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EFFECTS OF AGING AND LIFELONG EXERCISE ON THE METABOLIC FINGERPRINT AND FRAILTY STATUS OF RATS

PhD THESIS

by ANASTASIA TZIMOU

Graduate of the School of Physical Education and Sports Science, AUTH

Master in Sports Management, University of Technology, Sydney

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Anastasia Tzimou

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Examination Committee:

Professor Vassilis Mougios, supervisor

Professor Georgios Theodoridis, member of the advisory committee

Associate Professor Christos Galazoulas, member of the advisory committee

Professor Emmanuel Mikros, member of the examination committee

Professor Ioannis Taitzgolou, member of the examination committee

Professor Kosmas Christoulas, member of the examination committee

Assistant Professor Eleni Gkika, member of the examination committee

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EFFECTS OF AGING AND LIFELONG EXERCISE ON THE METABOLIC FINGERPRINT AND FRAILTY STATUS OF RATS

ABSTRACT

Life expectancy has increased over the past decades, resulting in an increase in the number of aged individuals. Aging comes with a multitude of physical and mental deteriorations that lead to age-associated diseases and frailty. Regular exercise is an important part of a healthy lifestyle, as it helps maintain a healthy weight and reduces the risk of chronic diseases. Also, it is available to everyone, simple, inexpensive and non-invasive. These characteristics make it a highly effective form of lifestyle intervention and treatment of age-related disease. Metabolomics is an important tool in understanding the mechanisms regulating the responses of biological systems to natural processes (such as aging) and stimuli (such as exercise), as well as for detecting specific and sensitive biomarkers. The aim of the present thesis was to examine the effects of aging and lifelong exercise on the rat blood and urinary metabolomes through a metabolomics approach, on insulin sensitivity and on the muscle, liver and brain fatty acid composition. Thirty-six male Wistar rats were divided into four equal groups: exercise during the 1st half of life (3–12 months, A), lifelong exercise (3–21months, B), no exercise (C), and exercise during the 2nd half of life (12–21 months, D). Exercise consisted in swimming for 20 minutes, five times a week. Blood and urine samples were collected at 3 months of age (before the beginning of exercise), at 12 months and at 21 months. Urine samples were additionally collected before and 1 h after an exercise session at 3 and 12 months of age. Insulin sensitivity was assessed by the blood glucose response to an insulin bolus injection on the 21st month, one week after the last exercise session of the exercising groups. The lifelong and no-exercise groups were afterwards subjected to the same exercise acutely and insulin sensitivity was assessed 1, 3, 5 and 7 days post-exercise. Seventeen days after the last exercise session of group D, the rats of groups A and D were euthanised and their quadriceps, liver and brain were collected. The same was done with groups B and C, 3 months after the last exercise session of group B. A total of 101 deproteinized whole-blood samples were analysed by proton nuclear magnetic resonance, which identified 39 metabolites. A total of 141 urine samples were analysed using targeted metabolomic analysis by liquid chromatography – mass spectrometry, which identified 51 metabolites. Twenty-four samples of quadriceps, 24 samples of liver and 22 samples of brain were analysed for the fatty-acid composition of triacylglycerols and phospholipids by thin-layer chromatography – gas chromatography. Data were analysed by multivariate methods (PCA, PLS-DA and OPLS) and by univariate methods (repeated-measures ANOVA and Student's *t* test). The groups

that exercised during the 2nd half of life weighed less than the groups that did not. Exercise had an orexigenic effect during the 1st half and an anorexigenic effect during the 2nd half. Multivariate analysis did not show any discrimination between groups at any of the three ages, in either blood or urine. However, it showed a clear discrimination between ages when the groups were treated as one, in both biofluids. Univariate analysis showed different effects of exercise and aging on metabolites involved in carbohydrate (glucose, pyruvate, acetylcarnitine), lipid (glycerol, acetylcarnitine) and protein (several amino acids and their derivatives) metabolism. Interestingly, although exercise had a profound effect on the metabolome at an early age (i.e., between 3 and 12 months), this effect tended to fade at an older age (i.e,, between 12 and 21 months). Insulin sensitivity did not differ among groups, suggesting either no prolonged effect of exercise or no effect at all. Moreover, compared to baseline, insulin sensitivity was higher at 1 and 3, but not at 5 or 7 days post-exercise. Multivariate analysis showed a discrimination between groups A and D in quadriceps triacylglycerol fatty acids and in both phospholipid and triacylglycerol fatty acids of the liver. Univariate analysis showed a decrease in some saturated and monounsaturated fatty acids, and an increase in polyunsaturated fatty acids. On the other hand, multivariate analysis did not show any discrimination between groups B and C on any lipid parameter. In conclusion, moderate-intensity exercise for life or half-life had strong and potentially healthful effects on body weight and (partly) appetite, as well as on some blood and urinary metabolites. The effects of aging on the blood and urinary metabolome seemed to be stronger than those of exercise. Furthermore, exercise appeared to exert an acute, not chronic, effect on insulin sensitivity which wore off between 3 and 5 days post-exercise. Finally, exercise during the 2nd half seemed to enhance the abundance of the vital polyunsaturated fatty acids. The opposite effect on saturated and monounsaturated fatty acids may be due to a higher utilisation of these fatty acid categories in exercise. Three months of detraining, however, annihilated the effects of exercise on the fatty-acid profiles of aged rats.

Keywords: aging, blood metabolome, exercise, insulin sensitivity, urinary metabolome

ΕΠΙΔΡΑΣΕΙΣ ΤΗΣ ΓΗΡΑΝΣΗΣ ΚΑΙ ΤΗΣ ΔΙΑ ΒΙΟΥ ΑΣΚΗΣΗΣ ΣΤΟ ΜΕΤΑΒΟΛΙΚΟ ΑΠΟΤΥΠΩΜΑ ΚΑΙ ΣΤΗ ΓΕΡΟΝΤΙΚΗ ΑΔΥΝΑΜΙΑ ΕΠΙΜΥΩΝ

ΠΕΡΙΛΗΨΗ

Το προσδόκιμο ζωής έχει αυξηθεί τις τελευταίες δεκαετίες, με αποτέλεσμα να αυξηθεί ο αριθμός των ηλικιωμένων ατόμων. Η προχωρημένη ηλικία συνοδεύεται από πληθώρα σωματικών και νοητικών αλλοιώσεων, που οδηγούν σε ασθένειες σχετιζόμενες με τη γήρανση και τη γεροντική αδυναμία. Η τακτική άσκηση είναι αναπόσπαστο μέρος ενός υγιεινού τρόπου ζωής, καθώς βοηθά στη διατήρηση υγιούς σωματικού βάρους και μειώνει τον κίνδυνο εμφάνισης χρόνιων παθήσεων. Επίσης, είναι διαθέσιμη σε όλους, απλή, φθηνή και μη παρεμβατική. Αυτά τα χαρακτηριστικά την καθιστούν μια πολύ αποτελεσματική μορφή παρέμβασης στον τρόπο ζωής και θεραπείας ασθενειών που σχετίζονται με την ηλικία. Η μεταβολομική είναι ένα σημαντικό εργαλείο για την κατανόηση των μηχανισμών που ρυθμίζουν τις αποκρίσεις των βιολογικών συστημάτων στις φυσικές διεργασίες (όπως η γήρανση) και φυσικά ερεθίσματα (όπως η άσκηση), καθώς και για την ανίχνευση συγκεκριμένων και ευαίσθητων βιοδεικτών. Σκοπός της παρούσας διατριβής ήταν η εξέταση των επιδράσεων της γήρανσης και της δια βίου άσκησης στο μεταβόλωμα του αίματος και των ούρων αρσενικών επιμύων μέσω μιας μεταβολομικής προσέγγισης, στην ευαισθησία στην ινσουλίνη και στη σύσταση λιπαρών οξέων του μυός, του ήπατος και του εγκεφάλου. Τριανταέξι αρσενικοί επίμυες της φυλής Wistar χωρίστηκαν σε τέσσερις ίσες ομάδες: άσκησης κατά το 1^{ο} μισό της ζωής (3-12 μήνες, A), δια βίου άσκησης (3-21 μήνες, B), μη άσκησης (C) και άσκησης κατά το 2^{ο} μισό της ζωής (12-21 μήνες, D). Η άσκηση περιλάμβανε κολύμβηση για 20 λεπτά, πέντε φορές την εβδομάδα. Δείγματα αίματος και ούρων συλλέχθηκαν σε ηλικία 3 μηνών (πριν από την έναρξη της άσκησης), 12 μηνών και 21 μηνών. Δείγματα ούρων συλλέχθηκαν επίσης πριν και μία ώρα μετά από μια συνεδρία άσκησης σε ηλικία 3 και 12 μηνών. Η ευαισθησία στην ινσουλίνη αξιολογήθηκε μέσω της απόκριση της γλυκόζης του αίματος σε μια ένεση ινσουλίνης τον 21ο μήνα, μία εβδομάδα μετά την τελευταία συνεδρία των ασκούμενων ομάδων. Η ομάδα δια βίου άσκησης και η ομάδα μη άσκησης υποβλήθηκαν κατόπιν στην ίδια οξεία άσκηση και η ευαισθησία στην ινσουλίνη αξιολογήθηκε 1, 3, 5 και 7 ημέρες μετά την άσκηση. Δεκαεπτά ημέρες μετά την τελευταία συνεδρία άσκησης της ομάδας D οι επίμυες των ομάδων A και D θανατώθηκαν και συλλέχθηκαν ο τετρακέφαλος μυς, το ήπαρ και ο εγκέφαλός τους. Το ίδιο πραγματοποιήθηκε και με τις ομάδες B και C, 3 μήνες μετά την τελευταία συνεδρία άσκησης της ομάδας B. Συνολικά 101 δείγματα αποπρωτεϊνωμένου αίματος αναλύθηκαν με τη μέθοδο του πυρηνικού μαγνητικού συντονισμού πρωτονίου, με την οποία ταυτοποιήθηκαν 39 μεταβολίτες. Συνολικά 141

δείγματα ούρων αναλύθηκαν χρησιμοποιώντας στοχευμένη μεταβολομική ανάλυση με υγρή χρωματογραφία – φασματομετρία μάζας, όπου ταυτοποιήθηκαν 51 μεταβολίτες. Εικοσι τέσσερα δείγματα τετρακεφάλου, 24 δείγματα ήπατος και 22 δείγματα εγκεφάλου αναλύθηκαν ως προς τη σύσταση λιπαρών οξέων των τριακυλογλυκερολών και φωσφολιπιδίων με χρωματογραφία λεπτής στιβάδας – αέρια χρωματογραφία. Τα δεδομένα αναλύθηκαν στατιστικά με πολυμεταβλητές μεθόδους (PCA, PLS-DA και OPLS) και με μονομεταβλητές μεθόδους (ANOVA με επαναλαμβανόμενες μετρήσεις και δοκιμασία *t* του Student). Οι ομάδες που ασκήθηκαν κατά το 2^{ο} μισό της ζωής τους ζύγιζαν λιγότερο από τις ομάδες που δεν ασκήθηκαν. Η άσκηση είχε ορεξιγόνα δράση κατά το 1^{ο} μισό και ανορεξιγόνα κατά το 2^{ο} μισό. Η πολυμεταβλητή ανάλυση δεν διαχώρισε τις τέσσερις ομάδες σε οποιαδήποτε από τις τρεις ηλικίες, ούτε στο αίμα, ούτε και στα ούρα. Ωστόσο, διαχώρισε ξεκάθαρα τις τρεις ηλικίες, όταν οι ομάδες αντιμετωπίστηκαν ως μία, και στα δύο βιολογικά υγρά. Η μονομεταβλητή ανάλυση έδειξε διαφορετικές επιδράσεις της άσκησης και της γήρανσης σε μεταβολίτες που εμπλέκονται στον μεταβολισμό των υδατανθράκων (γλυκόζη, πυροσταφυλικό, ακετυλοκαρνιτίνη), των λιπιδίων (γλυκερόλη, ακετυλοκαρνιτίνη) και των πρωτεϊνών (αμινοξέα και τα παράγωγά τους). Ενδιαφέρον προκαλεί το ότι, παρότι η άσκηση είχε έντονη επίδραση στο μεταβόλωμα σε νεαρή ηλικία (δηλαδή μεταξύ 3 και 12 μηνών), αυτή η επίδραση έτεινε να εξασθενεί σε μεγαλύτερη ηλικία (δηλαδή μεταξύ 12 και 21 μηνών). Η ευαισθησία στην ινσουλίνη δε διέφερε μεταξύ των ομάδων, γεγονός που υποδηλώνει ότι δεν υπήρχε παρατεταμένη επίδραση της άσκησης ή ακόμα και καθόλου επίδραση. Επίσης, συγκριτικά με τις αρχικές τιμές, η ευαισθησία στην ινσουλίνη ήταν υψηλότερη την 1^{η} και 3^{η}, αλλά όχι την 5^{η} ή 7^{η} ημέρα μετά την άσκηση. Η πολυμεταβλητή ανάλυση διέκρινε τις ομάδες A και D ως προς τα λιπαρά οξέα των τριακυλογλυκερολών του τετρακεφάλου και τα λιπαρά οξέα των φωσφολιπιδίων και τριακυλογλυκερολών του ήπατος. Η μονομεταβλητή ανάλυση έδειξε μείωση ορισμένων κορεσμένων και μονοακόρεστων λιπαρών οξέων και αύξηση των πολυακόρεστων λιπαρών οξέων. Από την άλλη πλευρά, η πολυμεταβλητή ανάλυση δεν διέκρινε τις ομάδες B και C ως προς οποιαδήποτε λιπιδική παράμετρο. Συμπερασματικά, η δια βίου ή μακροχρόνια άσκηση μέτριας έντασης είχε ισχυρές και δυνητικά ωφέλιμες για την υγεία επιδράσεις στο σωματικό βάρος και (εν μέρει) στην όρεξη, καθώς και σε ορισμένους μεταβολίτες του αίματος και των ούρων. Οι επιδράσεις της γήρανσης στο μεταβόλωμα του αίματος και των ούρων φάνηκε να είναι ισχυρότερες από εκείνες της άσκησης. Επιπλέον, η άσκηση φάνηκε να ασκεί οξεία, αλλά όχι χρόνια επίδραση στην ευαισθησία στην ινσουλίνη, ευαισθησία που εξασθένησε μεταξύ της 3^{ης} και 5^{ης} ημέρας μετά την άσκηση. Τέλος, η άσκηση κατά το 2^{ο} μισό φάνηκε να αυξάνει την αφθονία των ζωτικών πολυακόρεστων λιπαρών. Η αντίθετη επίδραση στα κορεσμένα και μονοακόρεστα λιπαρά οξέα ίσως οφείλεται σε υψηλότερη χρήση των συγκεκριμένων κατηγοριών λιπαρών οξέων κατά την άσκηση. Μετά

όμως από τρεις μήνες αποπροπόνησης, οι επιδράσεις της άσκησης στο προφίλ λιπαρών οξέων των ηλικιωμένων επιμύων εξαφανίστηκαν.

Λέξεις ευρετηρίου: άσκηση**,** γήρανση, ευαισθησία στην ινσουλίνη, μεταβόλωμα αίματος, μεταβόλωμα ούρων

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LIST OF ABBREVIATIONS

AMP: adenosine monophosphate

ADP: adenosine diphosphate

ATP: adenosine triphosphate

BCAA: branched chain amino acid

CoA: coenzyme A

CVD: cardiovascular disease

GC: gas chromatography

HMDB: human metabolome database

IMP: inosine monophosphate

LC: liquid chromatography

MS: mass spectrometry

MUFA: monounsaturated fatty acid

NMR: nuclear magnetic resonance

OPLS: orthogonal projections to latent structures

PCA: principal component analysis

PL: phospholipid

PLS-DA: partial least squared discriminant analysis

PUFA: polyunsaturated fatty acid

SFA: saturated fatty acid

T2D: type 2 diabetes

TG: triglyceride

TMAO: trimethylamine-n-oxide

UFA: unsaturated fatty acid

INTRODUCTION

Aging

Over the past two centuries human life expectancy has more than doubled, resulting in a dramatic increase in the number of older individuals (Pollock et al. 2015). Hansen & Kennedy (2016) have named the 21st century the century of aging, as it is expected that soon up to 20% of the global population will be over 60 years. Specifically, in Greece, life expectancy has increased approximately 3 years since 2000, being 81.4 years in 2017 (OECD 2018). Healthy life expectancy, termed 'the healthspan,' is not keeping pace with average life expectancy (Pollock et al. 2015). There is no evidence connecting the increasing lifespan with an increasing healthspan. Aging comes with a multitude of physical and mental maladies, including common metabolic, inflammatory, cardiovascular and neurodegenerative diseases, which will eventually reduce the healthspan (Beard et al. 2016; Hellsten et al. 2016; Houtkooper et al. 2011). Therefore, the aforementioned increase in lifespan will bring new challenges to health care systems, as the years spent with poor health and disabilities at old age will increase. This is also supported by OECD (2018), whose statistics show that people aged 65 and over in Greece are expected to live only about 40% of their lives without chronic diseases and disabilities, corresponding to at least 2 years less of a healthy life compared to the average of EU countries. Some of the main reasons for the increased risk of chronic diseases and disabilities are extreme weight gain, obesity, diabetes, cancer and lack of physical activity. Health spending in Greece increased between 2000 and 2009 at a rapid rate of 6.1% per year, accounting for at least 10% of gross domestic product (OECD 2012, 2018). Then it fell in 2010 and the years after that due to the reduction in public spending on health as part of government efforts to reduce the budget deficit. The legacy of the crisis in Greece, too, has weighed heavily on health and welfare within the last 10 years.

Aging is an inevitable part of life, characterised by a general decline in cellular function, which will ultimately result in a loss of homeostatic mechanisms. This often leads to the development of age-associated diseases and frailty, one of the major geriatric syndromes which will be discussed below (Gargiulo et al. 2016; Houtkooper et al. 2011; Lara et al. 2015; Pollock et al. 2015; Viña et al. 2016). Two types of aging, primary and secondary, have been described. Primary aging is the inevitable, irreversible and progressive deterioration of physical structure and biological function, independent of disease or the environment (Shoveller et al. 2018). On the other hand, secondary aging is the acceleration of primary aging caused by factors such as genetics, diseases, physical activity and diet (Booth et al. 2011; Lazarus & Harridge 2018; Shoveller et al. 2018; Viña et al. 2016).

Although not a disease in itself, aging increases vulnerability to disease and can change its threshold, severity and prognosis by affecting its pathophysiological process (Bai 2018; Seals et al., 2016). Aging is characterised by an increase in chronic, low grade inflammatory status, indicated as 'inflammaging' (Collino et al. 2013). A related severe condition is sarcopenia, which causes a decrease in skeletal muscle mass and strength, with negative influence on a person's autonomy to perform tasks of daily life (Navas-Enamorado et al. 2017). Sarcopenia is the most common disorder in the elderly in terms of frailty, disability and morbidity, as it affects approximately 25-50% of the non-active seniors aged 70-80 (Forbes et al. 2012). Sarcopenia is the main cause of falls in the elderly, with research showing that more than one-third of people aged over 65 tend to fall, resulting in loss of independence and increased health care costs (Freiberger et al. 2012). Atherosclerosis, the major cause of cardiovascular disease (CVD), is another leading cause of death and disability. It is often attributed to lifestyle factors such as smoking, lack of exercise and diets rich in saturated fatty acids (Wishart 2019). Apart from its physical impact, aging is also widely recognized to adversely affect mental and social functions. Therefore, it is important to maintain healthy cognitive aging for independence and well-being in the elderly (Proitsi et al. 2018).

The aging phenotype is diverse and can be described as a complex mosaic resulting from the interaction of a variety of environmental, lifestyle and genetic-epigenetic factors (Bakin et al. 2013; Collino et al. 2013). Behavioural and environmental lifestyle choices may contribute up to 50% to the variability in human lifespan, as they result in increased susceptibility to numerous diseases (Rea 2017). Consequently, age-related changes also progress in a nonlinear manner, showing a certain degree of individuality (Bai 2018). To better understand this, one can bring to mind frail individuals at the age of 70 and more physically fit individuals at the age of 80. Aging is, thus, a multi-faceted phenomenon, making its study complex and necessitating the use of multiple methodologies and platforms (Mukherjee et al. 2014).

Understanding how we age and finding ways to ameliorate the negative physical, mental and social effects of aging and achieve optimal longevity, maintain physiological function and slow down the track towards frailty is crucial and has become a major global challenge (Jackson et al. 2016; Pollock et al. 2015). Much of the research on age-related changes in humans has focused on certain disease processes, which are often quite complicated and are influenced by numerous factors unrelated to human aging (Bakun et al. 2013). One of the most important hallmarks of aging research is the identification and analysis of biomarkers of aging that can provide a more accurate calculation of the biological age of an individual. This knowledge will aid in obtaining individualised evaluation and developing programmes and interventions that will eventually delay or even prevent the incidence, accumulation, clinical evolution, and functional consequences of chronic age-related diseases (Bai 2018; Justice et al. 2018). One

such intervention is lifelong exercise, and studies examining its effects are scarce. The benefits of exercise as a universal therapeutic 'pill', as named by Navas-Enamorado et al. (2017), for healthy aging will be discussed below.

Frailty

The context of frailty is not fully understood, and it has been shown to be multidimensional and dynamic (de Vries et al. 2012). Frailty is mainly the loss of physical function and the dependence on assistance in performing activities of daily life. It negatively impacts physical, psychological and social functions, resulting in hospitalisation, reduced longevity and poor quality of life (Chou et al. 2012). Failing mobility and falls are the main, obvious symptoms of frailty (Faber et al. 2006). Both aging and frailty are associated with multimorbidity, that is, the presence of two or more long-term disorders (Beard et al. 2016; Hanlon et al. 2018). There is no established measure of frailty, which hampers its study. However, Fallah et al. (2011) have created a frailty index as the number of deficits present in an individual divided by the total number of deficits (40) considered, which categorises individuals as non-disabled, disabled and severely disabled, as well as deceased. The main features of frailty are unintentional weight loss, self-reported exhaustion, weak grip strength, slow walking speed and low physical activity (Chou et al. 2012; Hubbard et al. 2009). Pre-frailty is when 1 or 2 out of the 5 features are present, whereas frailty is when 3 or more are present (Faber et al. 2006). Frailty, however, can be reduced or increased, and exercise and physical activity seem to be very important interventions to prevent, delay or reverse its level (de Vries et al. 2012).

Exercise

Physical activity and exercise are readily available to everyone and are a simple, inexpensive, non-invasive and highly effective form of lifestyle intervention (Rea 2017). The beneficial effects of exercise and physical activity on numerous aspects of health have been scientifically documented for over half a century and are nowadays well known and generally accepted (Viña et al. 2016; de Vries et al. 2012). Participation in sports and exercise can lead to beneficial modifications in many interrelated biochemical pathways (Floegel et al. 2014; Morris et al. 2013; Pechlivanis et al. 2015). There is a proven impact of regular exercise on various phenotypes associated with aging, with a clear anti-aging effect through reduced morbidity and mortality risks (Navas-Enamorado et al. 2017). Although no amount of physical activity can stop biological aging, there is evidence that regular exercise can minimize the physiological detriments of a sedentary lifestyle (Chodzko-Zajko et al. 2009). Literature shows no connection between aging and sedentarism; however, both are related to the occurrence of diseases. It is a fact that the modern environment has increased sedentarism, especially in western populations, having significant detrimental effects on physiological function and

health, specifically by increasing the proclivity to obesity, insulin resistance and adverse cardiometabolic effects (Booth et al., 2011, Harridge & Lazarus, 2017; Kujala et al. 2013; Morris et al. 2013). The current obesity epidemic is ultimately due to an imbalance between energy intake and energy expenditure (Kujala et al. 2013). Moreover, the occurrence of type 2 diabetes (T2D) has also increased dramatically over the past 30 years, mainly because of increases in the prevalence of a sedentary lifestyle and obesity. Consequently, chronic hyperglycaemia develops, and impairments in fat and protein metabolism are observed. Therefore, it is of critical importance to identify lifestyle interventions to lessen the repercussions of inactivity.

Lifestyle interventions, such as physical activity and exercise, contribute to disease prevention and recovery from illness and are the most cost-effective strategies for improving one's health (Gerber et al. 2014; Rea 2017). Regular exercise and physically active lifestyles are known to improve mobility, reduce visceral fat, blood glucose, blood pressure and dyslipoproteinaemia. They also reduce the prevalence of metabolic syndrome and contribute to the prevention of various chronic diseases such as diabetes, CVD, obesity and cancer (Daksalaki et al. 2014; Kuhl et al. 2008; Mukherjee et al. 2014; de Vries et al. 2012). Habitual activity, even light one, has also been associated with the delayed onset of coronary heart disease (Arbeev et al. 2016). Most tissues and organs are affected by the pleiotropic effects of exercise, both at a metabolomic and transcriptomic level (Navas-Enamorado et al. 2017). The effects of exercise on biological parameters can be either acute, lasting a few seconds to days after the last bout, or chronic, persisting for much longer (Pechlivanis et al. 2015). A deterioration of body composition is another hallmark of the aging process that can be retarded with regular exercise by increasing lean mass and decreasing fat mass (Chodzko-Zajko et al. 2009; Hayes et al. 2013). Exercise mainly affects skeletal muscle by modulating key metabolic pathways and is also the only intervention to successfully prevent sarcopenia (Navas-Enamorado et al. 2017). There is also evidence for psychological and cognitive benefits accruing from regular exercise in older adults, as it enhances learning and memory and it protects against neurodegenerative disorders, such as dementia and anxiety (Chodzko-Zajko et al. 2009; Lazarus et al. 2018; Mukherjee et al. 2014; Rea 2017). Moreover, exercise also improves the quality of sleep (Dishman et al. 2006).

Maximal oxygen consumption (VO_2max) describes an individual's capacity to utilise oxygen during maximal aerobic exercise and is an accepted marker of aerobic and cardiovascular fitness. Regular endurance and resistance exercise during maturation have shown to contribute to higher lifetime peak VO_2max and peak skeletal muscle power, respectively, whose declines are the main symptoms of physiological aging (Booth et al. 2011). Low levels of aerobic fitness are associated with an increased risk of metabolic diseases and CVD,

whereas high levels of aerobic capacity are associated with a reduction in all-cause and cause-specific traditional risk factors (Berry et al. 2013; Lustgarten et al. 2013). Chorell et al. (2012) speculate that persons with better fitness levels acquire an increased cardiorespiratory, immune and antioxidant defence system. On the contrary, lower fitness levels in healthy, middle-aged adults are associated with a marked increase in hospitalization due to heart failure in later life (Berry et al. 2013).

Older participants can benefit as much from exercise programmes as younger groups and may even have more to gain (Rea 2017). Physical exercise, especially of high intensity, improves physical function in the impaired elderly (de Vries et al. 2012). Strength, balance and endurance training can enhance physical performance for up to 24 months, resulting in improved independence and well-being (Freberger et al. 2012). Even light physical activity, such as regular walking or cycling, in middle-aged men can result in an approximately 50% lower risk of fatal or nonfatal coronary heart disease (Jefferis et al. 2014). Research on same-sex twins, looking into the effects of prolonged physical activity on metabolic and gene expression links, found numerous differences in the circulating metabolome between physically active and inactive individuals, reflecting better cardiometabolic health in the physically active twin (Kujala et al. 2013).

Lifelong exercise is a relatively new and evolving area of study with limited information on its effects (Gries et al. 2018). Research on lifelong exercise shows that it appears to limit age-related cardiac stiffening, resulting in reduced risk for heart failure (Berry et al. 2013). As far as skeletal muscles are concerned, over 50 years of aerobic exercise, regardless of intensity, has been found to fully protect capillarization and enzymes involved in aerobic metabolism (Gries et al. 2018). The same authors found that, following a maximal cycle test, a hierarchical pattern was observed showing that young exercisers performed best, followed by lifelong exercisers and old healthy non-exercisers.

Excessive sitting time (television time, screen time or leisure time spent sitting) and chronic exposure to physical inactivity have deleterious health effects, especially on physiological function; in cases of forced inactivity, like bed rest, they may even accelerate the aging process (Pollock et al. 2015). Physical inactivity in older adults is a risk factor for frailty and mortality and is now blamed for 6% of deaths globally (Sampson et al. 2014; de Vries et al. 2012). Inactivity increases the risk of all-cause mortality, CVD, metabolic syndrome, cognitive impairment and cancer and is also associated with adverse changes in circulating lipids and insulin sensitivity, thus increasing the risk of T2D (Biswas et al. 2015; Duvivier et al. 2013; Rea 2017; Sampson et al. 2014). These effects generally decrease in magnitude among persons who had higher levels of physical activity compared with those who had lower levels (Biswas

et al. 2015). A logical assumption would be that people with a sedentary lifestyle will actually have more to gain when they start training compared to already active people, since they are starting from zero. According to a study, however, exercise among sedentary older adults is beneficial in increasing fitness but translates into only trivial differences in adaptations in muscle of the heart (Berry et al. 2013). Only long-term participation in physical activity can help to reduce the increased cardiometabolic risks associated with sedentary lifestyles (Kujala et al. 2013). It is also noteworthy that, according to Duvivier et al. (2013), one hour of daily physical exercise cannot compensate for the negative effects of inactivity on plasma insulin and lipid concentrations if the rest of the day is spent sitting. Inactivity results in removal of activity-induced stressors and stimuli, resulting in adaptations towards decreased cardiorespiratory fitness, adverse changes in blood lipids and insulin sensitivity, less bone mineral density and muscle mass, and increased inflammatory activity in adipose tissue (Chorell et al. 2012). Thus, reducing inactivity by increasing the time spent walking or even standing is highly desirable.

Different types of exercise are potentially beneficial not only for the elderly but also for people suffering from chronic diseases associated with aging (Navas-Enamorado et al. 2017). For example, acute exercise has been found to induce increases in the production of free radicals in elderly subjects, together with an increase in antioxidant defences, which has also been noticed following endurance and resistance exercise (Liguori et al. 2018). Endurance training, however, is the only type of exercise that induces a decrease in the production of free radicals (Liguori et al. 2018). Therefore, establishing a combination of personalised treatments and exercise programs could lead to the effective prevention or delayed onset of muscle dysfunction and wasting that occur with aging (Navas-Enamorado et al. 2017).

Based on all the aforementioned benefits of exercise, the American College of Sports Medicine (ACSM) and the American Diabetes Association (ADA) recommend that individuals should exercise at least 150 min/week for health and weight maintenance (Gerber et al. 2014; Kuhl et al. 2008). Endurance, muscle strengthening and flexibility exercises should be included in a week of training. However, it is generally difficult to motivate people to exercise, and emphasis should be placed on finding ways to provide and maintain motivation. Unfortunately, the inclination for inactivity increases with age, resulting in fewer than half of the adults aged 65-74 and about one-third of the adults aged 75 and over meeting the current recommendations for exercise (Rea 2017).

Metabolism-Related Terms

The following metabolism-related terms are used in this dissertation.

Metabolites are low-molecular weight organic or inorganic compounds. Thus, metabolites can be lipids, amino acids, peptides, nucleic acids, sugars, alcohols and organic acids, all essential for growth, development and physiological functions (Wishart 2019). They are present in biological samples such as biofluids, tissues and cellular extracts (Nieman et al. 2013).

Metabolome is the complete collection of the metabolites found in a living organism or part of it (Heaney et al. 2017). The metabolome of an individual is sensitive to many internal and external variables including age, sex, diet, geographical location, environment, time of day and genetics (Wishart 2019). The characterization of the human metabolome has evolved rapidly over the past two decades, and the Human Metabolome Database (HMDB) now contains over 114,000 detected metabolites from all body matrixes, with approximately 25,000 found in human serum and about 4,000 found in urine (Wishart et al. 2018). Each metabolite in the database is presented with a 'metabocard' that demonstrates chemical, biochemical, clinical and enzymatic data details (Heaney et al. 2017).

Metabolic fingerprinting and *metabolic footprinting* are two terms used when sample classification is achieved through global, rapid analysis, used as a screening tool to discriminate between samples of different biological status or origin (Ellis et al. 2007). Metabolic fingerprinting refers to cells, blood, plasma or serum, whereas metabolic footprinting refers to biological excreta, such as urine, feces and sweat (Wishart 2019). The metabolite composition and concentration of all biofluids and excreta can vary depending on the site of sampling (for example, venous or arterial blood), the time of day, diet, age, gender, lifestyle and other variables (Wishart 2019). Therefore, metabolic fingerprinting and footprinting have proven to be important additions to the battery of classical tools for understanding the mechanisms controlling the response of biological systems to different stimuli (Pechlivanis et al. 2015).

A *biomarker*, or *biological marker*, is a quantifiable compound used as an indicator of the biological state, that is, to objectively assess the body's physiological or pathological processes (Palacios et al. 2015). Metabolite concentrations in body fluids reflect phenotypes including cardiorespiratory fitness and obesity (Floegel et al. 2014). In exercise settings, the concentration of a biomarker depends on factors such as training status; degree of fatigue; type, intensity and duration of exercise; age and sex (Palacios et al. 2015). Biomarkers are key parameters to assess the impact of exercise on different systems, tissues and organs, acutely or in the long term, and they offer a means of monitoring maintenance of or progression towards health (Palacios et al. 2015; Sampson et al. 2013). The discovery of aging- and exercise-related biomarkers will, therefore, aid in the early diagnosis and therapy of diseases (Kim et al. 2014). For this reason, in recent years, there is a growing interest in finding

biomarkers that could be used to evaluate health-related aspects that can be altered by regular physical activity and sport (Palacios et al. 2015; Sebastiani et al. 2017). Nevertheless, the investigation of the biological impact of physical activity and the association between chronological age and most biological functions is extremely complex, as changes in thousands of molecules need to be considered and interpreted (Pollock et al. 2015; Sampson et al. 2014).

Metabolomics is the large-scale, comprehensive study of the metabolome in terms of metabolite identity, quantity, and function (Mougios 2020). Metabolomics is an important tool in understanding the mechanisms regulating the responses of biological systems to natural processes (such as aging) and stimuli (such as exercise) and in detecting specific and sensitive biomarkers. It is a rapidly evolving field of life sciences that uses advanced analytical-chemistry techniques in conjunction with sophisticated statistical methods to simultaneously measure and comprehensively characterize numerous metabolites (Heaney et al. 2017; Morris et al. 2013; Wishart 2019). Metabolomics has been primarily used to diagnose diseases or to detect pathological conditions; every person under the age of 25 has probably had a metabolomic-based test as a newborn for the detection of inborn errors in metabolism (Wishart 2019).

Metabolomics can employ both targeted and non-targeted strategies. Targeted analysis can be applied to identify known metabolites related to specific biological pathways, and it usually focuses on identifying and quantifying a small subset of metabolites (50-500, Heaney et al. 2017; Wishart 2019). Non-targeted analysis, on the other hand, employs a wide-scope analytical collection and measurement of all detectable metabolites (Heaney et al. 2017). Incidentally, there are two other types of metabolomic analyses, fluxomics and metabolite imaging. The former measures metabolite reaction rates and monitors the movement of isotopic labels through metabolic intermediates, whereas the latter involves in vivo or in vitro detection and visualisation of metabolites in tissues (Wishart 2019). Global metabolite profiling, namely, untargeted metabolomics, allows for new discoveries linking cellular pathways to physiological function (Lustgarten et al. 2013). Methods used to identify exercise-induced changes in the metabolome usually employ the measurement and comparison of the concentrations of a limited number of metabolites already known to play distinct roles in exercise metabolism (e.g., glucose, lactate; Pechlivanis et al. 2013). Therefore, the use of metabolomics in exercise and aging research constitutes a novel and interesting method for identifying biomarkers related to performance and health.

A typical workflow for a metabolomics researcher begins with biofluid collection and/or tissue isolation and extraction. Then comes chemical analysis, which results in spectra that are

further analysed to produce long lists of compounds, which are then analysed with multivariate statistical, clustering and classification techniques in order to be interpreted and translated into logical results. The most commonly employed analytical techniques of metabolomics are nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry (MS), tandem MS (MS/MS), liquid chromatography (LC) and gas chromatography (GC). The most commonly used statistical analyses are principal component analysis (PCA, a clustering technique), partial least square discriminant analysis (PLS-DA) and orthogonal projections to latent structures (OPLS), the latter two being classification techniques.

Blood vs. Urine

Research on physical activity biomarkers focuses mainly on blood plasma or serum, which bathe all organs and tissues and serve as relatively good representatives of whole-body metabolism (Wishart 2019). In exercise and sports research, cardiorespiratory fitness and obesity are generally more strongly linked to serum metabolite networks associated with diet and physical activity (Floegel et al. 2014). However, it is important to understand that people are unlikely to be willing to provide blood samples on a regular or even semi-regular basis (Sampson et al. 2014). Moreover, organ and tissue extraction is highly invasive; so, unless it involves a muscle or selective biopsy, which still depends on the willingness of a subject to provide it, it is generally limited to studying animal models (Wishart 2019). Therefore, during the past years there has been a preference in non-invasive samples like urine and saliva. A well-known urinary test is the performance-enhancing-drug test conducted in elite sport. Urine is a readily available biological material that lacks homeostatic mechanisms, which makes it a better source of biomarkers than other biofluids (Pechlivanis et al. 2010). Since it results from plasma filtration through the kidneys, changes in plasma composition may be magnified in urine (as long as a metabolite is not reabsorbed by the kidneys) and reflect exercise-induced changes occurring in muscle (Wu & Gao 2015). Thus, urine analysis has become a rapidly evolving analytical tool that is intended to establish new diagnostic methods for performance and health. Urine metabolomics has also presented rapid advances in the prediction, detection, understanding and monitoring of human disease (Zhang et al. 2012).

Gaps in the Literature

The metabolic effects of aging and lifelong exercise have not been adequately explored. It is understandable that testing the effects of lifelong exercise through longitudinal studies on human subjects is a difficult task, and this is why most data in this area derive from cross-sectional studies, despite limitations emanating from different genetic backgrounds and hard-to-control lifestyle factors (Lazarus et al. 2018). Little information is available regarding metabolite changes linked to acute bouts of exercise or long-term exercise training with lower

workload volumes (Sakaguchi et al. 2019). Another gap in the literature on metabolomics and physical activity is the absence of information on the metabolomic profiles associated with sedentary behaviour and with the starting age of physical activity (Xiao et al. 2016).

To circumvent these problems and motivated by the finding of great similarities between humans and rats in the responses of the blood biochemical profile to exercise (Goutianos et al. 2015), we applied a lifelong exercise model on rats, which have a life expectancy of about 2 years when fed ad libitum, reach adulthood at 3 months, and age at about 21 months. Therefore, the aim of this thesis was to explore the effects of aging and lifelong exercise training on rat metabolism through a metabolomics approach. It was also examined whether training during the 1st or 2nd half of life would produce different results. The main hypothesis was that training would modify the effects of aging on the metabolome.

