Metabolomics in Exercise and Sports: a Systematic Review

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Key points

- Metabolomics can improve the understanding of the physiological responses to exercise training and determinants of sport performance.
- While untargeted metabolomics studies with small sample sizes, invasive sampling methods and a focus on short term effects of exercise have dominated exercise metabolomics studies, there is currently a trend towards longer term, biomarker-based studies with larger numbers of subjects using noninvasive sampling.
- Improvements in study design, method standardization and data integration/interpretation will be key to improving the quality of exercise metabolomics studies

Abstract

Background: Metabolomics is field of omics science that involves the comprehensive measurement of small metabolites in biological samples. It is increasingly being used to study exercise physiology and exercise-associated metabolism. However, the field of exercise metabolomics has not been extensively reviewed or assessed. Objective: This review on exercise metabolomics has three aims: (i) to provide an introduction to the general workflow and the different metabolomics technologies used to conduct exercise metabolomics studies; (ii) to provide a systematic overview of published exercise metabolomics studies and their findings and; (iii) to discuss future perspectives in the field of exercise metabolomics. Methods: we searched electronic databases including Google Scholar, Science Direct, PubMed, Scopus, Web of Science and SpringerLink academic journal database between January 1st 2000 and September 30th 2020. Results: Based on our detailed analysis of the field, exercise metabolomics studies fall into five major categories: 1) exercise nutrition metabolism; 2) exercise metabolism; 3) sport metabolism; 4) clinical exercise metabolism and 5) metabolome comparisons. Exercise metabolism is the most popular category. The most common biological samples used in exercise metabolomics studies are blood and urine. Only a small minority of exercise metabolomics studies employ targeted or quantitative techniques, while most studies used untargeted metabolomics techniques. In addition, mass spectrometry was the most commonly used platform in exercise metabolomics studies with about 54% of all published studies. Our data indicates that biomarkers or biomarker panels were identified in 34% of published exercise metabolomics studies. Conclusion: Overall, there is an increasing trend towards better designed, more clinical, mass spectrometry-based metabolomics studies

involving larger numbers of subjects/patients and larger numbers of metabolites being identified.

1. Introduction

Traditionally, exercise physiologists have only been able to study selected or small numbers of genes, proteins and/or metabolites and their response/adaptation to exercise or training (1, 2). Indeed, the standard approach to gathering metabolic data in exercise physiology studies has typically required the use of tissue or muscle biopsies (3-5). This highly invasive approach limits the number of subjects and the number of samples that can be analyzed, which further restricts the number of analytes that can be meaningfully measured. While these highly targeted and invasive approaches have been successful for identifying key physiological pathways (6, 7), they do not provide a comprehensive, unbiased approach to detect all exercise-induced changes in all tissues or all metabolic pathways (2). Because exercise leads to substantial, often profound changes in the metabolism of many organs and many tissues, the understanding of exercise-induced changes to large numbers of metabolites and large numbers of metabolic pathways is particularly interesting and challenging (3-5). Current estimates place the number of metabolites in the human body at more than 110,000 different compounds (8) and the number of metabolic or metabolite signaling pathways in the human body at more than 46,000 (9). Given that most exercise metabolism/physiology studies simultaneously measure fewer than a dozen metabolites and examine only 1-2 pathways at a time (10-12), it is clear that only a tiny fraction of what can be measured is currently being explored. This narrow view of human metabolism has limited our understanding of the relationship between exercise, physiology and metabolism.

To address these limitations in metabolic measurements, a route for better, more comprehensive and less invasive approaches has long been sought by exercise physiologists. In this regard, the emergence of metabolomics over the past 20 years has enabled the possibility of performing comprehensive, high-throughput, minimally invasive or non-invasive metabolic studies in a large number of fields, including exercise physiology. Metabolomics is defined as the comprehensive characterization of small molecules (molecular weight<1500 Da) called metabolites in biological samples (13). These small metabolites can serve as sensitive sentinels of genomically driven processes or states. Indeed, a single base change in a single gene can lead to a 10,000-fold change in the concentration of a metabolite in the metabolome (14). Because metabolites are effectively the end products of complex interactions occurring inside the cell (the genome, the transcriptome, and the proteome) and events or phenomena occurring outside the cell (the environment), the comprehensive measurement of metabolites (via metabolomics) allows one to measure interactions between genes and environment. This means

that metabolomics offers an ideal route for measuring both phenotype and physiology (15, 16) (Fig 1).

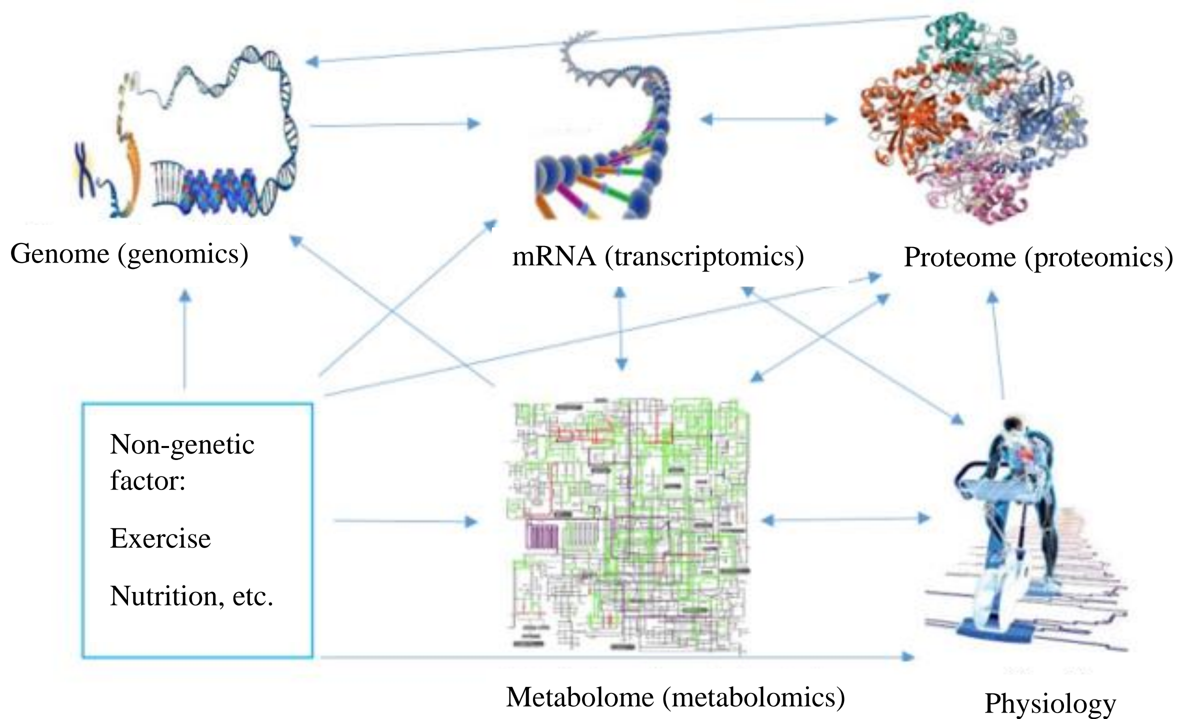

Fig 1. The flow of information from genes to metabolites, their connections and omics related fields (Modified from Kasture et al (17), with permission).

Metabolomic techniques have gained increasing traction in many fields of life sciences, including medicine and exercise physiology due to their ability to simultaneously probe both environmental and genetic interactions. One of the reasons for the growing popularity of metabolomics within biomedicine lies in the fact that metabolomics studies can be conducted in a variety of ways. They may be conducted on subjects to: 1) obtain "healthy" reference value measurements; 2) compare "cases" versus "controls" to explore how different perturbations can affect the metabolome; or 3) collect data over short or long periods of time to explore longitudinal effects. This makes metabolomics ideal for probing processes as they occur in real time or over the time scale of seconds, minutes, hours or even days (15, 18). This ability to probe an organism's phenotype or physiology is the reason why metabolomics is increasingly being used by exercise physiologists (19, 20). As a result, a new branch of exercise physiology, called "exercise metabolomics" has emerged that brings metabolomics together with exercise research. Given its rapid growth over the past few years, we believe that a review of the field and an assessment of where it has been and where it is going is both timely and important.

