Fakultät für Sport - und Gesundheitswissenschaften

Metabolite and protein signatures of exercise, physical fitness, and athletic phenotypes in healthy humans

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Abbreviations

Δ $\dot{V}O_2$max / $\dot{V}$O2peak	Trainability of maximum oxygen uptake capacity
ABL	Abelson murine leukemia viral oncogene homolog 1 encoding a tyrosine protein kinase ABL1
ACSL1	Acetyl-CoA synthase long-chain member 1
ANOVA	Analysis of variance
BCR	Breakpoint cluster region protein
BMI	Body mass index
CK	Creatine kinase
EDTA	Ethylenediaminetetraacetic acid
EPOR	Erythropoietin receptor
FDR	False discovery rate
IL-22	Interleukin-22
LC-ESI-MS	Liquid chromatography-Electrospray ionization-Mass spectrometry
LC	Liquid chromatography
LDH	Lactate dehydrogenase
M.	Musculus (Muscle)
MB	Myoglobin
MS	Mass spectrometry
PICO	Population, Intervention, Control, Outcome
PLS-DA	Partial least squares discriminant analysis
ROC	Receiver-Operator-Characteristics
SNP	Single nucleotide polymorphism
TCA	Tricarboxylic acid
TNNI	Troponin I
$\dot{V}O_2$max / $\dot{V}O_2$peak	Maximum oxygen uptake capacity, a measure of cardiorespiratory fitness

Summary

During the last 20 years, exercise as a science has gained in importance in answering health and performance related questions in sports medicine, competitive sports and for the health of an increasingly inactive and ageing society. One important finding during these years was that one exercise does not fit all and can lead to highly variable adaptation between individuals, even to worsening of performance. As adaption is the key driver of athletic performance, risk factor reduction and even mortality, there is a need for individualization of exercise training to ensure physiological adaptation. So called *omics* methods enable a granular picture of exercise-associated changes in human metabolism by quantifying hundreds to thousands of metabolic molecules at the same time. In that way, the first steps towards individualized exercise can be made.

Through incorporation of the existent literature and two experimental studies, this cumulative thesis aims to answer the first set of questions that arise during an individualization in exercise science.

In a first step, a systematic literature review of studies looking at global metabolite concentration changes after an acute bout of exercise were summarized. The result is a comprehensive overview on how and at which time after exercise, metabolite classes like lipids, amino acids or TCA cycle metabolites change their concentration in human blood, urine, saliva and sweat. Amongst others, key pathways of energy metabolism were reflected in the summarized metabolite concentration changes. The review is designed as a resource for researchers on the expected metabolite changes after exercise in general and to aid future exercise omics study designs.

After having global overview on metabolite changes in the systematic review, Study 2 (MetaExtreme study) experimentally investigates the specific metabolite changes after long-term exercises. For that we measured targeted and untargeted metabolomics in three groups of competitive athletes (natural bodybuilders, endurance athletes and sprinters) plus an untrained control group (n=35) before and after a maximum graded exercise test to exhaustion. Athletes represented the upper extremes of a particular spectrum of bioenergetic capacity (high anabolism, high oxidative capacity, high glycolytic capacity). Multivariate statistical analysis of the targeted dataset (n=18, ~200 metabolites) revealed that endurance athletes and natural bodybuilders have distinct metabolite profiles before and after exercise, whereas sprinters and controls were more similar. Some of the key metabolites that separate endurance athletes and natural bodybuilders from others were related to different bioenergetic capacities in oxidative metabolism, anabolism, and athlete-specific nutrition. In a first analysis of the untargeted dataset (n=35, ~800 metabolites) we replicated the main results of the multivariate analysis and found additional metabolites that are different between athlete groups. Further we found novel associations between metabolites and phenotypic traits (e.g. muscularity), which all remain to be published.

A second experimental study (Study 3) was done to test if blood molecules can be used to predict the individual physiological adaptation to training. Predictability of an individual response to training is key to make individualized training decisions. Here, we used a set of >600 blood samples from the HERITAGE family study during my research residence at the Beth Israel Deaconess Medical Center in Boston. A set of ~5,000 blood proteins were quantified with an aptamer-based assay and associated with $\dot{V}O_2max$ and $\dot{V}O_2max$ trainability ($\Delta\dot{V}O_2max$) after 20 weeks of endurance exercise. Proteins significantly associated with $\dot{V}O_2max$ spanned a wide range of the known organ systems incorporated into $\dot{V}O_2$ (e.g. muscle, vascularization) and also revealed novel associations (e.g. proteins involved in bone metabolism). Finally, 56 out of the measured ~5,000 proteins were used in a prediction model of $\Delta\dot{V}O_2max$ changes with exercise, reaching ~80% positive predictive accuracy.

In summary, metabolite changes upon exercise in general can reflect key pathways of energy metabolism (e.g. fat oxidation). However, not all metabolite changes can be generalized (e.g. amino acids). The thesis showed that sport-specific exercise training for years, favorable genetics and other factors like nutrition result in characteristic blood metabolite concentrations in athletes, many of which are relevant in metabolic diseases. With further validation, this knowledge could be used to individualize exercise training to change disease-associated blood metabolites in a targeted way. Secondly our study on subjects representing extreme phenotypes (Study 2) is another step into biomarker identification for exercise-related traits like muscle strength or cardiorespiratory fitness which are associated with future health and mortality. In the future, biomarkers for these traits could be used in standard clinical diagnostics and facilitate and fasten lifestyle decisions for the broad public. Protein concentrations measured in Study 3 can predict training responses and could help to identify individuals where exercise is beneficial to improve health and others for whom other interventions (e.g. pharmacological or dietary) may be more suitable to improve health.

Zusammenfassung

Sport als Wissenschaft hat vor allem in den letzten 20 Jahren an Bedeutung gewonnen, um Antworten auf gesundheits- und leistungsbezogene Fragen in der Sportmedizin, im Spitzensporttraining und für die Gesundheit einer zunehmend inaktiven und alternden Gesellschaft zu finden. Eine wichtige Erkenntnis der Sportwissenschaft ist, dass dieselbe sportliche Aktivität oder Sportart nicht für alle Individuen gleich gut geeignet ist und zu stark unterschiedlichen physiologischen Anpassungen (z.B. Ausdauerleistungsfähigkeit oder Muskelmassewachstum), ja sogar zu einer Verschlechterung der Leistung führen kann. Da die physiologische Anpassung der Treiber sportlicher Leistung, der Verringerung von gesundheitlichen Risikofaktoren und sogar der Sterblichkeit ist, muss sportliches Training individualisiert werden, um die physiologische Anpassung zu gewährleisten. Mit so genannten Omics-Methoden können Hunderte bis Tausende von Stoffwechselmolekülen gleichzeitig im menschlichen Blut gemessen werden, was ein detailliertes Bild der mit dem Training verbundenen Veränderungen des menschlichen Stoffwechsels ermöglicht. Auf diese Weise können die ersten Schritte in Richtung eines individualisierten Trainings unternommen werden.

Durch die Einbeziehung der vorhandenen Literatur und zweier experimenteller Studien, zielt diese kumulative Doktorarbeit darauf ab, einen ersten Teil der Fragen zu beantworten die bei einer Individualisierung sportlichen Trainings auftreten.

In einem ersten Schritt wurden in einer systematischen Literaturarbeit Studien zusammengefasst, die Änderungen der Metabolitenkonzentrationen nach einer akuten sportlichen Belastung zeigen. Das Ergebnis ist ein umfassender Überblick darüber, wie und zu welchem Zeitpunkt nach sportlicher Belastung sich Metabolitenklassen wie Lipide, Aminosäuren oder TCA-Zyklus-Metaboliten ihre Konzentrationen im menschlichen Blut, Urin, Speichel und Schweiß verändern. Die Arbeit zeigt, dass sich in den Veränderungen der Metabolitenkonzentration, Schlüsselwege des menschlichen Energiestoffwechsels widerspiegeln. Sie soll Forschern als Ressource dienen, um zu erwartende Metabolitenänderungen nach sportlicher Aktivität im Allgemeinen zu kennen und somit die Planung künftiger Omics-Studien zu unterstützen.

Nachdem in der systematischen Literaturarbeit ein globaler Überblick über Metabolitenänderungen gegeben wurde, untersuchte Studie 2 experimentell, wie spezifisches, jahrelanges Training zu Metabolitenänderungen führt. Dazu wurden targeted (~200 Metaboliten) und untargeted (~800 Metaboliten) Metabolomics in drei Gruppen von Leistungssportlern (Natural Bodybuilder, Ausdauersportler und Sprinter) sowie einer untrainierten Kontrollgruppe (n=35) vor und nach einem maximalen Ausdauerbelastungstest gemessen. Die rekrutierten Leistungssportler repräsentierten extreme menschliche Metabolismen ihrer jeweiligen antrainierten bioenergetischen Kapazität (hoher Anabolismus, hohe oxidative Kapazität, hohe glykolytische Kapazität).

Die statistische Analyse der targeted Daten ergab, dass Ausdauerathleten und Natural Bodybuilder einzigartige Metabolitenkonzentrationen in Ruhe sowie nach der maximalen sportlichen Belastung haben, wobei sich die Metabolitenkonzentrationen von Sprintern und Untrainierten mehr ähnelten. Die wichtigsten Metaboliten, die Ausdauersportler beziehungsweise Natural Bodybuildern von allen anderen unterscheiden, hängen wahrscheinlich mit den unterschiedlichen bioenergetischen Kapazitäten im oxidativen Stoffwechsel, im Anabolismus und in der Ernährung zusammen. In einer ersten Auswertung der untargeted Daten konnten die Hauptergebnisse reproduziert werden sowie weitere Metaboliten und Metabolit-Phänotyp Assoziationen gefunden werden, welche in Zukunft ebenfalls publiziert werden.

In einer zweiten experimentellen Studie wurde getestet, ob Blutmoleküle zur Vorhersage der individuellen physiologischen Anpassung an sportliches Training genutzt werden können. Die Prädiktion der Anpassung ist für die individualisierte Trainingsplanung wichtig. Für Studie 3 wurde an über 600 Blutproben der HERITAGE Familienstudie im Rahmen eines Forschungsaufenthaltes am Beth Israel Deaconess Medical Center in Boston durchgeführt. Dabei wurden ~5.000 Proteine mit einem Aptamer-basierten Verfahren gemessen und mit der $\dot{V}O_2max$ und der $\dot{V}O_2max$ Trainierbarkeit ($\Delta\dot{V}O_2max$) nach 20-wöchigem Ausdauertraining assoziiert. Die Proteine, die signifikant mit der $\dot{V}O_2max$ assoziiert waren, deuteten auf die bekannten Organsysteme, welche die VO_2 bedingen (z.B. Muskulatur, Gefäße) sowie auf noch nicht bekannte Assoziationen (z.B. Knochenstoffwechsel) hin. 56 der ~5.000 gemessenen Proteine konnten schließlich Modell zur Vorhersage der $\Delta\dot{V}O_2max$ verwendet werden, welches eine positive Vorhersagegenauigkeit von ~80% erreichte.

Zusammenfassend spiegeln ein Teil der Metabolitenänderungen nach sportlicher Aktivität Schlüsselwege des Energiestoffwechsels (z. B. die Fettoxidation) wider. Jedoch können nicht alle Metabolitenändeurngen nach sportlicher Aktivität verallgemeinert werden (z.B. Aminosäuren).

Weiterhin hat diese Doktorarbeit gezeigt, dass jahrelanges sportartspezifisches Training, günstige genetische Voraussetzungen und andere Faktoren wie Ernährung, zu charakteristischen Blutmetaboliten-Konzentrationen bei Leistungssportlern führen. Durch weitere Validierung der Ergebnisse und umfangreichere Studien, könnte das Wissen um sportartspezifische metabolische Anpassung zur Individualisierung beitragen und neue Biomarker für metabolische Anpassung (z.B. Muskelmasse oder Reduktion der Risikofaktoren) gefunden werden.

Letztlich zeigte diese Arbeit auch, dass mit Hilfe von Blutproteinen, eine gesundheitsrelevante Anpassung an sportliches Training vorhergesagt werden kann und besser ist als die Vorhersage durch Standardparameter (z.B. BMI, Geschlecht, Alter). Mit einer Vorhersage können einerseits Personen identifiziert werden, für die Sport eine gesundheitsfördernde Lebensstilintervention darstellt, und andererseits Personen, für die andere Interventionen (z.B. Ernährung oder Medikamente) besser geeignet sind, um ihre Gesundheit zu verbessern.

I. INTRODUCTION

I-1. Exercise science and its role in improving health

I-1.1. A brief history of exercise science

Exercise science is a rather young field of research and was not regarded as ‘a true science discipline’ by politics, society, or other disciplines until the late 1960ies in Germany (Grupe 1996). Tackling exercise related questions scientifically was seen as ‘unnecessary’ back then and recommendations for exercise training were mostly based on practical experience by coaches. In the 1970ies, exercise science was institutionalized in the German academic system (Grupe 1996). Since then, plenty of sub-disciplines have emerged, for example biomechanics, sports psychology, didactics, sports medicine, sports informatics, or exercise biology, which all unite knowledge of exercise science with their respective mother discipline.

Politics, society, and other disciplines have more and more valued exercise science since then, not only for supporting national athletes with evidence-based recommendations but also for answering health-related questions that became increasingly important in society (Grupe 1996): “How can elderly people exercise safely after a myocardial infarction?”, “Is exercise beneficial or harmful during a chemotherapeutic treatment in cancer?”, or “Can young females run marathons without compromising fertility?”

Exercise science has for several years now succeeded in answering all and more of these questions and continues to face novel challenges with societal and behavioral change like an ageing population and a sedentary lifestyle: “Can exercise slow down metabolic ageing?” (Viña et al. 2016), “Is a time and cost-efficient form of exercise called ‘exercise-snacking’ (exercising for <1 minute but very intense bouts several times per day) sufficient to improve cardiometabolic health?” (Islam et al. 2021), “Can I counteract the negative metabolic consequences of sleep loss with exercise?” (Saner et al. 2018; Saner et al. 2021). Can we predict the individual training response after endurance training by blood tests (Robbins et al. 2021) ? These novel challenges reflect the increasing need to individualize exercise to fit our personal needs and circumstances like a limited time or travelling with the overall aim to reach health- or performance-oriented goals.

I.1.2. Individualization as the turning point

Individualization per se is established in other fields of research, for example in medicine, where individualized therapies have been developed for many diseases like cancer and where their application improves therapeutic outcomes or even survival. As an example, patients with a certain type of blood cancer called chronic myeloic leukemia (CML), are genetically screened for a mutation called BCR-

ABL[1], which if present, leads to an enzymatic over-expression of tyrosine-kinase, promoting cell proliferation and therefore the CML. Patients with the BCR-ABL mutation can be treated with imatinib which inhibits the overly active tyrosine-kinase in a targeted way and leads to cancer remission in over 80% of eligible patients (Savage and Antman 2002).

Not only genes like in CML, but also other biological features like immune cells in blood are used to decide upon treatment e.g. for COVID patients, natural killer T-cell levels can be used to predict severe courses of disease at hospital admission (Kreutmair et al. 2021).

In medicine, the rationale for individualization is that patients respond different to the same therapy and that an individualized therapy, based on biological features of the patients (e.g. genetic variants) leads to the desired treatment success.

In exercise science, the same rationale for individualization applies: the same exercise leads to different responses (i.e. outcomes in physiological adaptation) between individuals. An example for this is the variance in endurance capacity changes among individuals of the HERITAGE family study (Bouchard et al. 1999). Bouchard and colleagues showed, that endurance capacity (measured as maximum oxygen uptake capacity, $\dot{V}O_2max$) changes to the same 20-week-long training ranged from very few super-responders, who increased their $\dot{V}O_2max$ by over >1000 ml/min, over to a lot of average responders (between 200-800 ml/min increase) and few non-responders to very few adverse responders, who worsened their $\dot{V}O_2max$ by endurance training (**Figure 1**) (Bouchard et al. 1999). This specific example highlights that standardized exercise programs over a defined time window, lead to highly variable adaptations of $\dot{V}O_2max$ between individuals. Furthermore, it underlines that some individuals do not benefit at all from such a generalized exercise program.

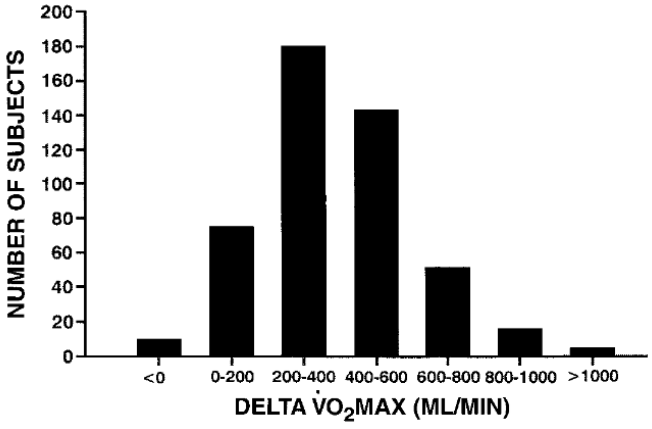


Figure 1. Distribution of $\dot{V}O2max$ changes (delta $\dot{V}O2max$) of 481 individuals by 20 weeks of endurance training. Adapted from Bouchard et al. (1999). Blood samples of this study were used in study 3 of this thesis (Robbins et al., 2021)

[1] A fusion-gene originating from the fusion of the two genes BCR and ABL leading to production of altered proteins, which escape the regular cell cycle and have continuously enhanced activity of signaling pathways which lead to enhanced cell proliferation and increase the risk of oncogenesis

Not only the physiological adaptation to endurance training ($\Delta\dot{V}O_2max$) but also the adaptation to resistance training, which is measured by the increase in muscle cross-sectional area (CSA, i.e. muscle size) is highly variable between individuals (Ahtiainen et al. 2016; Hubal et al. 2005) and follows, like $\dot{V}O_2max$, a normal distribution (**Figure 1**). A study by Hubal et al, showed that the M.biceps brachii size adaptation after 12 weeks of resistance training varied from none or adverse responders with loss of 2% of CSA (i.e. no muscle size change) over the average responders (increases of 10-25% in CSA) to super responders who increased their CSA by 59% after 12 weeks of resistance exercise (**Figure 2**).

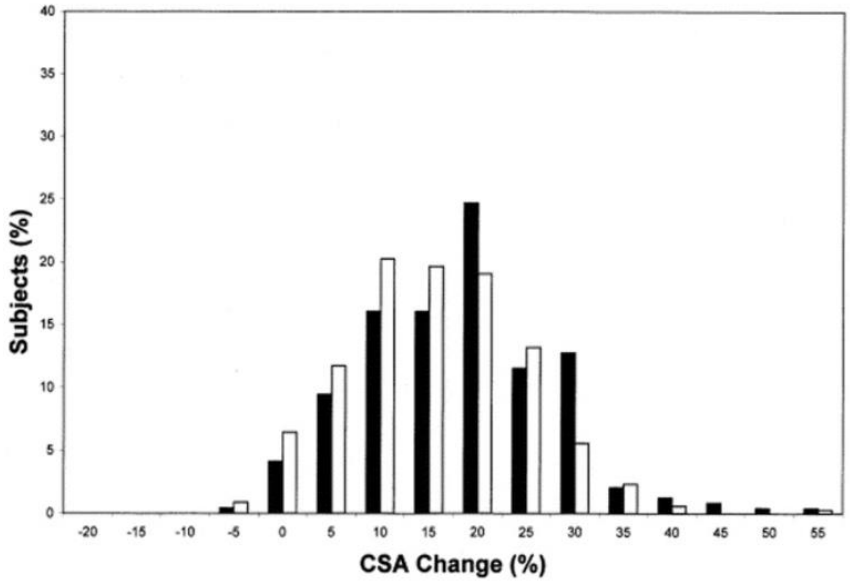


Figure 2. Muscle cross-sectional area (CSA, i.e. muscle size) adaptation in % change from baseline to 12 weeks of resistance training (M. biceps brachii and M. triceps brachii) shows responses in 243 men (■) and 324 women (□). Adapted from (Hubal et al. 2005).

Taken together, the evidence that not one exercise fits all, shows the need for individualization in exercise science. However, contrasting to medicine, where biological features (or biomarkers) measured in patients' blood are already used to build an individualized therapy to reach positive outcomes, exercise science has just started to make use of biological feature-driven individualization (e.g. lactate) and has so far only tapped the full potential of large-scale biological feature detection that exists today.

I-1.3. Individualization to improve long-term health

Individualization of exercise must target physiological adaptation that is meaningful for either performance (e.g. of athletes) or health (e.g. the general population or patients). The variability in physiological adaptation shown by Hubal and Bouchard shows the necessity for a differentiated exercise training. Practically this could mean that non-responders (0 ml/min/kg of delta $\dot{V}O_2max$ (**Figure 1**) or 0% of CSA change (**Figure 2**)) would either need prolonged training periods, longer regeneration breaks

in between sessions, other training modes or an entirely different sport, to reach a meaningful physiological adaptation. As an example, a meaningful increase of $\dot{V}O_2$max would be >15% of an individuals' baseline value, as this has been shown to decrease the risk of cardiovascular diseases (Ross et al. 2016b).

In general, the degree of physiological adaptation is strongly associated with future health (**Figure 3A and 3B**) and is not only limited to muscle but also to systemic changes in the body that lead to improvements in health: Endurance exercise increases heart volume, blood volume, glucose uptake, insulin sensitivity, fat oxidation, and mitochondrial content and efficiency, consequentially increasing endurance capacity ($\dot{V}O_2$max or $\dot{V}$O2peak). Resistance exercise elevates muscle protein synthesis (above protein breakdown) which increases muscle fiber size and trains neuromuscular activation of skeletal muscles. Functionally, this results in higher strength, power, and speed. Both adaptation examples, $\dot{V}O_2$max and muscular strength, are associated with reduced mortality (Blair 2009; Kodama et al. 2009; Ladenvall et al. 2016; Ruiz et al. 2008). Subjects in the middle and upper thirds of muscular strength were shown to have 1.46-1.50 times lower mortality compared to subjects in the lowest third (**Figure 3A**). Similarly, subjects with higher $\dot{V}O_2$max (tertile 1) had lower mortality (75%) compared to subjects with lower $\dot{V}O_2$max (tertile 3) (90% mortality) after 45-years of follow-up analysis (**Figure 3B**).

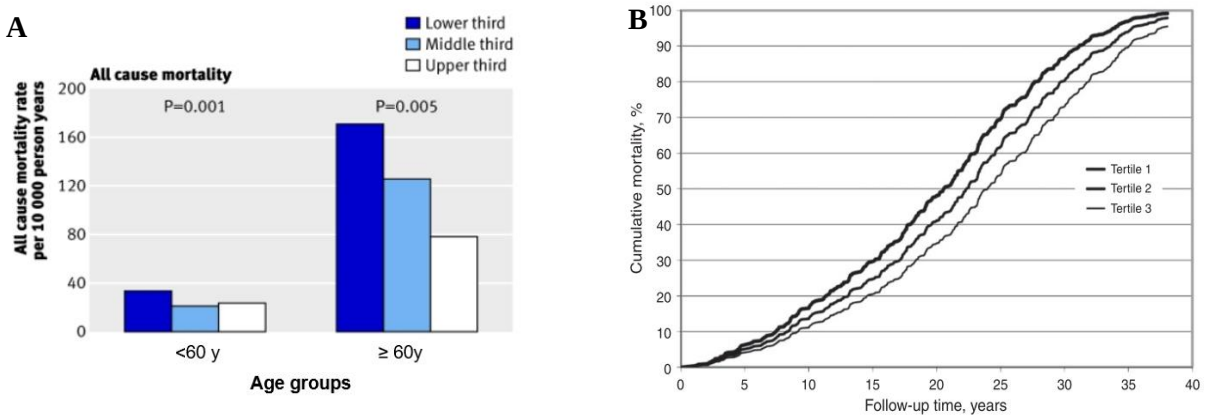


Figure 3 A. Age adjusted all-cause mortality rates by thirds of muscular strength separated by age from an 18-year follow up study (adapted from Ruiz et al. 2018). **B.** Cumulative all-cause mortality in relation to tertiles of predicted peak oxygen uptake capacity ($\dot{V}$O2peak) from a 45-year follow up study: $\dot{V}O_2$ Tertile 1: 2.0 l/min; $\dot{V}O_2$ Tertile 2: 2.26 l/min; $\dot{V}O_2$ Tertile 3: 2.56 l/min (adapted from Ladenvall et al. 2016).

In summary, there is substantial evidence that exercise is beneficial for health (Pedersen and Saltin 2015) and that exercise adapted individuals have a reduced mortality (Ladenvall et al. 2016; Ruiz et al. 2008). However, there is also evidence that the adaptation to exercise is individual and that not one exercise fits all (Bouchard et al. 1999; Hubal et al. 2005). But only through either metabolic (e.g. increased insulin sensitivity) and/or phenotypic (e.g. increased muscle mass) physiological adaptation to exercise, positive health effects will be reached. Consequentially it is necessary to individualize exercise for society and for competitive sports. Advantages of such an individualization could be a better health and a more permanent commitment to exercise in society and better athletic performance in competitive sports.

I-2. Steps towards individualizing exercise

This thesis aims to answer a first set of questions that come along in the process of an individualized exercise science.

The first step is to collect information on the metabolic changes through exercise *in general*. Whereas the phenotypic adaptation in the average exercising individual is well known for certain sports (**Figure 1 and 2**), we know less about the *metabolic* adaptation that happens after exercise. Exercise scientists' focus has been limited to single metabolic molecules like lactate (for endurance capacity at a given workload) (Mader 1976). Through modern omics, there are thousands of molecules measurable in the human body which entail metabolic information. Through this information, a global metabolic picture of exercise can be obtained.

The second step is to collect information on the metabolic changes through *specific exercise modes* like resistance or endurance exercise, including the differentiation between chronic (several weeks to years) and acute exercise (one or few exercise sessions).

The third step is to collect information the metabolic changes *within specific exercise modes*, different training intensities (which all have differing metabolic demand) like aerobic training, threshold training or high-intensity interval training, including differing exercise duration and regeneration times in between.

Through such a granular fragmentation of exercise, scientists will better understand the short and long-term metabolic changes through exercise and will be able to decide (after further validation) which exercises will be considered for which subjects or for reaching which goals in health and competitive sports.

A granular, metabolic fragmentation of exercise can be determined with so called "omics" technologies. The term omics covers different sub-disciplines: genomics, transcriptomics, proteomics, and metabolomics and refers to the measurement of *all* respective biological features (e.g. molecules like

genes, transcripts, proteins or metabolites) within a biological sample (e.g. blood, urine, saliva, skeletal muscle a.s.o.), which can range from few hundreds to several thousands. Omics methods have already yielded biomarkers, which are biological features whose concentration indicates a certain phenotype (i.e. a diseased), mostly in a clinical context.

Twenty years ago, discovery of such a great many features was difficult and only a single or a few features were measurable. Methods used in omics research like mass spectrometry (MS) have advanced technologically and nowadays enable the quantification of hundreds up to thousands of molecules in blood. Mass spectrometry today is quick, efficient, and more affordable than several years ago, making large-scale biological features detection possible for exercise scientists.

I-3. Omics to measure biological features in exercise science

I-3.1. Metabolomics

I-3.1.1. Definition

The term "*omics"* describes methods such as genomics, transcriptomics, proteomics, and metabolomics, which quantify or characterize hundreds or thousands of molecules such as genes, transcripts, proteins, and metabolites, in one biological sample. *Omics* methods are part of the unbiased discovery science (Aebersold et al. 2000) where as much data as possible of a biological system (e.g. an human) are generated and then analyzed with computational models e.g. to discover novel biomarkers.

Metabolites are low-weight (up to 1500 Da) endogenous or exogenous molecules and are measurable in various human body fluids like blood, urine, saliva, cerebrospinal fluid or sweat and in tissues like muscle fibers or adipose tissue. Endogenous metabolites include lipids, amino acids, nucleotides, vitamins and cofactors, carbohydrates, and TCA cycle metabolites. Exogenous metabolites, so called xenometabolites or xenobiotics enter the body via food, drinks, drugs, or pollutants. In metabolomics, all (or as many as possible) metabolites within a biological sample are measured and are termed, the metabolome.

Metabolites include the end products of human metabolism and therefore incorporate information from genes over transcripts, proteins, and environmental influences (Artati et al. 2012). From all molecules, they reflect human phenotypes best and inform about functional activities, transient effects, and endpoints of metabolic processes (Artati et al. 2012).

Omics methods were established in the late 90ies (Humphery-Smith and Blackstock 1997; Tweeddale et al. 1999) and another 20 years later, the first exercise scientists used metabolomics (Yan et al. 2009). Exercise scientists can use metabolomics to analyze the metabolic response of human subjects to acute and long-term exercise or monitor metabolism during exercise.

I-3.1.2. Measurement approaches

Metabolites can be quantified using a targeted or a non-targeted approach. Targeted metabolomics quantifies the absolute concentrations of a known and pre-defined set of metabolites (**Table 1**). Targeted metabolomics is used to measure a set of metabolites of interest where measurement accuracy and reliability is the priority.

In contrast, non-targeted methods quantify metabolites relatively, meaning that no absolute concentrations of metabolites are given. For the same metabolite, researchers can only conclude a higher or lower value between two measured probes (Adamski and Suhre 2013). Non-targeted methods capture all signals of metabolites within a sample (Adamski and Suhre 2013) and thereby aim to discover new or "unexpected" metabolites or metabolic pathways as potential biomarkers for a human phenotype (e.g. for a certain disease) (**Table 1**).

To measure metabolites, mainly mass spectrometry (MS) or nuclear magnetic resonance spectroscopy (NMR) are used as technologies. Only MS will be described in more detailed, as all experimental metabolomics data incorporated in this thesis were measured with MS. Details on the targeted approach used in thesis are found in the methods section of Study 2 (**II-2.**).

In targeted and non-targeted MS, metabolites are first extracted from the biological sample (e.g. blood plasma or serum, urine, or tissue like muscle) and then separated by gas chromatography (GC) or liquid chromatography (LC), which improves analytical sensitivity (Artati et al. 2012). Afterwards, metabolites are ionized, which is required for subsequent mass spectrometry. Ions and their fragments are resolved in an electromagnetic field according to their mass to charge (m/z) ratio. A spectrum showing the quantity of ions with the m/z ratio of the molecule ion and its fragment is specific to every metabolite and ultimately used for identification and quantification.

Table 1. Scientific approaches and main features in targeted and non-targeted metabolomics (adapted from Schrimpe-Rutledge et al. (Schrimpe-Rutledge, 2016 #754) and Adamski et al. (Adamski and Suhre 2013). FIA flow-injection-analysis, GC gas chromatography, NMR nuclear magnetic resonance spectroscopy.

	Targeted	**Non-targeted**
Scientific approaches	○ Hypothesis-driven ○ Subset of metabolites analyzed ○ Correlated to reference standards ○ Compounds known ○ Absolute quantification ○ → Validation/quantification	○ Hypothesis-generating ○ Global analysis of the metabolome ○ Correlated to libraries/databases ○ Qualitative identification ○ Relative quantification ○ → Discovery

Metabolite coverage	10-100	300-1200
Advantages	Absolute quantification of metabolites	Diverse set of distinct metabolites
Disadvantages	Many metabolites not covered	Only semi-quantitative determination of concentration
Main use	Pathway analysis, kinetics studies, quantitative estimation of challenge impact, diagnostics	Unbiased search for differences, discovery

I-3.2. Proteomics

I-3.2.1. Definition

Proteins formed through amino acids chains, interconnected by peptide bonds. Endogenous proteins include e.g. structural proteins that form and stabilize cells like collagen, contractile proteins, storage proteins, transporter proteins, protective proteins like antibodies in the immune system, or hormones that regulate metabolism or work as messenger substances like erythropoietin (EPO) and enzymes (**Table 2**). As metabolites, proteins are produced endogenously and can be ingested via food or drinks. As metabolomics, proteomics studies the entire set of proteins, the proteome, within a biological system at a given timepoint.

I-3.2.2. Measurement approaches

Proteins (for proteomics) can be measured using mass spectrometry or affinity-based proteomics, where either antibodies or other binding reagents (e.g. aptamers) specifically bind to target proteins in the experimental samples.

Proteins are ten to thousand times heavier than metabolites (**Table 2**). To measure proteins using MS, they must be digested into smaller pieces before injection into the mass spectrometer. During digestion, proteins are split by using protein-cutting enzymes like trypsin, which splits proteins' peptide bonds at specific amino acid sequences. This controlled splitting leaves amino acid and/or peptide fragments that are specific for the original protein and can be analyzed with MS.

High or medium abundant proteins (e.g. if measured in human blood) can overlay proteins with lower abundance and can reduce measurement sensitivity for those. This can be problematic because functionally important, low abundant proteins, e.g. regulatory hormones will be missed.

In this thesis, affinity-based proteomics, more precisely aptamer-based proteomics was used, which is why only this method is described. Detail can be found in the publication of Study 3 (**II-3**.)

Aptamers are molecules built from nucleic acids and fold into a specific 3-dimensional structure **(Figure 4).** Aptamers are added to the research samples and their structure is designed to bind specifically to a target protein (**Figure 4**). In this way, every protein in the research sample is targeted individually. The method is highly sensitive and can bind proteins within a wide concentration range up to the femtomolar concentration (10^{-15}) including low abundant proteins (Gold et al. 2010; Smith and Gerszten 2017).

Aptamers for protein identification were first developed in the early 2000's (Elrick et al. 2006). Aptamer-based methods have some limitations like unspecific binding of the aptamers to random proteins, which is why its outcomes should be validated by an independent method. So far, the technique was successfully applied in biomarker discovery in clinical settings e.g. for kidney health (Ngo et al. 2020), cardiovascular diseases (Jacob et al. 2018), or muscle wasting in Duchenne's disease (Hathout et al. 2015).

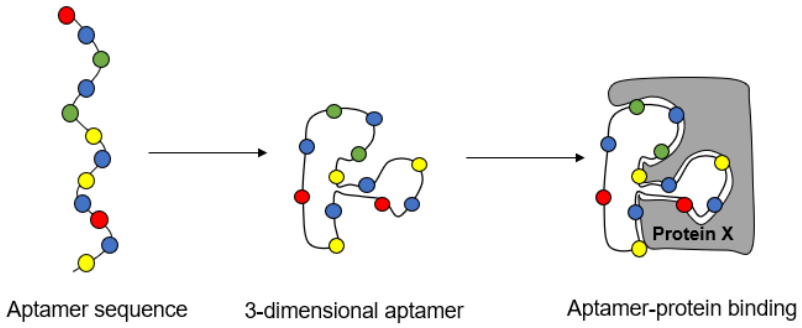


Figure 4. Simplified aptamer-protein binding process. The aptamer has a specific nucleotide sequence (colored circles) and is folded in a 3-dimensional structure that specifically binds to the protein of interest ("Protein X") like an antibody.

Table 2. Overview and methodology in metabolomics and proteomics

Omics technology	Metabolomics	Proteomics
Molecular weight	• Up to ~1.5 kDa	• ~13 - 3000 kDa
Complete set of molecules	• Metabolome	• Proteome
Classes	• Carbohydrates (e.g. hexose) • Lipids (e.g. fatty acids) • Amino acids and peptides (e.g. leucine) • TCA cycle metabolites (e.g. citrate) • Nucleotides (e.g. guanine) • Vitamins and cofactors (e.g. pantothenate) • Xenometabolites (e.g. caffeine or acesulfame)	• Structural proteins (e.g. collagen) • Contractile proteins (e.g. myosin) • Storage proteins (e.g. ferritin) • Transport proteins (e.g. hemoglobin) • Immune function proteins (e.g. antibodies) • Hormones (e.g. insulin) • Enzymes (e.g. creatine kinase)
Databases	• Virtual metabolic human database (Noronha et al. 2018) • Human metabolome database (HMDB) (Wishart et al. 2018)	• The human protein atlas (Uhlén et al. 2015) • Uniprot (Consortium 2017)
Measurement technologies	• Liquid-chromatography followed by electrospray-ionization and mass spectrometry (LC-ESI-MS) • Other technologies exist (e.g. GC-MS, NMR)	• LC-MS with upstream trypsin digestion (and other pre-analytical steps) • DNA aptamers[2]

Measurement approaches	• Untargeted (see **Table 1**)
	  ○ >1000 metabolites quantifiable
	  ○ Discovery research
	• Targeted (see **Table 1**)
	  ○ Pre-defined set of few hundreds of Metabolites quantifiable
	  ○ High measurement accuracy
		• LC-MS:
		  ○ Several hundreds of proteins quantifiable
		  ○ Only high to medium abundant proteins
		• DNA aptamers (see **Figure 4**):
		  ○ >5000 proteins quantifiable
		  ○ High to very low abundant proteins (Davies et al. 2012; Gold et al. 2010)

[2]DNA-aptamers are oligonucleotides of ~50 base pairs length and bind to proteins like antibodies

I-4. Biological features in exercise science

I-4.1. Molecules that determine human exercise phenotype

I-4.1.1. Genes: the stable basis of exercise phenotypes

Genes determine the basic structure of human phenotypes (Wood et al. 2014), that is all observable and measurable characteristics of an individuum (e.g. body height) (**Figure 5**). 99.9% of the genes in the human genome are identical between individuals, leaving a 0.1% difference. Variations in phenotype such as body height, insulin sensitivity but also exercise capacity or the capacity to adapt to exercise depend on a combination of the variable genetic difference of 0.1% plus environmental stimuli (e.g exercise training, diet, lifestyle, or diseases) (Frazer et al. 2009). Among the 0.1% of genetic variability, the most common genetic variations are so called single nucleotide polymorphisms (SNPs) where one base pair within a gene differs between the individuals of a population. An SNP can change the amino acid sequence of a protein, which can, depending on the protein encoded, alter downstream metabolites and the phenotype or phenotypic traits (**Figure 5**) in many ways.

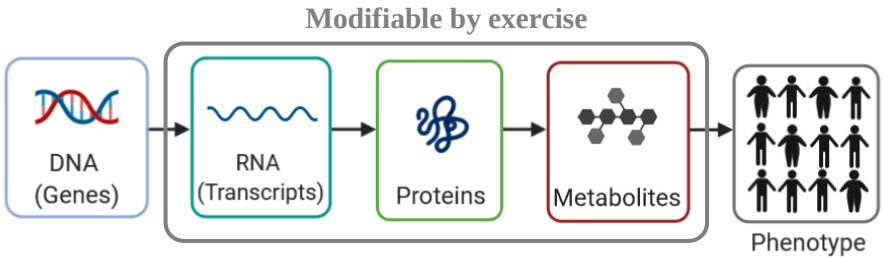


Figure 5. Molecules that determine human (exercise) phenotypes. Changes on every molecular level can cause downstream alterations and finally alterations in phenotype. Genes contain all genetic information of an individual (Lander et al. 2001). Transcripts contain the manual to build proteins (synthesis, transcription, and translation). Proteins are built from amino acid chains and catalyze metabolic reactions. Metabolites are the end products of human metabolism and are direct read outs of biochemical processes within the body (Patti et al. 2012).

An example of such a genetic variant causing a favorable exercise phenotype is that of the cross-country skier Eero Mäntyranta. Mäntyranta had an SNP on the gene that encodes the erythropoietin receptor (*EPOR*) (de la Chapelle et al. 1993), which led to an increased production of erythrocytes. Mäntyranta's blood contained ~20 g/dl of hemoglobin, whereas the normal range in men is 16-18 g/dl, allowing him to transport high amounts of oxygen to his working muscles. Through this favorable genetics and exercise training, Mäntyranta won seven medals in Olympic games and five medals in the World Championships in the 1960s.

In fact, genes can determine up to half of an exercise associated phenotype, which is relevant for performance and the adaption to exercise (Georgiades et al. 2017)

Another example of genetically influenced exercise phenotypes was shown by Bouchard et al. (1998 & 1999) (also see **I-1.3.**). In over 400 individuals (~100 two-generational families) Bouchard and colleagues showed that traits like intrinsic $\dot{V}O_2$max (Bouchard et al. 1998) or an individuals' capacity to train $\dot{V}O_2$max ($\Delta\dot{V}O_2$max or $\dot{V}O_2$max trainability) are determined by genes by ~50% (Bouchard et al. 1999). Among Bouchard's study participants, 21 SNPs in the genome were responsible for the variations in $\Delta\dot{V}O_2$max with the strongest association for a variant in the acyl-CoA synthase long-chain member 1 (*ACSL1*) gene, which accounted for ~6% of the differences in $\dot{V}O_2$max trainability (Bouchard et al. 2011).

More genes that determine endurance performance and whose manipulation has shown to increase endurance capacity by up to 1800% in mice were recently reviewed (Yaghoob Nezhad et al. 2019). Among the 31 genes identified in the review by Nezhad et al. was *PPARGC1A* which encodes the PGC1-alpha protein. PGC1-alpha stimulates mitochondrial biogenesis and can increase oxidative fiber (slow twitch type 1) content in skeletal muscle (Liang and Ward 2006), leading to the development of an endurance phenotype.

In summary, studies that systematically investigated the effect of genes on exercise phenotype found at maximum ~50% of the phenotype to be causally related to genes, so inherited. As a result, downstream molecules like transcripts, proteins, and metabolites, which partly influenced by the environment, are responsible for the other 50% of the phenotype. To describe an exercise phenotype or phenotypic trait, the stable (genes) and the modifiable (transcripts, proteins, and metabolites) parts are similarly important.

I-4.1.2. Metabolites and proteins: the modifiable part of exercise phenotypes

In contrast to genes, the concentrations of transcripts, proteins, and metabolites can change in response to environmental stimuli like exercise, diet, disease, or medication. As the experimental part of the thesis covers proteins and metabolites only, transcripts are not discussed in detail.

Proteins are encoded by genes and built from transcripts, containing information on genetics and the genetic regulation (transcripts) plus information on the environment (**Figure 5**). In exercise science, few proteins are established as biomarkers, e.g. creatine kinase (CK) as a read-out or marker for skeletal muscle damage after exercise or CK-MB, the myocard-specific form of CK, which indicates heart muscle damage (e.g. in myocarditis). Other muscle-specific proteins indicating muscle damage in disease include myoglobin (MB) itself and skeletal muscle troponin I (TNNI2) (Hathout et al. 2015). Though CK-MB, MB and TNNI2 are all clinical examples, their usage shows that proteins released from skeletal muscle, one of the most metabolically active organs during exercise, can be found in blood and that their levels are directly associated to (muscle) phenotype.

Not only can proteins inform about phenotypic traits like muscular properties but also about ongoing exercise adaptation. For example follistatin, which is secreted by the liver after exercise and inhibits myostatin (Murphy et al. 2020) which is a growth regulator of skeletal muscle mass (Schuelke et al. 2004). Through myostatin inhibition, follistatin can increase muscle mass by 10% as shown in rats (Schumann et al. 2018).

From an exercise perspective, proteins that are secreted from skeletal muscle during contraction and that have systemic effects on several other organs or on the whole organism (so called myokines) are very interesting to study. An example is interleukin-6 (IL-6) (Ostrowski et al. 1998), which is released from contracting muscles, has anti-inflammatory qualities (Starkie et al. 2003) and can help maintain glucose homeostasis during exercise (Febbraio et al. 2004).

Numerous other examples exist for proteins involved in metabolic communication during (Murphy et al. 2020; Pedersen et al. 2007; Pedersen and Febbraio 2012), in regulating the adaptation to (Egan and Zierath 2013) or in showing health-beneficial effects of exercise. Despite their importance in exercise, few studies have used proteomics to measure proteins and their changes through exercise. Due to the proven importance of some proteins in exercise contexts, it is likely that a comprehensive measurement of hundreds up to thousands of proteins by using proteomics could yield novel biomarkers for any of the metabolic processes during or after exercise.

Proteomics studies in an exercise context are limited, mostly to ones that use protein levels to predict health or performance changes after exercise. For example, a large cohort study (17,000 individuals from different sub-cohorts) by Williams et al. (2019) developed protein prediction models for different health conditions (e.g. liver fat) or future health risk (e.g. probability of a cardiovascular event within the next 5 years) with the vision for an "individual liquid health check" via blood proteins (Williams et al. 2019). Another study, relating proteins to $\dot{V}O_2$max was done by Santos-Parker et al. (2018) who compared aerobically trained with untrained participants and found that proteins related to $\dot{V}O_2$max training status are involved in metabolic pathways like apoptosis, glucose-insulin signaling, and immune response (Santos-Parker et al. 2018). One of the few studies on protein changes after acute exercise was done by Guseh et al., who showed that ~1,300 plasma proteins change their concentration in response to an acute bout of exercise (5-mile run) and that the magnitude of change depends on the intensity of the exercise (Guseh et al. 2020). Despite not many, the studies in the field of proteomics and exercise show that proteomics can yield readouts of metabolism after exercise and in the prediction of exercise adaptation.

At the end of cascade of molecules that determine exercise phenotype are metabolites. They are potential candidates for biomarkers as they change quickly in response to external stimuli like exercise (e.g. lactate in response to exercise) or fasting (e.g. blood glucose decline) and therefore directly reflect the functioning of the body.

Like the phenotype itself, metabolite concentrations are influenced by genetics (up to ~75% of the blood metabolite concentration can be inherited (Shin et al. 2014)) and the above mentioned environmental stimuli (Suhre and Gieger 2012).

During acute exercise, metabolites change quickly, and their concentration changes can be e.g. used to show fuel use during exercise (e.g. increased fat oxidation).

Metabolites that are changed after long-term exercise training could indicate exercise adaptations such as a decreased resting lactate concentration.

Cross-sectionally, metabolites could be used as biomarkers for a certain physiological phenotype (e.g. trained vs. untrained), in a similar way as they are used in the clinic for pathological phenotypes (e.g. health vs. disease).

In exercise science, single metabolites such as lactate (e.g. to monitor endurance capacity) (Brooks 2018) or glucose (e.g. to determine glucose availability during exercise) are established and applied as biomarkers. Attempts to discover novel biomarker with omics have started in 2009, when the first study used metabolomics to investigate metabolite concentration changes in response to exercise (Yan et al. 2009). Since then, several exercise omics studies found that exercise changed the concentrations of many metabolites like lipids, carbohydrates, amino acids or TCA cycle intermediates (Andersson Hall et al. 2016; Berton et al. 2017; Breit et al. 2015; Chorell et al. 2012; Coelho et al. 2016; Danaher et al. 2015; Daskalaki et al. 2015; Enea et al. 2013; Enea et al. 2010; Hall et al. 2016; Hooton et al. 2016; Howe et al. 2018; JanssenDuijghuijsen et al. 2017; Karl et al. 2017; Krug et al. 2012; Messier et al. 2017; Muhsen Ali et al. 2016; Mukherjee et al. 2014; Neal et al. 2013; Nieman et al. 2013; Peake et al. 2014; Pechlivanis et al. 2010; Pechlivanis et al. 2015; Prado et al. 2017; Ra et al. 2014; Samudrala et al. 2015; Sun et al. 2017; Valerio et al. 2017; Wang et al. 2015; Zauber et al. 2012), all summarized in Study 2 of this thesis (Schranner et al. 2020). Metabolite changes in these studies differed between exercise protocols and/or between subjects. This suggests that metabolomics is sensitive enough to distinguish exercises with different intensities, durations, or of different kind and to distinguish metabolic changes in-between subjects or subject groups.

The high sensitivity of metabolomics for comparing different exercise modes was recently also shown by a cross-over study. (Morville et al. 2020). The study found that within a group of ten healthy young males, resistance and endurance exercise provoke distinct changes in ~10% of all measured metabolites (~700). Most metabolites (~600) changed directionally similar after resistance and endurance exercise. Unsurprisingly, both exercises increased energy metabolism but to a different extent: resistance exercise led to higher increases in lactate, pyruvate, malate, and alpha-ketoglutarate, whereas endurance exercise led to higher increases in succinate.

Further, studies using metabolomics have shown that it is possible to track individual (Brennan et al. 2018; Contrepois et al. 2020) and inter-individual (Krug et al. 2012) metabolite concentration changes during exercise and in the recovery phase (which is particularly informative in terms of adaptation signaling). Contrepois et al. for example showed, that metabolite changes throughout exercise and in the post-exercise phase depend on individual capabilities of metabolism like glucose metabolism. This strongly suggests that metabolite changes during or after exercise could indicate bioenergetic capacities of an individual or between individuals (e.g. endurance athletes having high fat oxidation rates). Taken together, the susceptibility of metabolites to change quickly depending on the metabolic demands, makes it possible not only to find biomarkers for e.g. basal energy metabolism (e.g. mitochondrial ß-oxidation) or energy metabolism during exercise (e.g. glucose metabolism) but also metabolites that potentially signal the adaptation to exercise in the recovery phase (e.g. mitochondrial biogenesis or protein synthesis).

Though research on both proteomics and metabolomics in exercise has intensified in the last ten years (proteomics Pubmed articles 2010: 29 vs. 2020: 107[3] and metabolomics Pubmed articles 2010: 15 vs. 2020: 175)[4], omics-driven biomarker discovery in exercise science is still in its beginnings.

I-5. Aims of this PhD thesis

In summary, there is a need for individualized evidence-based exercise interventions for (competitive) sports and society (prevention and therapy of lifestyle related diseases). The overall aim of an individualized exercise science would be to achieve or to accelerate meaningful physiological adaptation meeting individual health or performance goals (e.g. risk factor decrease, decrease in mortality, $\dot{V}O_2max$ increase, muscle mass increase, a.s.o.). In the process of individualization, a knowledge base on the global and the specific metabolic effects of exercise is necessary, which is possible in-depth by using omics technologies.

Therefore, the first aim of this PhD thesis was to summarize the existing literature on the effects of exercise on metabolite concentrations in humans. 33 studies from 2009 to 2019 were summarized in a systematic literature review on the effects of exercise on blood, urine, saliva and sweat metabolite concentration changes in healthy human subjects(Schranner et al. 2020).

The second aim of this PhD thesis was, to investigate, how years of different exercise training in competitive athletes shape the blood metabolome at rest and after exercise. Highly trained athletes reflect the physiological extremes of human bioenergetic capacity: Years of selective training, increase concentrations of metabolic enzymes in the musculature e.g. endurance athletes have higher levels of

[3] For the search term *"proteomics AND exercise"*
[4] For the search term *„metabolomics AND exercise"*

succinate-dehydrogenase which is involved in fatty acid oxidation. Therefore, we assume that the bioenergetic capacity (e.g. high capacity to oxidize fat) of highly trained athletes is selectively shaped to their training and leads to changed blood metabolite levels. Furthermore, many metabolic enzymes are activated by exercise, which is why differences in bioenergetic capacity may only become apparent after exercise.