The thesis will continue with a literature review on, mainly, metabolomics-based studies, showing the effects of aging and exercise on both blood and urinary metabolites. Moreover, I will briefly present the effects of exercise on insulin sensitivity and the effects of both aging and exercise on gut microbiota and fatty acid metabolism. I will continue with the methods used in our research, followed by presentation of the results. Finally, I will thoroughly discuss all results and finish with the conclusions.

LITERATURE REVIEW

Since the publication of the very first article using the term ‘metabolome’ (Oliver et al. 1998), the field of metabolomics has experienced rapid growth. In 1999, only two papers were published on the subject, whereas, in 2018, there were more than 5,000 (Wishart 2019).

The purpose of this literature review is to survey the aging- and exercise-induced changes in blood and urinary metabolites, including fatty acids. Mainly metabolomics-based published research will be presented, firstly, on the effects of aging on the metabolic fingerprint (blood metabolome) and metabolic footprint (urinary metabolome) of both humans and animals. Then, the effects of acute, long-term and lifelong exercise on both the metabolic fingerprint and footprint of humans and animals will be presented. To facilitate understanding of the aforementioned effects, a presentation of each metabolic pathway will be manifested and the effects of aging and exercise on each one will be discussed. Specifically, the metabolic pathways that will be discussed are those involving amino acids and their derivatives, carbohydrates, lipids, Krebs cycle metabolites, nucleotides, redox pairs and gut microbiome. In the last part of each section, titled *Other metabolites*, changes in metabolites that do not fall into any of the classical metabolic clusters will be discussed, although relatively little might be known about the biological functions of some of these metabolites.

Effects of Aging on the Blood and Urinary Metabolome

Several biomarker signatures, mainly those associated with amino acid and lipid metabolism, change with aging, while abnormalities in metabolites related to physical function, disability, chronic diseases, morbidity and mortality might affect healthspan (Johnson et al. 2018; Liu et al. 2018; Sebastiani et al. 2017). The elderly have generally lower basal rates of fat oxidation and an age-related decline in basal metabolic rate (Slupsky et al. 2007). Muscle mass is estimated to account for about 30 percent of whole-body protein turnover in young adults, whereas in elderly subjects it accounts for 20 percent or even less (Young 1990). The elderly and especially those who suffer from chronic diseases show altered ratios between protein degradation and synthesis. Although protein degradation remains stable and is not affected by advancing age (in the absence of hypercatabolic stimuli such as inflammation), there is an attenuation in muscle protein synthesis resulting in a progressive depletion of total body protein with aging (Pasini et al. 2018; Timmerman & Volpi 2008). Consequently, there is an age-related progressive loss of muscle mass called sarcopenia, which is considered to be due to reduced basal metabolic rate (Slupsky et al. 2007). Therefore, there are expected changes in amino acid metabolism, which can, however, be reversed with exercise.

Musculoskeletal changes alter not only muscle function, but also serum markers related to bone density and regeneration (Siddharth et al. 2017). An age-dependent bone loss is noticed

in humans and several animal species, including rodents (Refaey et al. 2017). Moreover, in the elderly, the kidneys tend to shrink and decline in functionality; thus, there are some similarities between kidney aging and chronic kidney disease, which was documented on several metabolites mainly involved with amino acid metabolism (Wu & Gao 2015). Decreased antioxidant production and increased inefficiency of urea metabolism may also be present in aged subjects (Chaleckis et al. 2016). Oxidative damage of proteins, lipids and nucleic acids is one of the hallmarks of aging (Leeuwenburgh et al. 1999). Therefore, aging and age-related diseases are associated with increased oxidative stress and changes in mitochondrial energy metabolism (Salminen et al. 2014; Slupsky et al. 2007). There are also alterations in nutrient availability, including altered vitamin production and uptake, as well as altered carbohydrate and lipid metabolism (Siddharth et al. 2017). Thus, it has been proposed that the best metabolites to predict and capture the specificity of biological aging are in the areas of amino acid, carbohydrate and lipid metabolism, redox homeostasis, inflammation, endocrinology, haematology and nutritional status (Houtkooper et al. 2011; Liu et al. 2018; Sebastiani et al. 2017). The effect of aging on the main metabolic pathways will be discussed below.

Amino Acid Metabolism and Aging

To depict the quantity of proteins and amino acids in the human body, Wagenmakers (1998) gave an interesting example in which he mentions that, in the body of a 70 kg man, proteins weigh about 12 kg and free amino acids is 200 g. Of the latter, 120 g are in skeletal muscle, which accounts for 40-45% of the total body mass. In the circulation, however, only 5 g are free amino acids.

Apart from serving as the building blocks of proteins, amino acids serve as sources of energy, carbohydrates, lipids and other biomolecules, depending on the metabolic demands (Mougios 2020). Therefore, they are considered 'totipotent' molecules, essential for cellular activity (Pasini et al. 2018). They are a source of nitrogen for the synthesis of purine and pyrimidine bases, which are parts of ATP, its degradation products and nucleic acids, such as DNA and RNA (Pasini et al. 2018). Amino acids can be categorised as essential for humans and rats (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine), which cannot be synthesised in the body and have to be introduced with diet; and non-essential (alanine, arginine, asparagine, aspartate, cysteine, glutamate, glutamine, glycine, proline, serine and tyrosine), which are synthesised within the body from carbohydrates and lipids. Amino acids are also classified as glucogenic (alanine, arginine, asparagine, aspartate, cysteine, glutamate, glutamine, glycine, histidine, methionine, proline, serine, threonine and

valine), ketogenic (leucine and lysine) and both glucogenic and ketogenic (isoleucine, phenylalanine, tryptophan and tyrosine), according to their ability or inability to yield glucose.

Amino acid metabolism alters significantly during aging. Under conditions, such as injury, surgery, or chronic diseases, there is increased demand for amino acids due to higher resting energy expenditure (Pasini et al. 2018). Their availability is also important for maintaining brain function, as essential amino acids are precursors of the neurotransmitters serotonin, dopamine and norepinephrine, while the aromatic amino acids, phenylalanine, tryptophan and tyrosine, as well as aspartate and glutamate are themselves brain neurotransmitters (Dato et al. 2019). Amino acids are significantly associated with age and some, such as methionine, are indicators of healthspan (Johnson et al. 2018). Some of the plasma amino acids that are negatively correlated with aging include alanine, aspartate, glutamine, methionine, phenylalanine, proline, serine, tryptophan and tyrosine (Houtkooper et al. 2011; Johnson et al. 2018).

Aspartate, which is associated with VO_2max, decreased with aging in humans, possibly indicating lower aerobic capacity (Johnson et al. 2018). Glutamine, which is the most abundant free amino acid in plasma and a key regulator of amino acid-controlled cell growth, is essential for the maintenance of skeletal muscle function, and its availability decreases with aging and inflammation (Dato et al. 2019). However, chronically high concentrations of glutamine may lead to cancer or metastasis (Wishart 2019). Studies have found increased alanine, isoleucine, tyrosine and valine in the plasma of aged female humans (Kochhar et al. 2006), while cysteine and glycine were found to be higher in the plasma of aged humans (Johnson et al. 2018).

Cysteine is used in the production of important metabolites including glutathione, sulphate and taurine (Dong et al. 2018). Cysteine, methionine, tryptophan and tyrosine are susceptible to oxidation, and their oxidised forms were implicated in producing anti-osteogenic metabolites (Leeuwenburgh et al. 1999; Refaey et al. 2017). Moaddel et al. (2016) have found an association between low muscle quality and high plasma concentrations of methionine and tryptophan. Chronically high serum concentrations of methionine may also cause atherosclerosis and CVD, whereas chronically high concentrations of phenylalanine may lead to diabetes and insulin resistance (Wishart 2019). Studies with restriction of single amino acids, mainly of methionine, were shown to effectively increase median and maximal life span (Dong et al. 2018; Shoveller et al. 2018). Serine is also needed for lipid metabolism, muscle growth and for maintaining a healthy immune system; thus, its age-related decrease in plasma was associated with inflammatory conditions (Collino et al. 2013).

Branched chain amino acids (BCAAs), namely isoleucine, leucine and valine, account for 20% of the total amino acids released from protein degradation (Rennie & Tipton 2000). BCAA catabolism plays a key role in energy production, glucose regulation and protein synthesis, and BCAA status is proposed as a potential biomarker of health and disease. A growing body of literature has linked higher plasma concentrations of BCAAs with obesity, insulin resistance, metabolic disorders, T2D, CVD, stroke and chronic kidney disease (Kujala et al. 2013; Xiao et al. 2016). Leucine, specifically, provides direct anabolic stimuli to skeletal muscle, promotes muscle glucose uptake and increases secretion of leptin, which suppresses appetite and body weight increase, thus contributing to improved whole-body glucose metabolism (Dato et al. 2019). Research, however, shows that aged muscle may be slightly less sensitive to the anabolic effects of amino acids, specifically leucine, compared with young muscle (Timmerman & Volpi 2008). This statement is supported by evidence that, as age increases, leucine becomes less effective in inducing protein synthesis (Dato et al. 2019). The effects of aging on BCAA metabolism are not clear, as there are no clean-cut trends in their aging-induced modifications. Isoleucine and leucine decreased in the blood of elderly subjects, and this decrease might be associated with the reduced muscle activity in the elderly (Chaleckis et al. 2016). However, a study by Houtkooper et al. (2011) found increased plasma concentrations of all three BCAAs in aged individuals. These findings agree with a more recent study, by Moaddel et al. (2016), who found that participants with low muscle quality had higher plasma concentrations of isoleucine and leucine. The same authors attribute the increased concentrations of BCAAs and, generally, amino acids to impaired transport and/or mitochondrial activity due to low muscle quality. Higher concentrations of circulating BCAAs have negative effects on several pathophysiological conditions by promoting oxidative stress and mitochondrial dysfunction, and they were noticed in individuals with obesity, impaired fasting glucose and T2D (Dato et al. 2019). Epidemiological studies have also shown that chronically high concentrations of BCAAs, specifically isoleucine and leucine, may be biomarkers of several metabolic diseases such as insulin resistance, T2D and obesity or excessive weight gain (Bifari & Nisoli 2017; Wishart 2019). Solon-Biet et al. (2019), however, contradict these findings by stating that BCAAs alone cannot predict metabolic dysfunction, as they can be elevated in both healthy and unhealthy obese and insulin resistant subjects.

The aromatic amino acids, histidine, phenylalanine, tryptophan and tyrosine, function as antioxidants, are anabolic for bone and may play a protective role in an aging environment (Refaey et al. 2017). Phenylalanine exhibits anti-inflammatory properties and is often used to treat arthritis and Parkinson's disease (Collino et al. 2013). Tryptophan circulates primarily bound to albumin and is present at the lowest concentration in the blood among the amino acids (Rafaey et al. 2017). With increasing age, there is a marked decrease in serum

phenylalanine, tryptophan and tyrosine concentration (Lustgarten & Fielding 2017; Refaey et al. 2017). Depletion of tryptophan, with a subsequent increase in serum kynurenine, were found in patients suffering from inflammatory diseases (Collino et al. 2013; Lee et al. 2017). Kynurenine metabolite levels represent the overall status of the central nervous system, and measuring tryptophan and tryptophan metabolites, such as serotonin and melatonin, could be useful biomarkers of central nervous system aging (Lee et al. 2017). Changes in the kynurenine pathway were described in various diseases such as ischemic stroke, Alzheimer's disease, Parkinson's disease and multiple sclerosis (Lee et al. 2017). However, following exercise both kynurenine and tryptophan serum concentrations increased in aged mice, suggesting a protective role of exercise on these metabolites (Lee et al. 2017). Contrary to the previous findings are the results of a study by Moaddel et al. (2016), who found increased plasma concentrations of tryptophan and serotonin in subjects with low muscle quality; however, this study measured total tryptophan rather than free tryptophan.

Some amino acid derivatives, such as α-aminobutyric acid, β-aminoisobutyric acid, citrulline, cystathione, 3-methylhistidine and ornithine, were found to increase in plasma with age (Johnson et al. 2018; Liu et al 2018). The presence of 3-methylhistidine, produced by histidine methylation, in urine shows the presence of proteolysis (Pasini et al. 2018). Citrulline, a metabolite of the urea cycle, increased in the blood of healthy elderly persons, suggesting impaired urea cycle (Chaleckis et al. 2016). Deficiencies of urea cycle enzymes are indeed known to cause accumulations of these metabolites (Chaleckis et al. 2016). Increases in citrulline and ornithine with age have also been associated with an increased risk for cardiovascular events and indicate improper function of the pathways responsible for restoring arginine concentrations (Johnson et al. 2018). Arginine, a key substrate for nitric oxide production, was found to mitigate the vascular inflammatory response. This may benefit the vascular endothelium, whose dysfunction is closely related to increased cardiovascular risk and cognitive and physical declines associated with aging (Dato et al. 2019). Both arginine and citrulline increase with co-morbidities of aging and have been linked with age-related changes in the brain (Liu et al. 2018). Therefore, due to their diagnostic power, arginine and citrulline have become promising biomarkers of aging. Another non-protein amino acid, homocysteine, which is a metabolite of the essential amino acid methionine and is metabolised to cysteine, accumulates with aging (Antikainen et al. 2017). Homocysteine, which is associated with cardiovascular risk, is highly related to dietary folate, vitamin B_6 and vitamin B_{12}, and it affects the development of atherosclerosis by damaging the inner lining of the arteries and by promoting blood clots (Crimmins et al. 2018).

Carbohydrate and Lipid Metabolism and Aging

Fasting blood glucose and insulin are used as measures of glucose homeostasis, insulin resistance and diabetic conditions (Bürkle et al. 2015; Crimmins et al. 2018). Serum glucose has been included in the measures of phenotypic aging and is positively correlated with it (Levine et al. 2018). A one-year increase of phenotypic age was found to result in a 9% increase in the risk of all-cause mortality, aging-related diseases and chronic lower respiratory disease, a 10% increase in the risk of CVD, a 7% increase in the risk of cancer and a 20% increase in the risk of diabetes (Levine et al. 2018). Chronically high concentrations of glucose may lead to cancer or metastasis (Wishart 2019).

Blood glucose concentration is a well-known and obvious marker of insulin function and glycaemic control. Specifically, fasting plasma glucose is positively associated with BMI and waist circumference, which are primary markers of obesity (Floegel et al. 2014). This may be a result of impaired glucose metabolism and insulin resistance in obese individuals (Floegel et al. 2014). Mannose has also been shown to have a strong link with glucose intolerance, metabolic syndrome and obesity, and its plasma concentration does not change significantly postprandially (Xiao et al. 2016). Therefore, it may also serve as a marker of glycaemic control. Mannitol is a compound naturally found in many foods, and xylose is a sugar found in some grains and vegetables; the latter is not degraded by gut bacteria (Slupsky et al. 2007). Both metabolites are related to diet, and their excretion in urine depends on the time of day due to food intake differences over the day (Slupsky et al. 2007). Pyruvate and lactate are major products of carbohydrate metabolism through the glycolytic pathway. Research has found a positive association between serum pyruvate and delayed verbal memory in females (Proitsi et al. 2018). Resting urine lactate concentration is usually decreased in older individuals (Slupsky et al. 2007).

With increasing age, lipid synthesis decreases, mainly in females, whereas lipid synthesis is predominantly preferred in males over protein turnover (Kochhar et al. 2006). Carnitine is essential for the transfer of fatty acids into mitochondria for β oxidation. In order to enter mitochondria, fatty acids must be converted into acylcarnitines, which are then converted into acyl coenzyme A (CoA) and undergo oxidation. The plasma acylcarnitine concentration is positively associated with obesity and, specifically, BMI and waist circumference; phospholipids, however, show opposite associations with obesity (Floegel et al. 2014). Acylcarnitines have been used as biomarkers of insulin resistance and help identify metabolic imbalances in fatty acid oxidation (Liu et al. 2018). Increases in plasma acylcarnitines are also observed in conditions of obesity, T2D and Alzheimer's disease (Liu et al. 2018). Acetylcarnitine is formed when there is abundant acetyl CoA, resulting from increased carbohydrate and lipid oxidation due to, for example, exercise (Mougios 2020). A decrease

in the concentrations of carnitine and acetylcarnitine is noticed in the urine of older individuals (Slupsky et al. 2007), whereas high acetylcarnitine concentration was found in the blood of the aged group (Johnson et al. 2018). Pantothenate, a precursor of CoA, is more abundant in the blood of healthy elderly individuals, suggesting that CoA synthesis may be slightly impaired in elderly subjects (Chaleckis et al. 2016). However, its excretion was found decreased in urine of aged female rats (Deda et al. 2017). Finally, the concentration of 3-hydroxybutyrate, a ketone body produced by the metabolism of fatty acids in the liver, is lower in older subjects (Psichogios et al. 2008).

Krebs Cycle Metabolites and Aging

Metabolomic research on age-related changes showed decreased urine concentrations of Krebs cycle metabolites, such as acotinate, fumarate and oxaloacetate, associated with diminished mitochondrial function (Schnackenberg et al. 2007). Differences in metabolites associated with mitochondrial energy metabolism, such as citrate, have also been observed between younger and older groups (Wu & Gao 2015). Research has indicated citrate as one of the most important metabolites in urine, allowing discrimination between age groups, since it increases in older subjects (Psichogios et al. 2008). Changes in urinary acotinate, citrate and fumarate concentrations were seen in liver and kidney disease and were correlated with general stress (Slupsky et al. 2007). The concentration of acotinate is specifically decreased in aged individuals (Slupsky et al. 2007). Cancer studies revealed that succinate and fumarate are important inhibitors of 2-oxoglutarate-dependent dioxygenases, which are critical players in the regulation of gene expression (Salminen et al. 2014). Other studies have shown that chronically high concentrations of succinate in a tissue may lead to cancer or metastasis (Wishart 2019).

Redox Metabolism and Aging

NAD^+ is a key energy-sensing metabolite that contributes to the maintenance of metabolic homeostasis and can promote lifespan and healthspan extension (Navas-Enamorado et al. 2017). NAD^+ decreased in the blood of elderly subjects, suggesting that redox metabolism in the elderly might be somewhat compromised (Chaleckis et al. 2016). The age-dependent decrease in NAD^+ may also be due to the increased activity of CD38, an enzyme that promotes mitochondrial dysfunction in mice (Navas-Enamorado et al. 2017). Another redox metabolite, glutathione, has also been found reduced in the elderly (Houtkooper et al. 2011). Research on the effects of aging on the urinary metabolome showed increased levels of oxidised antioxidants, which is associated with increasing levels of reactive oxygen species (Schnackenberg et al. 2007).

Gut Microbiome Metabolism and Aging

The gut microbiome composition of an individual is influenced by the genotype, diet, lifestyle and other factors. Aging also deeply affects the structure and composition of the human gut microbiome (Collino et al. 2013). Age-related changes of the gut microbiome have been associated with inflammatory and/or immune status, impaired nutrient and, specifically, amino acid digestion and absorption resulting in decreased plasma amino acid concentrations and, consequently, a decline of musculoskeletal function (Pasini et al. 2018; Siddharth et al. 2017). The colonic bacterial transformation of food polyphenols, which varies widely depending on the gut microbiome of each individual and interacts with one's endogenous metabolome, becomes part of the 'food metabolome' (Nieman et al. 2013). Thus, research on the microbial metabolism of food components is important, as it investigates the metabolism of carbohydrates, lipids, amino acids and vitamins, which could alter the metabolic status of the host and contribute to the physiological state in aging (Siddharth et al. 2017). The microbial polyphenol metabolism produces a relatively small number of metabolites, including simple phenols and derivatives of benzoic acid, phenylacetic acid, mandelic acid, phenylpropionic acid and cinnamic acid (Nieman et al. 2013). Coumaric acid is another gut-derived phenolic compound that is present in urine and faeces when food rich in it is consumed (Nieman et al. 2013). Hippurate, whose concentration is related to the microbial activity and micro-floral composition of the colon, has significantly higher values in older persons and animals (Psichogios et al. 2008; Schnackenberg et al. 2007).

Trimethylamine-*N*-oxide (TMAO) is one of the most important urinary metabolites, contributing to the discrimination between age groups, with higher concentrations in subjects 50 years and over (Psichogios et al. 2008). The increased excretion of both TMAO and dimethylamine at older ages possibly indicates a slight dysfunction of their osmotic role in renal medullary tissue (Psichogios et al. 2008). Chronically high serum concentrations of TMAO may cause atherosclerosis, CVD and obesity or excessive weight gain (Wishart 2019). TMAO has also been associated with reduced survival in conditions such as heart failure and myocardial infarction (Heaney et al. 2017). Moreover, the aging process deeply influences the gut microbiome, as indicated by increased urinary concentrations of phenylacetylglutamine and p-cresol sulphate (Wu & Gao 2015). Altered gut microbiome, as well as increased gut permeability and circulating microbial burden, are related to poor muscle composition in older adults (Lustgarten & Fielding 2017). This increased systemic microbial burden results in increased adipogenesis as an antimicrobial compensatory response (Lustgarten & Fielding 2017).

Other Metabolites and Aging

Creatinine is a waste metabolite, generated from muscle metabolism, which is transported through the bloodstream, filtered in the kidneys and finally excreted in the urine (Crimmins et al. 2018). It is one of the most important urinary metabolites, contributing to the discrimination between age groups, with younger subjects having higher concentrations (Psichogios et al. 2008; Wu & Gao 2015). Creatinine is also a renal function marker, and its decrease was linked with reduced muscle density (Lustgarten & Fielding 2017), although Sebastiani et al. (2017) state that it generally increases with older age. The creatine/creatinine ratio has also been found decreased in urine of aged female subjects (Kochhar et al. 2006). Creatinine clearance, which is measured by collecting urine samples over a 24 h period, was shown to predict stroke and cardiovascular mortality (Crimmins et al. 2018). Creatinine concentration has also been closely correlated with nutritional status (Schnackenberg et al. 2007). The decrease in the urinary creatinine concentration in aged subjects could be explained by the declining muscle mass and/or deficiency in nutrient availability. However, creatinine depletion from urine in older individuals could also be due to a decline in glomerular filtration rate (Slupsky et al. 2007). Creatine, which is synthesised from three amino acids, glycine, arginine and methionine, is steadily converted into creatinine within muscle tissue (Mougios 2020). It is absent from or detected in only trace amounts in normal human urine; however, its concentration is also age dependent, and it increased in urine with aging (Psichogios et al. 2008; Schnackenberg et al. 2007).

The polyamines, putrescine, spermidine and spermine, are involved in many functions including cell growth, cell survival and cell proliferation (Moaddel et al. 2016). Their biosynthetic pathway begins with the production of ornithine from arginine, and putrescine is produced from the decarboxylation of ornithine (Moaddel et al. 2016). The plasma concentrations of the aforementioned polyamines were shown to decline with age and in subjects with low muscle quality (Moaddel et al. 2016). Higher concentrations of polyamines, however, are correlated with reduced inflammation and greater longevity (Moaddel et al. 2016).

Taurine, a sulphated organic compound, is negatively associated with changes in waist circumference, and its decreased plasma concentrations were associated with obesity (Brennan et al. 2018). Taurine is also a marker of liver function and its excretion increased with aging in male rats, suggesting impaired liver function (Schnackenberg et al. 2007).

Effects of Exercise on the Metabolic Fingerprint

An overall change in fuel expenditure is observed during exercise. Exercise increases the rates of glucose, fatty acid and amino acid catabolism (Zhou et al. 2019). Older research on male rats has shown increased proteolysis and glycogenolysis to supply energy due to lower plasma insulin and higher epinephrine concentrations observed post-exercise (Tarnopolsky et al. 1990). Also, higher physical activity levels are associated with lower plasma concentrations of BCAAs and monosaccharides, which are metabolic signatures consistent with better cardiometabolic health (Xiao et al. 2016). Furthermore, research on endurance athletes has shown that, following prolonged and high-intensity exercise, they experience great shifts in blood metabolites, especially lipid metabolites (Nieman et al. 2014). In general, exercise results in changes in the rates of many metabolic pathways, such as amino acid metabolism, glycolysis, gluconeogenesis, Krebs cycle, fatty acid metabolism, purine metabolism and gut microbiome metabolism. The effect of exercise on the main metabolites found in blood will be discussed below.

Amino Acid Metabolism and Exercise (Blood Metabolome)

Amino acids are not important energy suppliers during exercise; however, they play an active role in the metabolic response of skeletal muscle to exercise, as they can influence the concentration of Krebs cycle metabolites (Gibala 2001). Exercise stimulates amino acid metabolism in skeletal muscle and plasma, and the metabolic response of each amino acid to exercise depends on a set of controlling factors and is tissue specific (Hood & Terjung 1990; Peake et al. 2014). For example, during exercise more hepatic amino acids are used for glucose synthesis (Mougios 2020). Following removal of its amino group, the carbon skeleton of an amino acid can be used by the liver for the production of glucose through gluconeogenesis (Pasini et al. 2018).

The circulating concentrations of two BCAAs, leucine and isoleucine (Brennan et al. 2018), as well as of glycine and serine (Floegel et al. 2014), are positively associated with cardiorespiratory fitness and physical activity. Changes in the plasma concentrations of glucogenic amino acids, such as alanine, proline and tyrosine, are positively correlated with changes in BMI (Brennan et al. 2018). Decreases after strenuous exercise were found in arginine, asparagine, histidine, lysine, methionine, ornithine, phenylalanine, proline, serine, threonine, tryptophan, valine and isoleucine (Chorell et al. 2012; Thysell et al. 2012). Plasma concentrations of many amino acids were lower in high-fitness groups (Morris et al. 2013) and following a single bout of resistance exercise (Berton et al. 2016). The exercise-induced decline in the glucogenic amino acids may reflect their use for gluconeogenesis. However, it

is possible that an increase in the plasma pool of amino acids may be noticed in response to exercise due to amino acid synthesis or protein breakdown in skeletal muscle.

Pechlivanis et al. (2013) have found an increase in alanine with short- and long-term exercise, which can be explained by a preceding increase in pyruvate, from which alanine is produced by transamination with glutamate. A higher alanine concentration in trained groups might be consistent with its role in the glucose-alanine cycle, which promotes gluconeogenesis (Daskalaki et al. 2014). In this cycle, glucose, synthesised in the liver from alanine, is transported through the bloodstream to muscle; alanine is then produced from glucose in the exercising muscle and is transported to the liver through the bloodstream (Mougios 2020). An increase in the plasma concentration of alanine was also seen after strenuous exercise (Thysell et al. 2012) and following high-intensity interval exercise (Peake et al. 2014). Sixty minutes of vigorous exercise (approximately 75% VO_2max) and short-term high-intensity exercise also resulted in an increase in alanine in both skeletal muscle and plasma, indicating increased glycolytic rate (Bloomstrand & Saltin 1999; Gibala 2001). In two rather old studies, the plasma alanine concentration rose by 20-25% following moderate-intensity exercise (Felig & Wahren 1971; Wagenmakers 1998). Increased plasma concentrations of alanine, as well as glycine and proline, following endurance exercise were associated with improved insulin sensitivity (Huffman et al. 2011).

Contrary to these findings are the results of a study that found decreased plasma concentration of alanine after three exercises of different loads and intensities (high amount high intensity, high amount low intensity, low amount low intensity, Brennan et al. 2018). Lower rates of alanine and glutamine release from the muscle were observed in endurance athletes at rest, following exercise, consistent with a lower requirement for gluconeogenesis, possibly due to a better balance between oxidative metabolism and glycolysis (Daskalaki et al. 2014). Another study showed mixed results regarding the plasma concentrations of both alanine and glutamine, that is, higher values after acute maximal treadmill testing, lower values after marathon running and a noticeable fall at rest after prolonged training (Lewis et al. 2010).

Glutamate is one of the few amino acids that can discard the amino group in the form of ammonium by deamination (Mougios 2020). It is a neurotransmitter and a key substrate for the rapid increase in muscle Krebs cycle metabolites that occurs at the beginning of moderate to intense exercise (Gibala 2001). Plasma glutamate was found higher in people with obesity, insulin resistance and high metabolic risk (Xiao et al. 2016). Therefore, its abnormal homeostasis may be an important contributor to the development of diabetes. During prolonged exercise glutamate was found to remain unchanged in muscle, although it tended to increase in plasma (Gibala 2001). Significant increases in plasma concentration of

glutamate were also found during high-intensity interval exercise (Peake et al. 2014). The authors speculate that this increase may have served to generate more Krebs cycle metabolites during high-intensity exercise.

Glutamate was also increased in the rat heart following endurance exercise (Starnes et al. 2017). However, its plasma concentration decreased in male rats following exhaustive swimming exercise, although female rats showed an increase after completing the same exercise protocol (Zhou et al. 2019). Glutamate also decreased during short-term high-intensity exercise in both skeletal muscle and plasma (Gibala et al. 2001) and following a single incremental test to exhaustion (Felder et al. 2017). These decreases may reflect the increased consumption of glutamate in skeletal muscle during the first few minutes of exercise, particularly when glycogen is low, in order to synthesise either alanine, aspartate or glutamine (Hood & Terjung 1990; Peake et al. 2014; Wagenmakers 1998).

Aspartate is a metabolite associated with the activity of the Krebs cycle and increased formation of oxaloacetate during exercise (Felig & Wahren 1971), and it is also used in the reamination steps of the purine nucleotide cycle (Hood & Terjung 1990). Its concentration decreased in the rat heart following endurance exercise (Starnes et al. 2017). The serum concentration of asparagine also decreased after multiple bouts of submaximal exercise (Pohjanen et al. 2007). Chronically high blood and tissue concentrations of asparagine may lead to cancer or metastasis; therefore, its exercise-induced decrease may be beneficial (Wishart 2019). Furthermore, serine decreased in rat serum following swimming until exhaustion (Felder et al. 2017) and in male rat plasma following a single incremental test to exhaustion (Zhou et al. 2019). Contrary to these findings, serine increased following three exercises of different loads and intensities (high amount high intensity, high amount low intensity, low amount low intensity, Brennan et al. 2018). Serine also increased in rat heart following endurance exercise (Starnes et al. 2017). Threonine, an essential amino acid, decreased after training in a study with diabetic humans (Kuhl et al. 2008).

Oxidation of BCAAs in skeletal muscle is generally increased with exercise due to increased proteolysis in skeletal muscle and activation of key enzymes involved in BCAA degradation, as a result of reduced availability of muscular glycogen (Chaleckis et al. 2016; Morris et al. 2013; Xiao 2016). The extent of leucine oxidation in muscle during exercise was greater in trained rather than untrained subjects (Rennie & Tipton 2000). Both leucine and valine decreased in plasma following exhaustive exercise (Zhou et al. 2019). A sustained decline in plasma concentrations of BCAAs was observed after moderate-intensity exercise, reflecting their increased transamination and their role in gluconeogenesis (Peake et al. 2014). Decreases in serum leucine-isoleucine and norvaline-valine were found following both high-

intensity interval exercise and resistance exercise, but not after continuous moderate-intensity exercise (Siopi et al. 2019). Pechlivanis et al. (2013) have also found decreased serum concentrations of BCAAs and products of their catabolism following 80 m sprint running, which they attribute to increased muscle uptake. It has also been hypothesised that a higher rate of BCAA catabolism may be an indicator of more efficient lipid metabolism and fatty acid oxidation, a metabolic phenotype associated with an active lifestyle (Xiao et al. 2016).

Sixty minutes of exercise resulted in an increase in the plasma concentration of the aromatic amino acids phenylalanine and tyrosine (Bloomstrand & Saltin 1999). Increased plasma concentration of tyrosine was also found during high-intensity interval exercise (Peake et al. 2014). This increase, when followed by a decrease in phenylalanine, could be attributed to the conversion of phenylalanine to tyrosine. Phenylalanine and tyrosine increased by 8-35% following moderate and severe exercise, which was attributed to altered splanchnic exchange rather than enhanced peripheral release (Felig & Wahern 1971). Lustgarten et al. (2013) have found a significant positive association between VO_2max and serum concentration of another aromatic amino acid, tryptophan. Hence, elevated plasma tryptophan may be associated with increased endurance. Additionally, Brennan et al. (2018) have found increased tryptophan concentration in the exercise groups compared to a non-exercising control group. Kynurenate, a tryptophan metabolite, also increased after half-marathon running (Lewis et al. 2010). Therefore, tryptophan and tryptophan derivatives are important markers of the effects of exercise.

Exercise has a different effect on two non-proteinogenic amino acids. The first is citrulline, which is a marker of nitric oxide formation and is present to a lesser extent in the plasma of fitter individuals (Daskalaki et al. 2015). The second is ornithine which, increased only in female rats following exhaustive exercise (Zhou et al. 2019).

Carbohydrate and Lipid Metabolism and Exercise (Blood Metabolome)

Skeletal muscle secretes metabolites that participate in the regulation of whole-body glucose and lipid homeostasis. The adaptation of skeletal muscle to endurance training can be described as an increase in its capacity and efficiency to utilise fuels for the generation of ATP, such as improved oxidation of fat and decreased demand for glucose and glycogen (Lehmann et al. 2010). Carbohydrate oxidation, which is the primary ATP-producing system in most exercises, increases with exercise intensity, resulting in higher levels of Krebs cycle metabolites (Peake et al. 2014; Zhou et al. 2019). Two monosaccharides, glucose and mannose, whose blood concentrations are closely correlated, are inversely associated with physical activity (Xiao et al. 2016). Physical activity contributes to glucose homeostasis through both acute and chronic mechanisms (Kujala et al. 2013). Baseline glucose decreased

in the circulation of T2D subjects after 12 weeks of regular physical activity (Kuhl et al. 2008). A study comparing active and inactive co-twins found lower fasting glucose concentrations among the active ones (Kujala et al. 2013). Swimming until exhaustion has also resulted in decreased glucose in male rats, which suggests increased energy expenditure (Zhou et al. 2019). However, high-intensity interval training, compared with continuous moderate-intensity training, resulted in higher carbohydrate oxidation and higher plasma glucose concentration (Peake et al. 2014).

During high-intensity maximal exercise, a major part of the energy is derived from the anaerobic carbohydrate catabolism, resulting in accumulation of pyruvate and lactate in muscle and blood (Mougios 2020). Pyruvate and lactate are more likely to increase following intense exercise and especially in individuals who are not endurance trained (Daskalaki et al. 2014). Exercised muscle releases lactate, which is converted to pyruvate and then to glucose in the liver. This process is important in maintaining glucose supply to muscles during prolonged exercise and constitutes the Cori cycle. Acute exercise is associated with the activation of gluconeogenesis and glycogenolysis in the liver; both processes increase the release of glucose into the bloodstream and meet the demands of the skeletal muscle (Navas-Enamorado et al. 2017). Endurance trained athletes have generally more efficient re-utilisation of lactate for both oxidative metabolism and gluconeogenesis (Daskalaki et al. 2014).

Since the anaerobic breakdown of carbohydrates to lactate is one of the main means of energy supply during maximal exercise lasting up to 1 min, the muscle and blood lactate concentration is associated with exercise intensity. Therefore, higher lactate release from muscle indicates increased glycolytic rate. A study comparing high-intensity interval training with continuous moderate-intensity exercise found that plasma lactate of the high-intensity group was markedly higher (Peake et al. 2014). Increases in both serum lactate and pyruvate were seen after a single bout of resistance exercise (Berton et al. 2016). Plasma pyruvate also increased in the exercise groups compared to the non-exercising control group (Brennan et al. 2018). Contrary to these findings, following exhaustive swimming exercise the plasma concentration of lactate in rats decreased, indicating a reduction in whole-body glycolytic rate (Zhou et al. 2019).

Glycerol is a three-carbon molecule participating in triacylglycerol and glycerophospholipid structure. When triacylglycerols are catabolised, glycerol is released into the circulation. Long-term endurance training that resulted in increased VO_2max had different effects on serum glycerol. Petibois & Déléris (2003) found elevated resting concentration of serum glycerol following long-term endurance training, whereas Lustgarten et al. (2013) found

negative association between serum glycerol and VO_2max (only when the values of male and female humans were examined separately, not combined). Other studies have shown contrasting exercise-induced changes in circulating glycerol, that is, decreased glycerol after three exercises of different loads and intensities (Brennan et al. 2018), and increased glycerol after multiple bouts of submaximal exercise (Pohjanen et al. 2007). Lewis et al. (2010) hypothesised that plasma glycerol might be indicative of general physical fitness, based on its lower concentration in unfit individuals, resulting in impaired lipid utilisation in response to exercise. Moreover, after intensified endurance training, metabolites related to lipid metabolism were elevated, and the elevation remained 14 hours after the last bout, suggesting a prolonged stimulation of lipid catabolism during recovery (Nieman et al. 2013). These ambiguous results can be explained by the fact that, through an increased energy demand, exercise training either promotes more efficient mitochondrial β oxidation or reduces lipolysis via enhanced insulin action (Huffman et al. 2011).

Fatty acid oxidation generally increases during exercise at intensities up to 65% VO_2max and decreases at higher workloads, at which carbohydrates become the main energy suppliers. A post-exercise elevation in serum acetoacetate and 3-hydroxybutyrate, which are ketone bodies utilised by tissues as energy sources produced by the catabolism of fatty acids in the liver, was found after a 3-day period of intensified exercise and can be explained by the substantial use of fatty acids (Nieman et al. 2013). Higher fatty acid oxidation during exercise has been identified in trained compared to sedentary subjects, despite lower adiposity levels (Morris et al. 2013). Acetoacetate increased with three different exercises (Brennan et al. 2018). Also, training in T2D subjects induced increases in resting plasma 3-hydroxybutyrate (Kuhl et al. 2008). Exhaustive swimming exercise in animals also resulted in increased serum 3-hydroxybutyrate (Zhou et al. 2019).

Unlike glycolysis, oxidative metabolism requires a supply of CoA. Studies have observed increased concentrations of the CoA precursor, pantothenate, in plasma following half-marathon running (Lewis et al. 2010). Pantothenate has also been positively correlated with fitness, and it was proposed that its increased concentration might indicate increased demand for CoA biosynthesis (Daskalaki et al. 2015).

The observed close links among physical activity, aerobic fitness, reduced fasting plasma glucose concentration and body adiposity could be the result of a long-term adaptation to increased oxygen demand and fatty acid usage, with a consequent reduction in fatty acid storage and insulin resistance (Kujala et al. 2013). The exercise-induced improvement in the coordination and utilisation of lipid and carbohydrate as energy sources may delay the onset of sarcopenia (Navas-Enamorado et al. 2017).

