively, these controls (recent exercise bout, dietary intake and repeated sampling across several days) are not unique to this thesis, they are uncommon in comparison to other work that is concerned with investigating the influence of exercise training on the presence of exercise factors or small EVs at rest and do increase the robustness of data discussed in subsequent sections of this chapter.

7.3. THE INFLUENCE OF EXERCISE TRAINING ON THE PROFILE OF THE CIRCULATING METABOLOME

A key finding of this thesis is that across three metabolomic analyses (performed on two 'samples' of participants) there was no indication that exercise training elicited changes in the resting 'profile' of targeted metabolite panels (with Chapters 3 and 4 using the same panel of metabolites and Chapter 5 using a slightly different panel) when latent variable structures (i.e. principal components that were inferred to be 'reflective' of the totality of the metabolite panel) were constructed using various forms of PCA. There are two obvious interpretations of these observations: firstly, that there are no distinct 'metabolic fingerprints' of exercise training

that manifest at rest; and secondly, that the collective 'profile' of metabolites as exercise factors was not largely different across each experiment.

It is entirely feasible that both of these concepts could be true simultaneously. i.e. some metabolites acting as exercise factors are differentially abundant at rest, while some metabolites, notionally without obvious 'bioactivity', are also differentially abundant as the result of altered metabolism at rest and therefore, contribute as residual signals to a 'metabolic fingerprint' of exercise training. However, techniques that involve the construction of latent variable estimates (i.e. PCA, PLSDA and other similar algorithms) (Mazzocca et al., 2012) are likely only to be appropriate for investigating whether there are 'metabolic fingerprints' of exercise training and not whether metabolites *as a collective group of exercise factors* are changed in response to exercise training (for reasons argued in the next subheading).

To this point, the results obtained in this thesis produce no evidence of either a 'generic' (i.e. in exercise-trained, compared to untrained – regardless of training history) or 'divergent' (different profiles that are associated with specific types of exercise training) metabolic fingerprints of exercise training that manifests at rest. This finding contrasts with several previous reports (summarised in Table 2.1). However, when only studies for which the presence of residual effects of an acute exercise bout can be discounted with any degree of certainty (Duft et al., 2017; Kuehnbaum et al., 2015; Schranner et al., 2021; Yan et al., 2009) are compared to the studies contained within this thesis; it is notable that the studies performed herein are currently the largest in terms of sample size (Chapter 3 and Chapter 4), most robust in terms of control criteria (e.g. proximity from exercise, dietary control and sampling participants on more than one occasion, Chapter 4), and employ less biased modelling strategies (as PCA does not assume data class structures, while PLSDA does) (Table 2.1.). These aforementioned factors are essential considerations for the design and quality assessment of metabolomics studies concerning exercise (Belhaj et al., 2021).

There is a physiological rationale for how metabolic processes that are altered at rest in response to exercise training such as fat oxidation (Jurasz et al., 2022; Smorawiński et al., 2001) and protein synthesis (Figueiredo, 2019) could contribute to a circulating 'metabolic fingerprint' of exercise training that manifests at rest. However, knowledge is lacking with respect to the extent to which metabolites that could signal changes to these metabolic processes (e.g. altered amino acid concentrations or fat oxidation by-products like acylcarnitines) permeate to circulation at rest (i.e. whether a tissue 'metabolic fingerprint' of exercise training manifests in blood), how durable such changes are, or the number of metabolites that would need to be differentially present or abundant for robustly different latent variable estimates of 'metabolic fingerprints' to be constructed. Regardless, the results of this thesis indicate that under well-controlled sample collection procedures, a 'metabolic fingerprint' of exercise training is not apparent in resting blood samples.

The approaches taken within this thesis for investigating a 'metabolic fingerprint' of exercise training is most prominently limited by the use of targeted metabolomics panels in Chapters 3 and 4. While the metabolite panel employed was the same (i.e. identical metabolites were 'screened' for), the concept of a 'metabolic fingerprint' is largely theoretical and often ambiguous (i.e. the types of metabolites that would contribute to a 'metabolic fingerprint' of a given physiological condition is rarely considered *a priori)* (Kosmides et al., 2013). A similar intellectual perspective was taken within this thesis, i.e. no theoretical assumptions were made about what metabolites may constitute a 'metabolic fingerprint' of exercise training. However, the use of targeted metabolomics panels mean that technical assumptions were made regarding which metabolites were to be identified and therefore included into PCA models that were seeking to identify a metabolic fingerprint. Therefore, it is unclear whether the lack of identification of any metabolic fingerprints of exercise training within this thesis are related to the physiological lack of such a phenomena existing, or the technical selection of a metabolite

panel that consists of an insufficient number of components (i.e. individual metabolites) of the 'true metabolic fingerprint' to enable its identification via PCA.

7.4. THE INFLUENCE OF EXERCISE TRAINING ON THE PRESENCE OF CIRCULATING METABOLITES

While multivariate techniques (e.g. PCA) are useful for determining whether the total landscape of a group of measured molecules have changed in response to exercise or exercise training (e.g. provide inference of a 'metabolic fingerprint'), multivariate approaches of this nature may not represent an appropriate statistical approach for considering whether metabolites (or other molecule types) as exercise factors are altered with exercise training at rest. The reason for this relates to an incompatibility with how PCA (and associated algorithms like PLSDA) models are constructed and how an 'exercise factor' response to exercise training may manifest. The aim of PCA is to decompose a set of correlated variables (e.g. metabolites, proteins, RNA) into a singular representative value ('a latent variable') that explains as much of collective variance of *all of the data* as possible. Using PCA all individual variables (in this case metabolites) included in the model receive a weighted estimate (called a loading score) that describes the extent of their contribution to the value of the latent variable (Bro and Smilde, 2014; Cozzolino et al., 2019). Importantly, this means a given metabolite's loading score is not necessarily based solely on the concentration value of that metabolite, but instead is based on the metabolite's concentration along with consideration of the concentration of *all other metabolites in the dataset* (Bro and Smilde, 2014).

In essence, this means if the loading score of a metabolite is changed (e.g. increased) in response to an independent variable (exercise training intervention) – that does not necessarily mean that the value of said metabolite changed considerably in response to exercise training. Instead, it means that the value of said metabolite now contributes more to the overall explained variance of the decomposed latent variable. In more simple terms, the extent to which a loading score for a single metabolite changes will be determined by how measures of *all*

metabolites in the data have changed and not whether *that individual metabolite changed considerably*. Therefore, when considering metabolites from the perspective of a group of exercise factors, multivariate approaches are arguably less useful, as in theory, a single metabolite could have a large change in concentration in response to exercise training and potentially see a decreased loading score if all other metabolites remained largely unchanged (because the singular metabolite altered by exercise training is now so 'different' from the rest of the data).

To investigate whether metabolites are altered at rest in association with exercise training from the perspective of exercise factors, univariate hypothesis testing was also performed in this thesis to examine whether the concentration(s) of certain metabolites were altered at rest in response to, or association with, exercise training. In Chapter 3, 11 fatty acids observed to have significantly lower concentration in response to 9 sessions of SIT training, whereas in Chapter 4 a total of 44 metabolites were determined to be differentially-abundant between CON, END and STR groups with the majority (36) of differentially-abundant metabolites being associated with lipids or lipid metabolism (i.e. belonged to Fatty Acid, Acylcarnitine or Phosphotidylcholine groups of metabolites). Interestingly, two fatty acids (Alpha-Linolenic Acid & Gamma-Linolenic Acid) that were observed to have lower concentration after SIT in Chapter 3 were also observed to be in lower concentration in both END and STR participants (compared to CON) in Chapter 4. However, these results are somewhat perplexing, as elevated Alpha-Linolenic Acid concentrations have been associated with anti-inflammatory and cardioprotective benefits (Stark et al., 2008) and n-6 Polyunsaturated Fatty Acids (including Gamma-Linolenic Acid) have been positively associated with VO_{2max} (Esmaili et al., 2023).

Overall, it is very difficult to infer whether any of the differentially abundant metabolites identified in Chapter 4 are acting as exercise factors at rest. Some circulating fatty

acids are associated with alterations in signal transduction (Glatz et al., 1995), but this may be less likely for acylcarnitines (McCoin et al., 2015) and phosphatidylcholines (Tan et al., 2020). One metabolite identified as differentially abundant in Chapter 4, namely homovanillic acid, which was observed to be decreased in both STR and END participants compared to CON and in END participants compared to STR, could potentially warrant further investigation as an exercise factor. Homovanillic acid is produced by the enzyme monoamine oxidise acting on dopamine (Lambert et al., 1993). Homovanillic acid traditionally represents a marker of metabolic stress and is a clinical predictor of schizophrenic episodes (Amin et al., 1992); the extent to which neuroleptic drugs reduce plasma homovanillic acid concentrations correlates moderately ($R^2 \sim 0.36$) with improvements in the brief psychiatric rating scale (a questionnaire used to measure anxiety, depression and unusual behaviour) (Davila et al., 1988). Homovanillic acid also displayed a 'good' ICC of 0.65 in Chapter 4, suggesting that differences in this metabolite between groups are likely to have been robust. There is unfortunately no work produced by this thesis exploring homovanillic acid as a 'candidate exercise factor', but there is evidence to suggest further work could be undertaken to explore whether it could be identified as such.

7.5. THE INFLUENCE OF EXERCISE TRAINING ON THE PRESENCE OF SMALL EVS

EVs, and in particular small EVs, are now routinely proposed as 'carriers' of exercise factors of all types (Chow et al., 2022; Murphy et al., 2020; Nederveen et al., 2021; Safdar et al., 2016; Vechetti et al., 2020). There is evidence across several studies employing a variety of EV identification approaches and techniques that suggests small EVs enrich in response to acute exercise (Nederveen et al.,2021 and table 2.1), in essence mimicking the response of many exercise factors (Contrepois et al., 2020) and lending credence to the notion that small EVs do play a participatory role in the acute 'exercise factor response'. Unlike acute exercise, the evidence regarding the response of small EVs is less robust, and the predicted response of

small EVs to exercise training is less intuitive to speculate on. The abundance of small EVs has been suggested to increase with exercise training at rest, with a concomitant shift in the 'profile' of small EV cargo (Nederveen et al., 2021). Chapter 5 of this thesis performed what is to date the most comprehensive identification of small EVs derived from exercise-trained individuals at rest in accordance with the MISEV guidelines. Collectively, no differences in the presence of small EVs was indicated between CON, END or STR participants across four methods of EV identification (AMNIS flow cytometry, Western blot of EV markers, NTA or TEM) or between visit 1 and visit 2 within each exercise training history group. This implies the presence of small EVs is similar between individuals with divergent exercise training histories.

There are three major limitations to the approach taken to the characterisation of small EVs from CON, END and STR participants taken in this thesis. The first is the absence of a platelet depletion strategy when separating plasma from whole blood samples. Circulating platelets continually release EVs but upon activation platelet EV release can increase to 15-20x that of inactivated platelets (Aatonen et al., 2014). While this may be desirable in circumstances where platelet derived EVs are the subject of study (Puhm et al., 2021), EVs derived from activated platelets can also produce a considerable amount of background noise, particularly when platelet activation is stimulated by experimental processing (e.g., leaving vacutainers of extracted blood to clot producing serum) or measurement procedures (e.g., the trauma of inserting/removing needles during venepuncture or mixing of blood with anticoagulants) (Théry et al., 2018). It is generally recommended that platelet activation is prevented when studying EVs through the mixing of blood with an anticoagulant (Théry et al., 2018), this was achieved in chapter 5 by mixing blood with the anticoagulant to produce plasma (ACD-A). However further steps can also be taken (e.g., re-centrifugation of plasma) to deplete plasma of platelets (whether they are activated or not) (Lacroix et al., 2012) with the idea that this

removes ‘background noise’ with respect to detecting non-platelet derived EVs. However, as previously stated, even inactive platelets contribute EVs to circulation (Aatonen et al., 2014) and platelets have been suggested to contribute to the ‘exercise responsive’ pool of circulating small EVs (Brahmer et al., 2019). Therefore, when designing the EV separation protocol for chapter 5, I opted to use platelet rich (as opposed to platelet-depleted) plasma, as provided platelets were largely inactivated, populations of EVs separated from platelet rich plasma would be more representative of the ‘normal’ resting circulating EV pool. It is notable that platelet depleted plasma does show evidence of a lower ‘amount’ of EVs (measured via a lower presence of CD9, CD63 and CD41a ‘EV markers’) in resting plasma (McIlvenna et al., 2023) and that the ACD-A anti-coagulant used in chapter 5 has recently been demonstrated to produce almost absent evidence of platelet activation (Dhondt et al., 2023). However, it could still be argued that even inactive platelets contributed large enough of a signal that some differences between CON, END and STR participants were not detected and therefore it is a limitation of chapter 5 that EVs separated from platelet rich and platelet depleted plasma was not compared. A second limitation of chapter 5 is that only a single method of EV separation (SEC) from plasma was applied. While this method is popular (Gardiner et al., 2016), it is also generally accepted that different methods of EV separation can yield divergences in the profile of separated EVs (with the inference being that different separation techniques may preferentially separate different EV ‘subtypes’) (Cocozza et al., 2020) and it has previously been suggested that studies endeavour to employ multiple methods of EV separation (Théry et al., 2018; Van Deun et al., 2017). Therefore, the fact that this thesis only employed a single method of small EV separation is a limitation. The third limitation is that this thesis did not explore a greater variety of small EV cargo(es), as there is most robust evidence available that the ‘profile’ of small EV cargo may be altered in response to exercise training (in contrast to small EV abundance) (Nederveen et al., 2021). While the metabolomic profile of small EVs was

examined in this thesis, identified metabolites were largely related to small EV membranes (discussed in Chapter 5). Therefore, this approach was arguably more of an additional method of vesicle characterisation than an investigation of vesicle cargo. As such, this thesis provides no inference as to whether the bioactivity or bioactive cargo of small EVs derived from CON, END or STR participants was different and this remains an important question for understanding whether the abundance of exercise factors (carried via small EVs) are altered at rest in association with exercise training .

7.6. THE BIOACTIVIY OF PLASMA DERIVED FROM INDIVIDUALS WITH DIVERGENT EXERCISE TRAINING HISTORIES

Despite studies employing 'omic' technologies identifying several hundred candidate exercise factors, either in whole plasma or separated preparations of small EVs (Contrepois et al., 2020; Whitham et al., 2018). However, the number of exercise factors with suggested or identified biological effects is comparatively few (Chow et al., 2022) and consequently there is little empirical evidence regarding the physiological effects of the large number of purported exercise factors. Plasma or sera extracted in acutely-exercised or exercise trained contexts represent the totality of the 'exercise factor environment' and thus represent a straightforward, but ambiguous, model to identify potential physiological effects associated with exercise factors. The majority of work examining the bioactivity of exercise-associated plasma/sera has been done using sera extracted from acutely-exercised individuals that has been used to condition media then cultured with prostate, colon or breast cancer cells (Metcalfe et al., 2021; Soares et al., 2021).

Prior to the work performed in this thesis, limited data was available with respect to plasma extracted from exercise-trained individuals under circumstances where participants were young and healthy, or where the acute influence of a residual exercise bout could be confidently discounted (discussed in Chapter 2 and Chapter 6). In Chapter 6, media conditioned with plasma from CON, END and STR participants generally conveyed benefits to BT-549

cells in comparison to non-human plasma control conditions (either SFM or FBS conditions – depending on the assay), with no differences apparent between human plasma groups on any functional assay. The results of Chapter 6 provided no firm evidence of 'anti-cancer' effects elicited by media conditioned with plasma derived from healthy humans at rest, irrespective of their exercise training history. These findings are in contrast with what had previously been speculated by some narrative reviews (Hojman et al., 2018; Metcalfe et al., 2021). With respect to the larger themes considered by this thesis, these results could imply that changes to 'exercise factor environment' that may appear in the circulation of exercise-trained individuals at rest do not convey meaningful physiological effects. However, the work performed in Chapter 6 is not without limitations and this inference, while worthy of consideration, should at present, be considered with scepticism and hesitance.

Firstly, the 'bioactivity' of human plasma examined within this thesis was only explored in one cell line, from one disease type (triple negative breast cancer), therefore the inferences made regarding the data obtained in this thesis can only be faithfully applied to these cells. It is unclear whether plasma from CON, END or STR participants would have different influence on other types of tumour-derived cells or cell-lines produced from other cell types (e.g. skeletal muscle cells). Secondly, the work within this thesis did not include the present of an acutely exercised plasma condition of human plasma, where 'anti-cancer' effects (presumably mediated by exercise factors) would have been more expected (Hojman et al., 2018; Soares et al., 2021).

7.7. FUTURE DIRECTIONS

The process of studying exercise factors is difficult and complex. There are a multitude of components that need to be considered and it is rarely possible that a single study, or even a set of studies can collect to provide a clear insight into even a small component of this topic. The work from this thesis has manifested in a way that is less than straightforward to report or

interpret and there remains considerable work to be done in the field in order to create a more clear understanding of the response and activity of exercise factors. Here I provide what I perceive to be the three most important avenues for future research.

7.7.1. A unified definition of exercise factors and of what constitutes an exercise factor response

While there has been steady progression in the development of the definitions of exercise factor 'sub-types' (e.g. 'myokines' and 'exerkines') over the past decade, there is no unifying consensus on what an exercise factor 'is' nor is there unifying consensus on the definitions of exercise factor subtypes. 'Progressions' in the definitions of terms relating to exercise factors have come largely in the form of prominent researchers arbitrarily updating definitions in narrative reviews that speculate on the potential physiological relevance and translational potential of exercise factors (Brooks et al., 2023; Chow et al., 2022; Murphy et al., 2020; Whitham and Febbraio, 2016). This practice of allowing relatively malleable definitions of exercise factor subtypes to coexist has become accepted in the field, this flexibility of terms is problematic, because it allows for different definitions of the same term to exist with minor (but important) differences that impair the legitimacy of the larger exercise factor concept from a physiological perspective. For example, a myokine has recently been defined as 'a cytokine or peptide that is produced and released by skeletal muscle and subsequently exerts paracrine or endocrine effects' (Murphy et al., 2020) by one research group, with lactate (a metabolite) then also being considered a 'myokine' by another research group (Brooks et al., 2023). While the classification of lactate being a 'myokine' is easily argued to be fair, as it meets all qualifying criteria *except* that of being a protein or protein associated molecule (Brooks et al., 2022). In my opinion, the acceptance of this change to the 'myokine' term *without consensus* is an issue because it creates a landscape where researchers freely have precedent to change exercise factor-related definitions to suit their perspectives

(and perhaps too, their data). This situation compounds to create a scenario where the understanding of the *physiological phenomenon* that terms were initially defined *to collectively describe* becomes muddied through conflicts or disagreements over what should or shouldn't be considered for inclusion in the collective understanding of an 'exercise factor response'.

The first important future direction for research revolves around establishing firm and agreed upon parameters for more fundamental concepts relating to this thesis. In essence, a more strict and unified definition of *what an exercise factor actually is* – this would include more consensus-style agreement of qualifying criteria regarding the extent to which a candidate exercise factor's appearance and uptake kinetics must be known, the type(s) and magnitudes of physiological effects that must be identified; the extent to which targeted tissue(s) or tissues of origin must be determined; and a more exact understanding of the relationship of exercise factors with 'carrier' particles like EVs. Establishing this definition would allow a more clear understanding of what an 'exercise factor' response manifests as physiologically and the legitimate extent to which exercise factors contribute to both exercise metabolism and exercise adaptation.

7.7.2. An increased understanding of the physiological relevance of exercise factors

Currently much of the research concerning exercise factors (this thesis included) is focused on the discovery of 'candidate' exercise factors using 'omic' technologies (Chow et al., 2022). This approach is fruitful in the sense that it has consistently yielded the observation that hundreds of factors simultaneously change in response to exercise (Contrepois et al., 2020; Morville et al., 2020; Schranner et al., 2020; Shah et al., 2017; Wei et al., 2023). However, there does come a point where the usefulness of simply 'identifying' candidate exercise factors becomes questionable, as 'omic' studies provide very little physiological insight beyond highlighting that the number of exercise factors is potentially numerous and at best can only provide 'thematic' insight into metabolic processes that 'clusters' of candidate exercise factors

may gather into; for example, using ontological techniques like pathway analysis. Arguably, the most important characteristic of an exercise factor is bioactivity and while some exercise factors are associated with (mostly *in vitro*) effects (Chow et al., 2022), the bioactivity of very few exercise factors (IL-6 and lactate being prominent examples) are understood in detail (Brooks et al., 2022; Severinsen and Pedersen, 2020). In order for the genuine physiological relevance of exercise factors to be understood, it is important to develop a more comprehensive understanding of *which physiological responses exercise factors contribute to* as well as the extent of that contribution i.e., are responses partially, or entirely mediated by exercise factors? There are many models that can be applied to this point, such as *ex vitro* models and (preferably) *in vivo* administration of whole plasma, individual exercise factors, or separations of EVs extracted from acutely exercised or exercise trained individuals. These models can be used to firstly identify relevant physiological effects (e.g. changes, if any, in blood glucose, blood pressure), and subsequently inform work examining molecular mechanisms (e.g., altered signal transduction, cellular redox status and/or metabolic rate) to gain a more comprehensive understanding of the actual activities of exercise factors.

7.7.3. The separation and connection of exercise factors to 'fingerprints'

A connected point to an arguably excessive focus on simply identifying exercise factors is that not all factors that are identified to change in response to exercise may actually be acting as exercise factors. In other words, it is plausible that on an individual level, not all molecules that are measured to have altered circulating abundance in response to exercise or exercise training may be directly related to eliciting a physiological response (and therefore are arguably not exercise factors) (Chow et al., 2022). A key concept of this thesis has been that of 'metabolic fingerprinting' – which is essentially the idea that all metabolites that shift in response to a stimulus are contributing to a distinct 'pattern' of metabolism that can be identified with multivariate statistical techniques (Kosmides et al., 2013).