This review on exercise metabolomics is separated into three parts. The first part is intended to provide a brief overview of metabolomics and to describe the different metabolomics technologies employed in exercise metabolomics. The second part provides a technical overview of previously published exercise metabolomics studies and attempts to identify existing or emerging trends in the field. This involved a detailed and systematic analysis using Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) standards, of more than 120 exercise metabolomics papers. This systematic review allowed us to identify some of the challenges that exercise metabolomics researchers may face and possible solutions to these challenges, The third part of this review is more speculative and uses the information compiled in the first two sections to discuss future directions in the field of exercise metabolomics.

2 Metabolomics and Metabolomics Methods

Metabolomics as a field of omics science first emerged in the late 1990's (21). However, the simultaneous analysis of multiple (typically 10s or 20s) metabolites in biological samples via techniques such as nuclear magnetic resonance (NMR) spectroscopy and gas chromatography (GC) mass spectrometry (MS) dates from the early 1970s (21). It has only been in the last 10-15 years, primarily through the introduction of liquid-chromatography (LC)-MS, that metabolomics techniques have appeared permitting the detection and/or putative identification of thousands of metabolites at a time (21). Therefore, depending on the technological platforms used, the number of metabolites measured in each metabolomics experiment can range from a few dozen to thousands of molecules at a time. The number of metabolites detected in a metabolomic experiment can also depend on the sample size and sample type. Indeed, the types of biological samples analyzed in a metabolomic study may range from cells to tissues, organs, biofluids (such as urine, serum, plasma, saliva and cerebrospinal fluid) or an entire organism. Given the diversity of platforms and the diversity of samples. it is not surprising to learn that different "flavors" of metabolomics have emerged over the past two decades. These include: 1) targeted metabolomics; 2) untargeted metabolomics; 3) metabolic fingerprinting; 4) metabolic footprinting; 5) metabolic profiling; and 6) lipidomics, along with several other varieties (22-24). Table 1 provides a brief description or definition of each of these metabolomic terms and how they can be differentiated from each other.

Table 1. List of different kinds of metabolomics-related terms and their definitions (22-24)

Word	Definition
Metabolome	The complete set of low molecular weight metabolites (primary metabolites, secondary metabolites, endogenous and exogenous compounds) that can be found in a cell, a tissue, a biofluid or an organism.
Metabolomics	The comprehensive characterization of metabolites (small molecules with molecular weight<1500 Da) and other chemical species (both exogenous and endogenous) in biological specimens in response to different perturbations or interventions.
Metabonomics	Often synonymous to metabolomics. Metabonomics is generally focused on the application of metabolomic methods to study metabolic responses to therapeutic interventions or genetic modifications.
Metabolic fingerprinting	The characterization of metabolites of internal biofluids needed to sustain a living cell or organism (e.g. cell cytoplasm, serum, plasma, tree sap). This metabolomic method is commonly used in whole organism metabolomic studies as well as in cell, cell culture or microbial studies.
Metabolic footprinting (Exo-metabolomics)	Characterization of metabolites secreted by a living organism (urine, feces, saliva and other excreta) or found in cellular growth media. This method is commonly used in microbiology and biotechnology.
Metabolic profiling	A synonym of metabolomics. It is a term that is normally reserved for metabolomic studies with a smaller, more defined set of metabolites that may have common physiochemical properties (such as carbohydrates, amino acids, organic acids and nucleotides) or are involved in specific metabolic pathways (such as glycolysis, gluconeogenesis, beta-oxidation, beta-oxidation, and Krebs-cycle).
Targeted Metabolomics	A branch of metabolomics that is focused on the identification (and often exact quantification) of a specific, pre-defined collection or category of metabolites in a tissue, biofluid or biological matrix. Targeted metabolomics is widely used in clinical or biomarker applications.
Lipidomics	A branch of metabolomics that involves the comprehensive analysis of all lipids, fatty acids, and lipid-like molecules in a biological or environmental sample.
Untargeted Metabolomics	A branch of metabolomics that involves the broad, unbiased identification of the maximum number metabolites or metabolic features in a tissue,

biofluid or biological matrix. Untargeted metabolomics is widely used in biological discovery or hypothesis generation applications.

Regardless of the type or "flavor" of metabolomics that is chosen for a given study, nearly all metabolomics experiments follow a similar workflow. This workflow is illustrated in Fig 2.

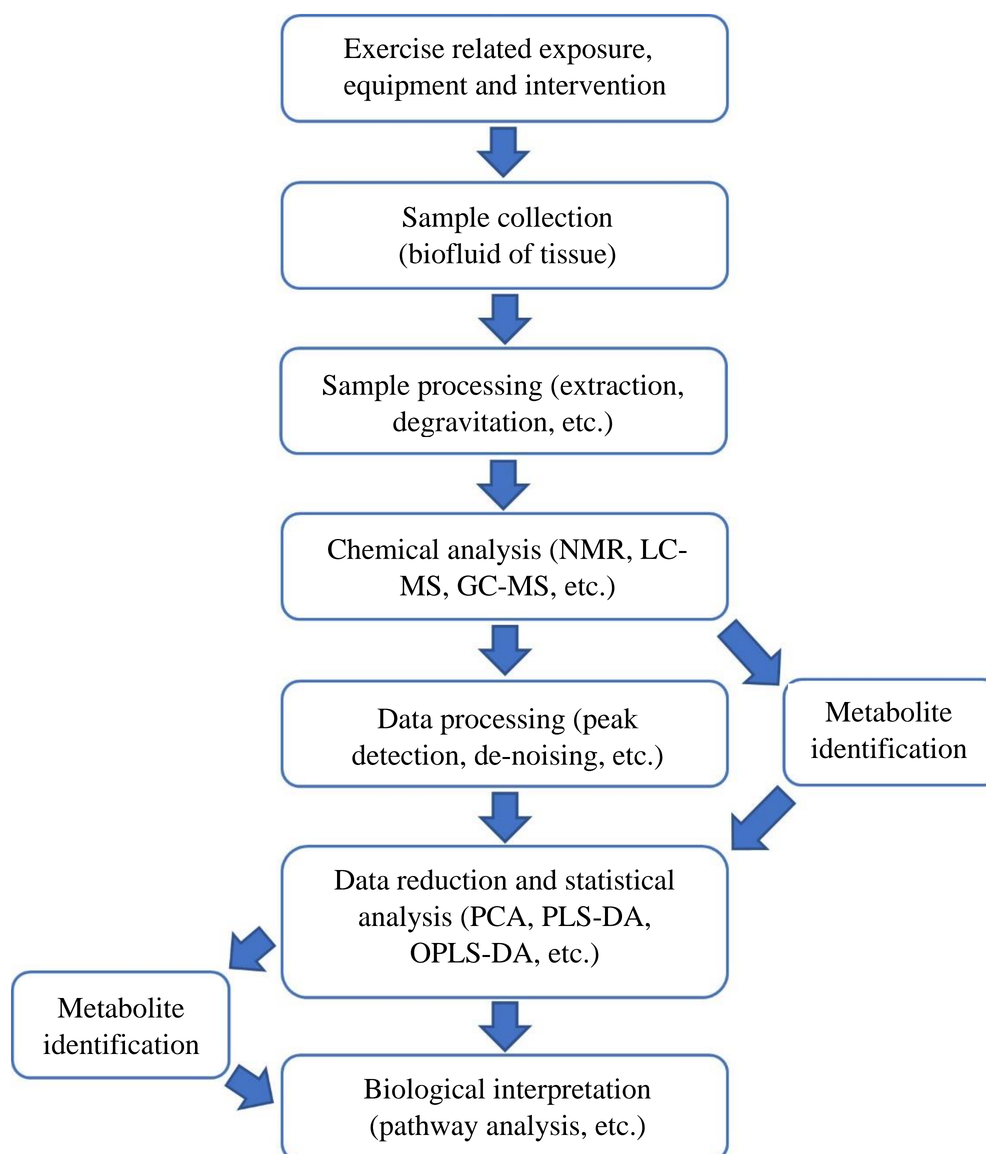

Fig 2. Metabolomics workflow of a metabolomic study. NMR: Nuclear magnetic resonance, LC-MS: Liquid chromatography–mass spectrometry, GC-MS: Gas chromatography–mass spectrometry, PCA: Principal component analysis, PLSDA: Partial least squares-discriminant analysis, OPSDA-DA: Orthogonal partial least squares-discriminant analysis

While it is not the intent of this review to provide a detailed summary of metabolomics methods and technologies (other publications have provided detailed information (21, 25)), we believe

a brief summary explaining the main points highlighted in Fig 2 would be helpful, especially in understanding the information presented in the second part of this review which involves a systematic PRISMA analysis of published exercise metabolomics studies.