Krebs Cycle Metabolites and Exercise (Blood Metabolome)

The Krebs cycle holds a central place in cellular energy production. Its metabolites are linked to glycolysis and many studies have found that they rise in blood post-exercise. The increase in Krebs cycle metabolites may reflect increased metabolite flux from glycolysis and β oxidation, making aerobic ATP production match the increased ATP demand with increasing exercise volume and intensity (Wagenmakers 1998; Gibala 2001; Zhou et al. 2019). Krebs cycle metabolites are more likely to be elevated in serum of trained individuals who have developed an increased aerobic capacity and who are able to oxidise pyruvate more efficiently (Daskalaki et al. 2014). Exercise resulted in increases in plasma Krebs cycle metabolites, specifically citrate and fumarate, in male rats after swimming until exhaustion (Zhou et al. 2019). Plasma citrate was identified as a marker of physical activity, since it is involved in the energy production from carbohydrates, lipids and proteins (Sampson et al. 2014). Citrate was also found increased in muscle following endurance exercise (Starnes et al. 2017).

Succinate increased in plasma in response to both moderate- and high-intensity interval training, with the response to high-intensity exercise being considerably higher than that to moderate-intensity exercise (Peake et al. 2014). These findings suggest an intensity-dependent change in Krebs cycle metabolites. Succinate also increased after 12 weeks of regular physical activity in the circulation of T2D subjects (Kuhl et al. 2008). A single bout of resistance exercise has also resulted in increases in serum succinate (Berton et al. 2016). A study comparing three trained groups (high amount high intensity, high amount low intensity, low amount low intensity) with a non-exercising group found decreased plasma concentration of acotinate and increased concentration of malonate in the trained groups (Brennan et al. 2018). Larger increases have also been observed in malate, fumarate and succinate than in oxaloacetate, isocitrate and α-ketoglutarate, 5 min after the cessation of exercise (Wagenmakers 1998). However, during prolonged exercise the pool of muscle Krebs cycle metabolites declines, and this was attributed to an increase in leucine oxidation (which relies on α-ketoglutarate) and/or local muscle fatigue (Gibala 2001).

Purine and Pyrimidine Metabolism and Exercise (Blood Metabolome)

Exercise was found to have many effects on purine metabolism. Specifically, adenine, which is one of the two purine bases of nucleic acids, decreased in plasma after training in a study with T2D subjects (Kuhl et al. 2008). Therefore, purine metabolites may be important markers of the effects of exercise. Zielisnki et al. (2012) found that the training mode of sprinters reduced the resting and post-exercise plasma purine concentration to a greater extent than in endurance-trained athletes. However, the same authors noticed a significant decline in plasma purine concentration after exercise in middle- and long-distance runners only in the

competition phase, when the training volume was reduced and its intensity increased, not during the general preparation period. Thus, purine metabolism may be affected by the intensity of exercise training.

The high ATP turnover rate in the exercising muscles increases AMP and IMP production (Pechlivanis et al. 2015). Following acute exercise, IMP is either reaminated to AMP to resynthesise ATP or is further degraded to inosine and hypoxanthine, which then enter the bloodstream from muscle, peaking a few minutes after cessation of exercise (Enea et al. 2010; Zielinski et al. 2013). Following 6 months of training plasma ATP was higher in the three exercising groups (high amount high intensity, high amount low intensity, low amount low intensity) compared to the non-exercising group, whereas AMP was lower in the three exercising groups (Brennan et al. 2018). Increased plasma inosine concentration was detected after strenuous exercise, reflecting the well characterized adenine nucleotide catabolism (ATP $\rightarrow$ ADP $\rightarrow$ AMP $\rightarrow$ IMP $\rightarrow$ inosine) that occurs in the working muscle during strenuous exercise (Thysell et al. 2012). However, after exercises of different loads and intensities, inosine decreased from baseline (Brennan et al. 2018).

Hypoxanthine is considered an index of tissue hypoxia and a marker of adenine nucleotide degradation (Zielisnki et al. 2011). The latter is an index of stress generated during exercise, a parameter of exercise intensity and a criterion of exercise classification (Zielisnki et al. 2011). Plasma hypoxanthine concentration during and after exercise is affected by several factors including exercise intensity, rate of reconversion of hypoxanthine to IMP, rate of excretion and activity of xanthine dehydrogenase, a rate-limiting enzyme in uric acid formation (Daskalaki et al. 2014, 2015). Another purine degradation product, xanthine, was lower in the plasma of three trained groups (high amount high intensity, high amount low intensity, low amount low intensity) compared to the non-exercising group, as well as after acute exercise (Brennan et al. 2018). Due to the production of xanthine and hypoxanthine, ammonia concentration might rise (Daskalaki et al. 2014). However, in the review of Daskalaki et al. (2014) it is mentioned that there may be less ammonia build-up from AMP deamination in trained individuals, due to more efficient purine utilisation.

Uridine is a pyrimidine nucleoside that forms a part of RNA and is necessary not only for the synthesis of nucleic acids, but also for the synthesis of glycogen via uridine diphosphate glucose (Dudzinska et al. 2013). A maximal-exercise-induced increase in blood uridine correlates with post-exercise increases in insulin and glucose concentrations; therefore, it is possible that high blood plasma uridine concentration plays some role in the development of insulin resistance (Daskalaki et al. 2014; Dudzinska et al. 2013). Uridine has also been linked

to stimulated glucose uptake and glycogen synthesis in isolated rat skeletal muscle (Dudzinska et al. 2013).

Redox Metabolism and Exercise (Blood Metabolome)

Training can have positive or negative effects on oxidative stress, depending on training load, training specificity and the basal level of training (Enea et al. 2010). Glutathione is one of the most important antioxidant metabolites, and the increase in 2-hydroxybutyric acid, 2-aminobutyric acid, glutamate, methionine and proline following prolonged and intensive exercise is indicative of enhanced glutathione production (Nieman et al. 2012). Another antioxidant compound produced in the body and protecting against reactive oxygen species generated during exercise is uric acid (Daskalaki et al. 2015). Chorell et al. (2012) found increased plasma concentrations of hypoxanthine and taurine (two metabolites also related to oxidative stress and muscle cell leakage) following strenuous exercise.

Other Metabolites and Exercise (Blood Metabolome)

Serum creatinine exhibits considerable inter-individual variability, whereas urinary creatinine shows fewer inter-individual differences and may therefore provide a more reliable index of kidney function (Crimmins et al. 2018). Blood creatinine, however, decreased after training in a study with T2D subjects (Kuhl et al. 2008), whereas it increased in the rat heart following endurance exercise (Starnes et al. 2017).

Betaine is an organic osmolyte and methyl donor, and metabolomics studies of dietary intervention on human subjects have reported that its blood concentration increases after weight reduction (Kim et al. 2013). Betaine has also been positively associated with physical activity (Xiao et al. 2016). However, a study on subjects with metabolic syndrome found exercise induced decreases in betaine following three different modes of exercise (high-intensity interval exercise, resistance exercise and continuous moderate-intensity exercise, Siopi et al. 2019).

Changes have also been detected in plasma 2-hydroxybutyric acid, itaconic acid and citraconic acid, all of which increased after high-intensity interval training (Peake et al. 2014). Itaconic acid may be derived from a Krebs cycle metabolite, aconitic acid, and citraconic acid may be a by-product of glutamine metabolism (Peake et al. 2014).

Effects of Exercise on the Metabolic Footprint

Urine contains considerable amounts of metabolites related to amino acid and fatty acid oxidation, the Krebs cycle, glycolysis, purine synthesis and the gastrointestinal microbiome (Wu & Gao 2015). Metabolites enter the bloodstream from tissues and are circulated to the kidneys, where, through renal filtration they pass into urine. Thus, urine analysis reflects the

natural homeostatic flux of tissue turnover during physical activity (Sampson et al. 2014). The application of urine metabolomic analysis to disease is an emerging field and shows great potential for biomarker discovery (Zhang et al. 2012). However, the effects of exercise on the urinary metabolome and the physiological variations of the urinary metabolome have not been extensively studied, since most of the research on exercise metabolomics has examined changes in blood serum or plasma.

Amino Acid Metabolism and Exercise (Urinary Metabolome)

Amino acids, such as lysine, methionine, phenylalanine, threonine, tyrosine and BCAAs, as well as the amino acid derivative 3-methylhistidine, are exercise biomarkers that are measured in urine to assess muscle protein breakdown (Sheedy et al. 2014). There is no clear effect of exercise on the urinary concentrations of several amino acids, especially alanine and glutamine, as they have either increased or decreased depending on the exercise modality in several studies mentioned in the review of Daskalaki et al. (2014). Alanine and glutamine are quantitively the most important gluconeogenic amino acids. Alanine increased in urine after brief maximal exercise (Pechlivanis et al. 2010). An approximately two- to three-fold increase in the excretion of alanine was found 30 min after the completion of short-term intensive exercise (Enea et al. 2010). The increase of urinary alanine after exercise is probably the result of the increase in pyruvate during glycolysis, since alanine is formed from pyruvate through transamination (Daskalaki et al. 2014; Enea et al. 2010; Pechlivanis et al. 2015). Glutamine and threonine excretion was found increased in rats following 16 months of swim-training, compared to non-exercising female rats (Deda et al. 2017), and increases in glutamine, lysine and threonine were also seen following high-intensity interval exercise in urine of healthy men (Siopi et al. 2017). Morris et al. (2013) found that amino acid excretion in urine was reduced in participants with high fitness. Similar are the results of a study by Mukherjee et al. (2014), who found that the fold change of the amino acid concentrations, specifically of glycine and tryptophan, were lower in athletes than non-athletes following an acute bout of submaximal exercise. Acutely or chronically high plasma or serum concentrations of glycine may damage the nervous system; therefore, its exercise-induced reduction in urine may serve therapeutic purposes (Wishart 2019). Although most of the previous studies found decreased excretion of amino acids following exercise, the excretion of lysine, methionine and tyrosine increased in older men after 18 months of training (Sheedy et al. 2014).

Differences in the findings concerning the changes in urinary BCAA concentrations with exercise are also observed. Specifically, leucine and valine have been found to either increase or decrease, which suggests an uneven response to exercise. Increased BCAA

degradation products in the urine of moderately trained males were found post-exercise (Daskalaki et al. 2015; Pechlivanis et al. 2010), suggesting increased BCAA breakdown. Lower resting BCAA concentrations were also noticed in a high fitness group compared to the low-fitness one (Morris et al. 2013). Contrary to these findings, Sheedy et al. (2014) found that after long-term training, the urinary concentrations of isoleucine, leucine and valine increased in older men.

The aromatic amino acids, histidine, phenylalanine, tryptophan and tyrosine, were found to fall in urine post-exercise, whereas their keto and hydroxy derivatives were found to increase (Pechlivanis et al. 2010). Tryptophan, however, increased in older men after 18 months of training (Sheedy et al. 2014). Lastly, the excretion of 3-methylhistidine was found increased after both high-intensity interval exercise and resistance exercise in healthy male subjects (Siopi et al. 2017).

Carbohydrate and Lipid Metabolism and Exercise (Urinary Metabolome)

Both urinary lactate and pyruvate were found increased 30 min after a single 30 s cycle ergometer sprint (Enea et al. 2010). Resting lactate excretion also increased after long-term exercise training in older men (Sheedy et al. 2014) and following vigorous exercise (Muhsen Ali et al. 2016).

Elevated glycerol excretion was observed after exercise in several studies mentioned in the review of Daskalaki et al. (2014), consistent with increased lipolysis. Moreover, measurement of urinary acylcarnitines is used to detect inborn errors of fatty acid metabolism, which result from a defect in one of the steps of β oxidation (Daskalaki et al. 2015). An increase in urinary acylcarnitines was found after vigorous physical exercise, which was attributed to the decreased demand for fatty acids during exercise in which there is a switch towards glycolysis in order to maintain a high ATP turnover rate (Muhsen Ali et al. 2016). However, no changes were found in urinary acylcarnitines in a study examining the effects of brief maximal exercise, possibly because interventions of higher intensity and duration are required (Pechlivanis et al. 2015).

Acetylcarnitine excretion increased in urine of female rats following 16 months of swimming training (Deda et al. 2017) and 4 h after high-intensity interval exercise in healthy male (Siopi et al. 2017). Two products of acetyl CoA metabolism, acetate and 3-hydroxybutyrate, were found elevated in the post-exercise urine samples (Pechlivanis et al. 2015). Acetate is produced directly from acetyl CoA, while 3-hydroxybutyrate is a ketone body (Pechlivanis et al. 2015). The rise in the concentrations of these two metabolites may be attributed to the rise in the level of acetyl CoA as a result of increased pyruvate, from which it is produced (Pechlivanis et al. 2015). A significant increase in acetate excretion was also observed after

short-term intensive exercise (Enea et al. 2010). Interestingly, trained subjects showed a smaller increase than untrained ones (Enea et al. 2010). A study conducted in older men also found that, following 18 months of training, urinary concentration of acetate increased (Sheedy et al. 2014). The excretion of pantothenate, which is a CoA precursor, was found elevated after one-hour endurance exercise, possibly reflecting a decreased demand for CoA and a possible switch to glycolysis instead of fatty acid degradation (Daskalaki et al. 2015). Increased pantothenate excretion was also observed in urine of female rats that had trained for 16 months (Deda et al. 2017).

Krebs Cycle Metabolites and Exercise (Urinary Metabolome)

Excretion of five compounds of the Krebs cycle exhibited mixed responses to brief maximal exercise in a study conducted by Pechlivanis et al. (2015). Citrate and succinate decreased, fumarate increased, and 2-oxoglutarate and oxaloacetate did not change significantly (Pechlivanis et al. 2015). The decrease of citrate was linked to high urinary lactate concentration and may be connected to renal malfunction due to lactic acidosis caused by maximal exercise (Pechlivanis et al. 2010). This is supported by other findings of the same study, in which metabolites correlated with renal malfunction, such as creatinine, glycine, hippurate and 4-aminohippurate, decreased post-exercise. A review by Sampson et al. (2014) also notes increased concentrations of fumarate and 2-oxoglutarate in urine following exercise. Greater excretion of fumarate was also seen following an acute bout of submaximal exercise in urine of athletes, compared to untrained, minimally active individuals, a finding consistent with the expected differences in succinate oxidation and overall Krebs cycle turnover (Mukherjee et al. 2014). Moreover, the rise in fumarate may be partly accounted for by either the concomitant decrease in tyrosine, since part of the carbon skeleton of the latter is incorporated in the former, or the catabolism of phenylalanine (Pechlivanis et al. 2015). Enea et al. (2010), however, found increased excretion of succinate 30 min after a single 30 s cycle ergometer sprint. Furthermore, malonate excretion was greater following an acute bout of submaximal exercise in a group of minimally active individuals, compared to the trained one (Mukherjee et al. 2014).

Purine and Pyrimidine Metabolism and Exercise (Urinary Metabolome)

Higher concentrations of metabolites involved in purine metabolism, such as guanine, hypoxanthine and inosine, are generally noticed in urine after vigorous physical exercise (Muhsen Ali et al. 2016). Hypoxanthine and inosine, which are metabolites in the catabolic pathway of purines, are important discriminators of resting and exercise states (Pechlivanis et al. 2015). The clearance of hypoxanthine and its urinary excretion in the recovery phase are considerably increased, even 20-fold, after high-intensity or prolonged exercise in untrained

healthy individuals (Sheedy et al. 2014). Sheedy et al. (2014) describe the clearance of hypoxanthine as a 'delayed echo' of muscle and blood concentrations. Greater excretion of hypoxanthine in athletes is attributed to greater ATP turnover, although there is no explanation regarding the mechanism responsible for removing hypoxanthine from blood via the kidney and gut (Mukherjee et al. 2014; Sheedy et al. 2014).

Small Aliphatic Acids and Exercise (Urinary Metabolome)

Formate in the body can be derived from a variety of sources. It is primarily produced in the mitochondria from serine, glycine and two metabolites of choline catabolism, *N,N*-dimethylgycine and sarcosine, through folate-dependent reactions (Pechlivanis et al. 2015). Brief maximal exercise caused a drop in the urinary formate concentration (Pechlivanis et al. 2015). Similarly, Mukherjee et al. (2014) found that formate excretion was greater following an acute bout of submaximal exercise in a group of minimally active individuals, compared to the trained one.

Gut Microbiome Metabolism and Exercise (Urinary Metabolome)

Post-exercise increases in a range of products of gut microbiome metabolism have been reported (Daskalaki et al. 2015; Muhsen Ali et al. 2016). Hippurate is normally found in human urine and its concentration is related to the microbial activity and microfloral composition of the colon (Psichogios et al. 2008). Changes in hippurate excretion are typically associated with the gut microflora, whose activities may play important roles in energy metabolism and immune function of the whole body (Neal et al. 2013; Schnackenberg et al. 2007). Following moderate-intensity exercise, hippurate excretion decreased (Neal et al. 2013).

Dimethylamine is also related to gut microflora, as it is produced by degradation of dietary choline to trimethylamine, which is subsequently converted to TMAO (Slupsky et al. 2007). Dimethylamine is also a metabolic by-product of dimethylarginine, which is involved in nitric oxide synthesis, and is reported to be elevated in urine of patients with hypertension and cardiac dysfunction but decreased in urine of exercise-trained patients (Sheedy et al. 2014). Increased concentrations of dimethylamine may be related to low blood glucose (Slupsky et al. 2007). Urinary dimethylamine excretion is thought to reflect intermediary metabolism and increased following moderate-intensity exercise (Neal et al. 2013). Dimethylamine excretion was also found increased after an 18-month training protocol in older men (Sheedy et al. 2014). Moreover, dimethylamine excretion is increased in aged, compared to young, subjects (Kochhar et al. 2006; Psichogios et al. 2008).

Trimethylamine and TMAO excretion decreased by 20% after exercise (Pechlivanis et al. 2015). TMAO is a metabolic product of trimethylamine and choline and shows high inter-

individual variation in both humans and control animals (Psichogios et al. 2008). Enea et al. (2010) also found decreased urinary concentration of TMAO after exercise. The negative effects of exercise on the urinary concentration of TMAO may be related to health benefits (Pechlivanis et al. 2015). Trimethylamine, however, increased following long-term training in older men (Sheedy et al. 2014).

Other Metabolites and Exercise (Urinary Metabolome)

Creatinine is an abundant urinary metabolite that correlates with overall body size and muscle mass, while exhibiting little excretory variation in healthy individuals (Sheedy et al. 2014). No significant changes were observed in creatinine excretion after exercise, although a difference between trained and untrained subjects was seen, with the trained ones having a higher increase (Enea et al. 2010). This difference is reasonable, since trained subjects generally have a higher muscle mass than untrained ones. Urinary creatinine excretion, however, increased in another study following moderate-intensity exercise (Neal et al. 2013). Interesting are the results of Sheedy et al. (2014), who found decreased excretion of creatinine in older men following 18 months of multi-component training.

Choline is a building block of the neurotransmitter acetylcholine (Sheedy et al. 2014). It also acts as methyl donor and is central to preventing endothelial damage caused by homocysteine for homeostatic regulation of intracellular methyl balance, which can be negatively affected by exercise (Sheedy et al. 2014). Choline excretion increased after 18 months of training in older men (Sheedy et al. 2014).

2-hydroxybutyric acid is usually elevated in urine of patients with lactic acidosis and ketoacidosis, which suggests that it plays some role under conditions of metabolic stress (Peake et al. 2014). A 2-fold increase of this metabolite was found following sprint running (Pechlivanis et al. 2015).

γ-Aminobutyric acid (GABA) is a compound whose acutely or chronically high concentrations may cause damage to the nervous system (Wishart 2019). Urinary GABA concentration decreased after vigorous exercise (Muhsen Ali et al. 2016). However, its excretion was found increased in urine of female rats that had trained for 16 months (Deda et al. 2017).

N,N-dimethylglycine, a product of choline metabolism, increased following training in older men (Sheedy et al. 2014). Likewise, methylamine excretion increased (Sheedy et al. 2014), which is supported by another study examining the effects of long-term exercise on the urinary metabolome of female rats (Deda et al. 2017).

Fatty Acids and Aging

Lipid metabolism is significantly associated with age, with specific fatty acids being indicators of healthspan (Johnson et al. 2018). Phospholipids are inflammatory mediators with atherogenic properties, and their altered concentrations are linked to age-related physiological changes (Collino et al. 2013). Specific fatty acids have also been associated with VO_2max (Johnson et al. 2018).

Saturated fatty acids (SFAs) activate a number of micro RNAs which impair insulin signalling and suppress the expression of the insulin receptor, leading to insulin resistance; therefore, SFAs have been associated with increased risk of T2D (Wishart 2019). Other studies have linked SFAs with increased risk of inflammation (Corsetto et al. 2019) and cardiovascular disease (Kawanishi et al. 2018). In particular, palmitic acid (16:0) and stearic acid (18:0) have been positively associated with inflammation and negatively associated with interleukin-6 (Corsetto et al. 2019). Age-related increases were observed in myristic acid (14:0) and 16:0 (Johnson et al. 2018). High levels of 14:0, 16:0 and 18:0 in plasma phospholipids are specifically associated with increased risk for T2D (Ma et al. 2015; Wishart 2019). Total free plasma SFAs were also found increased with age (Pararasa et al. 2016). Interestingly, in a very recent study, extreme longevity was associated with a higher phospholipid SFA content (Johnson & Stolzing 2019).

The serum concentration of monounsaturated fatty acids (MUFAs) was positively associated with low-density lipoprotein (LDL) and negatively associated with high-density lipoprotein (HDL) and cognition (Proitsi et al. 2018). Total free plasma MUFAs were also found increased with age (Pararasa et al. 2016). Age-related increases were observed in free serum, palmitoleic acid (16:1ω7) and oleic acid (18:1ω9, Johnson et al. 2018), as well as *cis*-vaccenic acid (18:1ω7) and eicosenoic acid (20:1ω9) in brain phospholipids (McNamara et al. 2008). Although 18:1ω7 has been associated with older age (Djoussé et al. 2014; McNamara et al. 2008), it has been inversely associated with the risk of T2D (Ma et al. 2015; Weir et al. 2019), coronary heart disease (Djoussé et al. 2012) and heart failure (Djoussé et al. 2014).

Serum polyunsaturated fatty acids (PUFAs) of both the ω3 and ω6 series were positively associated with HDL (Proitsi et al. 2018). ω3 PUFAs are considered beneficial for the cardiovascular system in adults, play vital structural and functional roles in the central nervous system and are associated with cognitive performance and brain function during general aging (Denis et al. 2015; Proitsi et al. 2018; Úbeda et al. 2012). Moreover, ω3 fatty acids have antioxidant (Tutino et al. 2018) and anti-inflammatory properties and mitigate age-related muscle loss, resulting in reduced risk of developing sarcopenia (Dupont et al. 2019; Stella et al. 2018). In particular, linoleic acid (18:2ω6), α-linolenic acid (18:3ω3) and docosahexaenoic

acid (22:6ω3) collectively protect against coronary heart disease (Gollasch et al. 2019). 22:6ω3 and arachidonic acid (20:4ω6) are important components of neuronal membranes, while 22:6ω3, 20:4ω6 and eicosapentaenoic acid (20:5ω3) also affect cardiovascular health and inflammation (Janssen & Kiliaan 2014). Higher proportions of 18:2ω6 have also been associated with a lower risk of T2D (Wu et al. 2017). 18:3ω3 is considered one of the best predictors of age in muscle, with increases observed with aging (Houtkooper et al. 2011; Johnson et al. 2018) and high levels in blood being associated with high risk of sudden cardiac arrest (Lemaitre et al. 2009), hypertension (Tsukamoto & Sugawara 2017) and unhealthy aging (Lai et al. 2018). However, increases in plasma 18:3ω3 through diet have been linked with lowered blood pressure (Caligiuri et al. 2017). Age was inversely correlated with total ω6 content, 20:4ω6, docosapentaenoic acid (22:5ω6) and 22:6ω3 in brain phospholipids (McNamara et al. 2008). Decrease in brain 22:6ω3 with age have been linked with deterioration of memory and other cognitive functions (Mohajeri et al. 2015; Thomas et al. 2015; Weiser et al. 2016). Total ω3 fatty acids were found depleted in the aged brain (Thomas et al. 2015) and aged plasma (Lauretani et al. 2007). Total ω6 PUFAs were also found decreased with age in plasma (Lauretani et al. 2007) and red blood cells (Harris et al. 2013), mainly due to the decrease in their most abundant member, 18:2ω6. A high negative correlation between plasma ω6 fatty acids and age has also been observed in another study (Corsetto et al. 2019). A decline in total plasma PUFA concentration was also found with age (Lauretani et al. 2007). Surprisingly though, increased concentration of free PUFAs in serum was found with age (Pararasa et al. 2016) and has been implicated in the pathogenesis of chronic diseases (Collino et al. 2013). On the contrary, lower PUFA contents were observed in extreme longevity (Johnson & Stolzing 2019).

Fatty Acids and Exercise

Changes in plasma fatty acids during exercise represent the balance between fatty acid release from adipose tissue and fatty acid uptake into skeletal muscle and liver (Peake et al. 2014). SFAs are preferentially diverted to storage, PUFAs and, specifically, ω6 fatty acids are preferentially oxidised, whereas ω3 fatty acids are usually used as building blocks for plasma membranes (Bradley et al. 2007). Increased concentrations of fatty acids and ketone bodies in the plasma of trained, compared with untrained, individuals are consistent with increased lipolysis to meet the energy demands of training (Daskalaki et al. 2014; Zhou et al. 2019). Long-term physical activity may have beneficial effects on the oxidation and desaturation of fatty acids, manifested by increased percentage of ω3 fatty acids and decreased serum concentration of SFAs (Kujala et al. 2013). Exercise intensity, however, does not seem to affect fatty acid oxidation and total serum fatty acids, as seen in a study comparing high-intensity interval training with continuous moderate-intensity training (Peake et al. 2014).

There is a gap in the literature concerning the effects of different modalities of exercise on different lipid classes in different tissues. In the results presented below, muscle will not be categorised according to muscle fibre type, since research has shown that it is not a major determinant of the fatty acid composition of skeletal muscle (Nikolaidis et al. 2006).

Resting muscle 18:0 increased in high-intensity trained animals compared to low-intensity trained ones (Starnes et el. 2017). 18:0 also increased after 12 weeks of endurance training, combined with strength exercises in T2D subjects (Kuhl et al. 2008). 14:0 increased after 3 days of intensified running in healthy subjects (Nieman et al. 2013). No significant differences in 14:0 or 16:0 were observed in plasma following exercise until exhaustion, as well as after competitions (Lyudinina et al. 2018). Contrary to these findings, Chorell et al. (2012) found that highly fit individuals, compared to low-norm subjects, had lower plasma concentration of 16:0 and 18:0, which is supported by the findings of another study in which trained rats had lower 14:0, 16:0 and 18:0 in muscle triacylglycerols, although the molar percentage of the latter was higher in the trained group (Petridou et al. 2005). The percentages of the same fatty acids in the serum free-fatty-acid fraction of humans decreased after prolonged exercise (Mougios et al. 1995; Mougios et al. 1998; Mougios et al. 2003). Free 16:0 was also found decreased in blood following 6 weeks of training (Shojaee-Moradie et al. 2007), and the same fatty acid, as well as the total content of SFAs, were also found decreased in blood phospholipids following one month of physical activity (Corsetto et al. 2019). Although there is no consensus in the findings related to SFAs, a main conclusion could be that exercise results in an increase in 18:0 and a decrease in 16:0, as well as the total content of SFAs.

Acute exercise increases, on average, by 16% the free MUFAs in plasma in both animals and humans, whereas chronic exercise decreases their molar percentage (Nikolaidis et al. 2004; Nikolaidis & Mougios 2004). A very large increase of 18:1ω9 and 16:1ω7 was found following 3 days of intensified running (Nieman et al. 2012, 2013). 18:1ω9 also increased in high-intensity trained animals compared to low-intensity trained ones (Starnes et el. 2017), in animals that performed exhaustive swimming (Zhou et al. 2019) and in humans following prolonged exercise (Mougios et al. 1993, Mougios et al. 2003). Increased concentration of 18:1ω9 following endurance exercise has been associated with improvements in insulin sensitivity (Huffman et al. 2011). However, contrary to the findings of the previous studies, high-fit individuals, compared to low-norm subjects, had lower concentrations of 18:1ω9 and 16:1ω7 (Chorell et al. 2012). 16:1ω7 in liver phospholipids and in both muscle phospholipids and triacylglycerols, 18:1ω9 and 18:1ω7 in muscle triacylglycerols, as well as total liver MUFAs have also been found decreased in the molar percentage distribution of trained compared to untrained rats (Petridou et al. 2005). Eicosenoic acid (20:1ω9) is the only MUFA whose concentration and molar percentage was higher in trained compared to untrained rats

in muscle phospholipids, although it was lower in the triacylglycerols of the trained group (Petridou et al. 2005). Furthermore, 18:1ω7 was found increased in human muscle phospholipids following 4 weeks of training (Helge et al. 2001).

Chronic exercise seems to increase the molar percentage of PUFAs and ω6 fatty acids in adipose tissue, as well as the relative amount of unsaturated fatty acids (UFAs) in liver lipids in both animals and humans (Nikolaidis et al. 2004). The molar percentage of ω6 fatty acids in phospholipid and of ω3 fatty acids in triacylglycerols were also higher in the muscle of trained compared to untrained rats (Petridou et al. 2005). It has also been noted that at moderate intensities primarily ω6 PUFAs are oxidised rather than SFAs (Bradley et al. 2007). Linoleic acid (18:2ω6), an essential fatty acid, is the most common ω6 PUFA in human diets and mammalian tissue (Nieman et al. 2014). Its oxidised metabolites (13- and 9-hydroxy-octadecadienoic acids) were linked to pathological conditions including atherosclerosis, diabetes, Alzheimer's disease, non-alcoholic steatohepatitis, psoriasis, chronic inflammation, obesity and cancer (Nieman et al. 2014). These hydroxy acids may serve as oxidative stress markers during exercise (Daskalaki et al. 2014). Increased 18:2ω6 in serum was observed after 3 days of running (Nieman et al. 2013). Starnes et al. (2017) also found increased 18:2ω6 in muscle following high-intensity endurance exercise. 18:2ω6 and 20:4ω6 also increased following exhaustive swimming exercise in plasma of female rats (Zhou et al. 2019). The phospholipid 18:2ω6, γ-linolenic acid (18:3ω6) and 18:3ω3 contents were higher in the liver of trained compared to untrained rats, with 18:2ω6 being increased in muscle phospholipids as well, although the 18:2ω6, 18:3ω3 and 20:4ω6 contents of triacylglycerols, as well as the total triacylglycerol fatty acids, were lower in the muscle of the trained group (Petridou et al. 2005). In the same study, the molar percentage of another ω3 fatty acid, 20:5ω3, was higher in muscle triacylglycerols of the trained compared to untrained rats (Petridou et al. 2005).

In the review of Daskalaki et al. (2014) high fitness levels are associated with high levels of 22:6ω3 in serum. This is supported by the results of a study showing that highly fit individuals, compared with low-norm subjects, had higher levels of 22:6ω3, and contrary to previous studies, lower levels of 18:2ω6 in plasma (Chorell et al. 2012). Helge et al. (2001) have found increased levels of 22:6ω3 and a decreased ratio of ω6/ω3 fatty acids in human skeletal muscle phospholipids following four weeks of one-leg exercise training. The exercise-induced decrease in the ω6/ω3 ratio is considered highly beneficial, as within the past decades there has been an increase in the consumption of ω6 fatty acids and a decrease in ω3 fatty acids, resulting in increased risk for obesity, chronic inflammation and comorbidities (Powell et al. 2015). Western-type diets have an ω6/ω3 ratio of 15-20, when the optimal ratio is 2 or even 1 (Tutino et al. 2018).

Exercise increases, on average, by 22% the UFA to SFA ratio in the plasma of both athletes and untrained individuals, and the longer it remains elevated, the higher its impact on human metabolism will be (Mougios et al. 2003; Nikolaidis & Mougios 2004). It is reasonable that, since UFA are more beneficial to health than SFA, a higher ratio is more desirable than a lower one. However, in muscle triacylglycerols the same ratio decreased in trained compared to untrained rats following 8 weeks of running training (Petridou et al. 2005). Contrary to the aforementioned studies, Peake et al. (2014) found that the ratio of UFA to SFA in plasma free fatty acids does not change immediately, 1 h and 2 h after high-intensity interval training or moderate-intensity continuous exercise.

In summary, changes in the abundance of individual fatty acids are noticed with acute and chronic exercise, raising the question as to the factors that determine the preference for mobilisation of specific fatty acids depending on exercise modality, tissue and lipid class. Although the mechanism of changes in fatty acid selectivity with exercise is not clear, it is speculated that this could be due to altered transport across cellular membranes (noticeably, there are five different fatty acid transport proteins that are expressed in skeletal muscle), rate of production through triacylglycerol, phospholipid and cholesterol ester hydrolysis, rate of oxidation and rate of triacylglycerol, phospholipid and cholesterol ester biosynthesis (Nikolaidis et al. 2006; Petridou et al. 2005). By reviewing the aforementioned results, one can notice that, among fatty acid categories, aging induces changes mainly in phospholipid SFAs, MUFAs and PUFAs, whereas exercise induces changes mainly in triacylglycerol SFAs and MUFAs. However, both phospholipid and triacylglycerol PUFAs seem to change. These conclusions could imply a larger effect of aging on structural lipids and a larger effect of exercise on storage lipids.

From the studies described in this literature review, it is clear that both aging and exercise have effects on major metabolic pathways. Aging seems to decrease blood amino acids, as well as blood and urinary metabolites related to carbohydrate oxidation, fat oxidation and redox metabolism. Also, aging is characterised by a decline in Krebs cycle metabolites in urine. Exercise, however, seems to mainly increase amino acids in blood, as well as blood and urinary metabolites related to carbohydrate and fat oxidation. Also, exercise is characterised by an increase in Krebs cycle metabolites in blood. Although many studies have examined the acute and chronic effects of exercise on the metabolic fingerprint of various tissues and biological fluids, research on how lifelong exercise influences the blood and urinary metabolome, insulin sensitivity and the fatty acid content of tissues in either humans or experimental animals is scarce. The scarcity of such studies is possibly due to the difficulty of monitoring living organisms from youth through old age, especially when this requires decades, as in humans. Therefore, the aim of the present study was to examine the effects

of aging and exercise on the blood and urinary metabolome, insulin sensitivity, as well as the quadriceps, liver and brain fatty acids in male rats.

METHODS

Animals and Ethics

Thirty-six male Wistar rats were initially obtained from the Veterinary Faculty of the Aristotle University of Thessaloniki at the age of 3 months (estimated onset of adulthood). The animals were caged in groups of 3 at temperatures ranging from 18 to 29 °C according to the seasonal fluctuation of indoor temperatures in temperate climates. Relative humidity ranged from 25 to 65%. The cages and watering equipment were cleaned once a week, the bedding was also changed once a week, and food and water were supplied daily. Furthermore, the room and cages were disinfected once a month. The rats had free access to water and rodent chow (ELVIZ SA, Greece). They were weighed weekly on a digital scale to the nearest gram. Food intake was measured on the same scale on a weekly basis and was expressed as average daily intake for each animal. Fig. 1 shows the housing department of the animals (a), the room where the cages were placed and where exercise was conducted (b), and a cage with the water and chow that was provided for the rats (c).

The experiment took place between June 2013 and March 2015. All procedures were conducted in accordance with the European Union regulations for care and use of laboratory animals. Additionally, all procedures were approved by the Department of Rural Economy and Veterinary Medicine, Prefecture of Central Macedonia, Hellenic Republic (Code: EL54BIO10, Protocol no.: 449161/4835).

Fig. 1 Housing department of animals (a), room with cages and exercise area (b), and cage with water bottle and rodent chow (c).

Animal Grouping and Labelling

The rats were divided randomly into four equal groups. Group A exercised during the first half of their adult life, that is, from the 3rd to 12th month of age (n = 9); group B, the lifelong exercise group, exercised from the 3rd to 21st month (n = 9); group C did not exercise at all (n = 9); and group D exercised during the second half of their adult life, that is, from the 12th to 21st month (n = 9). The animals were caged in groups of 3. Animals in each cage belonged to the same group. Each cage was marked with a label, and the 3 rats in it were identified as follows: #1

was not marked, #2 was marked in the area of the spine just below the head with permanent hair dye of organic origin, and #3 was marked on the back area just above the tail with the same dye. The designation of each blood, urine, or faeces sample taken from the rats consisted of a letter corresponding to the sampling time, a first number corresponding to the cage, and a second number corresponding to the animal. For example, sample A1.2 was the sample taken at 3 months from the 2nd rat of the 1st cage.

Exercise Training

The rats exercised by swimming individually in water tanks with a surface of 1000-1500 cm^2, depth of at least 51 cm, and water temperature of 33-36 ^oC. The lifelong and long-term training protocols consisted of swimming for 20 min/day, 5 days/week (Monday to Friday) with a lead fishing weight of 2% of each animal's body weight initially attached to its tail with adhesive tape (Durapore™, 3M, Germany). The intensity of such exercise is considered moderate (Jones 2007). As time went by, the weight load was reduced to 1% and, finally, removed in accordance with the declining ability of the aging animals to sustain the desired exercise duration and frequency. Other than that, exercise was well tolerated by the animals. According to the literature, swimming is pleasant to rats and may be used for the detection of physiological and biochemical responses and adaptations to either acute or long-term exercise (Jones 2007).

The acute exercise protocol consisted of one session of 20 minutes of swimming. The young rats swam with an additional weight of 2%, whereas the aged rats swam without additional weight. At the end of the exercise, the rats were dried and returned to the cages. Fig. 2 shows an exercising rat (a) and a rat about to be dried after completing the exercise bout (b). To ensure active swimming (rather than just floating around), the water was shaken frequently. To control for any effect of ambient temperature on metabolism, we kept the non-exercising rats in shallow water of the same temperature and for the same time as the exercising ones.

To have an adequate number of animals for statistical power, we initially decided to end the experiment once we had reached the minimum number of 5 rats per group. Also, all rats stopped exercising on their 21st month of age, as we wanted to control the effects of detraining for the next 3 months, that is, until they reached their 24th month, which was our limit for all experimental procedures. However, on the 22nd month, group D remained with 5 rats. Therefore, we euthanised both groups A and D, which were the groups with the half-life intervention and the least animals at this age (7 and 5, respectively), for statistical purposes, that is, to be able to compare the groups in pairs of most interest. Hence, only groups B and C, which were the groups with the lifelong intervention (i.e., lifelong exercise and no exercise, respectively), continued up to the 24th month.