Importantly, the concept of a ‘metabolic fingerprint’ is not concerned with how each individual metabolite contributes to the ‘fingerprint’ e.g., whether the metabolite appears as higher/lower abundance due to altered reaction rates, has stable concentration, or has lower abundance due to being increasingly used as an intermediary. However, understanding the concept of ‘metabolic fingerprinting’ is useful when considering the exercise factor response, because it widens the perspective to consider that metabolites (and possibly other molecule types e.g. ‘protein fingerprints’ or ‘transcript fingerprints’) that shift in response to exercise (for example, acylcarnitines) may only acting as a residual ‘signals’ for altered metabolism and not mediating discrete individual bioactive signalling (McCoin et al., 2015). However, this perspective is also made more complicated by the fact that it is certainly clear that individual molecules, such as lactate being a prominent example, can act as signals for altered metabolism (in the case of lactate ‘anaerobic metabolism’) (Poole et al., 2021), while also demonstrating the capacity to act as an exercise factor through the exertion of direct metabolic and signalling effects (Brooks et al., 2022).

Therefore, within the context of exercise factor research, an important topic of future work is identifying whether the individual molecules that ‘cluster’ to create associations with metabolic ‘themes’ or processes when ontological techniques are combined with ‘omics’ technologies are functioning as exercise factors, or are simply acting as residual intermediaries that are only contributing to ontological shifts. In more simple terms, future research must endeavour to identify what proportion of molecules, out of the totality of those that shift in response to exercise, actually have function as exercise factors, instead of simply being residual signals of tissue metabolism.

7.8. CONCLUDING REMARKS

In conclusion, this thesis has produced a body of work that provided some insight into elements of the ‘exercise factor’ response to exercise training in resting blood samples. A

particular focus was placed on metabolites as a family of exercise factors and small EVs as a family of exercise factor 'carriers'. While changes in the presence of some metabolites were observed in association with exercise training, it is not entirely clear whether this represents a shift in the presence of metabolites that are also acting as exercise factors with relevant bioactivity, or whether these are residual signals of tissue metabolism. Irrespective of exercise training history, the presence of small EVs appears to be consistent across separate days, provided measurement conditions are similar, although it is not clear whether the bioactive 'cargo' of small EVs is different. Lastly, human plasma largely conveys 'nourishing' effects to BT-549 cells in a manner that does not appear to be influenced by exercise training history. Therefore, collectively, this thesis does not support a paradigm wherein the 'exercise factor environment' at rest is largely different between healthy individuals with divergent training histories as assessed in terms of metabolite profile, small EV presence, or bioactive effects on BT-549 cells.

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APPENDIX A : Exercise Training and Small EV Review

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REVIEW
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Exercise Training and Circulating Small Extracellular Vesicles: Appraisal of Methodological Approaches and Current Knowledge

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In response to acute exercise, an array of metabolites, nucleic acids, and proteins are enriched in circulation. Collectively termed "exercise factors," these molecules represent a topical area of research given their speculated contribution to both acute exercise metabolism and adaptation to exercise training. In addition to acute changes induced by exercise, the resting profile of circulating exercise factors may be altered by exercise training. Many exercise factors are speculated to be transported in circulation as the cargo of extracellular vesicles (EVs), and in particular, a sub-category termed "small EVs." This review describes an overview of exercise factors, small EVs and the effects of exercise, but is specifically focused on a critical appraisal of methodological approaches and current knowledge in the context of changes in the resting profile small EVs induced by exercise training, and the potential bioactivities of preparations of these "exercise-trained" small EVs. Research to date can only be considered preliminary, with interpretation of many studies hindered by limited evidence for the rigorous identification of small EVs, and the conflation of acute and chronic responses to exercise due to sample timing in proximity to exercise. Further research that places a greater emphasis on the rigorous identification of small EVs, and interrogation of potential bioactivity is required to establish more detailed descriptions of the response of small EVs to exercise training, and consequent effects on exercise adaptation.

Keywords: endurance, exercise factors, exerkines, exosomes, myokines, skeletal muscle

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INTRODUCTION

When individuals undertake repeated bouts of exercise (i.e., exercise training), acute molecular responses and chronic adaptive changes occur that result in functional changes at the levels of cells, tissues, organs, and systems. These changes ultimately produce improvements in health status, and/or exercise capacity and performance, amongst other phenotypic changes. Currently, the totality of processes that regulate exercise adaptation are incompletely understood, and uncovering novel mechanisms that contribute to, or are characteristic of, this process represents a central topic of research for exercise physiologists (Egan and Zierath, 2013). Acute exercise induces the

enrichment in circulation of a vast array of factors including metabolites, several RNA species, and peptides/proteins (Contrepois et al., 2020). These may be derived from a variety of sources that include contracting skeletal muscle, among other prominent examples such as endothelial, cardiac, hepatic, and adipose tissues (Estébanez et al., 2020; Murphy et al., 2020). The physiological relevance of the enrichment of many of these factors is mostly unknown, but they may contribute to regulation of homeostasis and substrate metabolism during and after exercise (Murphy et al., 2020), and/or serve as the initiating signals for the adaptations that occur in response to repeated bouts of exercise (Hoffmann and Weigert, 2017). Under specific conditions, some of these factors have been classified with established labels, namely "myokine" for peptides and proteins released from skeletal muscle in response to exercise (Murphy et al., 2020), and "exerkine" for protein or RNA factors enriched in response to exercise, but with ambiguous tissue origin (Safdar et al., 2016). However, these terms are often used interchangeably, or in a manner that is misaligned to their definition (Eckel, 2019). Therefore, to avoid conflicting terminologies, within this article we operationally refer to all circulating molecules that are evidently responsive to exercise with the collective term of "exercise factors" (Goldstein, 1961; Severinsen and Pedersen, 2020). This term is generic and all-encompassing to relevant molecule species (i.e., metabolites, nucleic acids, and proteins) from any cellular source. We also consider a circulating exercise factor as any factor that is reported to demonstrate responsiveness to either acute exercise or chronic exercise training. This approach enables acknowledgment of a variety of potential responses across molecules and conditions, without having to provide excessive clarifications or redefinitions when divergences between individual molecules are present.

Extracellular vesicles (EVs) are a family of lipid bilayer encapsulated molecules that have regulated released from all nucleated cell types and are measurable in most common biofluids (e.g., blood, sweat, and urine; van Niel et al., 2018). EVs contain heterogenous bioactive molecules or "cargo" (e.g., metabolites, nucleic acids, and proteins), which are derived from their cell of origin, and are capable of being taken up into cells that are either proximal or distal to their site of release. These features have led to the assumption that the primary function of EVs is to serve as communicative "messages" between cells (Yáñez-Mó et al., 2015). Recently, it has been reported that EVs undergo circulating enrichment in response to acute exercise [reviewed by (Estébanez et al., 2020) and (Nederveen et al., 2021)] and this has produced speculation that EVs may represent a medium of transport for exercise factors (Safdar et al., 2016; Murphy et al., 2020). Under current models, the principal idea considered is that the mechanism by which many exercise factors appear in circulation is packaged as cargo within released EVs (Estébanez et al., 2020). This is suggested to serve as a means of protecting certain factor types (e.g., RNA) from degradation, while also explaining how proteins devoid of secretory peptide sequences may still enrich in circulation in response to exercise (Safdar et al., 2016). Interestingly, some subtypes of exercise factors have been reported to exhibit differential profiles between exercise-trained and sedentary individuals under resting conditions using metabolomic and proteomic analyses (Wei et al., 2018; Monnerat et al., 2020). Here we define "profiles" as the detection of individual factors as present or absent, and/or changes in their estimated abundance(s) in circulation. The observation of differential profiles suggests that exercise training may induce changes to the circulating milieu that are somewhat durable, rather than only transient and present during, and soon after, exercise.

Much of the current interest regarding the response of EVs to exercise has been focused on a subfraction of EVs in smaller size ranges (diameters of 50–150 nm), termed "small EVs" (Estébanez et al., 2020; Vechetti et al., 2020; Nederveen et al., 2021), with several studies having now investigated the effect of exercise training on the circulating profile (particle number, concentration/abundance, cargo, and/or cargo density) of small EVs (Chaturvedi et al., 2015; Bei et al., 2017; Bertoldi et al., 2018; Ma et al., 2018; Hou et al., 2019; Barcellos et al., 2020; Castaño et al., 2020; Nair et al., 2020; Xiang et al., 2020; Estébanez et al., 2021; Gao et al., 2021).

The primary aim of this review is to critically appraise whether changes in the profile of small EVs under resting conditions is expectable as a characteristic response to exercise *training*. To achieve this aim, we will outline the general paradigm of exercise factors and their response to acute exercise and chronic exercise training, in addition to discussion of small EVs in the same context, and what is currently known about the bioactivity of small EVs altered by exercise training. We will also highlight key methodological considerations for both the identification (separation and characterization) of small EVs, and the interpretation of exercise training studies given the critical importance of these aspects to our aim.

OVERVIEW OF THE RESPONSE OF EXERCISE FACTORS TO ACUTE EXERCISE

For each subcategory of exercise factors (i.e., metabolites, nucleic acids, and proteins), the number of individual molecules that are reported to change in response to acute exercise is often estimated to be hundreds (Fernández-Sanjurjo et al., 2018; Sakaguchi et al., 2019; Guseh et al., 2020). A small number of molecules may decrease in abundance in response to acute exercise, but it is generally considered that the majority of changes in exercise factors are in the form of increased circulating abundance(s; Fernández-Sanjurjo et al., 2018; Son et al., 2018; Murphy et al., 2020; Severinsen and Pedersen, 2020). Some individual factors are well-described and understood in terms of the kinetics of their response to exercise and subsequent metabolic and/or molecular effects, such as the metabolite lactate (van Hall, 2010; Brooks et al., 2021), and the myokine interleukin-6 (IL-6; Pedersen and Febbraio, 2008). Mention of the latter molecule is of particular importance as an illustration of a prototypical exercise factor. During and soon after a bout of aerobic exercise, circulating IL-6 is robustly (and sometimes substantially) increased, is mostly derived from contracting skeletal muscle (Pedersen and Febbraio, 2008), and exerts relevant effects during (e.g., enhanced hepatic

glucose output and adipose tissue lipolysis) and after (e.g., enhanced insulin sensitivity in skeletal muscle and enhancing pancreatic β-cell mass) exercise (Severinsen and Pedersen, 2020).

Collectively, these observations regarding IL-6 are seminal, as they established an intellectual foundation for the paradigm of how many exercise factors are now generally presumed to function. However, for the majority of exercise factors, limited information is available beyond (sometimes inconsistent) reports that indicate a change in circulating concentration(s) in response to acute exercise. The details regarding subcategories of exercise factors (e.g., potential bioactivities, variability within and between subcategories, and variability in response to different types of exercise) are beyond the scope of this review, but are discussed elsewhere in relation to metabolites (Sakaguchi et al., 2019; Kelly et al., 2020), RNAs (Sapp et al., 2017; Fernández-Sanjurjo et al., 2018), and proteins (Hoffmann and Weigert, 2017; Eckel, 2019; Severinsen and Pedersen, 2020). As EVs are proposed as potential carriers of many of these exercise factors (Safdar et al., 2016), there is an implication that the circulating response of EVs to acute exercise should mimic the general response of exercise factors. In the case of an exercise-induced "increase," this change would manifest through a combination of an absolute increase in the circulating abundance of EVs, a change in cargo profile, and/or an increase in cargo density per EV.

WHAT ARE EXTRACELLULAR VESICLES?

Extracellular vesicle is a generic term used to describe any lipid bilayer-encapsulated particle that is naturally-released by cells and is incapable of independent replication (Théry et al., 2018). At rest, EVs are continually released and removed from circulation (Matsumoto et al., 2020), but can demonstrate enhanced release in response to physiological stimuli; for example, in response to hypoxia or shear stress in vascular endothelial cells (Hromada et al., 2017). Circulating EVs elicit bioactivity through uptake and delivery of their molecular cargo to recipient cells, often different from the tissue of origin, and are implicated in the regulation of physiological processes, such as coagulation and antigen presentation (Yáñez-Mó et al., 2015).

For exercise physiologists, interest in EVs developed greater traction with the observation that many circulating proteins as candidate exercise factors did not contain signal (secretory) peptide sequences, and yet were present in online EV expression databases (Safdar et al., 2016). Currently, rigorous investigations of EVs in the context of response to acute exercise are limited, but reports are often interpreted as indicating EVs respond in a manner that mirrors that of exercise factors more broadly. Specifically, that EVs are transiently-enriched in circulation during and after exercise, and elicit relevant bioactivity through the delivery of their cargo to recipient cells (Vechetti et al., 2020; Nederveen et al., 2021).

Extracellular vesicles are broadly separated into three subtypes, which are delineated by their mechanism of biogenesis, namely (i) exosomes, (ii) ectosomes (a.k.a. microparticles or microvesicles), and (iii) apoptotic bodies (van Niel et al., 2018). Exosomes represent released intraluminal vesicles (ILVs), which are derived from multi-vesicular bodies (MVBs) that originate with inward budding of the plasma membrane. Because of their mechanism of origin, exosomes are expected to fall within the size range of MVB-associated ILVs, i.e., ∼50–150 nm (van Niel et al., 2018; Kalluri and LeBleu, 2020). Ectosomes are defined as EVs which originate via direct shedding from the plasma membrane. The exact mechanism of ectosome shedding in non-apoptotic cells is less well-described, but is suggested to involve altered membrane asymmetry via cytoskeleton remodeling mediated by Ca^{2+}-sensitive aminophospholipid translocases ("floppases" and "flippases"). Ectosomes have a broader reported size range of 50 nm to 1 μm (van Niel et al., 2018). Exosomes currently dominate the general interest in EVs in the domains of exercise physiology, metabolism and adaptation (Safdar and Tarnopolsky, 2018; Vechetti et al., 2020). As such, throughout this review, we will only consider reports which have endeavored to separate and characterize EVs that are within the size-range of exosomes (i.e., 50–150 nm). However, as EVs with similar physical characteristics to exosomes can bud directly from the plasma membrane (and therefore are not exosomes; van Niel et al., 2018) and technical challenges regarding the isolation of "pure" samples of individual EV subtypes (*vide infra*), hence we refer to these particles using the more collective term of "small EVs" (Théry et al., 2018).

SEPARATION AND CHARACTERIZATION OF EXTRACELLULAR VESICLES

Pertinent to any discussion of EVs is acknowledgment that there are currently no routinely-used direct methods for the characterization or quantification of EVs from biofluid samples. Instead, separation of biofluid fractions and multiple methods for identification and characterization are required to identify both the presence and quantity of EVs in a sample. This has implications regarding the inferential value of individual experimental studies concerning small EVs in any context. To enable critical discussion of relevant exercise studies, a brief overview of how EVs are currently recommended to be identified and analyzed by the International Society of Extracellular Vesicles (ISEV) via the MISEV2018 Guidelines is provided subsequently and are summarized in **Table 1**. Readers are also referred to the comprehensive position stand which details these guidelines (Théry et al., 2018).

Most contemporary EV separation techniques involve separation of particles from a biofluid sample (e.g., blood, sweat, milk, and saliva) based on either physical properties (such as size and density), specific expression of EV marker proteins, or a combination of both [reviewed by (Doyle and Wang, 2019)]. Currently numerous methods are used to separate small EVs from biofluids, the most common of which are differential ultracentrifugation, density gradient separation, size exclusion chromatography, and immunoprecipitation (Cocozza et al., 2020).

While these common techniques all may be used individually to separate small EVs, some may also be employed in

TABLE 1 | Summary of the minimal information for the study of extracellular vesicles (MISEV) guidelines.

Small EV characteristic	MISEV recommendation	Approaches	Analytic considerations
Quantity of EVs in a sample	- A quantified estimate of both the source of EVs (e.g., extracted whole blood/plasma volumes) and EVs themselves should always be provided	- Particle quantification (e.g., NTA, flow cytometry) - Total protein quantification (e.g., SDS page) - Total lipid quantification (e.g., SDS page)	- None of these methods exclusively quantify EVs - EV quantification is improved when methods are used in conjunction and to provide ratios indicative of purity, e.g., protein/particle or lipid/particle
EV marker identification	- At least three positive protein markers associated with EVs; including at least one transmembrane protein and one cytosolic protein - A purity control such as proteins that are identified as common contaminants (e.g., lipoproteins in plasma)	Traditional methods of protein identification (e.g., Western blot)	- "Mixed" signal may be determined across positive markers and therefore it is important multiple markers are used - High contaminant signal may have implications for interpretation of some quantification methods (e.g., NTA)
Characterization of single vesicles	- At least two different, but complimentary, methods of vesicle visualization should ideally be employed	- Microscopic-based techniques (e.g., electron or atomic force microscopy) - Single particle analyzers (e.g., NTA)	- Most microscopy techniques are not interchangeable in terms of the information they provide - Different techniques may need to be employed depending on the EV size range of interest

Modified from Théry et al. (2018).

combination for additive effects (Théry et al., 2018). However, the parameters by which these techniques work to separate particles tend to overlap across EV subtypes and various other molecules, thus it is often considered unavoidable that preparations of EVs will contain a heterogenous mix of particles, e.g., several EV subtypes with similar physical or expression characteristics (Théry et al., 2018; Cocozza et al., 2020), and non-EV molecules such as argonaute proteins (Arroyo et al., 2011), lipoproteins (Jamaly et al., 2018), and exomeres. The latter are secreted, bioactive and non-membranous protein complexes with diameters of ∼35 nm (Zhang et al., 2018, 2019). Accordingly, ISEV recommends referring to EVs within separated samples using clearly-defined operational terms based on identified physical and/or biochemical characteristics of particles within said samples. For example, instead of referring to EVs by the labels of specific subtypes, isolated EVs should be classified as "small" or "medium/large," and fixed size ranges for each term be declared, which can be determined at researchers' discretion. Alternatively, EVs can be termed based on the positive detection for specific markers (e.g., $CD63^+$ EVs), or the use of multiple defined parameters together such as "small $CD63^+$ EVs" (Théry et al., 2018).

Once small EVs have been separated from biofluid, it is then essential to validate the presence of small EVs and provide an estimate of small EV quantity, particularly in circumstances where enrichment is presumed to occur (e.g., exercise; Safdar et al., 2016; Vechetti et al., 2020). Broadly, the MISEV2018 guidelines recommend use of three principal indicators to identify the extent of small EV presence in a biofluid sample (**Table 1**). These are the quantification of particles in a small EV size range; the detection of marker proteins associated with small EV membranes and contaminants; and the visualization of small EVs derived from a sample (Théry et al., 2018).

The first indicator is the quantification of small EVs involving the estimation of the size range and concentration of particles within the sample. This enables determination of the distribution of particle diameters contained within a sample, and subsequent estimation of the concentration of particles that fall within the size range of small EVs (50–150 nm). Several methods are available for determining physical characteristics of these particles, but the most common technique employed in exercise studies is nano-particle tracking analysis (NTA). This technique measures the Brownian motion of individual particles via detection of scattered light, and by employing the Stokes-Einstein equation to determine diffusion coefficients can estimate distributions of particle concentration and diameter (Doyle and Wang, 2019). Traditional or modified methods of flow cytometry are also sometimes employed for the quantification of EVs by particle count (Welsh et al., 2020). Additionally, estimating total quantity of protein or lipid is useful as a global indication for determining the relative abundance of specific small EV markers or common contaminants (e.g., lipoproteins). These are recommended to be estimated via standard approaches, e.g., BCA assay or global protein stain of SDS-PAGE, and can also be usefully combined to estimate sample characteristics like unit protein per particle (Théry et al., 2018). The second indicator required for small EV enrichment is the characterization of common markers of EV status, which are generally "validated" small EV surface marker proteins such as CD63 (van Niel et al., 2018). Identification and quantification of at least three markers is recommended, encompassing an EV-associated protein and/or cytosolic protein, and at least one negative protein marker. The third, and final, indicator is the visual characterization of single vesicles, which is most commonly accomplished by using transmission or scanning electron microscopy, but may also employ techniques that can visualize vesicle topography, such as atomic force microscopy (Théry et al., 2018; Doyle and Wang, 2019).

Importantly, when considering the presence or bioactive effects of small EV cargoes, endeavoring to achieve a reliable indication of high abundance of small EVs in the sample is important, because some non-EV molecules that co-exist

in separated samples may also serve as carriers for factors that are proposed as bioactive cargo of small EVs, such as microRNA (miRNA) and protein (Arroyo et al., 2011; Zhang et al., 2019). Subsequently, to determine any roles or responses of small EVs to acute exercise or exercise training, it is essential that a rigorous characterization of small EVs is made. The technical approach (e.g., the number and types of methods) required to characterize the presence of EVs in a sample is extensive, and may be challenging to accomplish. However, when considering the question of whether small EVs represent an important "carrier" of exercise factors, the extent to which any given report demonstrates the presence/quantity of small EVs will have substantial influence on the inferential value of the reported results.

CHANGES IN THE SMALL EXTRACELLULAR VESICLE PROFILE IN RESPONSE TO ACUTE EXERCISE

When investigating the response to acute exercise, the profile of small EVs would then ideally be compared between pre-, during and/or post-exercise samples using a multi-methods approach based on ISEV guidelines (**Figure 1**). Namely, enrichment would be evident as greater particle concentration (e.g., a greater NTA or flow cytometry signal), and quantities of total protein and small EV protein markers would be measurable in post- compared to pre-treatment (e.g., exercise) samples, together with a consistent (and ideally low) indication of contamination within preparations of small EVs from both pre- and post-treatment (Théry et al., 2018). The confidence with which enrichment could be inferred would then be based not on the signal of a single indicator, but instead on the general signal across multiple independent methods (**Figure 1**).