2.1 Sample Collection

As seen in Fig 2, the first (and perhaps the most important) step involves the collection and storage of biological samples. In human metabolomics studies, the most commonly selected sample types are serum/plasma, urine, saliva, sweat, and stool samples. Muscle biopsies and other kinds of tissues biopsies are less frequently collected (26). The choice of sample type, the number of samples and the collection/storage conditions for samples are critical to the success of every metabolomics experiment. Indeed, the failure to properly plan or design the first step is the reason for the failures of many metabolomics studies. The selection of sample types should be made on the basis of sample accessibility (invasive, non-invasive, expensive or inexpensive) and the appropriateness of sample for the question being asked (27). As a general rule, in a metabolomics experiment biofluids or samples that bathe or surround the organ/tissue of interest should be chosen (28, 29). A summary of the advantages and disadvantages of different sample types that are typically used in exercise metabolomics studies is given in Table 2.

Table 2. Advantages and disadvantages of the most commonly used biological samples in exercise and sport metabolomics studies.

Type of biological sample	Advantages	Disadvantages
Blood (Plasma/Serum)	Contains all molecules secreted or excreted by different tissues Primarily includes endogenous metabolites Indicates and tracks temporal and physiological changes well Useable in all methods of analysis	Contains high amounts of proteins and lipoproteins Invasive collection method Rapid reaction of blood analytes with enzymes in the sample (metabolic degradation) Difficult to detect small metabolites by NMR
Urine	Contains stable compounds Generally free of proteins and other macromolecules	High salt and urea concentration (problematic in MS analysis)

	Contains higher concentrations of waste, disease or toxic compounds Contains endogenous and exogenous metabolites Non-invasive collection methods available Large amounts of samples are readily available Simple storage and shipment possible	Can be contaminated with bacteria Can be significantly affected by environmental/diet conditions Metabolically complex
Saliva	Provides a good reflection of the physiological conditions of the body Has a wide range of low molecular weight molecules Non-invasive collection methods available Simple storage and shipment possible	Can be contaminated with bacteria and high molecular weight proteins Limited amounts of samples are available Has lower concentration of endogenous metabolites than blood Affected by oral intake, physiological and pathological conditions of the mouth
Tissue	Provides high concentrations of measurable metabolites Provides the most accurate representative of local metabolic conditions Composition can vary greatly depending on the sampling site May contain endogenous metabolites only	Very invasive sampling Limited amounts of samples are available Often contaminated with high molecular weight proteins

NMR: Nuclear magnetic resonance, MS: Mass spectrometry

The total number of samples used for a metabolomics experiment is best determined using a power analysis (28), however for many kinds of discovery-based studies a power analysis is not possible or reasonable. As a general rule, at least 30-40 cases and 30-40 controls are

required to get sufficiently robust data. However, if the signals or differences are particularly strong, it is possible to work with much fewer (e.g. 5 to 10) samples (30).

2.2 Sample Processing

After collection of biological samples for a metabolomics experiment, they must typically be further processed or extracted to get them into a suitable state for further chemical analysis. The processing and extraction steps are dependent on the type of sample and the analytical platform being used. Solid samples (tissue, feces, cells, etc.) must typically be frozen, then grounded to powder (while frozen) and extracted with both hydrophobic (chloroform) and hydrophilic solvents (water and/or methanol) so that liquid extracts can be obtained (30). Liquid extracts are essential for metabolite analysis via standard analytical chemistry platforms such as NMR spectrometers or mass spectrometers. For samples that are already liquefied (serum, plasma, urine, saliva, etc.) the biofluid must usually be filtered to remove protein and cellular debris (or other macromolecules). The removal of proteins also prevents enzymatic reactions from occurring which may alter metabolite levels during metabolite analysis. An alternative to filtration, especially for samples to be analyzed by LC-MS or GC-MS, is solvent extraction. Solvent extraction, using organic solvents, is one of the most effective ways of precipitating proteins and extracting certain classes of metabolites. Polar solvents such as methanol or methanol/water (1:1) can be used to extract polar metabolites from serum, plasma, saliva and most tissues, while non-polar solvents such as chloroform (mixed with methanol/water 2:2:1.8 (v/v/v)) can be used to extract lipids or non-polar metabolites from most biofluids or tissues. Solvent extraction is an effective way of reducing the chemical complexity of both biofluids and tissue, which makes subsequent analytical steps somewhat easier. Solvent extraction also ensures that samples are protein and/or enzyme free, which is critical to ensuring that no further enzyme-mediated metabolic processes can take place during analysis (i.e., metabolic quenching). For certain kinds of metabolomic analysis (such as GC-MS or certain LC-MS methods), samples must also be chemically derivatized. Chemical derivatization involves chemically reacting metabolites with certain chemical moieties to either enhance their volatility (for GC-MS) or to isotopically label them for improved LC separation and enhanced MS differentiation (30).

2.3 Chemical Analysis

The two most common chemical analysis methods used in metabolomics are MS and NMR spectroscopy (15). MS is a chemical analysis method that has been used for more than a century

for measuring the mass-to-charge ratio (or m/z) of molecules or atoms. Most MS methods used in metabolomics incorporate at least one or several chemical separation steps prior to injecting the sample into the mass spectrometer. This is done to reduce the complexity of the mixture while at the same time increasing the sensitivity and enhancing the ability of the MS instrument to detect individual metabolites. The most common chemical separation methods are GC, LC, and capillary electrophoresis (CE) (31). Each of these methods separates molecules over time and space based on their physiochemical properties. GC separates molecules based on their boiling point, mass, polarizability, and molecular shape. LC separates molecules based on hydrophobicity, charge and size while CE separates molecules primarily on the basis of charge (30). Therefore, different molecules have distinct and uniquely characteristic retention times, the time it takes for a molecule to reach the detector from the chromatographic system entrance (15). GC-MS is commonly used for the detection and separation of lower molecular weight, less easily ionized metabolites while LC-MS or CE-MS are used for higher molecular weight and more easily ionized or charged metabolites (15).

In addition to MS techniques, NMR spectroscopy is another commonly used chemical analysis method to identify and quantify metabolites. NMR measures the response of atomic nuclei to radio-frequency perturbations under strong magnetic fields (32). In NMR-based metabolomics, the separation of peaks due to chemical shift differences among different molecules means that the analysis of chemical mixtures by NMR does not require chromatographic or electrophoretic separation or chemical derivatization. This makes NMR-based metabolomics somewhat faster and easier than MS-based metabolomics (15). Nevertheless, the identification and analysis of thousands of NMR peaks in a complex biofluid such as urine or serum is challenging. Furthermore, unlike MS, NMR is not a very sensitive technique (15).

Both MS and NMR can be used in targeted and untargeted metabolomics. MS is generally more sensitive while NMR provides more structural details and greater quantitative accuracy. Each of these methods have their strengths and weaknesses, which are outlined in Table 3-.

Table 3. Advantages and disadvantages of MS and NMR techniques (15, 33-35).