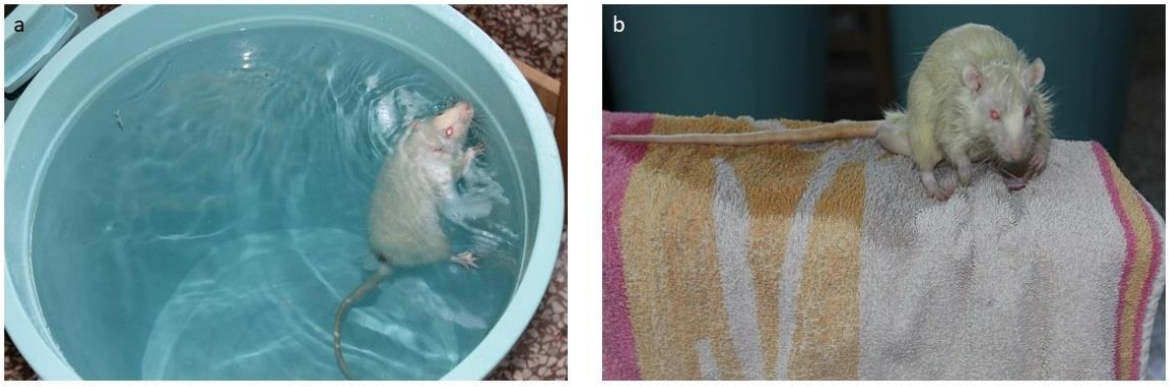


Fig. 2 An exercising rat (a) and a rat about to be dried after completing the exercise bout (b).

Acclimatisation

The exercising rats were acclimatised for 5 days before the beginning of the training protocol by starting with 5 min of swimming with no additional weight on the 1st day. Then, both the swimming duration and weight load were progressively increased until the desired duration and weight load were reached. In detail, on the 2nd day they swam for 10 min, on the 3rd day they swam for 10 min with 1% additional weight, on the 4th day they swam for 15 min with 1% additional weight, and on the 5th day they swam for 20 min with 2% additional weight.

Sampling Schedule

Blood, urine, and faeces samples were collected for metabolomic analyses. Urine samples for the analysis of the acute effects of exercise were collected one hour after the first bout of exercise from the 27 animals of groups A, B, and D. In addition, blood, urine, and faeces samples were collected from all animals every three months from the 3rd until the 21st month of life. Pre-training samples (at 3 months for groups A and B and at 12 months for group D) were obtained three to four days before the first acclimatization day. Samples obtained during or immediately after training were obtained on a Monday morning, that is, three days after the last training session, to eliminate any acute effects of exercise on the metabolome.

After the 21st month, sampling was conducted every 6 weeks. Upon completion of 22 months (for groups A and D; precisely, 17 days after the last bout of exercise of group D) or 24 months (for groups B and C, that is, 3 months after the last bout of exercise of group B), the rats that had not died naturally were euthanised by trained veterinarians, using the method of cervical dislocation, and samples of tissues, as well as blood, urine, and faeces, were collected for analysis. The difference in time of death between groups is due to the small number of animals that had remained in groups A and D. Fig. 3 summarizes the study design.

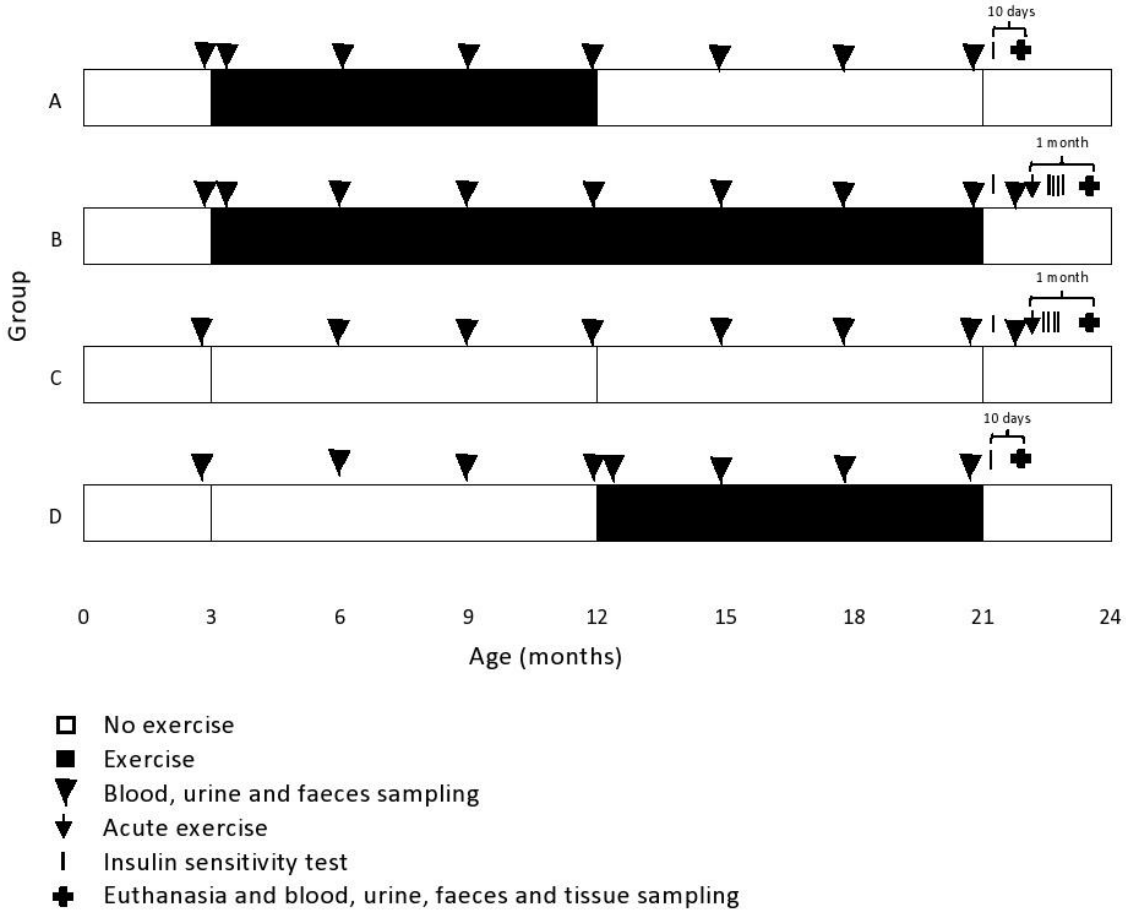


Fig. 3 Study design showing the interventions on the four groups of rats. Solid bars denote exercise training and open bars denote no exercise. Arrowheads denote sampling of blood, urine, and faeces. Arrows denote acute exercise, whereas short vertical lines denote insulin sensitivity tests. Lastly, the cross denotes euthanasia and sampling of blood, urine, faeces, and tissues.

Blood Sampling

Blood samples were collected by puncturing the tail vessels (Fig. 4). This method was chosen as it provided enough blood volume, it had quick recovery, and it was the least stressful for the animals. However, it caused haemolysis; therefore, we decided to immediately cause total haemolysis and precipitate all proteins by adding ice-cold methanol and collect blood lysates instead of plasma. Specifically, approximately 350 µl of blood obtained in this way were placed in an 1.5-ml Eppendorf tube and were immediately mixed with 1050 µl of ice-cold methanol (1:3). All tubes collected in a day were kept at 4 ºC until centrifugation at 10,000 *g* for 15 min at 4 ºC. Then, the supernatant lysate was collected and placed into two 500 µl Eppendorf tubes, which were stored at -80 ºC until analysis. The final collection of blood was made after euthanasia by cardiac puncture, and both blood lysates and serum were collected. Blood

lysates were collected using the aforementioned method, whereas for the collection of serum the rest of the blood was placed in a tube containing clot activator, left to clot and then centrifuged at 5,000 *g* for 10 min at 4 ºC. The supernatant was collected and placed in an 1.5 ml Eppendorf tube, which was then stored at -80 ºC.

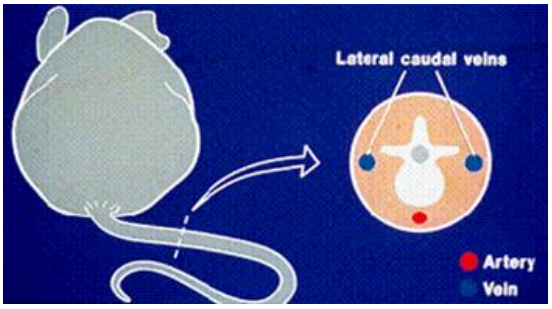


Fig. 4 Cross-section of a rat's tail. Accessed on 23/04/2018. http://www.hanbio.net/en/articles/item/590

Urine Sampling

To collect urine, a slight pressure was applied to the pubic area and the rat was left to urinate on a clean surface. Urine was collected with the use of a pipette and was immediately placed in a 0.5-ml Eppendorf tube. The procedure was repeated so as to get two samples, each of about 150-200 µl. All samples were stored at -80 ºC.

Faeces Sampling

To collect faecal samples, each rat was placed in an empty cage for several minutes, until he defaecated. Faeces were then collected and placed in an 1.5 ml Eppendorf tube. All samples were stored at -80 ºC.

Tissue Sampling

After euthanizing the rats, we collected one quadriceps at random, heart, brain, liver, lungs, left and right kidney, caecum and colon. All tissues and organs were washed in saline before being placed in plastic sachets and stored at -80 ºC. Fig. 5 shows the tissue collection procedure and all tissues and organs collected.

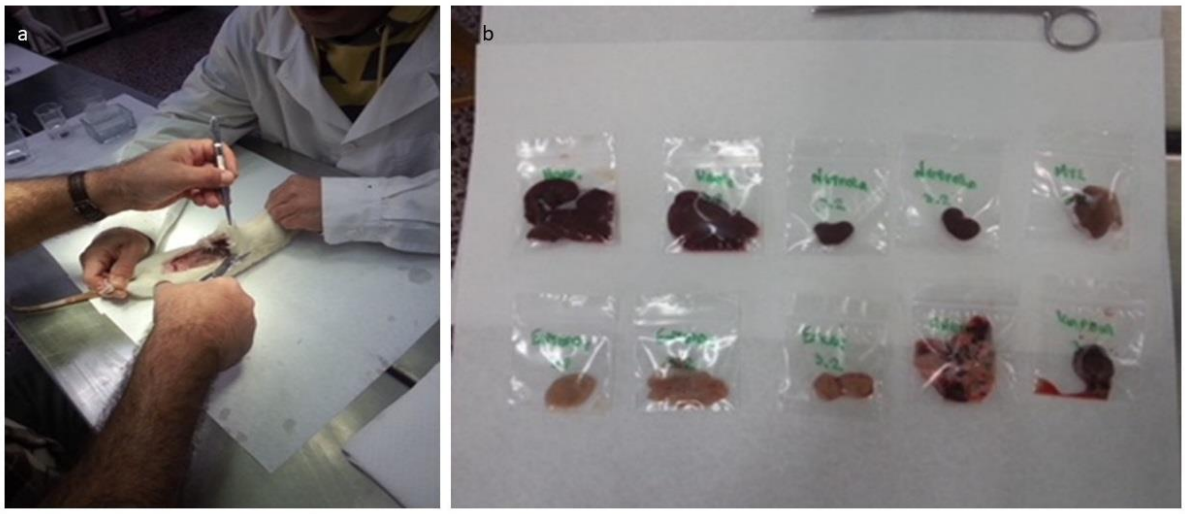


Fig. 5 Tissue and organ collection (a) and all tissues and organs collected and placed in plastic sachets (b).

Insulin Sensitivity Test

Twenty- six rats at the age of 21 months (6 from group A, 7 from group B, 8 from group C and 5 from group D) took part in five insulin sensitivity tests (IST). The first test, comparing all four groups, was performed one week after groups B and D had stopped exercising, in order to eliminate any acute effects of exercise on insulin sensitivity. Thus, this test aimed at examining the chronic effect of exercise on insulin sensitivity. One week later, the "extreme" and most numerous groups, that is, B (lifelong exercise) and C (no exercise), performed one bout of exercise, and insulin sensitivity was assessed through four IST performed 1, 3, 5 and 7 days post-exercise. These tests, in turn, aimed at examining the acute effect of exercise on insulin sensitivity. In the course of these seven days, two animals (one from each group) died, so this part of the study was completed with 13 animals.

The rats fasted overnight (for 10-12 hours) before each test. On the day of each IST, insulin (Humulin R) was diluted (1:20) with saline, and 1 U/kg body weight was injected into each animal intraperitoneally. Glucose was measured in blood taken from the tail with a portable analyzer (Bayer Contour XT) before injection and 15, 30, 60, 90, 120, 150, and 180 minutes after injection. The insulin dosage (Bae et al., 2014; Flores et al., 2006), the fasting time and timing of the measurements were selected after a thorough review of the literature referring to similar tests conducted on the same species (Castro et al., 2013; Flores et al., 2006; Motshakeri et al., 2013; Richardson et al., 2014). In order to evaluate insulin sensitivity, the area under the glucose concentration-*vs.*-time curve (AUC) was calculated using the trapezium rule. The higher the insulin sensitivity is, the lower the AUC.

Urine Metabolite Analysis by Liquid Chromatography – Mass Spectrometry

Liquid chromatography coupled with mass spectrometry (LC/MS) is an analytical technique that combines the separating power of high-performance liquid chromatography (HPLC) with the detection power of mass spectrometry. HPLC involves two main phases, the mobile and the stationary ones. The mobile phase consists of the solvent, which in our case was acetonitrile-water, and the stationary phase consists of a capillary column packed with very small particles. Mass spectrometry is an analytical technique in which chemical compounds are ionised to generate ions, whose mass-to-charge ratio is then measured. The ions are separated according to this ratio, their signal is channelled to a computer and is then processed into mass spectra. Fig. 6 shows a schematic representation of LC/MS, and fig. 7 shows an example of the resulting spectrum.

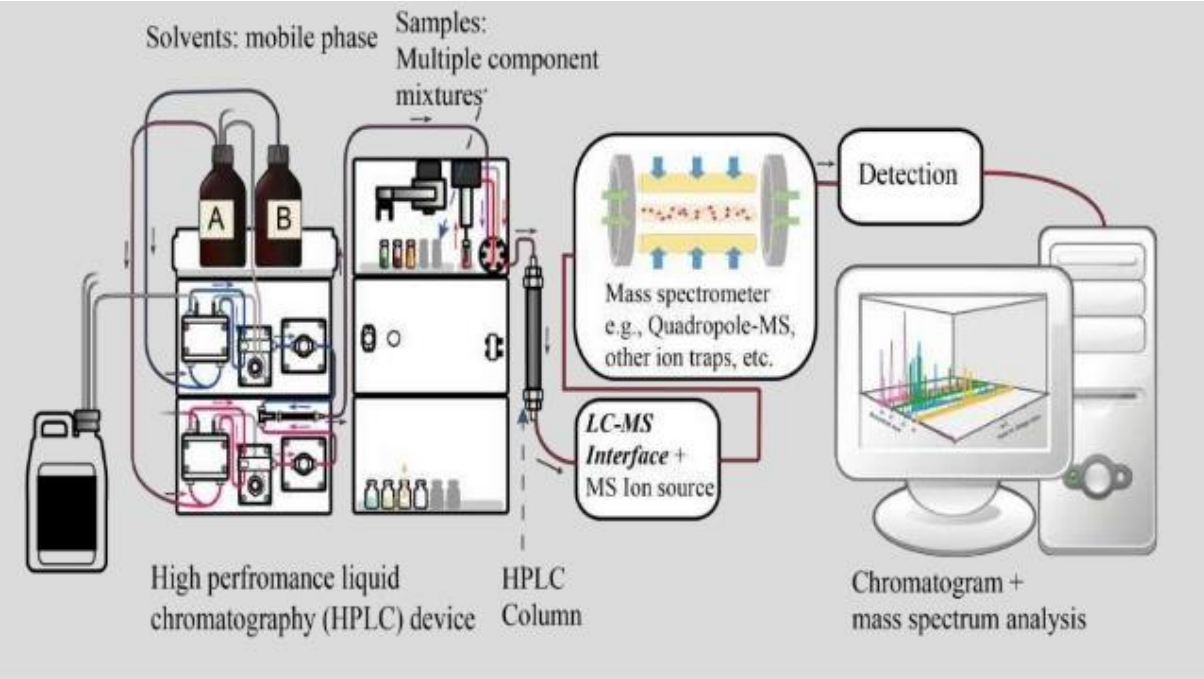


Fig. 6 Schematic representation of LC/MS. Choudary BM (2017). Accessed on 24/04/2018. https://www.slideshare.net/bhaveshchoudhary549/lc-ms-82987688

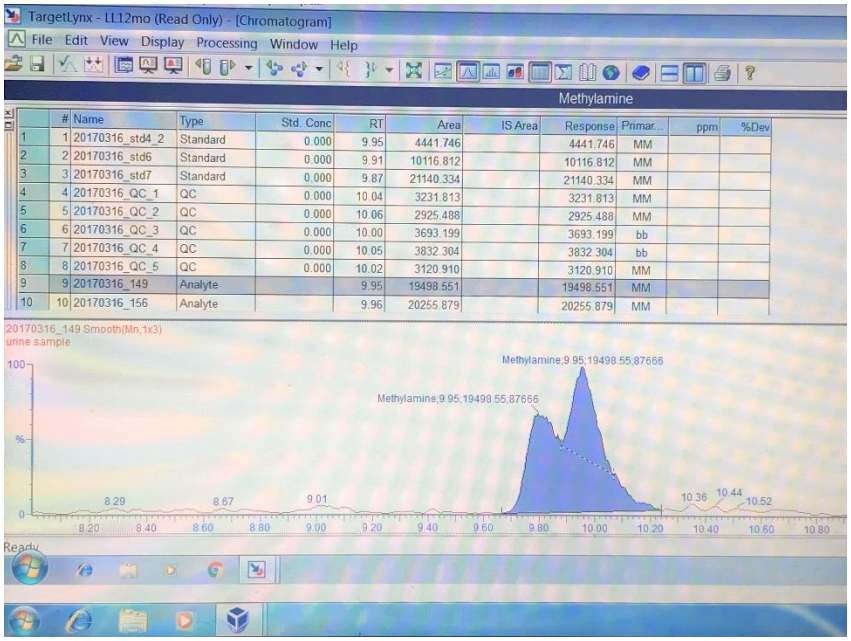


Fig. 7 Example of a mass spectrum.

141 samples of urine were analysed using targeted metabolomics by LC/MS in the laboratory of Toxicology at the School of Medicine of the Aristotle University of Thessaloniki. For the effects of acute exercise 40 samples were analysed: 11 pre- and post-exercise at 3 months of age, and 9 pre- and post-exercise at 12 months of age. The remaining 137 samples were as follows: 36 were at 3 months of age (9 from each group), 36 were at 12 months of age (9 from each group), and 29 were at 21 months of age (7 from group A, 9 from group B, 8 from group C and 5 from group D).

The LC/MS analysis of urine metabolites was based on the method described by Virgiliou et al. (2015). 20 μl of urine were placed in an 1.5-ml Eppendorf tube. Then 60 μl of ice-cold acetonitrile were added. A quality control (QC) sample was also prepared, consisting of 10 μl of all samples, which were then vortexed. 20 μl of the mixture were removed into a separate Eppendorf tube and 60 μl of acetonitrile were added to it. The QC sample was used to check the method's precision and accuracy. All tubes were vortexed for 5 min and then centrifuged at 18,000 *g* for 15 min at 6 ^{o}C. The supernatants were removed and filtered through 13-mm syringe filters before being transferred to LC/MS inserts, which were then placed inside 2-mL screw top tubes. These were closed with septum and screw cap and loaded in the autosampler tray that was maintained at 10 ^{o}C for the duration of the analysis. Working standards were prepared from a stock solution that had already been prepared in concentrations of 1000, 5000 or 10000 mg/L in a suitable solvent depending on the analyte.

Liquid chromatography was performed on an Acquity BEH Amide Column (2.1 mm x 150 mm, 1.7 μm) protected by an Acquity UPLC Van-Guard precolumn (Waters Ltd., Elstree, UK), using

an Acquity UPLC System (Waters Corporation, Millford, USA), as described (Virgiliou et al., 2015). Temperature was maintained at 40°C. Separation was performed under gradient elution using a binary mobile phase system consisting of solvent A [acetonitrile-water 95:5 (v/v)] and solvent B [water-acetonitrile 70:30 (v/v)]. Both solvents contained 10 mmol/l ammonium formate. Gradient elution started with a 4-min isocratic step at 100% A, then rising to 40% B linearly over the next 21 min and finally reaching 85% B linearly over the next 5 min. The column was equilibrated for 10 min in the initial conditions. Flow rate was 0.5 mL/min.

Mass spectrometry was carried out on Xevo TQD system (Waters Ltd., Esltree, UK), as described (Virgiliou et al., 2015). Electrospray ionization was operating at positive and negative modes (polarity switching mode) during a single analysis. Capillary voltage was set at +3.5 kV or –3.5 kV. Block and desolvation temperatures were 150 °C and 350 °C, respectively, desolvation gas flow rate was 650 L/h and cone gas was set at 50 L/h. Cone voltage and collision energy were optimized for each analyte in direct infusion mode. Multiple reaction monitoring (MRM) mode was applied for the detection and quantification of all compounds. In total, 101 transitions were monitored in specific time windows for total analysis of 40 min. Dwell time was set automatically from the software for most of the analytes, while longer dwell time was allocated to compounds with low signal-to-noise ratio. Data analysis for the integration and identification of the analytes was performed by Waters MassLynx version 4.1 (SCN 882) and TargetLynx (Waters Ltd., Elstree, UK). The normalization method of total ion count was used, in which the signal of each metabolite in a sample is divided by the sum of signals of the ions detected in the sample and, for practical purposes, is multiplied by 1000. Fig. 8 shows the equipment used for the LC/MS analysis.

Fig. 8 Liquid chromatographer and mass spectrometer.

Blood Lysate Analysis by ^{1}H Nuclear Magnetic Resonance Spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is a technique used to determine the structure of complex molecules. It provides a 'map' of the entire structure, including the carbons and hydrogens, of an organic compound. All nuclei with an odd number of protons (e.g., ^{1}H) and all nuclei with an odd number of neutrons (e.g., ^{13}C) exhibit magnetic properties. The nuclear spins of the nuclei are oriented, in the absence of an external magnetic field, in a random manner. When, however, a sample containing these nuclei is placed between the poles of a powerful magnet, as is done in NMR spectroscopy, the nuclei acquire specific orientations. A spinning nucleus can be either aligned with the external field (lower energy), or against it (higher energy). When the aligned nuclei are irradiated with appropriate frequency of electromagnetic radiation, energy absorption takes place and the lower energy state changes to the higher energy state (spin reversal). When this reversal occurs, it is said that the nuclei are in resonance. All nuclei, however, are shielded from the applied magnetic field by their electrons surrounding them. Thus, nuclei in different groups of a compound are in a somewhat different electronic environment and, as a result, the actual applied magnetic field is not the same. Depending on the sensitivity of the NMR instrument, these small differences can be detected, so we get a different signal for each chemically distinct nucleus of a molecule. Therefore, the ^{1}H or ^{13}C NMR spectrum of an organic compound efficiently maps the carbon-hydrogen bonding network. On the resulting one-dimensional (1D) spectra, the horizontal axis records the chemical shift of the specific nucleus (^{1}H or ^{13}C) in ppm, while the vertical axis shows the intensity of the energy absorbed. Fig. 9 shows a schematic representation of NMR spectroscopy, and fig. 10 shows an example of the resulting 1D spectra.

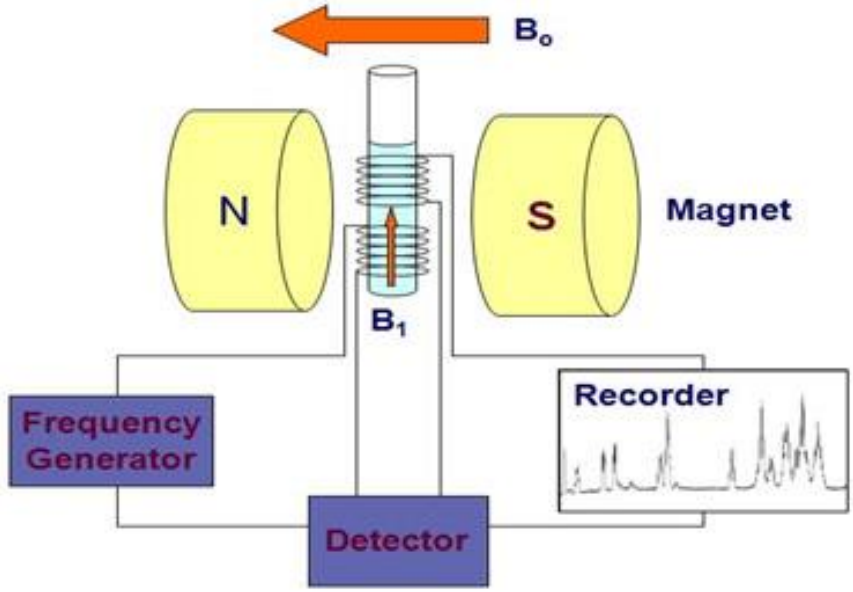


Fig. 9 Schematic representation of NMR spectroscopy. Agilent (2013). Accessed on 25/04/2018. https://www.agilent.com/labs/features/2013_101_nmr.html

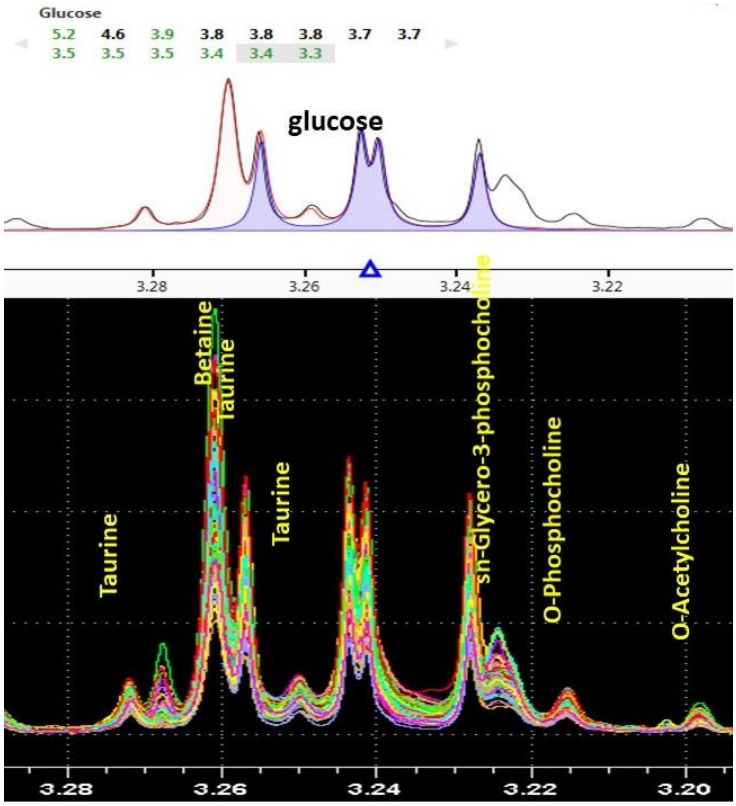


Fig. 10 Example of 1D spectra.

One hundred one samples of blood lysates were analysed by ^{1}H NMR spectroscopy at the Faculty of Pharmacy of the National and Kapodistrian University of Athens. These were 36 (9 from each group) at the age of 3 months, before starting exercise training, 36 (9 from each group) at the age of 12 months and 29 (7 from group A, 9 from group B, 8 from group C and 5 from group D) at the age of 21 months.

The ^{1}H NMR metabolomic analysis of blood lysates was based on the method described by Benaki & Mikros (2018). All samples were first lyophilised in a centrifugal vacuum concentrator at 30 ^{o}C (Eppendorf 5301, Hamburg, Germany) to remove any solvent. Then, a buffer to dissolve the lyophilisates was prepared. For 50 mL of buffer, 10.2 g KH_2PO_4, 50 mg of sodium 3-(trimethylsilyl)-(2,2,3,3-$^{2}H_4$)-propionate, and 6.5 mg NaN_3 were added in 40 mL D_2O and dissolved in an ultrasonic bath. The pH was adjusted to 7.4 with KOH pellets. D_2O was then added to a final volume of 50 ml, and the solution was stored at 4 °C. Then, 340 μl of the buffer were added in the-500 μl Eppendorf tube containing the sample and then vortexed for 1 min to ensure that the samples were brought to a stable pH. They were then placed in an ultrasonic bath for 5-7 min. A 200-μl pipette was used to remove all contents from the 0.5-ml Eppendorf tube into an 1.5-ml Eppendorf tube, and another 340 μl of D_2O-buffer were placed in the tube. Then, the Eppendorf tubes were placed in the ultrasonic bath for another 5-7 min and then centrifuged at 4,000 *g* for 10 min at 4 ^{o}C. The supernatant was finally placed

in a 5-mm-diametre NMR tube and closed with a cap. In order to identify any contamination, a sample of the solvent was analysed at the beginning and at the end of each day of analysis. The blood lysates were analysed in a Bruker Avance 600 MHz NMR spectrometer (Karlsruhe, Germany) equipped with a z-gradient PABBI probe and an automatic sample changer of 60 positions (B-ACS60, Bruker Biospin). The following actions were fully controlled by the Bruker IconNMR automation software (v. 5.0.7 Build 43 for TopSpin v. 3.5, Bruker BioSpin 2015): sample insertion in the magnet, temperature stability, optimization of field homogeneity, pulse calibration, data acquisition, as well as data processing, including axis calibration, phase, and baseline correction. Both 1D (Carr-Purcell-Meiboom-Gill sequence with solvent presaturation, Bruker library) and (for confirmatory purposes) pseudo two-dimensional (2D) J-res spectra were recorded for each sample. The J-resolved pseudo 2D spectra are of utmost importance, as they offer the possibility to resolve signals in overlapped regions of the ^{1}H 1D spectra. Fig. 11 shows the NMR spectrometer and the samples placed in the automatic sample changer, and fig. 12 shows an example of the resulting pseudo 2D J-res spectra.

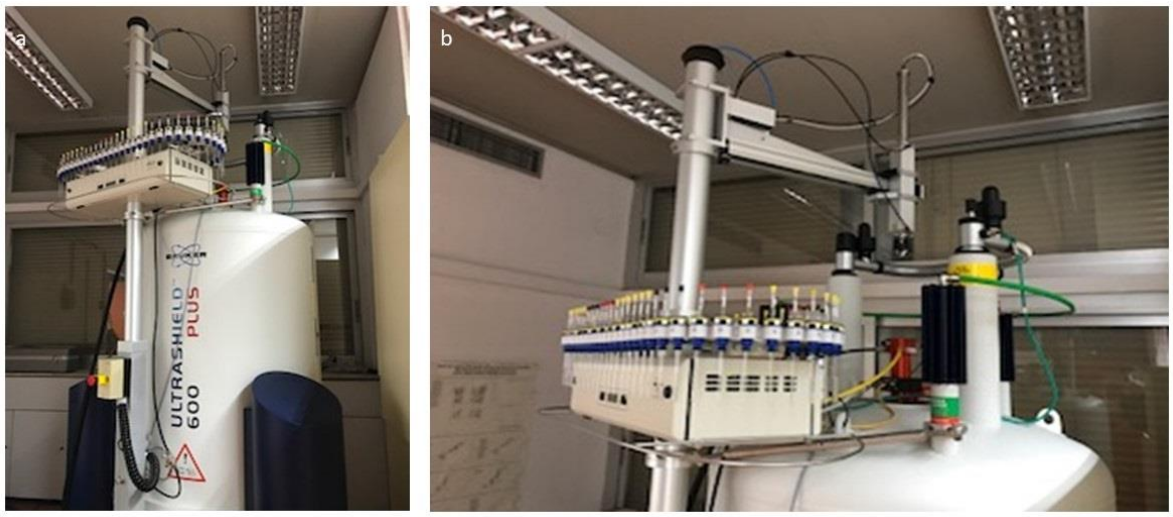


Fig. 11 NMR spectrometer (a) and samples placed in the automatic sample changer (b).

The 1D spectra were segmented in buckets of 0.02, 0.01 and 0.005 ppm using the AMIX software (Analysis of Mixtures; version 3.9.12, Bruker Analytische Messtechnik) and were integrated and normalized using the total spectrum integral (i.e., each bucket integral was divided by the sum of the bucket integrals and, for practical purposes, multiplied by 1000). The metabolites were identified with the aid of Chenomx profiler software (CHENOMX NMR Suite v. 8.1, Edmonton, Alberta, Canada) and Human Metabolome Database v. 4.0 (www.hmdb.ca; Wishart et al. 2018). The spectral regions corresponding to the residual water (5.16-4.68 ppm) and methanol (3.37–3.30 ppm), which was probably left from the precipitation of proteins and contained non-specific signals, were excluded from the analysis.

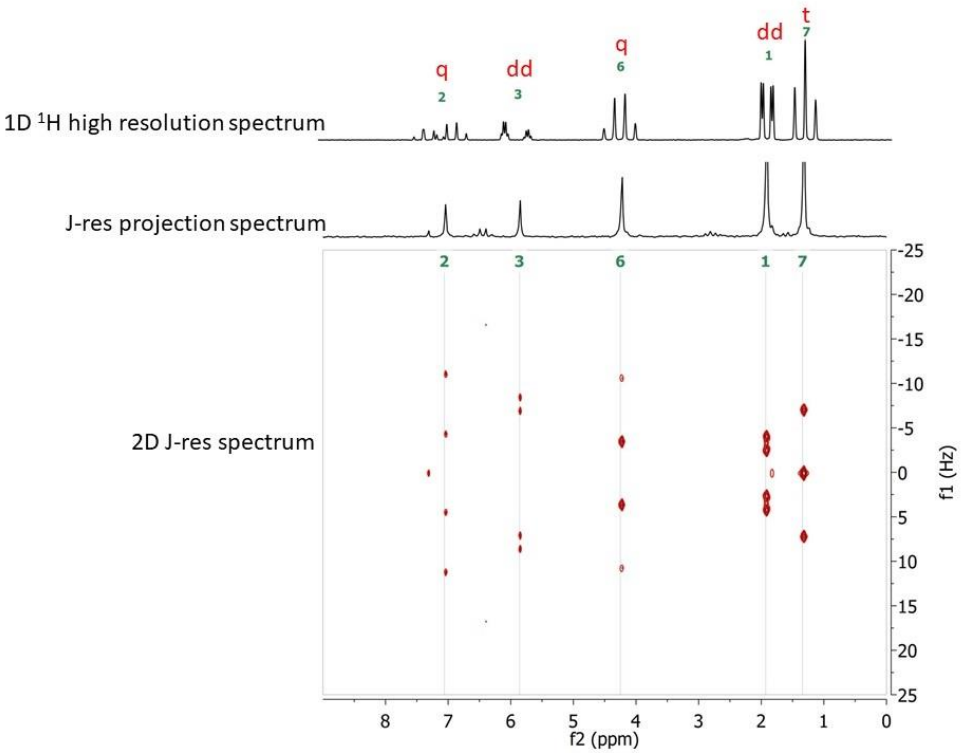


Fig. 12 Example of 2D J-res spectra.

Tissue Fatty Acid Analysis by Gas Chromatography

24 samples of quadriceps and 24 samples of liver (6 from each group), as well as 22 samples of brain (6 from groups A and C each and 5 from groups B and D each) were analysed. As mentioned under *Sampling,* groups A and D were euthanised on their 22nd month of age, whereas groups B and C were euthanised at 24 months of age. Hence, not all four groups were compared in the same statistical test; rather, group A was compared with group D, and group B was compared with group C. Each sample was analysed in triplicate. Analysis consisted of three parts: i) lipid extraction, ii) thin-layer chromatography and fatty acid methyl ester preparation, and iii) fatty acid methyl ester analysis by gas chromatography.

Lipid Extraction

The lipid extraction procedure was based on the method described by Mougios & Petridou (2012) for skeletal muscle. However, we noticed that the optimal amounts of tissue and lipid internal standards varied widely among tissues. Therefore, after getting some indicative values from the literature regarding the phospholipid (PL) and triacylglycerol (TG) content of each tissue, we conducted pilot experiments, which are described below, to establish the amounts that had to be used. Table 1 shows the particular figures for each tissue, which will be described below.

Table 1 Amounts referring to the lipid analysis of muscle, liver and brain.

		Muscle	Liver	Brain
Approximate lipid content (%)	PL	0.8	1.7	5
	TG	3.5	1.4	very low
Internal standard (µl)	PC	10	10	30
	TG	40	10	-
Tissue (mg)		60	30	30
Extract volume spotted on TLC plate (µl)		7	10	7
Hexane volume to dissolve FAME for GC (µl)	PC	80	80	80
	TG	240	80	-

FAME, fatty acid methyl ester; GC, gas chromatography; PC, phosphatidyl choline; PL, phospholipid; TG, triacylglycerol; TLC, thin-layer chromatography.

To begin with, all tissues were pulverized with mortar and pestle, and the powder was kept at -80 °C. Then, for the quadriceps and liver analysis, 10.5 mg of triheptadecanoyl glycerol (17:0 TG, Sigma, St. Louis, MO), as triacylglycerol internal standard, and 10.5 mg of diheptadecanoyl phosphatidyl choline (17:0 PC Larodan, Solna, Sweden), as phospholipid internal standard, were weighed in a screw top tube kept on ice, to which 1.5 ml of chloroform were added to produce a concentration of 7 mg/ml each. For the brain analysis, only dinonadecanoyl phosphatidyl choline (19:0 PC, Larodan) was used as internal standard due to the presence of very low TG, again at a concentration of 7 mg/ml in chloroform.

Using a glass microsyringe with pointed needle, an aliquot of each internal standard was dispensed at the bottom of a glass tube depending on the tissue, according to Table 1. After the solvent had evaporated, the tube was placed in an analytical balance and the appropriate amount of pulverized tissue was placed in the tube and weighed to the fourth decimal place of a gram. Then, 19 volumes (precisely, 19 µl per mg of tissue) of the lipid extraction solvent [chloroform–methanol 2:1 (v/v), 0.005 % (w/v) butylated hydroxytoluene (BHT) as antioxidant] were immediately added and vortexed briefly. After 5 min, 4 volumes of distilled water were added, and the mixture was vortexed vigorously for 1 min. Then, the test tube was spun for 2 min at 1500 *g,* room temperature, to produce two clear phases with the tissue debris at the interphase. Lipids were contained in the lower phase. With the use of a glass Pasteur pipette, the lower phase was aspirated and transferred to a 3-ml glass test tube and was left overnight to evaporate at room temperature.

Thin-Layer Chromatography and Fatty Acid Methyl Ester Preparation

Thin-layer chromatography (TLC) is a chromatographic technique used to separate non-volatile mixtures. After the sample is instilled onto a suitable material (such as silica), which has been laminated to a glass, plastic or metal plate, a solvent is raised to the plate by capillary movement. Because different analytes climb the plate at different rates, separation is achieved.