Currently several studies have investigated elements of the response of circulating small EVs to acute aerobic (Frühbeis et al., 2015; Guescini et al., 2015; Bertoldi et al., 2018; D'Souza et al., 2018; Lovett et al., 2018; Oliveira et al., 2018; Whitham et al., 2018; Brahmer et al., 2019; Yin et al., 2019; Karvinen et al., 2020; Rigamonti et al., 2020; Gao et al., 2021; Neuberger et al., 2021; Zhang et al., 2021), resistance (Annibalini et al., 2019; Just et al., 2020; Vechetti et al., 2021) or combined plyometric and downhill running (Lovett et al., 2018) exercise. Most of these studies have attempted to characterize circulating small EVs separated from plasma (Frühbeis et al., 2015; Guescini et al., 2015; D'Souza et al., 2018; Lovett et al., 2018; Whitham et al., 2018; Annibalini et al., 2019; Brahmer et al., 2019; Yin et al., 2019; Just et al., 2020; Rigamonti et al., 2020; Gao et al., 2021; Neuberger et al., 2021; Vechetti et al., 2021), although four studies have separated small EVs from serum (Bertoldi et al., 2018; Oliveira et al., 2018; Karvinen et al., 2020; Zhang et al., 2021).

An important point is that platelets are purported to be the largest contributors to the circulating EV pool (Yáñez-Mó et al., 2015). Therefore, results between studies that separate EVs from serum and plasma, which are rich in and depleted of platelets, respectively, will have inherit differences in their EV profiles, particularly as platelets may contribute to the exercise-associated small EV pool (Brahmer et al., 2019). This contribution is likely to be more apparent in preparations of small EVs separated from serum, and may influence differences in indications of small EV responses to exercise (e.g., enrichment) between studies.

In addition to differences in biofluid sources, these studies have not had consistent methodological approaches, which hampers broad conclusions on the nature of the response of circulating small EVs to acute exercise. For example, four studies have only examined changes in the concentration of specific miRNAs from plasma samples taken before and after aerobic exercise (Guescini et al., 2015; D'Souza et al., 2018; Yin et al., 2019; Karvinen et al., 2020). The inference was that these miRNAs represent small EV cargo. However, in each of these studies, miRNA concentrations (transcript abundance) were quantified in both pre- and post-exercise samples, but additional methods of small EV characterization were only applied to post-exercise samples (Guescini et al., 2015; D'Souza et al., 2018; Karvinen et al., 2020) or not at all (Yin et al., 2019). Problematically, this type of technical approach provides no indication as to whether the abundance of small EVs was in fact changed by the exercise bout and cannot associate changes in the concentration of these miRNAs with a concomitant change in the estimated abundance of small EVs. Given the heterogenous nature of preparations of small EVs, it is difficult to conclude that the data from these studies are definitively indicative of a change in small EV profile in response to acute exercise.

Ten studies have employed a single methods approach for determining small EV enrichment using either NTA (Lovett et al., 2018; Annibalini et al., 2019; Just et al., 2020; Rigamonti et al., 2020; Vechetti et al., 2021), flow cytometry (Bei et al., 2017; Gao et al., 2021), or Western blot (Bertoldi et al., 2018; Neuberger et al., 2021; Zhang et al., 2021) to estimate small EV enrichment in response to acute aerobic (Bei et al., 2017; Bertoldi et al., 2018; Rigamonti et al., 2020; Gao et al., 2021; Neuberger et al., 2021), resistance (Annibalini et al., 2019; Just et al., 2020; Vechetti et al., 2021), or combined plyometric and downhill running (Lovett et al., 2018) exercise. Each of these studies, except one (Lovett et al., 2018), observed an increase in their respective measures in within preparations of small EVs taken after exercise, which potentially indicates an exercise-induced enrichment in small EVs. However, using only measures of particle count is problematic because these only provide an indication of the concentrations of particles with specific size characteristics, which in practice would have low specificity for individual subtypes of EVs. For example, the use of NTA alone can reduce the accuracy of results as this measure is highly sensitive to factors such as nutrition status (Mørk et al., 2016; Jamaly et al., 2018; Brahmer et al., 2019).

Four studies have employed multi-method approaches in response to acute aerobic exercise (Frühbeis et al., 2015; Oliveira et al., 2018; Whitham et al., 2018; Brahmer et al., 2019). Two of these studies have seen increases in both NTA signal and protein concentration within preparations of small EVs derived from human plasma (Frühbeis et al., 2015; Whitham et al., 2018). One study employing a proteomics approach observed an exercise-induced increase in the abundance of 299 proteins (some of which were markers of small EVs such as CD81), which coincided

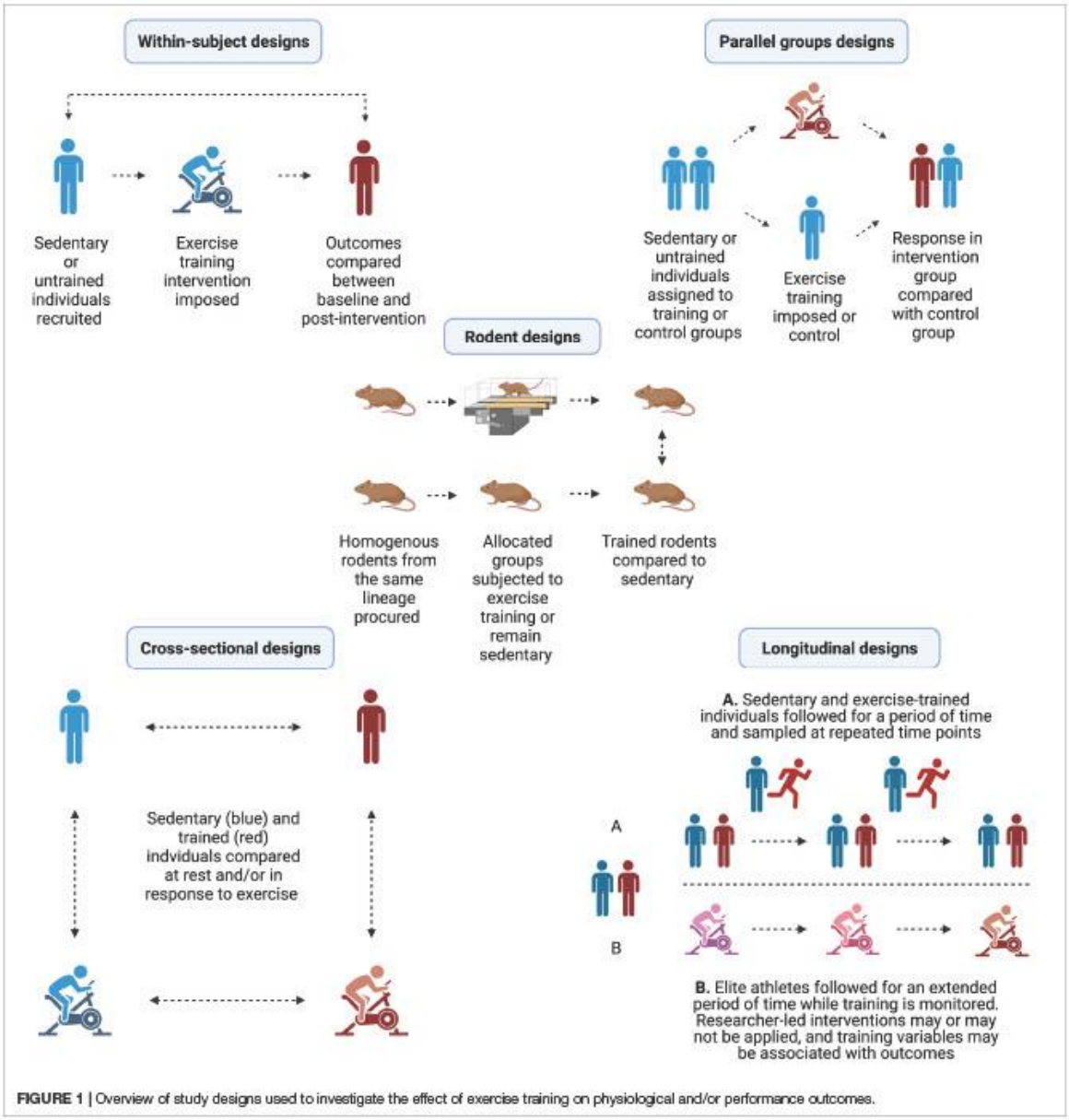

FIGURE 1 | Overview of study designs used to investigate the effect of exercise training on physiological and/or performance outcomes.

with an increase in NTA signal within preparations of small EVs (Whitham et al., 2018). Another study estimated an increase in the small EV marker proteins FLOT1 and HSP70 via Western blot, which also coincided with an increase in NTA signal comparing pre- and post-exercise samples (Frühbeis et al., 2015).

Small EV preparations from Wistar rats exposed to acute low, moderate or high intensity exercise demonstrated greater concentration of small EV-sized particles, total protein and CD63 abundance compared to samples derived from a sedentary control group. Importantly, APOIV, a lipoprotein and indicator of contamination preparations of separated EVs, was present but had similar abundance across all conditions (Oliveira et al., 2018). Another study using a pre-post analysis of acute exercise in humans with minimal lipoprotein contamination used NTA and Western blot analysis of small EV markers (i.e., CD9, CD63, Syntenin, CD41b, TSG101, and CD81), or a novel multiplex array that enables concurrent detection of 41 surface EV proteins (Brahmer et al., 2019). These methods produced

mixed results by observing no differences in small EV-sized particle concentration by NTA, but increases in multiple markers of small EVs via Western blot and multiplex assay approaches (Brahmer et al., 2019).

In this section, we have focused on appraising the approaches employed for determining whether small EV enrichment occurs, rather than focusing on the specifics of the exercise bouts, sample timing, or the specific changes in cargo or concentration during and after exercise. These specific details have been the subject of several recent reviews (Estébanez et al., 2020; Vechetti et al., 2020; Nederveen et al., 2021), and it is now often accepted that small EVs are enriched in circulation in response to acute exercise (Safdar et al., 2016; Whitham and Febbraio, 2016; Murphy et al., 2020; Vechetti et al., 2020), despite the methodological limitations of studies to date. Nevertheless, rigorously measuring small EV enrichment in response to exercise remains challenging to accomplish, as multi-method approaches are required and agreement across selected methods may not always be apparent (Brahmer et al., 2019). However, the majority of studies to date have not provided sufficient characterization of small EVs to definitively conclude that enrichment has occurred. That said, of the limited number of studies where multi-method approaches have been applied, acute exercise arguably does induce an enrichment of small EVs in circulation. This is consistent with the general response of exercise factors, and therefore the proposed role of small EVs as a medium through which exercise factors are transported in circulation.

STUDY DESIGN CONSIDERATIONS FOR INVESTIGATING THE ADAPTIVE RESPONSE TO EXERCISE TRAINING

Prior to considering whether exercise factors and/or small EVs are responsive to exercise training, it is salient to consider the various study design approaches that are employed to study exercise adaptation (**Figure 1**). Describing the features, advantages and disadvantages of each of these designs is pertinent to the primary aim of this review, as acknowledgment of the limitations of study designs also informs the extent to which inferences can be made regarding measured changes in small EV profiles associated with exercise training. There are three broad study designs that can be employed to examine adaptive changes in response to exercise training, namely pre-post intervention studies, cross-sectional studies, and longitudinal/prospective cohort studies.

Pre-post Intervention Study Designs

Pre-post intervention study designs represent a direct approach for investigating change in physiological and performance phenotypes in response to exercise training. There are numerous ways that these designs can be implemented including approaches of randomized or non-randomized control trials of parallel groups, single group designs, and detailed $n = 1$ case studies, amongst others as detailed elsewhere (Hecksteden et al., 2018). However, the common feature within this design category is the exposure of participants to a period of structured exercise training focused on changing some aspect of health or fitness, and a subsequent comparison of physiological and/or performance outcomes. In human trials, this outcome is achieved generally by either a within-group pre-post comparison (single group design), or between-group comparison to a sedentary control group (parallel group design).

There are also well-established models of exercise training in rodents, which provide the advantage of a high degree of control over homogenous groups, and arguably can provide more detailed mechanistic insight into adaptations to exercise. The advantages provided by control over ambulatory activity, feeding times and dietary composition, environmental conditions, sleep/wake cycles, and compliance with training often not possible in human studies. However, these experimental advantages must be tempered with the caution that innate differences (e.g., morphological and metabolic) between rodents and humans can make it difficult to reproduce some findings between species (Fuller and Thyfault, 2021). Additionally, within-subject designs in rodents are often impossible to conduct for invasive measures such as muscle and blood sampling. This is principally due to methodological limitations such as requiring the excision of whole skeletal muscles, or physiological limitations such as blood volumes required for downstream analyses. For example, rats have a total blood volume of ~10 to 25 mL on average depending on bodyweight (Lee and Blaufox, 1985), whereas mice have generally less than 2 mL (Riches et al., 1973). Considering the volume of plasma required for many assays, including for the analysis of small EVs, where multiple independent assays are required and thus large blood volumes are desirable (Théry et al., 2018), sampling at serial timepoints in an individual rodent is unfeasible.

Therefore, exercise training studies in rodents typically involve randomized groups selected from a homogenous inbred lineage either subjected to a period of forced daily exercise training (e.g., treadmill running or swim training), or allocated as a group of sedentary controls. Groups are subsequently compared after being euthanized at the same time-point corresponding to the end of exercise training intervention. An important point to note is that with such a design, any differences observed between groups are technically cross-sectional in nature, i.e., differences between trained and untrained groups (see section "Cross-sectional studies"). Therefore, results should be described as characteristic of the trained state, rather than as training-induced changes in a phenotype or outcome variable.

In general, the primary advantage of training intervention studies in rodents or humans is that phenotypic changes can be attributed to certain characteristics of the exercise training stimulus controlled as independent variables such as the frequency, intensity, duration and type of exercise bouts. However, these interventions also suffer from notable limitations. For example, most training studies employ sedentary participants and relatively short intervention durations (e.g., several weeks to months; Hecksteden et al., 2018). However, even short (e.g., 2 weeks) interventions can result in substantial increases in aerobic fitness, and induce marked changes in transcripts and proteins in skeletal muscle (Perry et al., 2010; Egan et al., 2013). Sedentary individuals are the most responsive to the

onset of an exercise training intervention, and therefore the changes that often occur in short interventions are unlikely to be reflective of what would continue to occur were the training intervention planned appropriately and continued in an extended fashion. Short-term training studies cannot be assumed to represent the same adaptive processes present in individuals who have extensive exercise training histories (e.g., years/decades), which are typically assessed in cross-sectional study designs (section "Cross-Sectional Studies"). Nor can the time course for the changes in physiological responses and/or performance outcomes that are observed in short-term training studies being interpreted as continuing in a linear manner.

A final consideration is that especially in studies of the molecular regulation of adaptation in humans, control groups are often absent and single group within-subject designs are employed. This type of design has implications regarding the extent that pre- to post-intervention differences can be accurately quantified because the absence of a control group does not allow analyses to account for factors such as the regression to the mean artifact, and/or random variability, ever-present and uncontrollable, in biological measurements between- and within-individuals (Atkinson and Batterham, 2015).

Cross-Sectional Studies

Cross-sectional studies encompass a form of observational research that broadly involves an isolated comparison of measurements representative of traits of interest between members of distinct population groups (Levin, 2006). For effects of exercise training, cross-sectional studies tend to involve recruiting and stratifying participants into exercise-trained and sedentary or untrained groups, based on standardized criteria of fitness (e.g., maximal oxygen uptake, VO_{2max}; maximal power output, W_{max}; one repetition maximum, 1RM), training history or competitive status (e.g., elite cyclist or powerlifter), and/or exercise performance (e.g., personal best running a set distance, or weight lifted in competition). Subsequently, differences in relevant resting measurements and/or physiological responses and performance outcomes are compared between groups. In these reports, group differences are then often inferred as the indications of the consequences of exercise training, with the reasoning that when other major confounders (e.g., age, sex, indices of health status) are controlled for, the largest determinant of difference between groups is the regular participation in specific type(s) of exercise training.

The advantage of this type of study design is that it is often less expensive, less of time burden, and relatively simple to perform in comparison to training interventions. This generally allows the collection of larger sample sizes at lower cost, and sometimes the recruitment of a higher caliber of trained participant such as elite athletes. The more likely participation of elite athletes in cross-sectional studies is due to having lower time commitments and generally not interfering with athletes' training regimes. However, cross-sectional studies are limited mostly by the fact they only provide a "snapshot" at a specific moment in time, and therefore indicate the prevalence of traits or responses between exercise-trained or untrained groups at the time of measurement (Sedgwick, 2014). These studies are not capable of providing information on *how* a parameter of interest has changed over time to eventually qualify a participant for inclusion in the trained group. Additionally, some performance-associated traits such as VO_{2max} may be relatively-stable (Edgett et al., 2018), and thus can provide some reliable ecological insight from a single timepoint measure.

This case is potentially less for many discrete biological measurements (such as circulating factors), which may have an inherent within-individual biological variability, often day-to-day and that is often unknown, or unaccounted for. For example, both of the exercise factors IL-6 and Fibroblast growth factor 21 (FGF21) are reported to have both innate diurnal and inter-individual variations in resting circulating concentrations (Sothern et al., 1995; Yu et al., 2011). Under these designs, the standardization of preparation for participants is paramount including, but not limited to, preceding days' dietary intake, preceding night's sleep, duration of fasting and morning ambulation for morning fasted samples, and time since last bout of exercise. Therefore, in cross-sectional studies where repeated experimental measures are not employed (e.g., duplicate or triplicate sampling of participants under similar resting or experimental conditions across several laboratory visits), the results of some measures may be less reliable, which could reduce inferential utility.

While in many cases between-group differences may most obviously be the consequence of differences produced by prolonged exercise training, quantifying the exact contribution of training to individual phenotypes is not possible in cross-sectional studies. Importantly, there is often no consensus on minimum performance or physiological thresholds that can be used to delineate an individual as "exercise-trained." For example, one excellent approach is the physiology of road cyclists, wherein the categories of trained, well-trained, elite, and world class have been delineated based on training status, estimated by the weekly frequency of training sessions, the total volume of weekly training and the total year spent training; racing status estimated by the number of competition days per year and international ranking of the rider; and physiological capacity, estimated by common physiological parameters associated with performance (e.g., W_{max} and VO_{2max}; Jeukendrup et al., 2000). However, this approach is a rare exception as many studies opt to use different and sometimes arbitrary minimum thresholds for their trained participants. This lack of consistency can have the effects of making comparisons between studies more difficult and making the trained groups somewhat heterogenous and not divergent enough from the untrained group in order reveal meaningful between-group differences. For example, if a study categorizes a participant as trained if they demonstrate a $VO_{2max} > 60$ mL kg^{-1} min^{-1}, this approach may subsequently produce a group with individuals with VO_{2max} values ranging 60–80 mL kg^{-1} min^{-1}. This group would represent a mixed population group of trained, well-trained and elite athletes of similar physiological phenotypes, but in fact, they would likely be heterogenous on parameters such as training volume, lactate threshold, and mechanical efficiency. Indeed, obvious physiological differences between individuals within this example range have been reported, e.g., in citrate synthase activity, an

indication of mitochondrial mass (Jacobs and Lundby, 2012). Lastly, cross-sectional studies often request that participants do not engage in moderate-to-vigorous exercise for a fixed period of time (e.g., ~24 to 48 h) before coming to the laboratory, but it is often difficult to validate adherence to this recommendation, with the problem being that the residual effects of acute exercise may also be present in these samples, i.e., the "last bout effect," which describes residual, but transient, physiological changes induced by acute exercise that extend beyond the cessation of exercise (e.g., for 24–48 h), but do not manifest as an adaptive response to long-term exercise training (see section "'Last Bout Effect': The Importance of Sample Timing").

Longitudinal and Prospective Cohort Study Designs

Like cross-sectional designs, longitudinal study designs are a form of observational research, with the principal difference being that participants are monitored over an extended period of time, and re-sampled at various intervals (Caruana et al., 2015). Some studies may also employ overlapping longitudinal and interventional designs, although these reports tend to be rare in exercise science (Hecksteden et al., 2018).

Longitudinal studies possess the principal advantages of cross-sectional studies (i.e., easier to measure larger sample sizes compared to a structured/supervised training intervention) and some of the limitations (heterogenous groups of participants, limited standardization of participant preparation prior to measurements, arbitrary thresholds of group qualification). However, this design may provide additional insight into the reliability of measures and the stability of traits within- and between-groups. Such between-group comparisons would require recruitment of a control or comparator group with the design then being a prospective cohort study.

Longitudinal and prospective cohort designs are often also applied to measure the physiological and performance development of well-trained and elite individual athletes and teams preparing for competitive events (Jones, 1998; Gabbett, 2005; García-Pallarés et al., 2010). While the latter is not specifically a training intervention *per se* (as the researchers often have no direct control over the training of the participants), these types of observational design does provide insight into long-term development that can occur in conjunction with regular, intensive exercise training. However, these studies can be confounded by concurrent interventions employed by athletes (e.g., nutrition and recovery strategies), and fluctuations in training strategies across monthly or annual cycles that may not necessarily be tracked extensively by researchers. These confounders can make it more challenging to associate which specific elements of the exercise training process have the largest proportional influences on specific physiological or performance outcomes.

"Last Bout Effect": The Importance of Sample Timing

An important consideration across all types of study purporting to measure adaptive changes with exercise training is the proximity of measurement of the outcome interest to the final exercise bout of the training intervention, or most recent exercise bout in the case of cross-sectional designs. This consideration is especially important for outcomes with short half-lives of response/decay, such as changes in circulating parameters, but less important for outcomes with longer half-lives of response/decay. An example of the latter is that one effect of prolonged resistance exercise training is an increase in muscle mass (Egan and Zierath, 2013), which can be said to be a chronic adaptation given that it persists for several days and weeks after training cessation before declining steadily over several months (Mujika and Padilla, 2000a,b).