Analytical platform	Advantages	Disadvantages
Nuclear magnetic resonance (NMR)	Quick analysis High resolution technique No need for derivatization Easy preparation	Low sensitivity Has a very small library of reference compounds

	High reproducibility Inexpensive Allows determination of structure Can be fully automated Non-destructive method	More than one peak per metabolite Limited to detection of hydrophilic molecules Expensive instrumentation
Gas chromatography-mass spectrometry (GC-MS)	High sensitivity High distinguishing power High linear absorption range Suitable for volatile compounds (especially nonpolar) Has a large commercial and public library of reference spectra High level of reproducibility Can be mostly automated Less expensive instrumentation	Lower throughput Usually needs chemical derivatization Unsuitable for thermally unstable compounds with high molecular weight Unusable for compounds that cannot be volatilized Complex preparation process Destructive method
Liquid chromatography-mass spectrometry (LC-MS)	Usually, no need for derivatization Can work with many separation methods Allows one to simultaneously analyze many samples Applicable for a wide range of compounds (polar and non-polar) The most sensitive metabolomics technique Good automation capability	Lower throughput Limited reference spectral libraries Expensive instrumentation Requires chromatographic separation Need for high level of training Destructive method
Capillary electrophoresis–mass spectrometry (CE-MS)	High separation power Very sensitive	Limited spectral reference library

	Can be highly automated Quick separation and analysis Ability to analyze neutral compounds, cations, and anions Usually, no need for derivatization	Destructive technique Instrumentation is challenging to work with

2.4 Data Analysis Methods

Regardless of the metabolomics platform used (MS or NMR) or approach employed (targeted or untargeted), all metabolomics data must go through a data analysis step that involves spectral cleaning, baseline correction, de-noising, peak picking and/or peak binning (see Fig 2). After this step, as shown in Fig 2, there are two options that branch off either for compound identification or further statistical analysis. As seen on the right side of Fig 2, metabolite identification for targeted metabolomics occurs after spectral processing or spectral “cleaning” and prior to statistical analysis and data reduction. As seen on the left side of Fig 2, metabolite identification for untargeted metabolomics occurs after the spectral processing step and after the statistical analysis and data reduction step. Indeed, the difference between targeted and untargeted methods is greatest when it comes to the metabolite identification step. In targeted metabolomics, all or nearly all of the metabolites that are targeted are typically identified and quantified. After the metabolites have been fully identified and quantified, then the task of data reduction, statistical analysis and data interpretation can take place (30).

On the other hand, untargeted metabolomics uses data reduction and feature selection techniques prior to metabolite identification. This is because the amount of data generated by untargeted methods is many times greater than the amount of data generated by targeted methods. Untargeted metabolomics, especially LC-MS-based metabolomics, produces thousands to tens of thousands of spectral features for any given sample in a study. Statistical analysis and data reduction typically reduces this number down to a few dozen statistically significant features. Once this feature list is winnowed down, the task of metabolite identification of these significant features can begin. It is important to note that the spectral features initially identified via untargeted metabolomics methods are only “potential metabolites” – they are not metabolites. Metabolite identification in untargeted metabolomics

follows a similar process to metabolite identification in targeted metabolomics. However, the spectral databases are less complete and therefore it is rare to identify more than 10% of the significant features in an untargeted metabolomic study. Furthermore, with untargeted metabolomics identified, metabolites cannot be absolutely quantified, and only relative quantification can be attained. After the metabolite identification step is completed in untargeted metabolomics, the task of data interpretation can begin. Both untargeted and targeted methods use similar methods for data or biological interpretation (14).

One of the most important steps in metabolomic data analysis is statistical analysis and data reduction step (see Fig 2). The statistical methods used in this step can broadly be divided into two categories: supervised and unsupervised methods (36). In unsupervised methods, the data are not labeled, and the statistical methods are designed to find naturally existing clusters of samples sharing similar features, similar peak intensities or similar metabolite concentrations. Principal component analysis (PCA) is the most common unsupervised method used in metabolomic studies. PCA is a dimensional reduction technique that produces small numbers (two or three) principal components (PCs) that capture the main features explaining a dataset's variance. PCA data is typically displayed as a two- or three-dimensional plot. A PCA scores plot typically shows multiple clusters of data points that share some degree of feature similarity. On the other hand, a PCA loadings plot displays the features (metabolites or spectral bins) that are most strongly differentiating between clusters. In this way, PCA plots may be used to extract the most important differentiating spectral or metabolite features in a metabolomic study. This reduces hundreds or thousands of features or metabolites to a manageable number of features (36, 37).

Supervised methods require that the data (metabolite concentrations or feature intensities) be labeled or explicitly identified (such as case or control) prior to data reduction. Supervised methods are widely used in metabolomics as in most metabolomics studies samples can be easily labeled (cases vs. controls, healthy vs. sick). Supervised methods are primarily used for classification (which is different from clustering). Supervised methods "learn" to identify the main features, such as metabolites or spectral features, that differentiate certain groups (e.g. cases and controls) from one another. In this regard, supervised methods are much more effective at detecting subtle differences between apparently similar samples than simple clustering methods such as PCA. The most common supervised methods used in metabolomics are partial least squares-discriminant analysis (PLS-DA) and its optimized form, orthogonal PLS-DA (OPLS-DA) (36, 38). It is important to notee that classification methods need careful

validation and testing to confirm that the classification model is not being over fitted (38). Furthermore, biomarker discovery requires other complementary analyses, including tests of accuracy, sensitivity, and specificity.

2.5 Biological Interpretation

The last step in the workflow shown in Fig 2 involves biological interpretation. Many metabolomics researchers use pathway analysis as a part of the biological interpretation process. In particular, pathway databases such as Kyoto Encyclopedia of Genes and Genomes (KEGG) (39), Reactome (40), MetaCyc Suite (41), and PathBank (9) are widely used to facilitate biological interpretation. MetaboAnalyst (42) is another commonly used web-resource that provides a large number of tools for more sophisticated biological interpretation, multi-omic integration and general biomarker analysis. We would refer readers to several excellent reviews that provide easy-to-understand overviews of how biological interpretation in metabolomics is best done (43-46).

3. Systematic Review of Exercise Metabolomics Papers

The second part of this review is intended to provide an overview of previously published exercise metabolomics studies and to identify existing or emerging trends in this relatively young field. In conducting this review, we systematically surveyed all of the major bibliographic databases using the PRISMA guidelines (47). The bibliographic databases included Google Scholar, Science Direct, PubMed, Scopus, Web of Science and SpringerLink academic journal database. The period of the review spanned from Jan. 1, 2000 to Sep. 30, 2020. The following keywords were searched in each of the bibliographic databases: 'Exercise/exercise nutrition and metabolomics/metabonomics', 'Physical activity/physical activity and nutrition and metabolomics/metabonomics', 'sport/sport and nutrition and metabolomics/metabonomics and sportomics'. The search was restricted to original peer-reviewed studies published in English, with review articles excluded. Titles and abstracts were screened by two independent reviewers, potential conflicts or discrepancies identified, and any conflicts resolved by discussion with a third reviewer. The article selection process is described in Fig 3. Selected studies were thoroughly examined, and the information listed in the headings for Electronic Supplementary Material Table S1were compiled. Two different reviewers independently extracted data and disagreements regarding the selected information were

resolved by a third reviewer and discussion. As seen in Fig 3, initially a total of 312 articles were identified during the first screening phase. This was reduced to a final set of 130 studies after duplicate removal, title screening/filtering and abstract screening. This meant that publications that did not use the words “exercise”, “sport” or ”physical activity” in their title were excluded. Thereafter, we read the selected manuscript abstracts and removed those papers that did not study physical exercise, but rather psychological or other types of exercise, or were not written in English. In addition, some review papers that passed the previous steps were also manually removed because we did not include review papers. Another 8 articles were removed due to the lack of access to full text manuscripts. This left us with a total of 122 papers with full text access that met all our inclusion criteria.