A third, experimental publication of this thesis covers a future field of an individualized exercise science, namely predicting individual physiological adaptation by using blood proteins via proteomics. We decided on the most well-studied trait of exercise physiology, $\dot{V}O_2$max, which is also a biomarker for endurance capacity. As shown in the introduction, $\dot{V}O_2$max and its trainability are highly relevant for future health and vary greatly in-between individuals. Genome-wide association studies have so far not yielded robust genetic biomarkers especially for $\dot{V}O_2$max trainability and there has been no study to explore whether there are blood protein biomarkers whose concentration is associated with $\dot{V}O_2$maxand its trainability.

The aims of each study were addressed in the following research questions:

Study 1: How does one bout of acute exercise globally affect blood metabolite concentrations in healthy humans?

Study 2: What is the effect of long-term specific exercise training/metabolism (highly anaerobic, aerobic, and anabolic metabolism) on blood metabolite concentrations at rest and after maximum exercise?

Study 3: Are there blood protein biomarkers of intrinsic $\dot{V}O_2$max and the trainability of $\dot{V}O_2$max and can we use blood proteins to predict $\dot{V}O_2$max trainability?

II METHODS

II-1. Systematic literature review (Study 1)

For compiling a systematic literature review on metabolite concentration changes after an acute bout of exercise in healthy humans, I followed the guidelines described in the PRISMA statement for systematic reviews and meta-analyses (Moher et al. 2009). These guidelines include the reporting of the workflow from literature identification, literature screening, verifying study eligibility criteria and finally the inclusion of studies and the checklist of items to include within a systematic review or meta-analysis.

Briefly, significant changes of metabolite concentrations in eligible studies were noted in a table and sorted according to the following metabolite subgroups and their underlying metabolism: carbohydrates and tricarboxylic acid (TCA) cycle intermediates; lipids; amino acids, their derivates and peptides; nucleotides; cofactors and vitamins, and xenometabolites (i.e., non-human metabolites). Metabolite concentration changes were further classified according to post-exercise sample timing and the biofluid in which they were measured. This quantitative analysis of the existing data in literature made it possible to compare very heterogenous studies, identify similarities within studies (e.g. increases in the concentration of most lipids) and find differences between studies in the metabolite response within 24 hours after acute exercise.

II-1.1. Search term building

The search terms were built applying a reduced form of the PICO (Population, Intervention, Comparison, Outcome) search strategy, using only Population, Intervention and Outcome, as there was no need for a control group within the screened studies. I conducted two main searches separated by 12 months (search 1: March 2018, search 2: March 2019) in different databases including PubMed, Web of Science, Scopus and MetaboLights database. According to the requirements of each database, the search term was slightly adjusted. For more comprehensive search results, the words “exercise” (for the intervention) and “metabolomics” or “metabolome” (for the outcome) were included as MeSH (Medical Subject Headings) terms. MeSH terms are words that are hierarchically mapped to a number of sub-terms and facilitate database searches as only the MeSH word needs to be used to cover all words that are mapped to it. For example, the hierarchical structure that is covered by the MeSH term “exercise” is shown below (**Figure 6**), covering all different kinds of exercises, whereas each of them in turn covers another subset of more granular definitions of e.g. “running”.

MeSH term	Subterms included, level 1	Subterms included, level 2
Exercise		
	Cool-down exercise	
	Gymnastics	
	Physical conditioning, human	
		Endurance training
		High-intensity interval training
		Plyometric exercise
		Resistance exercise
	Running	
		Jogging
		Marathon running
	Swimming	

Figure 6. Examples of the sub-structure of the MeSH term "exercise" as listed in PubMed (https://www.ncbi.nlm.nih.gov/mesh/68015444). Bold words have sub-terms included.

The initial MeSH terms were subsequently verified and expanded by PubMed Pubreminer (https://hgserver2.amc.nl/cgi-bin/miner/miner2.cgi), a webtool that enables detailed analysis of a PubMed query's results and which lists all MeSH terms and regular phrases that fit the respective query. With this, the term "sportomics" was also added to the search string, resulting in the final search term: *(("metabolomics" [MeSH Terms] OR "metabolome" [MeSH Terms]) OR sportomics [All Fields]) AND "exercise" [MeSH Terms*]

II-1.2. Identification and screening of studies

The two main searches resulted in 716 abstracts that were retrieved from the respective databases and other sources such as reference list from the publications used. After removing duplicates, applying eligibility criteria, and reading full-text, 27 articles remained for quantitative analysis.

II-1.3. Eligibility criteria

Peer-reviewed articles, written in English language were rated as eligible. The outcome of the eligible studies needed to be quantitative metabolite concentration changes (e.g. fold-change or % change) occurring within 24 hours after a bout of any exercise. Subjects that were investigated within these articles had to be older than 18 years of age, healthy and normal weight or minimally overweight with a BMI between 18 and 28 kg/m². Metabolite measurement techniques had to be one kind of a metabolomics method, where many metabolites were simultaneously quantified, such as nuclear

magnetic resonance spectroscopy (NMR) or GC-MS or LC-MS. Physical activity or exercise of any kind or duration completed in one bout /session had to be the primary intervention of the study. For metabolites to be reported in the manuscript, concentration changes within 24 hours after exercise had to be reported as significant or had to have a nominal p-value of below 0.05 in comparison to a value taken pre-exercise or at rest. From every study, the following information were extracted, if available: Author, year of publication, subjects (age, weight, BMI, bodyfat percentage, engagement in regular exercise, $\dot{V}O_2max$) specifics on the exercise intervention of the study (duration of fasting period before exercise, duration of the exercise itself, relative intensity of the exercise itself in % $\dot{V}O_2max$ or in % of heart rate maximum, allowance of drinks or food other than water during the exercise), and specifics on the samples (kind of sample like blood plasma, blood serum, urine, saliva or sweat, if blood: venous, capillary or arterial blood, timepoint of pre- and post-exercise sampling), details on metabolite quantification (instrumentation, number of quantified metabolites).

II-1.4. Quantitative data analysis

Extracted metabolite concentration changes were graphically summarized in horizontal dot plots per metabolite class and colored according to post-exercise sample timing and shaped according to the biofluid in which they were measured. The coloring according to sample timing was used as metabolite changes are highly dynamic and some metabolites/metabolite classes' changes are observable only in defined time windows post-exercise and/or differ in extent or direction depending on post-exercise timepoint. For example, this is the case for some nucleotides such as hypoxanthine and its related metabolites which are observable only between ~ 5 to 20 minutes after exercise. Furthermore, lipid changes are highest post-exercise but can still be observed 24 hours after exercise, gradually decreasing in extent (Schranner et al. 2020).

II-2. Experimental study on metabolite differences at rest and changes in response to exercise in human athlete phenotypes (Study 2)

In study two, I experimentally investigated metabolite differences in between three different human athlete phenotypes and an untrained control group at rest and after maximum exercise. Details on subjects are found under **II.2.2**.

Briefly, I designed questionnaires for training history, current training, diet, dietary supplement intake and medication which participants filled out for seven, 28 and 14 days before the study (**Figure 7, (1)**). 24 hours before the study, participants followed a standardized nutrition plan that was designed together with a nutritionist (**Figure 7, (2)**). On testing days, subjects reported to the laboratory at 7 a.m. after a 10 h overnight fast, followed by (**Figure 7,** (3)) physical examination and measuring anthropometry.

The first blood sample (**Figure 7,** (4)) was drawn ~20 minutes before the exercise (**Figure 7,** (5)). The second blood sample was drawn exactly 5 minutes after the exercise (**Figure 7,** (6)). Blood samples clotted for 30 minutes at 22° C and were then centrifuged at 15°C at 2460 g for 10 minutes after which supernatant serum (**Figure 7,** (7)) was harvested. A strength testing battery (**Figure 7,** (8)) was performed on every participant after a 90-minute break. The testing day was finished at approximately 11 am. Details of the experimental flow are found in **II-2.5 – 2.11**.

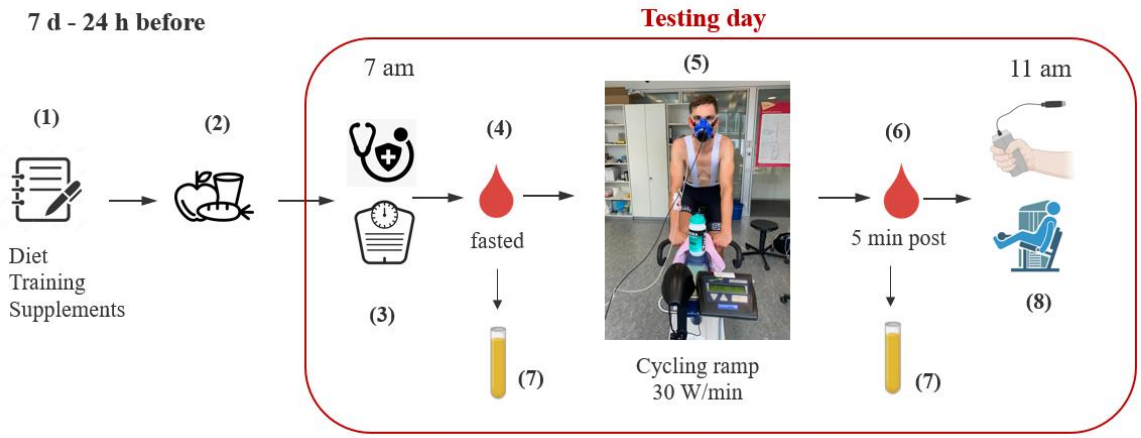


Figure 7. Experimental flow chart of the MetaExtreme study.

II-2.1. Ethical Approval for human studies

Ethical approval (reference number: 356/17S, cf. **VII-2.6.**) was obtained at the medical ethics committee of the Technical University of Munich, confirming that the study conforms to the Declaration of Helsinki for use of human subjects and tissue. Before the study, participants were fully informed about the aims, procedures, and possible risks of the study before they gave their written informed consent to participate. Participants were fully informed that their data will be made publicly available to the scientific community after anonymization.

II-2.2. Participant recruitment

For this study, 35 healthy males between the ages of 19 to 39 years were recruited. As blood metabolites are influenced by the female cycle, metabolomics measurements must be done in the first ten days of the cycle to prevent hormonal influences on the metabolome. Further, female participant recruitment would be limited to either females who do not use hormonal contraceptives at all and have regular menstrual cycles or females who use the same hormonal contraceptive for at least 3 months. Therefore, additional planning and term re-scheduling on short notice (e.g. when cycle changes occur) can be an issue when working with female participants, especially in smaller cohorts like this.

Natural bodybuilders were recruited via the ‘German Natural Bodybuilding and Fitness Federation’ (gnbf.net) according to their competitions and performances during the last two years. Eligible natural bodybuilders needed to have at least two national competitions per year during the last two years. Natural bodybuilders needed to train at least 8 hours of resistance or hypertrophy exercise per week, leading to high anabolic capacity and to high muscle mass and low bodyfat.

Endurance athletes were recruited via regional triathlon and road cycling clubs in the southern part of Germany according to their performance and partaking in at least two national or international competitions during the last two years. Triathletes were all training for long-distance competitions (3.8 km swimming / 180 km cycling / 42.1 km running), so called “ironman” triathlons with typically extensive but low intense training bouts (80-90%) and only a fragment of short and intensive training (~10-20%), leading to highly adapted fat metabolism and aerobic glycolysis, with low gains in muscle mass and minimal gains of anaerobic glycolytic capacity. Cyclists were all competing in long duration cycling events typically lasting ~3-8 hours with a similar training intensity distribution as triathletes. All endurance athletes needed to train at least 8 hours of endurance exercise per week.

Sprinters were recruited via regional track and field athletic clubs in the southern part of Germany. Track and field athletes were recruited as “sprinters” if they competitively trained for 400 m sprint or 400 m hurdles. These disciplines were selected according to their similarity in metabolic demands and typical loading times in training and competition of <60 seconds to few minutes. These short loading times with typically long breaks in between (up to ~20 minutes) lead to high muscular glycolytic capacity (anaerobic glycolysis) and to only little adaptation in aerobic glycolysis, endurance, and fat metabolism. In this way, we ensured maximum metabolic contrast between sprinters, natural bodybuilders, and endurance athletes. Sprinters needed to train at least 8 hours of sprint or speed training per week.

None of the athletes were allowed to currently take or have ever taken prohibited substances according to the National Anti-Doping Agency (WADA 2021), e.g. anabolic substances, ß2-agonists, or diuretics, unless they had a medical condition and a prescription from a physician for any of those substances.

Sedentary control subjects were recruited in different courses of the TUM and the LMU except exercise sciences. They were allowed to engage in a maximum duration of two hours of moderate exercise per week for at least 6 months before the study.

II-2.3. Exclusion / Inclusion criteria for human subjects

Exclusion criteria were cardiovascular, metabolic, respiratory, neoplastic, orthopedic, or acute illnesses that would exclude maximum exercise testing. Furthermore, subjects were excluded if they have had a long-distance flight (>3 time zones) during the last 4 weeks or were shift workers that include night shifts.

II-2.4. Pre-study questionnaires

Participants were asked to fill out the following questionnaires that I designed: history of training (**VII-2.1.**), including competitions, exclusion criteria questionnaire including questions on caffeine consumption, long-distance travels and diseases, a training diary for the last four weeks before the study (**VII-2.2.**), a diet diary of one week before the study (**VII-2.3.**), a nutritional supplement diary for two weeks (**VII -2.4.**) before the study and a medication diary for four weeks before the study (**VII -2.5.**). On the day before the study, participants needed to follow a nutrition plan, to wash out short term influences of food or drinks on the metabolome. Further, subjects needed to refrain from exercise, caffeine, or alcohol for 24 hours before the study. All subjects reported to the laboratory at 7 am on the testing day, after fasting for 10 hours. Due to expected circadian variations in some metabolites, time of day was kept the same for all subjects. Water and unsweetened tea were allowed until 30 minutes before the study. All questionnaires can be found in the **Supplementary material S2.**

II-2.5. Safety measures and anthropometry

Upon arrival at the laboratory, arterial blood pressure was measured on both sides via the cuff/ stethoscope auscultation method and a 12-channel resting ECG (custo cardio 400, custo med GmbH, Ottobrunn, Germany) was written. Resting ECGs was examined by a physician and a short anamnesis (allergies, familial diseases, history of diseases or accidents) including auscultation of heart and lung was done. All participants were cleared for exercise stress testing.

During exercise testing, participants wore a mobile 12-channel ECG (custo cardio 300, custo med GmbH, Ottobrunn, Germany) to detect any anomalies during exercise or in the immediate post-exercise phase (e.g. premature ventricular or supraventricular contractions or other arrythmias). Stress test ECGs were monitored throughout the course of the exercise and afterwards evaluated by a physician. Heart rate during exercise was collected by the ECG.

Anthropometric measures like weight, height, muscle circumferences of M. biceps brachii and M. quadriceps femoris together with subcutaneous fat measures with a caliper using the 7-point-calipermetry method by Jackson and Pollock (Jackson and Pollock 1978) were taken upon arrival.

II-2.6. Exercise testing (Spiroergometry)

Spiroergometry is the gold standard of cardiopulmonary performance diagnostics and is done to determine endurance capacity.

Endurance capacity is measured as the maximum oxygen uptake capacity ($\dot{V}O_2max$ or $\dot{V}O_2peak$) of an individual. Oxygen uptake ($\dot{V}O_2$) is a complex measure which incorporates information beyond the heart or cardiovascular system: it informs about metabolism, organ/ tissue specificities and cellular processes (**Figure 8**) of the whole organism.

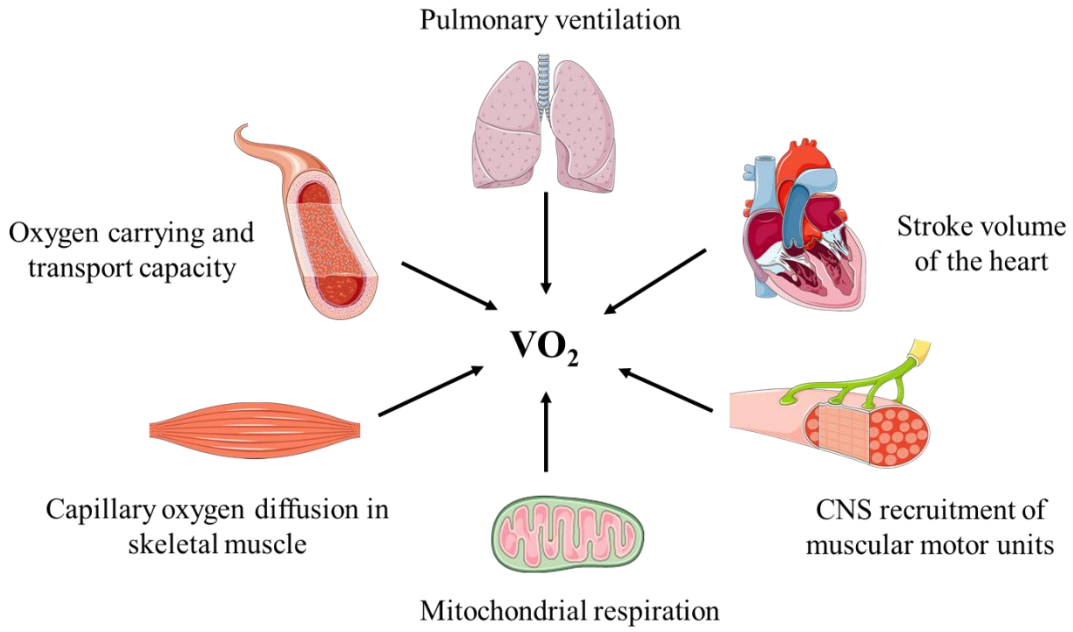


Figure 8. Metabolic, organ, tissue and cellular processes that are incorporated into oxygen uptake (V̇O2). The limits of these processes incorporated into $\dot{V}O_2$ are given by the measures of $\dot{V}O_2$max or $\dot{V}O_2$peak.

Endurance capacity ($\dot{V}O_2$max or $\dot{V}O_2$peak) can be measured by spiroergometric testing, where participants cycle on a stationary bike until exhaustion. In preparation for the ergometry of Study 2, the spirometric system (MetaLyzer 3B, CORTEX Biophysik GmbH, Leipzig, Germany) was warmed for ~30 minutes, gas calibrated with ambient air and volume calibrated using a 3-liter standardized calibration syringe.

After individual adjustments of the saddle and the handlebar reach, participants were seated on a Lode Excalibur Sport stationary bicycle ergometer (Lode B.V., Groningen, Netherlands). For the spiroergometry, an oro-nasal rubber mask (**Figure 9A**) (Hans Rudolph Inc., Shawnee, KS, USA) covering mouth and nose was fixed with a harness over subjects' neck and upper head. The mask was connected to a volume flow sensor, measuring the inhaled and exhaled volume of air in liters. An oxygen sensor was connected to the volume flow sensor (**Figure 9A**), measuring the concentration of oxygen in the inhaled and in the exhaled airstream. A 12 channel ECG to record heart rate was fixed to the upper body by disposable electrodes, 8 as chest wall leads (**Figure 9B**), 4 as limb leads. Limb leads during stress ECG were fixed above bone structures on the posterior upper chest area (acromion or spina scapulae, both sides) after palpation and on the posterior lower chest area on the spina iliaca posterior on both sides.

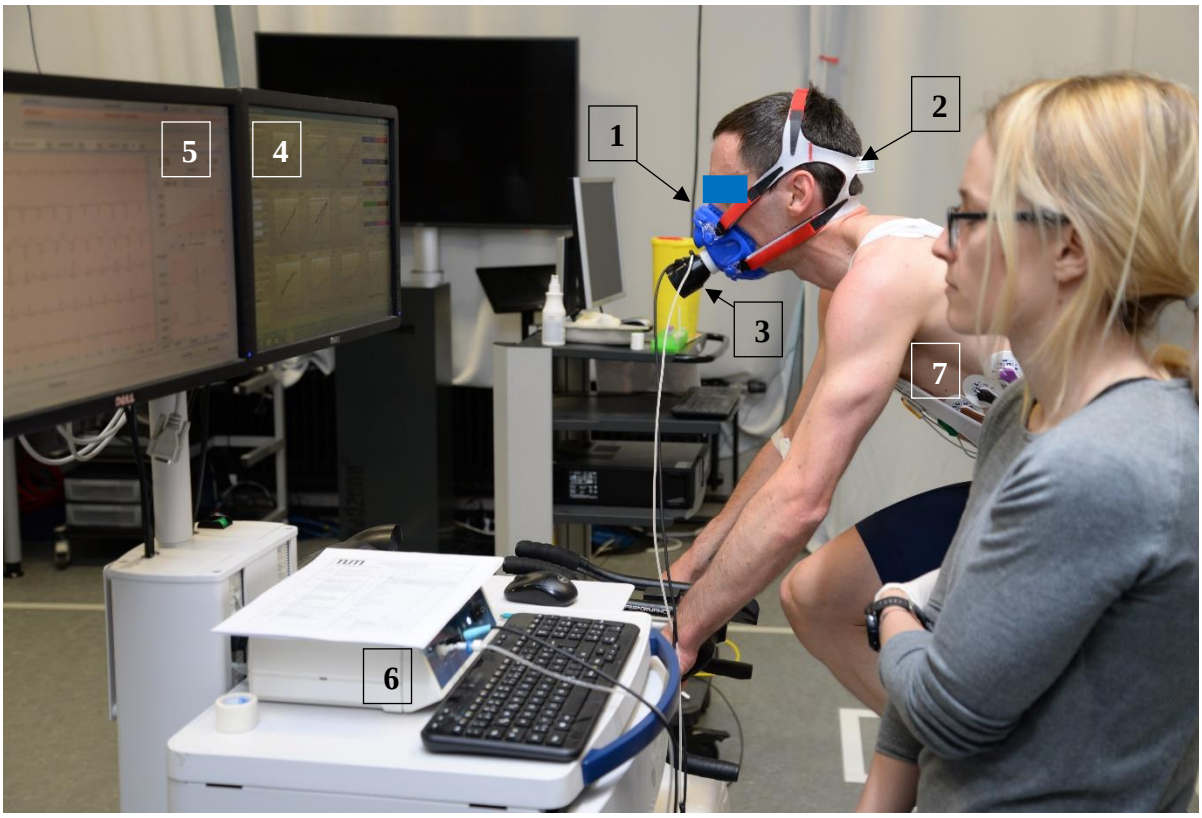


Figure 9 A. Spiroergometry setting showing: (1) Oro-nasal rubber mask, fixed with a harness (2) on the back and upper head. (3) Flow sensor measuring inspired and exspired air volumina with an attached oxygen sensor (white cable). Real-time monitoring of measured oxygen, carbondioxide and volumina (4) and real-time ECG (5). Measurement device for O_2, CO_2 and volumina (6). Disposable ECG-electrodes placed on the chest wall (7).

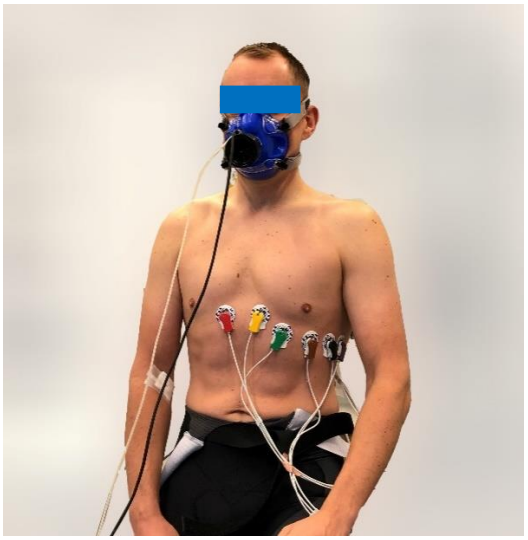


Figure 9 B. Detailed positioning of the 8 chest wall leads of the ECG during exercise.

The exercise protocol started with a 5-minute resting period, used to measure baseline gas exchange, and ensure proper operation of the spiroergometric system. After resting, subjects warmed-up for 3 minutes at 80 Watts load. Then, a so-called fast ramp protocol was automatically executed by the spiroergometry software (Meta Soft Studio, Cortex Biophysik GmbH, Leipzig, Germany), with load increasing linearly at a rate of 30 Watts per minute (**Figure 10A**). The protocol was stopped at voluntary exhaustion of the participant and tests were subsequently validated with the pre-defined objective exhaustion criteria (a ventilatory equivalent of oxygen (VE/VO2) >30.0 or a respiratory exchange ratio (RER) >1.0) (de Marées 2003). Participants were verbally encouraged throughout the final ~3 minutes of the exercise to make sure they reach maximum exhaustion. At maximum exhaustion, capillary lactate was sampled into 20µl capillaries from the earlobe at an interval of 2 minutes for 10 minutes after cessation of exercise. Immediately after exhaustion, the participant was asked to lay down and a physician or nurse drew the second blood sample at exactly 5 minutes after the end of exercise. Lactate capillaries were immersed in a buffer solution (EKF-diagnostic GmbH, Barleben, Germany) and inverted 10 times to prevent clotting. Lactate samples from every participant were measured within ~30 minutes at room temperature with an EKF BIOSEN C/S-line lactate analyzer (EKF-diagnostic GmbH, Barleben, Germany).

Results from the spiroergometry were analyzed using Meta Soft Studio (Meta Soft Studio, Cortex Biophysik GmbH, Leipzig, Germany). As the spiroergometry records gas concentrations and breathing volumes dependent on breathing frequency (so called breath-by-breath recording), obtained values were averaged with the software build-in 30-s moving average. The validity of the VT1 was verified using the ventilatory equivalent plot (**Figure 10A** or Panel 4 in **Figure 11**) and adjusted if necessary to the point where both equivalents start to approximate each other (**Figure 10B and C**). Manual validation can be necessary if e.g. participants change their breathing frequency unconsciously during exercise. Relevant parameters (RER, VT1, V̇O2max/ V̇O2peak, HRmax, and maximum Watts per kilogram bodyweight) were extracted from the 9-panel-plot (**Figure 11**) and the respective data sheets. Parameters were used for phenotypical description of participants, to validate exhaustion.

For the experimental studies of this thesis, two parameters of spiroergometry are essential: $\dot{V}O_2max$ or $\dot{V}O_2peak$ and the first ventilatory threshold (VT1). As mentioned earlier, $\dot{V}O_2max$ indicates the limits of all processes incorporated within, so maximum endurance capacity. However, if subjective voluntary exhaustion can be verified with a set of objective exhaustion criteria.

In endurance trained subjects objective and subjective exhaustion in general coincide well because they are used to exhaustive exercises. Verification of maximum exhaustion is indicated in all subjects but most important in non-exercise trained subjects. In our mixed cohort of study 2, cohort-adjusted objective exhaustion criteria of an RER >1.0 or a levelling-off of the oxygen uptake curve or reaching a ventilatory equivalent of oxygen (VE/V̇O2, **Figure 10B**) of >30.0 (de Marées 2003) were applied.

An RER >1.0 indicates primarily carbohydrate metabolism and hence metabolic exhaustion. Endurance trained subjects do not reach as high RERs than non-endurance trained subjects, as they have a higher proportion of fat metabolism during exercise (de Marées 2003).

A levelling-off of oxygen uptake (shown in **Figure 10A**) is shown by a plateau in $\dot{V}O_2$ uptake despite increasing exercise load (**Figure 10A**). Whereas this criterion is the most obvious for the investigator, it is only seen in ~ 40-50% of cardiopulmonary exercise tests.

Due to the limitations of RER and $\dot{V}O_2$ plateau as exhaustion criteria, a third objective criterion, the ventilatory equivalent of oxygen (VE/$\dot{V}$O2, **Fig. 10B**) of >30.0 was applied. The VE/$\dot{V}$O2 refers to the liters of ventilation per liter of oxygen consumed and indicates efficiency of ventilation (e.g. a VE/$\dot{V}$O2 of 25 means that 25 liters of air ventilating the lungs have to be inhaled to get one liter of oxygen). Normal values range between 23-28 at rest, with lower values referring to more efficient ventilation. During exercise, VE/$\dot{V}$O2 increases and at VT2 or later (depending on training status) exceeds 30. A VE/$\dot{V}$O2 of >30 is therefore used as the criteria for exhaustion.

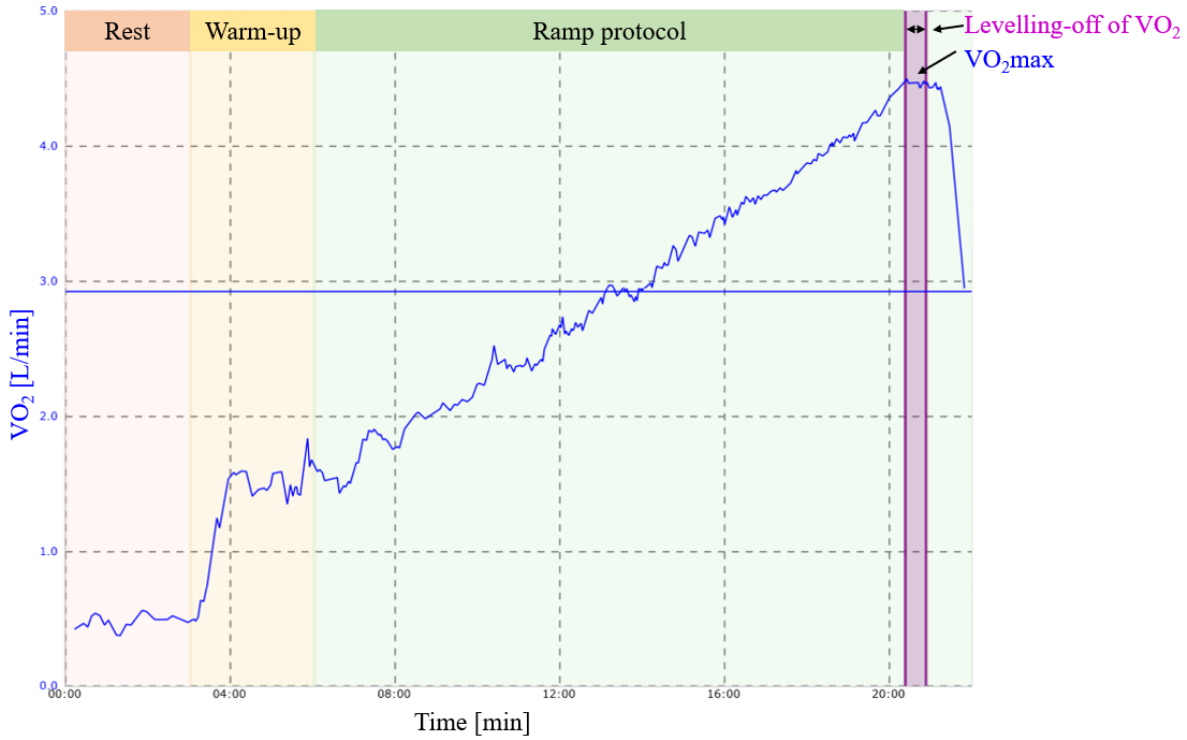
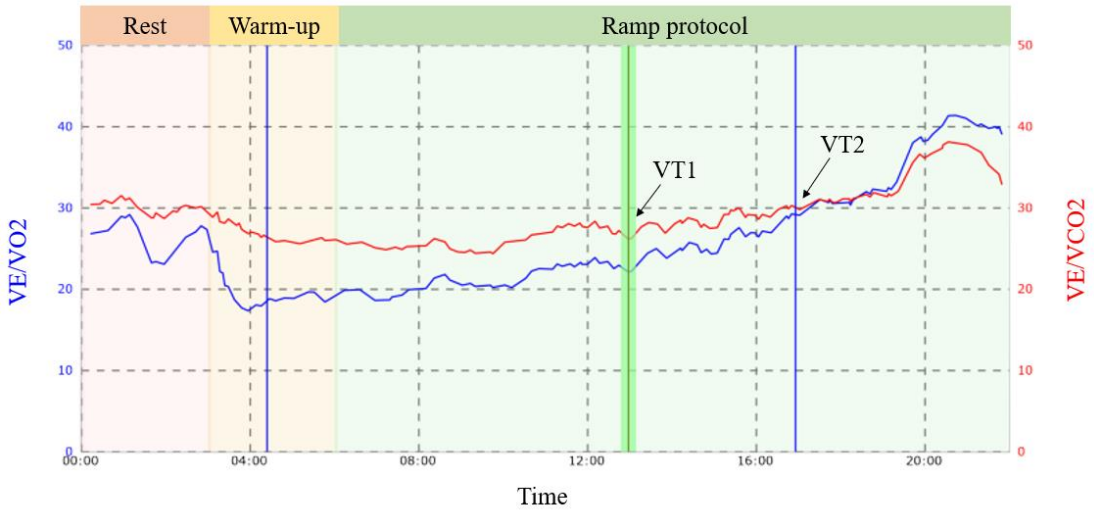


Figure 10 A. Absolute $\dot{V}$O2-uptake curve (in l/min) during rest (0 W, sitting), warmup (80 W) and the ramp protocol (increasing load of 30 W per minute). Levelling-off of $\dot{V}O_2$ in the purple area indicates $\dot{V}O_2$max. When no levelling-off is shown but other objective exhaustion criteria are met, maximum exhaustion is termed $\dot{V}O_2$peak.

B.



C.

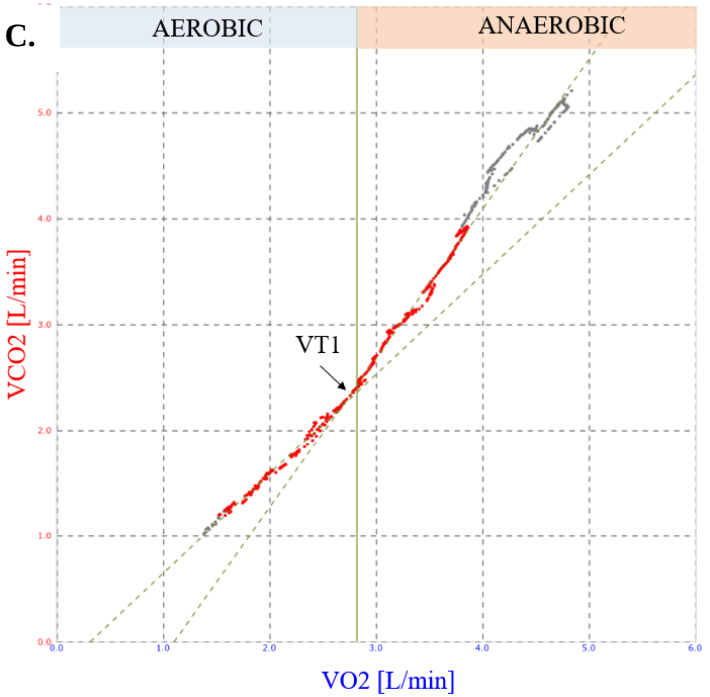


Figure 10 B. Panel 4 of the 9-panel plot (also see **Fig. 11**) showing the ventilatory equivalents of oxygen (VE/V̇O2) and carbondioxide (VE/CO2) during the ramp protocol of study 2. VE/V̇O2 of 30.0 seen slightly after VT2 in the graph was used as one of the objective exhaustion criteria. VT1 is determined via the approximation of the curves of VE/V̇O2 and of VE/VCO2 and verified using

Figure 10 C. Panel 3 of the 9-panel plot (also see **Fig. 11**) showing the slopes of O2 uptake vs CO2 production. Prior to VT1 the red slope of VCO2 vs VO2 has a smaller incline angle than after VT1. VT1 is at the point where the subsidiary lines for the slopes with differing inclination angles cross.

Next to oxygen uptake ($\dot{V}O_2$), other ventilatory gas exchanges like carbon dioxide production (VCO_2) in liters per minute as well as ventilation itself (VE) in liters per minute are measured. In addition, parameters like heart rate pulse ($\dot{V}O2/HR$) which quantifies how much oxygen is transported through the body per heartbeat or the ventilatory equivalents of oxygen ($VE/\dot{V}O2$) and of carbon dioxide (VE/VCO_2), which are calculated by the ventilation (l/min) divided by the O_2 uptake or CO_2 production, are calculated by these measures. Further the so-called respiratory exchange ratio (RER) is calculated, which is the ratio between CO_2 production and O_2 uptake. The RER is used to estimate the respiratory quotient (RQ) which indicates which fuels (carbohydrates, fats, mixed) are metabolized at each phase of the test. All the above information is monitored throughout and is used to qualitatively and quantitatively inform about endurance capacity, summarized in the 9-panel-plot or so-called Wassermann graph) (Wasserman K 2012).

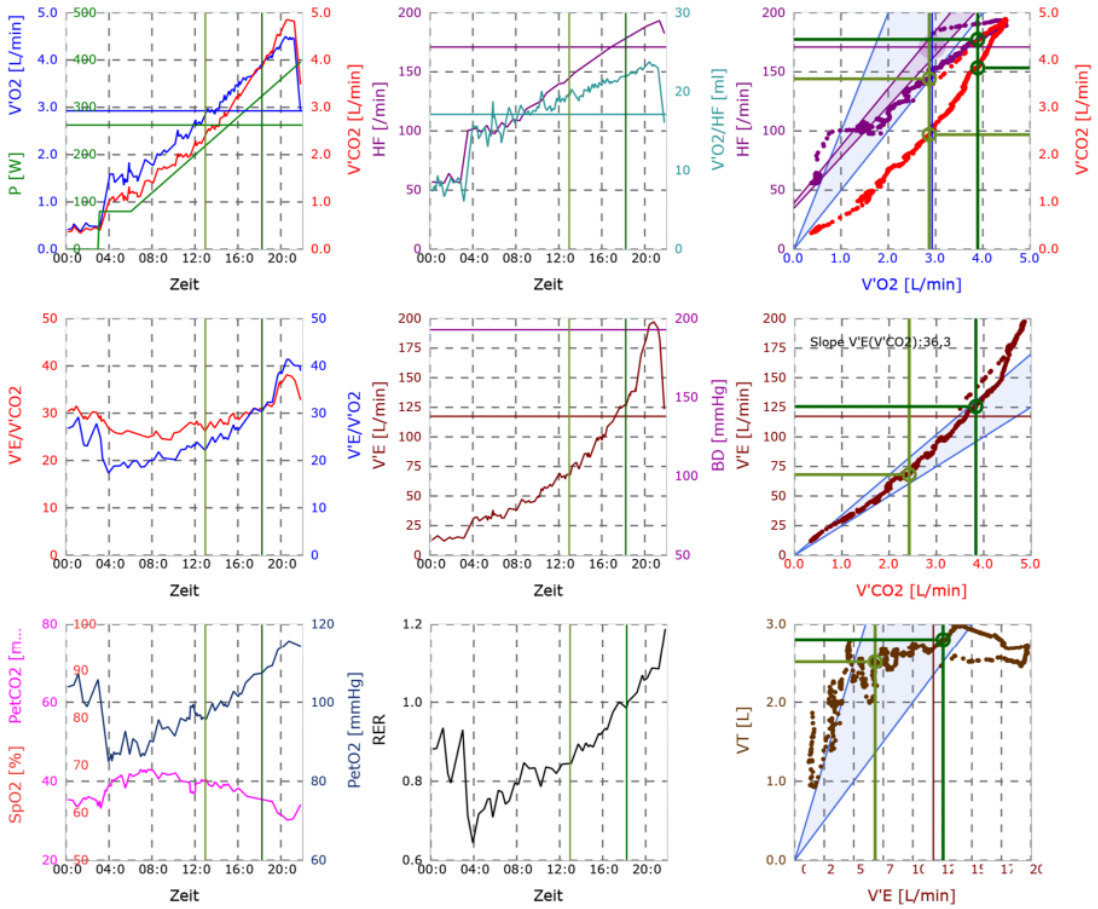


Figure 11. 9-panel-plot according to Wassermann during the maximum exercise testing of Study 2. All measures in this graph were obtained using spiroergometry and simultaneous heart rate monitoring. Most relevant panels for endurance capacity testing and analysis are in bold.

Panel 1: O_2 uptake plotted with CO_2 production during increasing load (P in Watts).

Panel 2: O_2 that is transported per heartbeat (VO_2/HF) plotted with heart rate

Panel 3: O_2 uptake plotted against CO_2 production and heart rate; slopes in this graph are used to determine key parameters of endurance capacity (ventilatory threshold 1 (VT1) and ventilatory threshold 2 (VT2); also see **Figure 10 C**)

Panel 4: Ventilatory equivalents of O_2 and CO_2 to determine VT1 (also see **Figure 10 B**)

Panel 5: Pulmonary ventilation (VE) over the time of the exercise (male endurance subjects reach up to 200 l/min, untrained male subjects reach up to 120-150 l/min; BD = blood pressure - was not measured during exercise)

Panel 6: Pulmonary ventilation plotted against CO_2 production

Panel 7: Oxygen and CO_2 partial pressures over time. SpO_2 = oxygen saturation was not measured.

Panel 8: RER over time indicating fuel use during exercise: 0.7- 0.8 primarily fat metabolism, 0.8-1.0 mixed metabolism, >1.0 primarily carbohydrate metabolism

Panel 9: Absolute pulmonary ventilation plotted against relative.

Panels 3, 6 and 9 also show VT1(light green) and VT2 (dark green).

II-2.7. Blood sampling, processing, and storage

We drew 6 x 9 ml venous blood at both time points (2 x EDTA Plasma, 4 x Serum) from an antecubital vein, totaling ~108 ml. Before every draw, a 2.7 ml discard sample was drawn to ensure no contamination of the samples by the disinfectant, skin remainders or the needle. After drawing, EDTA monovettes were gently inverted for 3 minutes and then centrifuged at 2460 g for 10 minutes at 20° C. Serum monovettes were placed on a rack in an upright position and allowed to clot for 30 minutes after which they were centrifuged at 2460 g for 10 minutes at 15°C. After centrifugation, tubes were immediately put on ice (~1-4°C) and only two thirds of the supernatant plasma or serum were collected, to minimize the risk of contamination of the probes by the buffy coat (for plasma) or the separating gel layer (for serum). Supernatants of all serum or plasma tubes were merged into 15 ml falcons, inverted twice and then aliquoted as 400 µl aliquots into 1 ml cryotubes and immediately put on dry ice. Serum tubes were discarded, and plasma tubes were filled with phosphate buffered saline (PBS) and then frozen. All materials were bought from Sarstedt AG & Co. KG (Nümbrecht, Germany).

II-2.8. Strength testing battery

After spiroergometric testing, participants had a 90-minute break. Before the strength testing battery, participants re-warmed on a stationary bicycle ergometer for 10 minutes at 100 Watts and were instructed to do several dynamic jumping and dynamic stretching exercises (~ 5 minutes) to prevent injuries in the strenuous tests that followed.

II-2.8.1. Reactive strength

Reactive strength was assessed with a drop jump from 30 cm height onto a force measurement plate (Kistler Germany). The drop jump is used in exercise science to test reactive strength of the leg musculature. The jump is executed from a defined height (e.g. 30 cm) where the hands rest in the hips for the entire jump, prohibiting active arm movement to support the jump (**Fig. 12A-1**). The jump itself is rather a fall or a passive jump, where subjects lean slowly forward on the jumping-off height, moving the body's center of gravity forward. The ground is covered with a force measurement plate (**Fig. 12A-2**) where subjects, landing on the toes only, need to jump as quick as possible from (**Fig. 12A-3**). The force plate records the ground contact time and calculates the jump height (**Fig. 12A-4**). Jump height is calculated via the gravitational force and the participants weight, recorded in a resting standing position after the jump (**Fig. 12A-5**). The reactive strength is calculated from jump height / ground contact time.

For study 2, participants were allowed 3 practice jumps from 10 cm height, 20 cm height and 30 cm height respectively. Three attempts from the measurement height of 30 cm were allowed after practicing and the jump with the best reactive strength index (RSI) was recorded.

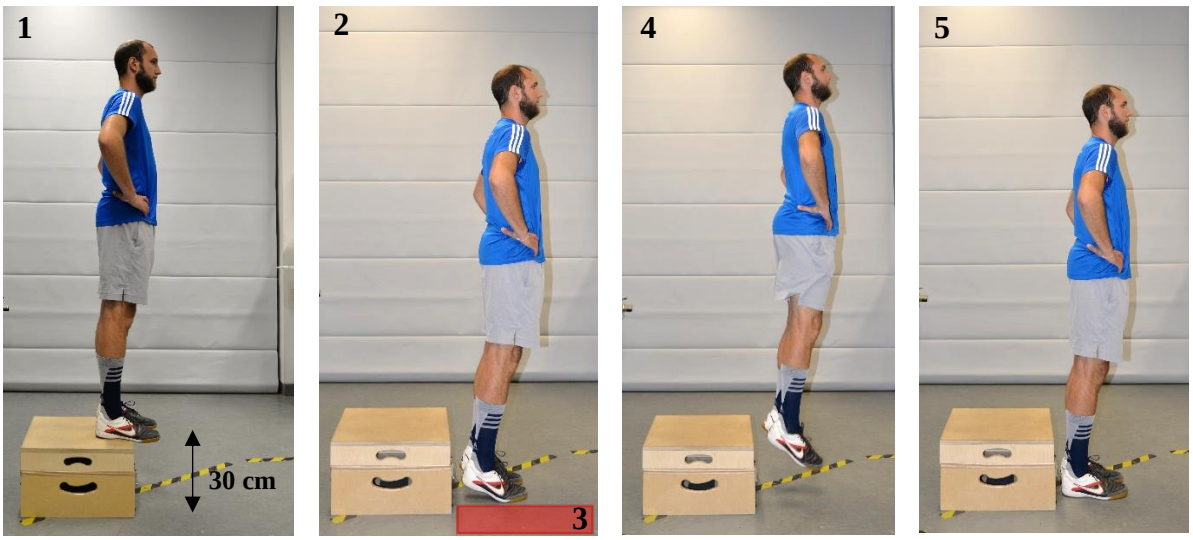


Figure 12 A. Time-course of a drop jump. Participants stand with hands resting in their hips on a 30 cm height (1), followed by a drop (2) onto a ground-integrated force measurement plate (3) and a jump (4) as high as possible. After the jump (5) weight of the participants is measured by the plate in a standing position.

II-2.8.2. Grip strength

Grip strength, of all strength measures in humans, is known to best reflect whole body strength (Trosclair et al. 2011) and is predictive of cardiovascular risk and all-cause mortality (Mearns 2015). We measured grip strength using a JAMAR dynamometer (JLW instruments, Chicago, IL, USA) in a seating position, with a knee angle of ~90° and the measurement arm hanging on one side with minimal elbow bent. Three attempts with verbal encouragement over 3 seconds of maximum voluntary grip were done on the dominant arm and the best out of three attempts was recorded. Between attempts a break of two minutes was allowed.

II-2.8.3. Isometric leg strength

Isometric leg strength was measured to quantify maximum ismetric strength of the M. quadriceps femoris. Isometric strength is characterized by the force that a muscle can develop without changing its length. Of all muscular contraction forms (concentric, eccentric, isometric), isometric strength (at a given angle of the muscle spanning joint) is the highest. For the M. quadriceps femoris, the knee angle with the highest force production is ~60° (Weineck 2009). We measured isometric strength of the quadriceps femoris on a stationary leg strength machine (Isomed2000, D. & R. Ferstl GmbH, Hemau, Germany) (**Figure 12B**). Before measurement, participants were seated and fastened on the machine and a specific warmup, including 20-30 movements over a movement spectrum of the knee joint angle of 60-90° was executed. Mechanical stops were set to prevent injury. Subjects were allowed 3 attempts of 3 seconds of voluntary maximum contraction at 60° knee angle and at 90° knee angle respectively with verbal encouragement. The highest isometric strength at both angles in Nm was recorded.

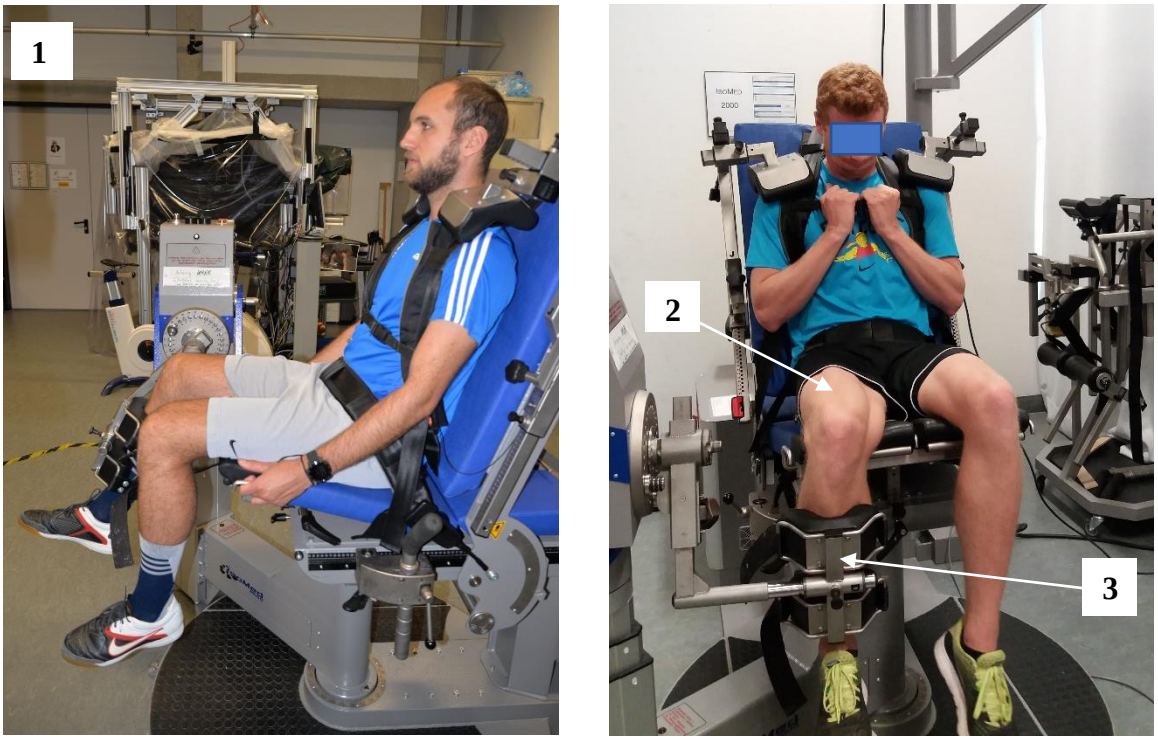


Figure 12 B. Experimental setup of the isometric leg strength measurement, side-view **(1).** Participants are fastened on the machine and asked to produce maximum force with the M. quadriceps femoris **(2)** against the lever arm **(3)**, front-view**.**

Together with the $\dot{V}O_2$max/$\dot{V}O_2$peak measures and the anthropometry recorded, strength measures were used to describe phenotypes of the athletes and to ensure maximum phenotypic contrast between the subject groups.