In contrast, exercise elicits obvious beneficial effects as improvements in glycaemic control and insulin sensitivity, but these are likely transient and related to the most recent bout of exercise. For example, a single bout of exercise modestly lowers blood glucose concentrations in the immediate post-exercise period in patients with type 2 diabetes (Minuk et al., 1981), but also improves whole-body insulin sensitivity for up to 48 h after exercise cessation (Mikines et al., 1988; Perseghin et al., 1996; Koopman et al., 2005). The additive effect of repeated bouts of exercise, i.e., training, over and above those that are seen in the aftermath of an acute bout of exercise in isolation is relatively modest (Perseghin et al., 1996). In cross-sectional terms, although well-trained athletes have markedly-enhanced insulin sensitivity compared to sedentary individuals (King et al., 1987), the habitual state of an athlete is between two individual exercise training bouts, thereby making it difficult to distinguish differences between acute responses and chronic effects of exercise. However, when well-trained or physically-active individuals cease training or reduce their daily activity, a decline in insulin sensitivity rapidly occurs toward sedentary levels at a time when declines in aerobic fitness or increases in adiposity are negligible (Heath et al., 1983; Krogh-Madsen et al., 2010). In turn, a single bout of exercise is sufficient to restore insulin sensitivity in these detrained individuals to their previously trained values (Heath et al., 1983), suggesting the effects of exercise for improved whole-body insulin sensitivity are mediated in large part by the acute effects of a single bout of exercise, rather than a generalized training effect, i.e., the absence of a synergistic effect.

Therefore, the physiological responses to a single bout of exercise, i.e., acute responses to exercise, can extend to 24 to 48 h after the cessation of an individual exercise bout, and thus some convergence exists between the effects of an acute bout of exercise and those effects associated with adaptation to exercise training. This phenomenon was coined by William L. Haskell (Haskell, 1994) as the "last bout effect" and proposes that some metabolic effects and purported health benefits of exercise (e.g., lowering of blood pressure or circulating lipoprotein profile) are attributable to the biological consequences of the most recent bout of acute exercise, rather than to a true training adaptation.

The overall implication is that in studies where the post-intervention, or cross-sectional, blood samples are taken within close proximity (e.g., <48 h) to the final, or most recent, training bout, respectively, it may be difficult to discriminate whether some outcomes represent a chronic adaptation to training, or an extended residual effect of the last exercise bout. This point

is particularly salient when interpreting current literature on the effect of exercise training on the resting profile of circulating small EVs given the research designs employed to date as discussed in section "Does Exercise Training Alter the Resting Profile of Circulating Small Extracellular Vesicles?"

DOES EXERCISE TRAINING ALTER THE RESTING PROFILE OF CIRCULATING EXERCISE FACTORS?

A pertinent question to our understanding of the biological importance and mechanistic consequence of exercise factors is whether these factors function as transient, beneficial responses exclusively related to the enrichment of factors induced by acute exercise, or whether exercise training induces more persistent changes to circulating exercise factors that are measurable distally from acute exercise. Clearly, exercise training alters the *resting* proteome (Holloway et al., 2009; Ferreira et al., 2014; Padrão et al., 2016), transcriptome (Pillon et al., 2019), and metabolome (Castro et al., 2019; Klein et al., 2020) of tissues such as skeletal (Holloway et al., 2009; Padrão et al., 2016; Pillon et al., 2019; Klein et al., 2020) and cardiac (Ferreira et al., 2014) muscle. Assuming that the internal biomolecular composition of a cell influences the host of factors it releases into circulation (Uhlén et al., 2019), exercise training-induced changes in the resting profile of exercise factors is a physiologically-plausible outcome. Conversely, to reiterate the example of IL-6, in healthy individuals exercise training does not appear to alter resting plasma concentrations of IL-6, nor the kinetics of an exercise induced circulating response (Fischer et al., 2004). There are numerous other experimental studies that have investigated the influence of exercise training on the resting abundance or concentration of a small number of selected candidate miRNA and protein targets in circulation. Detailed discussion of these reports is beyond the scope of this review, but collectively the training-induced response is equivocal; some factors increase after a period of exercise training, while others decrease, with occasional inconsistencies in individual factors across studies. Again, variations in experimental design and sample timing described in the section "Study Design Considerations for Investigating the Adaptive Response to Exercise Training" must be considered, and interested readers are referred to reviews with appropriately collated tables of these studies for miRNA (Fernández-Sanjurjo et al., 2018) and protein (Son et al., 2018).

An alternative approach to measurement of hypothesis-driven candidate targets is unbiased, hypothesis-free omics approaches surveying the broadest possible profile of exercise factors, albeit exercise training studies paired with these approaches are limited to date. One example is in the response to a 4 month treadmill training intervention (20 min at 12–15 m/s per day, 6 day/week), which produced numerous differences at 24 h post-intervention in the resting plasma proteome of male Sprague-Dawley rats (54 proteins in higher abundance, 47 proteins in lower abundance) in trained compared to sedentary controls (Wei et al., 2018). Similarly, cross-sectional studies have reported differences in the resting plasma proteomes of moderately-trained male and female endurance athletes (16 proteins in higher abundance, 23 proteins in lower abundance) compared to sedentary controls (Santos-Parker et al., 2018), and resting plasma metabolomes of elite (6 metabolites in higher abundance, 9 metabolites in lower in abundance) compared to less well-trained endurance athletes (Monnerat et al., 2020). Therefore, there is some indication that exercise training does result in alterations to resting concentrations of exercise factors in circulation. Given the proposed role of small EVs as carriers of these factors we will subsequently appraise the effect of exercise training on the resting profile of circulating small EVs in the next section.

DOES EXERCISE TRAINING ALTER THE RESTING PROFILE OF CIRCULATING SMALL EXTRACELLULAR VESICLES?

There are currently thirteen available reports (across twelve experimental studies) that have investigated the influence of exercise training on indicators of small EV abundance and/or cargo (summarized in **Table 2**). Under the assumption that small EVs are carriers of exercise factors and that exercise factors experience training-induced alterations in resting concentrations in circulation, there are several scenarios wherein *increases* could be represented. The first is alterations in the cargoes associated with preparations of small EVs, i.e., the same approximate quantity of circulating small EVs, but with individual molecules presenting as novel cargo, or as cargo of greater abundance, in exercise-trained states. In other words, an increase in the "density" of a specific cargo per EV, or novel molecules appearing in detectable abundances in circulating small EVs as a result of exercise training. The second is an increase in the quantity of circulating small EVs, i.e., a greater total abundance of cargoes in circulation, which is caused by an increase in the total quantity of circulating small EVs in exercise-trained states (i.e., an exercise training-induced increase in the concentration of circulating small EVs at rest). The third scenario would be some combination of these two scenarios (e.g., a greater quantity of both small EV concentration and cargo abundance). The next subsections will describe the relevant studies in both rodents and humans and whether any of these scenarios are observed.

Results From Rodent Studies

Nine studies have investigated the influence of exercise training on the small EV profile of rodents (Chaturvedi et al., 2015; Bei et al., 2017; Bertoldi et al., 2018; Ma et al., 2018; Hou et al., 2019; Barcellos et al., 2020; Castaño et al., 2020; Xiang et al., 2020; Gao et al., 2021). All of these studies were specifically interested in the miRNA cargo of small EVs, their differential expression and potential biological relevance, and employ an experimental design wherein animals subjected to aerobic exercise training are sampled after the cessation of training. Preparations of small EVs were then separated from plasma (Bei et al., 2017; Ma et al., 2018; Barcellos et al., 2020; Castaño et al., 2020; Xiang et al., 2020; Gao et al., 2021) or serum (Chaturvedi et al., 2015; Bertoldi et al., 2018; Hou et al., 2019), and compared to those of sedentary controls. Collectively, these studies have been inconsistent in

TABLE 2 | Studies in rodents or humans that have investigated the effect of exercise training on the profile of circulating small EVs either by examining the response to exercise training interventions or by performing cross-sectional comparisons of trained and untrained individuals.

Article	Study design and intervention	Sample time point after last exercise bout	Separation and characterization of small EVs	Abundance of small EVs	Changes in small EV cargo
			Training intervention studies		
Ma et al., 2018	- 8–10 week old male C57BL/6 mice - 4 weeks of Low or Moderate treadmill running compared to Sedentary controls (n = 4–6 per group) - Training: 60 min of either Low (5 m/min) or Moderate (10 m/min) intensity; 5 day/week	24 h	- Separation by differential ultracentrifugation, and magnetic microbead sorting of EPC-derived EVs by CD34/VEGFR2 - Particle size and concentration quantified by NTA - Western blot of positive EV markers CD63 and TSG101 and EPC markers CD34 and VEGFR2	- $CD34^+/VEGFR2^+$ EV particles greater in Low (↑1.9x) and Moderate (↑4.3x) compared to Sedentary and greater (↑~2.3x) in Moderate compared to Low	- miR-126 measured by qPCR - ↑1.5x in Low vs. Sedentary - ↑2.3x in Moderate vs. Sedentary - ↑1.5x Moderate vs. Low
Hou et al., 2019	- 6 week old male Sprague-Dawley rats - 4 weeks of swim training intervention compared to Sedentary controls - Training: 1 week of progressively increasing duration, then 3 weeks of 2 × 90 min; 7 day/week - Minimum of n = 6 per assay, but total sample size not reported	24 h	- Separation by differential ultracentrifugation - Particle size and concentration quantified by NTA - Western blot of positive EV markers CD81 and Tsg101 - Small EVs visualized with TEM	- No differences between groups	- 765 miRNAs assayed (Illumina HiSeq 2500) with 14 miRNAs differentially expressed ($> \pm 2$x and $P < 0.05$) - 12 miRNA confirmed by qPCR as differentially expressed in Trained vs. Sedentary - Increased: miR-3571, miR-1-3p, miR-342-5p, miR-122-5p, miR-196b-5p, miR-486, miR-208a-3p, miR-3591, miR-184, miR-760-3p, miR-99a-5p (all ↑~1.8-2.6x) - Decreased: miR-191a-5p (↓~60%)
Castaño et al., 2020	- 15 week old male C57BL/6 mice - 5 week treadmill running intervention compared to sedentary controls - Training: 15 × 2 min at 80% of maximal running speed with 2 min rest between efforts; speed increased by 2 m/min each week; day/week not reported - n = 5/6 per group	48 h	- Separation by differential ultracentrifugation - Particle size and concentration quantified by NTA, and abundance by acetylcholinesterase activity - Western blot of positive EV markers CD63, CD9 and HSP70 - Small EVs visualized with TEM	- No subjective differences in CD63, CD9 and HSP70 between groups - No differences in acetylcholinesterase activity in equal volumes of plasma between groups	- 378 miRNAs assayed by qPCR with 7 miRNAs differentially expressed in Trained vs. Sedentary - Increased: miR-133b-3p (↑11.0x), miR-205-5p (↑10.3x), miR-206-3p (↑9.6x), miR-133a-3p (↑9.5x), miR-19b-3p (↑2.7x), miR-30d-5p (↑2.2x) - Decreased: let-7g-5p (↓7.3x)
Estébanez et al., 2021	- Humans aged 70–85 year - 8 weeks of whole-body progressive resistance training (n = 28 M/F, 15/13) compared to Sedentary controls (n = 10; M/F, 1/9) - Training: 3 sets of 12–8–12 repetitions of 8 exercises of; 2 day/week	5 to 6 days	- Separation by differential ultracentrifugation - Western blot of positive EV markers CD9, CD14, CD63, CD81, Flot-1, and VDAC1	- Increase in CD63 was lower in Trained (6.8%) vs. Sedentary (42.5%)	- No difference between groups in miR-146-5p measured by qPCR
Garai et al., 2021	- Human males aged 23 ± 2 year (n = 14) - Concurrent aerobic and resistance exercise training three times per week for 6 months	At least 24 h	- Separation from pooled serum samples using Total Exosome Isolation reagent and traditional centrifugation - Particle size and concentration quantified by NTA, using unlabeled and labeled (CD63, CD81) preparations - Small EVs visualized with TEM	- No differences between groups	- 54 miRNA identified via NanoString multiplex array with 7 miRNAs differentially expressed in Post vs. Pre (FDR < 0.05) - Decreased: miR-21-5p (FC 0.73), miR-451a (FC 0.59), miR-130a-3p (FC 0.36), miR-15b-5p (FC 0.39), miR-199a/b-3p (FC 0.40), miR-223-3p (FC 0.45), miR-23a-3p (FC 0.49)

(Continued)

TABLE 2 | (Continued)

Article	Study design and intervention	Sample time point after last exercise bout	Separation and characterization of small EVs	Abundance of small EVs	Changes in small EV cargo
			Cross-sectional studies		
Hou et al., 2019	- 19–22 year old males - Rowers (n = 16) training for 1–2 h/day, 6 day/week, and sedentary controls (n = 16) provided resting blood samples	- Rowers: 24 h - Sedentary: ≥7 days	- Separation by differential ultracentrifugation - Particle size and concentration quantified by NTA - Western blot of positive EV markers CD81 and Tsg101 - Small EVs visualized with TEM	- No differences between groups	- miR-342-5p higher (↑1.8x) in Rowers compared to Sedentary when measured via qPCR
Nair et al., 2020	- >65 year old males - Trained (n = 5) or Sedentary (n = 5) stratified based activity history provided resting blood samples	48 h (personal communication)	- Separation by differential ultracentrifugation combined with commercial assay (ExoQuick) - Particle size and concentration quantified by NTA	- No differences between groups	- Small RNA-seq performed (Illumina MiSeq) with 7 miRNAs differentially expressed (> ± 2x and $P < 0.05$) in Trained vs. Sedentary - Higher: miR-206, miR-148a-3p, miR-486-5p, let7b-5p - Lower: miR-874-3p, miR-339-5p, miR-383-5p
Garai et al., 2021	- Sedentary humans aged 23 ± 2 year (n = 14; M/F, 2/12) compared to older males aged 62 ± 6 year (n = 11) identified as >25 year of training at least twice per week in aerobic and resistance exercise	At least 24 h	- Separation from pooled serum samples using Total Exosome Isolation reagent and traditional centrifugation - Particle size and concentration quantified by NTA, using unlabeled and labeled (CD63, CD81) preparations - Small EVs visualized with TEM	- No differences between groups	- 54 miRNA identified via NanoString multiplex array with 3 miRNAs differentially expressed in Trained vs. Sedentary (FDR < 0.05) - Decreased: miR-199a/b-3p (FC 0.40), miR-451a (FC 0.46), miR-23a-3p (FC 0.47)

Several other studies have been performed that purported to investigate the effect of exercise training on the profile of circulating small EVs (Chaturvedi et al., 2015; Bei et al., 2017; Bertoldi et al., 2018; Barcellos et al., 2020; Xiang et al., 2020; Gao et al., 2021), but as referred to in the main text have been excluded because of their sampling of blood <24 h after cessation of the last exercise bout (see sections "'Last Bout Effect': The Importance of Sample Timing" and "Results From Rodent Studies"). Abbreviations: EPC, endothelial progenitor cells; FC, fold change; FDR, false discovery rate; NTA, nano-particle tracking analysis; and TEM, transmission electron microscopy

their approaches toward separating and characterizing small EVs, have tended to measure only a limited number of specific miRNA as representative of small EV cargoes, and have reporting or methodological issues, which all combined, interferes with the inferential utility of these works.

For example, C57BL/6 mice subjected to 4 weeks of treadmill running at either a low (5 m/min) or moderate (10 m/min) speed, and sampled 24 h after the last exercise bout, had an increase in the resting abundance of both $CD34^+/VEGFR2^+$ small EVs and associated miR-126 cargo, in an intensity-dependent manner (Ma et al., 2018). These findings suggest that exercise training induces an increase in the resting concentration of some small EVs, namely those derived from endothelial progenitor cells based on their status as $CD34^+/VEGFR2^+$. However, in this study, the $CD34^+/VEGFR2^+$ small EVs were extracted from an initial separation of small EVs via differential ultracentrifugation using an additional procedure involving magnetic bead sorting. While a particle count assay (NTA) of these preparations estimated a ∼2 to 2.5-fold increase in particles in trained compared to sedentary groups, no difference was visually-apparent in the abundance of either CD34 or VEGFR2 markers in associated Western blots, and these markers were not quantified. As NTA is a non-specific method of small EV quantification, these results imply a disagreement between assays and call into question the inference of the increased resting concentration of small EVs in response to exercise training.

Additionally, Sprague-Dawley rats subjected to 4 weeks of swim exercise and sampled 24 h after the last exercise bout exhibited no change in NTA signal or the abundance of selected small EV markers (TSG101 and CD81) compared to controls, i.e., the basal concentration of small EVs was unchanged by training (Hou et al., 2019). Interestingly, however, 12 miRNA were observed to be differentially expressed (11 up-regulated, 1 down-regulated) in the small EV samples from the exercise-trained rats, suggesting that, even in the absence of a change in the resting concentration of small EVs, the cargo of small EVs may change with exercise training (Hou et al., 2019).

Four other studies have reported increases in signals indicative of particle enrichment and small EV marker abundance from small EV samples from exercise-trained rats (Bei et al., 2017; Barcellos et al., 2020) and mice (Chaturvedi et al., 2015; Gao et al., 2021). However, of these studies Barcellos et al. (2020) sampled 1 h after the last exercise bout, Gao et al. (2021) sampled immediately after the last exercise bout (personal

communication), and Bei et al. (2017) sampled on average 6 to 12 h after the last exercise bout (personal communication), i.e., each study sampled in the immediate post-exercise period, when acute exercise-induced enrichment may still be present. We were unable to ascertain the time point of sampling from the work of Chaturvedi et al. (2015) or Xiang et al. (2020). Regardless, in training studies where sampling is in close proximity to the end of the last exercise bout, it is difficult, or sometimes impossible, to parse out whether results are indicative of the effect of acute exercise, residual bout effects, a true training effect, or a combination of the three.

Other studies have waited 24 h before taking post-training samples (Ma et al., 2018; Hou et al., 2019), but residual influences of an acute exercise bout may still be apparent within these and shorter time periods (section "'Last Bout Effect': The Importance of Sample Timing"). Indeed, in humans, acute exposure to downhill running and plyometric exercise produced decreases of miR-31b in preparations of small EVs for up to 24 h after exercise (Lovett et al., 2018). To our knowledge, only one other study has investigated the influence of exercise training on circulating small EV profiles in samples extracted > 24 h after the last training bout (Castaño et al., 2020). Castano et al. subjected male C57BL/6 mice to 5 weeks of progressive high intensity treadmill running, and small EVs were isolated from plasma 48 h after the last training bout. No differences were observed in small EV abundance via an acetylcholinesterase assay, or subjective appraisal of Western blots of three small EV markers. Additionally, it should be noted, however, that acetylcholinesterase has been proven to not be a generic marker of EVs (Liao et al., 2019). Regardless, profiling of 378 miRNA transcripts by qPCR revealed seven differentially expressed miRNAs (6 miRNA with higher abundance, 1 miRNA with lower abundance) when comparing exercise-trained to sedentary groups (Castaño et al., 2020).

Collectively in these rodent studies, methodological limitations in terms of providing adequate indication of the presence of small EVs within samples separated from biofluids, and potential confounding by residual influences of the last exercise bout must be acknowledged, but some reports do indicate altered miRNA cargo within preparations of small EVs taken at rest after a period of exercise training (Hou et al., 2019; Castaño et al., 2020).

Results From Human Studies

To our knowledge, there are currently four reports describing the influence of prior exercise training on preparations of circulating small EVs derived from resting humans (Hou et al., 2019; Nair et al., 2020; Estébanez et al., 2021; Garai et al., 2021; **Table 2**), three of which separated small EVs from plasma (Hou et al., 2019; Nair et al., 2020; Estébanez et al., 2021) and one which has separated small EVs from serum (Garai et al., 2021). The first study reported 1.8-fold greater abundance of miR-342-5p at rest in preparations of small EVs derived from young (19 to 22 years) male rowers with at least 1 year of training experience compared to sedentary controls (n = 16 in each group). However, small EVs were not formally characterized in this study, despite a small EV separation protocol being applied to plasma samples, and miR-342-5p abundance measured as the only target of interest. The next study compared resting miRNA profiles (via RNA sequencing) of small EV samples derived from endurance-trained and sedentary older (~69 years) males, albeit with only n = 5 in each group (Nair et al., 2020). Seven differentially expressed miRNAs were identified between trained and untrained individuals, with 4 increased and 3 decreased in preparations of small EVs derived from the trained individuals. However, this study provided minimal information regarding the characterization of small EVs in samples by reporting only a single NTA result and making no comparisons between groups. Additionally, while the aerobic fitness of the participants was discordant (VO_{2max} of 34.4 ± 1.1 and 21.7 ± 1.2 ml kg^{-1} min^{-1} in trained and untrained groups, respectively), and the trained participants clearly had superior fitness, the average VO_{2max} for these individuals is still approximately half of what is commonly-reported for well-trained athletes of younger age (Jeukendrup et al., 2000; Jones et al., 2020). Therefore it is unclear whether a greater magnitude of fitness at younger age would create additional or alternative differences in the profile of resting small EVs between either the trained or sedentary group in the aforementioned study (Nair et al., 2020). The third study was in male and female older adults (~73 years) that compared an 8 week resistance exercise training intervention (n = 28) to a sedentary control group (n = 10; Estébanez et al., 2021). However, the analysis was limited to the presence of small EVs via quantification of total exosome protein, identification of six marker proteins of small EVs via Western blot, and one proposed miRNA cargo of small EVs (miR-146a-5p; Estébanez et al., 2021). Of these, only the small EV marker CD63 exhibited a differential pattern between groups with the increase of ~7% in the training groups being less than the ~43% increase in the sedentary control group. Therefore, an attenuation of small EVs expressing CD63 may be a response to resistance exercise training, but overall, the data from this study are also limited in their coverage.

The final study consisted of two pilot experiments that investigated the potential influence of exercise training on the circulating profile of small EVs at rest (Garai et al., 2021). The first experiment consisted of healthy participants (n = 14; M/F, 2/12) performing a concurrent aerobic and resistance exercise training intervention 3 days per week for 6 months. Resting blood samples were taken at baseline and after the end of the training intervention, with each sample being reported as taken at least 24 h after any vigorous exercise. In this experiment, no difference was observed in the number of particles within separated preparations of small EVs via NTA before and after exercise training, although seven miRNA were differentially-expressed (all decreased) when analyzed using a NanoString array (**Table 2**). The second experiment reported in this study was a cross-sectional study in which resting profile of circulating small EVs from older men (aged 62 ± 6 years; n = 11) self-reporting > 25 years of exercise training [and identified as "trained" via their responses to the International Physical Activity Questionnaire (IPAQ; Craig et al., 2003)], was compared to the baseline sedentary samples of the cohort in the first experiment (Garai et al., 2021). Again no difference was observed in the resting abundance of small EVs between the older trained men and younger sedentary individuals via NTA. However, three miRNA were differentially-expressed (all decreased) when analyzed using a NanoString array (**Table 2**). Given that the older

participants were defined as trained based on their response to the IPAQ rather than objective measures of exercise training history or physical fitness, arguably it is difficult to discern the extent to which these (minor) differences in miRNA were solely the result of divergent exercise training habits. Age and sex may also have been a factor given that the trained cohort in this study were on average ~39 years older than the sedentary participants and were male as opposed to mostly females in the sedentary group (Garai et al., 2021). The resting profile of circulating small EVs is influenced by biological sex and chronological age (Noren Hooten, 2020) and therefore are likely to be major confounders in these data.