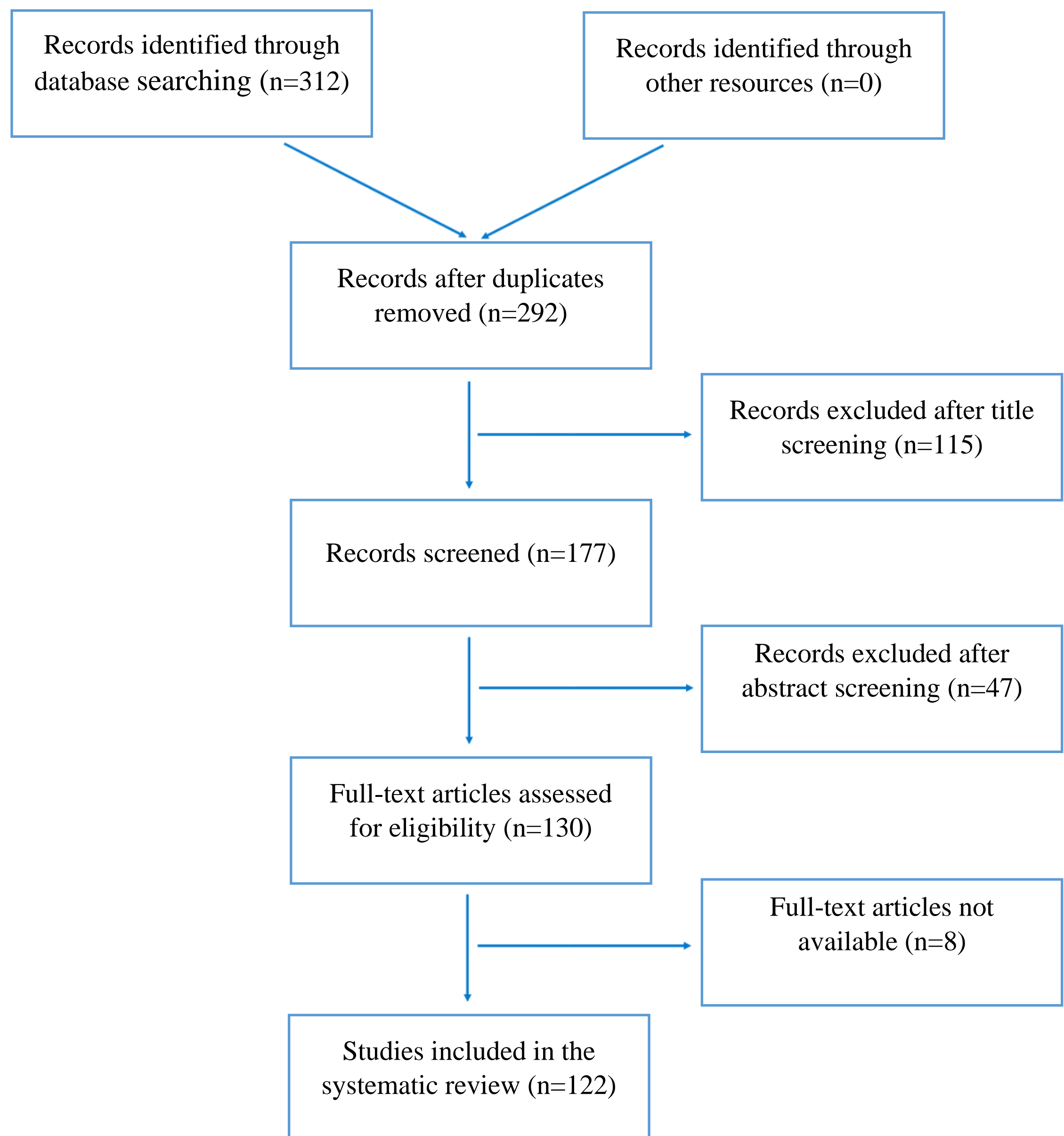

Fig 3. PRISMA flow diagram displaying the identification, screening, and selection of relevant studies in this systematic review. PRISMA: Preferred Reporting Items for Systematic reviews and Meta-Analyses'.

3.1 Study Types

Based on the 122 studies published between 2007 (first ever published manuscript) and September 30th, 2020, we classified the studies into five general categories (Fig 4):

1. Exercise nutrition metabolism (studies that examined the effect of any supplement or special diet on exercise metabolism)

2. Exercise metabolism (studies that examined metabolic responses/adaptations to a particular exercise protocol)

3. Sport metabolism (studies that examined metabolic responses to a particular sport or a specific exercise test)

4. Clinical exercise metabolism (studies that examined the effect of exercise on patients' metabolism)

5. Metabolome comparison (studies that compared athletes/patients/animals' metabolome)

The number of papers identified in each of these five categories is shown in Fig 4 for each year starting from 2007 to the end of the present systematic review (September 2020).

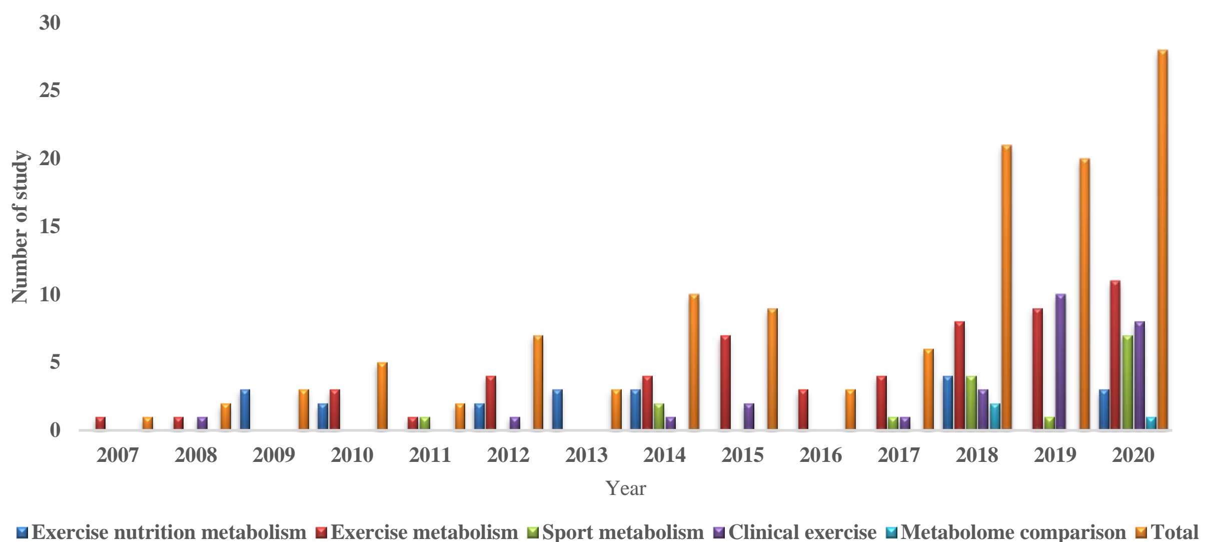

Fig 4. The number of studies published in each category in different years.

As seen in Fig 4, the total number of exercise metabolism studies has been increasing since 2007, with the highest number of studies being published in 2020. Indeed, the last 4 years (2017-2020) accounted for more than half of all exercise metabolomics studies published to date. This suggests that the field has experienced a rather significant surge of interest. Among the different categories of exercise metabolomic studies, we found that studies focused on "metabolome comparison" were quite rare, with one study reported in 2008 and 2020 and two studies in 2018 (for a total of four studies). Sport metabolism studies and exercise nutrition

metabolism studies were also relatively uncommon with just 16 and 20 studies, respectively. The two most common kinds of exercise metabolomics studies were exercise metabolism studies and clinical exercise metabolism studies with 55 and 27 studies, respectively. It is notable that the highest numbers of clinical exercise metabolism studies were published in 2019 (N=10) and 2020 (N=8) which shows an increasing tendency of exercise metabolomics researchers towards clinical or patient-centered studies.

3.2 Study Duration

We classified all 122 exercise metabolomics studies into short-term (one week or less) and long-term (more than one week) duration studies. There were 73 short-term studies and 46 long-term studies. Three studies used a mix of both short- and long-term methods. The relative ratio of short-term studies to long-term studies (roughly 2:1) appeared to remain constant throughout the time period assessed. The one exception appears to be for 2020 with 15 long-term and 15 short-term studies. Whether this is the beginning of a trend towards longer term studies is unclear.