II.-2.9. Targeted metabolomics

Details on measurement and statistics can be found in the publication of Study 2 **(III-2.3.)**. Briefly, sera of a subset of 18 participants were analyzed with a kit-based targeted metabolomics approach (Absolute*IDQ* p180 Kit; Biocrates Life Sciences AG, Innsbruck, Austria) applying liquid chromatography (LC) and flow injection analysis-tandem mass spectrometry (FIA-MS/MS) quantifying a set of 188 metabolites: free carnitine (C0), 39 acylcarnitines, 21 amino acids, 21 biogenic amines, hexose (sum, consisting of about 90-95 % glucose), 90 glycerophospholipids (14 lysophosphatidylcholines (lysoPC) and 76 phosphatidylcholines) and 15 sphingolipids. After quality control of the raw data, 151 metabolites remained for statistical analysis.

II.-2.10. Untargeted metabolomics (unpublished data)

To cover a broad spectrum of metabolic pathways, serum samples from all 35 participants, equaling 70 samples (35 baseline and 35 post-exercise) were analyzed using untargeted metabolomics on mass-spectrometry based platforms established by Metabolon Inc., Durham, USA. In total, ~1300 metabolites

were quantified in the complete sample set, from which ~900 remained after quality control. Quality control included the following steps: batch correction on metabolites with <70% missing values over all samples, filtering out metabolites with >30% coefficient of variation (CV) in reference samples, filtering out metabolites with >30% of missing values over all samples. Finally, remaining missing values were imputed using a k-nearest-neighbor approach (k=10) (Do et al. 2018). Quality control was performed using R (version 4.1.0) and R Studio (version 1.3.1093). In a preliminary analysis of the new data using PLS-DA we were able to replicate our findings on global metabolite differences between the four subject groups (**IV-2.5)**. Further analyses are ongoing.

II-3. Experimental study on proteins associated with V̇O2max (Study 3)

II-3.1. Summary of the HERITAGE family study

For study 3, blood samples on a subset of participants (n=654) from the HERITAGE (*HEalth, RIsk factors, exercise Training And Genetics*) family study were used. Human experiments for that study have been carried out between 1995 and 1999 as a multi-center, multi-ethnic study across the USA and Canada (Bouchard et al. 1995) and have been stored at -80° since then. Details on the participants and protocol of HERITAGE can be found elsewhere (Bouchard et al. 1995) and the main publications can be found in (Bouchard et al. 1999; Bouchard et al. 1998). Briefly, subjects were males and females, healthy, between the ages of 17 and 65 years and sedentary for at least 3 months before entering the study. The 654 individuals belonged to 130 families (90 Caucasian, 40 African American) consisting of parents and at least three of their biological children.

All subjects' cardiorespiratory fitness ($\dot{V}O_2max$) was tested (similarly as described in **II-2.6.**) before and after a 20-week endurance exercise intervention. Endurance exercise was done three times per week increasing from 30 to 50 minutes per session and from 55% to 75% of their individual $\dot{V}O_2max$ (**Figure 13**), to ensure continuous physiological adaptation. Blood plasma samples used for proteomics analyses were drawn from intravenous catheters on the arm before the first and 24 hours after completing the last exercise session.

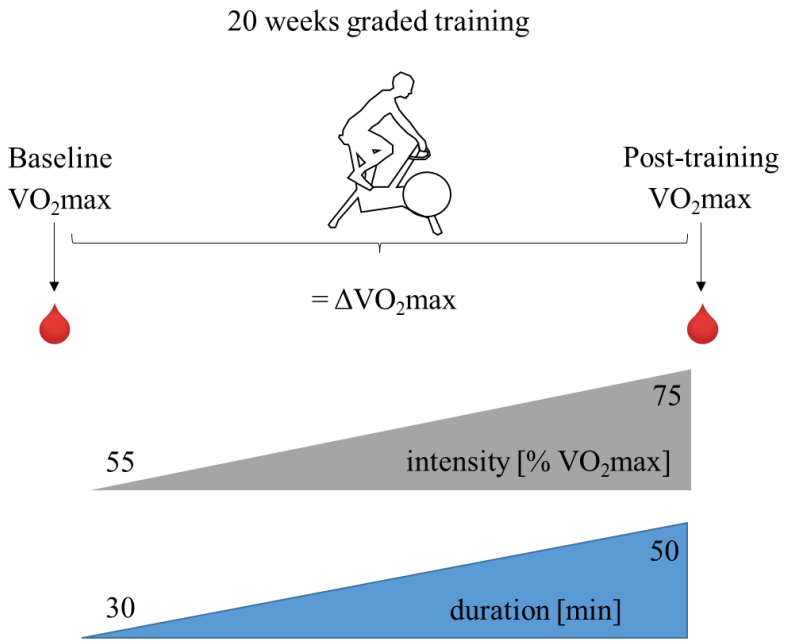


Figure 13. Study overview of the HERITAGE family study. Baseline VO2max testing was done before the first exercise session including baseline blood sampling. During the 20 weeks of exercise training, session intensities and durations gradually increased from 55% of baseline VO_2max to 75% of baseline VO_2max and from 30 to 50 minutes long. 24 hours after the last exercise session, post-training blood was sampled and post-training VO_2max was measured. The increase in VO_2max (Δ VO_2max) from baseline to post-training was calculated as post-training minus baseline.

II-3.2. Proteomics

Details on measurement with the aptamer-method can be found in the manuscript of Study 3 **(III-3.3.)**. Briefly, plasma samples were incubated with a mixture of fluorescently labeled single-stranded DNA aptamers (~5,000 SOMAmers™). Protein-aptamer complexes were isolated from unbound or nonspecifically bound proteins using a two-step, streptavidin bead-based immobilization process. Aptamers that were eluted from the target proteins were quantified using the degree of fluorescence on a DNA microarray chip. Samples were normalized to 12 hybridization control sequences within each microarray and across plates using the median signal for each dilution.

II-3.3. Statistical analysis

Details on statistics are provided in the methods part of publication 3 (Study 3). Briefly, two linear regression models were performed to determine the relationship between baseline plasma protein values and both baseline $\dot{V}O_2$max (ml $O_2 \cdot min^{-1}$) and Δ $\dot{V}O_2$max (=post-training $\dot{V}O_2$max minus baseline $\dot{V}O_2$max). Covariates in regression models included age, sex, race, and baseline values of body mass index (BMI), body fat percentage, fat free mass (kg), and $\dot{V}O_2$max (for the Δ model only). Benjamini-

Hochberg procedure was used to correct for multiple comparisons and a false-discovery rate (FDR) < 0.1 was used to determine statistical significance.

To find out if a prediction model of $\dot{V}O_2$max including clinical participant characteristics and a set of 53 proteins can predict meaningful $\dot{V}O_2$max changes (>15% increase in $\dot{V}O_2$max relative to baseline) within an individual, receiver-operating-characteristics analyses and a subsequent logistic regression were done.

Model 1: *relative $\dot{V}O_2$max change = age, sex, race, and BMI*

Model 2: *relative $\dot{V}O_2$max change = age, sex, race, BMI and >5,000 proteins*

Prediction model 1 is based solely on clinical traits, whereas model two incorporated over 5,000 proteins determined by aptamer proteomics. In several iterative steps, the initial panel of 5,000 proteins as predictors was reduced by a constraint-based features algorithm, resulting in a panel of 56 proteins. These 56 proteins were then used in a logistic regression model together with the standard clinical traits to predict relative $\dot{V}O_2$max change after training. Prediction of relative $\dot{V}O_2$max changes were based on a threshold of >15% due to the median change of the entire cohort being ~16% and because these 15% increase in $\dot{V}O_2$max have been shown to have clinical significance (e.g. risk factor reduction) in previous studies (Ross et al. 2016a).

II-3.4. Biological interpretation of the data

Proteins that were significantly associated with baseline $\dot{V}O_2$max or with $\Delta\dot{V}O2$max after 20 weeks of training were automatically annotated (pathways, diseases, and related genes) using Perseus version 1.6.6.0 (Max-Planck Institute of Biochemistry; maxquant.net/perseus/) (Tyanova et al. 2016). Subsequently, proteins associated (negatively and positively) with either baseline $\dot{V}O_2$max or $\Delta\ \dot{V}O_2$max were uploaded onto the string.db database (string-db.org), for building functional protein association networks from a list of uploaded proteins (Szklarczyk et al. 2021). In that way, proteins were clustered into the organ systems that are incorporated into $\dot{V}O_2$max (**II-2.7**), e.g. heart, muscle, mitochondria or cardiovascular system. Further, organ systems that are not actively known to be incorporated into $\dot{V}O_2$max were identified by this analysis (e.g. bone metabolism). For proteins that could not be annotated or clustered automatically, PubMed was searched for studies that related individual proteins to any of the organ systems incorporated into $\dot{V}O_2$max, to future health outcomes or endurance capacity itself.

II-4. Data availability

Data of Study 1 is available from the original manuscript at https://sportsmedicine-open.springeropen.com/articles/10.1186/s40798-020-0238-4

De-identified data of Study 2 is available at https://www.ebi.ac.uk/metabolights/MTBLS2104 (accession number MTBLS2104).

De-identified data of Study 3 is available at https://motrpac-data.org/related-studies/heritage-proteomics.

III PUBLICATION RECORD

III-1. Publication 1: Systematic literature review on metabolite changes after acute exercise

- **Title:** Metabolite Concentration Changes in Humans After a Bout of Exercise: a Systematic Review of Exercise Metabolomics Studies
- **Authors:** Daniela Schranner, Gabi Kastenmüller, Martin Schönfelder, Werner Römisch-Margl & Henning Wackerhage
- **Journal:** Sports Medicine Open. *2020;6(1):11. doi:10.1186/s40798-020-0238-4*
- **Publication date:** 10th February 2020
- **Citations:** 38, on 19th of December 2021
- **Accessions:** 9.928 (Sports Medicine Open), on 19th of December 2021
- **Citation:** Schranner D, Kastenmuller G, Schonfelder M, Romisch-Margl W, Wackerhage H. Metabolite Concentration Changes in Humans After a Bout of Exercise: a Systematic Review of Exercise Metabolomics Studies. Sports Med Open. 2020;6(1):11. doi:10.1186/s40798-020-0238-4.

III-1.1. Personal contributions

Personal contribution statements are written in the first person singular.

III-1.1.1. Defining the research question and search term building

I did an initial literature search to evaluate the need and the data for the systematic literature review. I built the search term and re-fined it.

III-1.1.2. Literature search

I performed two literature searches, defined eligibility criteria, and chose the studies that were eligible for the review.

III.1.1.3. Data analysis

I transcribed significant metabolite fold-changes from the written manuscript or from supplementary data from the respective studies into an excel sheet which was customized to summarize the number of studies reporting increases/decreases.

III.1.1.4. Data interpretation

I with the help of co-authors compared the findings and hypothesis for metabolic phenomena that are shown through metabolite changes after exercise with the literature.

III-1.1.5. Article

I drafted the article, drew figures, and wrote the first version of the manuscript, and compiled the supplementary material. Manuscript revisions and submission were done with the help of co-authors.

III-1.2. Summary Study 1

Exercise changes the concentrations of many metabolites, which are small molecules (< 1.5 kDa) metabolized by the reactions of human metabolism. In recent years, especially mass spectrometry-based metabolomics methods have allowed researchers to measure up to hundreds of metabolites in a single sample in a non-biased fashion. To summarize human exercise metabolomics studies to date, we conducted a systematic review that reports the results of experiments that found metabolite concentration changes after a bout of human endurance or resistance exercise. We carried out a systematic review following PRISMA guidelines and searched for human metabolomics studies that report metabolite concentrations before and within 24 h after endurance or resistance exercise in blood, urine, or sweat. We then displayed metabolites that significantly changed their concentration in at least two experiments. Twenty-seven studies and 57 experiments matched our search criteria and were analyzed. Within these studies, 196 metabolites changed their concentration significantly within 24 h after exercise in at least two experiments. Human biofluids contain mainly unphosphorylated metabolites, as the phosphorylation of metabolites such as ATP, glycolytic intermediates, or nucleotides, traps these metabolites within cells. Lactate, pyruvate, TCA cycle intermediates, fatty acids, acylcarnitines, and ketone bodies all typically increase after exercise, whereas bile acids decrease. In contrast, the concentrations of proteinogenic and non-proteinogenic amino acids change in different directions. Across different exercise modes and in different subjects, exercise often consistently changes the average concentrations of metabolites that belong to energy metabolism and other branches of metabolism. This dataset is a useful resource for those that wish to study human exercise metabolism.

III-1.3. Original manuscript

Sports Medicine - Open

SYSTEMATIC REVIEW Open Access

Metabolite Concentration Changes in Humans After a Bout of Exercise: a Systematic Review of Exercise Metabolomics Studies

Daniela Schranner[1], Gabi Kastenmüller[2], Martin Schönfelder[1], Werner Römisch-Margl[2] and Henning Wackerhage[1*]

Abstract

Background: Exercise changes the concentrations of many metabolites, which are small molecules (< 1.5 kDa) metabolized by the reactions of human metabolism. In recent years, especially mass spectrometry-based metabolomics methods have allowed researchers to measure up to hundreds of metabolites in a single sample in a non-biased fashion. To summarize human exercise metabolomics studies to date, we conducted a systematic review that reports the results of experiments that found metabolite concentrations changes after a bout of human endurance or resistance exercise.

Methods: We carried out a systematic review following PRISMA guidelines and searched for human metabolomics studies that report metabolite concentrations before and within 24 h after endurance or resistance exercise in blood, urine, or sweat. We then displayed metabolites that significantly changed their concentration in at least two experiments.

Results: Twenty-seven studies and 57 experiments matched our search criteria and were analyzed. Within these studies, 196 metabolites changed their concentration significantly within 24 h after exercise in at least two experiments. Human biofluids contain mainly unphosphorylated metabolites as the phosphorylation of metabolites such as ATP, glycolytic intermediates, or nucleotides traps these metabolites within cells. Lactate, pyruvate, TCA cycle intermediates, fatty acids, acylcarnitines, and ketone bodies all typically increase after exercise, whereas bile acids decrease. In contrast, the concentrations of proteinogenic and non-proteinogenic amino acids change in different directions.

Conclusion: Across different exercise modes and in different subjects, exercise often consistently changes the average concentrations of metabolites that belong to energy metabolism and other branches of metabolism. This dataset is a useful resource for those that wish to study human exercise metabolism.

Keywords: Metabolomics, Biomarker, Exercise, Physiology, Energy metabolism

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Key Points

- This study identified 196 metabolites that significantly change their concentration from pre to 24 h post endurance or resistance exercise in human blood, urine, or sweat in at least two metabolomics experiments.
- A bout of acute exercise typically increases the concentrations of lactate, pyruvate, fatty acids, acylcarnitines, ketone bodies, nucleotides; lowers the concentrations of bile acids; and has mixed effects on proteinogenic and non-proteinogenic amino acids.

Background

Living organisms are stable systems even though the molecules within organisms constantly change in a myriad of chemical reactions. Based on the Greek word "*metabole*" (English: change), "*metabolism*" is used to describe all the chemical reactions that change molecules in living organisms. Key metabolic discoveries by the early biochemists are the discovery and characterization of glycolysis by Pasteur, Embden, Meyerhof, and Parnas; the discovery of enzymes; the mapping of metabolic pathways; and discoveries linked to biochemical genetics [1, 2]. Our current knowledge of human metabolism is summarized in genome-scale metabolic reconstructions such as the Virtual Metabolic Human database, containing 17730 reactions and 5180 metabolites [3]. Metabolites are the molecules that change or react in metabolic reactions of a living being. Metabolites typically have a molecular mass of less than 1.5 kDa. In addition to our own, endogenous metabolites, metabolic databases also include exogenous metabolites that are produced by microorganisms, residing for example in our intestines, or that are derived from nutrients or drugs, which are termed xenometabolites [4].

How Does Exercise Affect Metabolism?

While each meal feeds our metabolic pathways with new metabolites, nothing quite changes the rates of metabolic reactions as much as a bout of intensive exercise [5]. On a whole-body level, oxygen uptake rises from 0.25 l/min at rest to 5 l/min during maximal exercise in a trained athlete. This is an energy turnover of ≈5 kJ/min at rest which equates to 0.3 g of glucose per minute to ≈100 kJ/min during maximal exercise which equates to 6 g of glucose per minute. The fact that there is only a total of ≈4 g of free glucose in a human being [6] demonstrates the challenge that such a rise of energy expenditure poses to the metabolism of the exercising individual.

The fold-changes of metabolic reactions in the working muscles are even greater. When changing from rest to exercise, the rate of adenosine triphosphate (ATP) hydrolysis especially by the force-generating myosin heads of a muscle fiber can increase by more than 100-fold [7]. Given that there are only ≈10 mM of ATP in a muscle fiber [8] and given that a major drop of the ATP concentration will cause *rigor mortis*, ATP-synthesizing reactions must immediately increase their rate so that ATP re-synthesis matches ATP hydrolysis within fractions of a second [9].

Exercise also affects hormone concentrations which is relevant as many hormones are technically classified as metabolites. Here, the best characterized exercise change is the increase of catecholamines [10, 11] that helps to increase heart rate and cardiac contractility as well as adjusts metabolism and blood flow. Finally, resistance exercise not only increases muscle protein synthesis for several days post exercise [12] but also elevates muscle protein breakdown [13]. While proteins are not classified as metabolites, the amino acids that constitute them are metabolites and either ingested or synthesized in metabolic reactions. Collectively, this demonstrates that the three major branches of metabolism, which are energy metabolism, anabolism, and catabolism, are profoundly changed in response to a bout of exercise.

How Are Metabolites Measured and What Is Metabolomics?

The concentrations of metabolites in biofluids such as blood, urine, and saliva have traditionally been measured one-by-one with enzyme assays followed by fluorometric or spectrophotometric detection [14]. This, however, has changed with the advent of metabolomics methods. Metabolomics describes methods that allow the high throughput quantification of hundreds of metabolites in a single sample. This is mostly achieved through the separation of metabolites via liquid or gas chromatography followed by the detection of individual metabolites through their specific mass-to-charge ratio (m/z) and their induced breakdown (fragmentation) in a mass spectrometer. The retention time from the chromatographic separation, the mass-to-charge ratio, and the fragmentation pattern are characteristic features for each ionized metabolite. This information can therefore be used to identify the detected metabolites through matching against databases of known metabolites [4]. While nuclear magnetic resonance spectroscopy-based metabolomics methods are also available and have specific advantages [15], mass spectrometry-based metabolomics methods dominate. Further variations of metabolomics are untargeted or global metabolomics which measures all detectable metabolites in a sample versus targeted metabolomics where a specific subset of metabolites is measured [4]. Collectively, the improvements in

metabolomics methods allow researchers to detect more and more metabolites in human body fluids [16].

While exercise physiologists have traditionally focused on measuring individual metabolites such as lactate [17], they have since 2009 used metabolomics methods to obtain a global view of how exercise changes metabolite concentrations [18]. The plethora of different metabolites and methodologies used in these studies makes it difficult to obtain a comprehensive overview over how a bout of exercise changes human metabolite concentrations in different body fluids and organs.

The aim of this project was therefore to conduct a systematic literature analysis to review all published studies where researchers used mass spectrometry or nuclear magnetic spectroscopy-based metabolomics to study the effect of exercise on metabolite concentrations. Specifically, we report and discuss metabolites that significantly change their concentration in mass spectrometry or nuclear magnetic resonance-based human metabolomics studies after a single bout of exercise. In our analysis, we found 196 metabolites that significantly change their concentration within 24 h after a bout of exercise in at least two studies within human blood or other body fluids.

Methods

Search Strategy

To identify publications that use a metabolomics approach to measure metabolite changes after a bout of exercise, we carried out a systematic review following the PRISMA guidelines [19]. We searched four different literature databases using the PICO (Population, Intervention, Comparison, Outcome) strategy [20]. This search strategy combines the parameters of the research question into one search string to find relevant studies (Fig. 1).

For the literature search, we only used the parameters "*intervention*" and "*comparison*" of the PICO search strategy in the search terms because the eligible studies did not need to have a control group. To get a first overview, we searched PubMed using the search term ("*metabolomics*" AND "*exercise*") and repeated this search in PubReMiner to extract additional, relevant search terms. We ended up with the search terms (("*metabolomics*" [*MeSH Terms*] *OR* "*metabolome*" [*MeSH Terms*]) *OR sportomics* [*All Fields*]) *AND* "*exercise*" [*MeSH Terms*]. With these search terms, we conducted two main searches on the third of August 2017 and on the first of June 2018 and searched PubMed (101

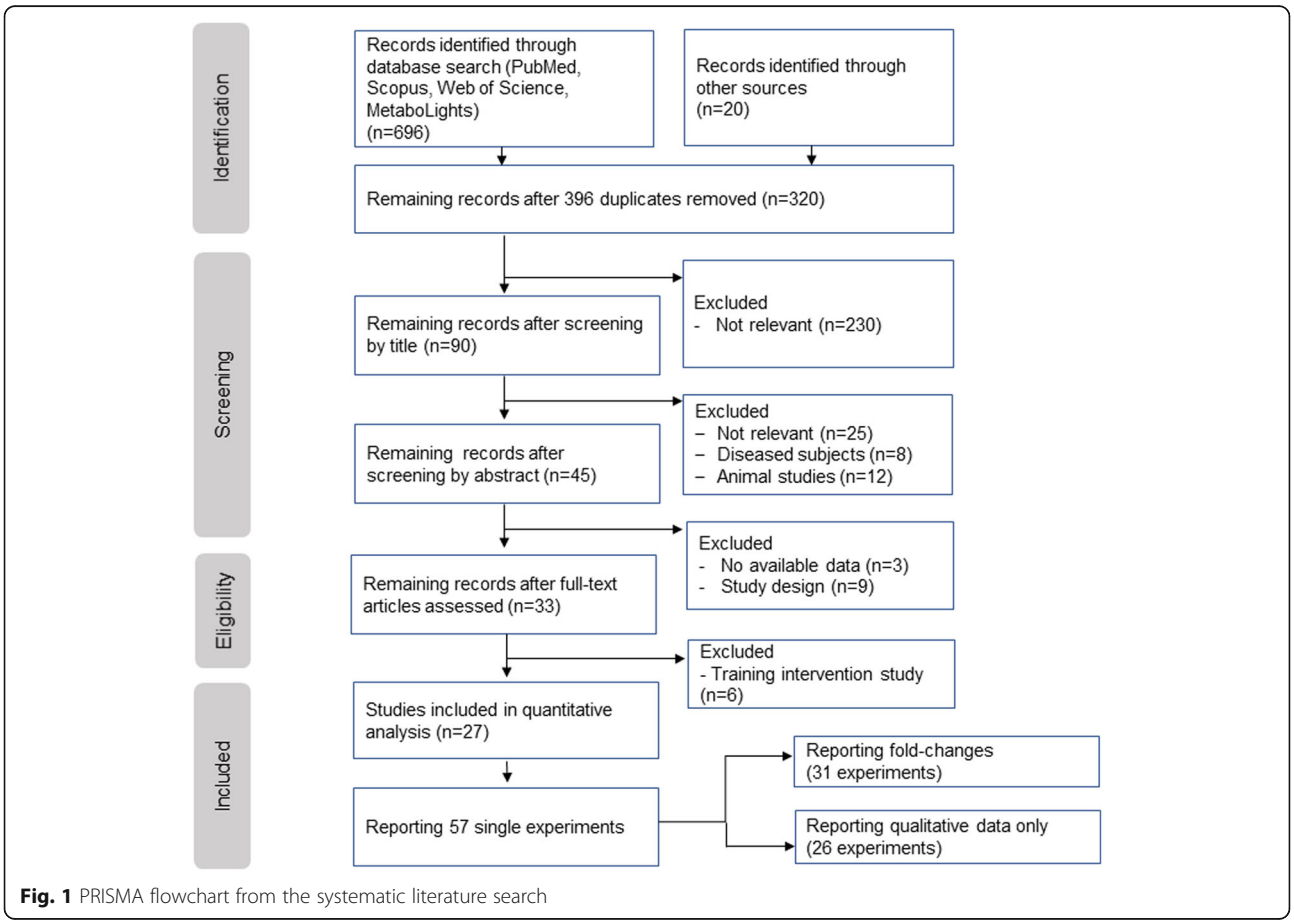

Fig. 1 PRISMA flowchart from the systematic literature search

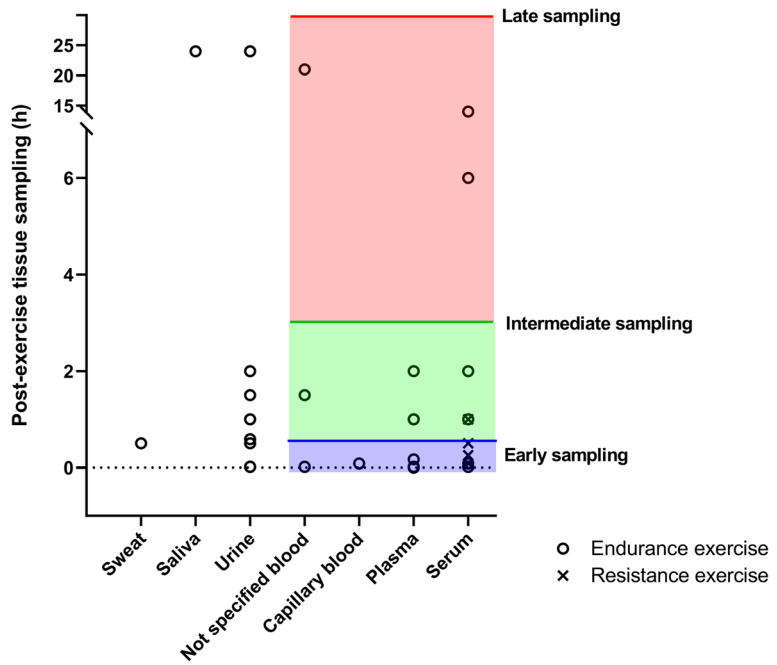

Fig. 2 Distribution of post-exercise sample type and timing across all 57 experiments. Note that only experiments with blood samples are color-coded and categorized into three timing categories. Blue: early samples; green: intermediate samples; red: late samples

abstracts), Web of Science (194 abstracts), Scopus (313 abstracts), and MetaboLights database (88 abstracts). The earliest study matching our search criteria was published in 2010. We included studies from that date onwards.

From the abstracts retrieved, we included articles from peer-reviewed journals, written in English that investigated metabolic changes in humans in response to a bout of exercise. Our eligibility criteria were as follows:

- Adult participants (> 18 years of age) without metabolic disease, malfunction or genetic disorder of metabolism (e.g., diabetes mellitus) and normal weight (BMI > 18 and < 28 kg/m^2)
- Metabolomics analysis technique based on either mass spectrometry or ^{1}H-nuclear magnetic resonance (^{1}H-NMR) applied to analyze metabolic changes
- Physical exercise of any kind had to be the primary intervention of the study
- Studies or experiments investigating changes of metabolite concentrations within 24 h after a bout of exercise
- Changes of metabolites had to be significant (raw p values of $p < 0.05$) and reported with respect to a resting value before exercise in fold-change, % change or as "decrease" or "increase." In cases with no fold-change values given, we contacted the corresponding author of the publication to request quantitative data
- Sampling specimen included in the review are serum, plasma, capillary blood, urine, saliva, and sweat

Studies with one or more of the following criteria were excluded:

- Article type: conference proceedings, reviews, comments or letters to editor
- Subjects: animal studies, studies on chronically or acutely ill subjects; studies on overweight, obese, individuals or individuals with eating disorders; studies on children
- Methods: studies where exercise was not the primary intervention and studies that used other methods than mass spectrometry or nuclear magnetic resonance metabolomics methods to measure metabolites (e.g., studies that used biochemical analyzer kits were excluded)
- Outcomes: metabolites within a study that showed no significant change after an exercise intervention
- Studies reporting changes of metabolite concentrations later than 24 h after a bout of exercise or after an exercise training intervention

From every study, the following information was extracted if available: Author, year of publication, subjects (numbers, age, sex, BMI or body fat percentage, training status or cardiorespiratory fitness (VO_{2max}), training load

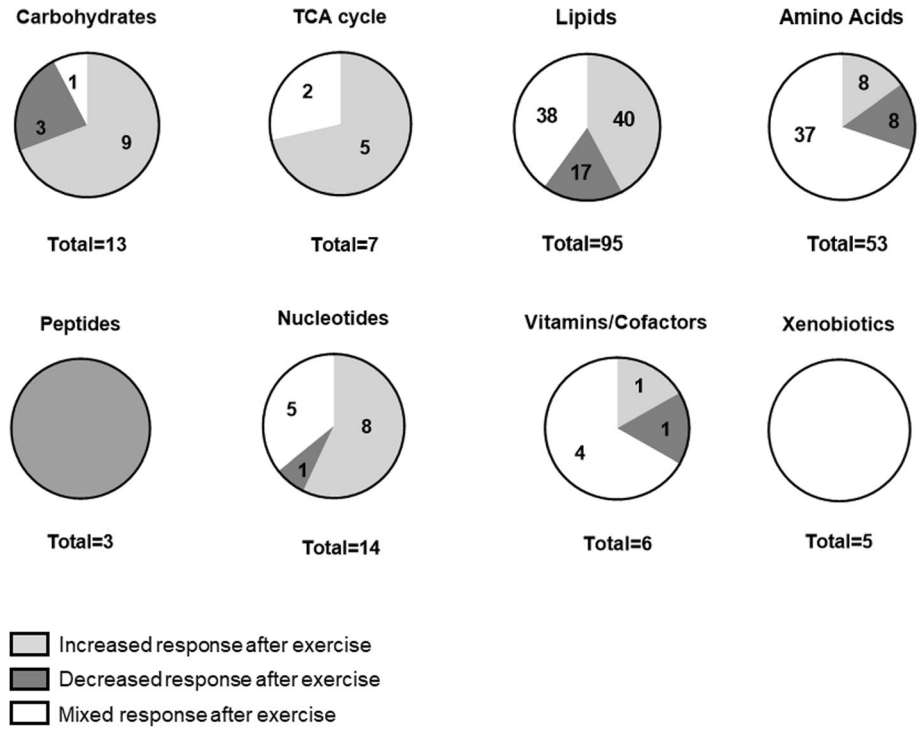

Fig. 3 Responses of each metabolite class within 24 h after a bout of exercise colored by direction of effects

per week, intervention of the study (intervention sessions, intensity (%VO_{2max}), duration, kind of exercise, nutrition during intervention), samples for metabolomics analysis (number of samples, tissue sampled, nutritional protocol before sampling, interval between exercise and post-exercise sample, fasting/no fasting before sampling, outcomes (number of metabolites detected, quantitative change of significantly altered metabolites, analyses method, database comparison)), and remarks.

Data Analysis

Significant changes of metabolite concentrations were noted in a table and sorted according to the following metabolite subgroups and their underlying metabolism: carbohydrates and tricarboxylic acid (TCA) cycle intermediates; lipids; amino acids, their derivates and peptides; nucleotides; cofactors/vitamins, and xenometabolites (i.e., non-human metabolites such as drugs or food dyes).

Results

After removing duplicates and applying exclusion criteria, we read 45 articles full-text of which 33 matched our eligibility criteria. Within these 33 publications, we further excluded six studies that were either exercise training studies or reported data that were not measured within the 24 h after a single bout of exercise. Within the remaining 27 publications [21–49], 57 single experiments were reported. Because only six out of these 57 experiments used resistance exercise as an intervention and 51 used endurance exercise, data are presented together but labeled separately.

Out of the 57 experiments, 26 experiments reported significant ($p < 0.05$) increase or decrease of metabolite concentrations qualitatively within 24 h after a bout of exercise without providing the corresponding fold changes. Thirty-one experiments were quantitative, reporting fold-change values for significantly ($p < 0.05$) changed metabolites in blood, urine, saliva, and sweat. The Additional file 1: Tables S1-5 summarize the results from all 57 experiments.

Subjects

Of 57 experiments, 45 investigated only male subjects (n = 307), ten investigated female and male subjects (n = 211) and two investigated only female subjects (n = 22) which is a ≈10-to-1 male bias that should be rectified in the future. In 23 of the 57 experiments, well-trained athletes were used as subjects. The remaining 24 experiments investigated heterogeneous groups ranging from sedentary to recreationally active subjects. Further details are in Additional file 2: Table S6.

Exercise Interventions

Fifty-one out of 57 experiments chose endurance exercise such as cycling or running as an exercise intervention in a controlled laboratory or outdoor setting.

Exercise duration ranged from 30 min to 96 h. Exercise intensities ranged from moderate (< 60% of VO_{2max}) to supramaximal (> 110–300% of the workload achieved at VO_{2max}) intensity. In 17 experiments, participants exercised at an intensity around the individual anaerobic threshold (corresponding to ≈60–80% VO_{2max}). In a further 13 experiments, participants did self-paced exercise, without a measurement of VO_2-uptake. Six experiments chose resistance exercise such as leg press as an exercise intervention on male subjects only. For details, see Additional file 2: Table S6.

Sample Type and Timing

Forty of 57 experiments used human blood (plasma, serum, capillary, or non-specified blood), thirteen used urine, three saliva, and one experiment sweat to determine metabolite concentration changes after exercise (Fig. 2).

With respect to timing, each included study compared one or more post-exercise samples with a baseline or pre-exercise sample. The earliest post-exercise samples across all tissue types were drawn immediately after exercise, the latest 24 h after exercise. Due to this heterogeneity in sample timing, we categorized all experiments with human blood samples into three categories: early (0–0.5 h after exercise), intermediate (> 0.5–3 h after exercise), and late (> 3–24 h after exercise) (Fig. 2).

Metabolites With Significant Concentration Changes After a Bout of Exercise

In total, 196 metabolites changed significantly in at least two out of the 57 experiments. We used this requirement to limit the number of metabolites that we report to a manageable level and to increase reliability. The 196 exercise-responsive metabolites belong to different metabolite classes. They include 13 carbohydrates, 95 lipids, seven tricarboxylic acid (TCA or Krebs) cycle metabolites, 53 amino acids and their derivatives, three peptides, 14 nucleotides, six vitamins and cofactors, and five xenobiotics. Out of 196, 106 metabolites changed in the same direction after exercise in all experiments: 71 metabolites were solely reported to increase, 35 solely to decrease. Ninety metabolites of 196 showed mixed responses between experiments (Fig. 3). Among these 90 mixed cases, 38 metabolites were determined in the same biofluid such as blood plasma. Twenty-two of the 90 mixed responses were sampled in different blood samples like serum, plasma, or capillary blood. Within the metabolite subgroups, amino acids were those that accounted for

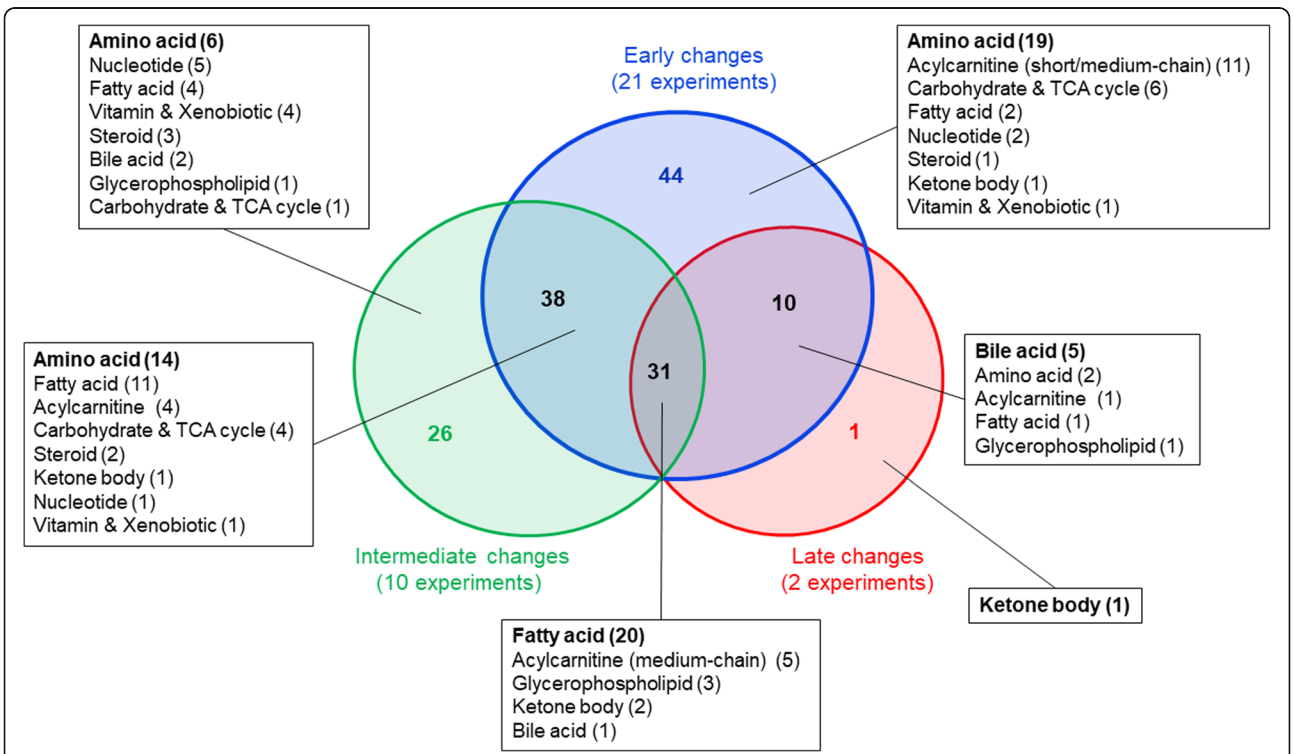

Fig. 4 Venn diagram showing the number of metabolites that are changed in relation to time point of sampling. Early changes: within 0.5 h after exercise; Intermediate changes: between > 0.5 and 3 h after exercise; Late changes: between > 3 and 24 h after exercise. The respective metabolites in each sample category (early to late) and the overlapping metabolites are shown in Additional file 3: Table S7. No metabolites were changed equally in intermediate and late only. For example, we found 31 metabolites that are changed at all sampling time points following exercise consisting of acylcarnitines and free fatty acids. Note that only blood metabolite changes of 33 experiments are shown in this diagram.

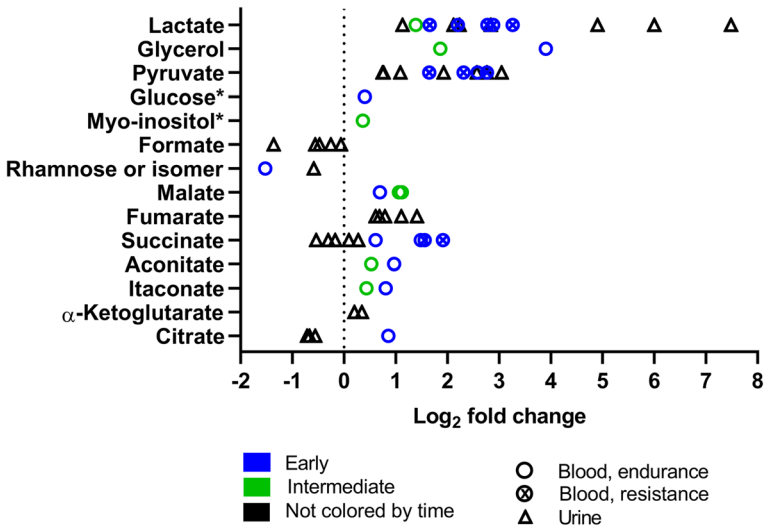

Fig. 5 Carbohydrate and TCA cycle intermediate changes in response to exercise (log_2 fold change versus rest). The graph shows seven metabolites of carbohydrate metabolism and seven TCA cycle intermediates reported with significant fold-changes in 20 (15 endurance, 5 resistance) and 11 (nine endurance, two resistance) experiments, respectively. One symbol represents one experiment. Rest = 0 (dotted vertical line). * fold-change values were only reported in one experiment. For detailed quantitative and qualitative changes of all carbohydrate metabolites, see Additional file 1: Table S1

most of the inconsistent findings. Within 37 mixed cases of amino acid responses after exercise, 19 were measured in the identical biofluid.

To identify if metabolite or metabolite class changes vary with post-exercise sample timing, we compared the results for all metabolites detected in blood samples (33 experiments) across the three timing categories (Fig. 4). Though not all metabolites were detected in each experiment and only two experiments were categorized as "late sampling," we found 31 metabolites that were changed at all sampling time points. The majority of these metabolites were lipids, with 20 fatty acids (mostly long-chain; three dicarboxylic; three odd-chain) and five acylcarnitines (mostly medium-chain) being significantly changed after a bout of exercise.

Thirty-eight metabolites—mostly amino acids and fatty acids—were affected in cases of early and intermediate sampling but not late sampling experiments. Ten metabolites among them mostly bile acids were affected in late and early sampling experiments. Early only changes are dominated by 19 amino acids, followed by 11 short- and medium-chain acylcarnitines, six carbohydrates, and TCA cycle intermediates, whereas intermediate changes show a variety of different metabolite groups (e.g., amino acids, nucleotides, vitamins/cofactors, and xenobiotics).

Comparison of Metabolite Fold Changes After a Bout of Exercise

To analyze the quantitative range of metabolite effects, we summarized the fold changes of all 31 experiments reporting this information for each metabolite class (Figs. 5, 6, 7, 8, 9, 10, 11, and 12). For simplification, we pooled the results from serum, plasma, capillary blood, and non-specified blood in our overview. The rationale for this is that metabolite concentrations in human serum and plasma correlate (r = 0.81), with concentrations being generally higher in serum [50].

Carbohydrate Metabolism and TCA Cycle

Figure 5 shows carbohydrates and TCA cycle intermediates. Carbohydrates are metabolized to synthesize ATP via glycolytic lactate formation or via their oxidative phosphorylation. In the context of exercise, lactate is the most measured metabolite as its concentration at a given exercise intensity is a measure for endurance capacity [51]. The concentrations of lactate and pyruvate increase in various body fluids, as expected, whereas formate, a by-product of ketone body synthesis, and the sugar rhamnose (hexose) decrease.

In several reactions, the TCA cycle uses acetyl-CoA derived from carbohydrates, fats, or amino acids especially for nicotinamide adenine nucleotide (NADH) and subsequent ATP synthesis. After a bout of exercise, TCA cycle intermediates mainly increase in blood and urine. In blood, TCA intermediates are upregulated, especially in the early phase (until 30 min after exercise) by both endurance and resistance exercise.

Lipids and Lipid-Derived Compounds

Lipids are hydrophobic molecules including fuels for energy metabolism such as triacylglycerols, signaling molecules such as steroids or phosphatidic acid, and

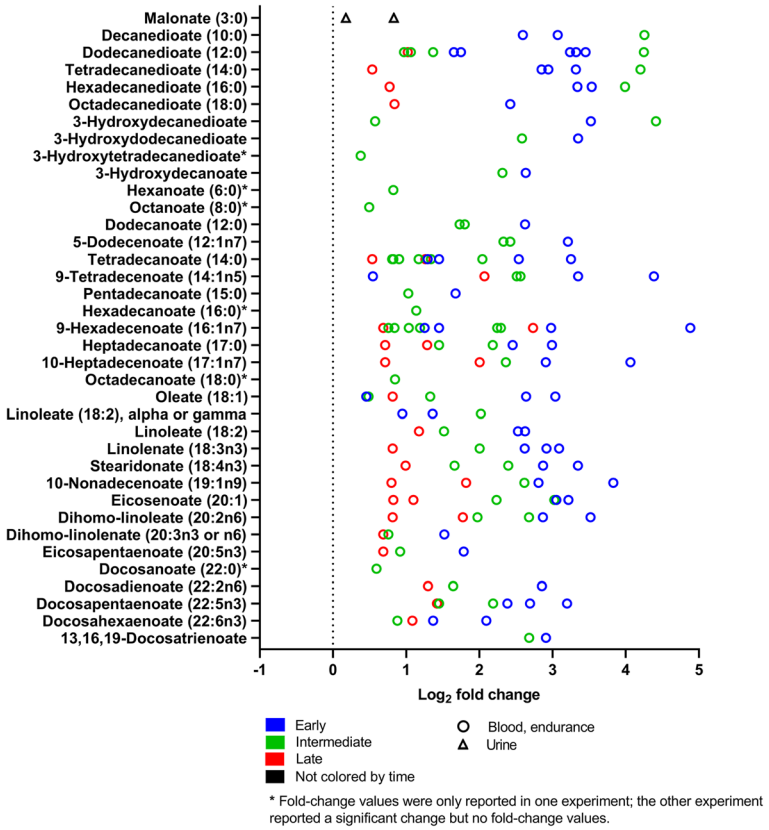

Fig. 6 Free fatty acid changes in response to exercise (log_2 fold change versus rest). The graph shows 37 fatty acids reported with significant fold-changes after exercise in 16 experiments (all endurance). One symbol represents one experiment. Rest = 0 (dotted vertical line). * fold-change values were only reported in one experiment. For detailed quantitative and qualitative changes of all fatty acids, see Additional file 1: Table S2

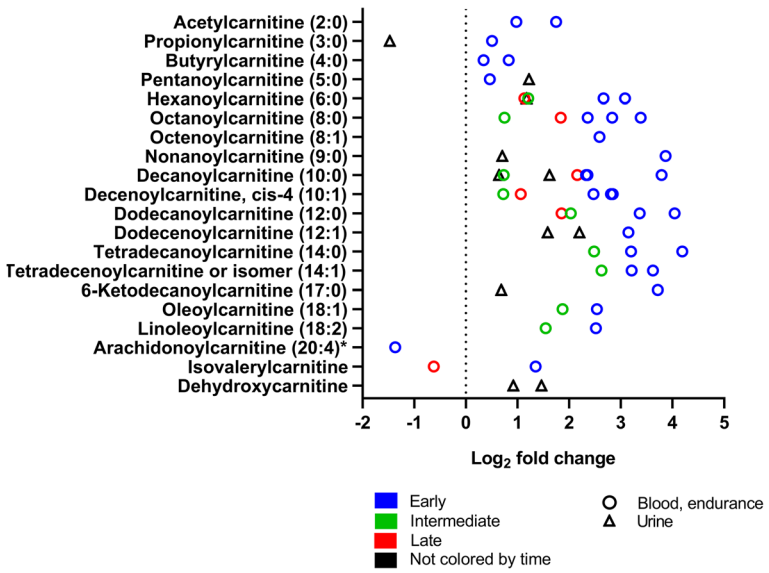

Fig. 7 Acylcarnitine changes in response to exercise (log_2 fold change versus rest). The graph shows twenty acylcarnitines reported with fold-changes after exercise in 10 experiments (all endurance). One symbol represents one experiment. Rest = 0 (dotted vertical line). * fold-change values were only reported in one experiment. For detailed quantitative and qualitative changes of all acylcarnitines, see Additional file 1: Table S2

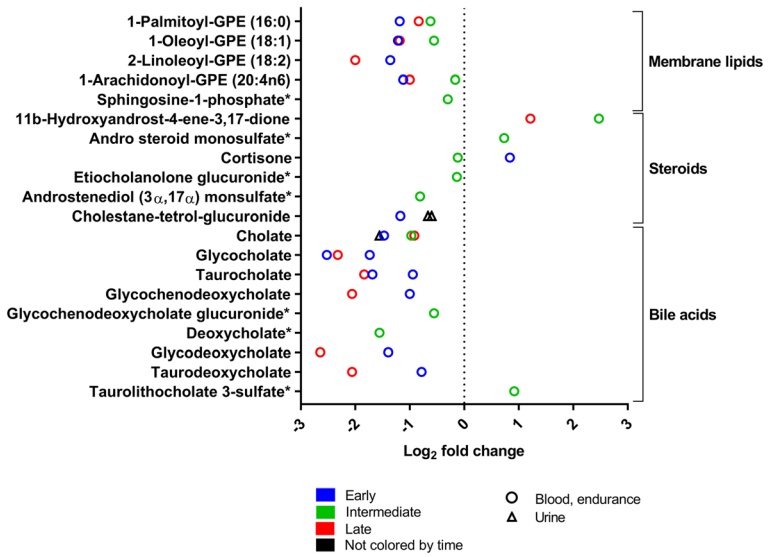

Fig. 8 Membrane lipids, steroids and bile acid concentration changes in response to exercise (log_2 fold change versus rest). The graph shows five membrane lipids (changed in three experiments), six steroids (changed in six experiments), and nine primary bile acids (changed in five experiments) significantly changed after exercise. Rest = 0 (dotted vertical line). One symbol represents one experiment. * fold-change values were only reported in one experiment; the other experiment(s) reported a significant change but no fold-change values. For detailed quantitative and qualitative changes of all ketone bodies, see Additional file 1: Table S2

structural components of cell membranes including phospholipids and sphingolipids. Here, we summarize the exercise-induced concentration changes of different subgroups of lipid metabolism or their derived compounds after exercise: free fatty acids, acyl-carnitines, ketone bodies, bile acids, steroids, sterols, sphingolipids, and glycerophospholipids.