TG were separated from PL in the tissue lipid extracts by 1D thin-layer chromatography based on the method described by Mougios & Petridou (2012). The dried lipid extract of each sample was dissolved in 30 μl of lipid extraction solvent and was then vortexed and rotated in order to retrieve all the extract from the wall of the test tube. An aliquot of each lipid extract, as shown in Table 1, was slowly spotted, with the use of a glass microsyringe with blunt needle and a spotting guide (Fig. 13), on a high-performance silica gel plate (Macherey-Nagel, Düren, Germany), cut to dimensions of 10 cm x 20 cm, 1.5 cm apart from each other and from the sides.

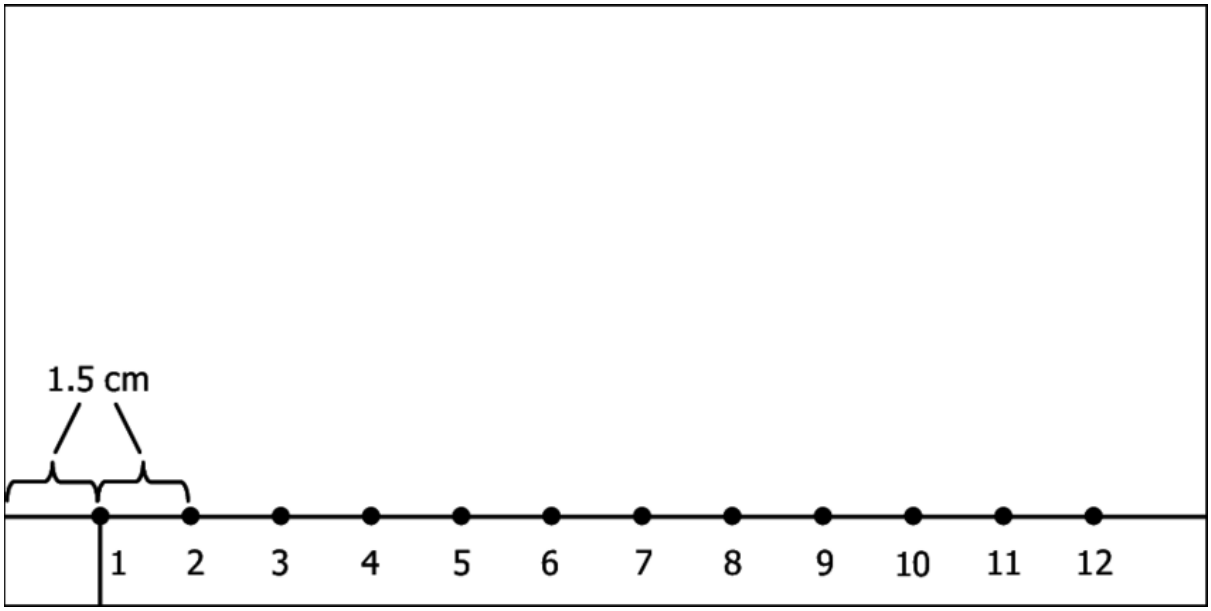


Fig. 13 Spotting guide for TLC. From Mougios & Petridou (2012) with permission.

On the first analysis of each tissue, 2 μl of the TG standard solution was spotted on a separate position in the middle of the plate to help us identify the TG spots in the samples after chromatography. PL remained at the origin (under the chromatographic conditions described below), thus they were easily located. By the end of spotting a yellow-brownish spot was formed on the plate. Once spotting was over, the plate was developed in a mixture of petroleum ether - diethyl ether - acetic acid, 80:20:1 (v/v/v) for approximately 25 min until the solvent front reached 1 cm from the top of the plate (Fig. 4a). After drying for about 15 min, the plate was sprayed with a solution of 0.2% (w/v) dichlorofluorescein (Sigma) in ethanol until

it acquired a faint orange colour without getting overly wet. Once more, the plate was left to dry completely for 15 min. Lipid spots were visualised under brief exposure to ultraviolet light. A typical TLC plate with the separated PL and TG spots is shown in Fig. 14b. The contour of the PL and TG spots was marked with pencil and the spots were scraped and transferred to screw-cap tubes (Fig. 14c). Fatty acid methyl esters (FAME) were produced by the addition of 1 ml of methanol - sulfuric acid 96:4 (v/v), vortexing and heating at 64 ^{o}C overnight. The originally colourless liquid turned yellow, as methanol extracted the dichlorofluorescein from the silica gel.

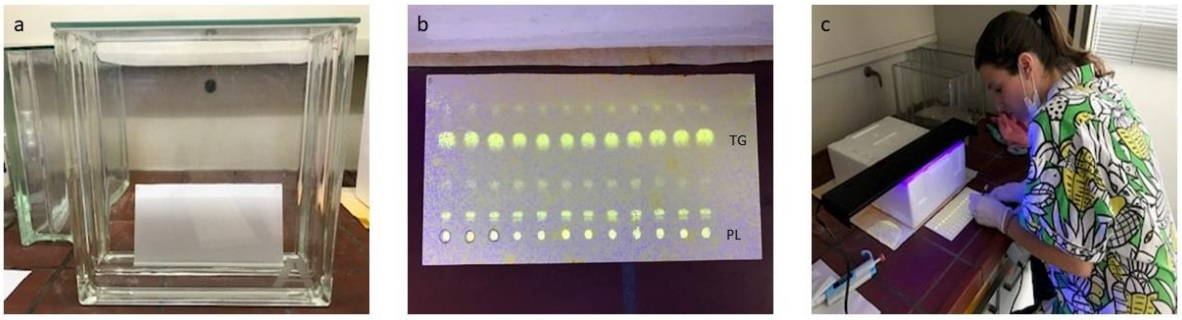


Fig. 14 TLC tank with plate (a), lipid spots visualised under ultraviolet light (b), and scraping of PL and TG spots (c).

Fatty Acid Methyl Ester Analysis by Gas Chromatography

Gas chromatography (GC) is the method that separates the components of a mixture based on their different distribution between a gaseous and a liquid phase. The liquid, or stationary, phase consists of a thick sticky substance, which is uniformly spread over the interior wall of a capillary column (a column with very small thickness). The gaseous, or mobile, phase comes from a gas tank, passes through a trap that withholds unwanted impurities and then enters the column. It is also called carrier gas and is usually helium. Due to the different chemical structure of the components of a sample, there is a different distribution between the stationary and mobile phase. Thus, the components move at different speeds in the column and exit at different times. The time from insertion of a substance to its exit from the column is called the retention time (RT). RT depends on the flow of the carrier gas and the column temperature program. A precondition for the use of GC is that substances are volatile or can be converted into volatile derivatives. In our case, acyl groups in TG and PL are converted into methyl esters.

The FAME produced in the previous section were extracted from the methylating medium by the addition of 1 ml of hexane and vigorous mixing for 1 min. After spinning the test tubes briefly in a centrifuge, the upper phase, which contained the FAME, was removed using a glass Pasteur pipette into a small glass test tube and was then evaporated under a stream of

nitrogen at room temperature. The dry residue in each test tube was dissolved in half the volume of hexane indicated in Table 1, then vortexed and transferred into a microvolume insert. The same procedure was repeated in order to have a more efficient transfer of the FAME. Once the insert was prepared, it was placed inside a 2-mL screw top tube, which was closed with septum and screw cap and placed in the autosampler, which was programmed to inject 3 µl at a time. The FAME were separated in an Agilent 7890 gas chromatograph (Santa Clara, CA), equipped with a 30 m-long AT-WAX capillary column (Alltech, Deerfield, IL) and flame ionization detector (FID) (Fig. 15a). In this column, RT of FAME increases with number of carbon atoms and double bonds. The column temperature was programmed from 140 to 270ºC at 40 ºC min^{-1} and the run was held at 270 ºC for 3.75 min. The total run time was 7 min and the carrier gas was helium at a flow rate of 1.6 ml min^{-1}.

Before analysing the FAME, a blank run was performed, in order to ensure that the column was clean, and then a hexane run was performed, to ensure that both the syringe and the hexane used were clean. Once all checks were completed, analysis of the samples commenced. While exiting the column, each FAME was burnt in the FID and the generated electrical signal was channelled to the computer. There, it was converted into a curve of signal intensity with respect to RT. This curve is called a chromatogram (Fig. 15b). In the chromatogram each FAME was depicted as a peak. The FAME were identified with the aid of the Agilent ChemStation software, based on the RT we had established from a mixture of 37 FAME (Supelco 37 Compontent FAME Mix, Sigma-Aldrich). The assumption we used to quantify FAME and, by extension, the lipids from which they were derived was that the mass of a FAME is proportional to the area under its peak. Therefore, we compared the area under their peaks to that of the internal standard, that is, methyl heptadecanoate, in the case of the quadriceps and liver, or methyl nonadecanoate, in the case of the brain.

Fig. 15 Gas chromatograph (a), and chromatogram (b).

To convert the peak integration data into tissue content values (i.e., μmol/g) a series of calculations were conducted. According to the aforementioned premise, the mass of a FAME equals the mass of the standard's FAME × area of FAME/area of the standard's FAME. Both areas are known from the chromatogram. To calculate the mass of the standard's FAME (in μg), we multiplied the volume of standard added in each sample (in μl) by its concentration (in μg/μl) by its purity, which, according to the manufacturer's label, was 0.99 in all cases. Then the result of this multiplication was divided by the standard´s M_r, multiplied by the number of acyl groups in each lipid class (3 for TG, 2 for PL) and, finally, multiplied by the M_r of the standard´s FAME. This produced the mass of the standard's FAME, which was entered in the expression stated above to produce the mass of a FAME in a sample (in μg). To express it in μmol, we divided it by its M_r. Finally, to get the desired tissue content in μmol/g, we divided the previous value by the amount of tissue (in g) that was used in the extraction process. In addition, we calculated the percentage distribution of fatty acids in each lipid class by dividing their amounts by the total amount of FAME and then multiplied by 100. All calculations were performed on an Excel worksheet.

As the fatty acid composition of tissue lipids is affected by the fatty acid composition of the animals' diet, we also determined the latter by following the aforementioned procedure except for internal standard addition and TLC. That is, we extracted the rodent chow's lipids and then produced FAME, which we analysed with GC. We then calculated the percentage distribution of fatty acids in the food. Fig. 16 shows a schematic representation of the GC analysis.

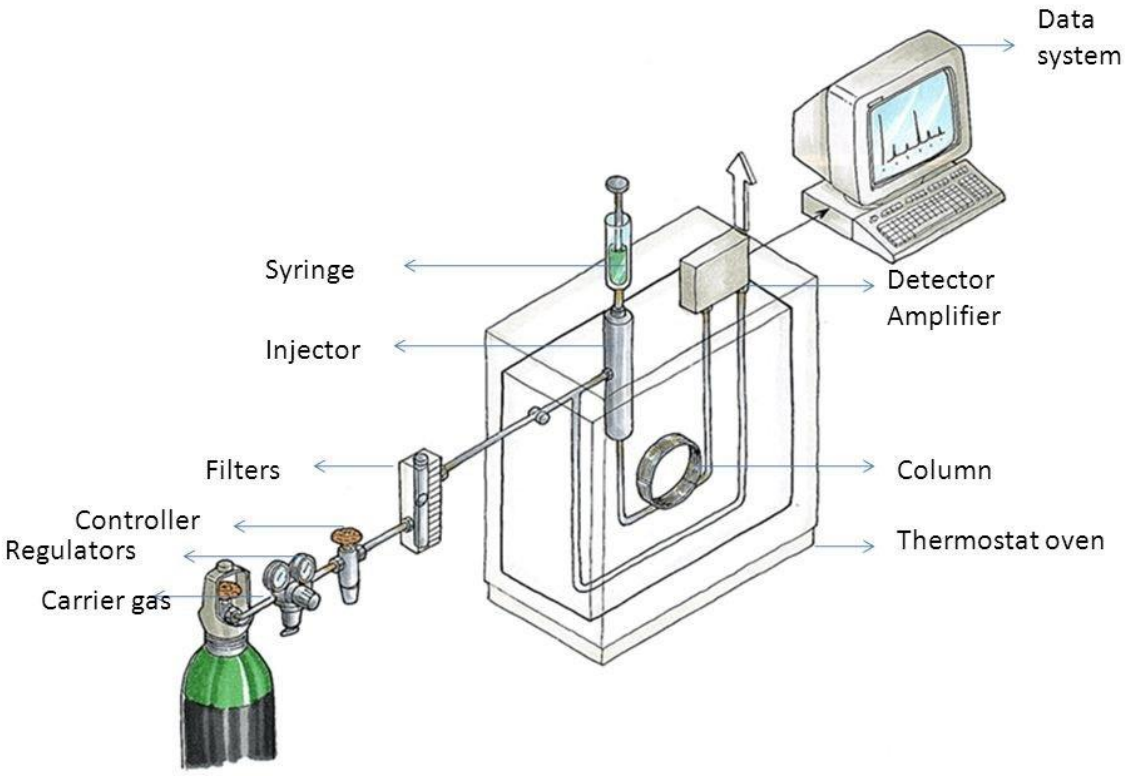


Fig. 16 Schematic representation of gas chromatography. Accessed on 23/04/2018. http://slideplayer.com/slide/10457195/

Pilot Experiments on Tissue Fatty Acid Analysis

As mentioned above, we conducted pilot experiments to establish the amounts of tissue and lipid internal standards for the fatty acid analysis. In each pilot experiment, 4 samples, one from each group were analysed. The amount of tissue initially used was 30 mg, while the amount of TG and PL internal standards was calculated depending on the lipid content of the tissue, according to the literature. For optimal peak integration in the gas chromatogram, we wanted the area of the FAME emanating from the standard to be around ⅓ to ½ the area of the most abundant endogenous FAME. If we didn't meet this criterion, we adjusted the amount of standard and repeated the analysis.

Another parameter that we had to adjust was the amount of lipid extract to be spotted on the TLC plate. We started empirically with about 10 µl and, after developing the plate, we checked whether there was a good separation between PL and TG (Fig. 17). If the separation was not good, depending on the look of the plate, we changed either the composition of the developer or the amount of sample spotted on the plate until we got a satisfactory result.

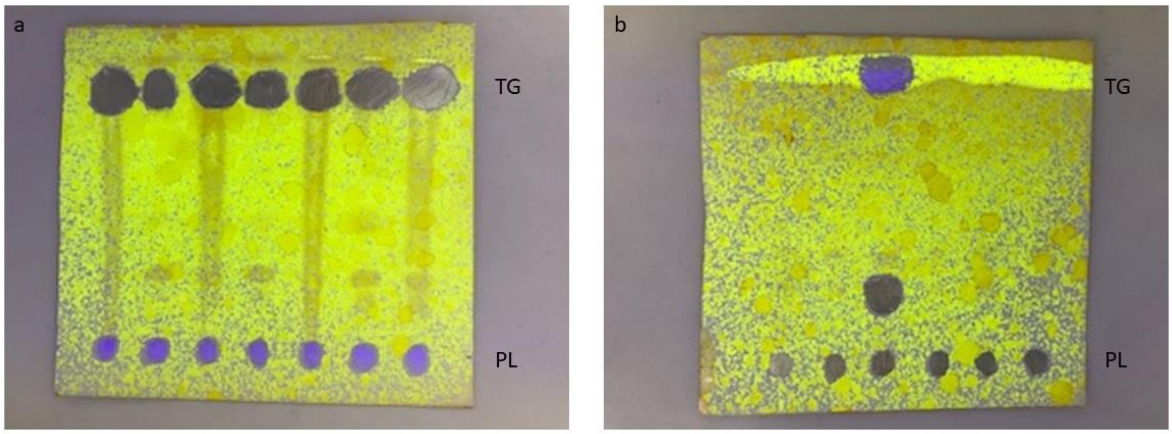


Fig. 17 (a) A not-so-good separation of PL and TG on a TLC plate, as there is an obvious smear connecting them. To avoid this, we spotted a smaller amount of sample. (b) Merging of TG spots from adjacent lanes due to movement with the solvent. To avoid this, we changed the composition of the developer.

An additional parameter that was examined in the pilot experiments was the amount of hexane the dried FAME would be dissolved in before being injected in the gas chromatograph. This was determined by the appearance of the chromatogram. We wanted clear, sharp peaks, not broad peaks with considerable noise.

After having concluded with the aforementioned parameters, we checked the repeatability of the analysis. This is where we experimented with the number of sample replicates and the amount of tissue to be used. As a criterion we used the coefficient of variation (CV) between replicate analyses, that is, the ratio of the standard deviation to the mean, multiplied by 100. We accepted any CV that was lower than 10%. Any value larger than this was re-examined, and the experimental procedure was changed. Muscle seemed to be a heterogeneous tissue, and this is why we doubled its amount for each repetition, as shown in Table 1. Apart from that, all tissues were analysed in triplicate to obtain an acceptable CV value.

Statistical Analysis

Multivariate analysis of the data from the metabolomic analysis of blood and urine samples with ^{1}H NMR spectroscopy and LC/MS, as well from the fatty acid analysis of tissues with GC, was performed through principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA) and orthogonal projections to latent structures (OPLS) by use of the SIMCA-P software (version 11.5, Umetrics, Malmö, Sweden). The PCA, a non-supervised method, was initially used to explore the existence of possible clustering and detect putative outliers. In the PLS-DA and OPLS, which are supervised methods, the user assigns the samples to predefined groups, which creates the risk of either biased clustering or overfitting.

In all cases, two scaling methods were applied that use the standard deviation (SD) as an entity to measure the data spread: a) the unit variance scaling (UV), where SD is the scaling factor, and b) Pareto scaling (Par), where the square root of SD is used as the scaling factor.

The aforementioned statistical methods are used to examine the effect of an intervention on a large number of variables (in our case, metabolites and fatty acids) simultaneously, rather than on a one-to-one basis. They consist in a linear transformation of the data that reduces the variables and, instead of having a space of a number of dimensions as many as the variables, it selects a new space of fewer dimensions in which the projection of the dispersion of the data is presented in a declining order from one dimension to the next. The outcomes of these analyses were scores plots showing whether there was a discrimination between groups. Moreover, for every discrimination noticed in the Par model, a loadings plot was produced. In addition, a variable importance for the projection (VIP) plot and list were brought out to check for the correlation of specific variables with the discrimination between groups. Values larger than 1.5, produced by the VIP plot and list, indicate “important” variables, whereas values lower than 1.5 indicate “unimportant” variables. Lastly, validity of every PLS-DA model was checked. The validation check compares the goodness of fit (R2, descriptive ability, and Q2, predictive capacity of the generated model) of the original model with the quality of fit of several models (depending on the number of permutations set). In our case, the validation check was conducted with 100 permutations, and the desired outcome was for the original, unpermuted, model to exhibit the higher R2 and Q2 (all left values corresponding to the random models to be lower than the original points to the right and the blue regression line of the Q2 points intersecting the vertical axis on the left, at or below zero).

As a complement to the multivariate analysis described above, univariate analysis on the sum of all peaks of each identified metabolite (except for glucose, whose only the anomeric peak at 5.23 ppm was used, being the clearest one, with no overlap with other metabolites) was performed using analysis of variance (ANOVA) on SPSS (version 25, IBM Statistics, Armonk, NY) and GraphPad Prism (version 6, GraphPad Software, San Diego, CA). Only data that passed the Shapiro-Wilk test for normality of distribution were analysed ($p > 0.05$). Data were also checked for sphericity with Mauchly’s test. When sphericity was not met the correction of Huynh-Feldt was used. Any outliers were treated according to Field (2009), who explains that an outlier can be adjusted by adding or subtracting the standard deviation times two or three to the mean. The previous operation was conducted only when the outliers were less than 5% of the total data; in any other case the data were considered not normal and did not enter any further statistical analysis. In detail, the chronic effect of exercise on insulin sensitivity was examined by comparing the area under the glucose-vs-time curve (AUC) in the first IST between groups by one-way ANOVA. The acute effect of exercise on insulin

sensitivity was examined through repeated-measures one-way ANOVA on the AUC in the five IST, that is, the one performed before acute exercise (on the animals that took part in the subsequent IST only) and the ones performed 1, 3, 5 and 7 days after exercise. Significant effects were followed up by Fisher's LSD test in order to identify significant pairwise differences. The acute effect of exercise on urine metabolites was examined by two-way ANOVA (age × time) with repeated measures on time, followed by simple main effect analysis for pairwise comparisons. The life-long effect of exercise on body weight, urine metabolites and blood metabolites was examined by three-way ANOVA, that is, age (with repeated measures) × training or not during the 1st half of life (referred to as "1st half" for brevity hereafter) × training or not during the 2nd half of life ("2nd half"). Significant interactions were followed up by simple main effect analysis for pairwise comparisons. Furthermore, the concentration and percentage of each fatty acid in each tissue was compared between groups with independent-sample Student's *t* test. Also, the following indices were calculated and compared in the same way: saturated fatty acids (SFA), unsaturated fatty acids (UFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), ω6 fatty acids, ω3 fatty acids, ω6/ω3 and UFA/SFA. To control for type I error, significant main effects and interactions were corrected with a 5% adjusted false detection rate (FDR) according to Benjamini & Hochberg (1995). Correlations between AUC and weight on the 21st month, as well as between ambient temperature and food intake, were examined through Pearson's correlation coefficient. Heat maps were created with MetaboAnalyst (www.metaboanalyst.ca; Chong et al. 2018) based on Pearson's hierarchical clustering, for the visualization of the changing patterns of metabolite profiles. Results are presented as the mean ± SD or median (interquartile range). The level of significance was set at $\alpha = 0.05$. Effect sizes (ES), as an index of meaningfulness, for main effects and interactions were estimated by calculating partial η^2 and were classified as small (0.01 to 0.058), medium (0.059 to 0.137) or large (0.138 or higher), according to Cohen (1988). Cohen's *d* was used to determine effect sizes in the *t test* analyses by calculating the difference of means divided by the pooled standard deviation [$\sqrt{((SD_1^2 + SD_2^2)/2)}$], and were classified as small (0.2), medium (0.5) or large (0.8, Cohen 1988).

RESULTS

Body Weight and Food Intake

A main effect of age ($p < 0.001$, ES = 0.91) was found on body weight (Fig. 18), reflecting the fact that the weight of the animals generally increased until the age of 17 months and either stabilized or declined thereafter. In addition, there was a main effect of 2nd half ($p = 0.008$, ES = 0.27), which showed that groups B and D (which trained during the 2nd half) weighed less than groups A and C (which did not train during the same period).

Repeated measures two way ANOVA (age x intervention) between groups B and C, which continued the experimental procedures until the 24th month, showed a main effect of group ($p < 0.021$, ES = 0.47), which showed that the lifelong exercising group (group B) weighed less than the lifelong non-exercising group (group C).

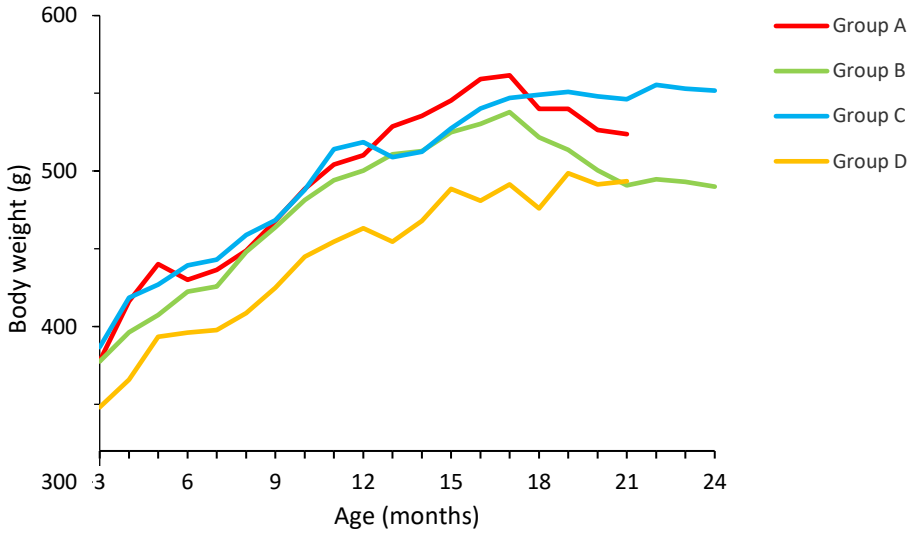


Fig. 18 Mean body weight of the four groups during the study.

A main effect of age ($p < 0.001$, ES = 0.79) was also found on food intake (Fig. 19), reflecting the fact that, during the warmer months (June to November), the rats consumed less food than they did during the cooler months (December to May). In fact, there was a negative correlation between ambient temperature and average food intake on a monthly basis ($r = –0.79$, $p < 0.001$). In addition, there was an interaction of age and 1st half ($p < 0.001$, ES = 0.22) and an interaction of age and 2nd half ($p = 0.001$, ES = 0.17). The former can be explained by the fact that, during the 1st half, groups A and B (which trained) started from lower intake and ended with higher intake than groups C and D (which did not train), indicative of an orexigenic effect of exercise. The latter can be explained by the fact that, during the 2nd half, groups B and D

(which trained) had lower food intake than groups A and C (indicative of an anorexigenic effect of exercise), with the differences being more pronounced during the warmer months.

However, repeated measured two-way ANOVA (age x intervention), from 21 to 24 months between groups B and C showed no differences in food intake between the two groups.

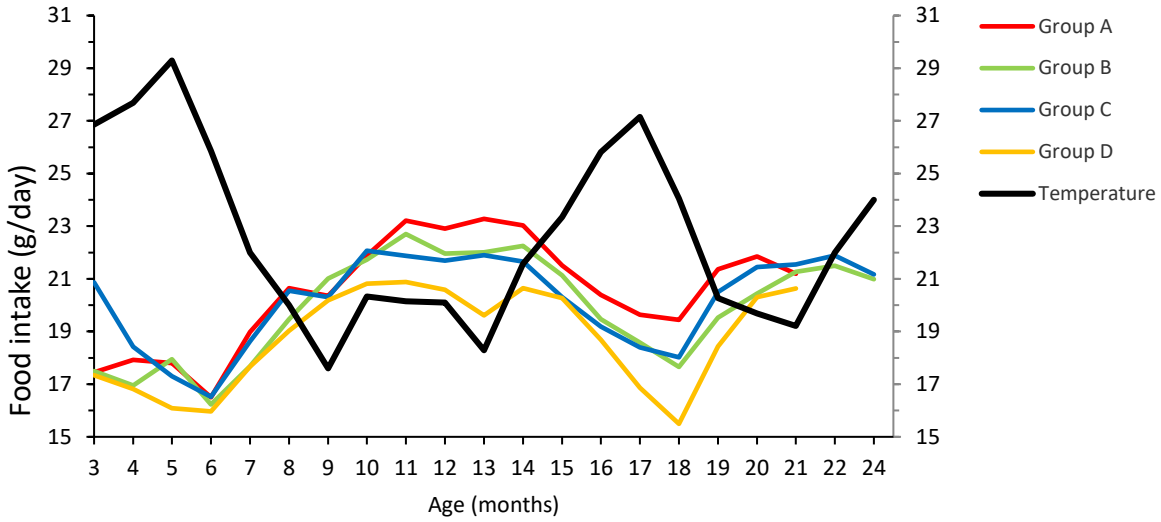


Fig. 19 Mean food intake by the four groups and ambient temperature during the study. Weekly data of food intake have been averaged on a monthly basis to facilitate comparison with the body weight data in Fig. 18.

Insulin Sensitivity Test

ANOVA showed that the IST performed one week after the last exercise session of groups B and D did not produce significant differences among the four groups, suggesting either no prolonged effect of exercise on insulin sensitivity or no effect of exercise at all (Table 2). There was no significant relationship between AUC and body weight (r = 0.25, P = 0.11).

Table 2 Area under the glucose concentration-vs.-time curve (AUC) in the first insulin sensitivity test and body weight of the groups on their 21st month of age. Values are mean ± SD.

Group	A (n = 6)	B (n = 7)	C (n = 8)	D (n = 5)
AUC (mmol min/L)	543 ± 176	502 ± 49	540 ± 93	454 ± 48
Weight (g)	524 ± 37	491 ± 36	546 ± 26	493 ± 41

In order to distinguish between the aforementioned possibilities, that is, whether there was no prolonged effect of exercise or no effect of exercise at all, we subjected groups B and C to one bout of exercise and repeated the IST 1, 3, 5, and 7 days post-exercise. ANOVA, which was conducted without discriminating the two groups (since they showed no differences in the previous procedure), showed a significant, large effect of time (p < 0.001, ES = 0.98). Specifically, the AUC at 1 and 3 days post-exercise was significantly lower than pre-exercise (p < 0.001 and p = 0.005, respectively, Fig. 20). Moreover, the AUC at 5 and 7 days was significantly higher than that at 1 day after exercise (p = 0.043 and 0.011, respectively). Finally, the AUC at 7 days was significantly higher than that at 3 days (p = 0.025). These findings suggest that the beneficial effect of exercise on insulin sensitivity lasted at least 3 days but not 5 days or more.

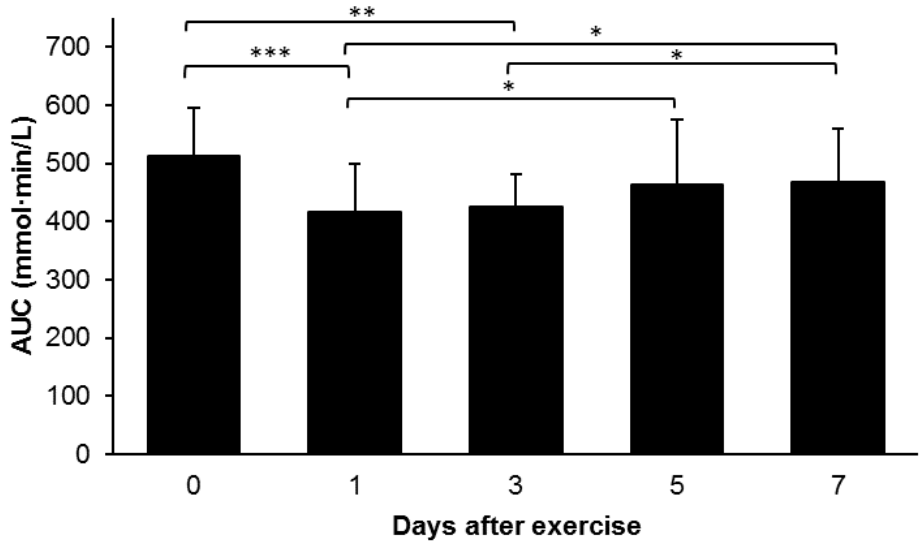


Fig. 20 Area under the glucose concentration-vs.-time curve before and 1, 3, 5, and 7 days after an acute bout of exercise. ***p < 0.001, **p < 0.01, *p < 0.05.

Effects of Acute Exercise on the Urinary Metabolome

Ninety metabolites can be identified by the LC/MS method used; however, 51 were found in the urine samples collected, with six of those presented in pairs (norvaline-valine, isoleucine-leucine and arabinose-xylose), since they could not be discriminated (Table 3).

Table 3 Metabolites detected in rat urine by LC/MS.

No.	Metabolite	Molecular formula	Retention time
1	Methylamine	CH_5N	10.14
2	Dimethylamine	C_2H_7N	8.20
3	Trimethylamine-*N*-oxide (TMAO)	C_3H_9NO	13.32

4	Putrescine	$C_4H_{12}N_2$	21.00
5	Sarcosine	$C_3H_7NO_2$	15.30
6	Alanine	$C_3H_7NO_2$	16.38
7	Cadaverine	$C_5H_{14}N_2$	20.79
8	Choline	$C_5H_{14}NO$	7.25
9	γ-Aminobutyric acid (GABA)	$C_4H_9NO_2$	17.19
10	Serine	$C_3H_7NO_3$	18.32
11	Hypotaurine	$C_2H_7NO_2S$	16.02
12	Cytosine	$C_4H_5N_3O$	7.80
13	Histamine	$C_5H_9N_3$	13.70
14	Creatinine	$C_4H_7N_3O$	4.80
15	Proline	$C_5H_9NO_2$	14.75
16	Betaine	$C_5H_{11}NO_2$	12.56
17	Norvaline-valine	$C_5H_{11}NO_2$	14.40
18	Threonine	$C_4H_9NO_3$	17.10
19	Taurine	$C_2H_7NO_3S$	14.73
20	Pyroglutamic acid	$C_5H_7NO_3$	15.16
21	Isoleucine-leucine	$C_6H_{13}NO_2$	13.70
22	Creatine	$C_4H_9N_3O_2$	16.30
23	Adenine	$C_5H_5N_5$	3.90
24	Glutamine	$C_5H_{10}N_2O_3$	18.12
25	Methionine	$C_5H_{11}NO_2S$	14.66
26	Guanine	$C_5H_5N_5O$	10.40
27	Tryptamine	$C_{10}H_{12}N_2$	5.46
28	Phenylalanine	$C_9H_{11}NO_2$	12.93
29	Cotinine	$C_{10}H_{12}N_2O$	1.13
30	Tyrosine	$C_9H_{11}NO_3$	14.86
31	Mannitol	$C_6H_{14}O_6$	14.47
32	Kynurenic acid	$C_{10}H_7NO_3$	10.12
33	Acetylcarnitine	$C_9H_{17}NO_4$	14.66
34	Tryptophan	$C_{11}H_{12}N_2O_2$	13.02
35	Pantothenic acid	$C_9H_{17}NO_5$	12.82
36	Thymidine	$C_{10}H_{14}N_2O_5$	2.26
37	Cytidine	$C_9H_{13}N_3O_5$	11.29
38	Biotin	$C_{10}H_{16}N_2O_3S$	8.49
39	Thiamine	$C_{12}H_{17}N_4OS$	12.02
40	Adenosine	$C_{10}H_{13}N_5O_4$	4.72
41	Inosine	$C_{10}H_{12}N_4O_5$	9.48
42	Riboflavin	$C_{17}H_{20}N_4O_6$	9.80
43	Pyruvic acid	$C_3H_4O_3$	7.47
44	α-ketoglutaric acid	$C_5H_6O_5$	16.21
45	Arabinose-xylose	$C_5H_{10}O_5$	8.72
46	Glucose	$C_6H_{12}O_6$	14.77
47	4-Hydroxyphenyllactic acid	$C_9H_{10}O_4$	10.15

48	Uridine	$C_9H_{12}N_2O_6$	4.93

Multivariate Analyses

PLS-DA UV (Fig. 21) showed a clear discrimination due to age and acute exercise. The permutations plot confirms the validation of the PLS-DA model.

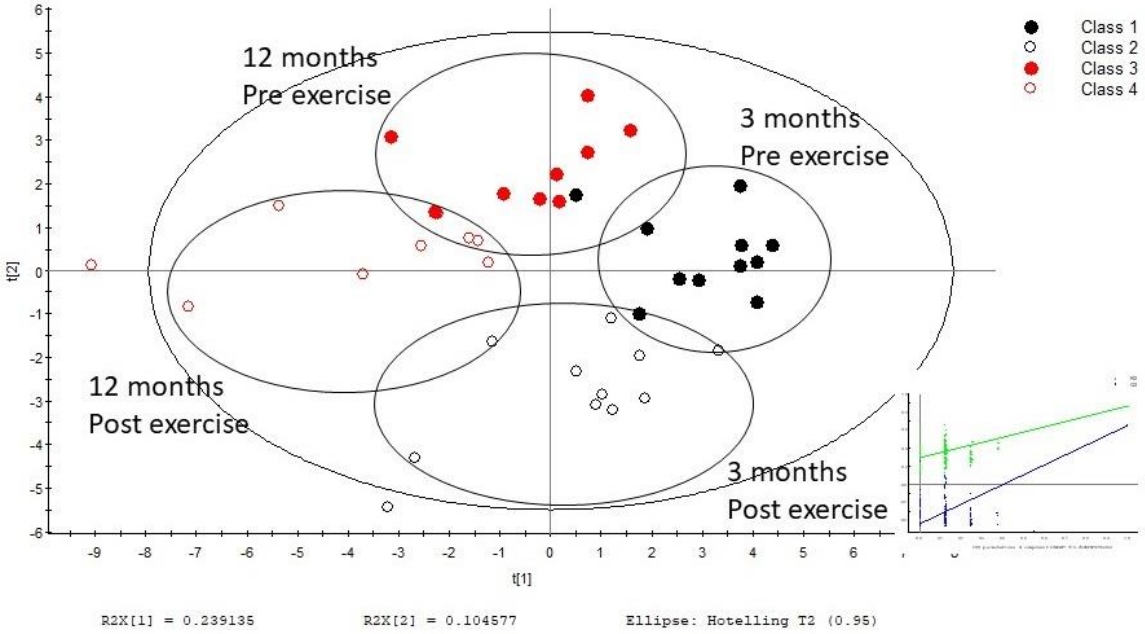


Fig. 21 PLS-DA UV scores plot with permutations plot as insert. Ellipse in a represents 95% confidence intervals of Hotelling's T^2 distribution.

In order to get a detailed insight of the age and exercise differences, the samples were investigated with OPLS-DA Par, and results are presented in Figs. 22a and 23a. According to the S-plots (Fig. 22b and 23b), discrimination between pre- and post-exercise samples was due to (in order of descending VIP value, shown in parentheses) thiamine (4.06), acetylcarnitine (2.89), creatinine (2.46), TMAO (1.86), isoleucine-leucine (1.72) and choline (1.58). Discrimination between 3 and 12 months was due to creatinine (3.99), thiamine (3.14), TMAO (2.47), histamine (2.15), putrescine (1.54) and isoleucine-leucine (1.46). Specifically, as shown in the graphical representations in Fig. 24, exercise resulted in an increase in acetylcarnitine at both ages, in creatinine at 3 months and in isoleucine-leucine at 12 months, whereas it resulted in decreases in thiamine and TMAO at 3 months and in choline at both ages. Aging, on the other hand, resulted in increases in creatinine, histamine and isoleucine-leucine (the latter only post-exercise), whereas it resulted in decreases in thiamine (only pre-exercise), TMAO and putrescine.