3.3 Sample Types

Our systematic review identified nine different types of biological samples that were used in these exercise metabolomics studies: plasma, serum, whole blood, urine, saliva, muscle/liver tissue, sweat, feces, and breath samples. The number of exercise metabolomics studies using each kind of sample were as follows: plasma: 41, serum: 26, urine: 21, muscle/liver tissue/other: 12, feces: 3, saliva: 4, sweat: 2, whole blood: 3, and mixed samples: 10. Ten studies reported using more than one sample type (such as serum and urine or tissue, exhaled breath condensate and plasma). The frequency with which a given sample type was selected aligns very closely to the frequency reported in other kinds of systematic reviews of human metabolomics studies (48). . Regarding the temporal trends in sample type selection, we found that plasma was the most frequently used sample over the past few years with the highest rates in 2019 (N=9) and 2020 (N=10). Interestingly, serum was not used very frequently until 2018 during which nine studies used it and it was also studied in 2019 (N=4) and 2020 (N=6). Urine has seen steadily increasing use with the first reported urine study being in 2009 and with an average of 3-4 studies per year employing urine between 2014 and 2020. We also noted that exercise metabolomics researchers only started to use muscular tissue in 2014, and sweat and feces in 2018 for metabolomic assessments.

3.4 Analytical Methods

MS-based metabolomics was the most common analytical platform in exercise metabolomics studies with 66 (about 54% of all studies) published manuscripts that exclusively used MS-based techniques. Among these 66 studies, we found that 39 studies used LC-MS and 27 studies used GC-MS. NMR was used exclusively in 22 studies (about 17%) while 12 studies used other types of analytical platforms such as clinical analyzers, or high performance liquid chromatography (HPLC) coupled with ultraviolet-visible spectrophotometry and 21 studies used a combination of LC-MS and NMR. In one study, the type of the used chromatography was not specified (49). The number of studies using MS-based methods (e.g. LC and GC) showed an increasing trend over the past 10 years with 2020 having the highest number of reported MS-based studies. This trend reflects the fact that MS methods offer exceptional sensitivity and that these methods maximize the detection of metabolites. Interestingly, we found that NMR was most frequently used to analyze urine samples. Another notable trend in terms of platform preference was the tendency of exercise studies to use multiple analytical platforms – especially in recent years (2018-2020). The combined use of GC-MS and LC-MS or NMR and LC-MS methods is known to broaden metabolite identification coverage (Fig 5).

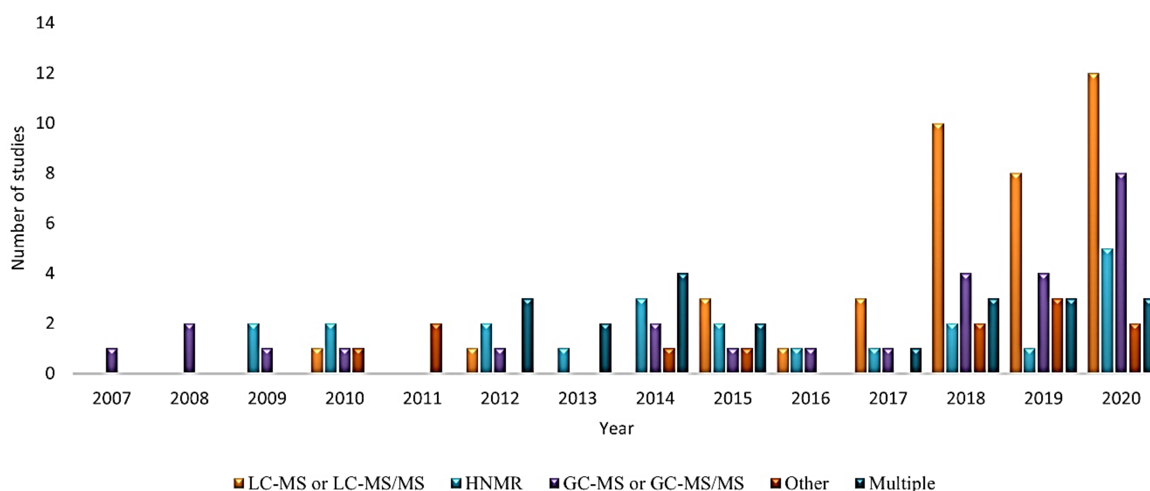

Fig 5. Different analytical methods used in exercise metabolomics studies from 2007 to September 2020. LC-MS: Liquid chromatography–mass spectrometry, LC-MS/MS: Liquid Chromatography with tandem mass spectrometry, HNMR: Hydrogen nuclear magnetic resonance, GC-MS: Gas chromatography-mass spectrometry, GC-MS/MS: Gas chromatography with tandem mass spectrometry, Other: Other analytical methods, Multiple: Multiple analytical methods

3.5 Targeted vs. Untargeted Methods

Our data indicates that 82 studies used untargeted methods (15 NMR-based studies, 63 MS-based studies and 4 studies that used other analytical methods), 33 studies used targeted methods (9 NMR-based studies, 22 MS-based studies and one using other analytical methods) and 7 studies used a combination of both targeted and untargeted methods. A total of just 25 papers provided absolute metabolite quantification while 97 provided only qualitative (or relative concentration) metabolite data. In the last three years (2018-2020), 51 out of 69 studies (74%) used untargeted methods, highlighting the increasing trend towards untargeted metabolomic methods over targeted methods in recent years. Preferences for untargeted/targeted studies in the 5 different exercise metabolomics categories was as follows: (i) sport metabolism: 9 untargeted vs. 6 targeted (one study used mixed methods), (ii) clinical exercise metabolism: 17 untargeted vs. 8 targeted (2 studies used mixed methods), (iii) exercise metabolism: 32 untargeted vs. 19 targeted (4 studies used mixed methods), (iv) sport nutrition: 14 untargeted vs. 5 targeted (one study used mixed methods), and (v) metabolome comparisons: 4 untargeted studies.

3.6 Metabolites and Pathways/Physiological Processes Identified

According to Electronic Supplementary Material Table S1, the average number of metabolites identified in the targeted exercise metabolomic studies was 31, while the average number of “features” having statistical significance in untargeted exercise metabolomic studies was 48. The biofluid or biological matrix that yielded the highest average number of metabolites or features was urine. The biofluid or biological matrix that yielded the lowest average number of metabolites or features was sweat. Generally, exercise metabolomic studies identified more metabolites in urine relative to serum or plasma. The lowest number of metabolites identified having a significant change in any exercise metabolomic study was two as reported by Arthur et al. (50) for a study looking at metabolic changes in saliva after 60 minutes of cycling (45 minutes at an intensity of 70% of maximum power (P_{max}) and greatest distance possible for a further 15 minute-period) in 11 healthy male athletes using targeted liquid chromatography flow injection analysis tandem mass/mass spectrometry (LC-FAIMS-MS). The highest number of metabolites identified in any exercise metabolomic study was 743, as reported by Al-Khelaifi et al. in 2018 (51) for a study looking at the serum metabolic profiles between moderate- and high-power and endurance elite athletes. Of the published studies, 68 (~56%) identified specific metabolic or signaling pathways. The average number of pathways identified as having significant metabolic changes in these exercise metabolomic studies was six. The lowest number of identified or significantly changed pathways was one, as reported

by five different studies (52-56). The highest number of significantly changed pathways identified was 23 as reported by Contrepois et al. (2020) (57), which was described earlier. As expected, these pathways were mostly related to energy, fatty acids, carbohydrates or amino acid metabolisms.

3.7 Statistical and Data Reduction Methods

Most of the reported exercise metabolomics studies used a mix of multivariate statistical methods including PCA and PLS-DA/OPLS-DA (N=51). However, 5 studies used PCA alone, 7 studies used hierarchical clustering analysis (HCA) alone, 10 studies used PLS-DA alone and 9 studies used OPLS-DA alone. Furthermore, 21 studies used univariate statistical analysis (such as Student's t-test and volcano plots) and 19 studies used other statistical methods. In addition, 41 studies identified specific metabolite biomarkers including 9 studies involving urine, 10 studies involving serum, 14 studies involving plasma and 8 studies involving other kinds of biological samples. Fifteen of the 41 biomarker studies provided sensitivity and specificity values while 26 studies provided receiver operating characteristic (ROC) curves or area under the ROC curve information.