Figure 6 shows the concentration changes of fatty acids. Fatty acids are carboxylic acids with an aliphatic chain and can be categorized according to their length and structure into short-, medium-, and long-chain, saturated and unsaturated fatty acids. Next to glucose, fatty acids are the major muscular energy fuel during exercise [52]. After a bout of exercise, the concentrations of

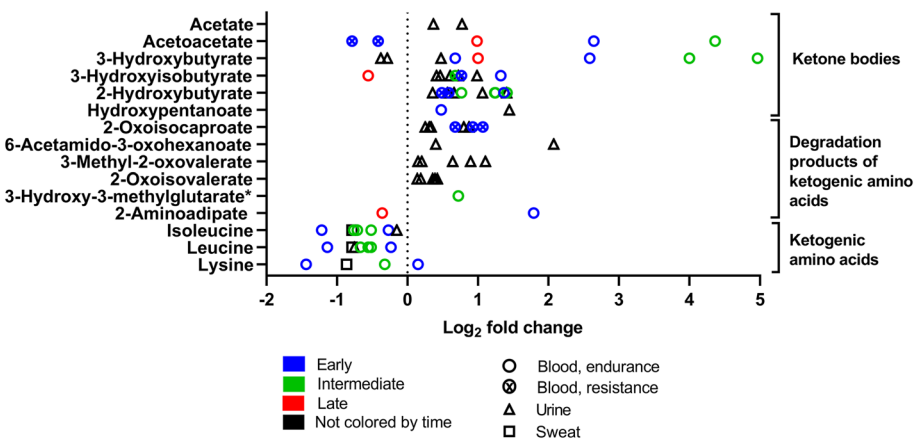

Fig. 9 Ketone bodies, ketogenic amino acids, and their degradation products changes in response to exercise (log_2 fold change versus rest). The graph shows 12 ketone bodies and ketogenic precursors of amino acid degradation and three ketogenic amino acids that changed significantly in 31 experiments (26 endurance, five resistance). Ketogenic amino acids are displayed for the overview but are also as part of Fig. 10. Rest = 0 (dotted vertical line). One symbol represents one experiment. * fold-change values were only reported in one experiment; the other experiment(s) reported a significant change but no fold-change values. For detailed quantitative and qualitative changes of all ketone bodies, see Additional file 1: Table S2

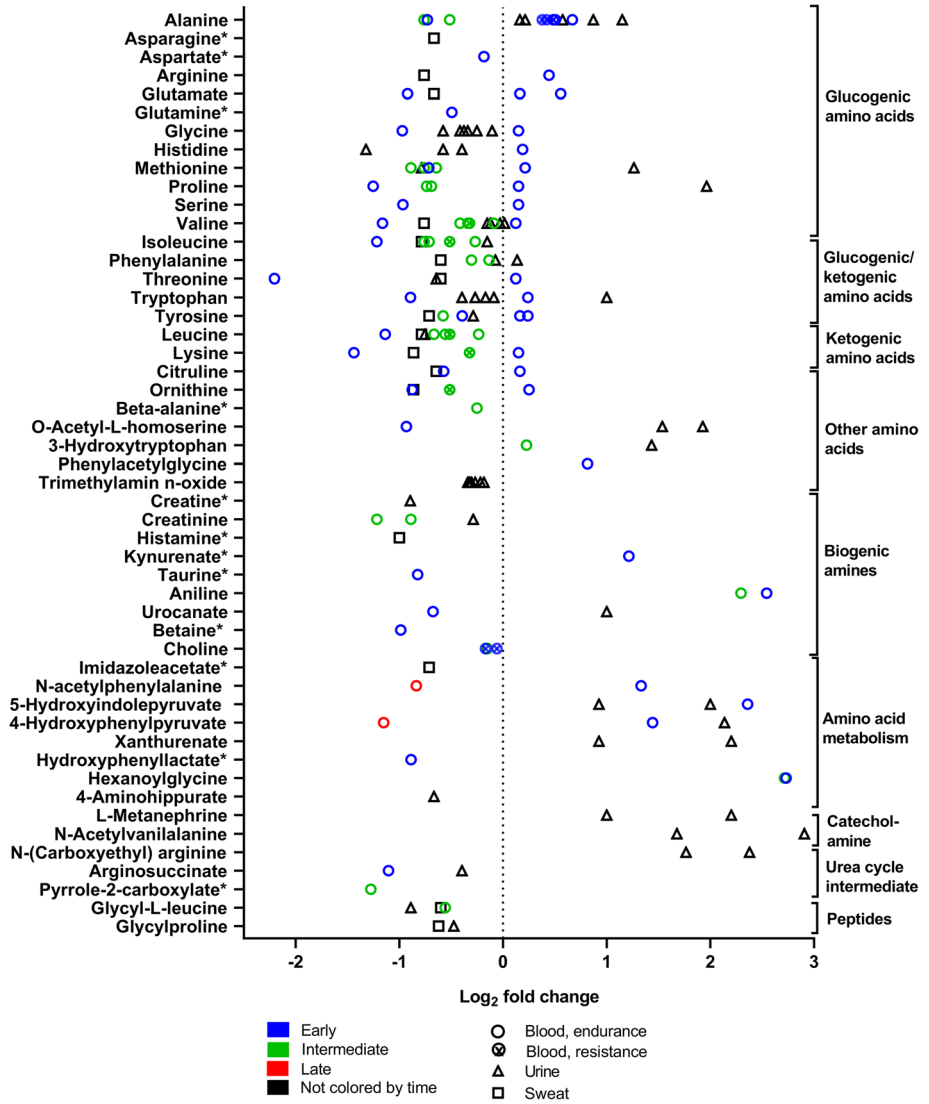

Fig. 10 Amino acids, their derivates and peptides changes in response to exercise. The graph shows 48 amino acids, their derivates, and two peptides that significantly changed in 32 (26 endurance, six resistance) and six (all endurance) experiments, respectively, after exercise. Rest = 0 (dotted vertical line). One symbol represents one experiment. * fold-change values were only reported in one experiment; the other experiment(s) reported a significant change but no fold-change values. For detailed quantitative and qualitative changes of all amino acids, see Additional file 1: Table S3

various free fatty acids increase in human blood as a consequence of exercise-induced lipolysis. The majority of free fatty acid concentrations are changed most early after exercise. Contrarily, four of six dicarboxylates were reported with highest fold-changes between > 0.5 and 3 h (intermediate) after exercise.

Figure 7 shows the concentration changes of acylcarnitines. Acylcarnitines are fatty acids bound to carnitine. They are fatty acid intermediates that are transported into the mitochondria but can leave cells to appear in blood and other biofluids. Similarly to other lipids, the concentrations of almost all acylcarnitines increase in blood and urine in response to a bout of exercise. Like fatty acids, they increase especially early after exercise. In contrast to fatty acids, some acylcarnitines are also detected in urine.

Figure 8 shows exercise-induced changes in bile acids, glycerophospholipids, sphingolipids, and steroids. Bile acids are synthesized in the hepatic cytosol out of cholesterol and help to digest dietary fat in the intestine. After a bout of exercise, the concentrations of several bile acids decrease mainly in blood. The highest fold-decreases are reported in late (> 3–24 h after exercise) sampling time points. Glycerophospholipids such as glycerophosphatidylethanolamines and sphingolipids are mainly associated components of human biological membranes. Overall, these lipid classes decrease their concentration in blood and

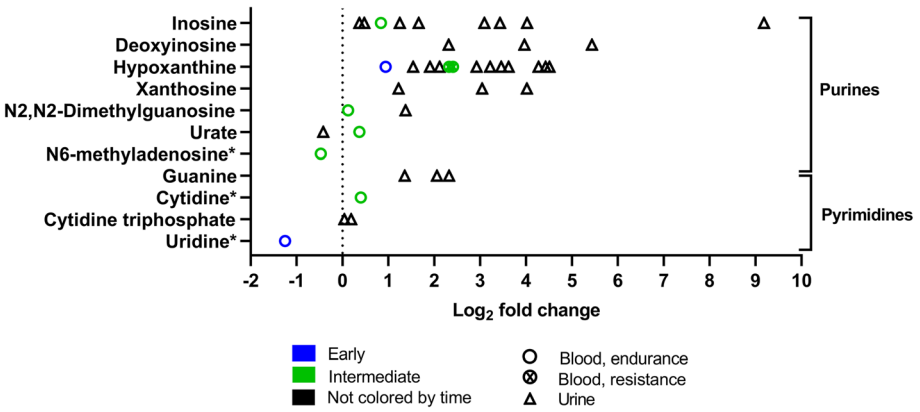

Fig. 11 Nucleotide changes in response to exercise. The graph shows eleven nucleotides that changed significantly in 14 experiments (12 endurance, two resistance). Rest = 0 (dotted vertical line). One symbol represents one experiment. * fold-change values were only reported in one experiment; the other experiment(s) reported a significant change but no fold-change values. For detailed quantitative and qualitative changes of all nucleotides, see Additional file 1: Table S4

urine after a bout of exercise, decreasing highest early after exercise. Steroids especially act as steroid hormones such as testosterone or cortisol and are derived from cholesterol [53]. A bout of exercise changes several steroids in blood but there is no uniform change of concentration.

Figure 9 shows the concentration changes in ketone bodies. Ketone bodies are "energy metabolites" synthesized from acetyl-CoA or ketogenic amino acids such as leucine in the liver. Ketone bodies are used in particular in brain and muscle when carbohydrates are limited, e.g., during fasting or prolonged exercise [54].

After an acute bout of exercise, the concentration of most ketone bodies and their precursors increases significantly in different human body fluids. 3-Hydroxybutyrate and acetoacetate, the classic ketone bodies, show higher increases in intermediate samples compared to early and late samples. In resistance exercises, acetoacetate even decreased early following exercise. Other ketogenic compounds that result from the degradation of branched chain amino acid (BCAA) like 2-oxoisovalerate or 3-methyl-2-oxovalerate do not show this timing-pattern

Amino Acids, Peptides, and Related Metabolites

Figure 10 shows amino acids and peptide changes after exercise. Amino acids comprise 20 proteinogenic amino acids encoded by deoxyribonucleic acid (DNA), non-proteinogenic amino acids, derivates, and amino acids that are modified in proteins and then degraded into modified amino acids such as 3-methylhistidine.

Amino acids also are part of the glucose-alanine cycle. The glucose-alanine cycle degrades amino acids to supply glucose to muscles. Here, the remaining amino groups are transported to the liver in the form of alanine to generate ammonia in the urea cycle [55]. The main finding is that an acute bout of exercise changes the concentration of amino acids and their degradation products significantly in different human body fluids (Fig. 10). In contrast to the results for most lipids, the findings for many amino acids are not consistent across experiments. While similar fold changes have been observed in the same tissue for amino acids such as glycine or trimethylamine-n-oxide, vastly different changes within the same body fluid were reported for amino acids such as alanine, valine, or tryptophan.

Organic bounds between amino acid monomers form peptides, which are reported as dipeptides (compounds of two amino acids) here. They can be a part of enzymes or signaling molecules in metabolism. Within peptides, especially the dipeptides of glycine with leucine or proline decrease in serum and plasma after a bout of exercise.

Nucleotides

Figure 11 shows nucleotide changes after exercise. Nucleotides are organic molecules that are the substrates for both DNA and ribonucleic acid (RNA) synthesis. Moreover, nucleotides such as ATP are key metabolites for energy metabolism and nucleotides such as cyclic adenosine monophosphate (cAMP) or guanosine triphosphate (GTP) are involved in cellular signal transduction. However, phosphorylated metabolites are rarely detected in blood and other biofluids because phosphorylation traps metabolites inside cells [56]. Furthermore, nucleotides such as coenzyme A and NAD can act as mediators of hormone and cofactor reactions. After one bout of exercise, many nucleotides as well as degradation products of nucleotide catabolism such as inosine and hypoxanthine mostly increase

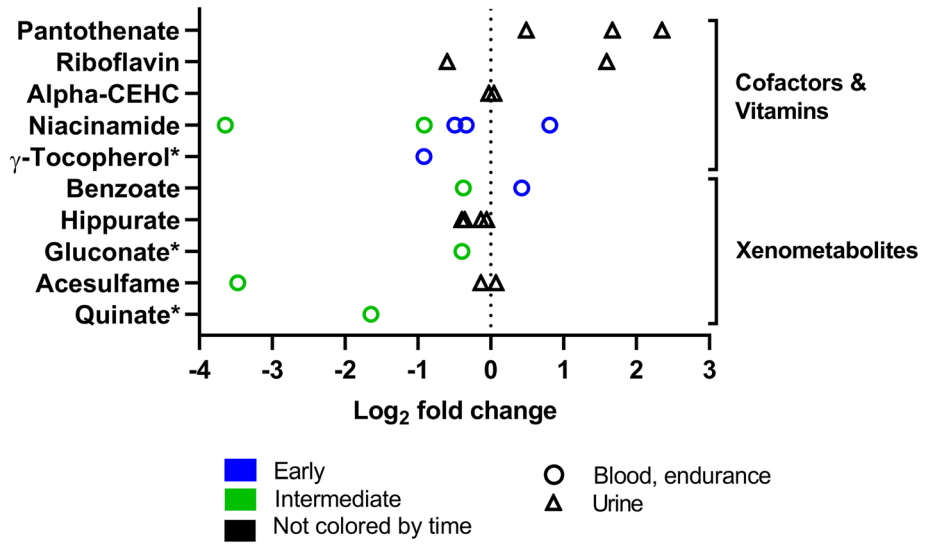

Fig. 12 Cofactor/vitamin and xenometabolite changes in response to exercise (log_2 fold change versus rest). The graph shows five cofactors or vitamins and six xenometabolites that changed significantly in 17 experiments (all endurance) Rest = 0 (dotted vertical line). One symbol represents one experiment. * fold-change values were only reported in one experiment; the other experiment reported a significant change but no fold-change values. For detailed quantitative and qualitative changes of all cofactors/vitamins and xenometabolites, see Additional file 1: Table S5

their concentrations in human urine and blood in the early and intermediate phase following exercise.

Cofactors and Vitamins and Xenometabolites

Figure 12 shows changes in cofactors, vitamins, and xenometabolites after exercise. The metabolism of cofactors and vitamins contains a variety of biochemical transformations. Organic compounds of non-proteinogenic origin, including some vitamins, assist these transformations. Observed changes of cofactors and vitamins differ between the here summarized experiments. Like cofactors and vitamins, xenometabolites are exogenous compounds. Xenometabolites can be drugs, food ingredients such as preservatives, plant components, or pesticides. Xenometabolites mostly decrease after a bout of exercise.

Discussion

In this review, we summarize how metabolite concentrations change in human blood and other biofluids within 24 h after a bout of exercise. Our analysis provides the first overview of results across metabolomics studies that use different human subjects, endurance, and resistance exercise; analyze different body fluids; utilize several analysis methods; and collect samples at different time points after exercise. Even though there are many differences in-between studies, the concentrations of many metabolites such as fatty acids or acylcarnitines often change similarly after exercise. There are, however, exceptions where metabolite concentrations change in different directions after exercise. This combined dataset illustrates such differences and may help researchers to identify the causes.

Exercise Alters the Concentrations of Metabolites that Are Involved in Energy Metabolism

Exercise is a major challenge to the body's homeostasis as it requires an immediate, large increase of ATP resynthesis. As a consequence, the flux of many energy metabolism reactions changes quickly with the onset of exercise. This changed flux then alters the blood and biofluid concentrations of metabolites involved in these reactions. These concentration changes reflect the mobilization, utilization, and conversion of energy metabolites such as carbohydrates and triacylglycerols (fats) to meet the ATP demand of the exercising muscles. Exercise studies also confirm that ketone bodies are generated, and amino acids are converted into glucose when carbohydrates are limited.

In the summarized studies, many of the metabolites that increased globally after exercise are lipids or related to lipid metabolism. These metabolites include glycerol (Fig. 5), free fatty acids (Fig. 6), and acylcarnitines (Fig. 7). During exercise, lipases split the triacylglycerols stored in adipose tissue into fatty acids and glycerol [57]. The fatty acids and glycerol are then released into the bloodstream, before being taken up and utilized for ATP synthesis by the exercising muscles. A new insight of this combined analysis is that all free fatty acids increase within 24 h after exercise no matter whether they are unsaturated or saturated, short, medium, or long. The earlier the post-

exercise sample is taken, the higher fatty acid increases in blood are (Fig. 6) [58].

Fatty acids that are taken up by muscle are then transported into the mitochondria in several steps that involve carnitine and the formation of acylcarnitines [52]. Even though acylcarnitines are formed within the cell, increased concentrations of acylcarnitines are detected after exercise in blood and other biofluids (Fig. 7).

During high-intensity exercise, blood glucose and muscle glycogen become the dominant sources of energy [59, 60]. They enter glycolysis and as a consequence, pyruvate and lactate concentrations are increased especially during and after high intensity exercise (Fig. 5). Blood pyruvate and lactate then decline within an hour after exercise but high urine concentrations are also measured 24 h after exercise [36, 37]. In contrast to pyruvate and lactate, glycolytic intermediates did only appear in blood in one study [34] and are therefore not shown in the graphs. Normally, glycolytic intermediates, which are phosphorylated are trapped inside cells [56].

Also, at high-intensity exercise, TCA cycle flux and the concentrations of TCA cycle metabolites such as malate increase (Fig. 5) which has been previously discussed in a review [52, 61]. In our analysis, the TCA cycle intermediates succinate and malate increased most in blood after high-intensity endurance and high load resistance exercise [40, 47] especially early after exercise (Fig. 5).

When carbohydrates run out during prolonged exercise or when fasted, then especially the liver is synthesizing new substrates for energy metabolism through ketogenesis and gluconeogenesis [62]. Liver synthesizes ketone bodies from ketogenic amino acids such as leucine or lysine and glucose from glucogenic amino acids such as valine or glycine [54]. The main ketone bodies 3-hydroxybutyrate and acetoacetate are then released into the blood [54] (Fig. 9) which explains their increased blood concentration after exercise. In parallel, the degradation products such as 2-oxoisovalerate increase too (Fig. 9). For gluconeogenesis, glucogenic amino acids are degraded to pyruvate and then transaminated to alanine. Alanine goes into the blood stream and blood alanine concentration increases (Fig. 10). In the liver, it is transformed to pyruvate again, and finally into glucose. Furthermore, the degradation products of glucogenic amino acids such as n-acetylphenylalanine and 4-hydroxyphenylpyruvate increase (Fig. 10).

After Exercise, the Concentrations of Nucleotide Degradation Products Increase, Whereas Bile Acid and Complex Lipid Concentrations Decrease

While nucleotides such as ATP or inosine monophosphate (IMP) molecules are trapped within cells [56], their unphosphorylated degradation products inosine, hypoxanthine, xanthine, and uric acid are detected in blood and other biofluids. After exercise, the concentrations of these nucleotide degradation products generally increases, especially in urine (Fig. 11). Generally, the concentrations of nucleotide degradation products increase most after high-intensity exercise [63] and most concentration changes occur between > 0.5 and 3 h after exercise [31, 43] (Figs. 4 and 11).

Exercise also lowers the concentrations of bile acids (Fig. 8). The primary bile acids cholic acid (cholate) and chenodeoxycholic acid are synthesized in the liver and secondary bile acids are then formed by intestinal bacteria [64]. The decrease of bile acids after exercise reported by metabolomics studies is in line with recent literature showing that both endurance and resistance exercise decrease the total bile acid concentration [65].

Fasting alone decreases bile acid concentrations [66] and bile acids decreased after exercise (Fig. 8), during which subjects did not eat. Therefore, lower bile acid concentrations after exercise are a result from exercise and fasting and not exercise alone.

Given that bile acid concentrations are associated with metabolic disease [67], this may identify exercise as an intervention that can modulate bile acid concentrations for therapeutic gains.

Additionally, complex lipids like glycerolipids and sphingolipids all decrease after exercise (Fig. 8). These lipids are not only important constituents of membranes but engage in signal transduction. For example, sphingosine-1-phosphate is released from cells by cell-specific transporters into the circulation. There, it can bind to five G-protein coupled receptors to regulate cellular behaviors such as survival and proliferation [68], e.g., through the modulation of Hippo signaling [69]. Whether the observed drop of sphingosine-1-phosphate after exercise (Fig. 8) can be exploited for the treatment of disease is currently unknown.

Different Study Protocols and Feeding Can Influence Metabolite Changes

Next to many consistent metabolite changes across studies, this analysis also showed metabolite changes in different directions between studies.

One example for variable concentration changes after exercise are amino acids and their derivates. Specifically, out of 53 amino acids, 37 changed in different directions after exercise (Figs. 3 and 10). One reason for these differences could be that amino acids are used or synthesized by many reactions and that these reactions may differ between different exercise and feeding protocols. For example, amino acids can contribute up to 10% to oxidative phosphorylation [70]. Amino acids are also used as substrates for gluconeogenesis, ketogenesis [54],

and protein synthesis especially after resistance exercise [71] (Fig. 10). The use of amino acids in all of these reactions will lower their concentration. Conversely, amino acids are generated by protein breakdown via the proteasome or autophagy [72] or enter the blood when proteins are digested and together breakdown and protein ingestion will increase the concentrations of amino acids in blood.

Compared to other metabolites such as fatty acids, post-exercise sample timing alone did not influence the variable changes in amino acids between the studies. What differs the most between studies is the duration of the exercise. The two studies [23, 31, 40, 47] that had the biggest difference in protocols and energy demand (a VO_{2max} test of ~ 10–15 min versus a simulated ultra-marathon of ~ 8–9 h) had the highest differences in fold-change after exercise (Fig. 10). Amino acid concentrations were lower after exercising with moderate intensity and for long duration. In contrast, amino acid concentrations were higher after exercise with high intensity but short duration.

Another factor that can influence amino acid concentrations and many other metabolites is pre- or post-exercise feeding. Especially carbohydrate intake reduces gluconeogenesis [66] and ketogenesis [54] and thereby reduces the usage of amino acids in these reactions so that the concentrations of these amino acids change less if subjects ingest carbohydrates.

Limitations

This systematic review has limitations. First, the studies summarized in this review combine many different exercise modes with variable intensity and duration and further vary in their nutrition and sampling times. Moreover, subjects are men and women, differing in their sex hormone concentrations and in the concentration of roughly one-third of all metabolites [73]. Furthermore, subjects were of different ages, differentially trained, and may have varied in their health and body composition. This is a key source of variation in this dataset. Thus, if metabolites such as fatty acids (Fig. 6) all increase their average concentration in response to different exercise modes and in different subjects then this suggests that the increase of fatty acid concentrations is a robust response to exercise.

A second limitation of our analysis is that not all included studies measured the same set of metabolites. When we report metabolites that only change in one of the three sampling phases (early, intermediate, late), it can be that this metabolite was measured only at this specific time point after exercise.

A third limitation is that both mass spectrometry and nuclear magnetic resonance methods have been used to measure metabolites. Also, since 2010, the metabolomics protocols have become more sensitive, allowing to detect and better quantify more metabolites. Thus, variable methodology is another source of variation in this combined analysis. We have made no attempt to control for the methods used but have indicated the methods used in Additional file 2: Table S6.

A fourth limitation is that this study reports average changes, not individual changes. This is an important limitation, because individual resting blood metabolite concentrations vary greatly in-between individuals and are strongly dependent on DNA sequence variation [74]. Moreover, the response of metabolites to exercise training varies too [75] and this individual variability is not reflected in this dataset as we only report mean concentration changes.

Conclusion and Outlook

Across different exercise modes and in different subjects, exercise often consistently changes the average concentrations of metabolites involved in energy metabolism and other branches of metabolism. This dataset should therefore be a useful resource for those that wish to study human exercise metabolism.

For the future, one important focus should be to use metabolomics to investigate whether individual metabolite concentrations or “metabolite fingerprints” (i.e., combinations of metabolites) are biomarkers for disease, metabolic function, trainability, or other “hard to measure” traits such as muscle fiber percentages. Here, it may be essential, similar to cardiovascular stress tests [76], to activate a system by exercise, as the capacity and function of many systems can only be assessed when the system is active and under stress. Many metabolic enzymes are inactive at rest and only become activated by exercise [77]. Therefore, the capacity of these enzymes may only be revealed by measuring metabolite concentrations during and after an exercise challenge. The best known example for this paradigm is of course lactate, as resting concentrations do not but exercise lactate concentrations do predict the capacity of aerobic metabolism [51].

Finally, while metabolite concentrations might be useful indicators of health or fitness-related phenotypes, they often do not report the flux or capacity of metabolic reactions. Here, the combination of stable isotope-labeled tracer molecules such as glucose or amino acids in combination with mass spectrometry analysis may in future allow the measurement of metabolic flux and this has been termed fluxomics [78]. Applying this technology to exercise studies is arguably the next methodological frontier of metabolic research in relation to sport and exercise.

Additional Files

Additional file 1: Table S1. Carbohydrate Metabolites and TCA cycle intermediates. **Table S2.** Lipids and intermediates of lipid metabolism. **Table S3.** Amino Acids and Peptides. **Table S4.** Nucleotides. **Table S5**. Cofactors/Vitamins and Xenometabolites.

Additional file 2: Table S6. Descriptive summary of 57 experiments that reported metabolites concentration changes after a bout of exercise.

Additional file 3: Table S7. Metabolites changed in relation to time point of sampling (with Fig. 4).

Abbreviations

ATP: Adenosine triphosphate; BCAA: Branched chain amino acid; BMI: Body mass index, calculated with bodyweight divided by body height (kg/m^2); cAMP: Cyclic adenosine monophosphate; DNA: Deoxyribonucleic acid; GTP: Guanosine triphosphate; H-NMR: Proton nuclear magnetic resonance; IMP: Inosine monophosphate; kDA: Kilo Dalton; mM: Millimolar; NADH/NAD: Nicotinamide adenine nucleotide; PICO: Population Intervention Control Outcome; RNA: Ribonucleic acid; TCA cycle: Tricarboxylic acid cycle; VO_{2max}: Maximum oxygen uptake capacity

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Author's Contributions

All author contributed to conception of the article. DS drafted the article and performed the literature search and analysis. All authors contributed to interpretation of the results. MS, WR-M, GK, and HW critically revised the work. All authors read and approved the final manuscript.

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Availability of Data and Materials

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

Ethics Approval and Consent to Participate

Not applicable.

Consent for Publication

Not applicable.

Competing Interests

The authors, Daniela Schranner, Gabi Kastenmüller, Martin Schönfelder, Werner Römisch-Margl, and Henning Wackerhage, declare that they have no competing interests.

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Publisher's Note

III-2. Publication 2: Experimental study on metabolome difference between athlete phenotypes

Title: Physiological extremes of the human blood metabolome: A metabolomics analysis of highly glycolytic, oxidative, and anabolic athletes

Authors: Daniela Schranner, Martin Schönfelder, Werner Römisch-Margl, Johannes Scherr, Jürgen Schlegel, Otto Zelger, Annett Riermeier, Stephanie Kaps, Cornelia Prehn, Jerzy Adamski, Quirin Soehnlein, Fabian Stöcker, Florian Kreuzpointner, Martin Halle, Gabi Kastenmüller & Henning Wackerhage

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III-2.1. Personal contributions

Personal contribution statements are written in the first person singular.

III-2.1.1. Study planning & schedule study physicians

I wrote the ethics proposal and obtained approval, did the laboratory setup, recruited study medical staff/physicians, and constructed the overall study protocol, the protocol for blood sampling, blood processing and storage, and developed participant questionnaires. As this study was not externally funded, I asked physicians of the Preventive and Rehabilitative Sports Medicine of the TUM to volunteer for the medical assessment and blood drawing.

III-2.1.2. Participant recruitment

I had sole responsibility over participant recruitment and communication. Due to high standards of athlete level, I recruited sprinters and endurance athletes individually, based on their achievements during the last 2 years in their respective sports via mail and natural bodybuilders via the president of the German Natural Bodybuilding Foundation (GNBF). Untrained control participants were recruited via flyers from different faculties of the TUM and LMU, excluding students of exercise sciences. I communicated with each participant before the study including the assessment of exclusion criteria, current training phases and scheduling study appointments.

III-2.1.3. Study execution

I spent ~7 hours on site per participant (in total 35 participants, manuscript includes first 18 participants only), lasting from 7.00 am until 3.00 pm and including anamnesis and initial blood drawing, together with physicians of the TUM Sports Medicine of Klinikum Rechts der Isar, blood handling and processing, resting ECG diagnosis together with physicians, anthropometry, execution of bicycle ergometry including spirometric testing, stress ECG diagnosis, blood lactate sampling from participants earlobe and subsequent analysis, post-exercise blood drawing together with physicians, grip strength testing, reactive strength testing and analysis, isometric leg strength testing, and supplying participants with endurance and strength test results after the study.

III-2.1.4. Data analysis

Quality control of the raw data including missingness analysis, outlier analysis, metabolite coefficient of variation analysis in quality control samples, (batch correction, done on full, untargeted dataset), missing value imputation, statistical analysis of quality-controlled data was done together with the Institute of Computational Biology of the Helmholtz Zentrum München.

III-2.1.5. Data interpretation

Comparison of results with literature and drawing conclusions was done with co-authors.

III-2.1.6. Article

I wrote the article draft, did the literature search for introduction and discussion, drew the figures, and compiled the supplementary material. Together with co-authors, I wrote and revised the manuscript.

III-2.2. Summary

Human metabolism is highly variable. At one end of the spectrum, defects of enzymes, transporters, and metabolic regulation result in metabolic diseases such as diabetes mellitus or inborn errors of metabolism. At the other end of the spectrum, favorable genetics and years of training combine to result in physiologically extreme forms of metabolism in athletes. Here, we investigated how the highly glycolytic metabolism of sprinters, highly oxidative metabolism of endurance athletes and highly anabolic metabolism of natural bodybuilders affects their serum metabolome at rest and after a bout of exercise to exhaustion. We used targeted mass spectrometry-based metabolomics to measure the serum concentrations of 151 metabolites and 43 metabolite ratios or sums in 15 competitive male athletes (6 endurance athletes, 5 sprinters, and 4 natural bodybuilders) and 4 untrained control subjects at fasted rest and 5 minutes after a maximum graded bicycle test to exhaustion. The analysis of all 194 metabolite concentrations, ratios and sums revealed that natural bodybuilders and endurance athletes had overall different metabolite profiles, whereas sprinters and untrained controls were more similar. Specifically, natural bodybuilders had 1.5 to 1.8-fold higher concentrations of specific phosphatidylcholines (e.g. PC aa C36:6) and lower levels of branched chain amino acids than all other subjects. Endurance athletes

had 1.4-fold higher levels of a metabolite ratio showing the activity of carnitine-palmitoyl-transferase I and 1.4-fold lower levels of various alkyl-acyl-phosphatidylcholines. When we compared the effect of exercise between groups, endurance athletes showed 1.3-fold higher increases of hexose and of tetradecenoylcarnitine (C14:1). In summary, physiologically extreme metabolic capacities of endurance athletes and natural bodybuilders are associated with unique blood metabolite concentrations, ratios, and sums at rest and after exercise. Our results suggest that long-term specific training, along with genetics and other athlete-specific factors systematically change metabolite concentrations at rest and after exercise.

III-2.3. Original manuscript

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ORIGINAL ARTICLE

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Physiological extremes of the human blood metabolome: A metabolomics analysis of highly glycolytic, oxidative, and anabolic athletes

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Abstract

Human metabolism is highly variable. At one end of the spectrum, defects of enzymes, transporters, and metabolic regulation result in metabolic diseases such as diabetes mellitus or inborn errors of metabolism. At the other end of the spectrum, favorable genetics and years of training combine to result in physiologically extreme forms of metabolism in athletes. Here, we investigated how the highly glycolytic metabolism of sprinters, highly oxidative metabolism of endurance athletes, and highly anabolic metabolism of natural bodybuilders affect their serum metabolome at rest and after a bout of exercise to exhaustion. We used targeted mass spectrometry-based metabolomics to measure the serum concentrations of 151 metabolites and 43 metabolite ratios or sums in 15 competitive male athletes (6 endurance athletes, 5 sprinters, and 4 natural bodybuilders) and 4 untrained control subjects at fasted rest and 5 minutes after a maximum graded bicycle test to exhaustion. The analysis of all

*Jointly directed the work.

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194 metabolite concentrations, ratios and sums revealed that natural bodybuilders and endurance athletes had overall different metabolite profiles, whereas sprinters and untrained controls were more similar. Specifically, natural bodybuilders had 1.5 to 1.8-fold higher concentrations of specific phosphatidylcholines and lower levels of branched chain amino acids than all other subjects. Endurance athletes had 1.4-fold higher levels of a metabolite ratio showing the activity of carnitine-palmitoyltransferase I and 1.4-fold lower levels of various alkyl-acyl-phosphatidylcholines. When we compared the effect of exercise between groups, endurance athletes showed 1.3-fold higher increases of hexose and of tetradecenoylcarnitine (C14:1). In summary, physiologically extreme metabolic capacities of endurance athletes and natural bodybuilders are associated with unique blood metabolite concentrations, ratios, and sums at rest and after exercise. Our results suggest that long-term specific training, along with genetics and other athlete-specific factors systematically change metabolite concentrations at rest and after exercise.

KEYWORDS

athlete, energy metabolism, exercise biomarker, exercise phenotype

1 | INTRODUCTION

Inactivity, overweight and its negative impacts on health are a world-wide problem (Afshin et al., 2017). In contrast, physical activity and exercise training are widely accepted as health-promoting factors (Afshin et al., 2017; Blair, 2009; Cohen et al., 2015; Pedersen & Saltin, 2015). Therefore, a major goal in exercise science is to understand how exercise triggers physiological adaptation (e.g., an increase in muscle mass or aerobic capacity) and how these adaptations can benefit health or mitigate disease (Pedersen & Saltin, 2015).

In athletes, years of training plus a unique genetic makeup (Sarzynski & Bouchard, 2020) result in metabolic adaptations: Endurance training increases mitochondrial content and activity of oxidative enzymes in skeletal muscle (Egan & Zierath, 2013), resistance training increases muscle fiber size (Mero et al., 2013), and anerobic training like sprint training increases glycolytic enzymes in skeletal muscle (Ross & Leveritt, 2001). Eventually, genetics and years of specific training in athletes result in physiologically extreme metabolic phenotypes.

Metabolites can serve as molecular read-outs of these metabolic phenotypes (Aebersold & Mann, 2016; Patti et al., 2012). The metabolome, which comprises all metabolites within an organism, is highly dynamic, and susceptible to external influences like exercise (Krug et al., 2012). Studies have shown that one bout of exercise (Contrepois et al., 2020; Morville et al., 2020; Schranner et al., 2020) and exercise training for several weeks (Felder et al., 2017; Neal et al., 2013) change hundreds of metabolites in blood.

A targeted change of specific metabolites through exercise could be directly relevant to diseases with dysregulated metabolism. Recently, Morville et al. showed that a short term, targeted change of metabolites is possible through different exercise modes. They showed that within one session, endurance exercise changes different metabolites than resistance exercise does (Morville et al., 2020). However, it is not clear if there is a long-term effect of different exercise modes on the metabolome.

While short-term metabolite changes after one exercise session in athletes were reported (Al-Khelaifi et al., 2018, 2019; Breit et al., 2015; Coelho et al., 2016; Hall et al., 2016; Howe et al., 2018), no study showed how years of metabolic adaptation to endurance, resistance, or sprint training affect metabolite changes to the same acute exercise.

Therefore, we wanted to find out how long-term physiological adaptation to different exercise training modes (endurance, resistance, sprint) affect the metabolome at rest and how these different adaptations affect the metabolic response to the same acute exercise. By comparing the serum metabolomes of glycolytic sprinters, oxidative endurance athletes, and anabolic natural bodybuilders before and after a maximum graded exercise test, we aimed to answer the following research questions:

1) Do sprinters, endurance athletes and natural bodybuilders have distinct blood metabolite concentrations? If so, the concentrations of which metabolites explain the differences in-between athlete groups?

2) Within these highly trained athletes, how does a bout of graded cycle exercise to exhaustion affect blood metabolite concentrations? And specifically, do metabolite

concentrations change differently after the exercise depending on the athlete group?

2 | MATERIALS AND METHODS

2.1 | Study cohort and human exercise testing

For this study, we recruited three groups of healthy male athletes ($n = 15$): 5 sprinters, 6 endurance athletes, 4 natural bodybuilders, and 4 healthy untrained males. All participants passed the inclusion criteria (Supplementary Table S1) and completed the study. Mean group characteristics (Table 1) and individual details (Supplementary Table S2) are provided. In preparation for the study, participants followed a standard diet (Supplementary Table S7) on the day before testing, refrained from exercise training for 24 hours and from dietary supplements for 48 hours before testing. Participants recorded their exercise training for 4 weeks (Table 1) and their intake of dietary supplements and medication for one week before the study (Supplementary Table S2).

Human exercise testing included three phases: baseline measurement, exercise testing, and recovery (Figure 1). To reduce circadian bias, all participants reported to the laboratory at 7 am after a 10 hour overnight fast. Upon arrival, we measured height, weight, body circumferences, and body fat including measurement of subcutaneous fat over Biceps brachii and Quadriceps femoris. Body fat was calculated from the thickness of seven skin folds (7-point-calipermetry) using the method by Jackson & Pollock (Jackson & Pollock, 1978). After resting for 10 minutes, we took blood samples from an

TABLE 1 Mean (SD) group characteristics of study participants showing significantly different groups ($p < 0.05$) in bold after correcting for multiple testing

	Control	Natural Bodybuilding[a]	Endurance	Sprint
Number of subjects	4	4	6	5
Age (years)	30 (2)	28 (6)	30 (3)	24 (3)
Resting heart rate (bpm)	70 (9)	56 (4)	51 (15)	59 (7)
Resting blood pressure (mmHg)	140/80 (12/7)	123/78 (10/4)	122/75 (11/7)	126/78 (9/9)
BMI (kg/m^2)	24.8 (2.3)	**26.5 (2.7)**[b]	22.1 (1.9)	21.9 (1.5)
Height (cm)	188 (4.5)	172 (6.6)	183 (3.8)	189 (7.0)
Body fat (%)	18.8 (7.7)	10.6 (1.2)	**7.5 (1.0)**[c]	**5.5. (0.7)**[c]
Upper arm circumference (cm)	30.1 (2.7)	33.1 (3.7)	28.1 (1.7)	28.3 (1.8)
Thigh circumference (cm)	54.2 (4.2)	59.5 (6.6)	51.4 (5.1)	55.4 (4.1)
Subcutaneous fat upper arm (mm)	**14.4 (7.2)**[b,d,e]	6.4 (0.5)	6.5 (2.1)	5.6 (0.9)
Subcutaneous fat thigh (mm)	**20.4 (7.3)**[b,d,e]	10.1 (2.7)	7.7 (3.4)	5.8 (1.7)
Ventilatory threshold 1 (ml/kg/min)	26.2 (2.1)	27.2 (3.0)	**47.9 (6.0)**[b,c,d]	27.3 (5.6)
VO_2max (ml/kg/min)	45.6 (4.7)	41.8 (2.0)	**63.6 (6.6)**[b,c,d]	52.6 (5.4)
Relative maximum workload (W/kg)	3.9 (0.5)	3.7 (0.2)	**5.9 (0.3)**[b,c,d]	**4.8 (0.2)**[c,d]
Lactate (mmol/l) 4 min after maximum workload	12.0 (1.0)	9.7 (0.9)	9.5 (2.6)	13.0 (1.5)
Lactate (mmol/l) 10 min after maximum workload	12.1 (1.7)	9.2 (2.7)	**5.9 (1.7)**[b,c]	11.4 (3.1)
Reactive strength (RSI)	111 (30)	125 (25)	164 (29)	**218 (37)**[c,d]
Hand grip strength (kg)	59.3 (7.2)	62.4 (6.6)	51.5 (2.8)	60.2 (5.1)
Endurance training (min/week)	41 (63)	80 (40)	**815 (317)**[b,c,d]	162 (65)
Resistance training (min/week)	0 (0)	**413 (227)**[c,e]	85 (50)	207 (56)
Speed training (min/week)	0 (0)	135 (201)	65 (84)	**294 (161)**[c]

[a]Natural bodybuilders are bodybuilders who abstain from performance enhancing drugs listed in the World Natural Bodybuilding Federation banned substances list e.g. steroid hormones (Liokaftos, 2018).

[b]significantly different from sprinters.

[c]significantly different from controls.

[d]significantly different from natural bodybuilders.

[e]significantly different from endurance athletes.

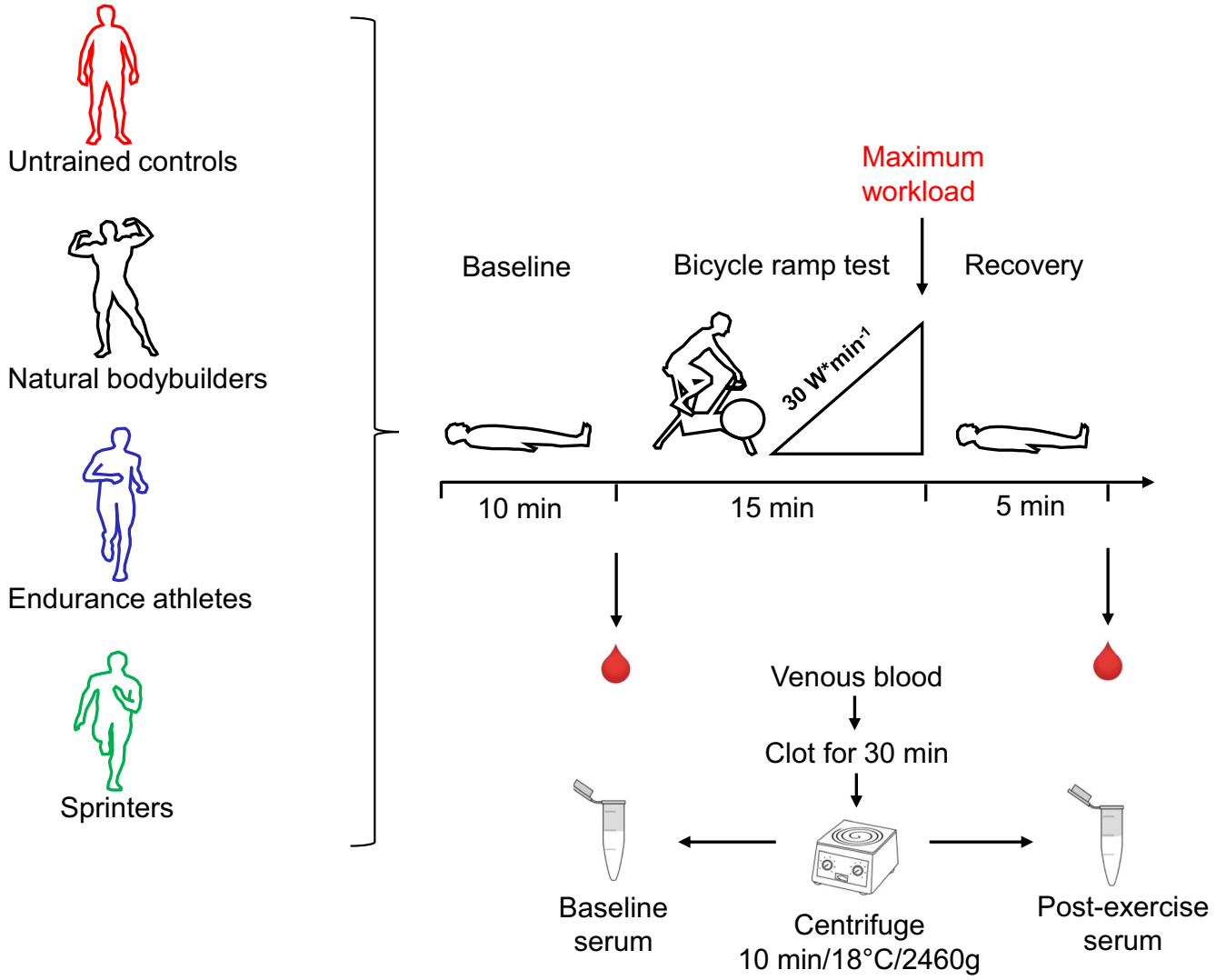

FIGURE 1 Overview of the study design where a standardized bicycle ramp test was performed with a continuously increasing load of 30 watts per minute until voluntary exhaustion

antecubital vein of the right arm in a supine position. After a three-minute warm up, subjects performed a ramp-test on a bicycle ergometer (Lode, Groningen, Netherlands) with power increasing linearly at a rate of 30 W per minute until voluntary exhaustion. During cycling, we continuously measured gas exchange with a stationary cardiopulmonary exercise testing system (Cortex, Germany). Out of 19 subjects, 18 met objective exhaustion criteria of either a respiratory exchange ratio (RER) >1.0 or a ventilatory equivalent of oxygen ($VEeqO_2$) of >30.0 (Aspenes et al., 2011). One endurance athlete, E1 did not meet these criteria. Despite endurance trained subjects have lower RER than non-endurance trained subjects in response to similar relative exercise intensity (Jeukendrup et al., 1997) we conclude subject E1 was not entirely physically exhausted. Five minutes after the end of exercise, we took a second blood sample from the antecubital vein of the left arm in a supine position. At maximum exhaustion, we started to sample lactate from the earlobe in 20 µl capillaries (EKF diagnostics, Germany) every 2 min for 10 min in total and analyzed samples immediately (Biosen S-Line Analyzer, EKF diagnostics, Germany). Then participants rested for 90 minutes and ingested drinks and foods ad libitum. After rest, participants re-warmed for ~15 minutes (10 min ergometry at 100 W and 5 min supervised jumping and dynamic stretching exercises). After re-warm, reactive strength was measured by a drop jump from 30 cm height with a force plate (Kistler GmbH, Germany). The best out of three attempts (highest RSI) was recorded. Afterwards, we measured maximum hand grip force with a hand grip dynamometer (Jamar, JLW instruments, USA) where the best out of three attempts was recorded as well.

2.2 | Blood sample preparation

We drew venous blood samples in four replicates into 9 ml serum S-Monovettes Z-Gel collecting tubes (Sarstedt AG und

Co KG, Nuembrecht, Germany) at each timepoint. Clotting was allowed at room temperature for 30 min in an upright position. After centrifugation (10 min / 18°C, 2460 g), we merged the serum replicates into one 15 ml Falcon tube (Greiner Bio-One GmbH, Kremsmuenster, Austria). Then, we aliquoted the serum into cryotubes (Sarstedt AG und Co KG, Nümbrecht, Germany), froze aliquots on dry ice for ~30 min and stored them at −80°C until analysis.

2.3 | Metabolomics measurement

Blood serum samples were analyzed at the Genome Analysis Center at the Helmholtz Zentrum München (Munich, Germany) with a kit-based metabolomics approach (Absolute*IDQ* p180 Kit; Biocrates Life Sciences AG, Innsbruck, Austria) applying liquid chromatography (LC-MS/MS) and flow injection analysis-tandem mass spectrometry (FIA-MS/MS) to measure a pre-defined set of 188 metabolites in a targeted fashion. Sample preparation and MS/MS measurements were performed according to the manufacturer's instructions (manual UM-P180) as described previously (Zukunft et al., 2013). Briefly, 10 µL blood serum were placed into the 96-well plate of the p180-kit and dried in a nitrogen stream for 30 minutes. For tagging amino acids and biogenic amines, samples were derivatized with an excess of 5% phenylisothiocyanate (Sigma-Aldrich, Steinheim, Germany). After drying under nitrogen, metabolites were extracted in 300 µL methanol (AppliChem, Darmstadt, Germany) containing 5 mM ammonium acetate (Sigma-Aldrich, Steinheim, Germany). After incubation for 30 min at room temperature and centrifugation, the eluate was split and diluted for the following MS/MS analyses. For sample preparation and MS/MS analysis, we used the following laboratory equipment: (i) Hamilton Microlab STAR^{TM} robot (Hamilton Bonaduz AG, Bonaduz, Switzerland) for liquid sample handling, (ii) Ultravap nitrogen evaporator (Porvair Sciences, Leatherhead, UK) for sample drying, (iii) 1200 Series HPLC (Agilent Technologies Deutschland GmbH, Böblingen, Germany) equipped with a HTC PAL auto sampler (CTC Analytics, Zwingen, Switzerland) for the liquid chromatography step, and (iv) API 4000 triple quadrupole (Sciex Deutschland GmbH, Darmstadt, Germany) operated using the software Analyst (version 1.6.2) for MS/MS analysis. For compound identification and quantification, the mass spectrometer was run in multiple reaction monitoring mode. Following the kit procedure, we applied the MultiQuant 3.0.1 (Sciex) and Met*IDQ*™ software to assess measurement quality and to calculate metabolite concentrations in reference to the corresponding isotope-labeled internal standards contained in the kit plate. Concentrations were reported in µM.

The assay allows simultaneous quantification of 188 metabolites: free carnitine (C0), 39 acylcarnitines (Cx:y), 21 amino acids (19 proteinogenic +citrulline + ornithine), 21 biogenic amines, hexose (sum, consisting of about 90%–95% glucose), 90 glycerophospholipids 14 lysophosphatidylcholines (lysoPC) and 76 phosphatidylcholines and 15 sphingolipids (SMx:y). The abbreviations Cx:y are used to describe the total number of carbons and double bonds of all fatty acid chains, respectively. PCs are labeled as either diacyl-phosphatidylcholines (PC aa) or alkyl-acyl-phosphatidylcholines (PC ae). This labeling is based on the assumption that even-numbered fatty acids and lower degrees of desaturation are more common than odd-numbered fatty acids or very high degrees of desaturation. For example, the labels PC ae C38:0, PC aa C37:0, and PC aa C38:7, all have the same mass and, thus, all would describe the same PC kit measure representing a mixture of these structurally different PCs; according to the assumptions above, the respective kit measure is labeled as PC ae C38:0 (for more details see (Quell et al., 2019)).

The values for limit of detection (LODs) of metabolites were calculated as three times the values of the zero samples, here phosphate-buffered saline. To assess the experimental variation of measurements, five aliquots of a pooled reference plasma (Haid et al., 2018) were measured on the same kit plate as the samples of interest and were used to calculate the coefficient of variation (CV) for each metabolite.

For a full list of all measured 188 metabolites and 44 calculated biologically relevant metabolite ratios or sums see Supplementary Table S3.

2.4 | Statistical analysis

2.4.1 | Data quality control and preprocessing

In total, 37 metabolites and 1 ratio were excluded from further analysis based on the following criteria: (i) missing values for more than 90% of the samples (c4-OH-Pro, Dopamine, Nitro-Tyr, Carnosine), (ii) CV of the five reference plasma samples (indicating the technical variation of measurements) exceeding 25% (Zhang et al., 2020) (DOPA, Histamine, PC aa C30:2, SM C22:3, PEA, Spermine, SM C26:0, Spermine/Spermidine, C5:1-DC), and (iii) values below specified LOD for more than 50% of samples (29 metabolites, including DOPA, PEA, Spermine, C5:1-DC). Parameters for quality control (missingness, CV, LOD) along with the mean concentrations of all measured analytes are provided in Supplementary Table S3.

As metabolite concentrations are mostly log-normally distributed, the 151 metabolites and 43 ratios remaining after quality control were log-transformed (log2) and missing values (PC ae C30:1, 5.3%; PC ae C38:1, 18.4%) were imputed using a k-nearest-neighbor approach (k=3) with variable selection (Do et al., 2018).