Summary

Appraisal of existing studies across rodents and humans regarding the influence of exercise training on the abundance of small EVs and their proposed miRNA cargoes leads us to conclude that the characterization of small EVs within samples is generally inadequate by way of employing insufficient methodological approaches. Therefore, while some data indicate that the resting concentration of small EVs may change with exercise training, which has been commented by others (Brahmer et al., 2020; Nederveen et al., 2021), arguably the methods employed do not produce sufficient information to confidently state this to be the case.

Aside from the difficulty in accurately characterizing small EVs and their cargo, it is pertinent to also consider physiological and teleological aspects of the proposition that resting profiles would indeed be changed by exercise training. Circulating concentrations of small EV are a dynamic balance, such that the concentration measured in a resting sample is indicative of the processes of small EV release and small EV uptake, and yet small EVs may have a relatively short half-life of ~7 min (Matsumoto et al., 2020). Thus, for enriched concentrations of small EVs to be observed in a resting sample distal to acute exercise, exercise training would have to induce augmented small EV release, attenuated small EV uptake, or both, at rest. To rigorously determine whether either or both occur would require determining which mechanisms govern systemic release and clearance of small EVs, and how exercise training either augments or attenuates these processes. Distinct from assessing small EV abundance, there is greater consistency across studies indicating that exercise training influences the miRNA profile of preparations of small EVs from resting samples. As exercise training can alter the transcriptomes (Pillon et al., 2019) and proteomes (Holloway et al., 2009; Ferreira et al., 2014; Padrão et al., 2016) of contractile tissues, a change in cargo profile is plausible independent of change in small EV abundance, because local changes to the biomolecular environment of a cell could alter the cargo that is released, even in the absence of an altered rate of EV release. However, it is important to reiterate that without adequate characterization of small EVs, the extent to which these differential miRNA profiles associate specifically to small EVs (even when small EV separations have been undertaken) is unknown. There remains the question as to why a persistent, as opposed to acute, change in resting profile of small EVs would occur with exercise training, and therefore, lastly we will consider the bioactivity of small EVs isolated from trained compared to untrained rodents and humans.

BIOACTIVITY OF SMALL EXTRACELLULAR VESICLES OBTAINED FROM TRAINED AND UNTRAINED INDIVIDUALS

Consideration of the bioactivity and/or beneficial physiological effects of small EVs may provide insight into whether training-induced changes in the resting profile is a potential mediator of the benefits of exercise training, or perhaps methodological artifact. Six studies to our knowledge have performed *in vivo*/*ex vitro* experiments to investigate such effects (Bei et al., 2017; Ma et al., 2018; Hou et al., 2019; Castaño et al., 2020; Wang et al., 2020). However, we have not included discussion of two of these studies due to the post-training sample time point being <24 h after the last exercise training bout (Bei et al., 2017; Gao et al., 2021).

Of the remaining studies, two have investigated the bioactive effects of preparations of small EVs derived from exercise-trained mice exclusively in cell lines (Ma et al., 2018; Wang et al., 2020), and the other two have investigated potential *in vivo* effects through injection of small EVs derived from exercise-trained rodents into sedentary control animals (Bei et al., 2017; Castaño et al., 2020). In the first study (Ma et al., 2018), preparations containing $CD34^+/VEGFR2^+$ small EVs were co-incubated with cultured brain endothelial cells that were exposed to 18 h of 25 mM glucose and/or 6 h of hypoxia (1% O_2, 5% CO_2) as a model of endothelial cell injury. Co-culturing of cells with small EVs derived from exercise-trained or sedentary C57BL/6 mice produced reductions in cell apoptosis of ~5, 10, and 20% for sedentary, low and moderate exercise-trained samples, respectively. Cell migration and tube formation was increased similarly in a condition-dependent manner. These effects were speculated to be mediated by miR-126, a transcript increased in preparations derived from the exercise-trained mice. In these cells, preparations of small EVs enhanced the expression of the angiogenic protein VEGF, while anti-miR-126 decreased VEGF expression and removed the described effects of small EV co-incubation (Ma et al., 2018). These results suggest that exercise training enhanced the abundance of specific transcripts within preparations of small EVs, which in turn are capable of enhancing the expression of local factors associated with some exercise adaptations, such as angiogenesis in this case.

Similar findings were reported in an *in vivo* model of cardiac injury induced in Sprague-Dawley rats (Hou et al., 2019). Sedentary rats received a direct intramyocardial injection of small EVs derived from exercise-trained rats 2 days prior to a surgically-induced myocardial infarct. Rats who had received injections of preparations of small EVs derived from exercise-trained counterparts demonstrated ~40% lower infarct size 24 h post-surgery, and enhanced cardiac function through a lower reduction in ejection fraction and fractional shortening 4 weeks

post-surgery, compared those injected with small EVs from sedentary controls. These results were similarly associated with the presence of a single miRNA in small EV samples (miR-342-5p), which was measured to reduce the abundance of some apoptotic signaling proteins (Caspase-9 and Jnk2), and potentiate proliferative Akt signaling, in cultured cardiomyocytes exposed to hypoxic damage (Hou et al., 2019). In follow-up work to Ma et al., Wang et al. again used preparations containing $CD34^+/VEGFR2^+$ small EVs derived from plasma from exercise-trained mice (60 min/d at 10 m/min, 5 day/week for 4 weeks). Co-incubation of these EV preparations with neuronal N2a cells subjected to hypoxia-reoxygenation injury resulted in protection against injury (increased cell viability, decreased apoptosis), and restored neurite length. These results coincided with greater secretion of brain-derived neurotrophic factor from these cells, and all effects were partially attenuated when inhibition of miR-126 or Akt-PI3K signaling was employed (Wang et al., 2020).

The work in each of these three studies should be noted as models that have potentiated recovery from injury in damaged cells or tissues (Ma et al., 2018; Hou et al., 2019; Wang et al., 2020) and therefore, these bioactivities do not necessarily translate into potentiating the function of healthy cells, or provide insight into mediating an exercise adaptation or training response in tissues. However, recent work by Castano et al. has attempted to address these questions. C57BL/6 mice were exposed to 4 weeks of high intensity interval training via treadmill running after which small EVs were separated from plasma collected 48 h after the last exercise training bout. These preparations, as well as preparations derived from sedentary control mice, were then injected intravenously daily for 4 weeks into separate groups of sedentary mice. At the end of the 4 week administration period, mice treated with exercise-trained small EVs demonstrated lower body mass (~6%) and epididymal fat mass, lower circulating triglycerides (~15%), improved lipid tolerance, and improved insulin sensitivity and glucose tolerance (glucose AUC ~35% lower), compared to the control mice (Castaño et al., 2020). Analysis of differentially-expressed miRNA between preparations of small EVs derived from exercise-trained or sedentary mice (discussed in section "Results From Rodent Studies" and **Table 2**) also yielded a thematic association with the regulation of the transcription factor forkhead box O1 (*FoxO1*), which in part regulates hepatic gluconeogenesis (Puigserver et al., 2003). Preparations of small EVs derived from the exercise-trained mice reduced hepatic *FoxO1* mRNA expression, as well as

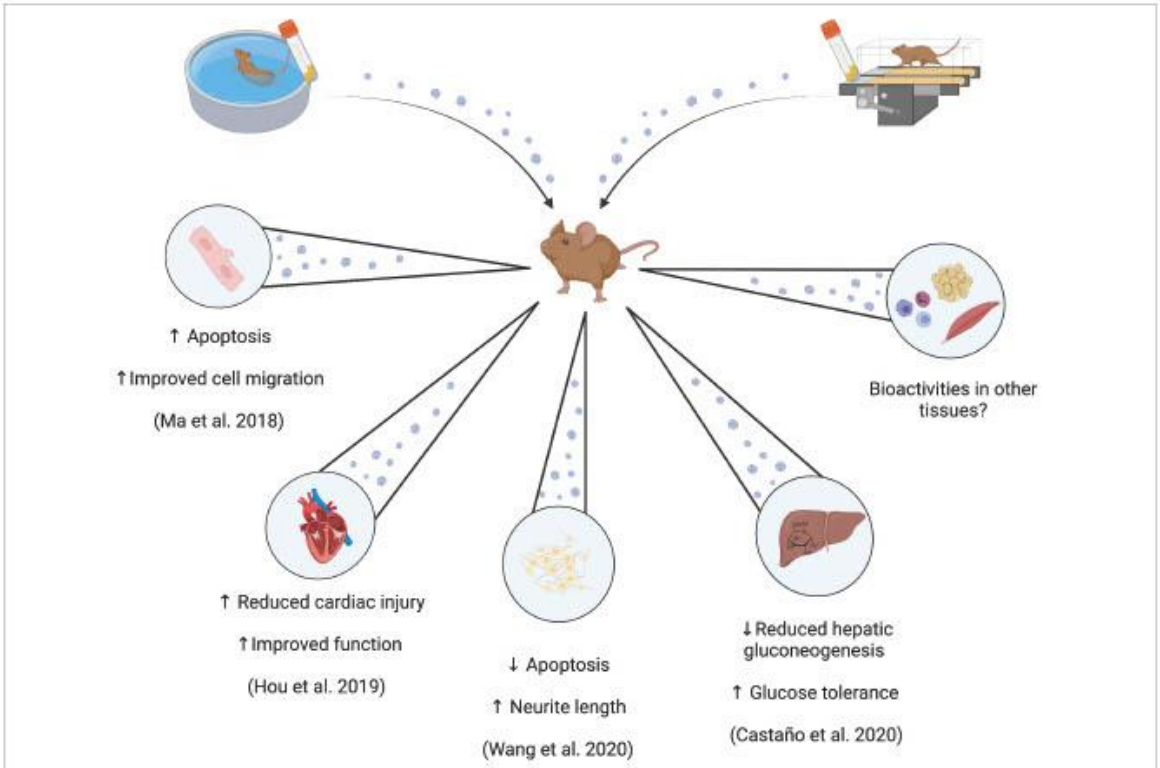

FIGURE 2 | Potential bioactivities of circulating small EVs derived from exercise-trained rodents. Administration of circulating small EVs prepared from blood samples from exercise-trained rodents are identified to induce effects both *in vivo* and *ex vivo* that may be associated with health primarily as enhanced glucose tolerance (*in vivo*), and enhanced recovery from ischemic injury (*in vivo* and *in vitro*).

transcripts of several gluconeogenic proteins associated with *FoxO1* signaling. Thus, extended administration of small EV samples derived from exercise-trained mice was proposed to enhance glucose tolerance through small EV-mediated delivery of miRNAs that attenuated hepatic gluconeogenesis (Castaño et al., 2020).

In summary, small EVs derived from resting blood samples of exercise-trained rodents appear to exert bioactive, and potentially beneficial, effects in several tissues compared to sedentary counterparts (**Figure 2**). These results suggest that exercise-trained small EV samples may offer therapeutic potential in specific use cases, or may play a role in exercise adaptation, and/or homeostatic maintenance. However, more research is required to determine aspects such as the consistency of specific metabolic effects, the type and time course of exercise training required to induce reliable changes in resting small EV profiles in humans, the duration for which these changes are sustained after training cessation, and whether it is the entire small EV sample or specific cargo within those samples that are the strongest determinants of observed bioactivity.

CONCLUDING REMARKS

The purpose of this review was to appraise the currently-available evidence regarding exercise-induced changes in the circulating profile of small EVs against an established biological paradigm of the response of their proposed cargo, i.e., exercise factors. On the whole, presently the small EV profile may indeed be changed both in response to acute exercise (via both altered small EV abundance and cargo), and at rest (via altered cargo profiles) in the exercise-trained state. However, working with small EVs is difficult and requires rigorous approaches to both separation and identification, which requires employing multiple complex methodologies. In this regard, the results of many studies are somewhat limited, as essential aspects of small EV characterization and/or quantification are often absent. This, in turn, has implications for the extent to which the role of EVs in exercise metabolism and adaptation can be understood.

Many pertinent questions remain, such as the proportional contribution of different cell types to the circulating small EV pool both at rest and with exercise, and by which mechanisms in cells a shift may occur in the cargoes that are packaged and released from small EVs during exercise or after training. Finally, investigation of the time course for which the circulating small EV profile at rest may change in response to exercise will inform whether training-induced changes in exercise factors are primarily residual artifacts, or an important component of the mechanistic basis for exercise adaptation. At present there are promising preliminary data that small EV preparations from exercise-trained samples do exert relevant bioactivity that may be important for the beneficial effects of exercise in organs beyond skeletal muscle.

AUTHOR CONTRIBUTIONS

ID and BE conceptualized the review. ID wrote and developed the initial drafts of the manuscript with feedback from BE. BE and ID wrote and developed advanced drafts of the manuscript with feedback from LO'D. BE developed and formatted the table and ID illustrated all figures, which were edited based on feedback from BE and LO'D. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

APPENDIX B : Short-Term Exercise Training and Serum Metabolome Article

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ORIGINAL ARTICLE

The resting serum metabolome in response to short-term sprint interval training

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Abstract

Purpose To investigate the response of a targeted fraction of (168 metabolites) of the resting serum metabolome to 9 sessions of sprint interval training (SIT).

Methods Thirty-four recreationally active males provided resting blood samples before (baseline) and 48–72 h after (post) a short-term (9 sessions) cycle ergometer-based SIT intervention. A targeted analysis of 168 metabolites was performed on serum using liquid chromatography mass spectrometry (LC–MS). 160 distinct metabolites were identified and combined with 4 calculated metabolite sums and 3 calculated metabolite ratios creating a panel of 167 individual factors. Data were analysed using principal component analysis and univariate testing of all factors classified into 5 metabolite subgroups.

Results SIT improved anaerobic capacity measured by average power output during a Wingate test ($p < 0.01$; mean difference = 38 W, 95% confidence interval [26, 51]) and aerobic capacity measured by average power output in a 20 min cycling test ($p < 0.01$; 17 W [12, 23]). Limited separation was discernible in the targeted serum metabolome between baseline and post-intervention when projected on the first and second principal component(s). However, univariate testing identified 11 fatty acids that had lower concentrations (false discovery rate < 0.05) in post-intervention samples.

Conclusions These findings demonstrate that this short-term SIT intervention had limited effect on the serum metabolome at rest, but a subfraction of fatty acids are potentially sensitive to short-term exercise training.

Keywords Exercise training · Metabolomics · Sprint interval training · Exercise metabolism

Abbreviations

ANOVA	Analysis of variance
FDR	False-discovery rate
^{1}H-NMR	^{1}H-nuclear magnetic resonance spectroscopy
LC–MS	Liquid chromatography mass spectrometry
PCA	Principal component analysis
SIT	Sprint interval training

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Introduction

Sprint interval training (SIT) is a term used to describe a method of exercise training composed of individual exercise bouts that are each characterised by sets of brief (i.e. < 30 s), highly intensive activity periods and extended inter-set recovery periods (i.e. > 2 min) (MacInnis and Gibala 2017). When discussed within the context of exercise training for the general population, SIT is often promoted as an effective and time-efficient approach (Gibala et al. 2012) to enhance physiological indices of aerobic fitness (e.g. VO_{2max}) (Gist et al. 2014) and/or health (e.g. blood pressure) (Gibala et al. 2012). Notably, even short-term SIT interventions (6 sessions performed over 2 weeks) have demonstrated increased time to task failure during continuous exercise trials with coincident increases in markers of physiological remodelling to exercise training, such as skeletal muscle maximal citrate synthase activity (Burgomaster et al. 2005). These observations imply that adaptations elicited by SIT are detectable in the early stages of a training intervention, and makes

short-term SIT a useful tool for studying systemic exercise adaptation.

Metabolomic analysis refers to the comprehensive systematic profiling of metabolites in a biological sample (Nicholson and Lindon 2008). Specific or collective metabolite concentrations represent the outcomes of metabolic reactions and, therefore, metabolomic analysis arguably provides the most informative insight into the general metabolic state of tissues at the time of sampling (Belhaj et al. 2021). In this regard, blood represents a tissue of particular interest. This is partially due to the mixed composition of the circulating metabolome i.e. the metabolite content of blood consists of contributions from many tissues, and thus may provide insight into metabolic shifts that are representative of the physiologic state of an organism as a whole; the study of blood is also practically appealing due to the ease with which samples can be obtained compared to other tissue types (Dunn et al. 2011), for example muscle biopsies—which require more invasive and technically challenging procedures to extract and tend to produce greater patient discomfort compared to blood samples (Ekblom 2017). Interestingly, changes to the circulating metabolome at rest can manifest as a consequence of exercise training (Sakaguchi et al. 2019). For example, 8 weeks of sprint training (repeats of 80 m running sprints) induced changes in the ^{1}H-nuclear magnetic resonance spectroscopy (^{1}H-NMR) metabolomic profile of serum of moderately trained men at rest that were primarily related to changes in circulating TCA intermediates, glucose and choline-containing molecules (Pechlivanis et al. 2013). Cross-sectional studies employing LC–MS have also demonstrated that exercise-trained individuals with different training backgrounds (bodybuilders or endurance athletes or untrained controls) each present with distinct serum metabolomic profiles at rest, with differences between groups in being driven by differences in metabolites related to Amino Acid and Hydroxysphingolipid and Phosphotidylcholine metabolism (Schranner et al. 2021); and that competitive middle distance runners (5000–10000 m) with a greater VO_{2max} present with divergent profiles of the plasma metabolome at rest compared to athletes with a lower VO_{2max} from the same competitive discipline, with differences between athlete groups primarily related to differences in metabolites associated with Alpha Linoleic and Glutathione metabolism and Carnitine synthesis (Monnerat et al. 2020).

Collectively, this provides indication that exercise training may exert some influence on the blood metabolome at rest, but it is currently unclear how the resting blood metabolome responds to short-term exercise training. Here, we present a secondary analysis on a subset of data derived from a larger trial, that has also been detailed in a previous publication (Aird et al. 2021), the aim of the current analysis is to provide insight into whether a short-term intervention of SIT (9 sessions performed over 3 weeks) induces changes to the serum metabolome at rest using a targeted analysis of a collective 167 individual metabolites and calculated metabolite sum/ratios.

Methods

Participants

Recreationally active males ($n = 34$, age = 25.2 ± 4.2 years, height = 1.8 ± 0.1 m, body mass = 83 ± 9.9 kg, BMI = 25.6 ± 4.2 kg/m^2, baseline VO_2max = 42.2 ± 4.9 mL kg min^{-1}, data presented as mean ± standard deviation, also available in Table S1) were recruited for this study and signed written informed consent forms prior to undergoing any experimental procedures. All the procedures performed in this study received ethical approval by the Faculty of Education and Health Sciences research ethics committee at the University of Limerick (Ireland) (Ethics No: 2016_18_11_EHS).

Exercise training intervention and performance measures

All participants successfully completed 9 sessions of SIT. Each session consisted of a fixed number of "Wingate"-style sprint intervals, where participants were asked to pedal with maximal effort on bicycle ergometer (Monark, 894E, Sweden) against a resistance equivalent to 7.5% of pre-intervention body mass for 30 s, before resting passively on the ergometer saddle for 4 min between each interval. At the beginning of each session, participants warmed up with 5 min of unloaded cycling at a cadence of 60–70 RPM and each session ended with a 3 min cool down of unloaded cycling at the same pedalling rate. The volume of each session was increased incrementally, whereby in sessions 1–3, participants performed 4 intervals, 5 intervals in sessions 4–6, and 6 intervals in sessions 7–9. Sessions were separated by 48–72 h (i.e. typically performed on a Monday, Wednesday and Friday) and participants were asked not to perform any vigorous physical activity outside of the prescribed training sessions, which was confirmed weekly with participants at the first training session of each week. For performance outcome measures, participants performed a single maximal effort 30 s Wingate test to measure anaerobic capacity, and a 20 min test to measure aerobic capacity. During the 20 min test, which was performed on a different mechanically braked cycling ergometer (SRM, Germany) cadence was fixed at 85 RPM while power adjusted in a variable fashion in accordance with participant's effort. Prior to the first assessment (the Wingate test) participants completed a standardised 5 min warm-up on a Monark cycle

ergometer that consisted of unloaded cycling at 60–70 rpm. These tests were performed in this order, 60 min apart, at both baseline, and 48–72 h after the final SIT session, respectively. Prior to beginning the 9 session SIT intervention, participants completed a 7 day weighed food diary which was entered to online software (Nutritics. Version 5.031). Participants were asked to maintain their habitual nutrition throughout the SIT intervention and prior to each training and lab testing session, participants performed a 24 h dietary recall with researchers, which was compared to initial 7 day weighed food diary to ensure habitual diet was maintained (Table S1). In addition, participants refrained from alcohol and caffeine intake for 24 h and 12 h, respectively, before baseline and post-intervention blood samples which were obtained in the fasted state.

These data are derived from a larger trial where participants were additionally randomised into one of four intervention groups whereby each training session was performed under different nutrition conditions, namely: fasted ($n=8$), carbohydrate-fed ($n=9$), whey protein concentrate-fed (WPC) ($n=8$), or whey protein hydrolysate-fed (WPH) ($n=9$). During this intervention, participants received a pre-prepared supplement drink 45 min before each exercise training session only; specific details of this intervention are available elsewhere (Aird et al. 2021). No significant difference in performance outcomes or evidence of differences in the profile of baseline/post-intervention serum metabolomes between the nutrition intervention groups were observed (Table S1). Therefore, for the present analysis, participants were pooled into a single collective group to increase statistical power.