3.8 Historical Review of Published Studies

All published exercise metabolomics studies that were reviewed in our study, are summarized in the Electronic Supplementary Material Table S1. This very extensive table includes details on each study's citation, duration and category, biological sample type, analytical platform, study protocol, subject types, number of metabolites identified or altered, number of pathways affected, study type (targeted or untargeted), and year of publication. As can be seen in this table, there is considerable diversity in the study types and study categories, participants (diabetic, elderly, elite athletes), the types of samples analyzed, the analytical methods and the breadth of metabolite coverage. Despite this diversity, some broad trends were detectable and worthy of a few brief comments. For instance, those studies that focused on endurance training consistently detected an increase in glycolysis products, TCA cycle intermediates, nucleotide metabolites, acylcarnitines and BCAAs. These classes of metabolites are frequently associated with aerobic energy production pathways. On the other hand, those studies that focused on resistance training showed a consistent increase in the levels of creatine, anabolic hormones (or their metabolites), choline, guanidinoacetate, and hypoxanthine along with a reduction in creatinine levels. These metabolites are typically associated with anaerobic energy production, muscle growth, intracellular buffering, and methyl-group regulation. We would also note that

a recent systematic review prepared by Schranner et al. (58) provides an excellent summary of the metabolite shifts seen after exercise in humans.

Rather than attempting to identify or summarize collective metabolite changes across all studies, in this section, we will briefly highlight some of the most notable exercise metabolomics studies and summarize some of the most interesting or important findings collected over the past 14 years. This is done to provide some historical context as well as to provide some perspective on the emerging trends and notable findings in exercise metabolomics studies.

The first study to bring metabolomics into exercise physiology was published in 2007 (2). In this early study, 24 healthy and active men performed 90 minutes of stationary bicycle pedaling (9 sets of 10 minutes as follows: 2 minutes at 40%, 6 minutes at 60% and 2 minutes at 80% of VO_{2max}). 420 potential metabolites were identified using a GC- time-of-flight (TOF)-MS platform and the authors noted significant changes in 34 of these metabolites. The authors focused on glycerol and asparagine as the most useful biomarkers. This study proved that untargeted GC-MS-based metabolomics could provide a thorough, unbiased approach to study the metabolic effects of exercise interventions (2).

Exercise nutrition metabolism researchers started to use metabolomics in 2009. In the study described by Kirwan et al. (59), participants were invited to a laboratory after overnight fasting to perform an exercise training protocol (rowing at 70% of maximal oxygen uptake (VO_{2max}) until exhaustion). In the first hour after exercise, participants received a dose of 4 g/kg (of body mass) carbohydrates. Participants also consumed 6 mg/kg caffeine immediately and 2 hours after the exercise period. Blood samples were collected before, immediately and 60, 120 and 180 minutes after exercise and analyzed using NMR spectroscopy. The authors identified a significant reduction in blood glucose and a significant rise in ketone bodies (3-hydroxybutyrate, acetoacetate and acetone) due to liver-derived ketogenesis. They also noted a significant rise in plasma levels of lactate and alanine, which are needed for gluconeogenesis (59).

Another notable exercise metabolism study in 2010 was undertaken by Enea et al. (60). In this study, they split their 22 participants cohort into two groups of trained and untrained women. Both groups performed the following protocols: 30 seconds of all-out exercise on a cycle ergometer followed by a test at 75% of their VO_{2max} until exhaustion on the same ergometer. Urine samples were collected at rest and 30 minutes after completing the protocol and analyzed

by NMR spectroscopy. The results showed that creatine, lactate, pyruvate, alanine, beta-hydroxybutyrate, acetate and hypoxanthine served as the most distinguishing metabolites between trained and untrained subjects. Urinary excretion of lactate, pyruvate, alanine, beta-hydroxybutyrate, and hypoxanthine also increased in both groups after 30 seconds of intensive exercise, but acetate excretion was lower in the trained group.

The term “Sportomics” was first used by Resende et al.in 2011 (61). Sportomics is the application of metabolomics in sports to investigate the metabolic effects of physical exercise on individuals, whether they are professional athletes or not (62). Here we classify this sportomics study as a sport metabolism study. In conducting this study, Cameron et al. (61) investigated the metabolic changes caused by two 30-minute windsurfing competitions, with a 30-minute rest in between. The same windsurfing tests and blood sampling were repeated (as with the pretest) after 3 months of training and nutritional intervention. The combined nutritional and training intervention produced an increase in plasma levels of branched-chain amino acids, aromatic amino acids, alanine, glutamate, and glutamine during exercise. Both training and nutritional interventions reduced plasma levels of ammonia, uric acid and urea. Furthermore, they found that appropriate nutritional supplementation could reduce the significant potassium drop seen during exercise.

In 2012, Neiman et al. (63) began using metabolomics in exercise nutrition metabolism studies. These authors examined the effects of banana consumption and isotonic carbohydrate supplementation in a 75-km cycling test on 14 participants. Urine samples were analyzed using a combination of LC-MS and GC-MS. In total, 103 metabolites were detected with 56 showing significant temporal changes (before, immediately and one hour after the sessions). Only dopamine showed significant variation between the banana-consumption and carbohydrate-consumption groups. These 56 identified metabolites were mostly related to carbohydrate, protein and lipid metabolism as well as liver glutathione production.

In 2014, Ra et al. (64) conducted the first large scale sport metabolism study, with 122 male soccer players participating in three soccer matches over a three-day period (one game per day). In this study, saliva samples were collected and analyzed by CE-MS methods. Salivary levels of 3-methyl histidine, glucose-1-phosphate, glucose-6-phosphate, taurine and several amino acids were significantly increased in the fatigued athletes compared to the non-fatigued athletes. These findings indicate that increased muscle breakdown, as well as glucose, lipid, amino acid metabolism, and overall energy metabolism was increased in the fatigued soccer

players. These metabolites were proposed as saliva-detectable metabolic indicators of fatigue in soccer (64).

In 2017, another sport metabolism study by Prado et al. (65) looked at the metabolic changes induced among semi-professional soccer players playing a soccer game. Urine samples were collected for a total of 30 soccer players and studied using untargeted LC-MS/MS, comparing pre- and post-game samples. This study was significant for the large number of metabolites measured and evaluated. A total of 1091 metabolites were identified of which 526 metabolites showed significant changes, with the most significantly upregulated metabolites being fatty acyls, carboxylic acids, steroids and steroid derivatives. Parallels studies that looked at capillary blood metabolites noted a significant increase in blood glucose, uric acid and urea along with a sharp decrease in potassium in response to exercise. Based on our data, this study appears to have been the most comprehensive metabolomic study undertaken in sport metabolism to date.

One of the most interesting and practical exercise metabolomics studies was published by Al-Khelaifi et al. in 2018 (51). In this study,191 elite athletes from different sports were divided into four categories (high endurance, moderate endurance, high power, and moderate power athletes) and blood samples were collected at rest to study the differences between blood metabolites among the four groups. Of the 743 metabolites detected, gamma-glutamylglutamate and gamma-glutamylvaline were significantly lower both in athletes with superior strength and with better endurance than their counterparts, indicating that a more active glutathione cycle was present in endurance-trained or strength-trained athletes. Serum levels of sex hormones, such as testosterone and progesterone, were higher, but levels of diacylglycerols and eicosanoids were lower in endurance-trained athletes. In addition, strength-trained athletes had higher levels of phospholipids and xanthine. Altogether, these findings showed that endurance-trained and power-trained athletes have significantly different metabolic profiles than their moderately trained or less-trained counterparts.