2.4.2 | Partial least squares discriminant analysis (PLS-DA)

To check if the four groups (control, endurance, sprint, bodybuilding) can be discriminated based on their metabolomic profiles at baseline and after exercise, we performed a PLS-DA analysis using the ropls R package (version 1.16.0). Prior to the analysis, metabolite concentrations were scaled (mean = 0, standard deviation = 1) over all samples. PLS-DA projects these z-scores of the 194 metabolite measures onto a reduced number of artificial components (here: components 1 and 2) that are linear combinations of the original variables which maximize the distinction of the pre-defined groups (i.e., the covariance between components built from the variables and response (grouping)) (Wold et al., 2001). R2X (0.258), R2Y (0.488), Q2Y (0.304) representing the fraction of explained variances (of variables and response) and the accuracy of prediction (Eriksson, 2002; Tenenhaus, 1998) are provided as measures for the quality of the resulting PLS-DA model in Supplementary Figure S1. To test for overfitting, we inspected R2Y and Q2Y from 20 PLS-DA models based on our data with random permutations of the group labels. Resulting empiric *p*-values for the achieved R2Y and Q2Y were below 0.05 (Szymańska et al., 2012). To identify key variables that drive the discrimination of athlete groups, we examined the loading vectors of the two PLS-DA components (i.e., the weights assigned to each metabolite in the linear combination that defines each component). The loadings along with the metabolites' variable importance in projection (VIP) scores (summarizing the loading weights for both components and how much the components explain the group distinctions) are given in Supplementary Table S4 and Supplementary Figure S1. To examine the influence of sums and ratios (as partially redundant variables) on the separation of groups in the PLS-DA, we repeated the analysis based on the 151 single metabolites and found the group differences to be stable (Supplementary Figure S2).

2.4.3 | Hypothesis testing for group differences in metabolite levels

For the metabolites that showed the five most extreme loading weights in negative and positive direction in the two PLS-DA components, we formulated a linear mixed effects model (assuming fixed effects for time (baseline/post exercise) and group and a random intercept for the subjects) and performed an ANOVA to obtain *p*-values for the group differences in the levels of these 2*10 selected metabolites (z-scored). Group effects were considered significant if $p < 0.0050$ ($\alpha = 0.05/10$ adjusted for ten tests). As component 1 separates the bodybuilding group and component 2 the endurance group from the other groups, we additionally tested for differences of these two groups against all other participants for the selected metabolites respectively, again using analogous linear mixed models.

2.4.4 | Hypothesis testing for effect of exercise and effect differences by group

To identify metabolites or ratios/sums that significantly change upon exercise, we performed a paired Student's t-test for each of the 194 variables. The resulting -log10(*p*-value) were displayed versus the means of metabolite fold changes within individuals of measured metabolite concentrations in a Volcano plot (generated using Prism 8.3.0, GraphPad). Log2 fold changes (means of fold change within individuals) and *p*-values for the full list of metabolites are provided in Supplementary Table S5. *p*-values were considered significant if $p < 2.58*10^{-4}$ ($\alpha = 0.05/194$ adjusted (Bonferroni) for multiple testing). For identification of group-specific effects of exercise, we performed a *t*-test for each metabolite comparing the mean log2 fold change (within individuals) of each group with the mean log2 fold change (within individuals) of all other subjects (Supplementary Table S6). Additionally, we performed non-parametric Wilcoxon signed rank/rank tests to ensure robustness of our results against potential outliers (Supplementary Tables S5 and S6).

All calculations were performed using R Studio (Version 1.2.5033, Boston, MA, USA) with R version 3.6.2. Single metabolite plots, the PLS-DA loading plot and the Volcano plot were generated using Prism version 8.3.0.

3 | RESULTS

We recruited three groups of healthy male athletes: 5 sprinters, 6 endurance athletes, and 4 natural bodybuilders. Four healthy sedentary males were recruited as a control group. All subjects met the inclusion criteria given in Supplementary Table S1 and completed the study. Mean group characteristics are listed in Table 1 and subjects' details are provided in Supplementary Table S2.

We analyzed 194 blood metabolite measures before and after the graded bicycle test to exhaustion to identify differences in the metabolomes of our four subject groups (Figure 1). These measures include 151 metabolites and 43 biologically relevant metabolite ratios and sum that remained after quality control of the analytical data (see Methods). We used a targeted metabolomics kit that mainly measures amino acids and lipids. Measured lipids include acylcarnitines, which are essential for fat metabolism and complex lipids such as sphingomyelins (SMs), and phosphatidylcholines (PCs), which are incorporated into membranes and carry two fatty acid residues. The kit also includes lysophosphatidylcholines (lysoPCs), which are degradation products of PCs. Over 70

PCs with different molecular weights are measured by the kit and are labeled as either diacyl-phosphatidylcholines (PC aa) or alkyl-acyl-phosphatidylcholines (PC ae), also known as ether lipids. This labeling bases on the assumption that even-numbered fatty acids and lower degrees of desaturation are more common than odd-numbered fatty acids or very high degrees of desaturation. Common violations of these assumptions and observable mixed molecular compositions of measured PCs in human blood have been discussed recently in more detail (Quell et al., 2019). The assessed biologically relevant ratios include e.g. the carnitine-palmitoyl-transferase-1 ratio (CPT1-ratio), calculated as the concentration ratio of the CPT-1 reaction products hexadecanoylcarnitine (C16:0) and octadecanoylcarnitine (C18:0) to the substrate free carnitine (C0). The CPT1-ratio is considered a proxy measure of ß-oxidation activity.

After quality control, the final set of 194 metabolite measures included 151 metabolites and 43 biologically relevant ratios or sums, which we used for all further statistical analyses (Supplementary Table S3).

3.1 | Do sprinters, endurance athletes and natural bodybuilders have distinct blood metabolite concentrations at rest and after exercise?

We calculated a partial least squares discriminant analysis (PLS-DA) by combining baseline and post-exercise samples and using all 194 metabolite measures. PLS-DA combines the large number of metabolite concentrations to yield two artificial components (component 1 and component 2) that are calculated to maximize the distance between the groups. Our PLS-DA revealed overlapping clusters of controls and sprinters suggesting more similar metabolite concentrations. Natural bodybuilders (along component 1) and endurance athletes (along component 2) appeared as distinct clusters from sprinters and untrained controls (Figure 2) suggesting unique metabolite concentrations.

3.2 | Which metabolites explain the differences in-between groups?

In a next step, we identified those metabolites that separated the clusters of natural bodybuilders and those of endurance athletes from the other groups. We inspected the PLS-DA loadings which show by how much each metabolite contributes to component 1 and component 2. We selected the five metabolites with the highest positive and negative contributions (i.e., largest absolute weights), respectively, for each component (Table 2). All 10 metabolites selected for component 1 (Table 2a) differed significantly in-between groups in an ANOVA. Out of the 10 metabolites selected for component 2 (Table 2b), four differed significantly in-between groups.

Isoleucine, leucine, BCAA, tryptophan and tetradecadienoylcarnitine (C14:2) were lower concentrated in natural bodybuilders (Figure 3a) when compared to all other groups. Five complex lipids including two hydroxy-sphingolipids (SM-OH), the total sum of hydroxy-sphingolipids (total SM(OH)) and two PCs (Table 2a) were higher concentrated in natural bodybuilders (Figure 3b) when compared to all others. Out of the 10 metabolites selected in component 1, PC aa C36:6, PC ae C38:0, and C14:2 differed significantly ($p < 5.0*10^{-3}$) between bodybuilders and all other groups when tested one by one (Table 2a).

Alpha-aminoadipic acid (alpha-AAA) and four PCaes (Table 2b, Figure 3d) were lower concentrated in the endurance athletes when compared to all other groups. In contrast,

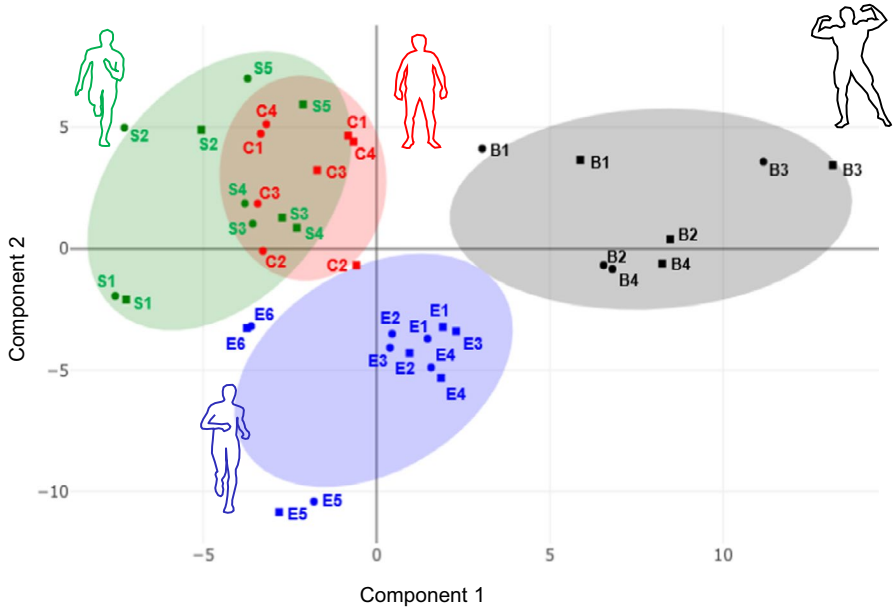

FIGURE 2 PLS-DA score plot showing baseline (●) and post-exercise (■) serum metabolite profiles within 75% confidence intervals (shading) of natural bodybuilders (B1-B4), endurance athletes (E1-E6), sprinters (S1–S5) and untrained controls (C1–C4)

TABLE 2 Metabolites that contributed most to the distinction of the natural bodybuilders in component 1 (a) and to the distinction of endurance athletes in component 2 (b) from all other subjects

(a) Metabolite, ratio or sum	Loading on component 1[a]	*p* value Overall group differences	*p* value Natural Bodybuilder versus others
Isoleucine	*−0.1500*	$2.7*10^{-6*}$	0.021
BCAA	*−0.1397*	$1.1*10^{-5*}$	0.014
Leucine	*−0.1375*	$6.5*10^{-5*}$	0.023
C14:2	*−0.1344*	$2.3*10^{-3*}$	$5.0*10^{-3*}$
Tryptophan	*−0.1269*	$3.8*10^{-6*}$	0.011
PC ae C38:0[b]	0.1603	$3.2*10^{-7*}$	$6.8*10^{-4*}$
Total SM-OH	0.1553	$4.8*10^{-5*}$	0.014
SM (OH) C22:2[b]	0.1501	$1.6*10^{-4*}$	0.0260
PC aa C36:6	0.1490	$2.9*10^{-7*}$	$2.2*10^{-3*}$
SM (OH) C22:1[b]	0.1488	$3.5*10^{-6*}$	0.015
(b) Metabolite, ratio or sum	**Loading on component 2[c]**	***p* value Overall group differences**	***p* value Endurance versus others**
LysoPC a C18:2	*−0.1426*	0.018	0.138
Kynurenine/tryptophan	*−0.1411*	0.050	0.054
LysoPC a C18:1	*−0.1370*	$6.9*10^{-3}$	0.083
Total lysoPC	*−0.1369*	0.020	0.073
CPT-I ratio[d]	*−0.1328*	$2.7*10^{-3*}$	0.042
PC ae C36:5	0.1758	$2.2*10^{-4*}$	$3.8*10^{-3*}$
PC ae C36:4	0.1722	$1.5*10^{-3*}$	$4.9*10^{-3*}$
PC ae C38:6	0.1491	$7.9*10^{-5*}$	0.018
PC ae C38:5	0.1427	0.017	0.023
Alpha-AAA	0.1418	0.060	0.059

[a]Negative *loadings* indicate lower concentration in natural bodybuilders. Positive loadings indicate higher concentration in natural bodybuilders compared to all other groups.

[b]PC ae C38:0 is isobar (same nominal mass) with PC aa C38:7. In human plasma of young healthy men, PC ae C38:0 is considered to contain considerable amounts of PC molecules that carry a fatty acid chain with 22 carbon atoms and 6 double bonds (C22:6), same as for the related measure PC aa C36:6 (Quell et al., 2019). SM (OH) C22:1 and SM (OH) C22:2 labeled as hydroxy-sphingolipids are isobar with odd-chain non-hydroxy sphingolipids (e.g. SM C23:0 and SM C23:1).

[c]*Negative loadings* indicate higher concentration in endurance athletes. Positive loadings indicate lower concentration in endurance athletes compared to all other groups.

[d]Sum of hexadecanoylcarnitine (C16:0) and octadecanoylcarnitine (C18:0) divided by free carnitine (C0).

*Significant comparison after correcting for multiple testing $p < 5.0*10^{-3}$.

three lysoPC measures, the kynurenine/tryptophan ratio and the CPT1-ratio (Figure 3c) which is a proxy measure for a rate-limiting step in ß-oxidation, were higher concentrated in endurance athletes than in all other groups (Table 2b). Out of the 10 metabolites selected in component 2, PC ae C36:4 and PC ae C36:5 differed significantly ($p < 5.0*10^{-3}$) between endurance athletes and all groups when tested one by one (Table 2b).

3.3 | How does a fasted, graded exercise test to exhaustion affect blood metabolite concentrations?

Next, we compared the concentrations of 151 metabolites and 43 metabolite ratios or sums between post-exercise and baseline. After exercise, a third of all metabolite measures (46 metabolites and 12 metabolite ratios or sums) significantly increased. In contrast, only ~5% of all metabolite measures (4 metabolites, 5 ratios or sums) significantly decreased after exercise (Figure 4, Supplementary Table S5).

In detail, the ratio spermidine/putrescine decreased most, while the ratio serotonin/tryptophan increased most across groups (tryptophan decreasing; serotonin increasing). Alanine concentration increased and had the lowest *p*-value (Figure 4a) of metabolites that changed by exercise. Among all amino acids, muscles mainly excrete alanine during fasting, and the blood transports it to the liver for gluconeogenesis (Adeva-Andany et al., 2016). After exercise, the ratio of short chain acylcarnitines to free carnitine (C2+C3/C0), the ratio of acetylcarnitine to free carnitine (C2/C0), short chain acylcarnitines (C2, C3) and

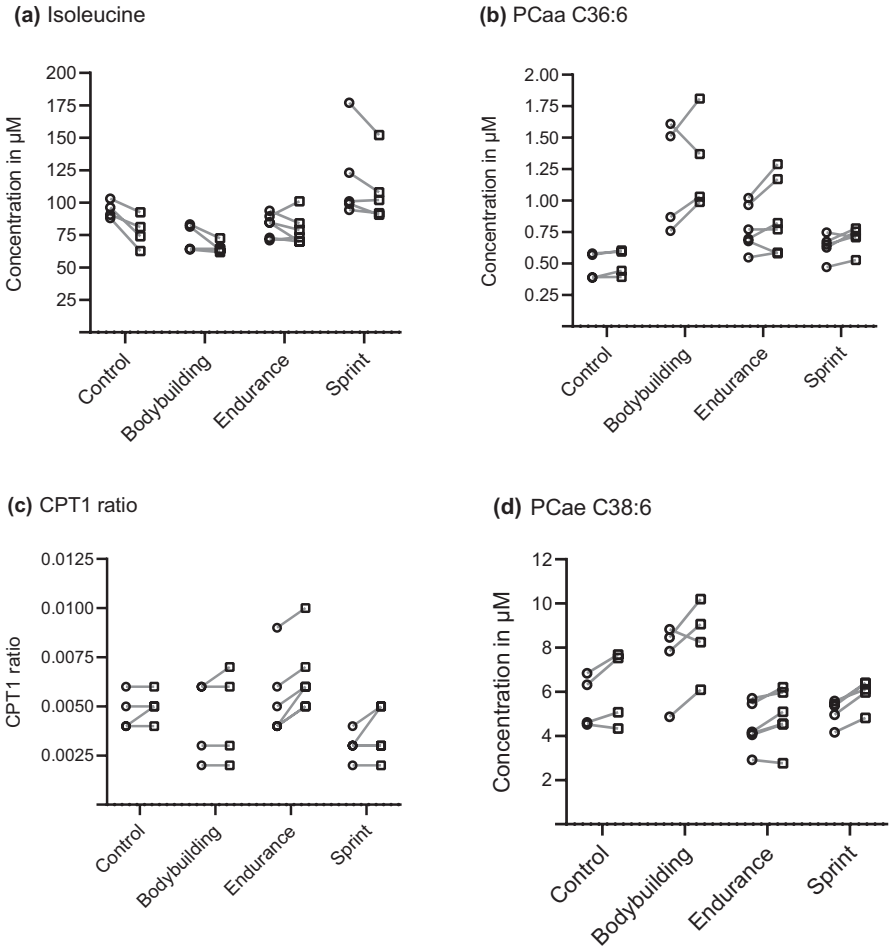

FIGURE 3 Concentration changes for every participant between *baseline* (○) and *post-exercise* (□) for isoleucine (a) and PC aa C36:6 (b), contributing most to the separation of natural bodybuilders and the CPT1-ratio (c) and PC ae C38:6 (d) contributing most to the separation of endurance athletes

the ratio of esterified to free carnitine (total AC/C0) increased, indicating higher ß-oxidation activity. Several complex lipids like PCs and SMs increased after exercise (Figure 4a), suggesting a general increase in blood complex lipids after exercise.

3.4 | Do metabolite concentrations change differently after exercise depending on the group?

Finally, we compared the log2 fold-change of each metabolite between groups (Supplementary Table S6). No metabolite change differed significantly between groups when correcting for 194 tests (151 metabolites, 43 ratios or sums). However, when using a raw *p*-value cut-off of $p < 0.01$, we identified metabolites with suggestive, group-specific responses to exercise.

In endurance athletes, hexose (Figure 5a), which mainly comprises glucose (fasting blood glucose concentration in healthy humans ranges between 4.0 and 5.9 mmol/l) (American Diabetes Association, 2014), butyrylcarnitine (C4), tetradecenoylcarnitine (C14:1), and tetradecadienoylcarnitine (C14:2), had higher fold-changes in response to exercise compared to all other groups. In natural bodybuilders, putrescine and taurine (Figure 5b) stayed almost at the same level form pre-to post exercise, whereas in all other groups, putrescine and taurine increased. In the control group, C14:1 and tetradecanoylcarnitine (C14:0) had lower fold-changes compared to all other groups. Notably, C14:1 was one of those metabolites which increased the highest in endurance athletes.

In sprinters, we found no metabolite that had a *p*-value below 0.01. The metabolites that differed most were serotonin ($p = 0.029$) and the serotonin/tryptophan ratio (Figure 4d) with higher, but not significant, increases in sprinters when compared to all other groups.

4 | DISCUSSION

The aim of this study was to investigate how the selectively adapted metabolism of aerobic, glycolytic, and anabolic athletes affects blood metabolomes at rest or after exercise and in response to exercise. We made the following main observations: First, endurance-trained athletes and natural bodybuilders had unique metabolite concentrations, ratios and sums when compared to sprinters and untrained controls. Second, endurance athletes had higher CPT1-ratios, higher lysoPCs C18:1 and C18:2 and lower levels of highly unsaturated alkyl-acyl-phosphatidylcholines than others. In contrast, natural bodybuilders had lower concentrations

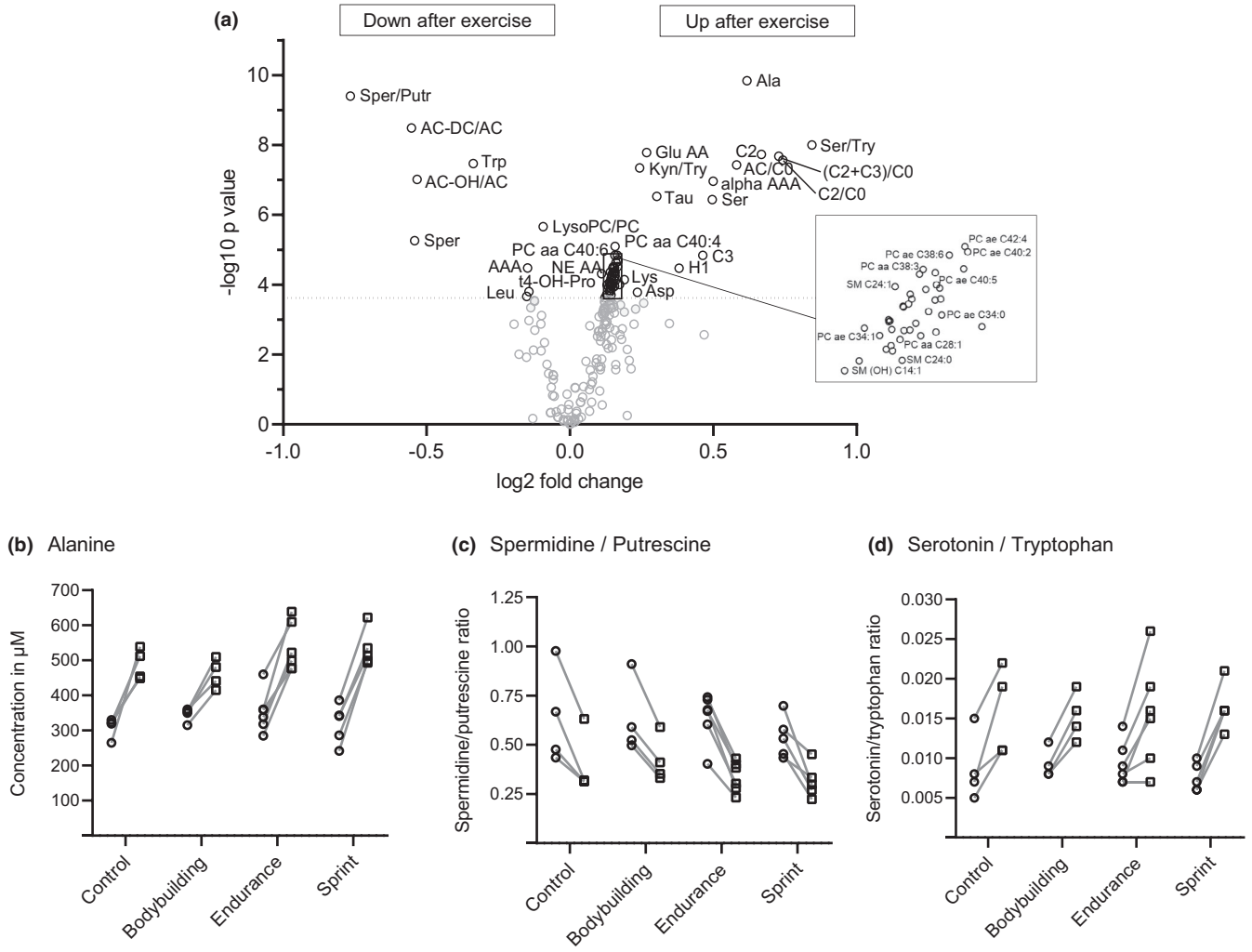

FIGURE 4 Volcano plot (a) showing significant metabolite changes (in black; $\alpha < 2.58*10^{-4}$) after graded cycle exercise in all participants and metabolites with the highest concentration changes from baseline (○) to post-exercise (□) including (b) alanine, (c) spermidine/putrescine and (d) serotonin/tryptophan

of BCAAs, lower tryptophan and higher concentrations of specific phosphatidylcholines and sphingomyelins. Third, ~30% of all serum metabolite measures increased 5 minutes after a graded bicycle ergometry test to exhaustion, whereas ~5% of all metabolite measures decreased. Fourth, some metabolites changed differently during exercise in-between groups but not significantly.

The first and the second main findings are discussed together, followed by the third and the fourth finding separately.

4.1 | Natural bodybuilders have a depleted blood BCAA pool, which might be caused by high muscle growth

Fasted, natural bodybuilders had lower concentrations of leucine, isoleucine, tryptophan, and BCAAs than the other groups. A likely explanation is that higher rates of protein synthesis (McGlory et al., 2017) and a greater muscle mass result in faster declines of amino acids including BCCAs. The standardized nutrition on the day before testing may have contributed to this finding: Based on their dietary reports, natural bodybuilders usually ingested higher amounts of protein (~36%, ~2.4 g/kg bodyweight) than all other groups (20–24%, ~0.9–2.0 g/kg bodyweight). On the day before testing, protein intake was standardized to 20% of total macronutrient intake for all participants. For bodybuilders, this reduced protein intake could have depleted BCAA and tryptophan in blood even faster because bodybuilders need more of these dietary essential amino acids than the other groups due to higher protein synthesis rates. A practical conclusion to the fast overnight depletion of blood amino acids could be that bodybuilders should consider ingesting protein pre-and post-sleep to avoid "running empty" on amino acids.

In general, habitual dietary protein intake can also influence amino acid levels in blood (Durainayagam et al., 2019; Seyedsadjadi et al., 2018). Durainayagam et al. report that doubling protein intake (from 0.8 g/kg bodyweight to 1.6 g/kg bodyweight) for 10 weeks increases tryptophan, creatine, and glutamine levels. Seyedsadjadi et al. report that the intake

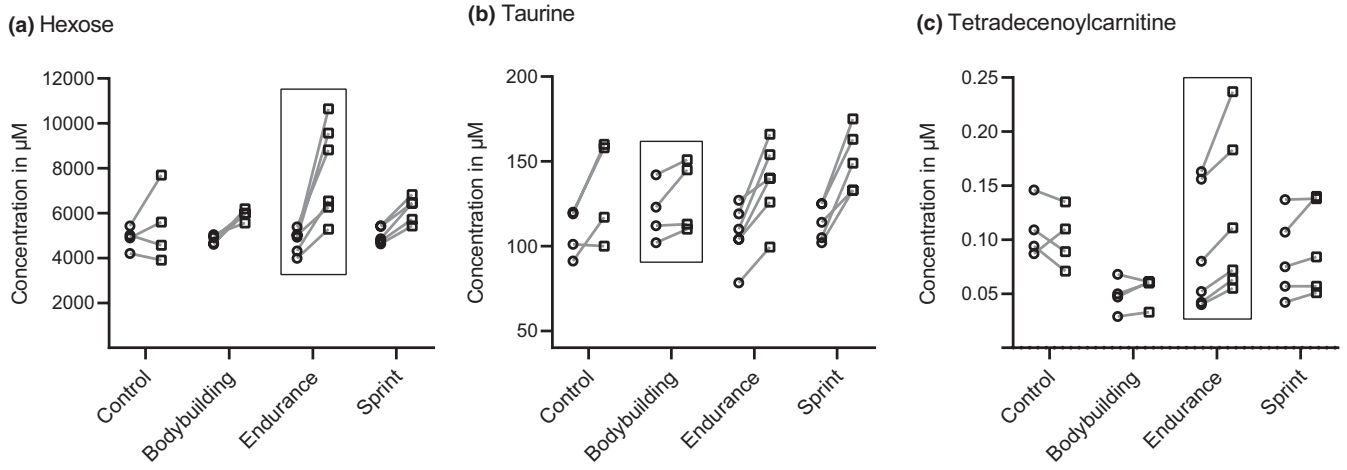

FIGURE 5 Among all 194 metabolite measures, hexose (a), taurine (b) and tetradecenoylcarnitine (c) showed suggestive group-specific responses between baseline (○) and post-exercise (□)

of ~94 g protein per day increases tryptophan and kynurenine but do not provide data on relative protein intake in g/kg bodyweight. Contrasting to both studies, tryptophan levels in bodybuilders were lower than in all other groups in our study. We are not aware of any study that has shown how the habitually high protein intake of the participating natural bodybuilders (~36%) affects the blood metabolome long-term.

4.2 | Natural bodybuilders have higher levels of two docosahexaenoic acid derivatives which may originate from supplemented fish oils

PC aa C36:6 and PC ae C38:0 concentrations were higher in natural bodybuilders when compared to all other groups, with the largest differences observed for PC aa C36:6. Quell et al. recently showed that blood PC aa C36:6 measured in healthy young men mainly comprises a derivative of DHA, a fatty acid with 22 carbons and 6 double bonds (C22:6). Quell et al. found PCaas that contain DHA as one, and tetradecanoic acid as the second fatty acid chain (PC 14:0_22:6) account for the major part (~88%) of measured PC aa C36:6 concentrations (Quell et al., 2019). They also suggested the second PCae that was higher in natural bodybuilders (PC ae C38:0) to be a DHA derivative. The measure labeled as PC ae C38:0 includes the concentrations of the isobaric (i.e., showing the same mass spectrometric signal) PC aa C38:7 with C22:6 as one of the two fatty acid chains. Natural bodybuilders may have higher levels of DHA-containing PCs as some bodybuilders supplemented fish oils. Fish oils are rich in omega-3 fatty acids including DHA and eicosapentaenoic acid (EPA, C20:5) and have been reported to increase muscle protein synthesis via increased mTOR and p70 S6 k signaling (Smith et al., 2011). Especially two natural bodybuilders (B3 and B4) who either ingested omega-3 capsules (B3, Supplementary table S3) or ate omega-3 rich oils (around 20 g daily), chia seeds (around 20 g daily), and fish (weekly) (B4) had high concentrations of DHA-containing PCs. Among the other participants, only endurance athlete E3 ate fish regularly. E3 had the highest baseline concentration in PC aa C36:6 next to B3 and B4, but no notable elevation in PC ae C38:0. No other participant reported rapeseed oil, linseed oil, or chia seeds or fish in their nutrition. Besides the DHA derivatives, the concentrations of two sphingomyelins which also contain fatty acids with 22 carbons were higher in natural bodybuilders than in all other groups. Whether this is similarly a result of the natural body builders diet or because other factors play a role is unclear. Collectively, the overall pool of 22 carbon fatty acid-containing molecules such as PCs or sphingomyelins is higher in natural bodybuilders, which might be in part because of their diets.

4.3 | Endurance athletes have higher CPT1-ratio, suggesting higher fat oxidation

Endurance athletes had higher CPT1-ratios than all others, especially sprinters. CPT1 is a mitochondrial transmembrane enzyme that catalyses a reaction essential for the transport of fatty acids from the cytosol into the mitochondria, where fatty acids enter β-oxidation (Lundsgaard et al., 2018). Metabolomics analyses allow to estimate the activity of the CPT1 reaction via its reaction products hexadecanoylcarnitine (C16:0) and octadecanoylcarnitine (C18:0) versus the concentration of free carnitine (C0). The CPT1-ratio could be a biomarker either for a higher capacity for fat oxidation or for acutely higher fat oxidation rates in endurance athletes. Supporting this assumption, endurance athletes had the lowest respiratory exchange ratios (RERs) of all groups, at rest and during the exercise test, indicating higher fat oxidation compared to the other groups (Supplementary Figure S3). Further studies should seek to clarify the association between

the CPT1-ratio, the capacity for fat oxidation, CPT1 activity and the acute rate of fat oxidation.

4.4 | Endurance athletes have higher lysoPC a C18:1 and C18:2 concentrations, which may be linked to cardiovascular fitness

Endurance athletes had higher concentration of lysoPC a C18:1 than bodybuilders and controls. Earlier studies have already associated lysoPCs containing 18 carbons with endurance traits (Felder et al., 2017; Schader et al., 2020). Specifically, lysoPC a C18:1 was, among other lysoPCs, reported to increase after several weeks of endurance training (Felder et al., 2017). Another lysoPC C18:2 was found to be elevated after a marathon race in subjects with high VO_2max (63.3 ± 5.2 ml/kg/min) that is similar to the VO_2max of our endurance athletes (Table 1), but not in subjects with low VO_2max (41.8 ± 5.5 ml/kg/min) (Schader et al., 2020) that is similar to the VO_2max of natural bodybuilders and controls (Table 1). Supporting the association between cardiovascular fitness and these lysoPCs, lysoPC a C18:0 and lysoPC a C18:2, were shown to be lower in patients with heart failure, who typically have a lower VO_2max (17.2 ± 7.2 ml/kg/min), than in healthy controls (Marcinkiewicz-Siemion et al., 2018). LysoPCs are generated by phospholipases A (PLA) from PCs. Overexpression of a specific PLA, phospholipase A2 type IIA (PLA2G2A), which is secreted to blood and expressed in skeletal muscle and adipose tissue (Prunonosa Cervera et al., 2021), increased the metabolic rate, and improved both insulin sensitivity and glucose tolerance in mice (Kuefner et al., 2017). Interestingly, mice expressing PLA2G2A compared to mice without PLA2G2A expression had higher uncoupling protein 1 (UCP-1) and higher peroxisome proliferator-activated receptor-gamma coactivator-1-alpha (PGC1-alpha) expression in adipose tissue, suggesting a role of PLA2G2A in adipose tissue browning (Kuefner et al., 2017). Among others PGC1-alpha is known as a major regulator for mitochondrial biogenesis after endurance exercise in humans. Furthermore, there is first evidence in humans that exercise can increase PLA2G2A expression in adipose tissue (Imam, 2019). Collectively, increased lysoPCs in endurance athletes may point to exercise-associated increases in specific phospholipases that are beneficial for metabolic health.

4.5 | Endurance athletes have lower levels of highly unsaturated PCaes, which are ligands of endurance adaptation regulators

Endurance athletes had lower concentrations of specific PCaes (PC ae C36:5, PC ae C36:4, PC ae C38:6) than all other groups. Functionally, PCae can act as ligands of signaling molecules like PPARγ (Dean & Lodhi, 2018), which is a known regulator of the mitochondrial biogenesis adaptation to endurance exercise. We therefore speculate that endurance exercise decreases certain blood PCaes, because they are needed in intramuscular signaling for signaling processes in adaptation.

4.6 | In all subjects, fasted, graded cycle ergometry to exhaustion affects energy metabolism

Consistent with other studies (Contrepois et al., 2020; Morville et al., 2020; Schranner et al., 2020), most metabolites that changed after exercise in all participants were energy metabolites related to glucose or fat degradation. Specifically, the concentrations of gluconeogenic and glycolytic metabolites such as alanine and hexose (mainly glucose) increased after exercise. Increases in blood glucose concentration during exercise probably result from hepatic glucose production via glycogenolysis and gluconeogenesis (Brooks, 2020; Kjær, 1998). Measures of overall fatty acid oxidation activity (C2+C3/C0), even-numbered fatty acid oxidation activity (C2/C0) and the concentrations of short chain acylcarnitines (C2, C3) all increased. Exercise is known to increase lipolysis in fat tissue and fat oxidation within mitochondria (Lundsgaard et al., 2018), leading to increased acylcarnitine levels in blood (Schranner et al., 2020).

4.7 | In all subjects, exercise increases tryptophan breakdown to serotonin and kynurenine, which links to mental health effects of exercise

Exercise increased the ratios of serotonin/tryptophan and kynurenine/tryptophan as well as increased the serotonin concentration and decreased the tryptophan concentration. In contrast, kynurenine concentrations did not change significantly. Thus, we assume that the increase in kynurenine/tryptophan is only because tryptophan decreased, suggesting that exercise shifted the tryptophan breakdown towards serotonin. Serotonin can be broken down to melatonin and positively regulate mood or sleep (De Crescenzo et al., 2017; Zimmer et al., 2016). Serotonin increases were shown to depend on exercise intensity (Zimmer et al., 2016), which explains the significant increase of serotonin after maximum exercise done in our study. Supporting our findings, Strasser et al. also found decreased tryptophan concentrations in athletes after exercise (Strasser et al., 2016). Collectively our data confirm that acute exercise alters metabolites that are associated with mood and mental health.

4.8 | In all subjects, exercise increases complex lipids, especially PCaes

Complex lipids can act as ligands for cell signaling and can be used for fat oxidation. We assume that increased concentrations of complex lipids after maximum exercise are a sign of increased lipolysis and oxidation in the fasted state. In line with that, total PCae concentrations increased in rat livers after acute exercise (Hoene et al., 2016), which could show a higher demand for muscular fatty acid oxidation. In contrast, several studies reported that complex lipids decrease after exercise in non-fasted humans (Karl et al., 2017; Nieman et al., 2013; Schader et al., 2020). Therefore, we assume that in fasted but not in non-fasted subjects, complex lipids like PCae or PCaa are used for fat oxidation during exercise.

4.9 | In all subjects, exercise increases polyamines, which are related to muscular hypertrophy

Spermidine and the spermidine to putrescine ratio decreased after exercise. As putrescine did not change, the observed decrease in the spermidine to putrescine ratio is mainly because of the decrease in spermidine. Polyamine concentrations in skeletal muscle are associated with hypertrophy (Cepero et al., 1998; Turchanowa et al., 2000) and muscle regeneration after injury (Kaminska et al., 1982) in rats. We assume that the blood spermidine pool decreases after exercise not because of changed spermidine synthesis, but because of higher spermidine demand in muscle after exercise e.g. to regenerate. Mechanistically, it is still unclear why muscular polyamine concentrations increase after exercise (Lee & MacLean, 2011) but it seems that androgens like testosterone, which also increase muscle mass and strength, regulate polyamine synthesis (Cyriac et al., 2002). Eventually, it is unclear if and how polyamine concentration changes after exercise in blood relate to intra-muscular processes.

4.10 | In endurance athletes, blood glucose concentration increased more after exercise than in all other groups

Despite the low sample size and lack of significance after stringent multiple testing correction, we briefly discuss metabolites that changed differently in-between groups after exercise. Specifically, we highlight the differences found in endurance athletes.

After exercise, hexose (mainly glucose) concentrations increased in all athletes but most in endurance athletes. Despite strenuous exercise, hexose increased only in 2 out of 4 untrained controls. Intensive exercise increases hepatic glucose production via glycogenolysis and gluconeogenesis by 2–3 fold (Brooks, 2020) to prevent hypoglycemia. Collectively, this suggests that endurance athletes either have a high ability for hepatic glucose synthesis and/or less muscular glucose uptake because of higher rates of fat oxidation during submaximal exercise.

4.11 | In endurance athletes, medium/long-chain acylcarnitines increased more after exercise than in all other groups

During exercise, the concentrations of C14:1 and C14:2, which are involved in the ß-oxidation of long-chain fatty acids, increased highest in endurance athletes especially when compared to untrained controls. We assume that this shows different usage or availability of long-chain fatty acids for fat oxidation. Recently, several other long-chain acylcarnitines have been associated with endurance exercise variables (Al-Khelaifi et al., 2018). Collectively, this suggests that endurance athletes may metabolize long-chain fatty acids differently than subjects who are not endurance trained.

4.12 | Limitations

This study has several limitations. First our study cohort was small, restricting the statistical power for group comparisons. We justify this small cohort with the large differences of glycolytic capacity, aerobic capacity, and anabolism in the four groups investigated. Following a hypothesis-free approach, we indeed observed significant differences in the metabolomes of these highly selective groups, despite the relatively low sample size and variations within groups. However, due to the limited statistical power in our study and because results of the PLS-DA might be biased towards the groups with the biggest differences observed (bodybuilders vs. all others and endurance vs. all others), we might have missed less pronounced differences, in particular potential differences between sprinters and controls. Therefore, we cannot draw any robust conclusion on the differences between these two groups. Still, we consider that the metabolomes of sprinters and controls are more similar than those of the other groups in this study. Moreover, the choice of PLS-DA might have biased the selection of metabolites for differential analysis (Ruiz-Perez et al., 2020). Second, we only used endurance exercise as an exercise mode, which activates only a subset of metabolic enzymes in the musculature. Other exercise modes such as resistance exercise would have stimulated other branches of metabolism (Morville et al., 2020) and may have revealed other group-specific changes in the

metabolome. With choosing a standardized maximum endurance test, we did not aim to report metabolite changes of endurance training per se but those of a metabolic challenge to metabolism. Contrepois et al. showed that such standardized maximum exercise testing is sufficient to show phenotypic differences in metabolism (Contrepois et al., 2020). Third, we measured a limited set of metabolite classes mainly lipids (e.g. acylcarnitines) and amino acids. We assumed that acute, fasted exercise particularly challenges lipid energy metabolism and shows differences between athlete groups with differently well-developed lipid metabolism. Furthermore, we assumed that especially natural bodybuilders, who have high protein synthesis, have different baseline amino acid levels than other athletes. Amino acids were also of interest because previous studies inconsistently reported amino acid changes after exercise (Schranner et al., 2020). Fourth, after study completion, subject E5 reported a nightly ingestion of ~1 mg of melatonin, which was against our inclusion criteria. Studies suggest that melatonin has effects on several organs (Opie & Lecour, 2016) besides the brain. Possibly, this influenced E5's metabolite levels at baseline, post-exercise or the level changes by exercise. Specifically, melatonin ingestion can affect metabolites of its related pathway, the tryptophan-serotonin pathway. In the PLS-DA, E5 appeared metabolically different from the cluster of E1-E4. However, this difference is not attributable to different serotonin levels, as E5 showed no conspicuous serotonin levels compared to E1-E4. Fifth, special nutrition or dietary supplements may influence certain metabolite concentrations long-term. As suggested by higher DHA levels in natural bodybuilders in our study, refraining from dietary supplements for 48 hours before a metabolomics analysis may not be long enough to eliminate all nutritional influences on certain blood metabolites. However, it is also questionable if longer restriction of supplements for several days is enough to wash out long-term dietary influences. Studies that investigated dietary effects on the metabolome assessed diets between 2 weeks and 6 months (Guasch-Ferré et al., 2018). Controlling supplementary intake that long is problematic when working with ambitious athletes. Sixth, we additionally provided 500–1000 kilocalories for athletes (Supplementary Table S7) on the day before the study as they have higher energy demand than sedentary controls. Increased caloric intake included all classes of macronutrients but slightly higher fat intake (~25%) when compared to controls (~20%). Dietary fat intake on the day before the study could have influenced acylcarnitine levels of the natural bodybuilders because they habitually ate low-fat (~14.7%). Low levels of C14:2 acylcarnitine have been associated with higher intake of fats such as butter (Floegel et al., 2013). Complex lipids like PCae's and PCaa's are not influenced by short term but by long-term fat intake over weeks and months (Saadatian-Elahi et al., 2004). Seventh, as expected in highly specialized athletes, we observed significant differences in body fat and suggestive differences in muscularity between groups (Table 1). A population-based study by Jourdan et al., (2012) found that a high fat free mass index (FFM kg/height²) which corresponds to low body fat, was associated with higher levels of BCAAs, acylcarnitines, and a shift in phosphatidylcholine composition, chain length and saturation (Jourdan et al., 2012). In the case of BCAA, we see the highest concentrations in the group with the lowest body fat (sprinters) but no consistent association, across our cohort.

5 | CONCLUSION

In conclusion, we found systematic differences in the concentrations of metabolites in-between highly trained glycolytic, aerobic, and anabolic athletes. Moreover, we observed different metabolite changes in-between groups that were not significant but worth of mentioning. The observed metabolic differences of years of training could give hints on which exercise mode can change specific metabolites or metabolite classes. However, influences on the metabolome are manifold and further studies are needed to disentangle the specific contributions of genetic variants, of adaptations to sports-specific exercise training or of special nutrition to the systematic metabolic differences between differently trained individuals.

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CONFLICT OF INTEREST

The authors declare that they have no competing financial or non-financial interests.

AUTHOR CONTRIBUTIONS

HW had the idea for the study. HW and MS designed the study. DS, MS, JS (Johannes Scherr), JS (Jürgen Schlegel), OZ, AR, SK, MH, QS, FS, and FK conducted human experiments and gathered the data. CP and JA measured the samples. DS, WRM, and GK analyzed the data. DS, MS, GK and, HW interpreted the results and wrote the manuscript. All authors proof-read and approved the final version of the manuscript.

ETHICAL APPROVAL STATEMENT

This study conforms to the Declaration of Helsinki for use of human subjects and tissue and was approved by the medical ethics committee of the Technical University of Munich (356/17S).

CONSENT TO PARTICIPATE

Participants were fully informed of the nature and possible risks of the study before they gave their written informed consent.

CONSENT FOR PUBLICATION

Participants were fully informed that their data will be made publicly available to the scientific community after anonymization.

DATA AVAILABILITY STATEMENT

The MS raw data and sample information of this study will be made available within the MetaboLights (Haug et al., 2020) repository under the accession number MTBLS2104 (https://www.ebi.ac.uk/metabolights/MTBLS2104).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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III-3: Publication 3: Experimental study relating blood proteins to cardiorespiratory fitness and to cardiorespiratory fitness gains after 20 weeks of exercise training

Title: Human plasma proteomic profiles indicative of cardiorespiratory fitness

Authors: Jeremy M. Robbins, Bennet Peterson, Daniela Schranner, Usman A. Tahir, Theresa Rienmüller, Shuliang Deng, Michelle J. Keyes, Daniel H. Katz, Pierre M. Jean Beltran, Jacob L. Barber, Christian Baumgartner, Steven A. Carr, Sujoy Gosh, Changyu Shen, Lori L. Jennings, Robert Ross, Mark A. Sarzynski, Claude Bouchard & Robert E. Gerszten

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III-3.1. Personal contributions

Personal contribution statements are written in the first person singular.

III-3.1.1. Data analysis

I did post-statistical pathway analysis using web-based pathway search machines and manual annotation using web-based protein databases.

III.3.1.2. Biological interpretation of the data

The main part of my work for this paper was the biological interpretation of the proteomics data, which included the identification of functional subunits of proteins related to specific organ systems of VO2max or mechanism relevant in the adaptation to endurance exercise and the comparison of these with relevant literature.

III.3.1.3. Manuscript writing

I wrote the parts of the manuscript which discuss muscle and bone proteins that we found to be related to cardiorespiratory fitness and drew the respective figures. I solely was responsible for compiling the supplementary data files.

III-3.2. Summary

Maximal oxygen uptake ($\dot{V}O_2max$) is a direct measure of human cardiorespiratory fitness and is associated with health (e.g. all-cause mortality). However, the molecular determinants of interindividual differences in baseline (intrinsic) $\dot{V}O_2max$, and of increases of $\dot{V}O_2max$ in response to exercise training ($\Delta\dot{V}O_2max$), are largely unknown. Here, we measure ~5,000 plasma proteins using an affinity-based platform in over 650 sedentary adults before and after a 20-week endurance-exercise intervention and identify 147 proteins and 102 proteins whose plasma levels are associated with baseline $\dot{V}O_2max$ and $\Delta\dot{V}O_2max$, respectively. Proteins positively associated with baseline $\dot{V}O_2max$ were related to angiogenesis (e.g. extracellular matrix protein 1), coagulation and hematopoiesis (e.g. tetranectin), lipid metabolism (e.g. apolipoprotein F) and included structural and functional muscle proteins (e.g. alpha-actinin 2, troponin-I, myosin light chain 3), and proteins involved in glycolysis (e.g. beta-enolase or lactate dehydrogenases A and B). Proteins with the strongest associations with $\Delta\dot{V}O_2max$ included 5'nucleotidase, IL-22 binding protein and fibromodulin. Only 5 proteins of those associated with $\dot{V}O_2max$ and $\Delta\dot{V}O_2max$ overlapped including insulin. Addition of a protein biomarker score derived from these proteins to a score based on clinical traits improved the prediction of an individual's $\Delta\dot{V}O_2max$. We validate findings in a separate exercise cohort, further link 21 proteins to incident all-cause mortality in a community-based cohort and reproduce the specificity of ~75% of our key findings using antibody-based assays. Taken together, our data shed light on biological pathways relevant to cardiorespiratory fitness and highlight the potential additive value of protein biomarkers in identifying exercise responsiveness in humans.

III-3.3. Original manuscript

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Human plasma proteomic profiles indicative of cardiorespiratory fitness

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Maximal oxygen uptake (VO_2max) is a direct measure of human cardiorespiratory fitness and is associated with health. However, the molecular determinants of interindividual differences in baseline (intrinsic) VO_2max, and of increases of VO_2max in response to exercise training (ΔVO_2max), are largely unknown. Here, we measure ~5,000 plasma proteins using an affinity-based platform in over 650 sedentary adults before and after a 20-week endurance-exercise intervention and identify 147 proteins and 102 proteins whose plasma levels are associated with baseline VO_2max and ΔVO_2max, respectively. Addition of a protein biomarker score derived from these proteins to a score based on clinical traits improves the prediction of an individual's ΔVO_2max. We validate findings in a separate exercise cohort, further link 21 proteins to incident all-cause mortality in a community-based cohort and reproduce the specificity of ~75% of our key findings using antibody-based assays. Taken together, our data shed light on biological pathways relevant to cardiorespiratory fitness and highlight the potential additive value of protein biomarkers in identifying exercise responsiveness in humans.

Oxygen uptake (VO_2) represents a measure of the body's capacity to supply oxygen to skeletal muscle to perform physical work. VO_2 reflects the integration of multiple organ systems and cellular processes, including pulmonary ventilation, oxygen carrying capacity and transport through the circulatory system, cardiac output, central nervous system recruitment of motor units, oxygen diffusion and extraction at the capillary–skeletal muscle level, as well as mitochondrial respiration. VO_2max defines the limits of these processes and is thus widely considered the gold-standard measure of cardiorespiratory fitness (CRF)[1,2].

It is thus not surprising that VO_2max (as a direct measure of CRF) has been firmly established as a powerful prognostic marker of cardiovascular disease (CVD) and all-cause mortality[3]. VO_2max's inverse relationship with CVD and mortality risk applies to both its baseline measure (intrinsic VO_2max[4,5]) and capacity to improve VO_2max through regular physical activity (acquired or adaptive VO_2max; ΔVO_2max)[6,7]. Consequently, there has been significant interest in characterizing the relative contributions of different organ systems to VO_2max. Several lines of evidence point to cardiac output and oxygen delivery as being the principal determinants of VO_2max[8,9]; however, even the precise contributions of these processes, including oxygen diffusion, convection and mitochondrial oxidative capacity, are not fully resolved[10,11].

Furthermore, both baseline measures of VO_2max and ΔVO_2max appear to vary greatly in the general population. In the HERITAGE Family Study, a subgroup of 429 apparently healthy but sedentary members of family units, who were of European descent, underwent direct measurements of baseline VO_2max through cardiopulmonary exercise testing (CPET) on 2 separate days, and the s.d. (9 ml O_2 kg^{-1} min^{-1}) was ~29% of the mean (31 ml O_2 kg^{-1} min^{-1}) after adjustment for age, sex, body mass and body composition[12]. Similarly, among 720 HERITAGE participants who completed the supervised 20-week endurance-exercise training programme, the s.d. was 53% of the mean change in VO_2max. Interestingly, there was no relationship between baseline and ΔVO_2max in this group ($r^2 = 0.011$). This suggests that these traits may have different biologic underpinnings and underscores our inability to predict VO_2max 'trainability' using existing clinical factors[13].