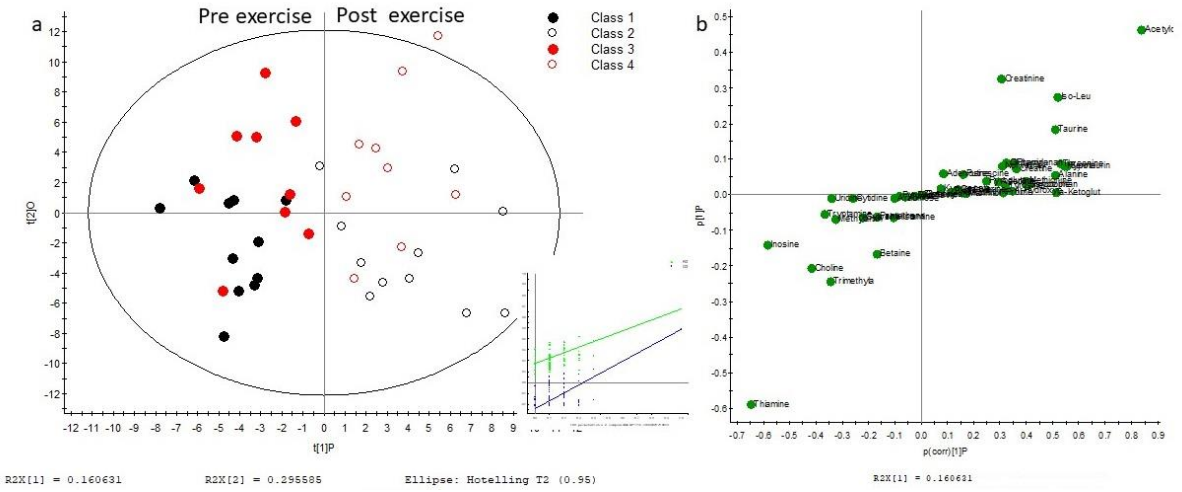


Fig. 22 (a) OPLS-DA scores plot pre- and post-exercise at both 3 (black) and 12 (red) months, with permutations plot as insert, (b) loadings plot of metabolites affecting the discrimination between pre- and post-exercise samples.

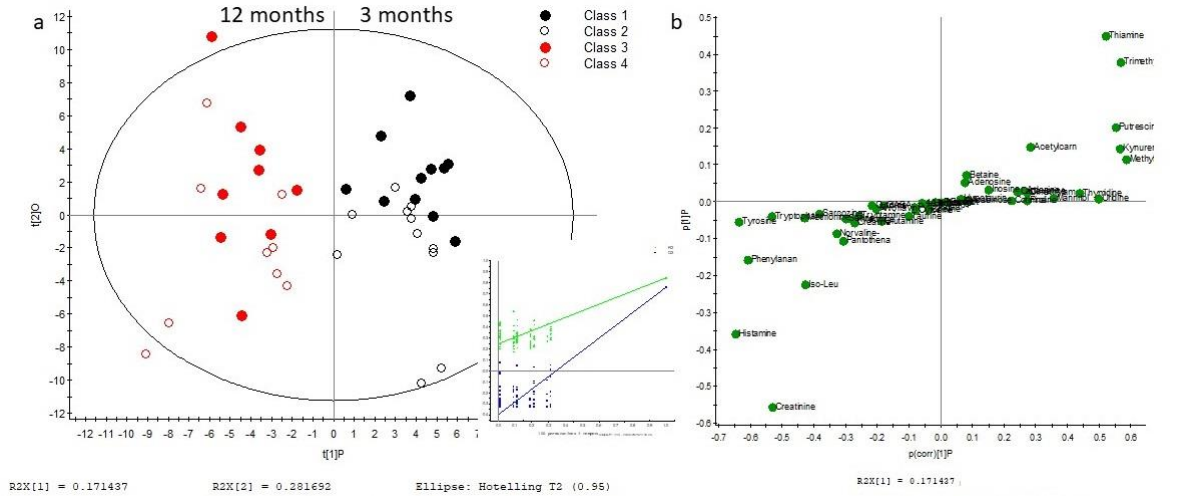


Fig. 23 (a) OPLS-DA scores plot between 3 and 12 months both pre- (dots) and post-exercise (circles), with permutations plot as insert, (b) loadings plot of metabolites affecting the discrimination between 3 and 12 months.

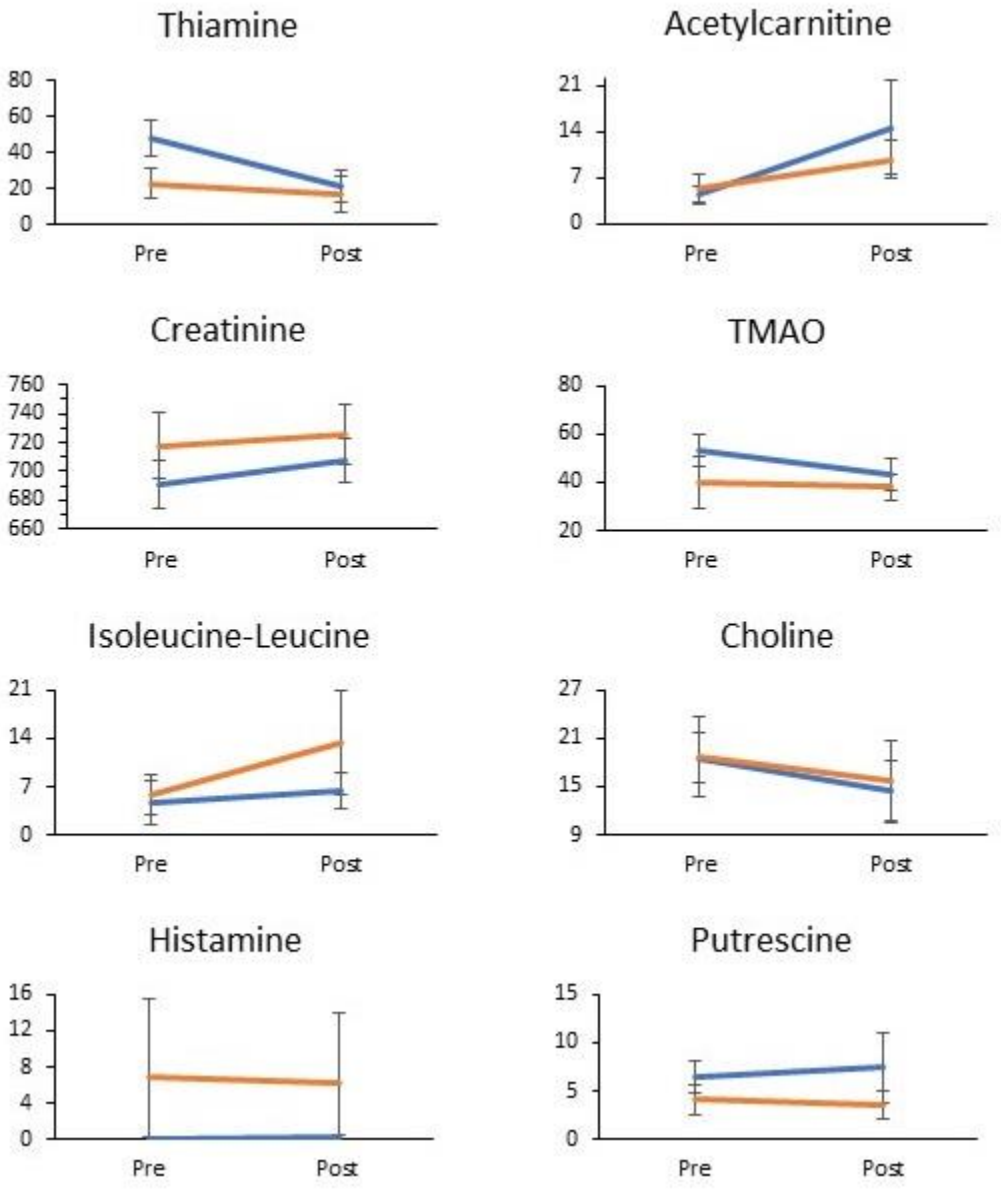


Fig. 24 Graphical representations of urinary concentration of metabolites contributing to the discrimination between 3 (blue) and 12 months of age (brown) pre- and post-exercise. The vertical axis shows peak area in arbitrary units. Results are presented as mean ± SD.

Figures 25 and 26 show metabolite changes with exercise and age, respectively, in the form of heat maps according to Pareto scaling and Pearson's hierarchical clustering. Metabolites are sorted by similarity of changes; however, in order to check for similarities between changes, samples were not reorganised. It can be seen that the younger rats had a better pre- and post-exercise separation, whereas the older rats created clusters of 1, 2 and 4 samples (Fig. 25). Moreover, the two ages had a better separation pre-exercise compared to post-exercise (Fig. 26).

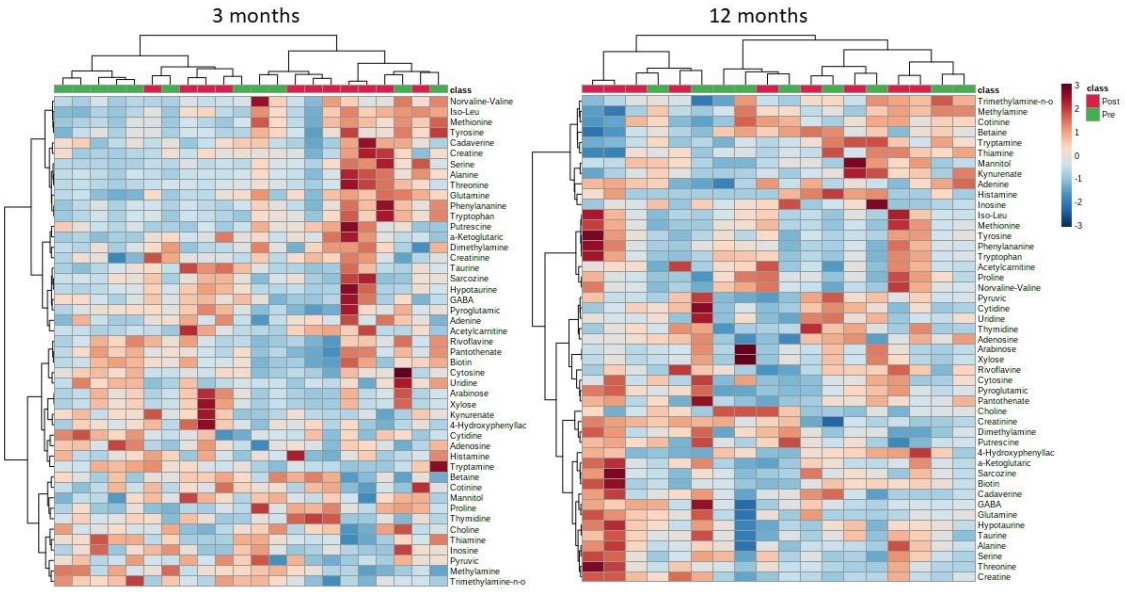


Fig. 25 Heat maps of changes in urinary metabolite peak areas pre- and post-exercise. Shades of red and blue show increase and decrease, respectively. Data are scaled by Pareto scaling, and motifs are ranked and sorted by Pearson's hierarchical clustering.

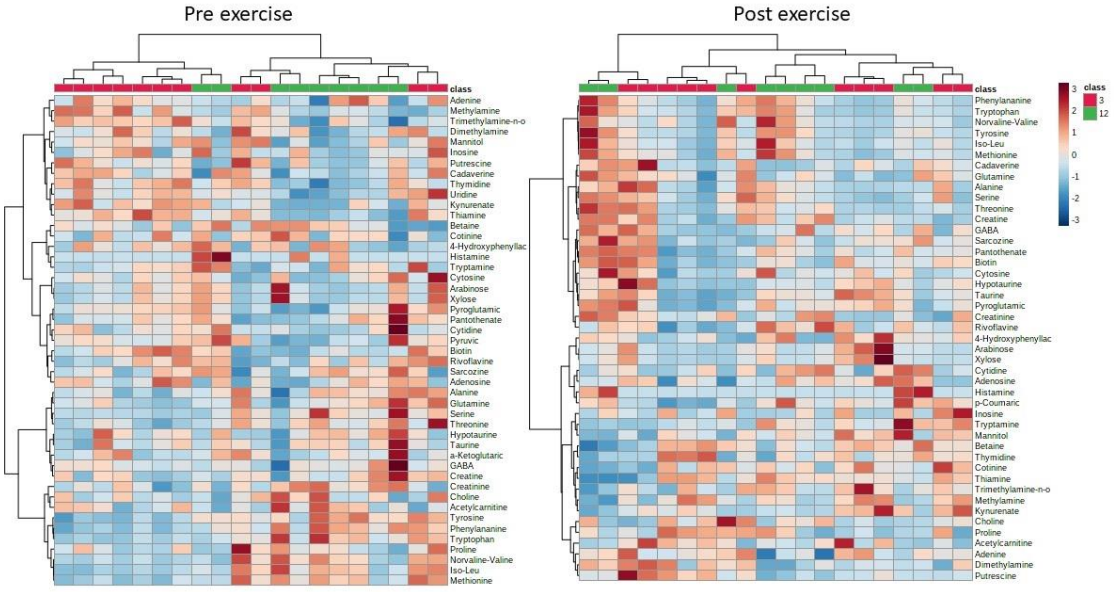


Fig. 26 Heat maps of changes in urinary metabolite peak areas between 3 and 12 months. Shades of red and blue show increase and decrease, respectively. Data are scaled by Pareto scaling, and motifs are ranked and sorted by Pearson's hierarchical clustering.

Univariate Analyses

The results of two-way ANOVA (age x exercise) are presented below and the corresponding descriptive statistics in Table 4.

Table 4 Peak areas of urinary metabolites at 3 and 12 months of age pre- and post-exercise. Values are mean ± SD.

	3 months (*n* = 11)		12 months (*n* = 9)	
Metabolite	Pre	Post	Pre	Post
Methylamine	2.67 ± 0.94	1.98 ± 0.57	1.68 ± 0.34	1.28 ± 0.42
Dimethylamine	1.88 ± 0.17	1.87 ± 0.24	1.71 ± 0.18	1.73 ± 0.19
TMAO	52.86 ± 6.64	43.25 ± 6.55	40.03 ± 10.50	38.14 ± 5.30
Putrescine	6.40 ± 1.69	7.42 ± 3.62	4.11 ± 1.60	3.59 ± 1.49
Sarcosine	0.11 ± 0.05	0.19 ± 0.12	0.17 ± 0.05	0.26 ± 0.18
Alanine	0.48 ± 0.12	0.66 ± 0.35	0.49 ± 0.12	0.71 ± 0.26
Cadaverine	0.74 ± 0.20	0.73 ± 0.37	0.47 ± 0.30	0.59 ± 0.29
Choline	18.55 ± 3.16	14.42 ± 3.72	18.70 ± 5.04	15.67 ± 5.07
GABA	0.17 ± 0.02	0.18 ± 0.04	0.19 ± 0.09	0.20 ± 0.05
Serine	0.24 ± 0.17	0.61 ± 0.49	0.41 ± 0.35	0.82 ± 0.37
Hypotaurine	0.33 ± 0.16	0.85 ± 0.68	0.44 ± 0.21	0.57 ± 0.26
Cytosine	4.12 ± 1.76	3.20 ± 0.91	4.21 ± 1.59	3.71 ± 1.43
Histamine	0.21 ± 0.09	0.22 ± 0.15	6.89 ± 8.63	6.33 ± 7.62
Creatinine	691.16 ± 16.35	707.59 ± 14.84	717.87 ± 22.48	725.73 ± 20.44
Proline	0.02 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.02 ± 0.01
Betaine	101.93 ± 14.45	109.96 ± 14.87	113.77 ± 15.31	95.18 ± 23.54
Norvaline-valine	2.03 ± 1.19	1.72 ± 0.62	2.22 ± 1.28	3.54 ± 1.57
Threonine	0.58 ± 0.24	0.92 ± 0.63	0.64 ± 0.16	1.28 ± 0.66
Taurine	4.96 ± 1.49	7.35 ± 3.31	5.83 ± 2.30	7.17 ± 2.00
Pyroglutamic acid	1.65 ± 0.27	1.77 ± 0.61	1.54 ± 0.59	1.91 ± 0.58
Isoleucine-leucine	4.75 ± 3.23	6.39 ± 2.69	5.82 ± 2.85	13.45 ± 7.51
Creatine	2.87 ± 0.39	3.14 ± 0.97	2.97 ± 0.64	3.80 ± 1.04
Adenine	0.83 ± 0.21	1.10 ± 0.21	0.68 ± 0.37	0.86 ± 0.29
Glutamine	4.72 ± 1.52	5.64 ± 1.26	4.95 ± 1.65	6.08 ± 1.35
Methionine	0.26 ± 0.16	0.25 ± 0.12	0.29 ± 0.11	0.54 ± 0.24
Tryptamine	0.53 ± 0.40	0.36 ± 0.26	0.73 ± 0.42	0.52 ± 0.41
Phenylalanine	1.34 ± 0.67	1.58 ± 1.09	2.23 ± 0.75	3.46 ± 1.57
Cotinine	0.03 ± 0.01	0.04 ± 0.02	0.03 ± 0.01	0.03 ± 0.01
Tyrosine	0.13 ± 0.07	0.12 ± 0.06	0.20 ± 0.06	0.35 ± 0.17
Mannitol	0.05 ± 0.01	0.05 ± 0.02	0.03 ± 0.02	0.04 ± 0.02
Kynurenic acid	3.33 ± 0.79	3.93 ± 1.57	1.95 ± 0.95	2.01 ± 0.93
Acetylcarnitine	4.42 ± 1.42	14.66 ± 7.13	5.35 ± 2.18	9.73 ± 2.83
Tryptophan	0.08 ± 0.06	0.11 ± 0.07	0.13 ± 0.08	0.24 ± 0.13
Pantothenic acid	5.53 ± 1.67	4.28 ± 2.28	6.73 ± 2.82	5.56 ± 2.18
Thymidine	0.17 ± 0.04	0.22 ± 0.08	0.10 ± 0.05	0.15 ± 0.07
Cytidine	0.04 ± 0.01	0.03 ± 0.01	0.05 ± 0.04	0.05 ± 0.02
Biotin	0.26 ± 0.11	0.23 ± 0.15	0.18 ± 0.07	0.24 ± 0.15
Thiamine	47.69 ± 9.84	21.30 ± 8.65	22.78 ± 8.50	16.69 ± 10.07
Adenosine	22.60 ± 14.09	21.65 ± 7.55	17.80 ± 14.01	23.27 ± 7.76
Inosine	2.00 ± 1.19	0.80 ± 0.78	1.51 ± 1.09	0.63 ± 0.52
Riboflavin	0.32 ± 0.15	0.27 ± 0.13	0.33 ± 0.10	0.39 ± 0.15
Pyruvate	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.01	0.02 ± 0.01
α-ketoglutaric acid	0.01 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.01
Arabinose-xylose	0.16 ± 0.09	0.16 ± 0.12	0.19 ± 0.12	0.11 ± 0.03

4-Hydroxyphenyllactic acid	0.03 ± 0.01	0.04 ± 0.03	0.03 ± 0.02	0.04 ± 0.02
Uridine	0.04 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.02 ± 0.01

i) Amino Acids and Amino Acid Derivatives

An interaction of age and exercise was found in tyrosine (p = 0.009, ES = 0.33) and betaine (p = 0.009, ES = 0.32). Specifically, exercise resulted in an increase in tyrosine in aged rats and a decrease in betaine in aged rats.

A main effect of exercise was noticed in tryptophan (p = 0.003, ES = 0.39), proline (p = 0.007, ES = 0.34), sarcosine (p = 0.008, ES = 0.33), glutamine (p = 0.012, ES = 0.30), 4-hydroxyphenyllactate (p = 0.017, ES = 0.28), phenylalanine (p = 0.017, 0.28) and choline (p = 0.017, ES = 0.28). Specifically, all amino acids and amino acid derivatives increased post-exercise, except for choline which decreased.

A main effect of age was found in tyrosine (p < 0.001, ES = 0.53), phenylalanine (p = 0.002, ES = 0.42) and tryptophan (p = 0.011, ES = 0.31). Specifically, all three amino acids increased from 3 to 12 months.

ii) Carbohydrate and Lipid Metabolism

A main effect of exercise was seen in acetylcarnitine (p < 0.001, ES = 0.63), due to an increase with exercise. Moreover, a main effect of age was found in mannitol (p = 0.018, ES = 0.27), due to a decrease from 3 to 12 months.

iii) Purine and Pyrimidine Metabolism

A main effect of exercise was found in adenine (p = 0.003, ES = 0.40), thymidine (p = 0.007, ES = 0.34) and uridine (p = 0.018, ES = 0.28). Specifically, adenine and thymidine increased post-exercise, whereas uridine decreased.

A main effect of age was noticed in uridine (p = 0.001, ES = 0.49) and thymidine (p = 0.010, ES = 0.32). Specifically, from 3 to 12 months both metabolites decreased.

iv) Gut Microbiome Metabolism

A main effect of age was found in TMAO (p = 0.001, ES = 0.47). Specifically, TMAO decreased from 3 to 12 months.

v) Other Metabolites

An interaction of age and exercise was found in thiamine (p = 0.011, ES = 0.31). Specifically, exercise resulted in a decrease in thiamine in young rats.

A main effect of exercise was noticed in taurine (p = 0.022, ES = 0.26) and methylamine (p < 0.001, ES = 0.56). Specifically, taurine increased post-exercise, whereas methylamine decreased.

A main effect of age was found in creatinine (p = 0.003, ES = 0.39), methylamine (p = 0.004, ES = 0.37) and thiamine (p < 0.001, ES = 0.72). Specifically, creatinine increased from 3 to 12 months, whereas methylamine and thiamine decreased.

Effects of Long-Term and Lifelong Exercise on the Urinary Metabolome

Multivariate Analyses

Multivariate analyses showed no discrimination between groups at any of the three ages of urine sampling. In contrast, PLS-DA UV (Fig. 27) showed a clear discrimination between young, middle-aged and old rats. The permutations plot confirms the validity of the PLS-DA model. In order to gain a detailed insight into the age differences, the three groups were investigated in pairs (3 vs. 12, 3 vs. 21 and 12 vs. 21 months) with OPLS-DA, and results are presented in Fig. 28. According to the S-plots (Fig. 28b, d, f), discrimination between 3 and 12 months was due to betaine (2.58), choline (2.46), TMAO (2.44) and thiamine (1.81); discrimination between 3 and 21 months was due to betaine (4.00), isoleucine-leucine (2.15), putrescine (1.89) and histamine (1.75); and discrimination between 12 and 21 months was due to TMAO (3.09), betaine (2.48), choline (2.17), cytosine (1.89) and acetylcarnitine (1.45). Specifically, as shown in Fig. 29, from 3 to 12 months, betaine and TMAO increased, whereas choline and thiamine decreased; from 3 to 21 months, betaine and histamine increased, whereas isoleucine-leucine and putrescine decreased; and from 12 to 21 months, betaine and choline increased, whereas TMAO, cytosine and acetylcarnitine decreased.

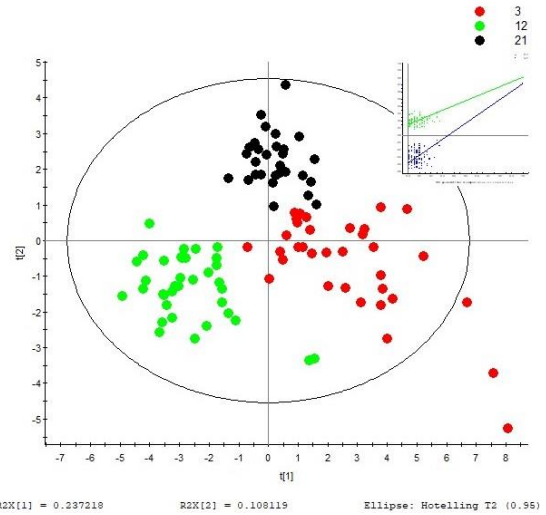


Fig. 27 PLS-DA UV scores plot with permutations plot. Ellipse represents 95% confidence intervals of Hotelling's T^2 distribution.

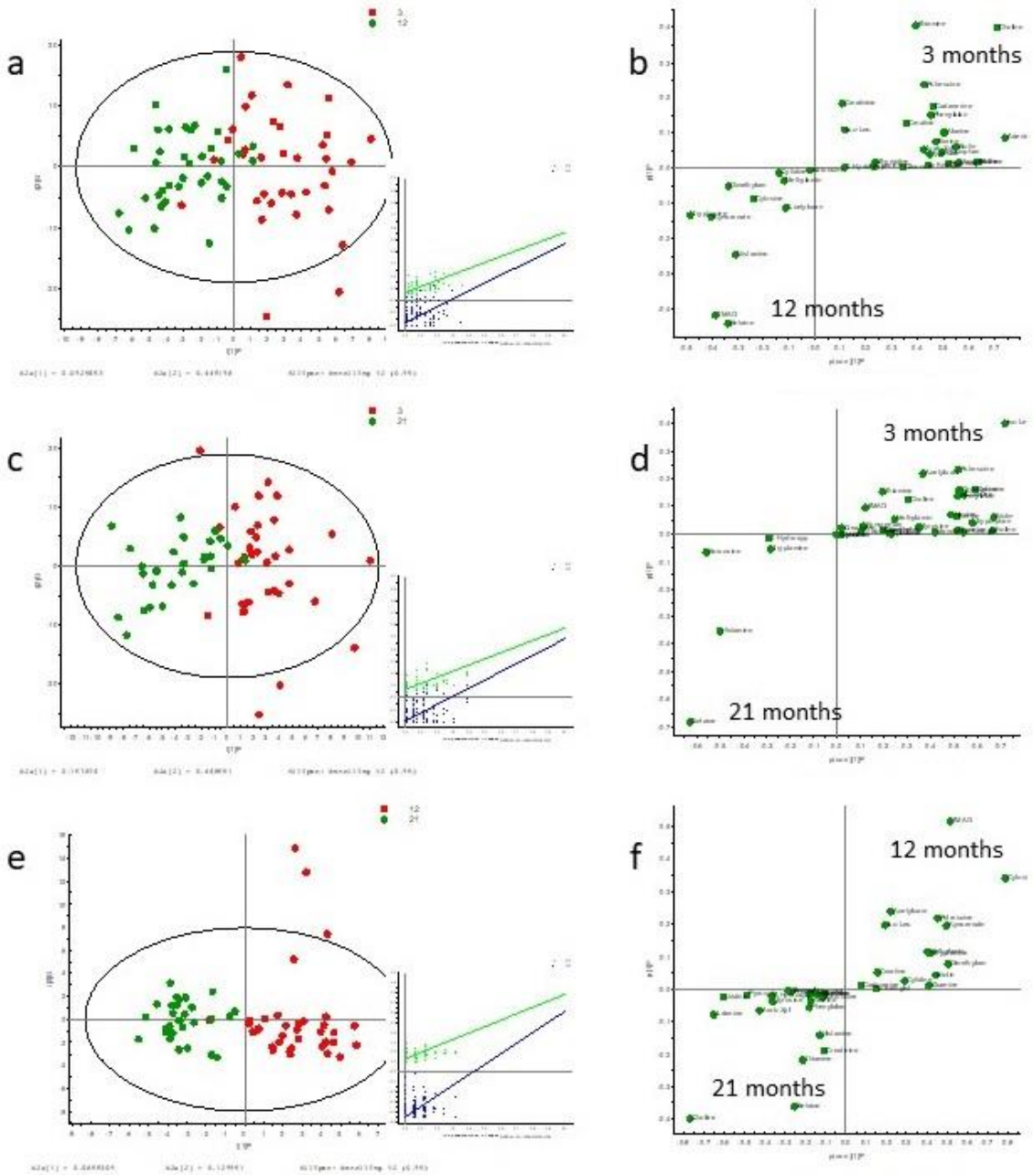


Fig. 28 (a) OPLS-DA scores plot between 3 (red) and 12 months (green) with permutations plot as insert, (b) corresponding S-plot, (c) OPLS-DA scores plot between 3 (red) and 21 months (green) with permutations plot, (d) corresponding S-plot, (e) OPLS-DA scores plot between 12 (red) and 21 months (green) with permutations plot, (f) corresponding S-plot.

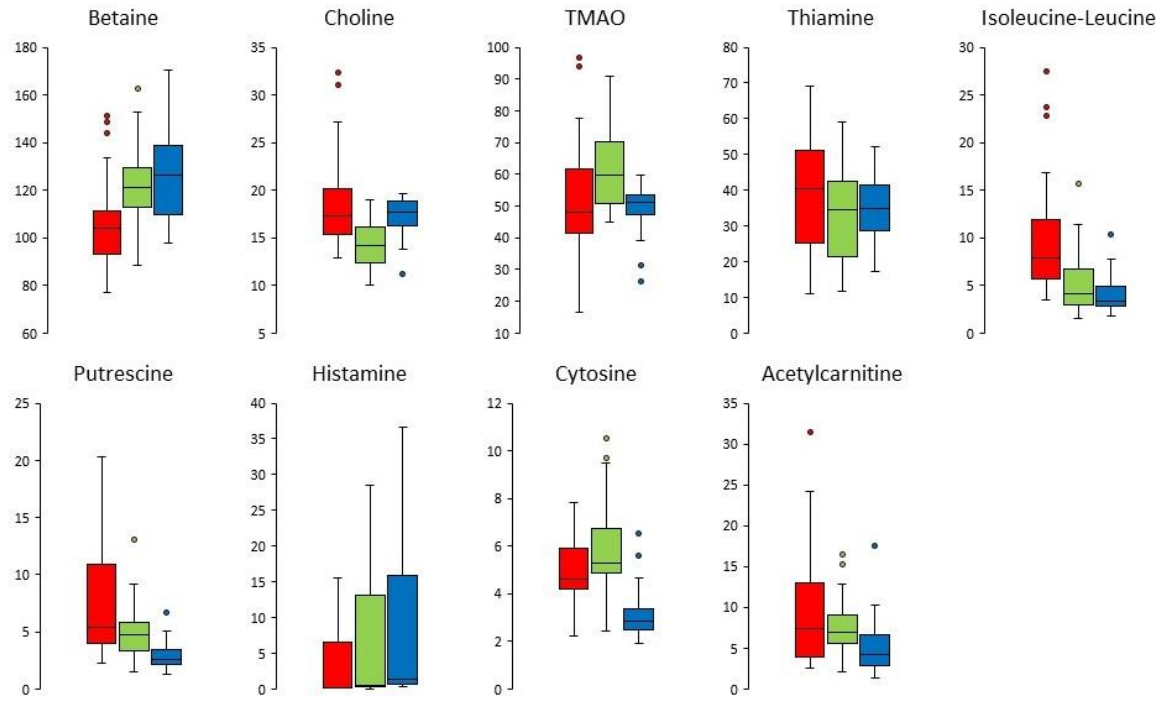


Fig. 29 Box plots of urinary metabolites contributing to the discrimination between 3 (red), 12 (green), and 21 months of age (blue). The vertical axis shows peak area in arbitrary units. Each box represents the interquartile range, and the center line represents the median. Whiskers are extended to the most extreme data point that is no more than 1.5 times the interquartile range from the edge of the box (Tukey style). Dots represent outliers.

Presentation of the observed metabolite differences between ages through heat maps illustrates the aforementioned findings. Specifically, based on Pearson's correlation, samples are clearly discriminated between ages in all three pairs (Fig. 30).

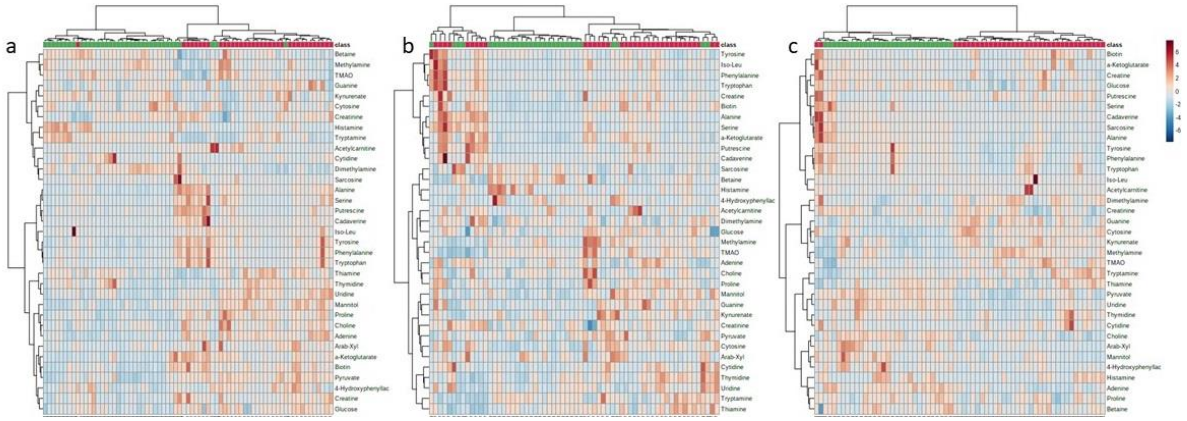


Fig. 30 Heat maps of differences in metabolite peak areas (a) between 3 (red heading) and 12 months (green heading), (b) between 3 (red heading) and 21 months (green heading), and (c) between 12 (red heading) and 21 months (green heading). Rows represent metabolites and columns represent different urine samples. Shades of red and blue provide a qualitative indication of the magnitude of difference according to the legend. Data were Pareto scaled, and motifs are ranked and sorted by Pearson's hierarchical clustering.

To reveal any hidden information related to exercise, we compared samples obtained at the same age depending on whether animals had exercised during the preceding period or not. Thus, samples at 12 months were compared between the animals that had exercised during the 1st half (groups A and B) and the ones that had not exercised during the same period (groups C and D). The OPLS-DA scores plot (Fig. 31) showed a moderate but still visible discrimination. S-plots could not be produced, as the OPLS-DA that produced results was UV (not Par) scaled. Hence, the metabolites that were responsible for the discrimination of the exercising and non-exercising groups could not be identified. At the age of 21 months, there was no discrimination between the groups that had exercised during the 2nd half (B and D) and the ones that had not exercised during the same period (A and C).

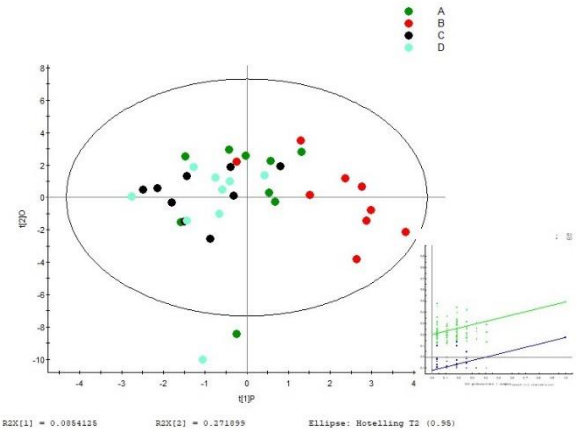


Fig. 31 OPLS-DA scores plot of the exercising and non-exercising groups at 12 months of age with permutations plot.

Univariate Analyses

The results of three-way ANOVA are presented below and the corresponding descriptive statistics in Tables 5 to 8.

Table 5 Peak areas of urinary metabolites in the samples of group A at three ages. Values are mean ± SD.

Metabolite	3 months (*n* = 9)	12 months (*n* = 9)	21 months (*n* = 7)
Methylamine	2.51 ± 1.25	2.27 ± 1.26	1.84 ± 0.97
Dimethylamine	2.05 ± 0.12	2.28 ± 0.29	2.11 ± 0.05
TMAO	64.79 ± 10.75	59.22 ± 8.21	53.99 ± 4.99
Putrescine	4.96 ± 1.58	5.66 ± 2.02	2.66 ± 0.84
Sarcosine	0.45 ± 0.33	0.38 ± 0.27	0.51 ± 0.16
Alanine	0.73 ± 0.27	0.39 ± 0.21	0.52 ± 0.10
Cadaverine	0.81 ± 0.35	0.21 ± 0.11	0.26 ± 0.03
Choline	0.45 ± 0.33	0.38 ± 0.27	0.51 ± 0.16
Serine	0.38 ± 0.33	0.19 ± 0.13	0.20 ± 0.04
Cytosine	3.99 ± 0.97	6.30 ± 2.03	2.88 ± 0.70
Histamine	0.24 ± 0.10	0.45 ± 0.14	2.45 ± 3.00
Creatinine	641.01 ± 43.89	677.01 ± 39.60	673.82 ± 22.27
Proline	0.03 ± 0.01	0.02 ± 0.01	0.02 ± 0.00
Betaine	113.77 ± 19.82	126.88 ± 13.89	122.03 ± 15.06
Isoleucine-leucine	113.77 ± 19.82	126.88 ± 13.89	122.03 ± 15.06
Creatine	0.37 ± 0.05	0.20 ± 0.07	0.35 ± 0.08
Adenine	0.37 ± 0.05	0.20 ± 0.07	0.35 ± 0.08
Guanine	0.03 ± 0.01	0.03 ± 0.01	0.02 ± 0.00
Tryptamine	1.16 ± 0.64	1.48 ± 0.73	1.38 ± 0.32
Phenylalanine	1.61 ± 0.21	1.70 ± 0.83	2.04 ± 0.34
Tyrosine	0.16 ± 0.07	0.12 ± 0.10	0.19 ± 0.04
Mannitol	0.03 ± 0.00	0.01 ± 0.01	0.02 ± 0.01
Kynurenic acid	2.46 ± 0.96	4.01 ± 1.68	2.77 ± 1.93
Acetylcarnitine	12.74 ± 8.45	6.83 ± 4.72	5.82 ± 2.92

Tryptophan	0.08 ± 0.02	0.07 ± 0.05	0.10 ± 0.03
Thymidine	0.18 ± 0.06	0.07 ± 0.02	0.15 ± 0.05
Cytidine	0.05 ± 0.02	0.07 ± 0.05	0.05 ± 0.02
Biotin	0.19 ± 0.08	0.14 ± 0.16	0.06 ± 0.03
Pyruvic acid	0.03 ± 0.00	0.01 ± 0.01	0.04 ± 0.01
α-ketoglutaric acid	0.19 ± 0.08	0.14 ± 0.16	0.06 ± 0.03
Arabinose-xylose	0.18 ± 0.07	0.23 ± 0.11	0.21 ± 0.15
Glucose	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
4-Hydroxyphenyllactic acid	0.03 ± 0.01	0.02 ± 0.01	0.05 ± 0.04
Uridine	0.04 ± 0.01	0.02 ± 0.00	0.04 ± 0.01

Table 6 Peak areas of urinary metabolites in the samples of group B at three ages. Values are mean ± SD.