In 2019, two exercise metabolomics studies stand out. One by Sato et al. (66) and another by Ezagouri et al. (67). Sato et al. (66) studied the time-dependent metabolic impact of exercise on skeletal muscle, revealing altered daily metabolic cycles after exercises that were specific to the time of day. Exercise in the morning was shown to robustly activate the hypoxia-inducible factor 1 alpha (HIF1α) pathway, followed by glycolytic activation, the use of alternative fuels, and adaptation of systemic energy expenditure. In the other study, Ezagouri

et al. (67) examined the daily variance in exercise capacity in both mice and humans. These authors found that the time-dependent effect of exercise was affected by exercise intensity and circadian clock proteins. This led to a distinct muscle transcriptomic and metabolic signature. Specifically, they demonstrated that 5-aminoimidazole-4-carboxamide ribonucleotide (ZMP), an adenosine monophosphate-dependent protein kinase (AMPK) activator, was induced by exercise in a daytime-dependent manner.

in 2020, Contrepois et al. (57) used a multi-omics approach (targeted and untargeted metabolomics, lipidomics, proteomics and transcriptomics) including an untargeted LC-MS metabolomics assay that analyzed more than 600 serum metabolites. Their metabolomics data showed that energy metabolism, oxidative stress, inflammation, tissue repair, and their regulatory pathways were significantly affected by exercise in diabetic patients. In addition, elevated plasma levels of interleukin 1beta (IL-1β), interleukin 5 (IL-5) and transforming growth factor-beta (TGF-β) as a "fitness inflammatory signature" were identified. Finally, these authors reported that while triacylglycerol and BCAAs are associated with low VO_{2max}, transporters of thyroxine and retinol transthyretin (TTR), hydroxy-fatty acids, corticosterone, hippuric acid, bile acids and leptin are associated with a high VO_{2max}.This work highlights the importance of molecular-based exercise adaptation study designs in both clinical and elite athlete studies as well as the advantage of using multi-omics techniques in such studies.

3.9 Limitations of previous studies and potential improvements

As with all other metabolomics disciplines, exercise metabolomics is constantly evolving and constantly improving. Based on our analysis of the published data, it is clear that many of the earlier exercise metabolomics studies lacked the statistical rigor (extensive use of multivariate statistics, under-powered, small sample sizes, no correction of false discovery rates) that is expected of most metabolomic studies published today. Similarly, the level of metabolite coverage of many early studies (prior to 2014) was often very modest with relatively few studies using more than one platform or providing sufficient information about the quality (via Metabolite Standards Initiative ratings) or certainty of their metabolite identifications. The trend with more recent studies towards greatly expanding their metabolite coverage, using more than one analytical platform and increasing their sample sizes is an encouraging sign. Overall, in conducting this review, we found a disturbing lack of metabolite quantification in many published exercise metabolomics studies. This lack of absolute quantification makes comparisons across labs, across studies or across platforms (NMR vs. LC-MS vs. GC-MS)

almost impossible. Certainly, if exercise metabolomics is to move beyond the "stamp collecting" phase of conducting highly specialized but largely irreproducible studies it will be important for the community to adopt more rigorous and more standardized approaches to metabolite identification and quantification as is now widely done in the clinical metabolomics field. Indeed, most of the clinical metabolomics field has moved exclusively to fully targeted, absolutely quantitative metabolomics studies.

Among the biomarker studies reported by most published exercise metabolomics studies published over the past 15 years, we found insufficient use (or reporting) of sensitivity, specificity and receiver-operating characteristic curves. These kinds of statistics have become standard in most biomarker studies in other disciplines, but metabolomics still seems to lag far behind. Likewise there appears to be almost no reporting of the logistic regression equations, cut off values (or thresholds) or parameters used to generate the biomarker performance curves. This omission not only limits biomarker reproducibility but it also limits biomarker utility. Once again, if the exercise metabolomics community could adopt the standards now widely used in clinical metabolomics or clinical biomarker fields, the quality and reproducibility of many exercise metabolomics biomarker studies could be improved.

Interestingly, we found that almost no exercise metabolomic study deposited its data into standard metabolomics data repositories, such as MetaboLights (68) or the Metabolomics Workbench (69). The tendency for many exercise metabolomics labs to avoid data deposition into public databases will likely come to haunt this field, especially as issues of scientific rigor and reproducibility become more of a concern for many other omics disciplines. Certainly if a database, specific to the field of exercise metabolomics, could be established then some of the issues concerning method standardization, proper experimental design (and reporting), statistical rigor and general scientific reproducibility could be addressed.

While it is clear that the study design for many sports metabolomics studies has significantly improved over the past decade, further improvements are certainly possible. In particular, sport metabolomics researchers need to pay much more attention to exercise-related parameters or measurements, such as intensity and duration, as these factors strongly influence the metabolic changes following exercise training. Including these parameters in the study design and quantifying them more consistently would enable more facile comparison between different studies. Additional development of more standardized data collection, data analysis, data deposition and data reporting protocols would also help improve the overall quality and

comparability of exercise metabolomics studies. Another important goal for exercise metabolomics studies will be the routine use and integration of additional omics (proteomics, genomics, transcriptomics) techniques in the study design. Metabolomics should not be an "island" and a true understanding of biology or physiology involves an understanding of the interplay of genes, proteins and metabolites with the environment. There is a clear trend for many recently published metabolomics studies to include multi-omics methods and to perform multi-omic data integration. Exercise metabolomics needs to embrace this concept if it wishes to evolve and grow. Other potential improvements and other encouraging trends in the field of exercise metabolomics are discussed below.

4. Conclusion and Future Perspectives

Overall, our data shows that there is an increasing trend towards better designed, more clinical, MS-based metabolomics studies involving larger numbers of subjects or patients and larger numbers of metabolites being identified. While the first exercise metabolomic studies mainly focused on finding biomarkers related to the effects of "simple exercise" on a stationary bicycle, improvements in experimental design and sample collection, combined with a generally improved understanding of the implications of various metabolites have gradually allowed the application of metabolomics to a wider range of areas both in sport and clinical settings. The most recent and best-designed exercise metabolomics studies now provide a very comprehensive metabolic picture enabling researchers to study metabolism more completely and more accurately, and to more thoroughly investigate the clinical consequences for prevention and treatment of metabolic disorders. It is also evident that metabolomics studies can be used to look at very specific sports or sport themes to better understand associated metabolic changes. This can provide much more practical data that can be applied to sport-specific conditioning programs. It is hoped that this information could enable coaches to design "precision sports training" programs to help maximize an athlete's performance and hopefully minimize injuries (62, 70).

Over the next five to ten years, we foresee an increasing trend for metabolomics studies being undertaken in both elite sport performance and clinical exercise settings. We also expect to see greater interest in the area of metabolomics and sport nutrition, with an emphasis on using the results to design personalized, precision nutrition regimens for maximizing the effects of both sport performance and exercise-based health benefits. Overall, the use of well-designed metabolomic or multi-omic studies would allow sport coaches to train their elite athletes more

efficiently and clinicians to refine and improve their exercise plans for patients or seniors. From a technical standpoint, we suggest that more emphasis in exercise metabolomics needs to be placed on human studies with more focus on practical-oriented and realistic designs using non-invasive sample collection methods focused on collecting urine and saliva. Such a trend would certainly make exercise metabolomics studies much more informative, much more welcomed by the subjects (c.f. non-invasive investigation), bringing newer, better and more actionable information to patients and physicians as well as athletes and coaches.

Declarations

Conflict of interests

Kayvan Khoramipour, Oyvind Sandbakk, Ammar Hassanzadeh Keshteli, Abbas Ali Gaeini, David Wishart and Karim Chamari declare that they have no conflicts of interest relevant to the content of this review.

Funding

No sources of funding were used to assist in the preparation of this article.

Ethics approval

Not applicable.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

No additional data are available.

Code availability

Not applicable.

Authorship Contributions

KKH, OS, AAG and KC designed the study. KKH and AAG conducted the literature searches and wrote the preliminary draft. KKH, AHK, DW, OS and KC revised the manuscript. AHK checked the data accuracy. All authors read and approved the final manuscript.

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