Given our incomplete understanding of the biologic basis of CRF and its close relationship to long-term health outcomes, uncovering the molecular determinants of VO_2max may provide insights into the mechanistic links between physical fitness and well-being. Indeed, this has become an important goal of the medical community. Prior efforts to characterize both baseline and acquired CRF at the molecular level have included genetic analyses, transcriptomic profiling of skeletal muscle and plasma metabolomics[14–16]. Although biochemical profiling of plasma proteins has yielded insights into differences in substrate metabolism among different fitness states in animal models[17] and has provided biologic 'snapshots' of human metabolism[18], few data exist regarding plasma

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Table 1 | HERITAGE cohort clinical characteristics

Clinical characteristics	Participants with baseline VO_2max ($n=745$)	Participants with baseline and post-training VO_2max ($n=654$)
Age, mean (s.d.), years	34.3 (13.4)	34.8 (13.6)
Female, *n* (%)	409 (54.9)	361 (55.2)
European descent, *n* (%)	457 (61.3)	424 (64.8)
BMI, median (interquartile range), kg/m^2	25.5 (22.4–29.7)	25.5 (22.5–29.7)
Maximal oxygen uptake, mean (s.d.), ml min^{-1}		
Baseline	2,345 (726)	2,348 (732.5)
Change after exercise training	–	383 (202.8)
SBP, mean (s.d.), mmHg	119 (12.0)	119 (11.8)
DBP, mean (s.d.), mmHg	69 (8.9)	68 (8.8)
Resting heart rate, mean (s.d.)	65 (8.9)	65 (8.9)

Mean (s.d.) and median (25–75%) values are shown.

proteomics profiling of CRF in humans, particularly in the context of exercise training. These limitations are in part due to the technical challenges involved in capturing the highly dynamic range of circulating proteins. Advancements in aptamer-based profiling methods now allow for the high-throughput measurement of over 5,000 proteins[19]. This technology spans a dynamic range of at least 7 orders of magnitude (~100 fM–1 μM) with demonstrated high assay reproducibility across both hospital- and population-based cohorts[20,21], and was recently applied in the HERITAGE study[22].

Here, we sought to compare the circulating proteomic profiles of baseline VO_2max as well as its adaptation to an exercise programme by applying a large-scale, affinity-based platform in more than 650 healthy but sedentary participants before and after a 20-week supervised endurance-exercise training intervention. We hypothesized that plasma protein signatures associated with VO_2max would reflect its integrative biology and highlight proteins related to skeletal muscle, hematopoiesis and the vascular system, among other determinants of CRF. Further, given that clinical traits are weakly correlated with VO_2max changes following exercise training, we anticipated that the addition of plasma proteins would improve the capacity to predict VO_2max responsiveness. Finally, given that both baseline VO_2max as well its capacity to change in response to exercise training are associated with future risk of death, we tested whether plasma proteins related to these measures would be associated with incident all-cause mortality in a separate population-based study.

Results

HERITAGE participant characteristics. The HERITAGE cohort was composed of adult parents and their biologic offspring. The mean (s.d.) age of the full cohort ($n=745$) used for baseline VO_2max analyses was 34.3 (13.4) years; 288 were African American (39%), 409 were women (55%) and 503 were offspring (68%). Mean (s.d.) baseline VO_2max was 2,345 (726) ml min^{-1}. Among the participants with VO2max measurements before and after exercise training ($n=654$), the mean ΔVO_2max was 383 (203) ml O_2 min^{-1} (Table 1).

Plasma proteins associated with baseline levels of VO_2max. We measured ~5,000 proteins using a multiplexed, single-stranded DNA aptamer (SOMAmers) assay (Supplementary Table 1). We first tested for age- and sex-adjusted protein associations with baseline VO_2max in the offspring generation ($n=503$) and then sought to replicate our findings in the parent generation ($n=242$). We identified 94 proteins that were associated with VO_2max in the offspring by using a false-discovery rate (FDR) threshold of <1%. Fifty of 94 proteins were associated with VO_2max in the parents at nominal significance ($P<0.05$) and 90/94 were directionally consistent (Fig. 1). We subsequently collapsed these subgroups for all further analyses.

In the full cohort, we identified 147 circulating proteins that were associated with baseline VO_2max (Fig. 2), including 85 proteins that were positively associated and 62 proteins negatively associated in analyses that were adjusted for age, sex, body mass index (BMI) and race (Supplementary Table 2). Proteins positively associated with baseline VO_2max spanned organ systems and biologic processes relevant to CRF including angiogenesis (for example extracellular matrix protein 1 (ECM1) and anthrax toxin receptor 2 (ANTXR2)), coagulation and hematopoiesis (for example, complement decay-accelerating factor (DAF) and tetranectin (TN)) and lipid metabolism (for example apolipoprotein F (APOF) and lipase member K (LIPK)). Interestingly, we found a large number of circulating proteins related to striated muscle structure and function (Fig. 3 and Supplementary Table 3). These included actin and myosin stabilizing molecules (for example, alpha-actinin 2 (ACTN2) and myomesin-2 (MYOM2)); proteins involved in muscle contraction (for example, troponin-I (TNNI2) and myosin-binding protein C (MYBPC1)); and two essential myosin light-chain elements (MYL3 and MYL6B) that regulate force production during muscular cross-bridge cycles. We also identified several muscle-isoform-specific enzymes involved in glycolysis in plasma, including beta-enolase (ENOB), ALDOA, phosphoglycerate mutase 1 (PGAM1) and 2 (PGAM2) and lactate dehydrogenase alpha (LDHA) and beta (LDHB).

These baseline cross-sectional analyses also identified several well-known markers of metabolic dysregulation known to be positively associated with adiposity, including leptin, CRP and insulin, which were inversely associated with baseline VO_2max. Thus, we adjusted for additional measures of body composition—body fat percentage and fat-free mass—to further examine the role of adiposity in our results. We found that the relationships between these proteins and VO_2max were no longer significant after adjustment for body fat percentage but remained significant after adjustment for fat-free mass (Supplementary Table 4). In contrast to these markers of metabolic dysregulation, the striated muscle proteins described above (and in Supplementary Table 3) maintained their correlation with baseline VO_2max after adjustment for body fat percentage but not fat-free mass, suggesting that their association with CRF may proceed through their relationship to lean body mass.

Among the 85 proteins positively associated with baseline VO_2max following multivariate adjustment, 25 were known to be secreted based on UniProt Consortium data (Supplementary Table 2). The group of secreted proteins included multiple proteins related to bone homeostasis, including members of osteoblast differentiation (SPARC-related modular calcium binding protein 1 (SMOC1)), bone metabolism via TGF-ß signalling (NOG, bone morphogenic protein 8B (BMP8B)) and structural components of hyaline cartilage (COL9A1, COMP, EPYC; Extended Data Fig. 1).

Test results for the interaction of generation, sex and race on protein–VO_2max relationships are shown in Supplementary Table 5. Although we identified 23 protein–generation interactions at nominal significance (P value < 0.05; highlighted in Supplementary Table 2), all were directionally consistent among parents and offspring. Similarly, all 20 protein X sex interactions were directionally consistent among males and females. Only Tartrate-resistant

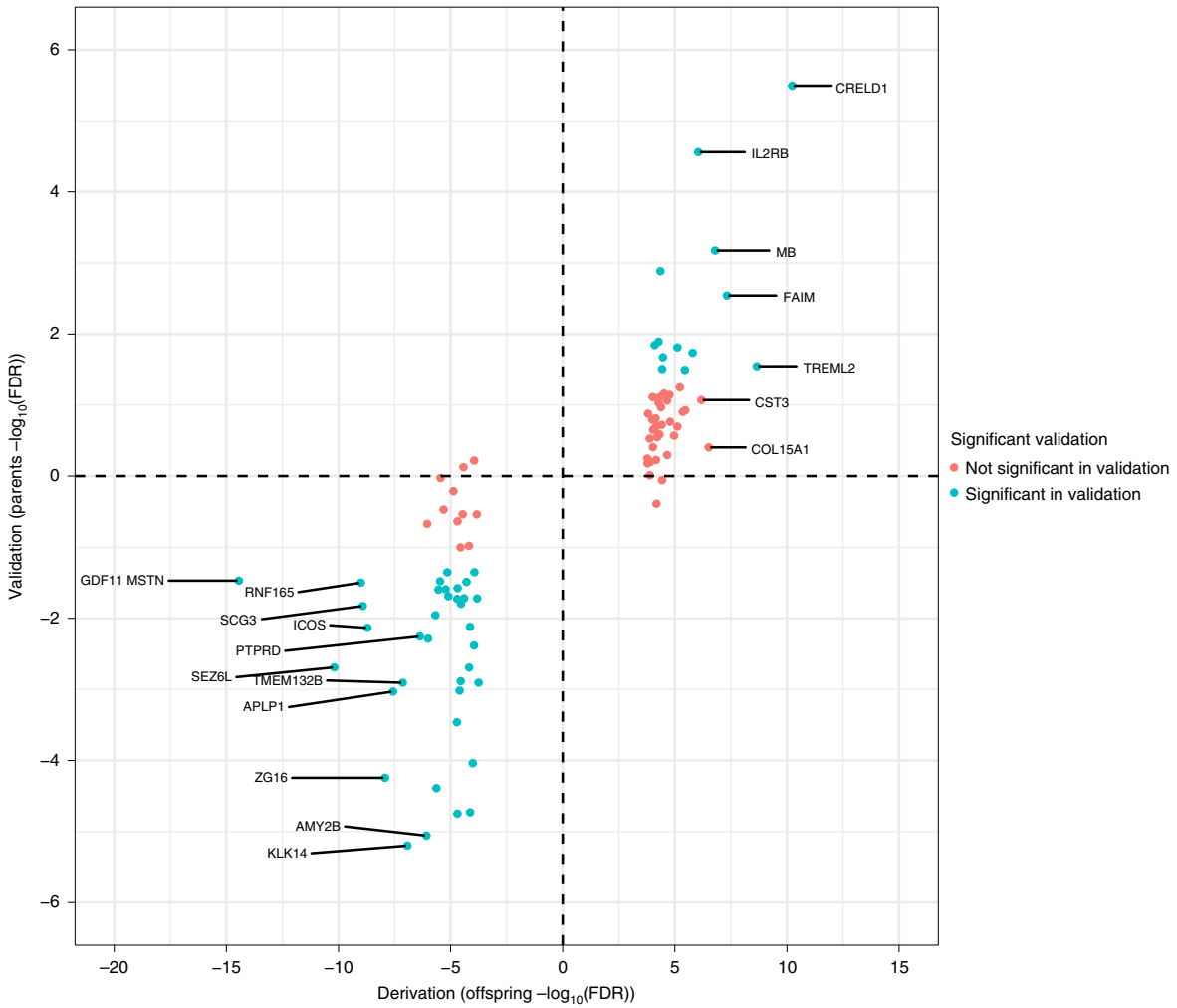


Fig. 1 | Proteins associated with baseline VO_2max among offspring and parents. Protein associations (FDR < 1%) using linear regression were determined first in the offspring cohort ($n = 433$). We subsequently validated 50/94 proteins in the cohort of parents ($n = 221$; $P < 0.05$). Ninety of 94 proteins were directionally consistent, indicated by quadrant (increase in parents and offspring, upper right; decrease in parents and offspring, lower left).

acid phosphatase type 5 (ACP5) and Neural cell adhesion molecule L1-like protein (NRCAM) were directionally different among the 29 protein-VO_2max associations that were different between racial groups, with both ACP5 and NRCAM having a positive association with VO_2max among African Americans and negative association among Caucasians (ACP5, $\beta = 2.5$ and -93.6, respectively; P for interaction = 0.003; NRCAM, $\beta = 21.1$ and -10.6, respectively; P for interaction = 0.02). All data have been made available and are available through the NIH Common Fund Molecular Transducers of Physical Activity Consortium (MoTrPAC; https://motrpac-data.org/related-studies/heritage-proteomics).

Validation of baseline VO_2max findings in an external cohort. To further assess the generalizability of our findings, we performed a similar proteomics screen in a separate cohort of abdominally obese individuals who were enroled in a dose–response trial of endurance exercise[23]. Participants in the validation study subgroup were older (mean age = 47) and had larger body mass (median BMI = 32.8) than HERITAGE participants. A higher percentage of the validation study subgroup was female (71%), and all participants were of European descent (Supplementary Table 6). Of the top 147 proteins associated with baseline VO_2max in HERITAGE, 107 were available in the validation dataset. Seventy-nine proteins were directionally consistent, and 24 met statistical significance in the validation cohort in a linear regression model adjusted for age, sex and BMI ($P < 0.05$; Supplementary Table 7).

Proteins associated with VO_2max changes to exercise training. We found 102 baseline proteins that were associated with ΔVO_2max in a linear regression model adjusted for age, sex, BMI, race and the baseline level of VO_2max (Supplementary Table 8). The proteins with the strongest associations with ΔVO_2max included: 5′ nucleotidase (NT5E), a cell-surface protein that hydrolyses extracellular nucleotides into membrane permeable nucleosides and in which cognate gene variants have been associated with premature arterial calcification[24]; IL-22 binding protein (IL22RA2), a soluble receptor whose ligand is involved in insulin and glucose homeostasis[25]; and fibromodulin (FMOD), a secreted protein that has been implicated in tissue repair and myogenic regulation through its interaction with myostatin[26].

A generation–protein interaction on ΔVO_2max was found for four proteins, with hepcidin (LEAP1) having directionally different associations among parents and offspring (Supplementary Table 9). Eleven proteins demonstrated a sex–protein interaction, with β-1,3-galactosyltransferase (B3GALT1) and triggering receptor expressed on myeloid cells 1 (TREM1) having directionally different associations among males and females. Among the 18 proteins that demonstrated a race–protein interaction on ΔVO_2max,

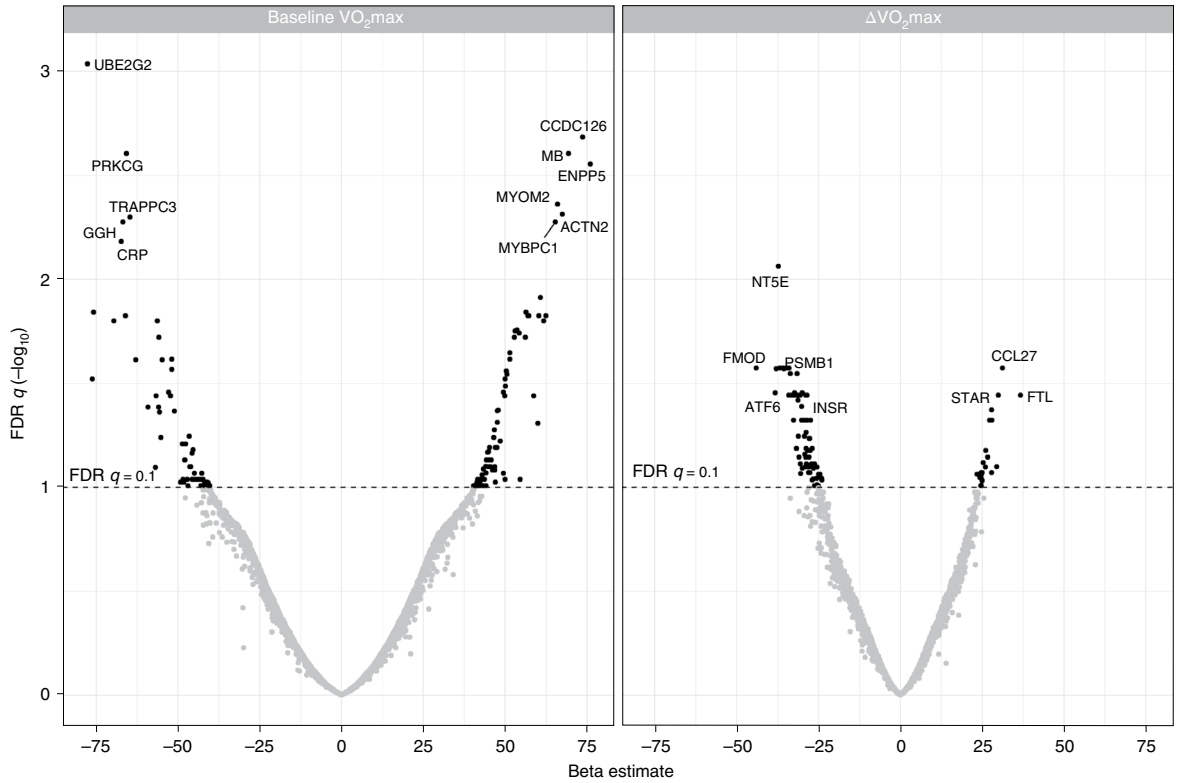
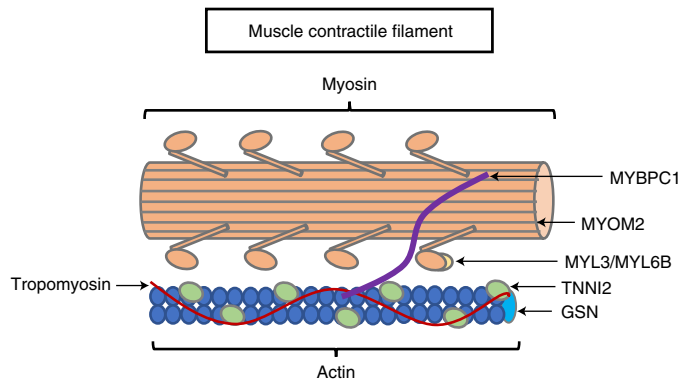


Fig. 2 | Plasma proteins associated with baseline and ΔVO_2max. Protein relationships to baseline VO_2max (ml O_2 min^{-1}) in a linear regression model adjusted for age, sex, BMI and race. The value of leptin's relationship with baseline VO_2max extends beyond the scale.



Gene symbol	Protein name
TNNI2	Troponin I, skeletal, fast twitch
MYOM2	Myomesin-2
MYBPC1	Myosin-binding protein C, slow type
MYL3	Myosin light chain 3
MYL6B	Myosin light chain 6B
ACTN2	Alpha-actinin 2
GSN	Gelsolin

Fig. 3 | Muscle proteins positively associated with baseline VO_2max. Left, muscle filament depiction highlighting the proteins positively associated with baseline VO_2max that participate in striated muscle structure and/or function. Myosin-binding protein slow-skeletal isoform (MYBPC1) regulates myosin–actin cross-bridge formation. Troponin I (TNNI2) inhibits actin-activated myosin ATPase activity. Gelsolin (GSN) is an actin severing protein. Myosin light-chain elements (MYL3 and MYL6B) regulate mechano-enzymatic function of myosin. Alpha-actinin 2 (ACTN2; not shown) and myomesin-2 (MYOM2) are actin and myosin stabilizing proteins. Right, table of names of proteins depicted at left and their associated gene symbols.

6 demonstrated directionally different associations among African Americans and those of European descent: C–C motif chemokine 27 precursor (CCL27), retinal rod rhodopsin-sensitive cGMP 3′,5′-cyclic phosphodiesterase subunit delta (PDE6D), phosphatidylinositol polyphosphate 5-phosphatase type IV (INP5E), plexin-A1 (PLXA1), pleiotropin (PTN), and EGF-like repeat and discoidin I-like domain-containing protein 3 (EDIL3).

We next performed gene set enrichment analysis (GSEA) to further elucidate biochemical pathways among this set of proteins, as well as those previously identified in the baseline VO_2max analyses (Supplementary Tables 10 and 11, respectively). Proteins negatively associated with ΔVO_2max were most enriched for ECM-related proteins (the 'matrisome')[27] (Fig. 4a,b). Positively associated proteins, however, were enriched for core signalling pathways that include platelet-derived growth factor receptor, neurotrophin and hepatocyte growth factor pathway signalling, among others (Fig. 4a,c,d). These biochemical pathways contrast with those enriched after GSEA was applied to proteins ranked by their association with baseline VO_2max (Fig. 4e).

We also compared the group of proteins associated with baseline VO_2max with those associated with adaptive VO_2max changes to exercise training and found minimal overlap between the two

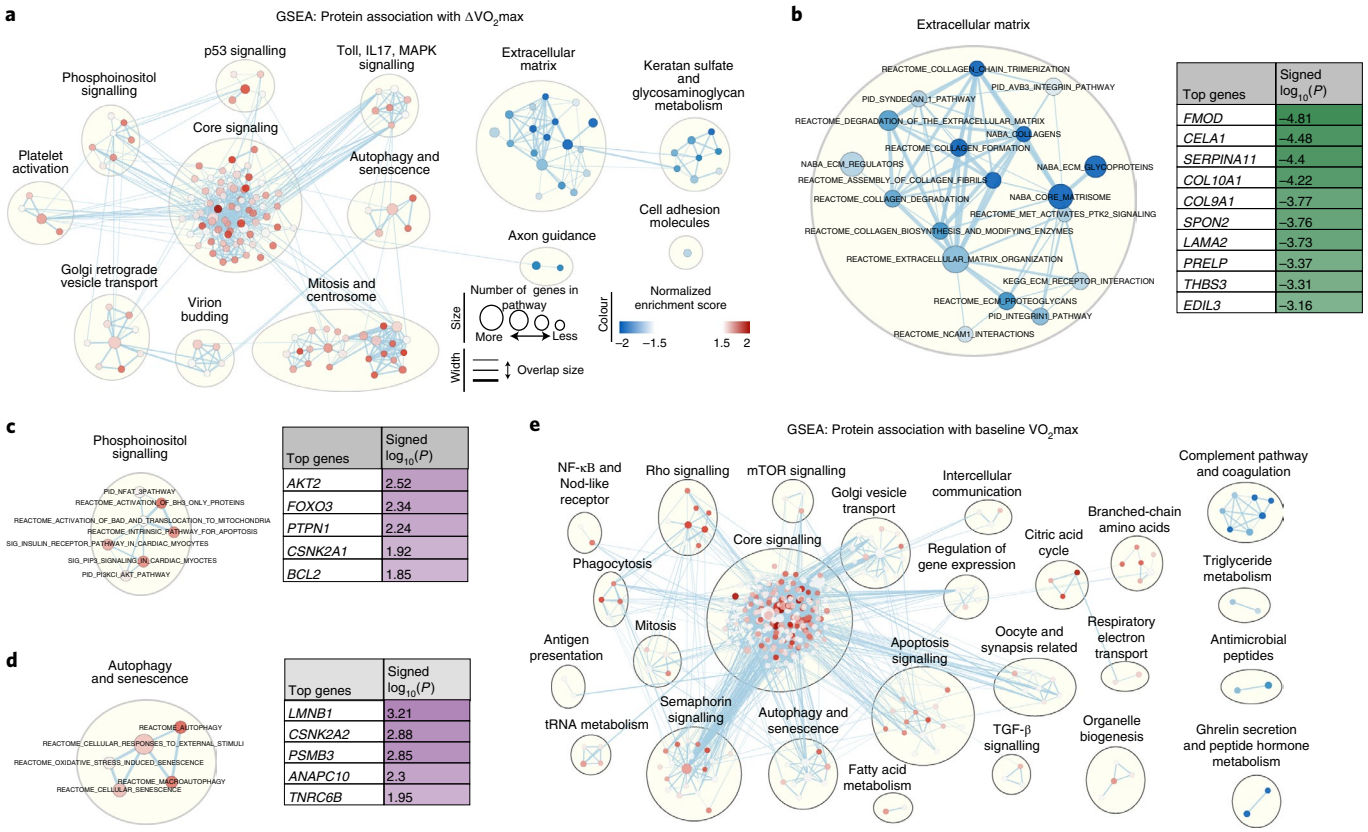


Fig. 4 | GSEA for proteins associated with ΔVO_2max or baseline VO_2max. Overview of overrepresented biological pathways and their connectivity using Cytoscape v3.7.1. **a**, Network visualization of GSEA results using the complete dataset of protein–ΔVO_2max associations. Red dots indicate pathways with over-represented positive protein–ΔVO_2max associations, and blue dots indicate over-represented negative protein–ΔVO_2max associations. A larger circle size denotes a larger number of genes in a pathway, and darker shades indicate a higher degree of enrichment. Clusters indicate biological pathways with shared proteins and biological function. **b**–**d**, Selected clusters of biological pathways with annotation, from **a**; the top contributing proteins to enrichment score are shown in a table. **e**, Network visualization of GSEA results using the complete dataset of protein–baseline VO_2max associations.

groups. Only five proteins, T132B, ATF6A, COL9A1, INS and PIANP, were associated with both baseline VO_2max and ΔVO_2max.

Plasma proteins improve prediction of ΔVO_2max responses. Given the vast heterogeneity in VO_2max changes that occur with exercise training, as described above, and that clinical factors account for a limited amount of the variance in VO_2max trainability[15], we sought to determine whether baseline plasma proteins could improve our ability to predict VO_2max changes in response to exercise training. Because baseline VO_2max and VO_2max changes with exercise training are minimally correlated, we tested to see whether proteins could help predict VO_2max changes relative to one's baseline VO_2max level (ΔVO_2max/baseline VO_2max). We selected a relative VO_2max change threshold of 15%, given that the median value among the cohort was ~16% (4.9 ml O_2 kg^{-1} min^{-1}) and a 15% change represented > 1 metabolic equivalent (1 MET), a clinically meaningful unit that has been related to >10% relative risk reduction in CVD and all-cause mortality in a series of longitudinal cohorts[3].

We first performed receiver–operating characteristic (ROC) analyses using a clinical trait model that included age, sex, race and BMI for relative VO_2max changes > 15%. The area under the curve (AUC) was 0.62 ($P = 0.91$) (Fig. 5). Feature selection and elastic net regression modelling of the 5,000 proteins yielded a final panel of 56 proteins (Supplementary Table 12). We next added our protein panel to the clinical trait model, and the AUC significantly increased to 0.81 ($P = 0.00018$). With regard to the operator characteristics, we found 79% sensitivity, 71% specificity, positive predictive value of 66% and negative predictive value of 83% for relative VO_2max changes > 15%. In a subsequent model that included the same clinical traits but only the group of proteins that both overlapped with an antibody-based proteomics platform (see 'Complementary data to support aptamer specificity') and demonstrated moderate to strong correlation between both platforms (7/10 proteins; SELE, TCL1A, COMP, CREG1, STC1, IL1RL2, LILRA2; $\rho = 0.41$–0.91), the operator characteristics were similar but performed slightly worse (AUC = 0.75, Extended Data Fig. 2), suggesting that there is added information provided by the remaining protein targets in our main model.

Association of VO_2max-related proteins and mortality. We previously performed proteomics profiling in the Framingham Heart Study (FHS) Offspring Study using first a 1.1 *k*-plex ($n = 821$ participants) and then an updated 1.3 *k*-plex version ($n = 1{,}092$) of the aptamer-based proteomics platform used in HERITAGE[28,29]. The clinical characteristics of the FHS sample are presented in Supplementary Table 13. Among the 102 proteins that were associated with ΔVO_2max in HERITAGE, 20 were available in both batches of FHS. Thirty-six out of the 147 proteins associated with baseline VO_2max were available in the FHS.

Of 1,909 FHS participants, 551 died after a mean (s.d.) follow-up of 13.6 (5.6) years. In age- and sex-adjusted analyses, 12 out of 36 proteins associated with baseline VO_2max and 9 out of 20 proteins associated with ΔVO_2max were also associated with incident

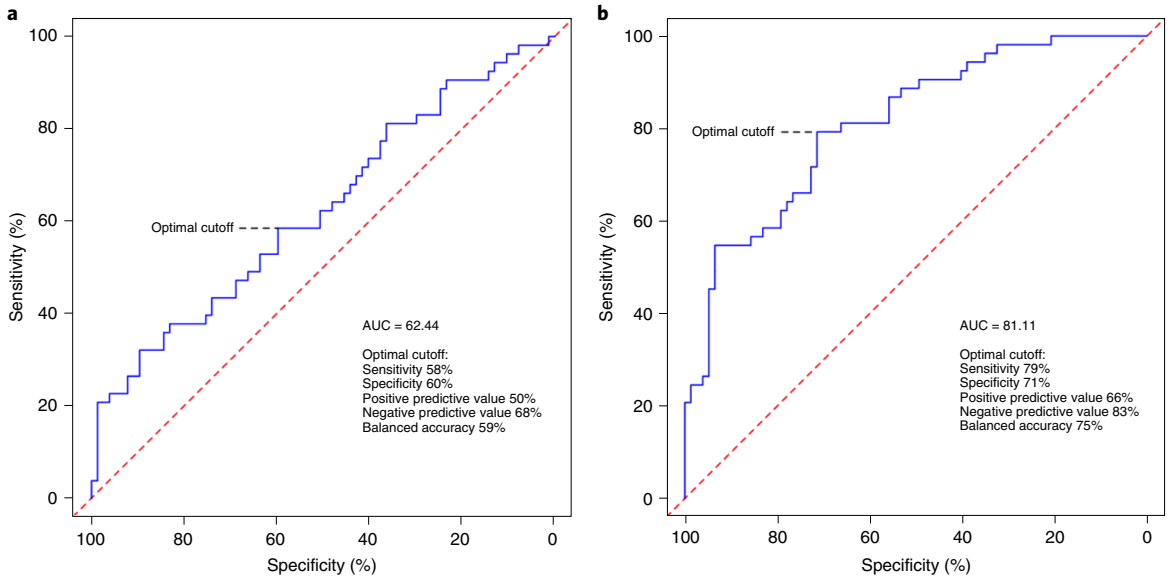


Fig. 5 | ROC curves for relative VO_2max changes with exercise training > 15%. a, The clinical trait score (age, sex, BMI and race) had a modest AUC. **b**, Addition of the protein score significantly improved the AUC. The sensitivity, specificity, positive predictive value, negative predictive value and accuracy at the optimal cutoff are included.

all-cause mortality (FDR $q < 0.1$; Table 2). We next performed stepwise regression using these protein sets (12 and 9 proteins, respectively) to estimate the percentage variation in all-cause mortality explained by each protein beyond age, sex and batch. Among the proteins associated with baseline VO_2max, gelsolin (GSN) was the most significantly associated with all-cause mortality (hazard ratio (HR), 0.71; FDR $q = 9.1 \times 10^{-13}$) and explained 3.4% of the variation beyond age and sex. Among proteins associated with ΔVO_2max, macrophage metalloelastase (MMP12) was the most significantly associated with all-cause mortality (HR, 1.34; FDR $q = 1.2 \times 10^{-7}$), explaining 1.8% of the variation in outcome.

Complementary data to support aptamer specificity. We tested the reproducibility of our top aptamer-based findings in HERITAGE specific samples using Olink's antibody-based proteomics platform (Olink Explore). Clinical characteristics of the random sample from HERITAGE are shown in Supplementary Table 14. Among the 21 proteins significantly associated with incident all-cause mortality, 12 protein targets were available on both platforms. Nine out of 12 of the protein targets were highly correlated. In addition, among the top protein targets associated with either baseline or ΔVO_2max that did not overlap with our all-cause mortality findings (Supplementary Table 15 and Tables 2 and 3 in Supplementary Data), an additional 13 proteins were available on both platforms. Ten of 13 assays demonstrated strong correlations. Taken together, 19 out of 25 of our top aptamer-based protein findings from HERITAGE were well correlated with an equivalent antibody-based assay (both sets of protein correlations shown in Fig. 6).

In addition, we leveraged mass spectrometry (MS)-based and genetic assays to support the specificity of the aptamer assays for our most significant findings. Among the 21 proteins significantly associated with incident all-cause mortality, genome-wide significant associations at *cis* loci (within 1 Mb of the transcription start site for the cognate gene of the protein) were identified for 17, consistent with the specificity of the aptamer–protein relationship. Aptamer specificity for two additional proteins (B2M and MB) was confirmed by MS[30]. Among the top 25 findings in both our baseline VO_2max and ΔVO_2max analyses, 23 and 24 were available for testing across genetic and MS-based analyses, respectively. The specificity of 11/23 proteins associated with baseline VO_2max and 12/24 proteins associated with ΔVO_2max was supported by these tests (Supplementary Table 15).

Discussion

VO_2max—as a direct measure of CRF—reflects the body's ability to transfer oxygen to skeletal muscle during sustained physical activity, and is thus a quantifiable measure of functional capacity. It has emerged as an important prognostic marker of future health risk that adds value beyond traditional risk factors[3]. While both baseline VO_2max and the adaptive changes in VO_2max in response to regular exercise provide valuable information about health status, these traits are largely unrelated to each other, a fact that underscores our limited understanding of their biologic basis and links to long-term health outcomes. Here, we performed large-scale plasma proteomic profiling in over 650 individuals with directly measured VO_2max before and after an endurance-exercise intervention to illuminate the biochemical features of baseline CRF and its adaptation to regular exercise. These analyses produced four notable findings. First, there was a broad and diverse set of circulating proteins associated with both baseline VO_2max levels and its changes in response to exercise training. Second, there was minimal overlap between the proteomic profiles of these distinct clinical traits. Third, the addition of a plasma protein score to baseline clinical traits improved the predictive accuracy of clinically significant improvements in VO_2max to exercise training. Finally, key proteins that are correlated with baseline VO_2max or ΔVO_2max were also associated with incident all-cause mortality in a separate population-based cohort.

Proteins are important regulators of biologic processes and, like CRF, reflect an individual's current health state as well as future risks[22]. The plasma proteome encompasses proteins from all tissues, making it an attractive medium to study the integrative biology of CRF. Indeed, we identified circulating proteins that spanned many of the organ systems involved in determining VO_2max, including the nervous, musculoskeletal, pulmonary, haematologic and circulatory systems. These included tissue-specific, structural and functional proteins (for example, striated muscle, Fig. 3) and proteins with signal peptide sequences (for example, secreted proteins;

Table 2 | Proteins associated with baseline or ΔVO$_2$max in HERITAGE and all-cause mortality in the FHS Offspring Study

Gene name	Protein name	Adjusted HR	95% CI		FDR *q* value	Variation explained by protein (%)
	Baseline VO$_2$max					
GSN[a]	Gelsolin	0.71	0.65	0.78	9.1×10^{-13}	3.00
CRP[a]	C-reactive protein	1.24	1.13	1.36	8.2×10^{-5}	
B2M[a]	β2-microglobulin	1.21	1.09	1.33	1.6×10^{-3}	1.00
ECM1[a]	Extracellular matrix protein 1	0.84	0.77	0.93	2.9×10^{-3}	
MB[a–c]	Myoglobin	0.87	0.79	0.96	1.7×10^{-2}	0.22
FCGR3B[a–c]	Low-affinity immunoglobulin gamma Fc region receptor III-B	1.13	1.04	1.23	1.7×10^{-2}	
ACP5[a–c]	Tartrate-resistant acid phosphatase type 5	1.14	1.03	1.27	3.1×10^{-2}	0.17
PLG[a]	Plasminogen	0.90	0.82	0.98	4.4×10^{-2}	0.45
NRCAM[a,b]	Neuronal cell adhesion molecule	0.90	0.83	0.98	4.6×10^{-2}	–
CFB[a]	Complement factor B	1.11	1.01	1.22	5.4×10^{-2}	–
ENPP7[a–c]	Ectonucleotide pyrophosphatase/phosphodiesterase family member 7	1.11	1.01	1.21	5.4×10^{-2}	–
NRXN3[a]	Neurexin-3-β	0.90	0.83	0.99	5.4×10^{-2}	–
ΔVO$_2$max						
MMP12[a–c]	Macrophage metalloelastase	1.34	1.22	1.48	1.2×10^{-7}	1.80
FAP[a–c]	Prolyl endopeptidase FAP	0.78	0.72	0.85	3.8×10^{-7}	
ANGPT2[a–c]	Angiopoietin-2	1.21	1.10	1.33	6.7×10^{-4}	0.47
STC1[a–c]	Stanniocalcin-1	1.19	1.09	1.30	1.8×10^{-3}	0.74
CCL27[a,b]	C-C motif chemokine 27	1.16	1.06	1.28	7.3×10^{-3}	–
IL11RA[a]	Interleukin-11 receptor subunit α	0.86	0.79	0.94	7.3×10^{-3}	0.54
ERBB3[a,b]	Receptor tyrosine-protein kinase erbB-3	0.86	0.78	0.94	8.3×10^{-3}	0.21
ACAN[a,c]	Aggrecan	0.87	0.80	0.96	1.7×10^{-2}	–
IMDH2	Inosine-5′-monophosphate dehydrogenase	1.12	1.03	1.23	3.3×10^{-2}	–

Cox proportional hazards analysis was performed for both the proteins associated with baseline VO$_2$max and those associated with VO$_2$max and all-cause mortality, adjusting for age, sex and batch. Proteins from each analysis that were statistically significant (FDR $q < 0.1$) were brought forwards in stepwise regression. The percent variation in all-cause mortality beyond age and sex is listed in the final column for those proteins retained in the final model. [a]Aptamer specificity supported by pQTLs and/or MS-based proteomics in population-based data (Supplementary Table 15). [b]Aptamer targets available for comparison on Olink Explore platform in HERITAGE subset ($n = 88$). [c]Proteins with Spearman correlation > 0.4 on aptamer and antibody-based platforms in HERITAGE subset.

Supplementary Table 2), as well as several proteins of uncertain function or not predicted to be secreted. Although these latter proteins may reflect tissue leakage or aberrantly secreted proteins, recent evidence suggests that traditional annotation methods may not fully account for proteins released into circulation via extracellular vesicles[31]. Indeed, our finding that a number of glycolytic enzymes, including fructose bisphosphate aldolase A (ALDOA), β-enolase 3 (ENO3) and lactate dehydrogenase (LDHB and LDHA), were present in the blood are consistent with those from Whitham et al.[31], who demonstrated a rise in plasma levels during acute bouts of exercise. The mechanistic relevance of these findings remains unknown, and additional research is needed to understand whether these enzymes have unanticipated functional effects in circulation or are biomarkers of physiologic states.

Among a group of classically secreted proteins, we identified several relevant to bone homeostasis that were positively associated with baseline VO$_2$max (Extended Data Figure 1). This group included BMP8B, an adipokine that regulates cartilage and bone development and has also been shown to induce brown-adipose-tissue thermogenesis[32] and adipocyte neurovascular remodelling[33], and SMOC1, a regulator of osteoblast differentiation relevant in physiologic cardiac hypertrophy[34]. We cannot localize the tissue origin of these circulating proteins, but our findings highlight the emerging paradigm of bone as an important endocrine organ involved in tissue crosstalk and exercise adaptation and motivate further interrogation of our data[35].

Few data describing the plasma proteomic profiles of baseline VO$_2$max exist[22,36], and to our knowledge this is the first study to investigate large-scale proteomic relationships with longitudinal VO$_2$max adaptations. Santos-Parker and colleagues[36] performed aptamer-based proteomics using a smaller-scale (1.1 *k*-plex) platform among a group of 47 sedentary or exercise-trained young men and women, and older men. The authors performed gene network and gene ontology (GO)-based annotation to identify biological processes associated with those in the exercise-trained state. More recently, Williams et al.[22] applied aptamer-based proteomic profiling in HERITAGE to generate a predictive model of cross-sectional VO$_2$max based on 115 proteins, using a training set that included 50% of samples from participants at baseline and 50% after completing exercise training.

While there was overlap among some of the broad biologic processes identified by Santos-Parker et al. (for example, autophagy and vasculogenesis) or individual proteins found by Williams et al. (~23% of our findings overlapped), our baseline VO$_2$max findings differed from these for several reasons. First, in contrast to these studies, our analyses were performed separately using only pretraining or post-training measures of VO$_2$max. Our baseline analyses did not include values obtained after the HERITAGE exercise intervention, which may reflect adaptive changes in VO$_2$max, a trait that is uncorrelated to its intrinsic value[13]. In addition, we used absolute values of VO$_2$max (ml O_2 min^{-1}) and adjusted for clinical characteristics in contrast to the univariate analyses of weight-adjusted

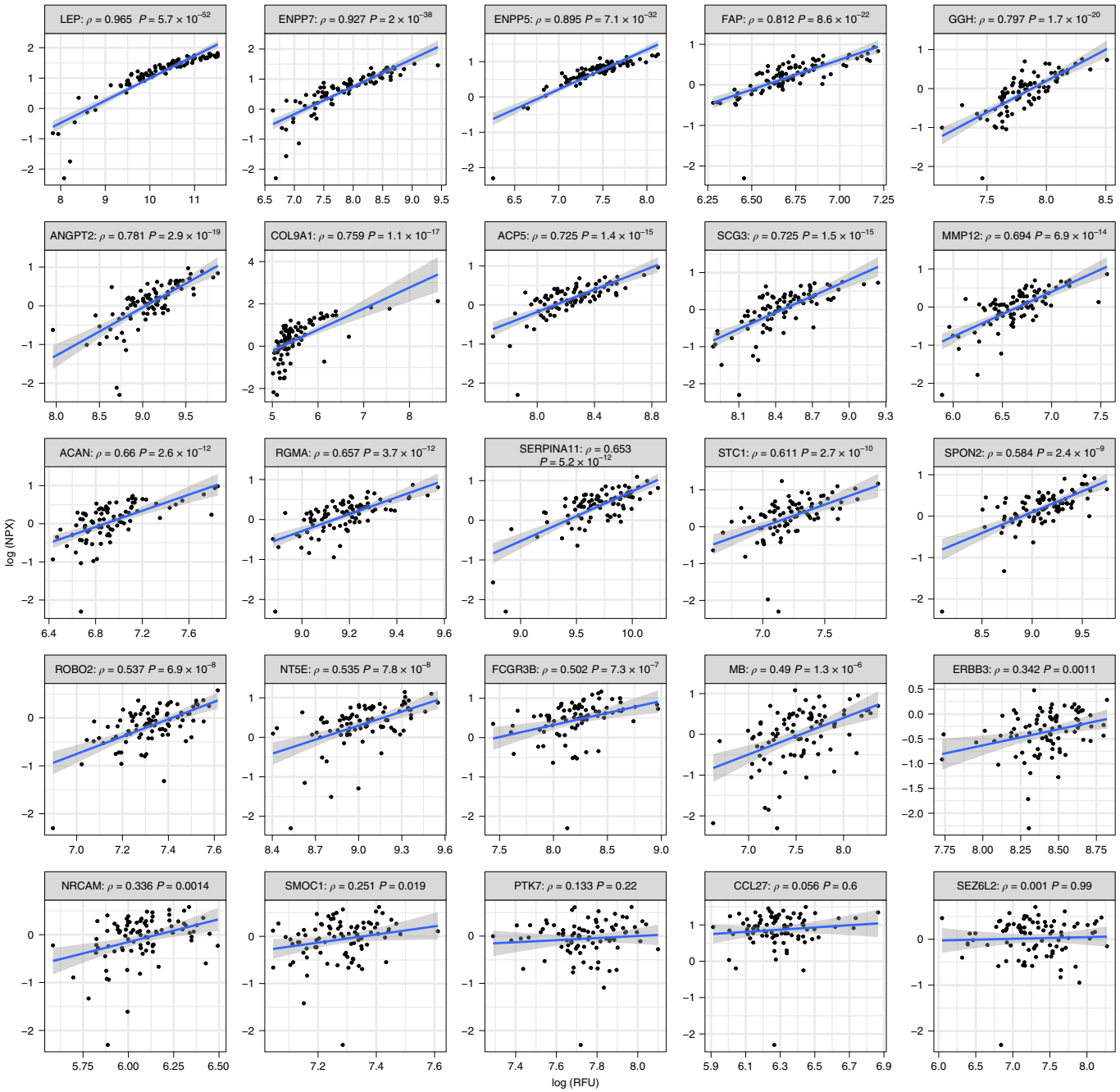


Fig. 6 | Spearman's correlations between aptamer-based and antibody-based assays among top findings. Spearman's correlations between protein levels measured by an aptamer-based method (log (RFU); *x* axis) and antibody-based method (log (NPX); *y* axis).

VO_2max (ml O_2 kg^{-1} min^{-1}) performed by Williams et al.[22]. Adjustments for age, sex and race probably significantly contributed to the differences between our groups' findings, owing to their relationships with CRF as previously documented and underscored in our interaction analyses[37–39].

Interestingly, when we performed additional adjustments for body-composition measures, we found that proteins closely associated with adiposity (for example, C-reactive protein, leptin and insulin) were no longer significant after adjusting for body fat percentage, but remained highly associated with VO_2max in a model adjusted for fat-free mass, similar to our main model using BMI. Although the main influence of body mass on VO_2max is mediated by fat-free mass, these data support prior findings that adipose tissue may contribute to VO_2max beyond differences in lean body weight[40]. Overall, there was modest overlap between the proteins related to baseline VO_2max in the models adjusted for body fat percentage and fat-free mass compared with the BMI-adjusted model (61 proteins, 48% overlap and 15 proteins, 56% overlap, respectively), whereas there was only one common protein (insulin-like growth factor binding protein 1 (IGFBP1)) among the fat-free-mass-adjusted and body-fat-percentage-adjusted models (Supplementary Table 4). These findings, coupled with the attenuation of striated-muscle-specific protein associations with baseline VO_2max after adjustment for lean body mass, highlight the importance of using standardized body size and composition adjustments for VO_2max when comparing results across studies.

We believe that the limited number of derivation proteins that achieved statistical significance in the external validation cohort

reflects the large differences in sample size between the two studies ($n=745$ in HERITAGE versus $n=91$ in the validation study) and the directional consistency of protein–VO_2max relationships (79/107) better reflects the stability of our findings across these studies. Further, given the known age- and body-size-related effects on proteomic profiles, as demonstrated in HERITAGE, we believe that large differences in the clinical characteristics between the two studies—even after restricting the validation cohort to age- and BMI-specific limits—impact the interpretation of our findings. We encourage additional validation of our findings; however, we are unaware of any other longitudinal, large-scale proteomic studies that include directly measured VO_2max at the moment.

The distinct proteomic profiles of baseline VO_2max and its exercise-induced changes that we observed are consistent with prior clinical observations demonstrating a lack of correlation between these traits[13,14]. The molecular mechanisms that underlie these differences are not well understood, and prior efforts to characterize CRF using candidate gene analyses[41], gene-expression data for skeletal muscle[42] and genome-wide association (GWAS) studies[43] have been limited by small sample sizes, lack of replication and the inherent challenges in applying reductionist strategies to describe a complex trait.

Using GSEA, we found nonrandom associations with baseline VO_2max in pathways related to hematopoiesis and angiogenesis (pathway participants included: chitinase 1 (CHIT1), haeme oxygenase 2 (HMOX2), cAMP-dependent protein kinase A (PRKACA), extracellular matrix protein 1 (ECM1)), the complement and coagulation systems (CD55, complement factor B precursor (CFB), cofilin-1 (CFI), plasminogen precursor (PLG), heparin cofactor 2 (SERPIND1)) and metabolic processes, including glycolysis, as described above (Supplementary Table 11a,b). These findings are consistent with those recently published from HERITAGE using integrative genomic analyses from GWAS and skeletal-muscle expression data in participants of European descent[44]. There, Ghosh et al. identified several gene loci that highlighted key determinants of CRF that we found using GSEA and through manual annotation (for example, skeletal muscle function (*SGCG*, *DMRT2*), cardiovascular physiology (*CASQ2*, *ATE1*) and hematopoiesis (*PICALM*)).

In contrast to our baseline VO_2max findings, we observed pathway enrichment reflecting proteins involved in extracellular matrix regulation (collagen alpha-1 (III) chain (COL3A1), COL9A1 COL10A1, aggrecan core protein (ACAN) and macrophage metalloelastase (MMP12)), key signalling pathways (for example, platelet-derived growth factor receptor B (PDGFRB) and hypoxia-induced factor 1 (HIF-1) signalling) and autophagy (for example, guanine nucleotide exchange factor (VAV3), cofilin-1 (CFL1)), among others, that were related to VO_2max responses to the exercise programme (Supplementary Table 10). These pathways were also present in a group of 16 over-represented Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways in GSEA previously performed using GWAS from HERITAGE[45]. While many of the proteins encoded by the relevant genes from HERITAGE genomic analyses were intracellular and were not captured on our plasma proteomics platform, our shared findings regarding relevant pathways point to possible biologic underpinnings that reflect or possibly mediate the differences between these two traits. Ongoing efforts to incorporate additional molecular profiling data in the study of fitness traits, including the NIH-sponsored initiative, Molecular Transducers of Physical Activity Consortium (MoTrPAC: NCT03960827), will further advance our understanding of these processes.

We also identified five circulating proteins that were associated with both VO_2max traits. Although variants in *TMEM132B* have been associated with lean body mass[46], and insulin may also be correlated with both traits through its relationship to body composition, the relationships of COL9A1, PIANP and ATF6A with VO_2max are unclear and remain the subject of future exploration.

Our protein biomarker analyses highlight the current lack of predictive capacity for exercise-induced VO_2max responses and the potential for large-scale plasma protein profiling for biomarker discovery. Although individual clinical traits such as age, sex, race and BMI have all been shown to influence VO_2max, their collective ability to predict a clinically meaningful response in VO_2max to exercise training was modest, and no other readily available biomarkers exist. The addition of our protein score helped identify at a high percentage (negative predictive value = 83%) those individuals unable to modestly improve their VO_2max despite undergoing a standardized, supervised exercise training programme. If validated in an external cohort, these findings would help with the early identification of individuals who may benefit from alternative lifestyle interventions or additional therapeutics to improve their CRF.

Finally, our observation that plasma proteins related to both baseline VO_2max and its trainability are also associated with future mortality risk highlights the potential value of biochemical profiling to better understand the mechanistic links between CRF and long-term health outcomes. The strongest relationship among both sets of proteins was gelsolin (Table 2), both a secreted and intracellular protein with multiple cellular functions. Gelsolin was positively associated with baseline VO_2max ($\beta=56.3$; FDR = 0.014) and inversely associated with incident all-cause mortality (HR = 0.71; 95% CI, 0.65–0.78), explaining ~3% of the variation in mortality after adjustment for age and sex in stepwise regression. Prior groups have linked lower plasma gelsolin levels to adverse outcomes in people with sepsis[47] and end-stage renal disease[48], and most recently higher gelsolin levels were associated with a decreased risk of congestive heart failure after adjusting for established risk factors[49]. Our data demonstrating its inverse association with all-cause mortality in a large population-based cohort extend these findings. Whether gelsolin is a biomarker or potential mediator of CRF and long-term health remains unclear. Gelsolin's most well-studied role relates to intracellular actin filament severing and cytoskeletal remodelling[50]; however, its secreted form predominantly comes from striated muscle and has been shown to function as an extracellular scavenger of actin[51] and inflammatory intermediates[52], as well as a participant in signal transduction pathways relevant to CRF, including the PI3K pathway[53]. Additional research into gelsolin's role in cardiometabolic health is warranted by these recent findings.