Metabolite	3 months (n = 9)	12 months (n = 9)	21 months (n = 9)
Methylamine	1.50 ± 0.52	2.26 ± 0.43	1.22 ± 0.70
Dimethylamine	2.04 ± 0.14	2.51 ± 0.21	2.07 ± 0.10
TMAO	49.37 ± 10.50	60.84 ± 6.84	48.83 ± 4.25
Putrescine	6.59 ± 3.67	3.38 ± 1.57	3.01 ± 1.03
Sarcosine	0.25 ± 0.10	0.45 ± 0.14	0.51 ± 0.22
Alanine	0.86 ± 0.39	0.19 ± 0.15	0.52 ± 0.12
Cadaverine	1.19 ± 0.88	0.12 ± 0.08	0.23 ± 0.04
Choline	0.25 ± 0.10	0.25 ± 0.14	0.51 ± 0.22
Serine	0.43 ± 0.30	0.14 ± 0.08	0.23 ± 0.10
Cytosine	4.98 ± 0.76	7.04 ± 1.95	3.49 ± 1.68
Histamine	0.16 ± 0.03	0.37 ± 0.30	1.77 ± 1.50
Creatinine	685.44 ± 34.52	701.34 ± 36.64	676.47 ± 16.43
Proline	0.03 ± 0.01	0.01 ± 0.00	0.01 ± 0.01
Betaine	100.41 ± 13.69	107.52 ± 16.40	118.29 ± 19.58
Isoleucine-leucine	100.41 ± 13.69	107.52 ± 16.40	118.29 ± 19.58
Creatine	0.48 ± 0.16	0.18 ± 0.06	0.34 ± 0.13
Adenine	0.48 ± 0.16	0.18 ± 0.06	0.34 ± 0.13
Guanine	0.03 ± 0.01	0.03 ± 0.01	0.01 ± 0.00
Tryptamine	0.98 ± 0.90	2.02 ± 0.87	1.17 ± 0.57
Phenylalanine	2.66 ± 1.37	1.81 ± 0.88	2.43 ± 0.92
Tyrosine	0.18 ± 0.07	0.11 ± 0.09	0.20 ± 0.08
Mannitol	0.03 ± 0.01	0.01 ± 0.01	0.02 ± 0.01
Kynurenic acid	3.08 ± 1.31	4.53 ± 1.19	2.46 ± 1.74
Acetylcarnitine	11.58 ± 10.37	7.09 ± 2.49	4.97 ± 2.62
Tryptophan	0.16 ± 0.09	0.06 ± 0.02	0.11 ± 0.03
Thymidine	0.17 ± 0.04	0.09 ± 0.05	0.12 ± 0.04
Cytidine	0.05 ± 0.02	0.05 ± 0.01	0.04 ± 0.02
Biotin	0.22 ± 0.08	0.19 ± 0.14	0.08 ± 0.04
Pyruvic acid	0.03 ± 0.00	0.02 ± 0.01	0.03 ± 0.01
α-ketoglutaric acid	0.22 ± 0.08	0.19 ± 0.14	0.08 ± 0.04
Arabinose-xylose	0.29 ± 0.12	0.16 ± 0.14	0.40 ± 0.30
Glucose	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00

4-Hydroxyphenyllactic acid	0.03 ± 0.01	0.02 ± 0.01	0.04 ± 0.03
Uridine	0.04 ± 0.01	0.02 ± 0.00	0.04 ± 0.01

Table 7 Peak areas of urinary metabolites in the samples of group C at three ages. Values are mean ± SD.

Metabolite	3 months (n = 9)	12 months (n = 9)	21 months (n = 8)
Methylamine	1.59 ± 0.45	2.19 ± 0.65	1.42 ± 0.27
Dimethylamine	2.04 ± 0.23	2.32 ± 0.25	1.95 ± 0.14
TMAO	47.14 ± 9.67	66.00 ± 15.49	51.19 ± 3.58
Putrescine	6.48 ± 4.95	4.70 ± 1.78	2.22 ± 0.54
Sarcosine	0.31 ± 0.13	0.19 ± 0.12	0.55 ± 0.15
Alanine	0.70 ± 0.33	0.37 ± 0.24	0.50 ± 0.07
Cadaverine	1.27 ± 1.02	0.17 ± 0.10	0.27 ±0.18
Choline	0.31 ± 0.13	0.19 ± 0.12	0.55 ± 0.15
Serine	0.30 ± 0.22	0.26 ± 0.11	0.17 ± 0.03
Cytosine	4.91 ± 0.14	5.63 ± 1.43	2.96 ± 0.35
Histamine	7.14 ± 5.76	15.30 ± 11.29	19.68 ± 13.90
Creatinine	679.18 ± 35.37	667.46 ± 31.30	668.31 ± 24.60
Proline	0.03 ± 0.01	0.02 ± 0.00	0.01 ± 0.00
Betaine	107.18 ± 24.35	135.28 ± 14.49	127.08 ± 14.33
Isoleucine-leucine	107.18 ± 24.35	135.28 ± 14.49	127.08 ± 14.33
Creatine	0.47 ± 0.13	0.26 ± 0.13	0.39 ± 0.11
Adenine	0.47 ± 0.13	0.26 ± 0.13	0.39 ± 0.11
Guanine	0.03 ± 0.01	0.02 ± 0.01	0.02 ± 0.01
Tryptamine	1.58 ± 0.93	2.12 ± 0.90	1.51 ± 0.25
Phenylalanine	1.95 ± 0.65	1.86 ± 0.74	1.93 ± 0.24
Tyrosine	0.15 ± 0.06	0.12 ± 0.06	0.20 ± 0.03
Mannitol	0.02 ± 0.01	0.01 ± 0.01	0.02 ± 0.01
Kynurenic acid	4.02 ± 1.39	4.11 ± 0.80	2.91 ± 1.26
Acetylcarnitine	5.97 ± 3.63	7.94 ± 1.62	3.59 ± 1.17
Tryptophan	0.11 ± 0.04	0.10 ± 0.07	0.09 ± 0.02
Thymidine	0.21 ± 0.09	0.12 ± 0.05	0.16 ± 0.06
Cytidine	0.09 ± 0.04	0.09 ± 0.07	0.06 ± 0.03
Biotin	0.30 ± 0.11	0.09 ± 0.06	0.08 ± 0.03
Pyruvic acid	0.03 ± 0.01	0.02 ± 0.00	0.03 ± 0.01
α-ketoglutaric acid	0.30 ± 0.11	0.09 ± 0.06	0.08 ± 0.03
Arabinose-xylose	0.26 ± 0.12	0.13 ± 0.07	0.50 ± 0.27
Glucose	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
4-Hydroxyphenyllactic acid	0.03 ± 0.02	0.03 ± 0.02	0.06 ± 0.06
Uridine	0.04 ± 0.01	0.02 ± 0.01	0.04 ± 0.01

Table 8 Peak areas of urinary metabolites in the samples of group D at three ages. Values are mean ± SD.

Metabolite	3 months (n = 9)	12 months (n = 9)	21 months (n = 8)
Methylamine	2.19 ± 1.81	1.61 ± 0.28	1.05 ± 1.06
Dimethylamine	1.97 ± 0.16	2.58 ± 0.33	2.01 ± 0.03
TMAO	47.95 ± 30.68	58.65 ± 11.73	44.42 ± 14.62
Putrescine	10.62 ± 6.99	6.46 ± 4.18	4.17 ± 1.64
Sarcosine	0.37 ± 0.12	0.42 ± 0.34	0.92 ± 0.35
Alanine	0.16 ± 0.47	0.32 ± 0.09	0.77 ± 0.37
Cadaverine	3.20 ± 3.09	0.17 ± 0.15	0.46 ± 0.25
Choline	0.37 ± 0.12	0.42 ± 0.34	0.92 ± 0.35
Serine	0.69 ± 0.41	0.20 ± 0.13	0.37 ± 0.21
Cytosine	5.55 ± 1.88	5.06 ± 0.16	2.90 ± 0.46
Histamine	4.34 ± 6.30	9.19 ± 12.22	10.53 ± 13.93
Creatinine	668.32 ± 29.50	690.44 ± 41.64	660.36 ± 31.56
Proline	0.03 ± 0.01	0.02 ± 0.01	0.02 ± 0.00
Betaine	106.05 ± 4.03	120.88 ± 5.83	151.49 ± 15.32
Isoleucine-leucine	106.05 ± 4.03	120.88 ± 5.83	151.49 ± 15.32
Creatine	0.65 ± 0.14	0.22 ± 0.13	0.52 ± 0.12
Adenine	0.65 ± 0.14	0.22 ± 0.13	0.52 ± 0.12
Guanine	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01
Tryptamine	0.42 ± 0.35	1.71 ± 0.64	0.80 ± 0.37
Phenylalanine	3.59 ± 1.74	1.54 ± 0.40	2.02 ± 0.75
Tyrosine	0.26 ± 0.11	0.09 ± 0.02	0.18 ± 0.06
Mannitol	0.03 ± 0.01	0.01 ± 0.01	0.02 ± 0.01
Kynurenic acid	2.23 ± 0.52	3.44 ± 1.37	1.58 ± 0.86
Acetylcarnitine	7.01 ± 1.67	10.41 ± 3.61	7.15 ± 6.15
Tryptophan	0.22 ± 0.14	0.10 ± 0.07	0.08 ± 0.04
Thymidine	0.11 ± 0.01	0.21 ± 0.12	0.17 ± 0.07
Cytidine	0.08 ± 0.04	0.23 ± 0.15	0.05 ± 0.02
Biotin	0.54 ± 0.22	0.15 ± 0.15	0.07 ± 0.03
Pyruvic acid	0.04 ± 0.00	0.02 ± 0.01	0.03 ± 0.00
α-ketoglutaric acid	0.54 ± 0.22	0.15 ± 0.15	0.07 ± 0.03
Arabinose-xylose	0.69 ± 0.47	0.07 ± 0.03	0.28 ± 0.22
Glucose	0.01 ± 0.01	0.01 ± 0.00	0.01 ± 0.00
4-Hydroxyphenyllactic acid	0.03 ± 0.02	0.02 ± 0.01	0.0 ± 0.03
Uridine	0.04 ± 0.01	0.03 ± 0.01	0.04 ± 0.01

i) Amino Acids and Amino Acid Derivatives

An interaction of age and 1st half was found in choline (p = 0.005, ES = 0.16). Specifically, the exercising groups showed a smaller decrease during the first half than the non-exercising groups.

An interaction of age and 2nd half was found in tryptophan (p = 0.006, ES = 0.15), betaine (p = 0.008, ES = 0.15) and alanine (p = 0.011, ES = 0.14). Specifically, all three metabolites increased in the groups that exercised during the 2nd half, whereas, in the groups that did not exercise, tryptophan did not change, while betaine and alanine decreased slightly.

An interaction of 1st and 2nd half was found in sarcosine (p = 0.007, ES = 0.26) and kynurenate (p = 0.005, ES = 0.27). During the 1st half, sarcosine decreased in the exercising groups more than in the non-exercising ones, whereas kynurenate increased in the non-exercising groups more than in the exercising ones. During the 2nd half, sarcosine increased, whereas kynurenate decreased in the exercising groups more than in the non-exercising ones.

A main effect of age was found in sarcosine (p < 0.001, ES = 0.52), alanine (p < 0.001, ES = 0.50), choline (p < 0.001, ES = 0.40), proline (p < 0.001, ES = 0.69), betaine (p < 0.001, ES = 0.38), isoleucine-leucine (p < 0.001, ES = 0.34), tyrosine (p < 0.001, ES = 0.25), tryptophan (p = 0.002, ES = 0.18), tryptamine (p < 0.001, ES = 0.41) and kynurenate (p < 0.001, ES = 0.37). Specifically, from 3 to 12 months, betaine, tryptamine and kynurenate increased, whereas proline, isoleucine-leucine, tyrosine and tryptophan decreased; from 12 to 21 months, sarcosine, alanine, choline and tyrosine increased, whereas tryptamine and kynurenate decreased; and, from 3 to 21 months, sarcosine, choline and betaine increased, whereas alanine, proline, isoleucine-leucine and tryptophan decreased.

ii) Carbohydrate and Lipid Metabolism

A main effect of age was seen in mannitol (p < 0.001, ES = 0.40), pyruvate (p < 0.001, ES = 0.62) and glucose (p < 0.001, ES = 0.30). Specifically, from 3 to 12 months, all three metabolites decreased; from 12 to 21 months, mannitol and pyruvate increased; and, from 3 to 21 months only mannitol showed a decrease.

iii) Krebs Cycle

An interaction of age and 2nd half was found in α-ketoglutarate (p = 0.005, ES = 0.16). Specifically, the exercising groups showed a decrease, whereas the non-exercising groups showed an increase.

Also, a main effect of age was found in α-ketoglutarate (p < 0.001, ES = 0.32). Specifically, it showed a decrease from both 3 to 12 and from 3 to 21 months.

iv) Purine and Pyrimidine Metabolism

An interaction of age, 1st and 2nd half was noticed in guanine (p = 0.005, ES = 0.16) and cytidine (p = 0.005, ES = 0.21). Specifically, during the 1st half, guanine decreased in the exercising groups less than in the non-exercising ones, while cytidine remained the same in

the exercising groups and increased in the non-exercising ones. During the 2nd half, guanine decreased less, whereas cytidine decreased more in the exercising groups than in the non-exercising ones.

An interaction of age and 1st half was seen in cytidine (p = 0.008, ES = 0.19). As mentioned above, the non-exercising groups showed an increase, whereas the exercising groups showed no change.

An interaction of age and 2nd half was seen in thymidine (p = 0.007, ES = 0.15). During the 2nd half, the exercising groups showed a decrease, whereas the non-exercising groups showed an increase.

A main effect of 1st half was found in adenine (p < 0.001, ES = 0.45) and cytidine (p < 0.001, ES = 0.44). Specifically, both metabolites had higher values at 12 months in the non-exercising groups than in the exercising groups.

A main effect of age was found in cytosine (p < 0.001, ES = 0.48), adenine (p < 0.001, ES = 0.47), guanine (p < 0.001, ES = 0.39), uridine (p < 0.001, ES = 0.59), thymidine (p = 0.002, ES = 0.15) and cytidine (p = 0.002, ES = 0.25). Specifically, from 3 to 12 months, cytosine and cytidine increased, whereas adenine, uridine and thymidine decreased; from 12 to 21 months, adenine and uridine increased, whereas cytosine, guanine and cytidine decreased; and, from 3 to 21 months, cytosine, adenine and guanine decreased.

v) Gut Microbiome Metabolism

A main effect of 2nd half was found in TMAO (p = 0.007, ES = 0.26). Specifically, TMAO decreased in the exercising groups more than in the non-exercising ones.

A main effect of age was found in dimethylamine (p < 0.001, ES = 0.73) and TMAO (p < 0.001, ES = 0.45). Specifically, from 3 to 12 months dimethylamine increased, and from 12 to 21 months dimethylamine and TMAO decreased.

vi) Other Metabolites

An interaction of age and 1st half was seen in biotin (p < 0.001, ES = 0.27). Specifically, during the 1st half, it decreased in the exercising groups less than in the non-exercising ones.

An interaction of age and 2nd half was seen in creatine (p < 0.001, ES = 0.23). Specifically, the exercising groups showed an increase, whereas the non-exercising groups showed a decrease.

A main effect of age was found in creatine (p < 0.001, ES = 0.29), creatinine (p = 0.001, ES = 0.20), methylamine (p < 0.001, ES = 0.29) and biotin (p < 0.001, ES = 0.54). Specifically, from

3 to 12 months, creatinine increased, whereas creatine decreased; from 12 to 21 months, creatine increased, whereas creatinine and methylamine decreased; and, from 3 to 21 months creatine and biotin decreased.

Effects of Long-Term and Lifelong Exercise on the Metabolome of Blood Lysates

Multivariate Analyses

Thirty-nine metabolites were identified in rat blood lysates (Table 9). Multivariate analysis of the spectroscopic data showed a clear discrimination between ages through both unsupervised (PCA; Fig. 32a) and supervised methods (PLS-DA, Fig. 32b). The permutation plot confirms the validation of the PLS-DA model. The major difference, projected on the first component, was between the samples at 3 months and those at 12 and 21 months. The latter were separated in the second component of the generated model, with the samples at 21 months showing higher variance than those at 3 and 12 months. In order to gain a detailed insight of the age differences the three groups were investigated in pairs (3 vs. 12, 3 vs. 21 and 12 vs. 21 months) with OPLS-DA and results are presented in Fig. 33. According to the S-plots (Fig. 33b, d, f), discrimination between ages was due to (in alphabetical order) acetate, ATP and ADP combined (the common peaks at 8.28 and 6.15 ppm), betaine (precisely, glycine betaine, or *N,N,N*-trimethylglycine), *N*,*N*-dimethylglycine, glucose, NAD^+, and serine. Specifically, as shown in the graphical representations in Fig. 34, acetate and glucose increased from 3 to 12 months and then decreased from 12 to 21 months. ATP and ADP (combined), betaine, *N,N*-dimethylglycine, and NAD^+ decreased from 3 to 12 months, whereas, from 12 to 21 months, they increased. Finally, serine decreased from 3 to 21 months.

Presentation of the observed metabolite differences between ages through heat maps illustrates the aforementioned findings. Specifically, based on Pearson's correlation, samples are clearly discriminated from 3 to 12 months (Fig. 35a) and from 3 to 21 months (Fig. 35b). From 12 to 21 months (Fig. 35c), the discrimination becomes less clear but is still visible.

To reveal any hidden information related to exercise, we compared samples obtained at the same age depending on whether animals had exercised during the preceding period or not. Thus, samples at 12 months were compared between the animals that had exercised during the 1st half (groups A and B) and the animals that had not exercised during the same period (groups C and D). The OPLS-DA scores plot (Fig. 36a) suggested a clear discrimination. The S-plot (Fig. 36b) showed that discrimination between exercising and non-exercising animals was due to (in alphabetical order) acetate, alanine, formate, glucose, glutamine, glycerol, 3-hydroxybutyrate, lactate, and succinate. Specifically, as shown in the graphical representations (Fig. 37), the exercising rats had higher values of acetate, alanine, formate,

glutamine, glycerol and succinate, whereas only lactate was higher in the non-exercising rats. The graphical representations of glucose and 3-hydroxybutyrate did not show a clear discrimination between the exercising and non-exercising groups. At the age of 21 months, there was no discrimination between the exercising (B and D) and the non-exercising groups (A and C).

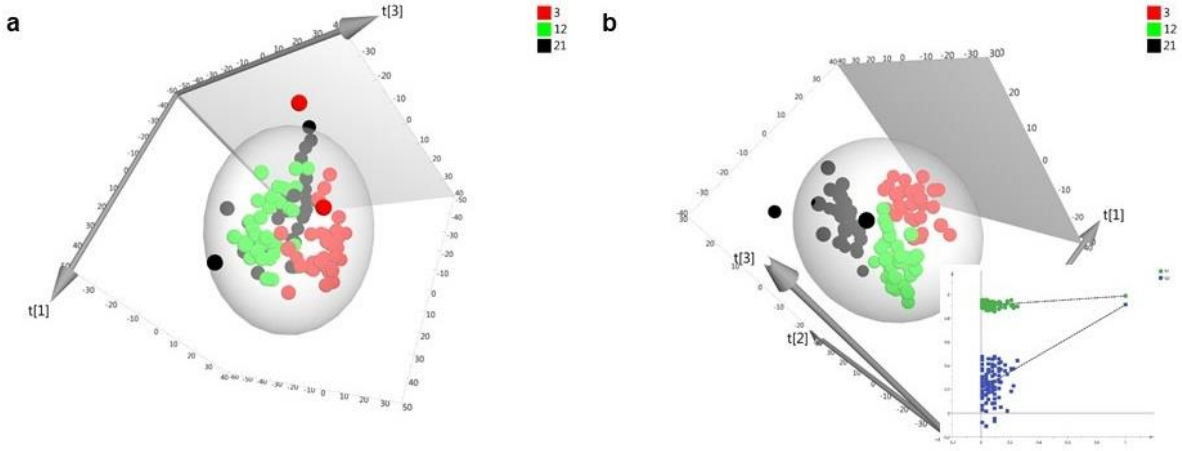


Fig. 32 PCA (a) and PLS-DA scores plot with permutations plot (b) of all samples collected at 3, 12, and 21 months of age (red, green, and black, respectively). Orbs represent 95% confidence intervals of Hotelling's T^2 distribution.

Table 9 Metabolites detected in the [1]H NMR spectra of blood lysates.

No.	Metabolite	Molecular formula	Chemical Shift (ppm)
1	Leucine	$C_6H_{13}NO_2$	0.96(d[1]), 0.95(d)
2	Isobutyrate	$C_6H_{12}O_2$	1.07(d)
3	3-Hydroxybutyrate	$C_4H_8O_3$	1.20(s)
4	Alanine	$C_3H_7NO_2$	1.48(d)
5	Acetate	CH_3COOH	1.92(s)
6	Acetone	C_3H_6O	2.23(s)
7	Pyruvate	$C_3H_4O_3$	2.37(s)
8	Succinate	$C_4H_6O_4$	2.40(s)
9	Glutamine	$C_5H_{10}N_2O_3$	2.48(m), 2.44(m), 2.15(m)
10	Aspartate	$C_4H_7NO_4$	2.81(d), 2.68(d)
11	*N,N*-Dimethylglycine	$C_4H_9NO_2$	2.92(s)
12	Asparagine	$C_4H_8N_2O_3$	2.95(dd), 2.86 (dd)
13	Lysine	$C_6H_{14}N_2O_2$	3.02(t), 1.74(m)
14	Malonate	$C_3H_4O_4$	3.11(s)
15	Betaine	$C_5H_{11}NO_2$	3.89 (s), 3.26 (t)
16	Taurine	$C_2H_7NO_3S$	3.42 (t), 3.26(t)
17	Glycine	$C_2H_5NO_2$	3.55(s)
18	Valine	$C_5H_{11}NO_2$	3.62(d), 1.04(d), 0.99(d)
19	Isoleucine	$C_6H_{13}NO_2$	3.66(d), 1.01(d), 0.93(t)
20	Glycerol	$C_3H_8O_3$	3.66(dd), 3.55(dd)
21	Glutamate	$C_5H_9NO_4$	2.34(m)
22	Glutathione disulphide	$C_{20}H_{32}N_6O_{12}S_2$	3.78(m), 3.30(m), 2.97(dd)
23	Creatine	$C_4H_{10}N_3O_5P$	3.92(s), 3.03(s)
24	Serine	$C_3H_7NO_3$	3.98(dd), 3.94(dd)
25	Lactate	$C_3H_6O_3$	4.10(m), 1.32(d)
26	Threonine	$C_4H_9NO_3$	4.25(m), 3.58(m), 1.32(d)
27	Glycerophosphocholine	$C_8H_{21}NO_6P$	4.32(m), 3.22(s)
28	Mannose	$C_6H_{12}O_6$	5.18(d)
29	Glucose	$C_6H_{12}O_6$	5.23(d), 4.64(d), 3.89(dd), 3.85-3.80(m), 3.76(dd), 3.72(dd), 3.71(t), 3.54(dd), 3.49(t), 3.48(m)-3.44(m), 3.42-3.38(m), 3.24(dd)
30	Tyrosine	$C_9H_{11}NO_3$	7.19(d), 6.90(d)
31	Phenylalanine	$C_9H_{11}NO_2$	7.42(m), 7.38(m), 7.33(m)
32	Tryptophan	$C_{11}H_{12}N_2O_2$	7.73(d), 7.54(d), 7.32(s), 7.28(m), 7.20(t)
33	Cytidine	$C_9H_{13}N_3O_5$	7.82(d), 6.06(d)
34	Histidine	$C_6H_9N_3O_2$	7.88(s), 7.09(s)
35	Methylhistidine	$C_7H_{11}N_3O_2$	7.94(s)
36	Formate	CH_2O_2	8.45(s)
37	Adenosine diphosphate (ADP)	$C_{10}H_{13}N_4O_8P$	8.53(s), 8.28(s), 6.15(d)
38	Adenosine triphosphate (ATP)	$C_{10}H_{14}N_5O_7P$	8.55(s), 8.28(s), 6.15(d)
39	Nicotinamide adenine dinucleotide (NAD^+)	$C_{21}H_{27}N_7O_{14}P_2$	9.33(s), 9.14(d), 8.83(m), 8.42(s), 8.19(m), 8.17(s), 6.09(d), 6.04(d), 4.54(m), 4.50(m), 4.48(t), 4.42(m), 4.38(m)

[1]d, doublet; dd, doublet of doublets; m, multiplet; s, singlet; t, triplet.

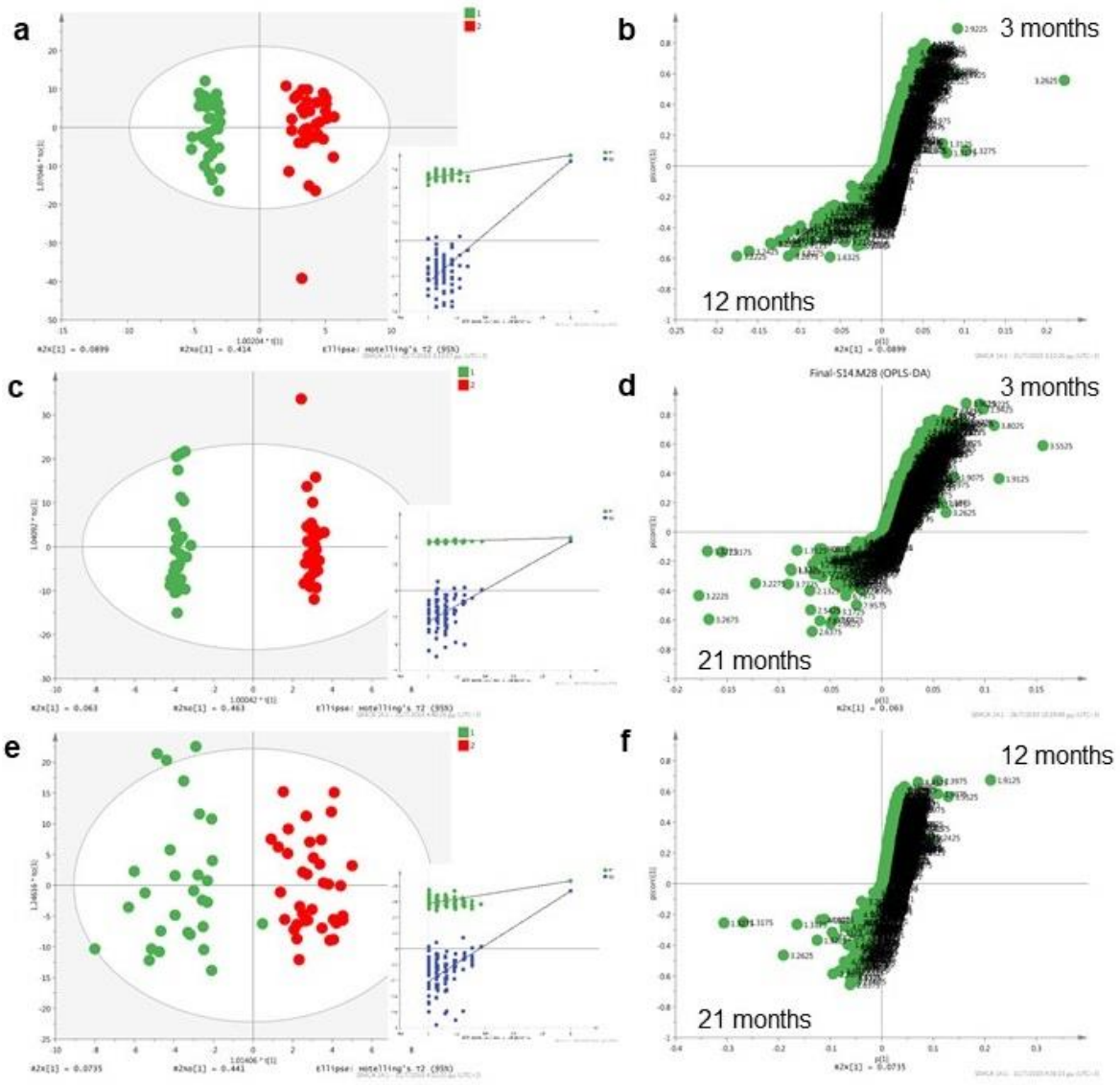


Fig. 33 (a) OPLS-DA scores plot between 3 (red) and 12 months (green) with permutations plot as insert, (b) corresponding S-plot, (c) OPLS-DA scores plot between 3 (red) and 21 months (green) with permutations plot, (d) corresponding S-plot, (e) OPLS-DA scores plot between 12 (red) and 21 months (green) with permutations plot, (f) corresponding S-plot.

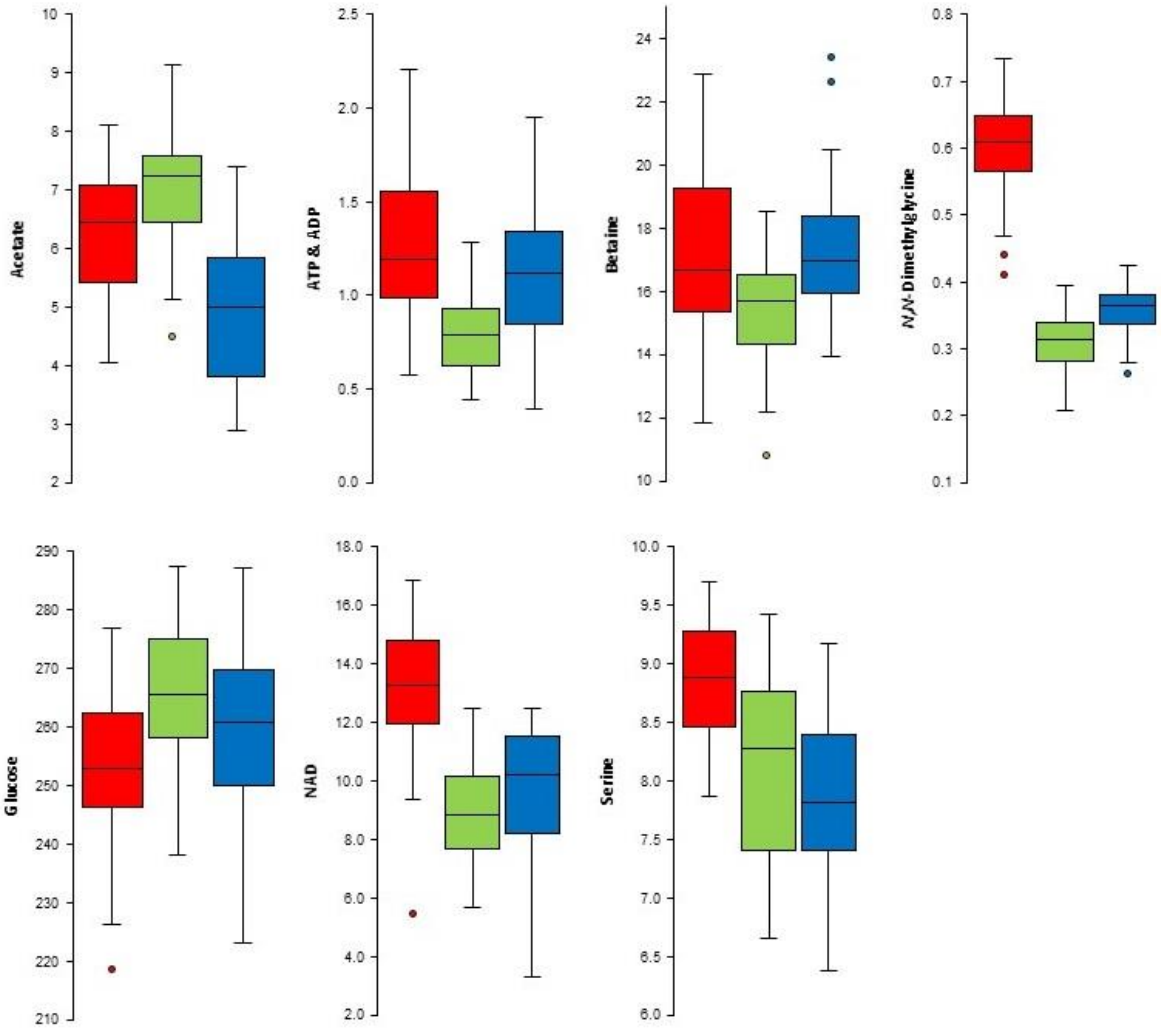


Fig. 34 Box plots of metabolites (in alphabetical order) contributing to the discrimination between 3 (red), 12 (green), and 21 months of age (blue). The vertical axis shows peak area in arbitrary units. Each box represents the interquartile range, and the center line represents the median. Whiskers are extended to the most extreme data point that is no more than 1.5 times the interquartile range from the edge of the box (Tukey style). Dots represent outliers.

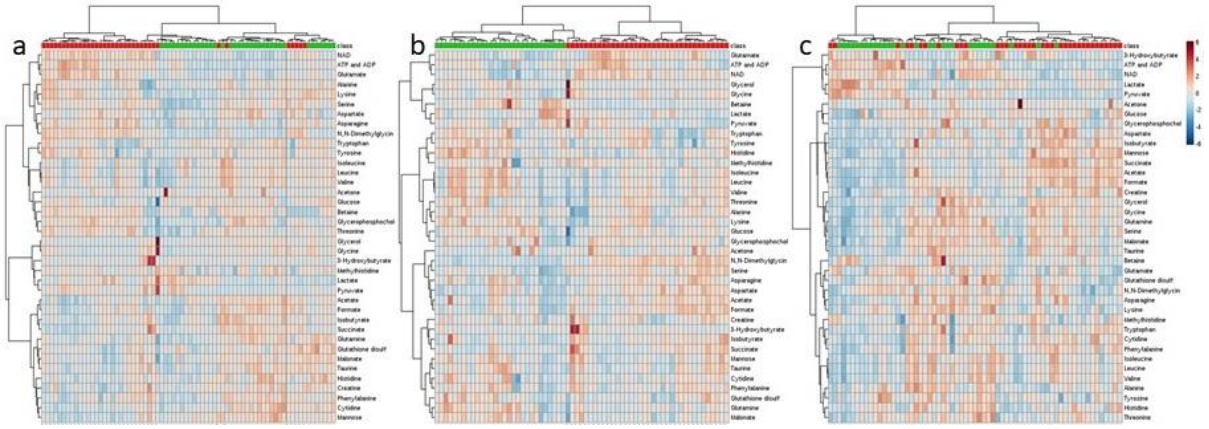


Fig. 35 Heat maps of differences in metabolite peak areas (a) between 3 (red heading) and 12 months (green heading), (b) between 3 (red heading) and 21 months (green heading), and (c) between 12 (red heading) and 21 months (green heading). Rows represent metabolites and columns represent different NMR samples. Shades of red and blue provide a qualitative indication of the magnitude of difference according to the legend. Data were Pareto scaled, and motifs are ranked and sorted by Pearson's hierarchical clustering.

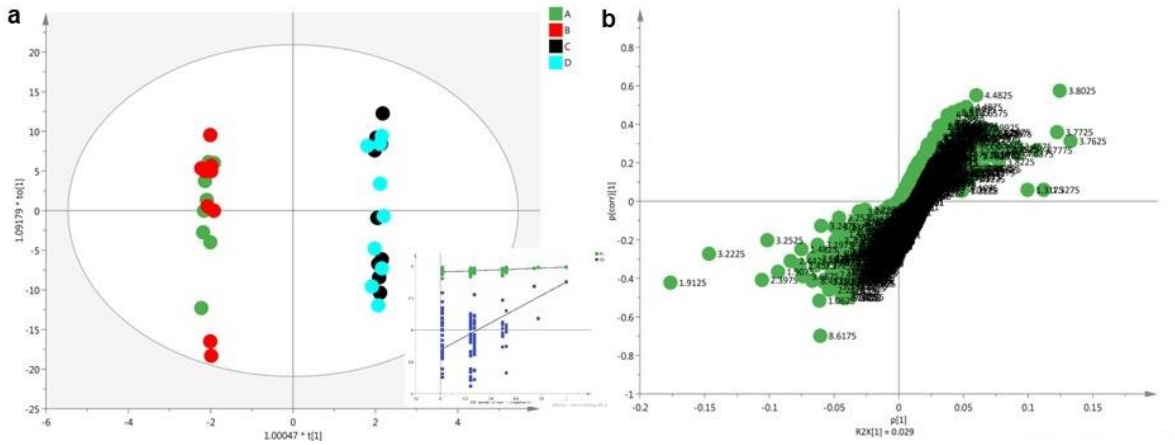


Fig. 36 (a) OPLS-DA scores plot of the exercising and non-exercising groups at 12 months of age with permutations plot, (b) loadings plot of metabolites affecting the discrimination of these groups.

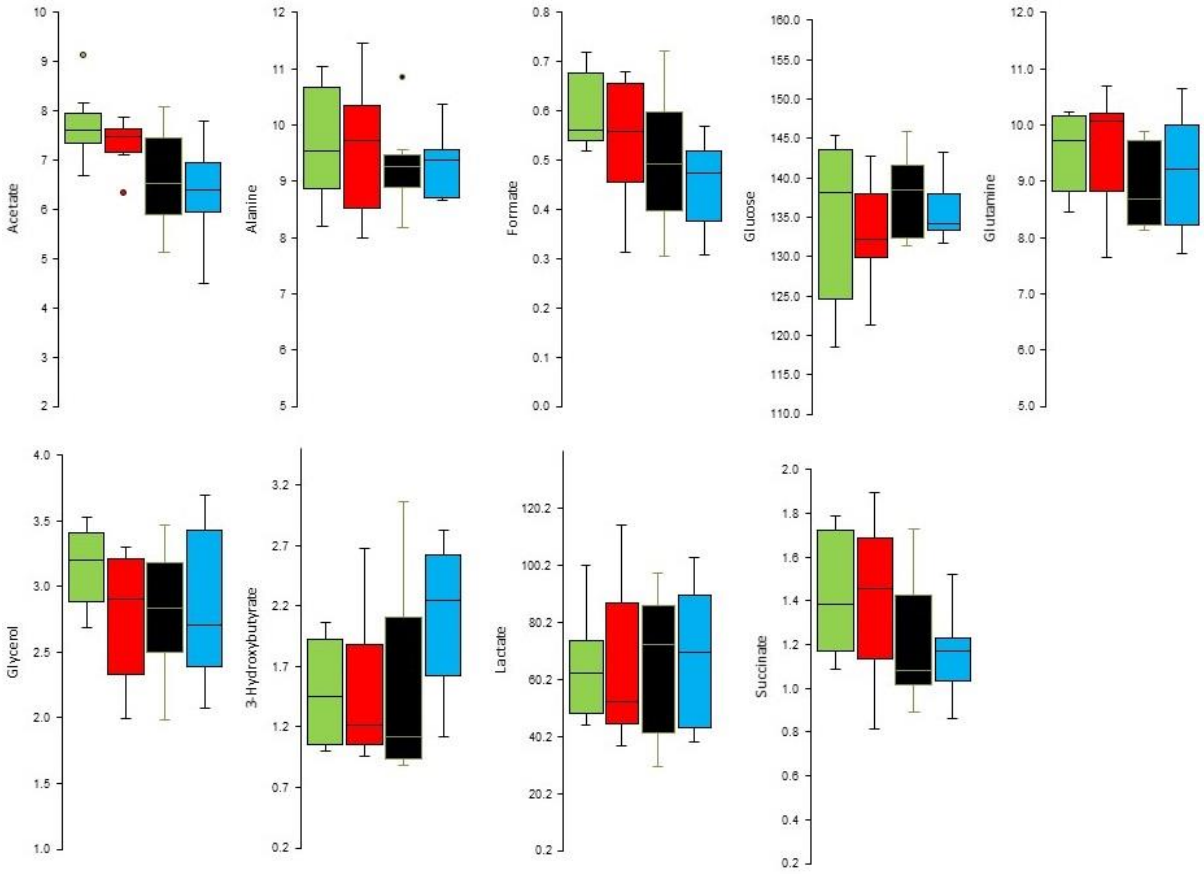


Fig. 37 Box plots of metabolites (in alphabetical order) contributing to the discrimination between the exercising groups (A, red, and B, green) and the non-exercising groups (C, black, and D, ciel) at 12 months of age. The vertical axis shows peak area in arbitrary units. Each box represents the interquartile range, and the center line represents the median. Whiskers are extended to the most extreme data point that is no more than 1.5 times the interquartile range from the edge of the box (Tukey style). Dots represent outliers.