Blood sample acquisition and storage

For blood extraction, a 9 ml blood sample was taken from an antecubital vein in an overnight fasted state at baseline and post-intervention (48–72 h after the final exercise bout) into Serum Monovette® vacutainer tubes (Sarstedt, Munich, Germany). All blood samples were extracted at a consistent time of day (between 7.30 and 8:30 am). To facilitate clotting, extracted blood samples were left to stand for 30 min at room temperature, samples were then centrifuged for 10 min at 2000 g (20 °C). Serum sample aliquots were stored at – 80 °C before being sent to an analytical facility for the subsequently described metabolomics analysis.

Serum metabolomics

All serum metabolomics analyses were performed by The Metabolomics Innovation Centre (TMIC, Calgary, Canada) using custom assays employing targeted methods. Each assay involved a combination of direct injection mass spectrometry with reverse phase liquid chromatography (LC–MS/MS) using an ABSciex 4000 Qtrap® tandem mass spectrometry instrument (Applied Biosystems/MDS Analytical Technologies, Foster City, CA, USA) coupled with an Agilent 1260 series UHPLC system (Agilent Technologies, Palo Alto, CA, USA). These instruments were used to quantify a targeted selection of up to 168 metabolites and can provide identification and quantification of numerous metabolite species (specifically, amino acids, acylcarnitines, biogenic amines and derivatives, uremic toxins, glycerophospholipids, sphingolipids and sugars, TMIC prime assay) and free fatty acids. Assays involved the derivatisation and extraction of analytes, and the selective mass-spectrometric detection using multiple reaction monitoring pairs and metabolites that are subsequently quantified using Isotope-labelled internal standards and other internal standards. This assay is an adapted method used for targeted analysis of metabolites in urine (López-Hernández et al. 2021) that have been detailed elsewhere (Zheng et al. 2020). Upon identification and quantification, metabolites were subsequently classified into four distinct metabolite groups (Amino Acids, Peptides and Analogues; Fatty Acids and Fatty Acid conjugates; Acylcarnitines; and Glycerophospholipids and Phosphosphingolipids) using the "ChemOnt" taxonomic classification technique (Feunang et al. 2016). Fourteen of our detected metabolites belonged to taxonomy groups for which < 3 metabolites were measured; these metabolites were gathered into a single group labelled "Others". We also calculated four metabolite sums (branched chain amino acids, gluconeogenic amino acids, essential amino acids, total acylcarnitine) and three metabolite ratios (acylcarnitine/carnitine, C2/C0 and kynurenine/tryptophan ratios, respectively), as this can reduce data variation and provide insight into whether metabolic processes may be altered when certain clusters of metabolites with similar bioactivities have changed in a collected fashion (Petersen et al. 2012). Eight metabolites in our targeted panel were below the limit of detection (histamine, cis-hydroxyproline, dopamine, carnosine, nitro-tyrosine, diacetyl-spermine, tyramine, phosphocreatine), which collectively left the total number of variables generated for analysis (individual metabolites and metabolite sum/ratios) at 167 (168 potentially identifiable metabolites on the targeted assay panel, minus 8 undetected metabolites, plus 7 calculated metabolite sums/ratios for a total of 167 variables prepared for analysis, Table S2).

Principal component analysis

Prior to dimension reduction, a Kaiser–Mayer–Olkin test of sampling adequacy and Bartlett's test of sphericity were performed on data to ensure they met the minimum standards for principal component analysis (PCA). Bartlett's test of sphericity produced a significant result ($X^2(166)=95{,}506$, $p<0.01$), while a Kaiser–Mayer–Olkin

test of mean sampling adequacy produced a result of 0.5, which is a low, but acceptable value (Kaiser and Rice 1974). Subsequently, a multilevel PCA was performed on all metabolites and metabolite sums and ratios using an orthogonal rotation and with factors mean zeroed and scaled for unit variance. Multilevel PCA refers to a modified analysis applicable to repeated-measures design and involves decomposition of within-subject variation after the values for each selected variable (in this case each individual metabolite, metabolite sum or ratio) are collectively decomposed into linear variables (termed principal components) each of which explain an independent (orthogonal) and descending (i.e. PC1 explains the largest individual amount of variance, PC2 the second largest amount of variance) of total data variance. This approach has been demonstrated to increase model accuracy for paired-sample data (Liquet et al. 2012). All PCA were performed using mixOmics (version 6.14.1), a curated analysis package designed for exploratory analysis/feature selection within the environment of the statistical programming language R (Rohart et al. 2017). The number of principal components used was seven, which was selected based on appraisal of the "elbow" of a scree plot estimating the point where the proportion of variance explained by each principal component becomes approximately level (Table S3).

Mixed ANOVA of performance outcomes and univariate analysis of metabolites

As this was a secondary analysis of data from a combined exercise and nutrient intervention, we first performed a mixed analysis of variance (time * nutrient intervention) (ANOVA) to determine whether there were performance differences between nutrient intervention groups at either baseline or post-intervention. Univariate testing was also performed on metabolite data, where a series of pairwise *t* tests with a Benjamini–Hochberg correction to adjust for multiple comparisons was applied to each of the five categorical metabolite groups (Amino Acids, Peptides and Analogues; Fatty Acids and Fatty Acid Conjugates; Acylcarnitines; Glycerophospholipids and Phosphosphingolipids and Others) in isolation (Table S4). To determine common metabolic processes associated with altered metabolites, significant results underwent pathway enrichment analysis using a free online web-platform (MetaboAnalyst) (Xia et al. 2009). All statistical tests were considered to have produced a significant result if a p value < 0.05 (or FDR < 0.05 for pairwise analysis of metabolites) was calculated. ANOVA results are reported with a partial-eta squared standardised estimate of effect size (η_p^2).

Results

Performance results

The results of 9 sessions of SIT on performance outcome measures are presented in Fig. 1. A significant main effect of time was observed for average Wingate power ($F = 41.88$, $p < 0.01$, $\eta_p^2 = 0.58$, baseline = 640 ± 74 W, post-intervention = 678 ± 75 W, mean difference = 38 W, 95% confidence interval = [26,51]) and average power during the 20 min test ($F = 41.29$, $p < 0.01$, $\eta_p^2 = 0.57$, baseline = 182 ± 30 W, post-intervention = 199 ± 30 W, mean difference = 17 W, 95% confidence interval = [12,23]).

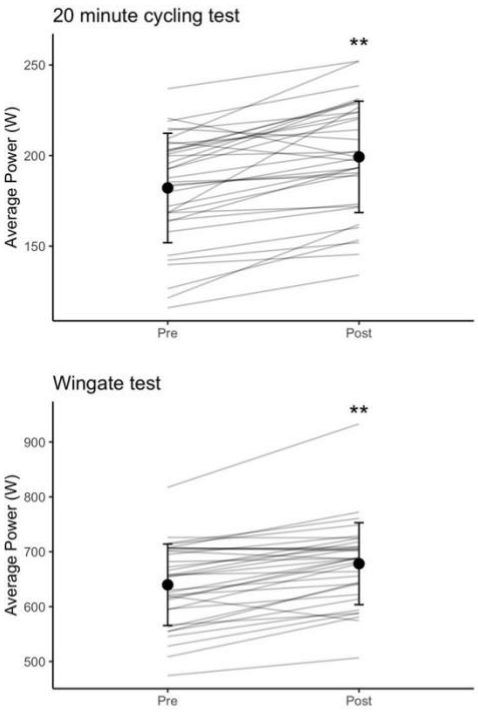

Fig. 1 Performance changes in response to 3 weeks of sprint interval training. Filled dots indicate collapsed group means, error bars represent standard deviations. Filled lines indicate individual participants. * indicates a significant main effect for intervention ($p < 0.05$) ** ($p < 0.01$)

Principal component analysis of the serum metabolome

The results of a multilevel PCA to investigate whether SIT intervention influenced any changes in the serum metabolome are reported in Fig. 2; there was no clear spatial separation between baseline and post-intervention samples, suggesting that 9 sessions of SIT did not influence the overall profile of this targeted analysis of the serum metabolome at rest.

Univariate analysis comparing baseline and post-intervention circulating metabolites

The results of univariate analysis of all metabolites within each categorical metabolite group comparing serum metabolites, metabolite sums and metabolite ratios at baseline and post-intervention are presented in Table S4. The following metabolites, all belonging to the Fatty Acids and Fatty Acid Conjugates group, had significantly lower circulating concentrations: Oleic acid (Fold Change (FC) = 0.73, p.adj = 0.001); cis-8, 11, 14 Eicosatrienoic acid (FC = 0.81, p.adj = 0.002); Alpha Linolenic acid (FC = 082, p.adj = 0.003), Myristic acid (FC = 0.73, p.adj = 0.004); Margaric acid (FC = 0.82, p.adj = 0.004); Gamma Linolenic acid (FC = 0.93, p.adj = 0.012); Linoleic acid (FC = 0.84, p.adj = 0.012); Stearic acid (FC = 0.90, p.adj = 0.016); Pentadecylic acid (FC = 0.93, p.adj = 0.023); Palmitic acid (FC = 0.90, p.adj = 0.023) and Palmitoleic acid (FC = 0.82, p.adj = 0.027) (Fig. 3). When the 11 total fatty acids with significantly lower concentrations were included in a subsequent enrichment analysis, 1 significant

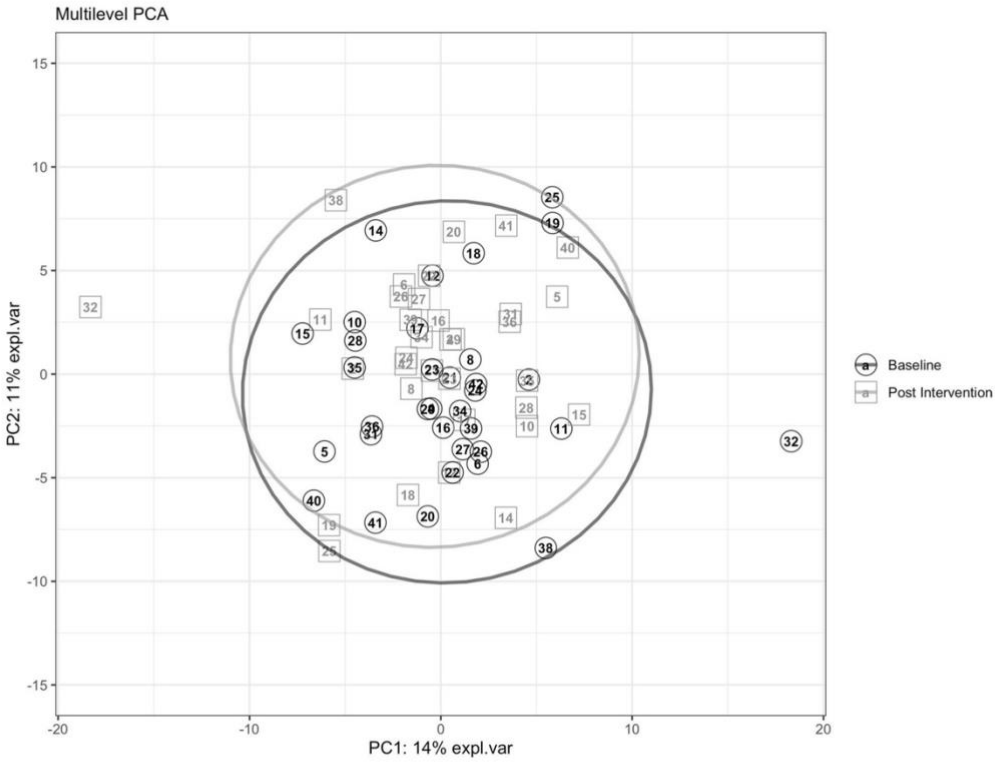

Fig. 2 Principal component analysis of resting serum metabolomes for all participants. Small panes represent simple analysis of participant's baseline ($R^2X = 0.58$) (**A**) and post-intervention ($R^2X = 0.56$) (**B**) metabolomes in isolation. Large pane (**C**) displays results of a multilevel analysis containing both participants' baseline and post-intervention samples. Individual numbers represent individual participants ($R^2X = 0.52$). Circles represent a baseline metabolome sample, while squares represent post-intervention metabolome samples; individual numbers represent participant IDs

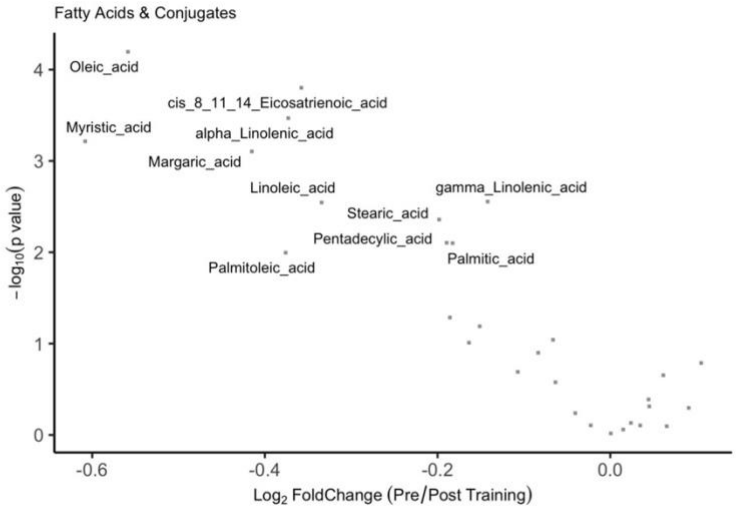

Fig. 3 Volcano plot of differentially abundant Fatty Acids, named metabolites are those which were found to be differentially (adjusted *p* value < 0.05) abundant between baseline and post-intervention samples

pathway—biosynthesis of unsaturated fatty acids (7/36 hits, *p*.adj < 0.01) was identified.

Discussion

The aim of this study was to investigate whether a short-term intervention of SIT induces changes in the serum metabolome of recreationally active males at rest using a targeted analysis of 167 metabolites and metabolite sums/ratios. Nine sessions of SIT improved the average power output of exercise tests indicative of anaerobic and aerobic capacity, with increases of 6% and 17% for the Wingate and the 20 min test, respectively. These results align with previous studies that have demonstrated this specific SIT protocol (multiple sets of 30 s all-out sprints, separated by 4 min recovery) can enhance measures of exercise and performance capacity (e.g. average Wingate power outputs, cycling time-trial performance(s)) (Hazell et al. 2010; Little et al. 2010) and physiological indices of aerobic capacity (e.g. VO_{2max} and skeletal muscle maximal citrate synthase activity) over short-term intervention periods (i.e. 6 training sessions) (Burgomaster et al. 2005; Hazell et al. 2010; Little et al. 2010).

After establishing the sufficiency of 9 sessions of SIT to increase performance, it was then investigated whether there was a coinciding change in the resting serum metabolome. An unsupervised dimension reduction technique adjusted for paired samples (multilevel PCA) was applied to the metabolomic profile of resting serum samples collected before the beginning of the SIT intervention and 48–72 h after the final performance test. PC1 and PC2 explained a low proportion of variance (14% and 11%, respectively) and no apparent separation of baseline and post-intervention metabolomes was discernible at the group level (Fig. 2). This implies the resting serum metabolome as measured by this targeted analysis as a whole was not altered by 9 sessions of SIT. The only other study to date of the effects of an exercise training intervention on the resting serum metabolome was almost three-times longer than the current study (24 exercise sessions, compared to 9) (Pechlivanis et al. 2013). In that study, participants were exposed to repeated 80 m running sprints, three times per week for 8 weeks with significant separation observed between the baseline and post-intervention resting serum metabolome profiles using a partial least squares-discriminant analysis (PLSDA). Separation between groups was observed to primarily be driven by metabolites classified as amino acids, monocarboxylates and some lipids (Pechlivanis et al. 2013). However, in addition to being a longer training intervention, this study also used ^{1}H-NMR to generate metabolomic profiles. ^{1}H-NMR is less sensitive than LC–MS and biases towards metabolites that are present in higher abundances (Gika et al. 2019). Therefore, while ^{1}H-NMR detects fewer metabolites compared to LC–MS, metabolites that are detected display lower variability, and larger mean differences in the concentration of metabolites may be more readily detected using this technique (Emwas 2015).

Two other studies using cross-sectional designs have investigated the effect of exercise training on the resting serum metabolome using PLSDA on metabolites identified via LC–MS and have demonstrated differences in the profile

of the resting circulating metabolome that may be related to exercise training history and ability levels (Monnerat et al. 2020; Schranner et al. 2021). First, in trained middle distance runners (Monnerat et al. 2020), group separation in the profile of the plasma metabolome between athletes with a low (61 ml kg^{-1} min^{-1}) or high (76 ml kg^{-1} min^{-1}) VO_{2max} at rest was driven primarily by metabolites associated with alpha linolenic acid metabolism, glutathione metabolism and carnitine metabolism (Monnerat et al. 2020). In another study by Schranner and colleagues, group separations in the resting serum metabolome of athletes from different sports and training backgrounds (sprinters, bodybuilders, endurance athletes and sedentary controls) were observed to primarily be driven by metabolites associated with amino acid metabolism, various phospholipids and metabolites associated with fatty acid oxidation. In addition, distinct metabolites or metabolite sum/ratios that were influential to PC1 and PC2 the PLSDA model produced in this study were also found to be in altered abundance between the groups; for example—compared to sedentary and endurance participants, bodybuilders had lower concentration of metabolites associated with amino acid metabolism (e.g. Isoleucine, Leucine, the sum of BCAAs). While markers of ß-oxidation (e.g. CPT-1 ratio and the Kyneurine/Tryptophan ratio) were significantly higher in endurance athletes (compared to bodybuilders and controls) (Schranner et al. 2021). However, cross-sectional studies are only capable of capturing "snapshots" of parameters that can be used to classify an individual's training status (e.g. VO_{2max}) (Darragh et al. 2021; Jeukendrup et al. 2000; McKay et al. 2022). Therefore, while cross-sectional studies can associate changes in the circulating metabolome at rest with a history of exercise training, these designs cannot directly quantify the isolated contribution of exercise training to parameters in resting blood samples at the time of sampling (Darragh et al. 2021). Cross-sectional studies investigating the resting blood metabolome may also be confounded by the contribution of additional factors that can coincide with a high level of exercise training, but are not directly induced by exercise adaptation itself, e.g. dietary habits (Burke et al. 1991).

Next, pairwise testing was performed to establish the effect of SIT on the abundance of individual metabolites. When pairwise testing was performed on the full set of identified metabolites within each of the 5 metabolite subgroups (Table S4) a total of 11 metabolites were identified to have lower concentrations (FDR < 0.05) after SIT intervention, all of which belonged to the Fatty Acids and Conjugates group (Fig. 3. Oleic acid, cis-8, 11, 14 Eicosatrienoic acid, Alpha linolenic acid, Myristic acid, and Margaric acid, Gamma linolenic acid, Linoleic acid, Stearic acid, Pentadecylic acid, Palmitic acid and Palmitoleic acid). Pathway analysis of these 11 fatty acids identified 1 significant pathway: biosynthesis of unsaturated fatty acids, which could suggest that SIT induced an alteration of this metabolic process that subsequently became evident in the resting serum metabolome. In addition, in Rats, aerobic training (90 min per day treadmill running, 6 × per week 11 week) (Scorpio et al. 1984) or 8 weeks of ad libitum wheel running (Petridou et al. 2005) has been reported to induce reduced rates of fatty acid biosynthesis in hepatic (Petridou et al. 2005; Scorpio et al. 1984), skeletal muscle and adipose tissues (Petridou et al. 2005). Lower hepatic rates of appearance of some of the identified fatty acids here (palmitic acid) have been reported in response to 18 sessions of continuous exercise training (20 min ergometer cycling sessions at power outputs estimated to elicit VO_2 responses of 60–80% of VO_{2max}) in sedentary men (Shojaee-Moradie et al. 2007). However, these studies involve intervention durations of 8 (Petridou et al. 2005), 11 (Scorpio et al. 1984) and 6 (Shojaee-Moradie et al. 2007) weeks, respectively. To our knowledge, no study to date has investigated whether these processes are attenuated in response to short-term exercise training. However, exercise training can also increase the content of transport proteins involved in the metabolism of medium/long chain fatty acids such as CD36 and fatty acid-binding protein (FABP) on both sarcolemmal (Perry et al. 2008) and mitochondrial (Perry et al. 2008; Talanian et al. 2010) membranes by approximately 16–20% in human skeletal muscle. This could potentially increase the capacity for the oxidation of medium/long chain fatty acids at rest, which is an alternative suggestion as to why decreased concentration of certain fatty acids at rest were observed in the current study. Previously, no change has been reported in the content of either CD36 or FABP in response to six sessions of SIT (Burgomaster et al. 2007) or in the activity of 3-hydroxyacyl-CoA dehydrogenase (βHAD, a proxy of total β-oxidation capacity) (Burgomaster et al. 2005). However, increases in these markers of fatty acid transport and oxidation have been reported in response to short-term training interventions employing other modes of exercise (namely high-intensity interval training consisting of 7 sessions of 10 × 4 min cycling intervals performed at 90% of VO_{2peak} performed every other day for 14 days) (Talanian et al. 2010). In addition, 15–20% increases in skeletal muscle βHAD activity were previously reported to have occurred in response to the 9 session SIT intervention that comprises the current study (Aird et al. 2021). Therefore, while the current study produced an ontological suggestion that short-term SIT is associated with a reduced biosynthesis of fatty acids, it is unclear whether this association was influenced by a decreased synthesis of fatty acids per se or an increased oxidation of medium/long chain fatty acids at rest. Monounsaturated fatty acids have previously been suggested to decrease in response to exercise training, although the concentration of polyunsaturated fatty acids may increase (Nikolaidis and Mougios 2004). The results of the current study partially

agree with these observations, given that the metabolite with the largest fold change decrease was Oleic acid (27%), which is the most abundant circulating monounsaturated fatty acid (Mashek and Wu 2015). Although contrary to this, the current study also observed significant decreases in several polyunsaturated fatty acids (e.g. Linoleic acid, Gamma linoleic acid, cis-8-11-4 Eicosatrienoic acid). Primary myotubes extracted from healthy men (40–62 years old) post-12 weeks of combined strength and endurance training (interval cycling training and whole-body resistance exercise) have been shown to increase Oleic acid uptake (~ 30%) and oxidation (~ 46%) (Lund et al. 2017) and 4 weeks of single leg extensor exercise training (60–120 min) has been shown to increase Oleic acid incorporation to skeletal muscle membrane phospholipids (~ 10%) also in healthy males (Helge et al. 2001). Together, these data suggest an altered "profile" of fatty acid oxidation as a result exercise training that may potentially result in lower circulating concentrations of select fatty acids.