There are several limitations to our work. First, HERITAGE is a single-arm study and thus VO_2max adaptations may reflect unmeasured factors beyond the exercise-training stimuli. Leisure-time physical activity was not measured; however, all participants were sedentary for 6 months prior to enrolment. The aptamer-based platform that we utilized targets ~5,000 proteins; however, this technology is biased towards circulating proteins and does not provide complete coverage of the plasma proteome. Further, affinity-based assays, such as aptamer technology, are subject to nonspecific binding and may have limitations in their performance in response to post-translational protein modifications[54]. To address these concerns, we measured protein levels of 25 of our top findings using an orthogonal, antibody-based platform in a random subset of 88 HERITAGE samples and found that 18 out of 25 protein targets were correlated with our aptamer-based results. Among the 7 proteins with a Spearman correlation < 0.5, 2 aptamer targets (SMOC1 and ERBB3) have variants in *cis* (located within 1 Mb of the transcription start site of the gene encoding the protein) that are highly associated with protein levels in internal HERITAGE genetic-protein analyses (*SMOC1*, $P=5.9\times10^{-8}$; *ERBB3*, $P=2.16\times10^{-6}$). In addition, five (CCL27, PTK7, SMOC1, NRCAM and ERBB3) aptamer measurements had *cis* genotype-protein quantitative trait loci (*cis*-pQTL) relationships from publicly available and existing population-based human genetics studies, and one protein (MB) was validated using a multiple-reaction-monitoring MS-based method (Supplementary Table 15). Although we cannot

resolve the reason for the lack of a stronger correlation between these target proteins, these additional data support the specificity of our aptamer-based findings. Ultimately, we recognize the need for additional confirmation to validate the remaining analytes in the platform. Efforts to do so are ongoing[22], and all of our primary data have been made available to the broader scientific community for subsequent efforts. The proteomics platform includes a broad group of proteins; however, we are unable to identify their tissue origin. We limited the number of adjustments in our analyses relating proteins to all-cause mortality because our central goal was to assess the presence of shared protein biology between CRF and long-term health outcomes, thus these findings cannot explain the specific mechanisms through which this occurs nor can they be used as biomarkers of risk prediction without additional work. We also limited our analyses of VO_2max changes to linear methods, thus there may be additional insights yielded by using nonlinear methods. Our tests for interaction among race, sex and generation among protein–VO_2max relationships may not have been sufficiently powered, and our use of nominal statistical significance may have yielded false positive results, particularly given that the great majority of interactions were directionally consistent between groups.

In summary, we identified a large number of circulating proteins that are associated with VO_2max and highlight distinct profiles that exist for its baseline state as well as its adaptation to endurance exercise training. While our findings highlight specific proteins and biochemical pathways associated with these traits, further analyses of these data should yield additional biologic insights and motivate studies in model systems to both identify the sources of these proteins and evaluate their functional significance.

Methods

HERITAGE Family Study. The HERITAGE Family Study design and its participants have been described[55]. Briefly, family units of African Americans and people of European descent, totalling 763 sedentary participants (62% of European descent) between the ages of 17 and 65 years, were enroled in a 20-week training study of graded endurance exercise training across 4 clinical centres in the United States and Canada. Participants were healthy but sedentary over the previous 3 months and were free from apparent cardiometabolic disease. A total of 745 participants who had baseline measures of VO_2max and plasma samples were included in cross-sectional analyses, whereas 654 participants who completed exercise training and had complete data were used for longitudinal analyses. Written informed consent was obtained from all participants in the HERITAGE Family Study. HERITAGE study consent was reviewed and the research performed in these analyses was approved by Beth Israel Deaconess Medical Center's institutional review board.

Cardiopulmonary exercise testing and VO_2max. Two maximal CPETs were performed on separate days, at least 48 hours apart, before and after the 20-week exercise training programme, using a cycle ergometer (model 800S, SensorMedics) connected to a metabolic cart (model 2900, SensorMedics). Standard gas-exchange measures were obtained as an average of 20-second intervals. The criteria used for the attainment of VO_2max were defined as: a respiratory exchange ratio >1.1, plateau in VO_2 uptake (change of <100 ml/min in the last 3 consecutive 20-second averages) and a HR within 10 beats/minute of the maximal level predicted by age. All participants met at least one of these criteria in one of the two tests[12], but most met two or more[56]. The average of the two measurements before and after exercise training were used as VO_2max unless the values differed by more than 5%, in which case the higher value was used. The correlation between VO_2max measurements between the two tests (r=0.97), coefficient of variations (CVs, 5%) and reproducibility among clinical centres were excellent[57]. We used absolute (ml O_2 min^{-1}) rather than weight-adjusted (ml O_2 kg^{-1} min^{-1}) measures of VO_2max so that body mass changes that occurred after exercise testing were not incorporated into our assessment of ΔVO_2max.

Exercise training protocol and plasma sampling. Participants exercised 3 times per week for 20 weeks, beginning at 30 minutes/session and increasing to 50 minutes/session for the final 6 weeks of the programme. Exercise intensity increased from the heart rate associated with 55% VO_2max obtained during baseline CPET to the heart rate associated with 75% VO_2max over the final 8 weeks of training. Cycle ergometers were electronically programmed to maintain a training heart rate by adjusting the power output. Each exercise session for all participants was continuously monitored by trained staff. Fasting plasma samples were collected in EDTA tubes from peripheral intravenous catheters prior to the beginning of the exercise training programme and at 24 hours following completion of the final exercise session.

Proteomic profiling. *Aptamer-based method.* Detailed analytic methods of the SOMAscan assay have been described[19–21]. Briefly, archived plasma samples stored at −80 °C from HERITAGE were diluted in 3 different concentrations (40%, 1% and 0.05%) and incubated with a mixture of fluorescently labelled single-stranded DNA aptamers (~5,000 SOMAmer). Plasma samples had either 0 freeze–thaw cycles or 1 freeze–thaw cycle prior to proteomics profiling. Protein–aptamer complexes were isolated from unbound or nonspecifically-bound proteins using a two-step, streptavidin-bead-based immobilization process. Aptamers eluted from the target proteins were quantified using the degree of fluorescence on a DNA microarray chip. Samples were normalized to 12 hybridization control sequences within each microarray and across plates, using the median signal for each dilution. We have previously reported median intra- and interassay CVs for the SOMAscan assay of ~5% (ref. [58]).

Antibody-based method. We subsequently performed additional proteomics profiling using an antibody-based technology (Olink) on a random sample (n=88) from the HERITAGE study to determine the reproducibility of our aptamer-based results. Briefly, the Olink plasma extension assay technology uses DNA oligonucleotide-labelled antibody pairs to bind target proteins; 384 assays are performed on 4 separate panels with different dilutions for different dynamic ranges of target proteins (total proteins assayed = 1,536). After incubation with plasma samples, the oligonucleotide pairs hybridize and are extended by DNA polymerase to create a unique DNA barcode that is subsequently read out using next-generation sequencing. The median intra-assay CV for the 1,536 proteins was 10.25%, as assessed by multiple replicates of a pooled sample included in the experiment.

Genome-wide association studies. We also leveraged existing GWASs of proteins to help to determine aptamer specificity. Genotypes were available for 1,421 participants in the Malmo Diet and Cancer Study and 759 participants in the FHS with existing SOMAscan data[59]. A meta-analysis of genome-wide association analyses was performed to identify variants associated with circulating protein levels within 1 MB of the cognate gene, which were considered *cis*. Analyses were conducted on unrelated individuals. The methods used to generate publicly available genetics analyses for SOMAscan data have been described[30,60,61].

Framingham Heart Study. Participants in the FHS Offspring cohort who attended the fifth examination (1991–1995) and who had previously underwent plasma proteomic profiling with the SOMAscan single-stranded DNA aptamer-based platform (1.1 or 1.3 k-plex assays) were included in this study[28,29]. A total of 1,909 participants were included in analyses. Clinical characteristics were obtained from FHS investigators.

Validation cohort. The clinical characteristics and methods to derive baseline VO_2max from this randomized clinical exercise trial have been described[23]. Briefly, 300 sedentary adults with abdominal obesity were randomized into 3 exercise arms and a control group. Of the 217 participants who completed the 24-week exercise intervention, 216 had baseline VO_2max data and were available as a validation cohort. Given substantial differences—by design—in clinical characteristics between the validation and HERITAGE study cohorts, we restricted our analysis to subjects in the validation study with BMI < 40 and age < 55 (n=91), to more closely approximate HERITAGE participants.

Statistical analysis. Baseline clinical characteristics of participants in the HERITAGE Family Study, validation study and FHS are reported as means ± s.d., proportions, or medians (interquartile range) according to visual inspection of normality. A two-sample Student's t-test was used to compare cases and controls in FHS. All protein values were natural-logarithmically transformed for subsequent analyses. Correlations between aptamer-based and antibody-based proteomics assays were assessed using the Spearman correlation coefficient. Linear regression was performed to determine the relationship between baseline protein values and both baseline VO_2max (ml O_2 min^{-1}) as well as the changes in VO_2max (ΔVO_2max, post-training VO_2max – pretraining VO_2max). Covariates in regression models included age, sex and baseline values of BMI, body fat percentage, fat-free mass (kg),and VO_2max (Δ model only). Protein levels were standardized to mean = 0 and multiples of 1 s.d. We used the Benjamini–Hochberg procedure to correct for multiple comparisons and employed a FDR < 0.1 to determine statistical significance for these hypothesis-generating analyses.

We tested for the interactions of generation, sex and race with protein level on baseline- and ΔVO_2max and adjusted for the other covariates, given previously reported differences in VO_2max trainability among these groups[13].

To evaluate the predictive utility of protein biomarkers for relative VO_2max changes (ΔVO_2max/baseline VO_2max) after exercise training, we performed the following analyses. First, we implemented a clinical trait model that included age, sex, race and BMI for relative VO_2max changes > 15%. We then added more than 5,000 proteins to train a more comprehensive model. The maximum number of missing values per protein within the entire dataset was ≤7, and the

total number of missing values was <2%. The data were randomly split into a training set (80% of cohort) that uses crossvalidation and a test set (20%) that was not used for model development. All preprocessing steps were first applied to the training set. The same steps were then carried out for the test set. We used a *k*-nearest neighbour algorithm to impute missing values ($k=10$)[62]. All continuous variables were zero-centred and scaled (s.d. = 1). Scaling in the test set was applied using the same scaling factors calculated from the training set. The initial set of more than 5,000 predictors (proteins, age, sex, race and BMI) was reduced using a constraint-based feature selection algorithm for identifying minimal feature subsets (MMPC algorithm[63]). We then fit elastic net logistic regression models on the basis of the remaining predictors. The hyperparameters of the elastic net were optimized for the AUC using a global optimization algorithm. Receiver–operating characteristics of the protein score were subsequently calculated, with sensitivity, specificity, positive predictive value and negative predictive value generated. The training performance in the results is the result of repeated tenfold cross validation within the 80% training datasets.

GSEA using the full proteomics dataset was performed using the Molecular Signatures Database canonical pathways collection (MSigDB, http://software.broadinstitute.org/gsea/msigdb/collections.jsp), which includes a total of 2,199 curated gene sets from domain experts[64]. Signed log-transformed *P* values were computed from the regression models using the coefficient estimates and *P* values for protein–VO_2max associations. The full proteomic dataset was then ranked by their signed *P* values and used as input for GSEA (v4.0.3, with default parameters). GSEA results were exported to Cytoscape for visualization with the Enrichment Map tool using the following thresholds for gene set significance ($P<0.05$, FDR $q<0.15$, overlap index >0.5)[65].

For the FHS participants, we performed Cox proportional-hazard regression to model all-cause mortality using the proteins that were significantly associated with baseline or ΔVO_2max and also available in FHS. In age-, sex- and batch-adjusted models, proteins that were associated with baseline or ΔVO_2max using a FDR $q<0.1$ were brought forward for stepwise regression to estimate the percentage variation in all-cause mortality explained by each protein. *Cis* variants were identified using a linear regression model to assess the associations of variants with proteins that had statistically significant relationships with baseline and ΔVO_2max; statistical significance was set at $P<5\times10^{-8}$. All statistical analyses were performed using R version 3.6.2 (R Core Team, R Foundation for Statistical Computing).

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Deidentified, individual-level proteomics and phenotypic data that support the HERITAGE findings within this paper are available at https://motrpac-data.org/related-studies/heritage-proteomics. Overlapping aptamer-based and antibody-based proteomics data on the HERITAGE sample are included Supplementary Data Table 1. GWAS summary statistics for FHS and JHS are available through restricted access via the database of Genotypes and Phenotypes (dbGaP), a publicly available resource developed to archive data from human studies of genotype–phenotype relationships and can be accessed here (https://www.ncbi.nlm.nih.gov/gap/; FHS accession number: phs000363.v19.p13; JHS accession number: phs000964). FHS proteomics data have also been deposited in dbGaP and are available through the same accession number. JHS proteomics data have been deposited in the JHS Data Coordinating Center and are being deposited in dbGaP (accession number: phs002256.v1.p1); pending its receipt in dbGaP, all JHS data are available from the JHS Data Coordinating Center on request (JHSccdc@umc.edu). In addition, proteogenetics findings (precise SNP IDs) included in Supplementary Table 15 from FHS/MDCS meta-analysis and JHS have been provided in Tables 2 and 3 in the Supplementary Data, respectively. Additional data supporting the findings of this study are available from the corresponding author upon reasonable request.

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Author contributions

J.M.R., M.A.S., C.B. and R.E.G. conceptualized the study. J.M.R., B.P., D.S., T.R., S.D., M.J.K., C.S., P.M.J.B, R.R. and R.E.G designed research, performed biochemical experiments and analysed the proteomics data. J.M.R., U.A.T. and D.H.K. performed genetics analyses. J.L.B., C.B., S.A.C., S.G. and L.L.J. provided technical assistance and/or conceptual advice. J.M.R. and R.E.G. wrote the manuscript with assistance from the coauthors.

Competing interests

The authors declare no competing interests.

Additional information

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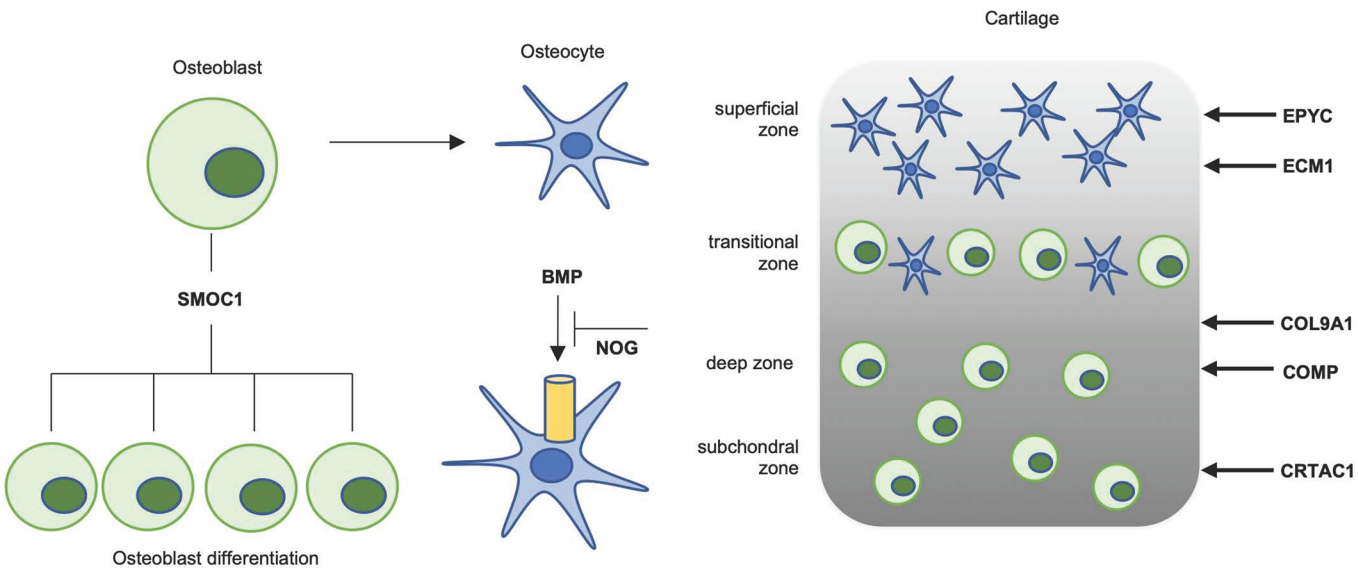
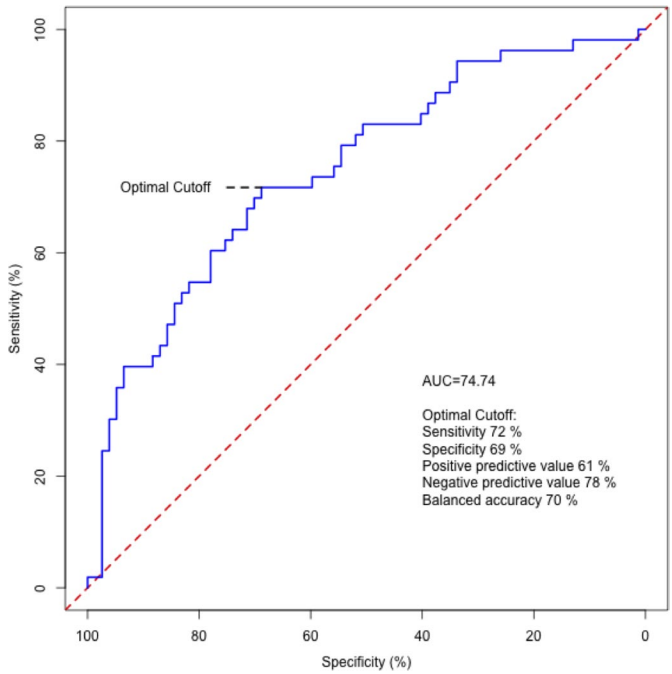


Entrez Gene Symbol	Target Protein Name
SMOC1	SPARC-related modular calcium-binding protein 1
BMP8B	Bone morphogenetic protein 8B
NOG	Noggin
ECM1	Extracellular matrix protein 1
EPYC	Epiphycan
COL9A1	Collagen alpha-1 (IX) chain
COMP	Cartilage oligomeric matrix protein
CRTAC1	Cartilage acidic protein

Extended Data Fig. 1 | Secreted proteins positively related to bone homeostasis and baseline VO_2max. Functional representation of proteins' role in bone metabolism and homeostasis. **Left and middle:** SMOC1 regulates osteoblast differentiation. BMPs are related to bone formation via the TGF-ß pathway and are mediated by extracellular signalling molecules such as NOG. **Right:** simplified schematic of proteins related to cartilage formation and their location within cartilage tissue.



Extended Data Fig. 2 | Receiver-operating characteristic curve for relative VO_2max changes with exercise training > 15% using overlapping targets between aptamer- and antibody-based proteomic platforms. 7/10 overlapping proteins on both platforms demonstrated moderate-strong correlation (SELE, TCL1A, COMP, CREG1, STC1, IL1RL2, LILRA2; $\rho = 0.41–0.91$) and were used in modeling.

IV. DISCUSSION

The following discussion includes a short summary of the results that from the original manuscripts. and puts them into the context of basic exercise science literature, exercise omics literature and the efforts to understand the routes by which exercise improves health.

IV-1. Study 1: An inventory of global metabolite changes in response to exercise

The aim of the systematic literature review was to summarize metabolite concentration changes in response to an acute bout of exercise. Studies that met eligibility criteria spanned a broad field of exercise modes, exercise intensities, and durations. Despite only studies with healthy participants were eligible and the majority of studies included only males, large differences in training status and or phenotypic characteristics (e.g. BMI or bodyfat) between participants, were present in the entire set of studies. Sample timing between studies varied from few minutes to 24 hours after exercise. Biofluids in which metabolite concentration changes were measured included blood, urine, saliva and sweat.

The systematic summary I did for this review with the categorization of post-exercise sample timing, made it possible for me to distinguish metabolites that change their concentration robustly meaning non-dependent on post-exercise sample time from other metabolites that show a recovery curve (i.e. high concentration changes after exercise and a slow return towards baseline levels) in the post-exercise time.

Overall study protocols and across all subject groups and sample time points, we found consistencies in many of the reported metabolite concentration changes after exercise: All fatty acids and almost all acylcarnitines measured, increased significantly following exercise; membrane lipids and bile acids decreased significantly. Ketogenic amino acids decreased, and degradation products of ketogenic amino acids and ketone bodies increased. Carbohydrates and TCA cycle metabolites increased. Only the effect directions reported for nucleotides, cofactors/vitamins, xenometabolites, and amino acids differed between studies.

The directionally consistent changes of lipids, carbohydrates, and TCA cycle metabolites mirror the activation and/or upregulation of key metabolic pathways relevant for skeletal muscle contraction during exercise (**Figure 14**) (Hargreaves and Spriet 2020). As an example, lactate and pyruvate increase, indicating glycolysis, fatty acids increase, indicating lipolysis, and malate, succinate and fumarate increase indicating activity of the TCA cycle (fueled by products of the ß-oxidation of fatty acids) (**Figure 14, cf. III-1.3.**).

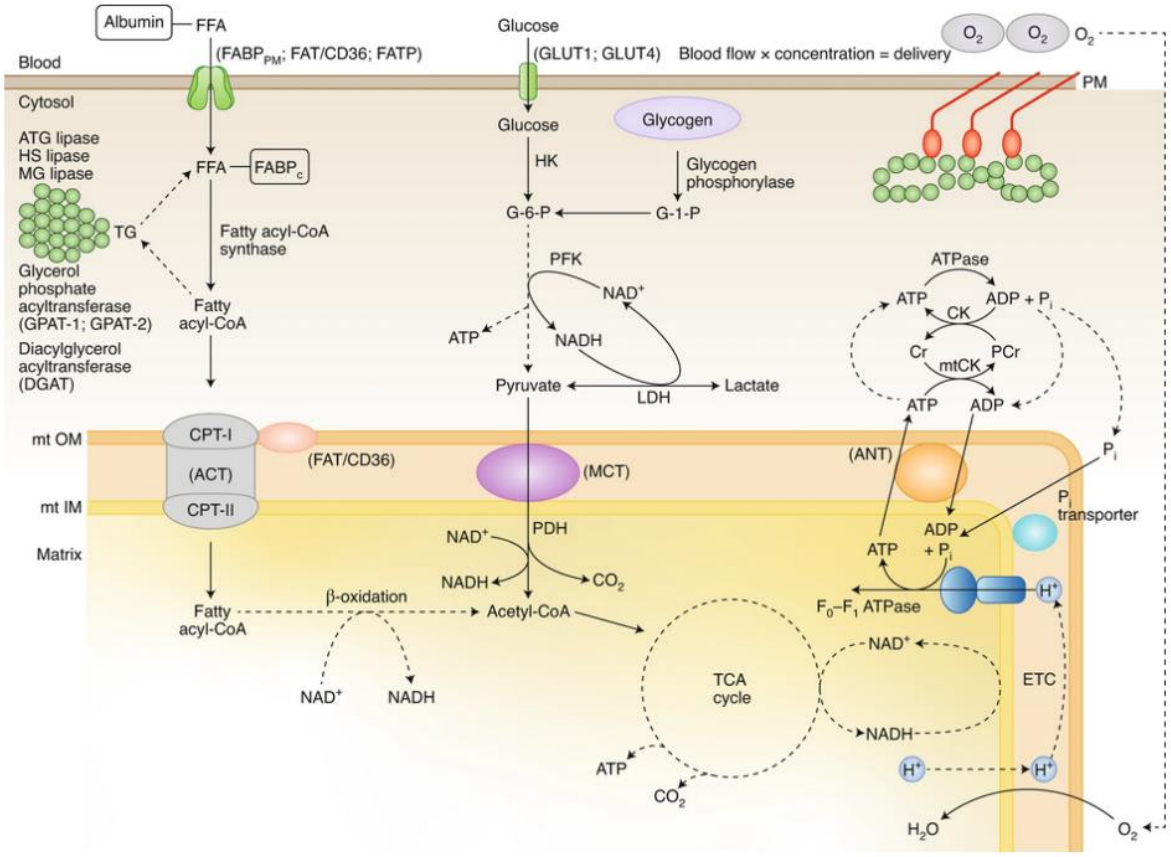


Figure 14. Metabolic pathways that play a key role in energy metabolism (ATP supply) of the working musculature during exercise. The graph is adapted from Hargreaves et al. (Hargreaves and Spriet 2020) and gives an overview of the major fuel avenues, enzymes and transport proteins (e.g. CPT-1, carnitine palmitoyl transferase 1) during exercise.
FFA, free fatty acids; PM, plasma membrane; FABPPM, plasma membrane fatty acid–binding protein; FATP, fatty acid transport protein; ATG, adipose triglyceride; HS, hormone sensitive; MG, monoglyceride; TG, triglyceride; FABPc, cytoplasmic fatty acid binding protein; HK, hexokinase; PFK, phosphofructokinase; LDH, lactate dehydrogenase; Cr, creatine; mtCK, mitochondrial creatine kinase; mt OM and mt IM, outer and inner mitochondrial membrane; ACT, acyl-CoA transferase; MCT, monocarboxylase transporter; ANT, adenine transport; PDH, pyruvate dehydrogenase; ETC, electron-transport chain.

This systematic literature makes it possible to show time-courses for different metabolite concentration changes from immediately to 24 hours post an acute bout of exercise, despite heterogenous study designs.

These time-courses could be used to monitor the extent of post-exercise upregulation of metabolites relevant for energy metabolism e.g. to compare the extent or the duration of upregulation after different exercise-training modes. For example, fatty acids could be used to demonstrate by what

extent lipolysis is upregulated or how long because fatty acid concentration changes were highly dependent on post-exercise sample timing with a 2 to 5-fold increases in the early (5-15 minutes) post-exercise phase, whereas this increase gradually decreased over the intermediate (30 minutes – 3 hours) post-exercise and the late (>3 – 24 hours) post-exercise phase, staying significantly elevated compared to baseline levels. The time course of fatty acids in this review shows the mobilization of free fatty acids following exercise that is known to increase and subsequently persist for 12-24 hours after exercise and to decline with progressing time (Magkos et al. 2009). Whilst this review gives a good overview over metabolite concentration changes with respect to post-exercise time, there were not enough studies to judge the differences regarding exercise mode or regarding differences in subject characteristics (e.g. training background).

IV-2. Study 2: Long-term specific exercise shapes the metabolome and influences the metabolic response to exercise

IV-2.1. The biological concept of extreme athlete phenotypes

The concept of Study 2 of "extremes of human metabolism" assumes that years of specific exercise training also cultivates specific bioenergetic capacity in different groups of athletes. It is known that different exercise modes (e.g. strength or endurance training) shape distinct exercise capacities (e.g. muscular maximal strength or VO_2max). The biological basis of these distinct capacities lie in the organ systems of the respective metabolic capacity or trait. For exercise, this is mostly skeletal muscle, its fibre type distribution, and the metabolic capacities that accompany fibre type. In 1976, Costill et al showed that sprinters and other glycolytic athletes have higher percentages of fast twitch type IIa/IIx fibres which have a high concentration of enzymes involved in glycolysis and glycogen breakdown, especially when compared to untrained subjects and endurance trained subjects (**Table 3**) (Costill et al. 1976a; Costill et al. 1987; Costill et al. 1976b). In contrast, endurance trained subjects have a higher proportion (~50-98%, mean 79%) of slow twitch type I muscle fibres (**Table 3**) compared to untrained subjects (27-73%) or sprinters (21-28%) (Costill et al. 1976a). Type I fibres have higher mitochondrial enzyme concentrations e.g. succinate dehydrogenase (Costill et al. 1987) and enzyme activities (Nemeth et al. 1986), favoring oxidative energetic capacity.

Table 3. Lateral gastrocnemius head enzyme activities of female and male track athletes, distance runners and untrained controls (Costill et al. 1976a; Costill et al. 1987; Costill et al. 1976b). Extreme values are in bold. F Female. M Male. Note that fibre distribution within the M. gastrocnemius is not uniform so some values may be expected.

Subjects	Phosphorylase ($\mu mol \cdot g \cdot min^{-1}$) glycogen breakdown	Lactate dehydrogenase ($\mu mol \cdot g \cdot min^{-1}$) glycolysis	Succinate dehydrogenase ($\mu mol \cdot g \cdot min^{-1}$) oxidative phosphorylation
Untrained controls	F 4.1-10.5 M 4.3-12.0	F 630-940 M 603-1192	F 5.4-14.9 M 5.2-10.1
Sprinters	F **16.5-23.5** M **12.8-17.7**	F **1340-1360** M **1048-1525**	F 10.1-10.7 M 12.4-13.4
Distance runners	F* 3.4-7.1 M 5.6-8.9 M 4.1-12.2	No data M 632-847 M 620-1000	F* 9.6-17.6 M 8.2-**20.9** M **15.8-31.9**

Next to endurance athletes and sprinters, extreme muscular properties are also seen in physique athletes such as natural bodybuilders, who aim to achieve high muscular anabolism. The cross-sectional area of a bodybuilders' muscle fibres can reach 12000 μm^2 in type 2 fibres (Alway et al. 1989) when compared to normal means of ≈3500 μm^2 in non-resistance trained controls (Lexell et al. 1988).

Taken together, scientific evidence on the biological "extremes" in human muscle between differently trained athletes exists and between athletes and untrained subjects already exists. But no study before ours has systematically investigated if and how the biological differences in muscle properties (enzymes or fibre size) translate into changed blood metabolites.

IV-2.2. Participant selection and scheduling

We selected athletes with a minimum training history of 3 years within their respective sporting discipline, to make sure we recruit extremes of their respective sporting discipline. We kept the within-group differences of training history, training volume and competitions at a minimum by prior interviewing/mailing with each athlete. For example, we recruited sprinters from the same training group. We limited our cohort to males only, due to metabolite fluctuations during the female cycle. When working with females in small study cohorts, metabolomics experiments are ideally limited to the early follicular phase (day 1.-10. of the cycle) because later, hormonal disturbances can make between-subject comparisons difficult. Despite requiring more planning and possible spontaneous re-scheduling, we included females in the study following this PhD project (MetaPerform).

We had to schedule athletes depending on their competitions. For example, we scheduled our tests at least two weeks before and one week after a competition, to allow for complete regeneration. It is known that acute strenuous exercise influences a variety of standard clinical blood parameters for at least 24 hours post-exercise, mainly due to tissue inflammation in skeletal and heart musculature, e.g. CK, CK-MB, cardiac troponin, white blood cell count and others (Gerth et al. 2002; Kratz et al. 2002; Seneczko 1983; Siegel et al. 2001), which might also influence blood metabolite levels. Time periods between competitions and testing were even longer in natural bodybuilders, as their preparation for competition can take over one week and includes several facets that might influence blood metabolites, like restriction in dietary carbohydrate intake, overdrinking, restriction in drinking, and fasting.

IV-2.3. Sports-specific factors that influence the metabolome in athletes

Next to exercise itself, other factors that are essential for specific adaptation (e.g. building muscle mass but not fat mass) can influence metabolite levels. In Study 2, this was shown by various DHA-containing phosphatidylcholines that were higher in bodybuilders who supplemented omega-3 fatty acids. Further, differing macronutrient intakes (**Table 4**) could have influenced the blood metabolome and/or the metabolome differences between groups. But as underlined in the introduction, an athlete or any human phenotype as a whole, is formed through a variety of internal (e.g. genes) and external (e.g. exercise, nutrition) factors. Information on nutritional supplements and diet should be carefully protocolled and taken into consideration when metabolite differences between different athlete phenotypes are interpreted, especially in small cohorts.

If we find out that these extreme physiological phenotypes and the distinct metabolomes in these athletes are beneficial for diseases or in the prevention of diseases, it is important to know which specific exercise was combined with which macronutrient intake and with which dietary supplements to shape these phenotypes and metabolomes.

Table 4. Relative macronutrient intake in grams per kilogram bodyweight in the cohort of study 2 according to a 7-day self-reported dietary intake. Extreme values are in bold.

Group	Carbohydrates (g/kg)	Proteins (g/kg)	Fats (g/kg)
Control	2.43	0.87	0.86
Bodybuilding	3.28	**2.40**	0.98
Endurance	4.23	1.59	1.30
Sprint	4.59	2.00	1.58

IV-2.4 Transferability of the results into exercise practice and sports medicine

Despite this was the first study to investigate metabolic differences between different athlete groups, some data can be valuable for athletes' exercise practice and give clues for future studies in sports medicine. One valuable result is that of bodybuilders having significantly lower blood amino acids, including branched chain amino acids which are essential for skeletal muscle growth (Wolfe 2017) after fasting overnight. To limit muscle protein breakdown, a practical suggestion for these athletes could be to refuel BCAAs in the morning after waking up or before going to bed at night, especially when fasting periods overnight are long. This result might also be transferrable to patient cohorts that suffer from muscle loss after diseases, age-associated muscle loss or muscle loss after injuries and need to increase their muscle mass. Resistance exercise leads to a known increase in muscle protein synthesis for up to 48 hours after exercise (McGlory et al. 2017), which could be mitigated in patients without proper refueling of amino acids. As shown in the introduction, muscle mass (CSA) is significantly associated with risk of mortality, and it has been shown that amino acid supplementation in people suffering from muscle loss can increase muscle protein synthesis (Rieu et al. 2006) or mitigate further muscle loss (English et al. 2016).

A second transferrable result is that of endurance athletes having higher CPT1-ratios at baseline and post-exercise. As shown in **IV.-1** and **Figure 14**, CPT1-ratio is a proxy for the transporter activity of CPT1 in mitochondrial membranes, which is a rate-limiting step in the oxidation of fatty acids (ß-oxidation). Higher levels in endurance athletes at both time points may show higher mitochondrial fat oxidation rates in general and/or acutely higher fat oxidation rates after exercise. Supposable reasons for this are an increased fat mobilization from adipose tissue and/ or more mitochondria in the skeletal muscle fibers (mostly type 1 fibers, cf. **IV-2.1**) also meaning more transporters like CPT1 that are incorporated into mitochondrial outer membranes and hence higher rates of fat transport and oxidation. That certain steps and the efficiency of mitochondrial energy metabolism can be approximated with blood metabolomics may be relevant for a number of diseases that either lead to mitochondrial dysfunction (e.g. Alzheimer's disease, muscular dystrophy, diabetes or cancer) (Bose and Beal 2016; Chow et al. 2017; Srinivasan et al. 2017) or such that might be in part caused by it (e.g. Alzheimer's disease) (Moreira et al. 2010). Short – and long-term exercise interventions that positively influence mitochondrial energy metabolism (e.g. by increased fat oxidation rates) could be tried out as a supplemental therapy in these diseases and changes in metabolites that approximate ß-oxidation efficiency can be monitored via metabolomics.

In general, exercise is recommended in diseases with mitochondrial dysfunction, and the sensitivity of metabolomics shown in previous exercise studies (Contrepois et al. 2020; Morville et al. 2020) and in this thesis (Schranner et al. 2020; Schranner et al. 2021) may also enable comparison of different exercise (e.g. exercise mode, intensity, duration and frequency) and their effect on proxies of mitochondrial ß-oxidation efficiency and maybe even on how long the effects of exercise persists.

A third transferrable result is that of higher increases of blood hexose (which mainly comprises glucose) following exercise in the blood of endurance athletes. Glucose stored in glycogen, is the main fuel for the working muscles during intense exercise, which is why hepatic glucose production is upregulated and glucose is released into the blood stream during exercise (Brooks 2018). We know that endurance athletes oxidize more fat during exercise compared to non-endurance trained subjects (as shown by the lower respiratory exchange ratios during the same exercise load, **Figure 15**) and therefore might need less glucose released from the liver.

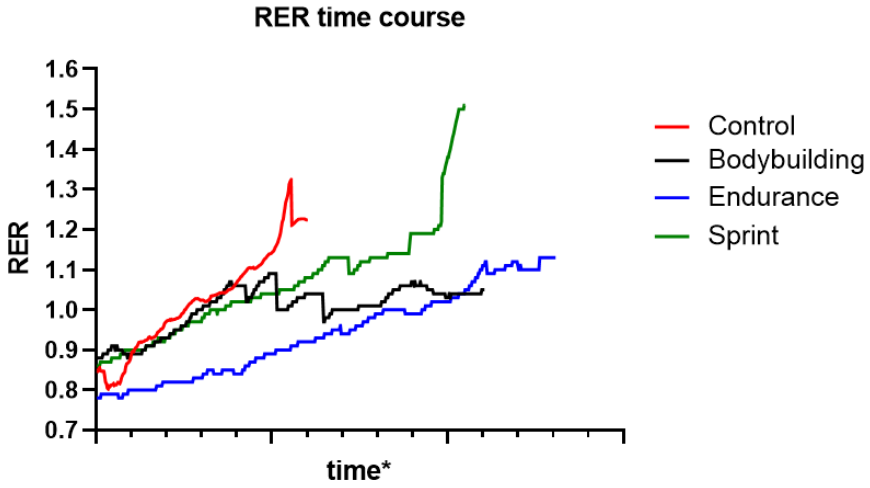


Figure 15. Respiratory exchange ratio curves during the course of the graded cycle ergometry test to exhaustion. *As gas exchange was measured breath-by-breath, every participant has an individual time stamp depending on breathing frequency. The x-axis is here only to show the courses of the RER and cannot be used to compare RER at different time points during the exercise. From Schranner et al. 2021

We also suggest that endurance athletes need less of the glucose in blood, because they have approximately two times higher intra-muscular glycogen stores compared to non-endurance trained subjects (**Table 5**) (Burke et al. 2017).

Table 5. Muscle glycogen content per kilogram wet muscle weight depending on endurance training status and diet (Burke et al. 2017).

	Endurance trained	Non endurance trained
Glycogen content, mixed diet (per kg wet muscle weight)	~120 mmol	80-85 mmol
Glycogen content, high carb diet (per kg wet muscle weight)	~200 mmol	-

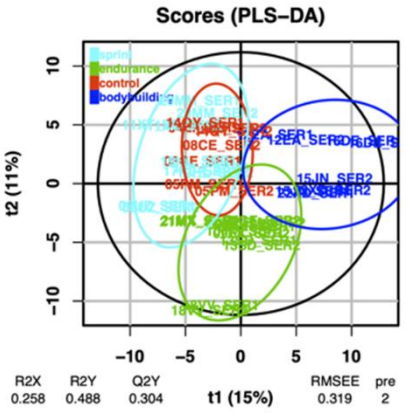
For the practice of competitive sports, initial glucose increases measured with metabolomics during short term, exhaustive exercise could inform (although in a limited way) about muscle and liver glycogen stores. For example, when glucose does not increase as expected during acute exercise, a depletion of glycogen stores could be the reason. Similarly non-increasing or a timely delay in increasing lactate during graded exercise is already used in performance diagnostics as an indication for glycogen depletion.

In that way, in the future, results from exercise metabolomics studies could inform about metabolic capacity of energy metabolism (e.g. transporters like CPT1 or fuel status like glycogen stores) and help in selecting exercise interventions in exercise practice of athletes and in various diseases.

IV-2.5. Reproducibility of global metabolome differences with untargeted metabolomics

We were able to reproduce the global metabolite differences of the targeted metabolomics that was done on a subset of all participants with untargeted metabolomics that was done on all participants (n=35). Briefly, we measured 70 serum samples of 35 participants on a non-targeted metabolomics platform (Metabolon Inc., Durham, USA). I did the quality control of the raw data (filtering metabolites with >70% missing values over all samples, followed by median batch correction, filtering for technical measurement variability using a CV cutoff of <30%, and filtering of metabolites with >30% missing values over all samples). Missing values for the remaining metabolites were imputed using the k-nearest-neighbor approach (k=10). After quality control, data was analyzed in the same way as the targeted data has been. In a first step we did a principal component analysis (PCA) followed by a partial least squares discriminant analysis (PLS-DA). Whereas the supervised PLS-DA (with a priori definition of the groups that should be separated) reproduced our findings of the targeted dataset well (**Figure 16A and B**), we also found the bodybuilders + endurance athletes to be separated from sprinters + controls in the unsupervised PCA (**Figure 16C**). In summary, we could reproduce the main findings in global metabolite differences between the four subject groups.

A.



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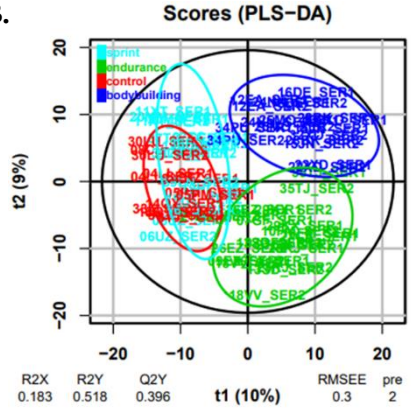

C.

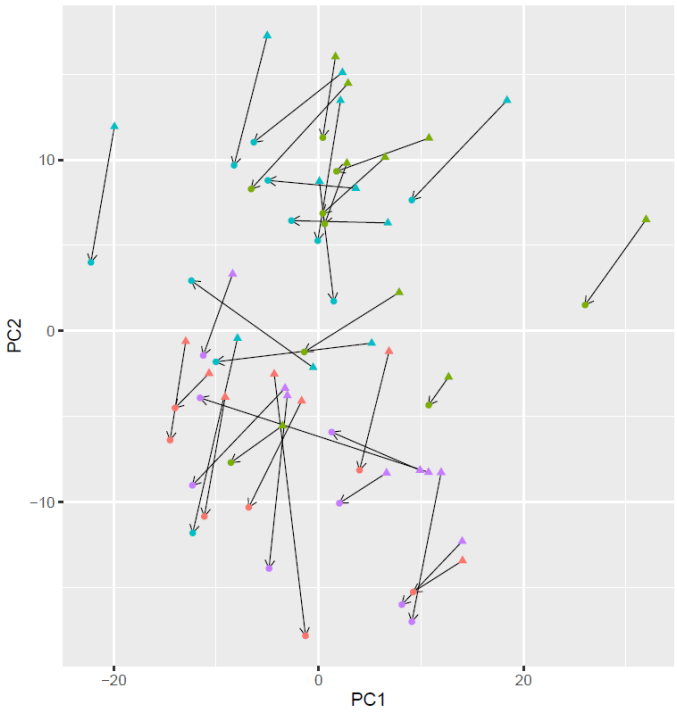

Figure 16 A. PLS-DA of the targeted dataset on a subset of 18 participants and the **B.** PLS-DA and **C.** PCA of the untargeted dataset on 35 participants. Lines in the PCA indicate changes from pre (▲) to post-exercise (●).

Color-code PLS-DAs: ●=control, ●=bodybuilding, ●=endurance, ●=sprint,

Color-code PCA: ●=control, ●=bodybuilding, ●=endurance, ●=sprint,

IV-3. Study 3: Predicting meaningful physiological adaptation by proteomics

Whereas studies 1 and 2 magnified the knowledge base on metabolite concentration changes after exercise in general and in specific, Study 3 has shown two things: First, a set of over 100 proteins is significantly associated with baseline VO_2max and with VO_2max increases after 20 weeks of exercise. Second, and relevant for future individualization in exercise, is that a set of 56 proteins (measured in the untrained state) and standard clinical factors can be used to predict meaningful changes in VO_2max after exercise. Despite the individual response to the same exercise is highly variable and follows a normal distribution (**Figure 1 and 2**), positive predictive accuracy of our model reached ~80% (Robbins et al. 2021). Though independent validation of this set of protein predictors has to be done, it still shows that minimally invasive measures like a blood draw can inform researchers, physicians, or the individual itself if it is beneficial for their health to engage in this kind of exercise. Such a prediction via blood proteins also offers the possibility to find those individuals who will not benefit sufficiently from an exercise intervention and need to do other things (e.g. medication or diet) to improve their health.

IV-4. Thesis outcomes in the context of exercise omics literature

The studies of this thesis contribute to the existing literature on exercise metabolomics and proteomics: Study 1 and Study 2 contribute to the literature on metabolite and protein changes after acute or chronic exercise. Study 3 contributes to studies that have reported protein or metabolite associations with either VO_2max or $\Delta\dot{V}O_2$max. For an overview, exercise omics studies found in the literature and the studies of this thesis are categorized depending on the molecules (genes, transcripts, proteins, metabolites) reported in each study (**Table 6**).

IV-4.1. Omics in acute exercise studies

Transcripts, proteins, and metabolites have all been studied in response to acute exercise, including 27 studies summarized in the systematic review of this thesis (**cf. II-1.3**) and others (Andersson Hall et al. 2016; Bassini and Cameron 2014; Berton et al. 2017; Breit et al. 2015; Chorell et al. 2012; Coelho et al. 2016; Danaher et al. 2015; Daskalaki et al. 2015; Enea et al. 2013; Enea et al. 2010; Floegel et al. 2014; Hall et al. 2016; Hooton et al. 2016; Howe et al. 2018; Huang et al. 2010; JanssenDuijghuijsen et al. 2017; Karl et al. 2017; Krug et al. 2012; Messier et al. 2017; Muhsen Ali et al. 2016; Mukherjee et al. 2014; Neal et al. 2013; Nieman et al. 2013; Peake et al. 2014; Pechlivanis et al. 2010; Pechlivanis et al. 2015; Prado et al. 2017; Ra et al. 2014; Samudrala et al. 2015; Schader et al. 2020; Sun et al. 2017; Valerio et al. 2017; Wang et al. 2015; Zauber et al. 2012). Formerly, others have reviewed metabolite concentration changes after exercise (Heaney et al. 2017) but only narratively reported exemplary results. In contrast, the systematic review of this thesis gives a comprehensive and quantitative summary of metabolite concentration changes after exercise, broken down into metabolite classes, sampling time and sample type. The given sample time informs the reader when after exercise a certain metabolite is expected to change and an help decide on sample timing in future studies (e.g. carbohydrates and TCA cycle intermediates mostly changed up to 30 minutes after exercise, whereas lipid concentrations changed from 5 minutes up to 24 hours after exercise) (Schranner et al. 2020). The review therefore recapitalizes metabolite changes after acute exercise and serves as a resource for scientists in the field of omics and exercise.

Metabolite changes after acute exercise depending on different exercise phenotypes are limited and respective studies classify phenotypes based on only one trait, e.g. $\dot{V}O_2$max (Chorell et al. 2012; Enea et al. 2010; Mukherjee et al. 2014; Schader et al. 2020). Being more comprehensive, in terms of athlete phenotype and its definition, in Study 2 of this thesis (Schranner et al. 2021), we looked at the baseline and post-exercise metabolite profiles of three highly specialized athlete cohorts and an untrained control group based on various phenotypic traits, e.g. endurance capacity, reactive strength, muscularity, muscle strength and athletes' training regimes. Study 2 therefore extends current exercise omics literature with the fact that athletic phenotypes can be discriminated based on their metabolite concentrations at rest and after exercise. Differing metabolite changes, which need to be

validated, in response to exercise also suggest that phenotypes can be discriminated by their metabolic reaction to the same exercise challenge (with certain metabolites). The metabolite differences after long-term specific training (at baseline), seen in Study 2, comply with data from Morville et al., who showed that only one bout of resistance or endurance exercise changes certain metabolites differently (Morville et al. 2020).

IV-4.2. Omics in chronic (long-term) exercise studies

In comparison to omics studies reporting acute metabolite changes after exercise, metabolomics in chronic exercise studies was done less (Brennan et al. 2018; Felder et al. 2017; Neal et al. 2013; Pechlivanis et al. 2013; Wang et al. 2015) (**Table 6**) and only Pechlivanis et al. compared different exercise modes and no study compared different phenotypes.

We recorded protein changes after chronic endurance training in the thesis' Study 3 (Robbins et al. 2021) however the data is not published yet. As one of the few studies that looked at protein changes after chronic exercise, Pillon et al. provide an online database on transcript and protein changes, separated into exercise modes (resistance exercise, aerobic endurance exercise, anaerobic endurance exercise and high-intensity endurance exercise) and duration (acute, chronic) (Pillon et al. 2020). Other than that, we are not aware of any other study investigating protein changes after chronic exercise in blood, only studies in skeletal muscle (Deshmukh et al. 2021; Oertzen-Hagemann et al. 2019; Petriz et al. 2017).

IV-4.3. Omics associations with $\dot{V}O_2$max or $\Delta\dot{V}O_2$max

Plenty of studies, mainly with data from the HERITAGE family study (Bouchard et al. 1995), investigated the association of genes with intrinsic $\dot{V}O_2$max and identified several SNPs that are associated with $\dot{V}O_2$max.

More recently, Al-Khelaifi et al investigated metabolites and genes in 490 world-class athletes (Al-Khelaifi et al. 2019a; Al-Khelaifi et al. 2018; Al-Khelaifi et al. 2019b). To find biomarkers associated with world-class athlete status, they compared their results to those of non-world-class athletes and found that metabolite profiles between non – and world-class athletes differ (Al-Khelaifi et al. 2019b). In a second study, Al-Khelaifi compared metabolite profiles of two different athlete phenotypes (high power vs. moderate power and high endurance vs. moderate endurance) and also found several metabolites that differed between phenotypes (Al-Khelaifi et al. 2018). Subsequently, they replicated 104 of these metabolites associated with world-class athlete status (high-endurance and high-power athletes) like higher androgenic steroids, higher monohydroxy fatty acid, lower diacylglycerols or lower acylcarnitintes, in an independent cohort (Al-Khelaifi et al. 2019a). Then, they tested if these metabolites are linked to genetic variants and found 19 genetic variants (17 known, 2 novel) that explained up to ~40% of metabolite levels in world-class athletes (Al-Khelaifi et al. 2019a) (**Table 6**). Despite the

metabolites associated with world-class athlete status, their causal relation to performance, need to be investigated. Al-Khelaifi et al.s' findings of ~40% of genetically explained variance in metabolite levels also supports Bouchard et al.s' ~47% of genetically explained variance of the phenotype (Bouchard 2012; Bouchard et al. 2011), as metabolites are known to be closely related to the phenotype.

Study 3 of this thesis gives first evidence that not only genes but also environmentally modifiable molecules like proteins are associated with intrinsic $\dot{V}O_2$max and increases in $\dot{V}O_2$max in a large and diverse cohort of over 650 Caucasian and African American individuals. For the majority of these 147 proteins, the directionality of the association with $\dot{V}O_2$max and the physiological function, points to real causal associations (e.g. positive associations between $\dot{V}O_2$max and proteins involved angiogenesis or in muscle function) that warrant validation in further studies. Compared to other exercise-proteomics studies by Contrepois et al., (~370 proteins quantified), Santos-Parker et al. (~1,100) (Santos-Parker et al. 2018) and Guseh et al. (~1,300 proteins quantified) (Guseh et al. 2020), we were able to quantify >5,000 proteins in Study 3 by using aptamer-based proteomics, which is novel amount and method in an exercise training context.

Plenty of literature on genetic associations with $\Delta\dot{V}O_2$max exists, likewise as with $\dot{V}O_2$max (**Table 6**), mainly coming from HERITAGE and summarized in systematic reviews (Rankinen et al. 2010; Williams et al. 2017). For $\Delta\dot{V}O_2$max, Bouchard et al. could even show causative SNPs (Bouchard et al. 2012; Bouchard et al. 2011; Ghosh et al. 2019).

Evidence on associations between transcripts, proteins, or metabolites to $\Delta\dot{V}O_2$max trainability is limited to a study by Castro et al. 2019, suggesting serum metabolites like lysine, phenylalanine, creatine, and glycerol are associated with $\Delta\ \dot{V}O_2$max after 8 weeks of aerobic endurance training or high-intensity interval training (Castro et al. 2019) (**Table 6**). Here Study 3 of this thesis can add 102 associations of proteins with $\Delta\ \dot{V}O_2$max (pending future validation). Notably, 9 out of 102 proteins associated with $\Delta\dot{V}O_2$max and 12 out of 147 associated with baseline $\dot{V}O_2$max were significantly associated with all-cause mortality in a separate cohort of the Framingham Heart Study (Ko et al. 2019), emphasizing the associations between $\dot{V}O_2$max and all-cause mortality described in the introduction. Despite the prediction model of $\Delta\ \dot{V}O_2$max, developed in Study 3, is modest in accuracy, it is unique so far and lays a foundation for predicting training responses by a set of protein biomarkers and other easy to obtain parameters.

Table 6. Classification of the PhD thesis outcomes in the context of existing literature on blood molecule changes in exercise omics studies.

	Genes	**Transcripts**	**Proteins**	**Metabolites**
Changes with acute exercise 	--	Contrepois et al. 2020; Pillon et al. 2020	Contrepois et al. 2020; Guseh et al. 2020; **Preliminary data, unpublished**	Contrepois et al. 2020; Morville et al. 2020; **Schranner et al. 2021***
Depending on the kind of exercise	--		Guseh et al. 2020	Morville et al. 2020
Depending on phenotype	--	Contrepois et al. 2020; Pillon et al. 2020	Contrepois et al. 2020	Contrepois et al. 2020; **Schranner et al. 2021**
Changes with chronic exercise 	--	Pillon et al. 2020	**Preliminary data, unpublished**	Pechlivanis et al. 2013; Felder et al., 2017; Neal et al., 2013; Wang et al., 2015; Brennan et al., 2018
Depending on the kind of exercise	--	Pillon et al. 2020	--	--
Depending on phenotype	--	Pillon et al. 2020	--	--
Associations with $\dot{V}O_2max$ or $\Delta\dot{V}O_2max$	Ghosh et al., 2019; Rankinen et al., 2010; Al-Khelaifi et al. 2019a; Al-Khelaifi et al., 2018; Bouchard et al. 1998; Bouchard et al. 2011; Bouchard et al., 2012; Williams et al., 2017[5]	--	**Robbins et. al. 2021;** Santos-Parker et al. 2018 (Santos-Parker et al. 2018; Williams et al. 2019)	(Schader et al. 2020) Al-Khelaifi et al. 2019a; Schader et al., 2020
Depending on the kind of exercise	--	--	--	--
Depending on phenotype	Al-Khelaifi et al. 2019a; Al-Khelaifi et al., 2018	--	**Robbins et al. 2021**	Al-Khelaifi et al. 2019a; Schader et al. 2020; Castro et al., 2019

* References of the summarized articles can be found in the original manuscript

IV-5. Feasibility of this thesis as foundation for further studies (MetaPerform, SportFATIGOM)

The outcomes of this thesis, namely that we could find global metabolite differences between exercise phenotypes and trace these differences back to statistically significant single metabolite differences which can be logically attributed to athlete's bioenergetic capacity, training or diet, led us to initiate two further studies: MetaPerform and SportFATIGOM. For both studies, I contributed significantly to writing the proposal and study design. In MetaPerform, I was lead scientist in recruiting participants, performing experiments during my PhD and will do the main part of data analysis.

The main rationale for a study like MetaPerform comes from an individualized exercise approach as well: No study in existing literature has looked at individual metabolite trajectories during prolonged constant exercise lasting >2 hours without nutritional fuel intake. Another novelty of MetaPerform is that metabolite changes are closely monitored every 30 minutes during cycling and one, two, and 24 hours post-exercise, totaling 11 timepoints per participant. From the experience of Study 1 and Study 2, we standardized pre-study exercise and nutrition for 24 hours before and after the test, protocolled nutrition, dietary supplements and training for one, two and four weeks before the study. We determined the load for the 3-hours cycling exercising for every subject individually by maximum exercise testing ($\dot{V}O_2$max testing) on another day that had to be at least three days before the 3-hours test. We set the load for the 3-hours test to 5% below the individual first ventilatory threshold (VT1-5%) to make sure that subjects can complete the test despite having no food. By the initial $\dot{V}O_2$max testing, we made sure that all subjects were sufficiently trained and have a very good to excellent $\dot{V}O_2$max/$\dot{V}O_2$peak for their age and sex (females >50 ml/min/kg; males >60 ml/min/kg) plus are used to endurance exercises that last for 3 hours and longer. The time of cycling of three hours was chosen, to challenge metabolism with fuel restriction as endogenous glycogen stores empty after ~90 minutes of exercising. We hypothesize to see a "switch" in metabolism from mixed metabolism of carbohydrates and fat (0-90 minutes) to primarily fat metabolism (after 90 minutes). During the last 5 minutes before each blood sample was taken, ventilatory gas exchanges were measured using spiroergometry (**Figure 16**). This was done, to make sure participants are exercising below their first ventilatory threshold, as duration of exercise increases, and to verify the metabolic switch from mixed metabolism to primarily fat metabolism. Depending on the individual, we further hypothesize that we see differences in metabolism or in metabolic compensation for the limited fuel availability, supposably also between females and males. The analyses of the MetaPerform blood samples are done using non-targeted metabolomics (Metabolon Inc, Durham, USA).

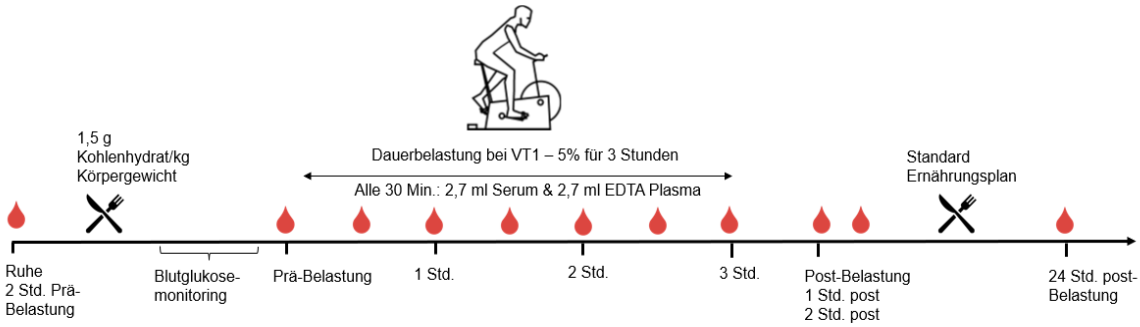

Figure 17. Study design of the MetaPerform study adding up to 11 timepoints of venous blood sampling.

IV-6. Limitations of metabolomics and proteomics in exercise science

Metabolomics and proteomics can inform exercise science researchers about metabolism at a given timepoint e.g. at rest, during exercise or in the recovery phase after exercise. Sensitivity of omics technologies allow to detect a plethora of molecules and to (at least in part) reflect biological processes within the body (especially metabolomics). However, researchers need to be careful to draw immediate mechanistic conclusions based on metabolomics or proteomics done in blood. Blood passes through the whole body and reflects the metabolic sum of all single organ or single tissue processes. Omics results from blood (especially in new fields of application like exercise) should ideally be compared in terms of plausibility with other direct metabolic measures e.g. respiratory gas exchanges by spiroergometry during exercise, which reliably states energy fuel usage.

Furthermore, careful considerations on the timing of blood samples during or after exercise should be made. Rapid changes of protein and metabolite concentrations in response to exercise can complicate interpretation of the results from only one or few time points. Even small timely variance (~10-15 minutes or even less) in sampling between subjects can lead to metabolite concentration changes, e.g. in nucleotides, which have been shown to change their concentration in the early post-exercise phase (5-20 minutes) but return to resting values quickly. As a classical exercise example, lactate changes in blood can be observed every minute when sampling from the earlobe during graded exercise and return to pre-exercise concentrations within minutes (depending on the exercise and the individual lactate clearance).

Practically, studies should be designed with at least two sampling time points within on subject and should cover different and precise metabolic states (e.g. pre-exercise, during exercise, post-exercise) to generate mechanistic hypotheses. Furthermore, researchers should verify metabolic states with other

measures (e.g. spiroergometry) and test mechanistic hypothesis in cell culture studies, mouse models, and ultimately, if feasible, in humans.

Thirdly, environmental factors like diet (e.g. protein-rich diet in strength athletes), dietary supplement intake (e.g. amino acid supplements), medication and exercise training (e.g. muscle damage) should be recorded and ideally standardized as they can influence metabolite concentrations in blood.

Finally, genetics contribute to metabolite and protein concentration differences between subjects (Lotta et al. 2021; Yousri et al. 2014) and can be responsible for up to 75% (for metabolites) of the metabolite concentration in blood (Shin et al. 2014). Similarly, athletic talent itself is influenced by genes (Sarzynski and Bouchard 2020). Advantageous traits for e.g. endurance performance like the trainability of $\dot{V}O_2$max, cardiac output or fat-free mass, and for strength-related performances like muscle strength are all in parts inherited (Bouchard 2012). Also, links between metabolites that differed between world-class and non-world-class athletes to genetic variants in world-class athletes have been reported (Al-Khelaifi et al. 2019a). Especially in small and highly specialized cohorts, genetics can influence outcomes in exercise studies.

IV-7. Conclusion

The variability and individuality of human metabolism, its associated phenotypes (from disease to athlete) and its capacity to adapt, calls for a granular and individualized compilation of exercise-associated metabolic changes. Within the field of exercise science, which has grown more and more important for a sedentary and ageing society, individualization can ensure the achievement of health and performance goal. Omics methods, which have been used in individualized medicine, enable such a granular compilation of metabolism and are since recently, attractive for exercise science research.

Metabolomics and proteomics studies in exercise science have become more and more since 2009 with the aim to discover metabolic read-outs or biological markers for metabolism or metabolic changes during or after short and long-term exercise interventions. The main issues in existing exercise omics studies were that a systematic summary on global metabolite changes after exercise was missing and that existing studies were to heterogenous in design to find out what the long-term effect of specific exercise modes on metabolite and protein changes was.

This thesis complements and extends existing basic exercise science and exercise omics literature with a global summary of exercise-associated metabolite concentration changes, the fact that the metabolome is shaped distinctly after to long-term specific exercise training and the finding that proteins can be used to predict exercise adaptation to training.

IV-8. Outlook and future directions

The valuable characteristic of omics methods for exercise or health science is the plethora of information about an individuals' metabolism they deliver. A targeted application of omics can lead to more individualization of exercise training in the future: the prediction of physiological adaptations and the monitoring of health and performance.

One specific hope is that omics measurements will yield biomarkers of a person's endurance, strength, or risk factor trainability that cope with the fact of individual variation in training or risk factor responses (Bouchard et al. 1999; Hubal et al. 2005). To avoid training for several weeks to find out that e.g. a risk factor has worsened, a key goal is to predict training responses at the planning stage e.g. by measuring biomarkers. In medicine, such an approach is known as precision, personalized or individualized medicine, where biomarkers are measured which are then used to select an intervention that works for the individual. Promising attempts to relate proteins or metabolites to endurance training status (Al-Khelaifi et al. 2019b; Robbins et al. 2021; Santos-Parker et al. 2018; Schader et al. 2020; Williams et al. 2017; Williams et al. 2019)) , world-class athlete status (Al-Khelaifi et al. 2018) or exercise phenotype have been made, including this PhD thesis (Robbins et al. 2021; Schranner et al. 2021). Still, these attempts need to be validated.

A proof-of-concept study that individual metabolic responses to external stimuli (here diet) can be calculated from multiple biomarkers was done by Zeevi et al. (2015). They used a combination of measures from the microbiome, dietary habits, anthropometry, physical activity, and blood parameters to predict the individual blood glucose response to different meals (Zeevi et al. 2015).

In relation to exercise, the prospective MoTrPac (*Molecular Transducers of Physical Activity*) studies aim to develop personalized recommendations for exercise (e.g. which kind of exercise and which intensity is most beneficial for an individual's health). Therefore, MoTrPac studies the systemic effects (genome, transcriptome, proteome, metabolome, adipose tissue, and muscle tissue) of acute and chronic exercise in untrained and trained subjects to identify molecules that cause adaptations to exercise. One specific goal is to find out if these molecules can be manipulated pharmacologically to mimic beneficial exercise effects (e.g. muscle mass gain or minimizing a risk factor) in people who cannot exercise (e.g. during bed rest) (Sanford et al. 2020).

In competitive sports, prediction of trainability would lay the fundament for future (training) decisions. Specifically, a future vision would be to shape or change the career of young athletes in several ways: First, for the individual itself, as performance goals are being reached in a reasonable time which increases the motivation to stick to the training. Secondly, it saves time for coaches as trainings are tailored to the athletes and training failures or injuries due to training that is not suitable fot he individual will get less frequent. Specifically, a prediction of trainability would include the identification of existing biomarker(s) for a trainability trait of interest, e.g. muscle mass gains or VO_2max in the blood of an individuum. Based on existing and validated computational models (e.g. ROC-analyses) for this trait of interest, the trainability can be predicted within a certain % accuracy. The limitation of such a prediction is that trainability prediction will be dichotomous in these models, meaning that a threshold value of the trait of interest e.g. muscle mass gain of 10% within a certain time frame is set and the prediction can only state if the subject can expect <10% or >10% of muscle mass gains. In most clinical settings, where improvements in health are the primary goal, such a dichotomous prediction (e.g. reduction in cardiovascular risk) would be sufficient, however in athletes or subjects who have specific performance goals a more detailed prediction (e.g. how much % muscle mass will I gain if I do training X for Y weeks?) is necessary. With current dichotomous prediction models being modest in predictive performance, such an envisioned detailed prediction might only be possible in the long run. Supposably, predictions would need to include a variety of standard physiological (BMI, sex, age, body fat, muscle mass), behavioral (exercise training, nutrition, lifestyle), exercise associated physiological (e.g. heart rate, performance tracking) and maybe even genetic predictors (SNPs) to reach a relevant predictive accuracy for application in competitive sports.

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VI. GLOSSARY

Aptamer	Nucleotide-based compounds with protein affinity, alternative to antibodies
Biomarker	Measurable biological indicator in body fluids like blood or urine that can be used for diagnosis or prognosis
Chromatography	method for protein separation from a mixture based on differential mobility through a medium
Duchenne's disease	A rare muscular dystrophy disease with muscle weakness and muscle loss
Electrospray ionization (ESI)	A technique to produce ions by spraying a liquid with molecules onto a laser to create an aerosol
Liquid chromatography (LC)	A separation technique applied in chemistry where molecules are dissolved in a liquid and are separated depending on their physical properties
Mass spectrometry (MS)	Method for metabolite or protein detection by mass and charge
Metabolome	All metabolites within one biological sample
Metabolomics	The study of all metabolites within one biological sample
Myokine	A protein with hormone-like effects that is secreted from skeletal muscle cells in response to muscle contraction and has various effects on other organs of the body (e.g. the liver)
PCG1a	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha. PCG1a is a protein that in humans is encoded by the PPARGC1A gene
Phenotype	All observable characteristics or traits of an organism (morphology, physical properties, structure, biochemical and physiological properties)
PPARGC1A	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
Proteome	All proteins within one biological sample
Proteomics	The study of all proteins within one biological sample
Sensitivity	Measure of assay ability to capture analytes with low abundance
Specificity	Measure of assay ability to measure a particular analyte rather than others
Tandem mass spectrometry (MS/MS)	A form of mass spectrometry using two or more mass analyzers in row to increase the sensitivity of molecule analysis (especially molecules with similar masses/charges)

Throughput	number of samples and/or analytes undergoing analysis in a certain timeframe
Intrinsic/baseline V̇O2max	the V̇O2maxan individual has without prior endurance training or after refraining from training for at least 3-6 months
Trait (phenotypic)	A characteristic of an organism

VII. SUPPLEMENTARY MATERIAL

VII-1. S1: Dynamic range of human plasma proteome and current detection methods

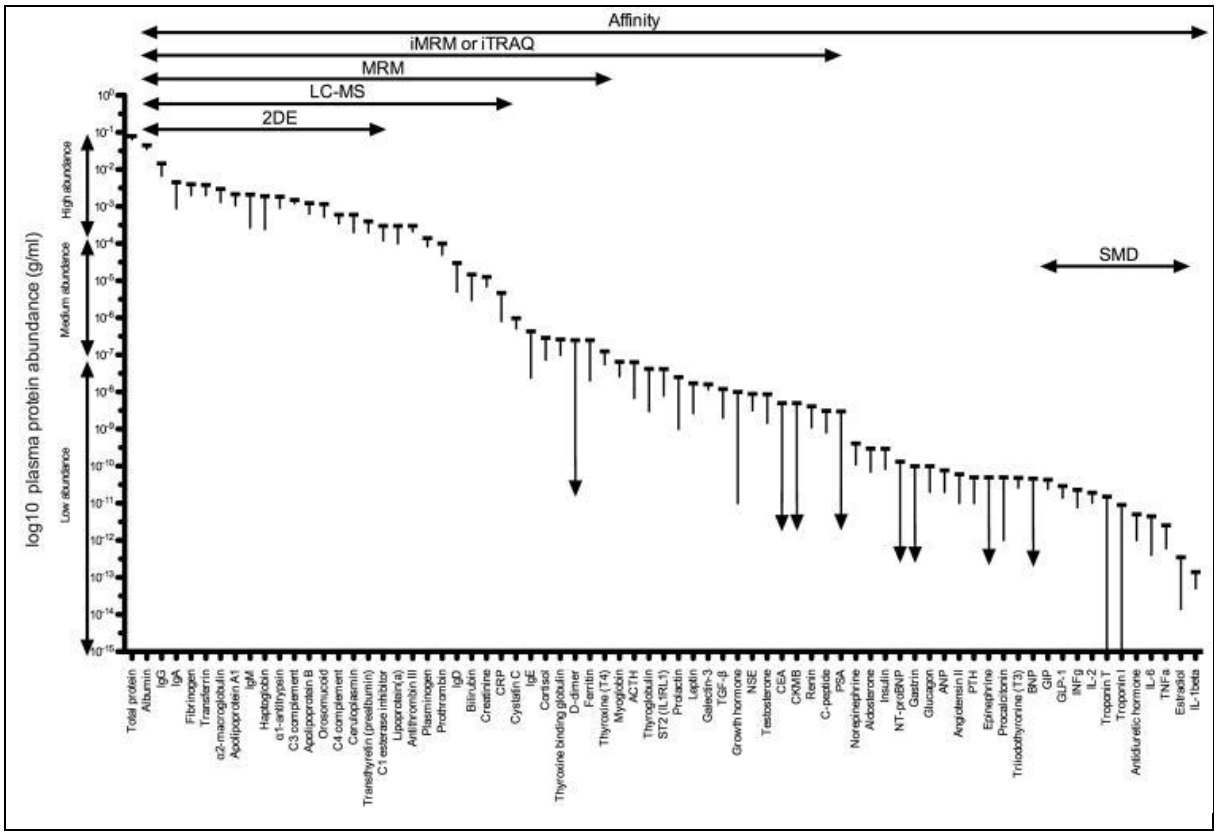


Figure S1. Dynamic range of the human plasma proteome and current detection methods.
Adapted from (Smith and Gerszten 2017).

Dynamic ranges for classical plasma proteins (high-medium abundance) and selected clinically relevant proteins. Intervals refer to published 95% reference ranges, or quartiles or ranges where reference intervals were unavailable. Lower ends of reference intervals have not been established for a few proteins (arrowhead). Affinity-based methods essentially capture proteins across the entire abundance spectrum but may be offset by specificity, whereas mass spectrometry (MS) has excellent specificity but is limited to proteins with high and medium abundance. Newer MS methods such as multiple reaction monitoring (MRM), immuno-MRM (iMRM) and isobaric tags (iTRAQ) may detect lower-abundance proteins and with additional separation steps reach as low as affinity methods. Ultrasensitive single molecule detection (SMD) assays are necessary to detect proteins at very low abundance, down to fg/ml. 2DE, 2-dimensional gel electrophoresis. LC-MS, liquid chromatography MS.

VII-2. S2: Pre-study questionnaires (in German)

VII-2.1. History of training

Referenznummer (ID): [redacted]

Fragebogen zur Trainingshistorie im Rahmen Studie

„Metabolomics und Sport“

an der Technischen Universität München:

Dank ihrer bereits absolvierten Blutabnahme, haben wir die Möglichkeit einen individuellen „Blut-Fingerabdruck“ von ihnen herzustellen. Da unser primäres Ziel ist, neue Biomarker-Kandidaten für den Sport zu entdecken, sind wir auf Informationen bezüglich ihrer sportlichen Karriere angewiesen. Warum?

Als Beispiel, ihr Trainingsalter definiert im großen Maße wie stabil metabolische Muster ausgeprägt sind – je höher das Trainingsalter, desto stabiler die angepassten biologischen Systeme, desto ausgeprägter der „Blut-Fingerabdruck“.

Wie Sie wahrscheinlich am eigenen Leib erfahren haben, braucht es ein diszipliniertes, zielgerichtetes und jahrelanges Training um ein erfolgreicher Athlet zu werden – genau diese Details versuchen wir mit diesem Fragebogen zu erfassen.

Dieser Fragebogen ist freiwillig, d.h. wenn sie sich dazu entscheiden ihn zu beantworten, bitte wir Sie ihn möglichst ehrlich und genau zu bearbeiten.

Alle hier erhobenen Informationen werden zur Auswertung und Archivierung pseudonymisiert und dann dauerhaft maschinell gespeichert. Dabei werden die geltenden gesetzlichen Bestimmungen des Datenschutzes eingehalten. Sie persönlich haben jederzeit die Möglichkeit Ihre Daten einzusehen.

1. Allgemein: Welche Sportart üben Sie aus?

Triathlon
Bouldern (1x mtl.)
Kampfsport (2-wöchentlich)

2. Allgemein: **Wie viele Trainingsstunden (ohne Wettkämpfe) absolvieren** Sie in der Woche im Durchschnitt?

13,2 (Saison 2017), 13,1 (Saion 2016), 15 (Saison 2105), 12 (Saison 2014), ca. 10 (2013)
aktuell (10-16h)

3. Allgemein: Wie viele freie („trainingsfreie“) Wochen haben Sie pro Jahr?

2017: 2 Wochen Saisonpause ohne Trainingsplan
2016: 3 Wochen
2015: 4 Wochen

4. Allgemein: Bitte listen Sie alle Wettkampfteilnahmen der letzten 3 Jahre auf, inklusive Platzierung.

2017: Duathlon Krailling 3., Trimotion Saalfelden 6., Triathlon Erding Bayern Liga 4.,
Allgäu Triathlon MD: 18.,Challenge Walchsee MD: 17., Zeitfahren King of the Lake 13.
2016: Duathlon Krailling 4., Triathlon Waging (BL) 37.,
Schlierseetriathlon 8., Challenge Regensburg 92., Ötztaler Radmarathon 664.
2015: Duathlon Krailling 14., Triathlon Ingolstadt 16., Triathlon Erlangen MD 27.
Challenge Walchsee 42.

5. Details: Bitte listen Sie alle ihre Verletzungen (ACHTUNG: keine Krankheiten) mit Zeitpunkt und Dauer auf:

Fraktur Zeh (III links) Mai 2012 2-3 Wochen Pause
Haarriss Nasenbein April 2015, 2 Wochen kein Schwimmen
Fraktur Zeh (rechts IV) Februar 2015, ca. 2 Wochen bis zu lockerer Belastung

6. Details: Bitte beschreiben Sie ihre verschiedenen Wettkampfphasen für dieses Jahr, und, in welcher Sie sich gerade befinden:

Vorbereitung I (KW 44-2): Schwerpunkt Krafttraining, Grundlagenausdauer
Vorbereitung II (KW 3-12): Schwerpunkt Grundlagentraining,
längere Umfänge, zunehmend Intervall Training, hauptsächlich (GA1/2)
Wettkampfvorbereitung I (KW 13-18): spezifische Intervalle verstärkt auch im EB, wenig Krafttraining
Wettkampfperiode I (KW 19-26): diverse Wettkämpfe, Training entsprechend angepasst
Wettkampfvorbreitung II (KW 27-31): Trainingszeit für Langdistanz in KW 32
Wettkampfperiode II (KW 32-38): Nach Langdistanz+Erholung, Schwerpunkt Zeitfahren
Übergangsperiode (KW 39-43): locker mit min. 2 Wochen Saisonpause)

7. A) Details: In einer regulären Trainingswoche, wie viele Stunden **Ausdauertraining** (z.B. Grundlagentraining, Intervalltraining, Spielformen, etc.), **Krafttraining** (z.B. Kraftausdauertraining, Maximalkrafttraining, Hypertrophietraining, Stabilitätstraining etc.), **Schnellkrafttraining** (Sprünge, Sprints, olympisches Gewichtheben, etc.) oder **Beweglichkeitstraining** (Dehnen, Mobilisation, Faszientraining, etc.) absolvieren Sie?

Ausdauertraining:	8-14 h varriert je nach Jahreszeit
Krafttraining:	2h in der Vorbereitung
Schnellkrafttraining:	-
Beweglichkeitstraining:	1-2 h

B) Details: Bitte beschreiben Sie detailliert, die oben angegebene Stundenanzahl:

Beispiel: OBEN = Ausdauertraining 14h pro Woche

Antwort: 8h Grundlagentraining, 2h langes Intervalltraining, 2h mittleres Intervalltraining, 2h Sprintintervalltraining

ODER:

Antwort: 10h Grundlagentraining, davon 5h Fahrrad, 3h Laufen, 2h Schwimmen; 3h langes Intervalltraining nur Laufen, 1h Sprintintervalltraining nur Fahrrad.

Reguläre Woche:
1-2 mal Schwimmen (1-3 h): GA, Intervalle (100er, 200er)
2-3 mal Laufen (3-5 h): eher lange Intervalle (10 min); eher lange Einheiten

2-3 Radfahren (6-12h): Grundlage 1-2, Schwellentraining ab

Intervalltraining idR. mit mittleren oder langen Belastungen

HIT-Intervalle selten

Intervalle sowohl beim Laufen als auch Radfahren, aber eine Disziplin als

Schwerpunkt jede Woche, die andere dann meistens mit lockereren Einheiten

Häufig Koppeltraining, Lauf direkt nach Radfahren (auch in dieser Jahresphase)

8. Bitte beschreiben Sie die letzten 4 Trainingswochen (mit den jeweiligen Wochenschwerpunkten) vor ihrer heutigen Teilnahme an unsere Studie:

KW7: Arbeitsbedingt eher lockere Woche

Schwerpunkt Krafttraining, Laufen und Radfahren mit Technik Anteil

KW 8: Schwerpunkt: Winterlaufserie 21,4km am Sonntag

Davor überwiegend lockeres Training beim Laufen, wenig Radfahren

Krafttraining für Maximalkraft, Temperaturen waren sehr kalt

KW9: Schwerpunkt: Grundlagenausdauer

Eher Laufintensiv, Rad und Kraft normal

Krafttraining auf Kraftausdauer umgestellt

KW 10: Schwerpunkt Grundlagenausdauer, längere Einheiten

Hauptanteil Radfahren

9. Details: Nur Sie kennen sich am Besten. Bitte beschreiben Sie sich als Athlet.

Beispielfragen: Was für ein Athlet sind Sie? Wo liegen ihre Stärken, bei welchen Trainings tun Sie sich leicht?
Bei welchen Einheiten fällt es ihnen schwer die geforderte Arbeit zu leisten?
Würden Sie sich eher als schnellen oder langsamen Athleten einschätzen?
Waren Sie als Kind/ Jugendlicher immer der schnellste in der Klasse?

Stärken:
Konsequentes und kontinuierliches Training anhand von einem Trainingsplan
Kann mich durch realistische Ziele gut Selbstmotivieren
Halbwegs robust, selten verletzt

Schwächen: Ernährung ist auf jeden Fall noch zu verbessern.
Am besten liegen mir mittlere Intervalle an der "Schwelle"
(~Mitteldistanz)
Bei langen Einheiten ist das Einteilen/Versorgen noch nicht ideal.
Schlechter verkrafte ich kurze und häufig wechselnde sehr Intensive
Intervalle
Absolute Endgeschwindigkeit beim Laufen könnte besser sein.
Ausdauersportarten lagen mir immer, sonstige Sportarten waren
ganz gut. Insgesamt viele Sportarten betrieben, dadurch halbwegs vielseitig.
Schulzeit: eher schlechter in Sprints, 400 m gingen schon eher,

Bitte kreuzen Sie zutreffendes an. Falls Sie „Ja“ ankreuzen, geben Sie bitte genauere Informationen.

Haben Sie innerhalb der letzten 4 Wochen vor Ihrem Termin für die Studie Melatonin oder Ähnliches eingenommen?	Nein ☑ Ja ☐

Hatten Sie innerhalb der letzten 4 Wochen vor Ihrem Termin für die Studie einen Interkontinentalflug (=mehr als 3 Zeitzonen)?	Nein ☑ Ja ☐
Konsumieren sie exzessiv Koffein/koffeinhaltige Produkte? (mehr als 750 mg pro Tag, vgl. 1 Tasse Kaffe entspr. ca. 40-120 mg Koffein)	Nein ☑ Ja ☐
Haben sie innerhalb der letzten 60 Tag vor Studienteilnahme Blut oder Blutbestandteile (z.B. Plasma, Thrombozyten o.Ä.) gespendet?	Nein ☑ Ja ☐
Nehmen Sie gleichzeitig noch an einer Studie teil?	Nein ☑ Ja ☐
Haben Sie die letzten 6 Monate an einer Studie teilgenommen?	Nein ☑ Ja ☐

Vielen herzlichen Dank, dass Sie sich die Zeit genommen haben.

Ihr Studienteam

VII-2.2. Training protocol

Endurance athlete, example for one week:

TUM Metabolomics Studie
Version 1, 24.11.2017

Woche ~~1~~ Trainingsprotokoll

Datum: 23.07. bis 29.07.18

Wochentage	Trainingsinhalt	Kommentar
Montag	12km lauf locker	
Uhrzeit 1.Training ___:___	4,4km H20 I (1:25h)	
Uhrzeit 2.Training ___:___	30min Stabi	
Dienstag	Rad 4x (20' I - 10' I II - 5' II i 10min locker)	
Uhrzeit 1.Training ___:___	Lauf 1:11h / 15,8km mit 6x80m II	
Uhrzeit 2.Training ___:___		
Mittwoch	1:20h 4,0km H20 I	
Uhrzeit 1.Training ___:___	2:30h Rad 72km	
Uhrzeit 2.Training ___:___	14km lauf I 1:01h	
Donnerstag	1:30h Rad nüg	
Uhrzeit 1.Training ___:___	30min koppellauf → Sis 3:30	
Uhrzeit 2.Training ___:___	30min Stabi 40min Freiwasser	
Freitag	1h H2O I 3km	
Uhrzeit 1.Training ___:___	40min Lauf I 9,4km	
Uhrzeit 2.Training ___:___		
Samstag	2h Rolle I+	
Uhrzeit 1.Training ___:___		
Uhrzeit 2.Training ___:___		
Sonntag	2:45 Rolle II : 3x5h II 5x1h II	
Uhrzeit 1.Training ___:___		
Uhrzeit 2.Training ___:___	17km lauf TV (3-1-22-1-3-2-2 i.1 locker)	

Natural Bodybuilder, example for one week:

TUM Metabolomics Studie
Version 1, 24.11.2017

Woche 1 Trainingsprotokoll

Datum: 25.06.18 bis 01.07.18

Wochentage	Trainingsinhalt	Kommentar
Montag Uhrzeit 1.Training: 17:45 Uhrzeit 2.Training: ___:___	Pull Kreuzheben Deload Deadlift 4x3 mit 80% Hex-Bar Deadlift 2x8 Latzug breiter Griff 2x10 Seal Rows Langhantel 2x10 Bizepscurl Sz-Stange 3x10 Reverse Flys Kurzhantel 2x15 Bizepscurl Kabelzug einarmig 2x12 Fahrradergometer 45min	alle Übungen mit 60% des Trainingsgewichts
Dienstag Uhrzeit 1.Training: 16:45 Uhrzeit 2.Training: ___:___		
Mittwoch Uhrzeit 1.Training: 18:00 Uhrzeit 2.Training: ___:___	Beine Leicht Kniebeuge ~~Low~~ High Bar 4x8 Frontkniebeuge 4x8 Ausfallschritte Langhantel 2x12 Toes to Bar 1x max Cable Crunch Kabelzug 3x12 Wadenheben einbeinig 4x15	
Donnerstag Uhrzeit 1.Training: 18:30 Uhrzeit 2.Training: ___:___	Push Brust Bankdrücken flach Langhantel 5x6 Bankdrücken schräg Langhantel 3x10 Seitheben 4x12 Schulterdrücken Kurzhantel 2x12 Flys/Fliegende Kurzhantel 2x12 French Press Sz-Stange 4x10	
Freitag Uhrzeit 1.Training: 18:30 Uhrzeit 2.Training: ___:___	Pull Klimmzug Klimmzug breiter Griff 5x8 T-Bar Rudern breiter Griff 4x10 Rudern Kabelzug eng 3x12 Bizepscurl Kurzhantel 3x10 Reverse Flys Kurzhantel 5x12 Konzentrationscurls Kurzhantel 3x12 Fahrradergometer 45min	
Samstag Uhrzeit 1.Training: 09:30 Uhrzeit 2.Training: ___:___		
Sonntag Uhrzeit 1.Training: 15:15 Uhrzeit 2.Training: ___:___	Beine Schwer Kniebeuge Low Bar 5x6, Backoff 70% 1x10 Ausfallschritte Langhantel 3x10 Toes to Bar 1x max Bulgarian Splitkniebeuge 3x15 Cable Crunch Seilzug 3x12 Wadenheben einbeinig 3x15	

VII-2.3. Nutritional protocol

Example for one day

TUM Metabolomics Studie

Version 1, 24.11.2017

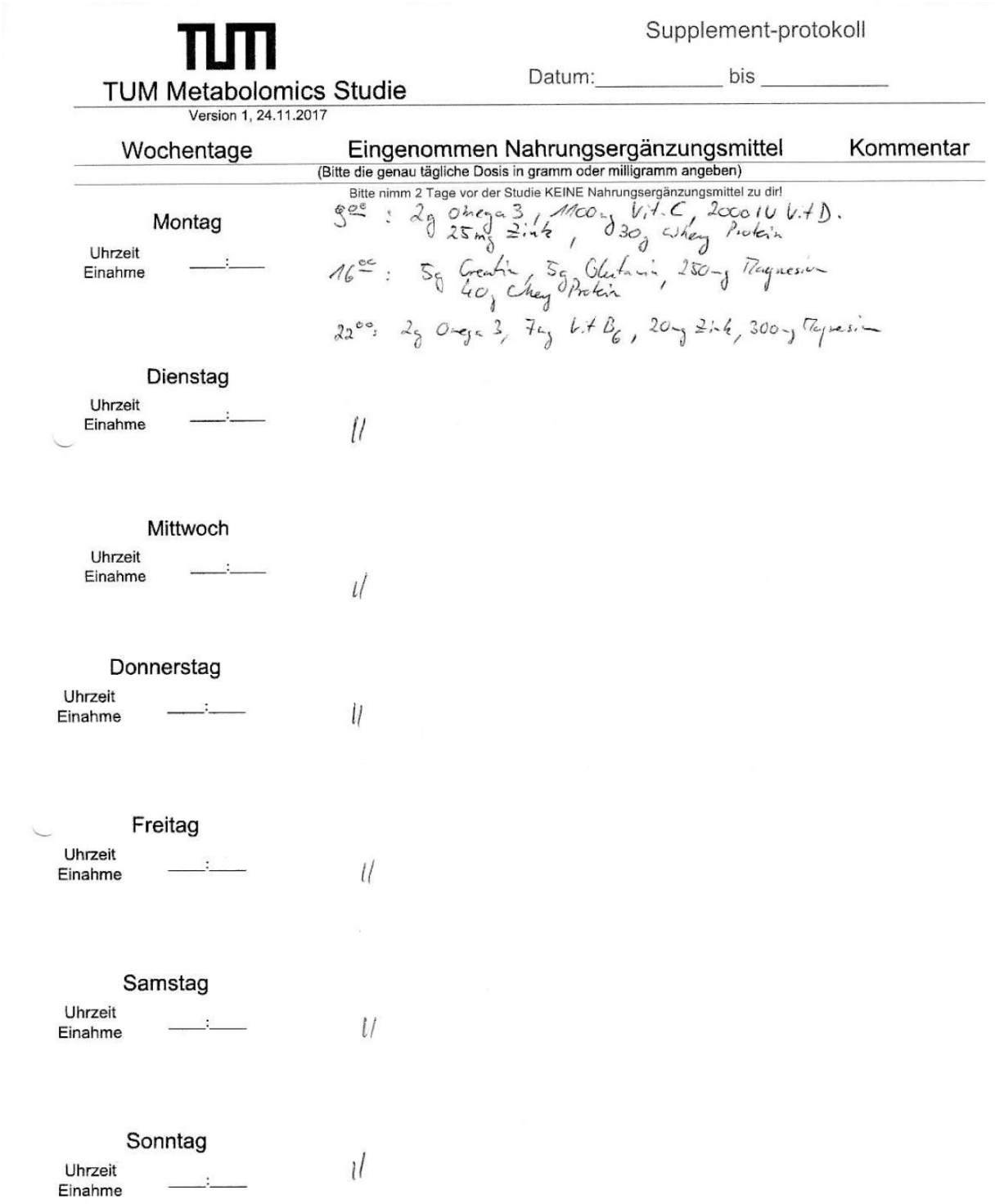
Tag 1 Ernährungsprotokoll

Datum: 01.03.2018

Mahlzeit	Nahrungsmittel & Getränke (Angaben in Portionen/ Gramm bei Essen, in Liter bei Getränken)	Kommentar
Frühstück Uhrzeit 08:00	150g Joghurt, 1 Apfel, 3 EL Walnüsse, 1 TL Honig, 4 EL Haferflocken, 2 Scheiben Toast, Margarine, Aprikosenmarmelade 1 Capuccino 0,5l Wasser	(Joghurt immer 3,5%)
Snack Uhrzeit :___		
Mittagessen Uhrzeit 13:00	Salat (50g Tomaten, 50g Gurke) Salatsauce 150g gegarte Kartoffeln, 100g gegartes Gemüse Tomatensauce 2 Hand voll Trauben 1l Wasser	(Gemüse-Mix aus karotten, Erbsen, Brokkoli, Bohnen)
Snack Uhrzeit 16:00	50g Studentenfutter, 0,5l Rote-Beete-Saft 0,5l Wasser 1 Capuccino	
Abendessen Uhrzeit 19:30	Salat (50g Tomaten, 50g Gurke) Salatsauce 200g Reis, 100g gegartes Gemüse Tomatensauce 0,25l orangensaft 1l Wasser	
Snack Uhrzeit :___		

VII-2.4. Dietary supplemental protocol

Example for one week



TUM Metabolomics Studie
Version 1, 24.11.2017

Supplement-protokoll

Datum:_____ bis _____

Wochentage	Eingenommen Nahrungsergänzungsmittel (Bitte die genau tägliche Dosis in gramm oder milligramm angeben)	Kommentar
	Bitte nimm 2 Tage vor der Studie KEINE Nahrungsergänzungsmittel zu dir!	
Montag	8^{00}: 2g Omega 3, 1100 mg Vit. C, 2000 IU Vit D, 25 mg Zink, 30 g Whey Protein	
Uhrzeit Einahme ___:___	16^{00}: 5g Creatin, 5g Glutamin, 250 mg Magnesium, 40 g Whey Protein	
	22^{00}: 2g Omega 3, 7 mg Vit B_6, 20 mg Zink, 300 mg Magnesium	
Dienstag	//	
Uhrzeit Einahme ___:___		
Mittwoch	//	
Uhrzeit Einahme ___:___		
Donnerstag	//	
Uhrzeit Einahme ___:___		
Freitag	//	
Uhrzeit Einahme ___:___		
Samstag	//	
Uhrzeit Einahme ___:___		
Sonntag	//	
Uhrzeit Einahme ___:___		

VII-2.5. Medication protocol

Example for one week

TUM Metabolomics Studie

Version 1, 24.11.2017

Medikamentenprotokoll

Datum:_____ bis _____

Wochentage	Eingenommen Medikamente (Bitte alle Medikamente in Milligramm (mg) angeben, mit dem jeweiligen Wirkstoff!)	Kommentar
Montag Uhrzeit Einahme ____:____		
Dienstag Uhrzeit Einahme ____:____		
Mittwoch Uhrzeit Einahme ____:____		
Donnerstag Uhrzeit Einahme ____:____		
Freitag Uhrzeit Einahme ____:____		
Samstag Uhrzeit Einahme ____:____		
Sonntag Uhrzeit Einahme ____:____		

VII-2.6 Ethical approval for Study 2

Unser Zeichen: **356/17 S**

Technische Universität München

Technische Universität München · Fakultät für Medizin · Ethikkommission
Ismaninger Str. 22 · 81675 München · Germany

Technische Universität München
Fakultät für Sport- und Gesundheitswissenschaft
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Fax +49 89 4140 4199

ethikkommission@mri.tum.de
www.ek.med.tum.de

München, 14.08.2017/S

Unser Zeichen: **356/17 S** (bitte bei Schriftwechsel angeben)

cc: Dr. Martin Schönfelder (per E-Mail)

Beratung nach § 15 Berufsordnung für Ärzte in Bayern

Studientitel: Ist die sportliche Leistungsfähigkeit mit dem BlutMetabolom in Ruhe oder nach maximaler Belastung assoziiert?
Antragsteller: Prof. Dr. Henning Wackerhage

Sehr geehrter Herr Prof. Wackerhage,

die Ethikkommission hat Ihren Antrag vom 09.08.2017 auf der Basis der vorgelegten Unterlagen geprüft.

Die Ethikkommission erhebt keine Einwände gegen die Durchführung der Studie.

Die ethische und rechtliche Verantwortung für die Durchführung dieser Studie verbleibt bei Ihnen.
Änderungen des Protokolls sind zur erneuten Prüfung einzureichen.
Das Studienende ist anzuzeigen und ein Kurzbericht über das Ergebnis der Studie ist vorzulegen.
Die Ethikkommission empfiehlt die Eintragung des Forschungsprojektes in ein WHO-anerkanntes Register.

Mit freundlichen Grüßen

Vorsitzender der Ethikkommission

Unser Zeichen: **356/17 S**

vorgelegte Unterlagen:
Antrag vom 09.08.2017
Anschreiben, 08.08.2017
Probandeninformation und Einverständniserklärung
Studienplan, Version 11, 04.08.2017
CV Wackerhage (Projektleiter), CV Schönfelder
Zustimmung Klinikdirektor, 09.08.2017; Finanzieller Interessenkonflikt unterzeichnet Wackerhage
Fragebogen

revidierte Unterlagen vom 11.08.2017; Eingang 14.08.2017
Finanzieller Interessenkonflikt unterzeichnet Schönfelder

VIII. COPYRIGHT AGREEMENTS

VIII-1. Sports Medicine - Open

Re: SMOA-D-19-00085R2

RL Rick Lamb <rick.lamb@biomedcentral.com>
An Schranner, Daniela

Antworten | Allen antworten | Weiterleiten | ...
Fr 11.06.2021 01:45

Sie haben am 11.06.2021 07:34 auf diese Nachricht geantwortet.
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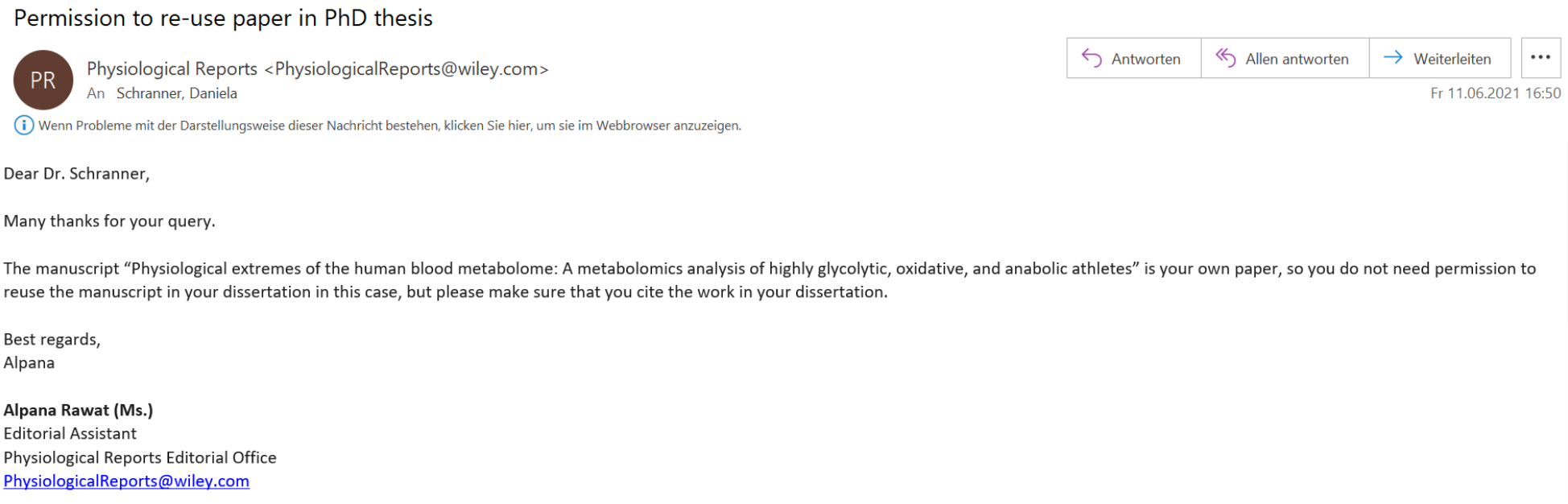
Best,
Rick Lamb
Production Editor,
Springer Nature

VIII-2. Physiological Reports



Permission to re-use paper in PhD thesis

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Physiological Reports <PhysiologicalReports@wiley.com>
An Schranner, Daniela

Antworten | Allen antworten | Weiterleiten | •••

Fr 11.06.2021 16:50

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Dear Dr. Schranner,

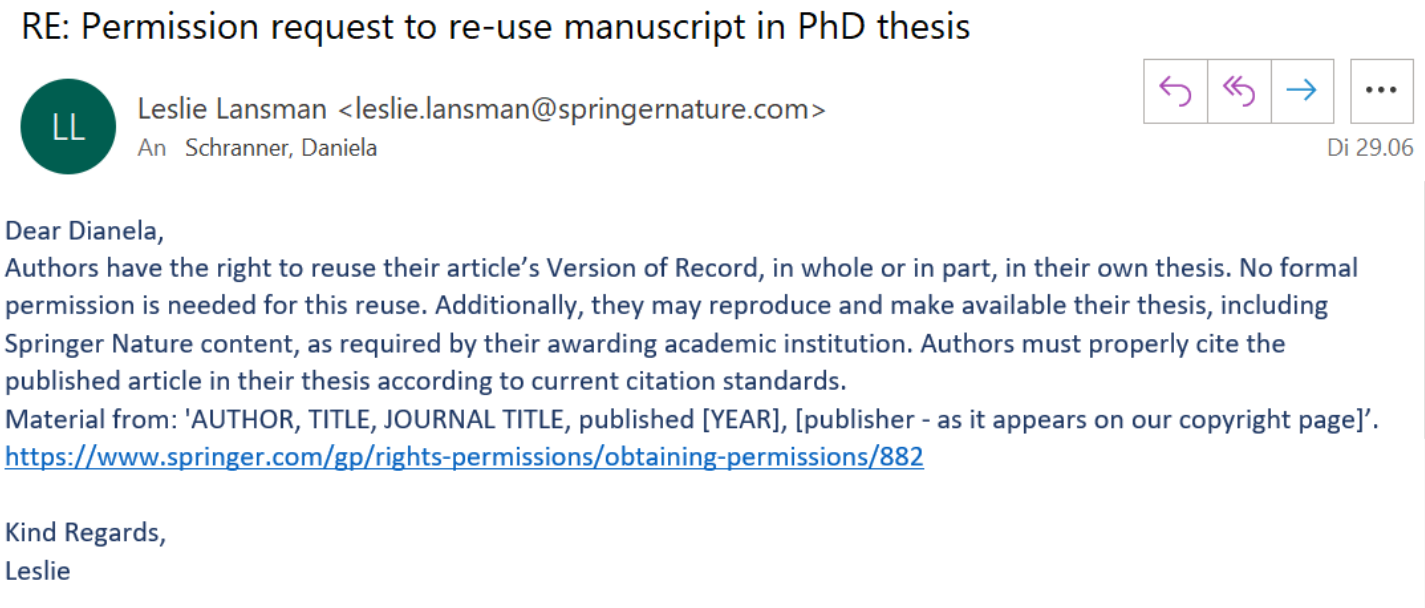
Many thanks for your query.

The manuscript “Physiological extremes of the human blood metabolome: A metabolomics analysis of highly glycolytic, oxidative, and anabolic athletes” is your own paper, so you do not need permission to reuse the manuscript in your dissertation in this case, but please make sure that you cite the work in your dissertation.

Best regards,
Alpana

Alpana Rawat (Ms.)
Editorial Assistant
Physiological Reports Editorial Office
PhysiologicalReports@wiley.com

VIII-3. Nature Metabolism



RE: Permission request to re-use manuscript in PhD thesis

LL

Leslie Lansman <leslie.lansman@springernature.com>

An Schranner, Daniela

Di 29.06

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Kind Regards,

Leslie