Limitations

The current study is limited by our use of a targeted approach for the identification of 168 (160 identified above the limit of detection) metabolites and 7 added metabolite sum ratios (167 analysed variables total). This approach identifies only a subfraction of the global serum metabolome, which has in some estimates been speculated to contain upwards of several thousand metabolites (Psychogios et al. 2011). In addition, the current study may be limited by the number of exercise training sessions employed, it is possible that larger changes in the resting serum metabolome may have been detected by an intervention of a similar length (3 weeks)—but containing a greater number of training sessions (i.e. > 9 training sessions). The current study may also be considered limited by the absence of a control group, whose inclusion would have enabled us to identify a causal relationship between the reduced concentrations of fatty acids identified via univariate analysis and short-term SIT. The current study is potentially limited by standardisation through the use of food diaries and dietary recall rather than prescribed nutrition pre- and post-intervention.

Conclusion

In conclusion, despite improving the anaerobic and aerobic capacity of recreationally active males, SIT did not induce changes in the serum metabolome at rest when analysed by PCA. However, univariate analysis did reveal decreases in the concentrations of some fatty acids in response to SIT. Therefore, changes in this metabolite subfraction could potentially represent an early shift in the resting serum metabolome. Further research regarding the influence of short-term exercise training interventions on physiological adaptations combined with metabolomic profiling are required to determine whether changes in circulating metabolites are reflective of an early adaptation to exercise training.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00421-022-05115-x.

Author contributions ID—analysed the data and wrote the manuscript; TPA—conceptualised the study, collected data and contributed to the manuscript; AOS—assisted with data analysis and contributed to the manuscript; BE—contributed to the manuscript; BPC—conceptualised the study and contributed to the manuscript.

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Data availability The data analysed in this study is available at the following link: https://osf.io/xj7ag/?view_only=2941ce2488a347ceb685afbc5ff8d2ce.

Declarations

Conflict of interest The authors declare that there is no conflict of interest.

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APPENDIX C : Exercise Training History and Metabolome Variability Article

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Human Kinetics
ORIGINAL RESEARCH

Within-Subject Variability and the Influence of Exercise Training History on the Resting Plasma Metabolome in Men

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This study investigated within-subject variability in the circulating metabolome under controlled conditions, and whether divergent exercise training backgrounds were associated with alterations in the circulating metabolome assessed in resting samples. Thirty-seven men comprising of endurance athletes (END; body mass, 71.0 ± 6.8 kg; fat-free mass index, 16.9 ± 1.1 kg/m^2), strength athletes (STR; 94.5 ± 8.8 kg; 23.0 ± 1.8 kg/m^2), and recreationally active controls (CON; 77.6 ± 7.7 kg; 18.1 ± 1.0 kg/m^2) provided blood samples after an overnight fast on two separate occasions controlled for time of day of sampling, recent dietary intake, time since last meal, and time since last exercise training session. A targeted profile of metabolites, performed using liquid chromatography and mass spectrometry on plasma samples, identified 166 individual metabolites and metabolite features, which were analyzed with intraclass correlation coefficients, a multilevel principal component analysis, and univariate *t* tests adjusted for multiple comparisons. The median intraclass correlation coefficient was .49, with 46 metabolites displaying *good* reliability and 31 metabolites displaying *excellent* reliability. No difference in the abundance of any individual metabolite was identified within groups when compared between visits, but a combined total of 44 metabolites were significantly different (false discovery rate <0.05) between groups (END vs. CON, 42 metabolites; STR vs. CON, 10 metabolites; and END vs. STR, five metabolites). Under similar measurement conditions, the reliability of resting plasma metabolite concentrations varies largely at the level of individual metabolites with ~48% of metabolites displaying *good*-to-*excellent* reliability. However, a history of exercise training was associated with alterations in the abundance of ~28% of metabolites in the targeted profile employed in this study.

Keywords: biomarker, endurance, metabolite, reliability, strength

Exercise training elicits a broad range of physiological adaptations in many tissues and organ systems (Egan & Zierath, 2013; Hawley et al., 2014). When a specific type of exercise training is emphasized, divergent exercise training phenotypes can manifest (e.g., endurance- or strength-trained athletes; Coffey & Hawley, 2017; Egan & Zierath, 2013), which include specific anthropometric and performance traits that can differ markedly in different athletic populations (Degens, 2019).

Metabolites are low molecular weight (mostly organic) chemicals that are usually the reactants, intermediates, or products of metabolic pathways (Dunn et al., 2011; Nicholson & Lindon, 2008). The metabolome represents the collective output of metabolic reactions and is arguably the most accurate representation of the phenotype of a sample at the time of measurement (Belhaj et al., 2021; Patti et al., 2012). The circulating metabolome is of interest as it represents metabolite contributions from all tissues and is an integrated snapshot of systemic metabolism (Dunn et al., 2011). Considering the extent and diversity with which exercise training can induce physiological remodeling, there is emerging interest in whether exercise training history or exercise training interventions alter the profile of the circulating metabolome (Khoramipour et al., 2022). Profiling of the circulating metabolome in a resting state using a cross-sectional design between groups with divergent histories of exercise training can infer the influence of exercise training while attenuating the confounding residual effects of recent exercise training sessions (Darragh et al., 2021).

To date, two cross-sectional studies have investigated the relationship between exercise training and the circulating metabolome at rest (Monnerat et al., 2020; Schranner et al., 2021), both of which suggest alterations associated with divergent exercise training history and/or performance characteristics. However, both studies comprised of only small sample sizes and the extent of control of dietary intake, and the last exercise training session, was unclear. Moreover, the studies acquired samples at only a single time point. When interpreting the durability of alterations to the circulating metabolome related to exercise training, these methodological issues are salient given the dynamic nature of the circulating metabolome and the evident within-subject variability in human samples (Agueusop et al., 2020; Breier et al., 2014; Floegel et al., 2011; Yin et al., 2022).

Therefore, using a targeted profile of metabolites measured in plasma samples taken at rest, the present study firstly investigated the within-subject variability in the circulating metabolome while controlling for time of day of sampling, recent dietary intake, time since last meal, and time since last exercise training session. Secondly, we investigated whether exercise training history was associated with alterations in the circulating metabolome by comparing samples from recreationally active controls and two groups of exercise-trained individuals with divergent training histories and performance characteristics.

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Methods

Participants

Men ($n = 38$) who were endurance-trained (END; $n = 13$), strength-trained (STR; $n = 13$), and recreationally active controls (CON; $n = 12$) were recruited for this study (Table 1 and Supplementary Table S1 [available online]). To qualify for the respective training group, participants self-reported being able to meet the following criteria: END, at least two of the following running performances, 5,000 m < 20 min, 10,000 m < 40 min, or 16,000 m < 64 min; and STR, a one repetition maximum for at least two of the following, squat ≥ 200 kg, bench press ≥ 140 kg, or deadlift ≥ 220 kg. Three recent performances were recorded (Supplementary Table S1 [available online]), and these were verified via social media accounts (e.g., Instagram), training logs (e.g., Garmin Connect, Strava), and/or public databases (e.g., www.worldathletics.org, www.openpowerlifting.org). Based on the recently proposed Participant Classification Framework (McKay et al., 2022), END was comprised of $n = 8$ Tier 3/highly trained athletes and $n = 5$ Tier 4/elite athletes, whereas STR was comprised of $n = 9$ Tier 3/highly trained athletes and $n = 4$ Tier 4/elite athletes. The CON participants were Tier 1/recreationally active but did not participate in intensive or sport-specific training.

Study Design

This study received ethical approval from the Research Ethics Committee of Dublin City University (DCUREC/2021/079) in accordance with the Declaration of Helsinki, and each participant provided written informed consent prior to participation. Participants visited the laboratory on two separate occasions under controlled conditions in an overnight fasted state and having not exercised for at least 24 hr beforehand (Table 1). Height was measured using a stadiometer (model 213, SECA), and body mass and body composition (fat mass, %body fat, and fat-free mass) were assessed by bioimpedance spectroscopy (SOZO, ImpediMed) (Esco et al., 2019). Participants lay supine for 10 min while answering questions to verbally confirm compliance with all previsit preparations including the timing of their most recent meal and exercise training session. A venous blood sample was then taken from a superficial forearm vein.

Dietary Control

Participants were provided with standardized meals (Gourmet-Fuel™) for consumption on the day prior to each visit, which were delivered by a member of the research team. The meals provided 30 kcal/kg body mass with a macronutrient ratio of 50/25/25 for carbohydrate, protein, and fat, respectively. Participants were permitted to consume caffeine during the day before each visit in doses that contained negligible additional nutrients (i.e., tea or coffee without milk), but were asked to consume only water on the morning of each visit. Participants were also asked to abstain from alcohol consumption in the 24 hr preceding each visit.

Blood Sampling, Processing, and Storage

Whole venous blood was drawn by inserting a 21G butterfly needle (Greiner, Bio-One) into an antecubital vein. The initial 4 ml of blood was drawn into a generic vacutainer and discarded. Subsequently ~50 ml of whole blood was drawn into six 9-ml blood collection tubes coated with ACD-A anticoagulant (Greiner, Bio-One). Blood samples were immediately placed on ice and centrifuged at 1500*g* for 15 min at 4 °C. Directly after centrifugation, plasma samples were separated into aliquots and stored at −80 °C. Samples were collected and stored between August and December 2021 and were analyzed in batch in May 2022.

Plasma Metabolomics

Plasma metabolomics analyses were performed by The Metabolomics Innovation Center using a targeted assay capable of detecting up to 172 metabolites and employing liquid chromatography and mass spectrometry methods. Further details of this method are detailed in Supplementary Material (available online) and have been described elsewhere (Zheng et al., 2020). One hundred and fifty-nine metabolites were detected by liquid chromatography and mass spectrometry methods and classified into five distinct metabolite groups (amino acids, peptides and analogs; fatty acids and fatty acid conjugates; acylcarnitines; glycerophosphocholines and phosphosphingolipids; and other) using the "ChemOnt" taxonomic classification technique (Feunang et al., 2016; Supplementary Table S2 [available online]). In addition, four metabolite sums (branched chain amino acids, gluconeogenic amino acids, essential amino acids, and total acylcarnitine) and three metabolite ratios (acylcarnitine/carnitine, C2/C0, and kynurenine/tryptophan) were calculated (Petersen et al., 2012), resulting in a total of 166 metabolite features included in the final analysis.

Principal Component Analysis

Prior to dimension reduction, a Kaiser–Meyer–Olkin test of sampling adequacy and Bartlett's test of sphericity were performed on data to ensure they met the minimum standards for principal component analysis (PCA). Bartlett's test of sphericity produced a significant result, $X^2(165) = 103{,}410$; $p < .01$, while a Kaiser–Meyer–Olkin test of mean sampling adequacy produced a result of 0.5, which is a low, but acceptable value (Kaiser & Rice, 1974). A multilevel PCA using an orthogonal rotation with features mean zeroed and scaled for unit variance was then performed on the full set of 166 metabolites, metabolite sums, and ratios using mixOmics (version 6.14.1), a package designed for use within the statistical programming language R (Rohart et al., 2017). Multilevel PCA refers to a modified analysis applicable to repeated-measures design that involves decomposition of within-subject variation prior to dimension reduction. This approach has been demonstrated to increase model accuracy for paired-sample data (Liquet et al., 2012).

Statistical Analysis and Calculation of Intraclass Correlation Coefficients

Data are reported as mean ± *SD* unless otherwise stated. All analyses were performed using the base version of the programming language R (4.2.1) or the "Rstatix" package (version 0.7.0). For variables in which a difference in outcomes between visits was possibly expected (body composition, timing of each sample, time since last meal, and exercise session), data were analyzed using a two-way (Visit × Group) mixed analysis of variance. No interaction effect was observed for any variable, but given our a priori interest in between-group differences, in the presence of a significant main effect for Group, post hoc pairwise comparisons were performed using the Bonferroni correction. In circumstances where visit was not a relevant independent variable (e.g., height,

Table 1 Participant Characteristics and Details of Control Measures for Visits 1 and 2

Variable	END ($n = 13$)	STR ($n = 13$)	CON ($n = 12$)	Interaction F	Interaction p	Interaction η_p^2	Group F	Group p	Group η_p^2	Visit F	Visit p	Visit η_p^2
Age (years)	30 ± 6	25 ± 5	26 ± 2				3.285	.05	.166			
Height (m)	1.82 ± 0.06	1.80 ± 0.07	1.78 ± 0.07				0.876	.426	.052			
Body mass (kg)	71 ± 7**	94 ± 9	78 ± 8**	1.634	.211	.093	27.259	<.001	.63	0.124	.727	.004
FFM index (kg/m^2)	17 ± 1**	23 ± 2	18 ± 1$^{\#}$·**	2.149	.133	.118	65.943	<.001	.805	0.671	.419	.021
Recent 5 km time (min:s)	16:30 ± 1:22											
Recent bench press (kg)		148 ± 30										
Recent back squat (kg)		228 ± 30										
Recent deadlift (kg)		253 ± 38										
Time training per week (hr)	9 ± 10	9 ± 2	4 ± 1$^{\#\#}$·**				51.992	<.001	.594			
Average time between visits (days)	12 ± 10	9 ± 6	9 ± 5				0.02	.98	.001			
Maximum time between visits (days)	42	26	21									
Minimum time between visits (days)	7	7	6									
Time since most recent meal, Visit 1 (hr)	12 ± 2	12 ± 2	12 ± 1	0.939	.401	.052	1.132	.334	.062	0.711	.405	.02
Time since most recent meal, Visit 2 (hr)	12 ± 1	12 ± 2	13 ± 1	0.939	.401	.052	1.132	.334	.062	0.711	.405	.02
Time since most recent exercise, Visit 1 (hr)	46 ± 23	66 ± 66	75 ± 84	1.945	.159	.103	0.974	.388	.054	0.376	.544	.052
Time since most recent exercise, Visit 2 (hr)	44 ± 12	42 ± 17	88 ± 132	1.945	.159	.103	0.974	.388	.054	0.376	.544	.052
Average time of sampling, Visit 1	08:32	09:16	08:44	1.559	.226	.089	1.322	.281	.076	3.672	.064	.103
Average time of sampling, Visit 2	08:17	09:18	08:32	1.559	.226	.089	1.322	.281	.076	3.672	.064	.103
Maximum difference between Visit 1 and Visit 2 time of sampling (min)	54	23	54	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Minimum difference between Visit 1 and Visit 2 time of sampling (min)	1	1	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Note. END = endurance; STR = strength; CON = control; FFM = fat-free mass; N/A = not applicable.
Significant difference from STR (**$p < .01$). Significant difference from END ($^{\#}p < .05$, $^{\#\#}p < .01$).

age, number of days between Visit 1 and Visit 2), data were analyzed using a one-way analysis of variance with post hoc pairwise comparisons again performed using the Bonferroni correction. All analysis of variance are reported with a relevant estimate of standardized effect size (η_p^2).

Univariate testing of metabolite data was performed using paired *t* tests with the Benjamini–Hochberg method to control for the false discovery rate of multiple comparisons. This approach was first applied to the full set of identified metabolite data (166 features) within each group. When no significant differences were observed between visits for each group, Visit 1 and Visit 2 data were averaged for participants in each group, and the same series of pairwise tests were performed to compare groups (i.e., END vs. CON; STR vs. END; END vs. STR). Intraclass correlation coefficients (ICC) for each individual metabolite were calculated using the ICC2 method (Shrout & Fleiss, 1979). The reliability of circulating metabolites was interpreted using arbitrary thresholds (<0.4, "poor"; 0.4–0.5, "fair"; >0.5–0.75, "good"; and >0.75 "excellent") that were established for analysis of psychometric test scores (Cicchetti, 1994), but have been employed to describe the reliability of circulating metabolites (Agueusop et al., 2020; Floegel et al., 2011; Li-Gao et al., 2019). All statistical tests rejected the null hypothesis at an alpha level of <.05, or a false discovery rate <0.05 for univariate tests.

Results

Descriptive and Visit Characteristics

Participants were of similar age and height (Table 1). Body mass and fat-free mass index of STR (body mass, 94.5 ± 8.8 kg; fat-free mass index, 23.0 ± 1.8 kg/m^2) were greater than that of END (71.0 ± 6.8 kg; 16.9 ± 1.1 kg/m^2) and CON (77.6 ± 7.7 kg; 18.1 ± 1.0 kg/m^2), whereas the CON had greater body mass and fat-free mass index compared to END (all $p < .05$; Table 1). STR (8.5 ± 1.8 hr) and END (8.6 ± 2.2 hr) reported exercising more hours per week (both $p < .05$) than CON (3.8 ± 1.3 hr). The time of day that the samples were drawn was similar between groups and visits, as was the number of days between each visit between groups (Table 1).

Reliability of Metabolite Concentrations Between Visits

The majority of the 159 detected metabolites displayed *fair* or better reliability between visits as evidenced by 98 metabolites (~62%) having ICCs ≥ .4, with 46 metabolites (~29%) displaying *good* reliability (ICCs > .5), and 31 metabolites (~19%) displaying *excellent* reliability (ICCs > .75) (Figure 1a; Supplementary Table S3 [available online]). ICCs varied between metabolite groups with glycerophosphocholines and phosphosphingolipids displaying the greatest reliability (83% of metabolites with an ICC > .5) and acylcarnitines displaying the lowest reliability (30% of metabolites with an ICC > .5) (Figure 1b).

Within-Subject and Between-Group Analyses

Multilevel PCA was performed to investigate whether clear spatial separations were apparent in within-subject or between-group analyses. This model produced no apparent clustering within groups or separation between groups, and different visits within participants tended to cluster closely implying the profile of a participant's metabolome was consistent between visits (Figure 2; Supplementary Table S4 [available online]).

The results of univariate within-subject analyses demonstrated that no metabolites displayed significantly different abundances between visits within any group (Figure 3). Univariate tests comparing the resting concentration of each metabolite between groups (END vs. CON; STR vs. CON; and END vs. STR) revealed a combined total of 44 metabolites with differences between groups (false discovery rate <0.05; Figure 4; Supplementary Table S5 [available online]). Boxplots for each of these 44 metabolites are presented in Supplementary Figure S1 (available online). The largest number of differentially abundant metabolites (42) was between END and CON, with 16 metabolites demonstrating lower abundance and 26 metabolites demonstrating higher abundance in END (Figure 4a). Of the 16 metabolites with lower abundance, four metabolites were in the acylcarnitine group, four metabolites were in the amino acid, peptide and analog group, six metabolites were in the fatty acid and fatty acid conjugate group, one metabolite was in the glycerophosphocholines and phosphosphingolipids, and one metabolite was in the others groups (Supplementary Table S5 [available online]). Of the 26 metabolites with higher abundance, six metabolites were in the acylcarnitine group, eight metabolites were in the fatty acid and fatty acid conjugate group, and 12 metabolites were in the glycerophosphocholines and phosphosphingolipids group (Supplementary Table S5 [available online]).

Comparing STR with CON (Figure 4b), 10 metabolites were differentially abundant, with five metabolites demonstrating higher abundance (four metabolites in the fatty acid and fatty acid conjugate group, and one metabolite in the Others group), and five metabolites displaying lower abundance (all in the fatty acid and fatty acid conjugate group) in STR (Supplementary Table S5 [available online]). Comparing END to STR (Figure 4c), five metabolites were differentially abundant with two metabolites displaying lower abundance (one in the amino acid, peptide and analog group, and one in the others group) and three metabolites displaying higher abundance (all in the glycerophosphocholines and phosphosphingolipids group) in END (Supplementary Table S5 [available online]).

Discussion

The present study employed a targeted profile of the circulating metabolome in resting plasma samples, first to investigate reliability when measured on two separate days. Under controlled conditions, namely time of day of sampling, recent dietary intake, time since last meal, and time since last exercise training session, the reliability of plasma metabolite concentrations varied largely at the level of individual metabolites. Specifically, ~48% of metabolites displaying *good*-to-*excellent* reliability in resting samples, whereas the remaining ~52% of metabolites displayed *fair*-to-*poor* reliability. Second, investigating whether divergent histories of exercise training were associated with alterations in the circulating metabolome revealed that the abundance of ~28% (44/159) of the metabolites detected in the targeted metabolite profile were altered in plasma between endurance- or strength-trained men compared with recreationally active controls.

Using ICC as an assessment of reliability, the median ICC across metabolites was .49, and ~62% of metabolites displayed an ICC ≥ .4. These data suggest that the majority of metabolites displayed *fair* or better reliability between visits, with ~48% displaying *good*-to-*excellent* reliability. However, the median ICC observed in the present study is lower than previously reported in

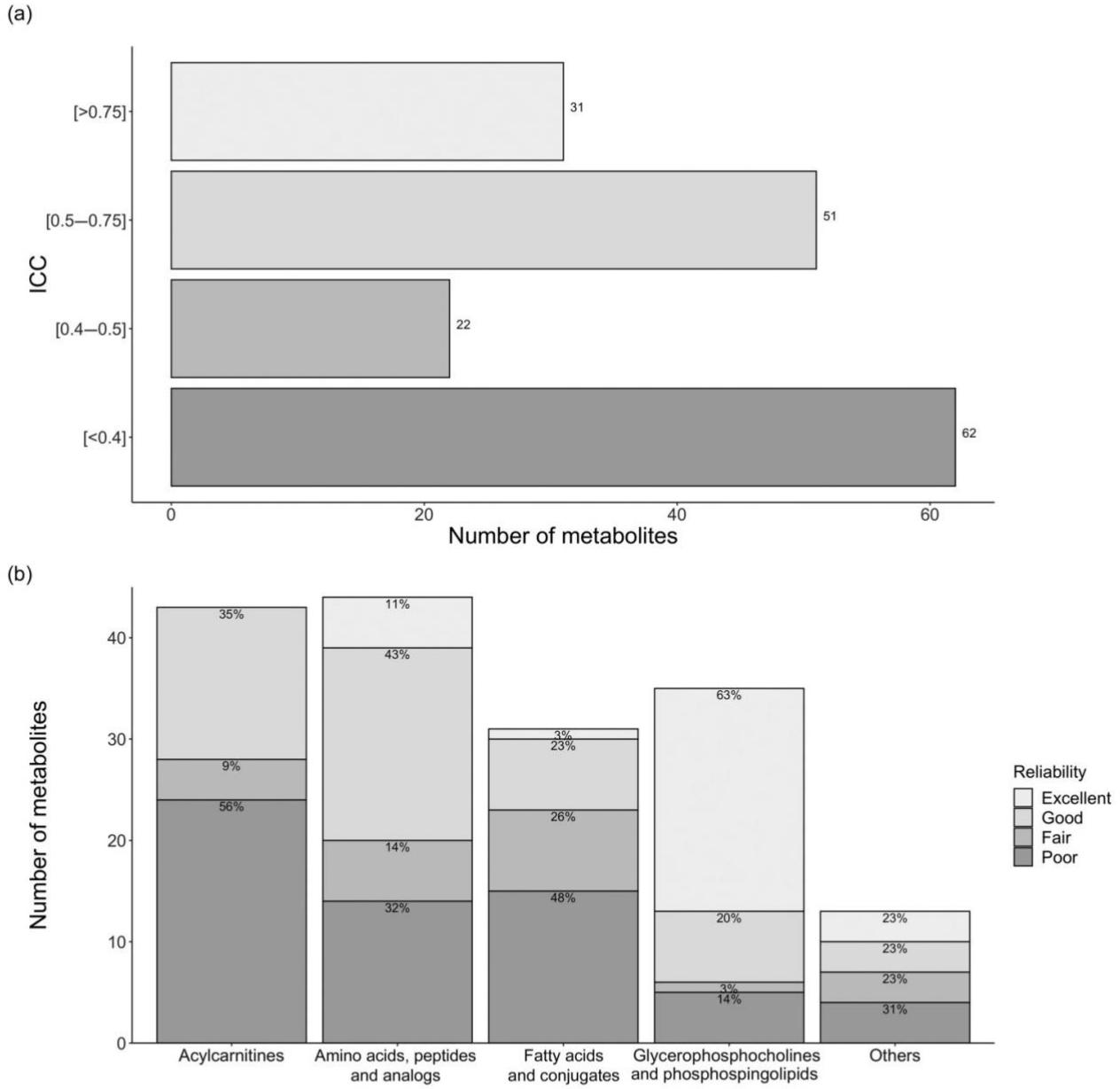

Figure 1 — The ICC of detected metabolites. (a) This distribution of all metabolites by ICC interval. (b) The number of metabolites in each group (*y*-axis) with the percentage of individual metabolites in each specific ICC interval. ICC = intraclass correlation coefficients.

other studies in blood samples taken from overnight fasted humans on multiple days, including both untargeted (median ICC of .65 for 1,438 metabolites identified in serum, Agueusop et al., 2020; median ICC of .66 for 148 metabolites identified in plasma, Li-Gao et al., 2019) and targeted (median ICC of .57 for 163 metabolites identified in serum, Floegel et al., 2011; median ICC of .62 for 138 metabolites identified in plasma, Yin et al., 2022).

When analyzed by metabolite group in the present study, the glycerophosphocholines and phosphosphingolipids group displayed the greatest reliability (median ICC of .78, with 63% of metabolites displaying ICCs > .75, and only 14% displaying a ICCs < .4), and the acylcarnitine group displaying the poorest reliability (median ICC of .28, with 60% of metabolites displaying ICCs < .4 and no metabolites displaying an ICC > .75). In similar studies, phosphocholines and sphingolipids have likewise displayed *good*-to-*excellent* reliability (median ICCs ~.6 to ~.8; Agueusop et al., 2020; Floegel et al., 2011; Yin et al., 2022), but our observation of *poor* reliability of the acylcarnitine group is in contrast to previous studies in overnight fasted humans (median ICCs ~.6 to ~.9; Agueusop et al., 2020; Breier et al., 2014; Floegel

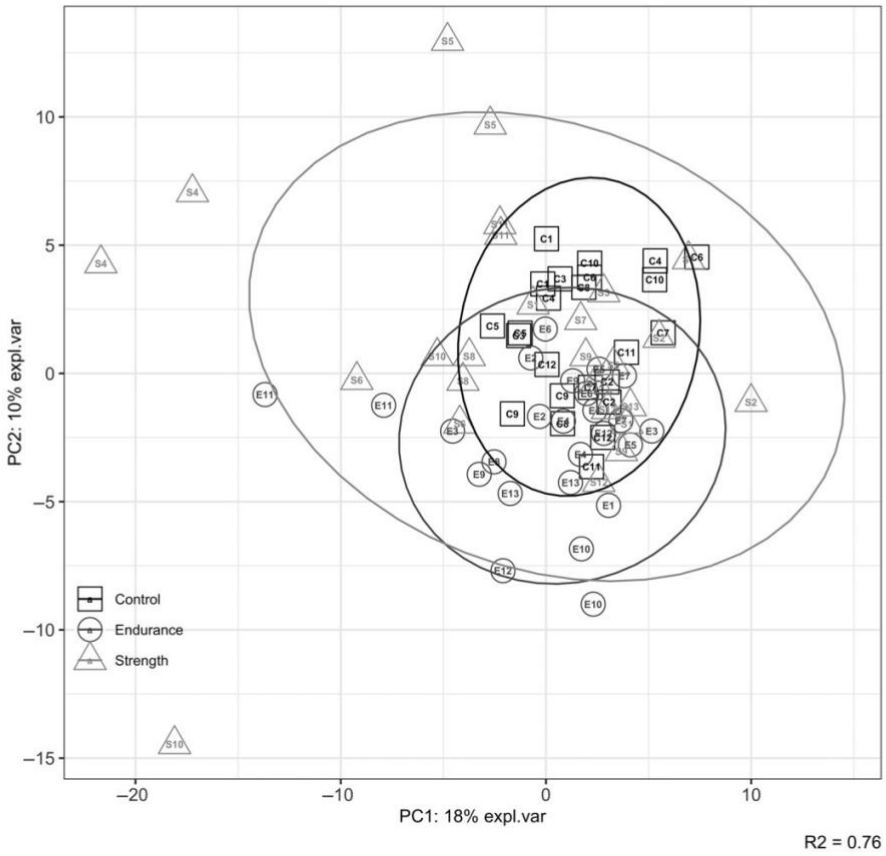

Figure 2 — A multilevel PCA of the collapsed profile of targeted metabolomes from the participants for both visits; circles represent endurance athletes, triangles represent strength athletes, and squares represent control participants. PC1/PC2 refers to the first and second principal components, that is, latent variable structures that explain the largest and second largest unique proportion of total model variance, respectively. expl.var = explained variance; PCA = principal component analysis.

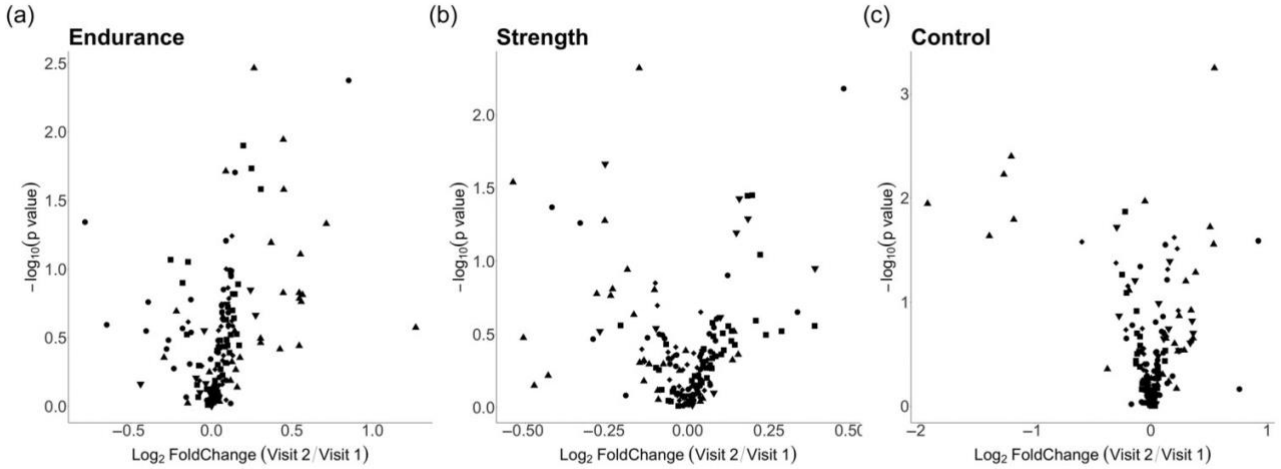

Figure 3 — Univariate analysis of metabolites within each group. (a) Endurance athletes, (b) strength athletes, (c) recreationally active controls. Shapes represent metabolites in specific metabolite groups with the following coding; squares = acylcarnitines; circles = amino acids, peptides, and analogs; triangles = fatty acids and conjugates; diamond = glycerophosphocholines and phosphosphingolipids; inverted triangle = others.

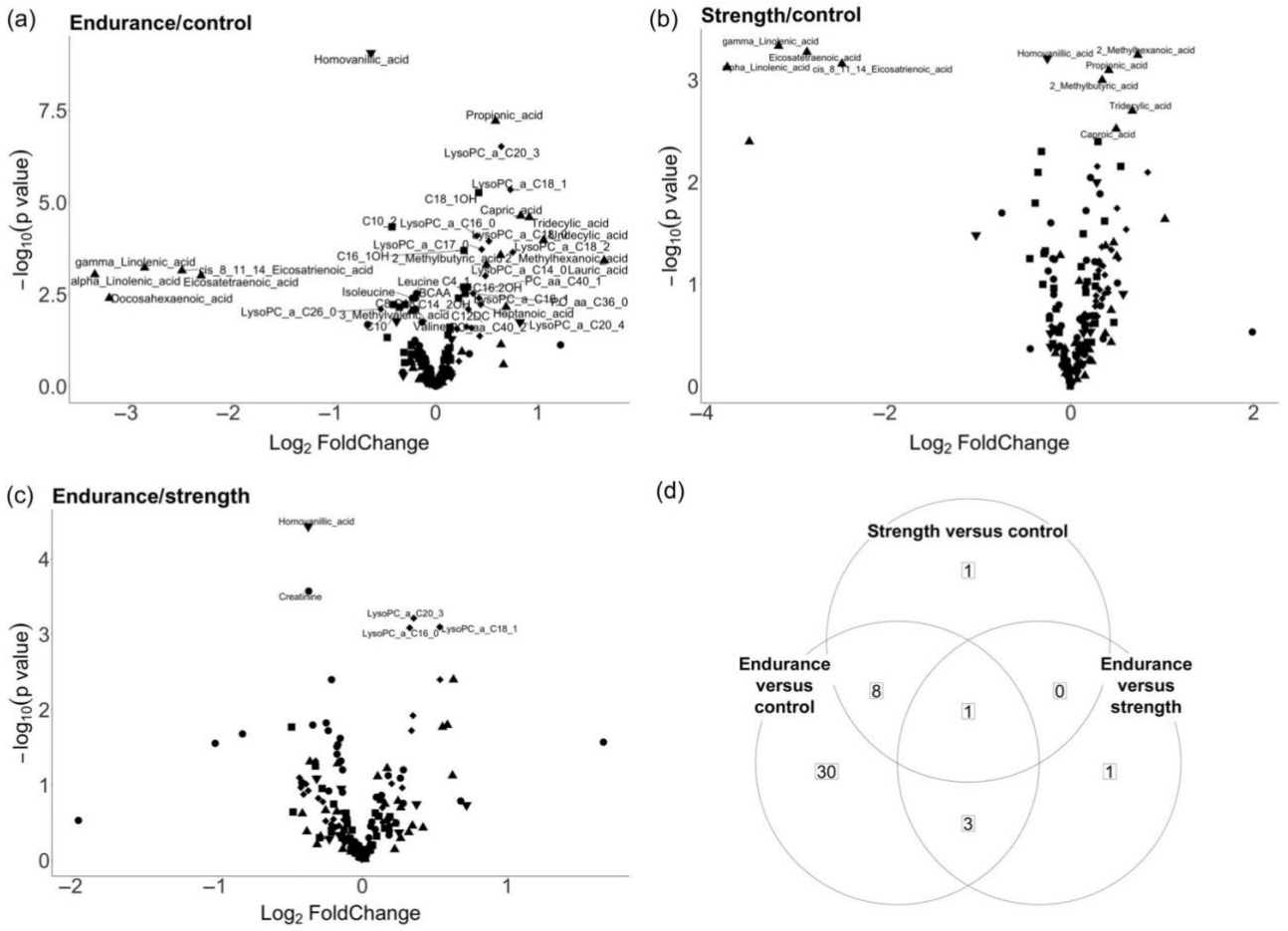

Figure 4 — Univariate analysis of metabolites between each groups. (a) Endurance athletes versus control, (b) strength athletes versus control, and (c) endurance athletes versus strength athletes. Annotated metabolites are differentially abundant (false discovery rate <0.05). (d) A Venn diagram showing the overlap of differentially expressed metabolites between individual group analysis. Shapes represent metabolites in specific metabolite groups with the following coding; squares = acylcarnitines; circles = amino acids, peptides, and analogs; triangles = fatty acids and conjugates; diamond = glycerophosphocholines and phosphosphingolipids; inverted triangle = others.

et al., 2011; Yin et al., 2022). The reliability of acylcarnitines may vary in a manner that is dependent on the length of the acylcarnitine chain with short-medium acylcarnitines displaying higher ICCs compared with longer chains (Breier et al., 2014; Floegel et al., 2011). A difference may also exist between plasma and serum as evidenced by acylcarnitines identified in serum having higher ICCs than those in plasma (Breier et al., 2014). The present study analyzed a mixed panel of 42 acylcarnitines ranging from C1 to C18, and measured metabolite concentrations in plasma, not serum, and at the level of specific acylcarnitines, some of the reliability data are similar to other studies (Floegel et al., 2011; Yin et al., 2022). For example, C4, which we observed to have an ICC of .70, has previously been reported to have *excellent* reliability with an ICC of .79 in plasma (Yin et al., 2022) and .81 in serum (Floegel et al., 2011). Possible explanations for the lower reliability observed in the acylcarnitines group in the present study may be related to the collection of plasma rather than serum, and to using a general classification of acylcarnitines rather than classifying acylcarnitines into separate categories of short or long, respectively. Overall these findings suggest, in agreement with others (Agueusop et al., 2020; Breier et al., 2014; Floegel et al., 2011), that the reliability of circulating metabolite concentrations can vary considerably within metabolite groups, even when measured under controlled conditions. Establishing the reliability of individual metabolites and specific metabolite groups is important within analyses that aim to investigate differences between groups or time points by providing an indication of whether potential differences in abundance are likely to be robust.

For the analysis of between-group differences, multilevel PCA revealed no separation between participant groups, implying that the decomposed metabolomic profile was not different between groups. However, both visits from individual participants tended to cluster closely together, suggesting that the metabolomes from individual participants projected similarly between each visit, which agrees with the reliability data described above. The observation of no clear separation between groups using a multivariate method is in contrast to that reported by two other cross-sectional studies investigating the influence of exercise training history and

performance characteristics on the circulating metabolome (Monnerat et al., 2020; Schranner et al., 2021). The first study compared elite long-distance runners with "low" (<65 $ml \cdot kg^{-1} \cdot min^{-1}$; $n = 7$) or "high" (>75 $ml \cdot kg^{-1} \cdot min^{-1}$; $n = 7$) values for maximal oxygen consumption (Monnerat et al., 2020), whereas the second study compared a control group ($n = 4$) and endurance ($n = 6$), sprint ($n = 5$), and bodybuilding ($n = 4$) athletes (Schranner et al., 2021). While both studies report clear separations between their athlete groups (Monnerat et al., 2020; Schranner et al., 2021), both studies also employed a partial-least squares discriminant analysis (PLSDA) modeling approach in contrast to the PCA approach employed in the present study. In contrast to PCA, PLSDA is a "supervised" method, wherein the model algorithm attempts to produce linear components that maximize the separation between predefined class structures (e.g., exercise training groups; Ruiz-Perez et al., 2020). This technical difference between methods is important because it means that PLSDA models will produce interclass separation, even in circumstances where no true class structure exists in the data (Ruiz-Perez et al., 2020). Therefore, the utility of a PLSDA model is based on a quality assessment of the model parameters (Szymańska et al., 2012). While neither model from the previous exercise training studies are reported as producing model overfitting (i.e., the training model over predicting the cumulative variance produced by the full data set; Monnerat et al., 2020; Schranner et al., 2021), both models did have evidence of underfitting (Q^2 values greater than R^2 of half of the full datasets across multiple permutations), which implies poor predictive power. In addition, the model produced by Schranner et al. (2021) explained a low cumulative variance ($R^2 = .5$). Therefore, the contrasting results between the present study and these prior studies (Monnerat et al., 2020; Schranner et al., 2021) in terms of broad between-group differences may largely be due to differences in the selection of model algorithm(s).

When pairwise univariate testing was performed on our data, no individual metabolite differed between visits within each group, again implying that metabolites detected in the targeted metabolite panel were consistent between visits. However, univariate comparisons between groups, that is, END versus CON; STR versus CON; and END versus STR identified a combined total of 44 metabolites with differential abundance between groups. The largest number of metabolites with differential abundance was between END and CON (42 metabolites), with a smaller number different between STR and CON (10 metabolites), and END and STR (five metabolites). The mechanisms that explain these between-group differences remain to be elucidated, and discussion at present is speculative. For example, increased abundance of numerous lysophospholipids was observed between END and both other groups (10 lysophospholipids higher vs. CON; three higher vs. STR). In plasma, lysophospholipids can reflect the activity of lecithin–cholesterol acyltransferase, an enzyme that is abundant in plasma and serves to facilitate the transport of cholesterol esters between high-density lipoprotein particles and hepatic tissue (Glomset, 1968; Tan et al., 2020). Aerobic exercise training can increase the presence of circulating high-density lipoprotein (Kodama et al., 2007). In addition, cross-sectional studies involving athletes with endurance training backgrounds have noted increased lecithin–cholesterol acyltransferase activity at rest (Gupta et al., 1993; Tsopanakis et al., 1988) and this activity is significant in the context of reverse cholesterol transport (Leaf, 2003). Therefore, one speculation would be that the increased abundance of lysophospholipids observed in END may indeed be related to this adaptation to aerobic exercise training.

Other notable results include the observation that of all metabolites identified to be differentially abundant, nine (2-methylbutyric acid; 2-methylhexanonic acid; alpha linolenic acid; cis,8,11,14 eicosatrienoic acid; gamma linolenic acid; homovanillic acid; propionic acid; and tridecyclic acid) were common to both athlete groups compared with CON in addition to these differences being directionally the same in both END and STR. Interestingly, one metabolite—Homovanillic acid, an endpoint of dopamine metabolism and marker of metabolic stress (Amin et al., 1992)—was also observed to be lower in END and STR compared with CON, but lower again in END versus STR. Ultimately, elucidating the importance of these differentially abundant metabolites will require further work, as would establishing whether these differences are definitively a consequence of exercise training given that a cross-sectional design can only establish association, rather than causation.

In athletes or exercise contexts, the present study includes the largest sample size profiled to date and is the first study to our knowledge to report reliability data for individual metabolites but is not without limitations. Exercise training history and current performance status of participants was established largely through self-reported methods, and thus, objective measures of the physical fitness of participants (e.g., VO_2max) are lacking. In addition, while our sample size is indeed the largest currently reported, some of our model parameters (e.g., the Kaiser–Meyer–Olkin score) are suggestive of us having low statistical power for multivariate modeling, a fact that may influence the results of the PCA. An important limitation that precludes the broader applicability of the results is that the groups comprised of only male participants, which is important given the influence of endogenous and exogenous sex hormones on metabolism, and that the responses of several genetic, metabolic, and physiological parameters to exercise differs between males and females (Ansdell et al., 2020; D'Eon et al., 2002; Fu et al., 2009; Landen et al., 2019; Maher et al., 2010). Lastly, we employed a targeted metabolomic approach; and therefore, our findings are restricted to only this subfraction of the circulating metabolome.

In conclusion, the present study found that when sampled under controlled measurement conditions, the resting plasma concentration of 166 metabolites and calculated metabolite sums and ratios did not differ overall between two visits, yet reliability was variable as evidenced by a large range in the average ICC within- and between-specific metabolite groups. In addition, divergent histories of exercise training were associated with alterations in the circulating metabolome at rest, but future work will be required to determine the importance of these differences, and whether these differences are definitively a consequence of adaptations to exercise training.

Acknowledgments

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APPENDIX D : Ethical Approval Letter

Ollscoil Chathair Bhaile Átha Cliath
Dublin City University

DCU

Dr. Brendan Egan
School of Health and Human Performance

Mr. Ian Darragh
School of Health and Human Performance

31st May 2021

REC Reference: **DCUREC/2021/079**

Proposal Title: **Investigating basal differences in the characteristics of circulating exosomes derived from exercise trained individuals with divergent training phenotypes**

Applicant(s): **Dr. Brendan Egan, Mr. Ian Darragh, Prof. Lorraine O'Driscoll**

Dear Colleagues,

Further to full committee review, the DCU Research Ethics Committee approves this research proposal.

Materials used to recruit participants should note that ethical approval for this project has been obtained from the Dublin City University Research Ethics Committee.

Should substantial modifications to the research protocol be required at a later stage, a further amendment submission should be made to the REC.

Yours sincerely,

Dr Geraldine Scanlon
Chairperson
DCU Research Ethics Committee

DCU
Research & Innovation

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