Univariate Analyses

The results of three-way ANOVA are presented below and the corresponding descriptive statistics in Tables 10 to 13.

Table 10 Peak areas of metabolites in blood lysates of group A at three ages. Values are mean ± SD.

Metabolite	3 months (*n* = 9)	12 months (*n* = 9)	21 months (*n* = 7)
Leucine	4.60 ± 0.45	5.18 ± 0.48	5.37 ± 0.66
Isobutyrate	0.58 ± 0.09	0.66 ± 0.09	0.58 ± 0.22

3-Hydroxybutyrate	2.40 ± 0.30	2.36 ± 0.50	2.28 ± 0.30
Alanine	9.93 ± 1.54	10.68 ± 0.90	11.43 ± 1.01
Acetate	6.17 ± 1.36	7.77 ± 0.75	4.59 ± 1.08
Acetone	0.94 ± 0.38	1.04 ± 0.41	0.83 ± 0.25
Pyruvate	0.73 ± 0.32	0.45 ± 0.19	1.01 ± 0.23
Succinate	1.51 ± 0.35	1.90 ± 0.30	1.16 ± 0.20
Glutamine	12.81 ± 1.02	13.30 ± 1.04	12.89 ± 1.72
Aspartate	1.25 ± 0.22	1.13 ± 0.24	0.83 ± 0.16
N,N-Dimethylglycine	0.58 ± 0.07	0.30 ± 0.06	0.33 ± 0.06
Asparagine	0.65 ± 0.06	0.58 ± 0.07	0.61 ± 0.13
Lysine	7.04 ± 0.80	6.79 ± 0.76	7.33 ± 0.57
Malonate	3.48 ± 0.50	3.64 ± 0.31	3.56 ± 0.33
Betaine	2.51 ± 0.34	2.22 ± 0.22	2.36 ± 0.26
Taurine	2.80 ± 0.55	3.03 ± 0.54	2.85 ± 0.43
Glycine	5.50 ± 0.59	5.81 ± 0.49	4.77 ± 0.70
Valine	10.18 ± 0.80	11.27 ± 1.02	11.51 ± 1.32
Isoleucine	1.61 ± 0.15	1.81 ± 0.24	1.87 ± 0.20
Glycerol	3.86 ± 0.58	4.88 ± 0.53	4.26 ± 1.20
Glutamate	5.51 ± 0.52	4.71 ± 0.30	5.09 ± 0.28
Glutathione	23.69 ± 2.98	23.98 ± 2.63	25.30 ± 2.42
Creatine	1.95 ± 0.42	2.30 ± 0.33	2.10 ± 0.25
Serine	8.57 ± 0.47	8.07 ± 0.69	7.71 ± 0.64
Lactate	136.15 ± 27.95	119.90 ± 32.01	151.89 ± 62.31
Threonine	4.95 ± 0.39	4.62 ± 0.27	4.92 ± 0.40
Glycerophosphocholine	1.48 ± 0.17	0.99 ± 0.19	1.17 ± 0.27
Mannose	0.53 ± 0.08	0.80 ± 0.06	0.52 ± 0.04
Glucose	17.86 ± 0.72	19.10 ± 1.31	18.29 ± 1.27
Tyrosine	2.72 ± 0.21	2.62 ± 0.21	2.92 ± 0.21
Phenylalanine	2.25 ± 0.39	2.64 ± 0.37	2.34 ± 0.35
Tryptophan	1.54 ± 0.27	1.68 ± 0.15	1.59 ± 0.16
Cytidine	0.77 ± 0.13	1.02 ± 0.26	0.81 ± 0.21
Histidine	0.43 ± 0.05	0.51 ± 0.08	0.56 ± 0.10

Methylhistidine	0.32 ± 0.09	0.31 ± 0.16	0.37 ± 0.09
Formate	0.47 ± 0.10	0.61 ± 0.08	0.30 ± 0.09
ATP and ADP	1.44 ± 0.46	0.76 ± 0.18	1.35 ± 0.20
NAD	14.07 ± 2.04	8.46 ± 1.56	10.84 ± 0.95

Table 11 Peak areas of metabolites in blood lysates of group B at three ages. Values are mean ± SD.

Metabolite	3 months (*n* = 9)	12 months (*n* = 9)	21 months (*n* = 9)
Leucine	4.90 ± 0.19	5.05 ± 0.47	5.18 ± 0.74
Isobutyrate	0.53 ± 0.08	0.61 ± 0.15	0.48 ± 0.06
3-Hydroxybutyrate	1.91 ± 0.21	2.30 ± 0.53	1.88 ± 0.24
Alanine	10.76 ± 0.89	10.51 ± 1.15	11.03 ± 0.69
Acetate	6.33 ± 0.85	7.35 ± 0.45	5.15 ± 1.25
Acetone	1.84 ± 0.94	2.38 ± 1.91	1.71 ± 1.29
Pyruvate	0.67 ± 0.22	0.44 ± 0.20	0.81 ± 0.30
Succinate	1.52 ± 0.21	1.90 ± 0.44	1.27 ± 0.18
Glutamine	12.62 ± 0.77	13.52 ± 1.30	13.87 ± 1.03
Aspartate	1.26 ± 0.15	1.19 ± 0.30	1.05 ± 0.30
N,N-Dimethylglycine	0.60 ± 0.06	0.29 ± 0.04	0.37 ± 0.03
Asparagine	0.72 ± 0.06	0.59 ± 0.11	0.66 ± 0.10
Lysine	7.11 ± 0.56	6.63 ± 0.64	7.62 ± 0.59
Malonate	3.45 ± 0.34	3.67 ± 0.24	3.79 ± 0.33
Betaine	2.66 ± 0.26	2.16 ± 0.22	2.58 ± 0.28
Taurine	3.00 ± 0.38	3.10 ± 0.58	3.21 ± 0.68
Glycine	5.22 ± 0.22	5.66 ± 0.59	4.94 ± 0.37
Valine	10.76 ± 0.50	10.86 ± 1.01	10.83 ± 1.14
Isoleucine	1.75 ± 0.07	1.77 ± 0.16	1.81 ± 0.21
Glycerol	3.56 ± 0.35	4.24 ± 0.74	4.37 ± 1.13
Glutamate	5.62 ± 0.40	4.86 ± 0.42	5.05 ± 0.37
Glutathione	24.49 ± 2.70	25.05 ± 1.68	24.38 ± 1.62
Creatine	2.00 ± 0.37	2.24 ± 0.18	2.22 ± 0.35

Serine	8.86 ± 0.55	8.21 ± 0.89	7.87 ± 0.65
Lactate	111.20 ± 17.92	113.98 ± 42.60	133.02 ± 34.34
Threonine	5.23 ± 0.30	4.75 ± 0.43	4.78 ± 0.27
Glycerophosphocholine	1.53 ± 0.20	1.52 ± 0.34	1.41 ± 0.41
Mannose	0.55 ± 0.06	0.72 ± 0.06	0.58 ± 0.08
Glucose	18.12 ± 0.89	18.96 ± 0.90	18.68 ± 1.18
Tyrosine	2.33 ± 0.30	2.39 ± 0.33	2.64 ± 0.10
Phenylalanine	2.50 ± 0.24	2.51 ± 0.27	2.25 ± 0.44
Tryptophan	1.13 ± 0.23	1.49 ± 0.28	1.35 ± 0.27
Cytidine	0.82 ± 0.07	1.00 ± 0.15	0.72 ± 0.28
Histidine	0.48 ± 0.06	0.51 ± 0.10	0.49 ± 0.09
Methylhistidine	0.37 ± 0.07	0.35 ± 0.18	0.30 ± 0.26
Formate	0.52 ± 0.09	0.54 ± 0.12	0.38 ± 0.11
ATP and ADP	1.37 ± 0.36	0.83 ± 0.25	1.03 ± 0.52
NAD	14.01 ± 1.46	8.72 ± 1.61	8.26 ± 2.83

Table 12 Peak areas of metabolites in blood lysates of group C at three ages. Values are mean ± SD.

Metabolite	3 months (*n* = 9)	12 months (*n* = 9)	21 months (*n* = 8)
Leucine	5.26 ± 0.35	5.00 ± 0.51	5.18 ± 0.61
Isobutyrate	0.51 ± 0.06	0.52 ± 0.09	0.42 ± 0.04
3-Hydroxybutyrate	2.18 ± 0.43	2.23 ± 0.86	2.21 ± 0.47
Alanine	11.25 ± 1.07	10.16 ± 0.82	10.49 ± 0.86
Acetate	5.80 ± 0.89	6.57 ± 0.96	4.79 ± 1.48
Acetone	0.96 ± 0.45	1.11 ± 0.42	0.92 ± 0.40
Pyruvate	0.65 ± 0.19	0.47 ± 0.23	0.76 ± 0.29
Succinate	1.26 ± 0.16	1.64 ± 0.35	1.29 ± 0.16
Glutamine	12.72 ± 0.73	12.70 ± 1.08	12.55 ± 1.37
Aspartate	1.19 ± 0.15	1.05 ± 0.30	0.81 ± 0.10
N,N-Dimethylglycine	0.63 ± 0.06	0.31 ± 0.03	0.35 ± 0.03

Asparagine	0.69 ± 0.06	0.60 ± 0.08	0.57 ± 0.10
Lysine	7.54 ± 0.76	6.42 ± 0.27	6.94 ± 0.14
Malonate	3.50 ± 0.25	3.70 ± 0.43	3.61 ± 0.38
Betaine	2.69 ± 0.39	2.20 ± 0.21	2.34 ± 0.25
Taurine	3.12 ± 0.37	3.26 ± 0.63	3.00 ± 0.47
Glycine	6.01 ± 0.81	5.70 ± 0.67	4.85 ± 0.68
Valine	11.47 ± 0.80	10.58 ± 1.09	11.03 ± 1.33
Isoleucine	1.82 ± 0.14	1.75 ± 0.19	1.82 ± 0.25
Glycerol	3.99 ± 0.45	4.43 ± 0.81	4.25 ± 0.69
Glutamate	5.42 ± 0.34	4.82 ± 0.34	4.75 ± 0.30
Glutathione	24.70 ± 1.29	26.10 ± 2.61	26.13 ± 2.63
Creatine	2.07 ± 0.41	2.29 ± 0.31	1.93 ± 0.10
Serine	8.97 ± 0.51	8.08 ± 0.88	7.63 ± 0.85
Lactate	105.92 ± 23.00	123.33 ± 42.59	146.82 ± 66.16
Threonine	5.21 ± 0.30	4.83 ± 0.59	4.98 ± 0.69
Glycerophosphocholine	1.40 ± 0.22	1.20 ± 0.24	1.17 ± 0.30
Mannose	0.54 ± 0.07	0.68 ± 0.12	0.61 ± 0.14
Glucose	18.79 ± 0.78	19.30 ± 0.71	18.71 ± 1.80
Tyrosine	2.81 ± 0.41	2.36 ± 0.21	2.64 ± 0.38
Phenylalanine	2.43 ± 0.24	2.44 ± 0.31	2.44 ± 0.41
Tryptophan	1.55 ± 0.31	1.34 ± 0.18	1.43 ± 0.22
Cytidine	0.74 ± 0.12	0.89 ± 0.22	0.80 ± 0.14
Histidine	0.48 ± 0.09	0.50 ± 0.09	0.51 ± 0.10
Methylhistidine	0.35 ± 0.07	0.38 ± 0.12	0.29 ± 0.06
Formate	0.48 ± 0.10	0.49 ± 0.14	0.34 ± 0.15
ATP and ADP	1.23 ± 0.38	0.77 ± 0.21	1.07 ± 0.24
NAD	13.53 ± 1.53	9.41 ± 1.19	10.04 ± 1.94

Table 13 Peak areas of metabolites in blood lysates of group D at three ages. Values are mean ± SD.

Metabolite	3 months (*n* = 9)	12 months (*n* = 9)	21 months (*n* = 5)
Leucine	4.73 ± 0.55	5.15 ± 0.45	5.16 ± 0.84
Isobutyrate	0.65 ± 0.12	0.55 ± 0.09	0.48 ± 0.07
3-Hydroxybutyrate	2.83 ± 1.78	2.87 ± 0.72	1.84 ± 0.16
Alanine	9.34 ± 1.66	10.12 ± 0.77	10.87 ± 1.37
Acetate	6.77 ± 1.53	6.43 ± 1.27	5.32 ± 1.65
Acetone	0.91 ± 0.17	0.88 ± 0.25	0.78 ± 0.36
Pyruvate	0.38 ± 0.10	0.61 ± 0.2	0.73 ± 0.33
Succinate	2.26 ± 0.69	1.64 ± 0.34	1.51 ± 0.34
Glutamine	12.94 ± 0.89	13.26 ± 1.43	14.06 ± 2.69
Aspartate	1.21 ± 0.15	1.12 ± 0.28	1.06 ± 0.22
N,N-Dimethylglycine	0.57 ± 0.13	0.33 ± 0.05	0.37 ± 0.05
Asparagine	0.65 ± 0.09	0.65 ± 0.10	0.64 ± 0.14
Lysine	6.75 ± 1.23	7.10 ± 0.95	7.42 ± 1.18
Malonate	3.68 ± 0.26	3.75 ± 0.54	3.74 ± 0.74
Betaine	2.42 ± 0.47	2.15 ± 0.16	2.46 ± 0.25
Taurine	3.66 ± 0.46	3.38 ± 0.57	3.30 ± 0.62
Glycine	6.68 ± 0.74	6.30 ± 0.98	5.48 ± 1.10
Valine	10.12 ± 1.31	10.99 ± 0.51	11.15 ± 1.82
Isoleucine	1.61 ± 0.17	1.77 ± 0.19	1.74 ± 0.26
Glycerol	4.28 ± 0.51	4.65 ± 0.99	5.51 ± 1.91
Glutamate	4.99 ± 0.30	5.18 ± 0.25	4.70 ± 0.36
Glutathione	26.58 ± 2.89	28.14 ± 2.96	27.13 ± 4.27
Creatine	2.58 ± 0.47	2.26 ± 0.30	1.94 ± 0.34
Serine	9.11 ± 0.73	8.10 ± 1.08	8.56 ± 0.19
Lactate	119.42 ± 46.30	109.86 ± 35.93	93.94 ± 10.49
Threonine	4.59 ± 0.39	4.73 ± 0.26	5.05 ± 0.85
Glycerophosphocholine	1.21 ± 0.28	1.09 ± 0.08	1.64 ± 0.43
Mannose	0.72 ± 0.12	0.72 ± 0.17	0.65 ± 0.12

Glucose	17.81 ± 1.30	19.11 ± 0.44	18.47 ± 0.68
Tyrosine	2.21 ± 0.42	2.55 ± 0.19	2.73 ± 0.43
Phenylalanine	2.56 ± 0.32	2.33 ± 0.16	2.43 ± 0.47
Tryptophan	1.50 ± 0.26	1.70 ± 0.16	1.70 ± 0.37
Cytidine	0.96 ± 0.15	0.96 ± 0.26	0.90 ± 0.28
Histidine	0.45 ± 0.05	0.46 ± 0.07	0.56 ± 0.09
Methylhistidine	0.32 ± 0.10	0.34 ± 0.21	0.36 ± 0.09
Formate	0.58 ± 0.14	0.48 ± 0.10	0.38 ± 0.14
ATP and ADP	1.08 ± 0.25	0.94 ± 0.22	0.98 ± 0.26
NAD	12.57 ± 1.12	10.27 ± 1.73	9.38 ± 2.23

i) Amino Acids

An interaction of age, 1st and 2nd half was found in alanine ($p = 0.003$, ES = 0.20). Specifically, during the 1st half groups A and B (which trained) showed an increase, whereas groups C and D (which did not train) showed a decrease, but during the 2nd half all groups showed an increase.

An interaction of age and 2nd half ($p = 0.005$, ES = 0.19) was found in tyrosine, which can be explained by the fact that, during the 2nd half, tyrosine increased in groups A and C (which did not train) more than it did in groups B and D (which trained).

An interaction of 1st and 2nd half ($p < 0.001$, ES = 0.39) was found in tryptophan. Specifically, during both halves the groups that trained showed an increase, whereas the groups that did not train showed a decrease.

A main effect of age was noticed in aspartate ($p < 0.001$, ES = 0.29), glutamate ($p < 0.001$, ES = 0.40), glycine ($p < 0.001$, ES = 0.41), histidine ($p = 0.002$, ES = 0.23), lysine ($p < 0.001$, ES = 0.28), serine ($p < 0.001$, ES = 0.39) and tyrosine ($p = 0.002$, ES = 0.22). Specifically, from 3 to 12 months, glutamate and serine decreased; from 12 to 21 months, lysine and tyrosine increased, whereas glycine decreased; and, from 3 to 21 months, aspartate, glutamate, glycine and serine decreased, whereas histidine and tyrosine increased.

Training resulted in significant amino acid changes only in the 1st half and only in glycine ($p = 0.003$, ES = 0.30). Specifically, from 3 to 12 months groups A and B showed an increase, whereas groups C and D showed a decrease.

ii) Carbohydrate and Lipid Metabolism

A main effect of age was seen in mannose ($p < 0.001$, ES = 0.46), pyruvate ($p < 0.001$, ES = 0.40), glycerol ($p = 0.002$, ES = 0.22), and glucose ($p = 0.007$, ES = 0.18). Specifically, from 3 to 12 months mannose, glycerol and glucose increased, whereas pyruvate decreased, and from 12 to 21 months mannose decreased, whereas pyruvate increased. In addition, an interaction of age and 1st half was seen in mannose ($p = 0.007$, ES = 0.18), owing to the fact that the increase in the groups that trained during the 1st half was higher than that in the groups that did not.

iii) Krebs Cycle

An interaction of age, 1st and 2nd half ($p = 0.006$, ES = 0.18) was found in succinate. Specifically, during the 1st half, groups A and B (which trained) showed an increase, whereas groups C and D (which did not train) showed a decrease. During the 2nd half, all groups showed a decrease, but the decrease in groups B and D (which trained) was smaller than that in the other two groups. Succinate also exhibited a main effect of age ($p < 0.001$, ES = 0.41), with values at 21 months being lower than at 3 or 12 months of age.

iv) Adenine Nucleotides

ATP and ADP combined showed a main effect of age ($p < 0.001$, ES = 0.35), decreasing from 3 to 12 months and increasing from 12 to 21 months. In addition, a main effect of age ($p < 0.001$, ES = 0.65) was found in NAD^+. Specifically, the values at both 12 and 21 months were lower than those at 3 months, although there was an increase from 12 to 21 months.

v) Small Aliphatic Carboxylic Acids

Acetate ($p < 0.001$, ES = 0.61), formate ($p < 0.001$, ES = 0.50) and isobutyrate ($p = 0.001$, ES = 0.24) showed a main effect of age. Specifically, acetate increased from 3 to 12 months. However, from 12 to 21 months, all three acids decreased, and their values at 21 months were lower than those at 3 months.

vi) Other Metabolites

An interaction of age and 1st half was found in creatine ($p = 0.002$, ES = 0.21), which can be explained by the fact that during the 1st half creatine increased in groups A and B (which trained) more than it did in groups C and D (which did not train). Training during the 2nd half did not have a similar effect (rather, creatine decreased in all groups). However, as a result of the effect of training during the 1st half, groups A and B ended up with higher creatine values

at 21 months compared to 3 months, whereas groups C and D ended up with lower values. Cytidine exhibited a main effect of age ($p = 0.002$, ES = 0.22). Specifically, it increased from 3 to 12 months but decreased from 12 to 21 months.

Betaine and N,N-dimethylglycine showed a main effect of age ($p < 0.001$, ES = 0.39, and $p < 0.001$, ES = 0.91, respectively). In both metabolites, the values at both 12 and 21 months were lower than those at 3 months, although there was an increase from 12 to 21 months.

Effects of Long-Term and Lifelong Exercise on Tissue Fatty Acid Content

Thirteen FAMEs were detected in considerable amounts (at least 0.001 µmol/g tissue) by GC in the PLs of quadriceps muscle (Table 14).

Table 14 Fatty acids in quadriceps PLs.

Abbreviation	Empirical name	IUPAC name
14:0	Myristic	Tetradecanoic
15:0		Pentadecanoic
16:0	Palmitic	Hexadecanoic
16:1ω7	Palmitoleic	*cis*-9-Hexadecenoic
18:0	Stearic	Octadecanoic
18:1ω9	Oleic	*cis*-9-Octadecenoic
18:1ω7	Vaccenic	*cis*-11-Octadecenoic
18:2ω6	Linoleic	*cis*-9,12-Octadecadienoic
20:3ω6	Dihomogamalinolenic	all-*cis*-8,11,14-Eicosatrienoic
20:3ω3		all-*cis*-11,14,17-Eicosatrienoic
22:0	Behenic	Docosanoic
22:4ω6	Adrenic	all-*cis*-7,10,13,16-Docosatetraenoic
22:6ω3		all-*cis*-4,7,10,13,16,19-Docosahexaenoic

Eleven FAME were detected in significant amounts in the TGs of quadriceps (Table 15).

Table 15 Fatty acids in quadriceps TGs.

Abbreviation	Empirical name	IUPAC name
14:0	Myristic	Tetradecanoic
15:0		Pentadecanoic
16:0	Palmitic	Hexadecanoic

16:1ω7	Palmitoleic	*cis*-9-Hexadecenoic
18:0	Stearic	Octadecanoic
18:1ω9	Oleic	*cis*-9-Octadecenoic
18:1ω7	Vaccenic	*cis*-11-Octadecenoic
18:2ω6	Linoleic	*cis*-9,12-Octadecadienoic
18:3ω6	γ-linolenic	all-*cis*-6,9,12-Octadecatrienoic
20:1ω9	Gadoleic	*cis*-9-Eicosenoic
20:3ω3		all-*cis*-11,14,17-Eicosatrienoic

Eighteen FAME were detected in the PLs of liver (Table 16).

Table 16 Fatty acids in liver PLs.

Abbreviation	Empirical name	IUPAC name
14:0	Myristic	Tetradecanoic
15:0		Pentadecanoic
16:0	Palmitic	Hexadecanoic
16:1ω7	Palmitoleic	cis-9-Hexadecenoic
18:0	Stearic	Octadecanoic
18:1ω9	Oleic	*cis*-9-Octadecenoic
18:1ω7	Vaccenic	*cis*-11-Octadecenoic
18:2ω6	Linoleic	*cis*-9,12-Octadecadienoic
18:3ω6	γ-linolenic	all-*cis*-6,9,12-Octadecatrienoic
20:0	Arachidic	Eicosanoic
20:1ω9	Gadoleic	*cis*-9-Eicosenoic
20:2ω6		*cis*-11,14-Eicosadienoic
21:0		Heneicosanoic
20:3ω6	Dihomogamalinolenic	all-*cis*-8,11,14-Eicosatrienoic
20:3ω3		all-*cis*-11,14,17-Eicosatrienoic
22:0	Behenic	Docosanoic
23:0		Tricosanoic
22:6ω3		all-*cis*-4,7,10,13,16,19-Docosahexaenoic

Thirteen FAME were detected in the TGs of liver (Table 17).

Table 17 Fatty acids in liver TGs.

Abbreviation	Empirical name	IUPAC name
14:0	Myristic	Tetradecanoic
15:0		Pentadecanoic
16:0	Palmitic	Hexadecanoic
16:1ω7	Palmitoleic	*cis*-9-Hexadecenoic
18:0	Stearic	Octadecanoic
18:1ω9	Oleic	*cis*-9-Octadecenoic
18:1ω7	Vaccenic	*cis*-11-Octadecenoic
18:2ω6	Linoleic	*cis*-9,12-Octadecadienoic
18:3ω6	γ-linolenic	all-*cis*-6,9,12-Octadecatrienoic
18:3ω3	α-linolenic	all-*cis*-9,12,15-Octadecatrienoic
20:1ω9	Gadoleic	*cis*-9-Eicosenoic
20:3ω6	Dihomogamalinolenic	all-*cis*-8,11,14-Eicosatrienoic
20:3ω3		all-*cis*-11,14,17-Eicosatrienoic

Fifteen FAME were detected in the PLs of brain (Table 18).

Table 18 Fatty acids in brain PLs.

Abbreviation	Empirical name	IUPAC name
14:0	Myristic	Tetradecanoic
15:0		Pentadecanoic
16:0	Palmitic	Hexadecanoic
16:1ω7	Palmitoleic	cis-9-Hexadecenoic
18:0	Stearic	Octadecanoic
18:1ω9	Oleic	*cis*-9-Octadecenoic
18:1ω7	Vaccenic	*cis*-11-Octadecenoic
18:2ω6	Linoleic	*cis*-9,12-Octadecadienoic
20:0	Arachidic	Eicosanoic
20:1ω9	Gadoleic	*cis*-9-Eicosenoic
20:3ω6	Dihomogamalinolenic	all-*cis*-8,11,14-Eicosatrienoic
20:3ω3		all-*cis*-11,14,17-Eicosatrienoic
20:5ω3	Timnodonic	all-*cis*-5,8,11,14,17-Eicosapentaenoic
24:0	Lignoceric	Tetracosanoic

22:6ω3	all-*cis*-4,7,10,13,16,19-Docosahexaenoic

The fatty acid composition of the rodent chow is presented in Table 19. MUFAs constituted 23.4% of the total fatty acids, PUFAs 49%, ω6 44.6% and ω3 4.3%. The ω6/ω3 ratio was 10.4 and the UFA/SFA ratio was 2.6. The most abundant fatty acids were 16:0, 18:1ω9 and 18:2ω6, constituting 88.8% of the total.

Table 19 Percent molar fatty acid composition of rodent chow.

Fatty acid	%
14:0	1.2
16:0	23.3
16:1ω7	1.2
18:0	3.2
18:1ω9	20.9
18:1ω7	0.4
18:2ω6	44.6
18:3ω3	2.7
20:1ω9	0.9
20:5ω3	0.7
22:6ω3	0.9
Total	100.0

Multivariate Analyses

Multivariate analyses showed no discrimination between groups B and C (whose members were killed at 24 months of age) in any of the tissues analysed. However, analyses showed discriminations between groups A and D (whose members were killed at 22 months of age) regarding muscle TG concentrations, muscle TG fatty acid composition, liver PL concentration, liver PL fatty acid composition, liver TG concentrations and liver TG fatty acid composition. Specifically, both PCA UV (Fig. 38a) and PCA Par (Fig. 38b) showed a discrimination between groups A and D with respect to muscle TG concentrations. PCA Par showed a discrimination between the same groups with respect to muscle TG fatty acid composition (Fig. 39). PCA UV showed a discrimination between groups A and D with respect to liver PL concentrations (Fig. 40). PCA Par showed a discrimination with respect to liver PL fatty acid composition (Fig. 41). Both PCA UV and PCA Par showed a discrimination between groups A and D regarding liver TG concentrations (Fig. 42). PCA par, PLS-DA Par and OPLS

showed a clear discrimination between groups regarding liver TG fatty acid composition. The permutations plot showed that the PLS-DA model was marginally valid (Fig. 43).

The PLS-DA loadings plot (Fig. 44a) and the OPLS S-line (Fig. 44b) showed that the discrimination between groups A and D regarding liver TG fatty acid composition was due to (VIP values in parentheses) 18:2ω6 (2.00), 16:0 (1.68) and 18:1ω7 (1.63). According to the graphical representations shown in Fig. 45, group A had higher values of 16:0 and 18:1ω7, whereas groups D had a higher value of 18:2ω6.

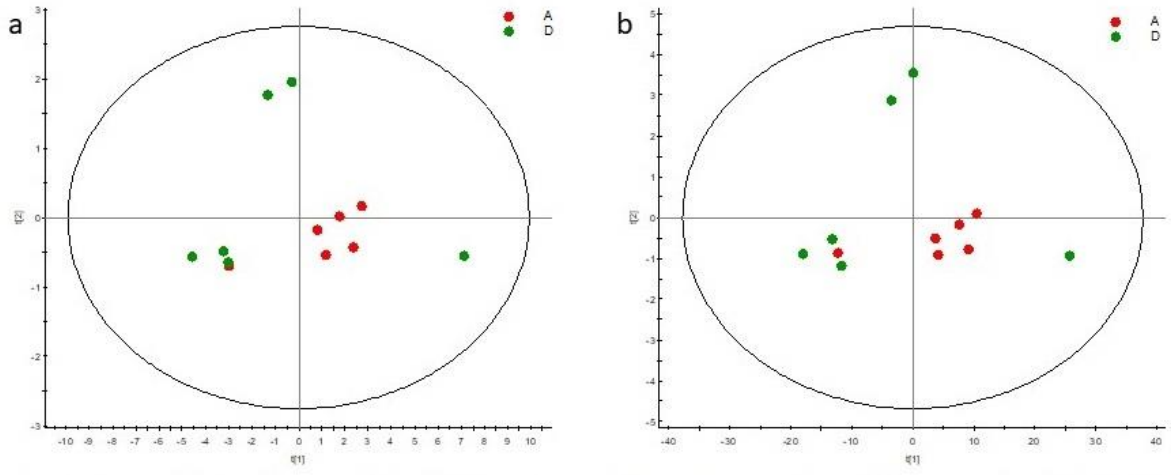


Fig. 38 PCA UV (a) and PCA Par scores plots (b) of muscle TG concentrations of groups A and D. Ellipses represent 95% confidence intervals of Hotelling's T^2 distribution.

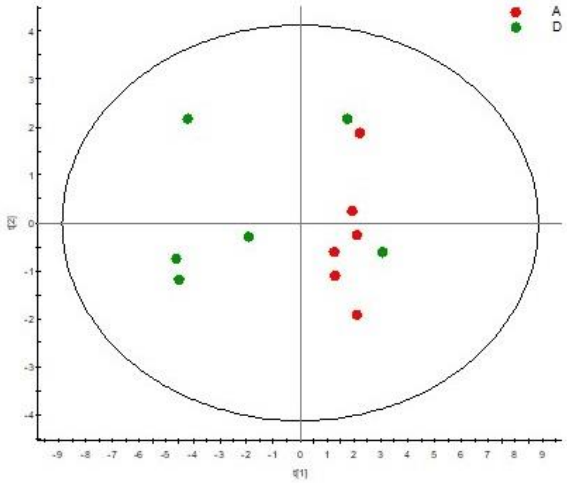


Fig. 39 PCA Par scores plot of muscle TG fatty acid composition. Ellipse represents 95% confidence intervals of Hotelling's T^2 distribution.

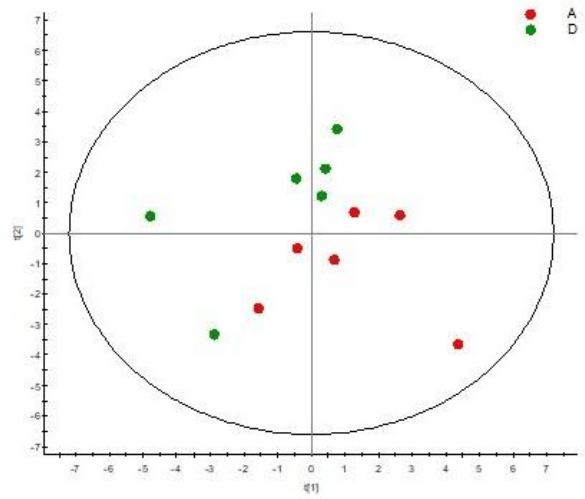


Fig. 40 PCA UV scores plot of liver PL concentrations of groups A and D. Ellipse represents 95% confidence intervals of Hotelling's T^2 distribution.

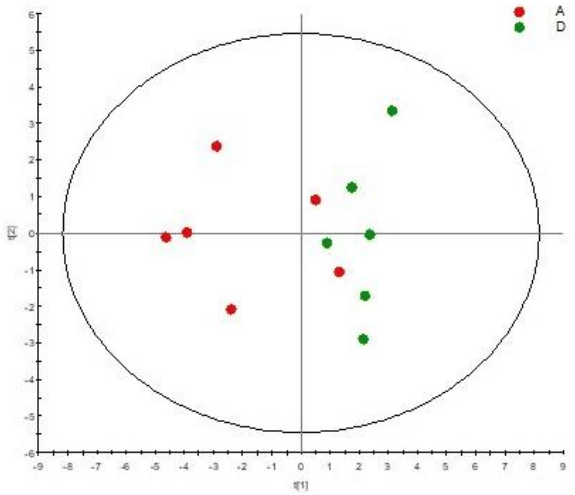


Fig. 41 PCA Par scores plot of liver PL fatty acid composition. Ellipse represents 95% confidence intervals of Hotelling's T^2 distribution.

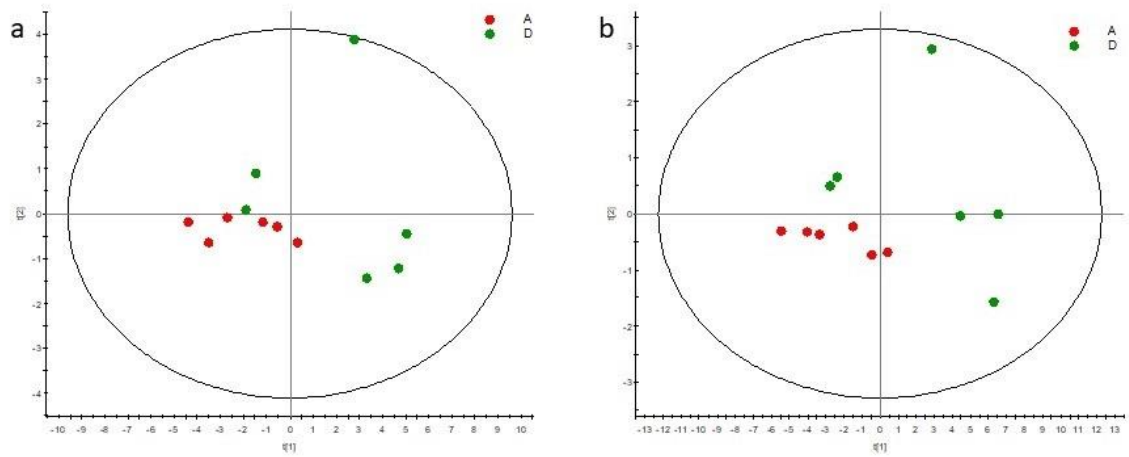


Fig. 42 PCA UV (a) and PCA Par scores plots (b) of liver TG concentrations of groups A and D. Ellipses represent 95% confidence intervals of Hotelling's T^2 distribution.

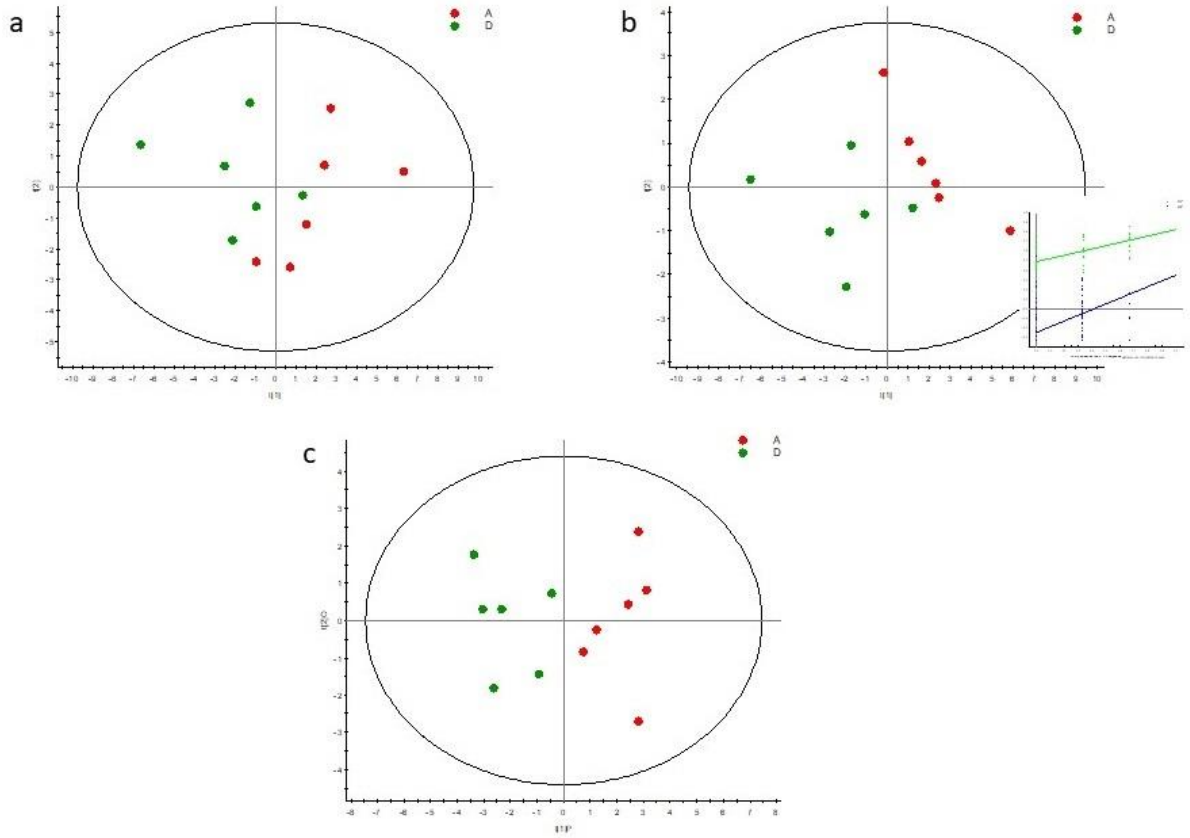


Fig. 43 PCA Par (a), PLS-DA Par with permutations plot as insert (b) and OPLS scores plots (c) of liver TG composition of groups A and D. Ellipses in a, b and c represent 95% confidence intervals of Hotelling's T^2 distribution.

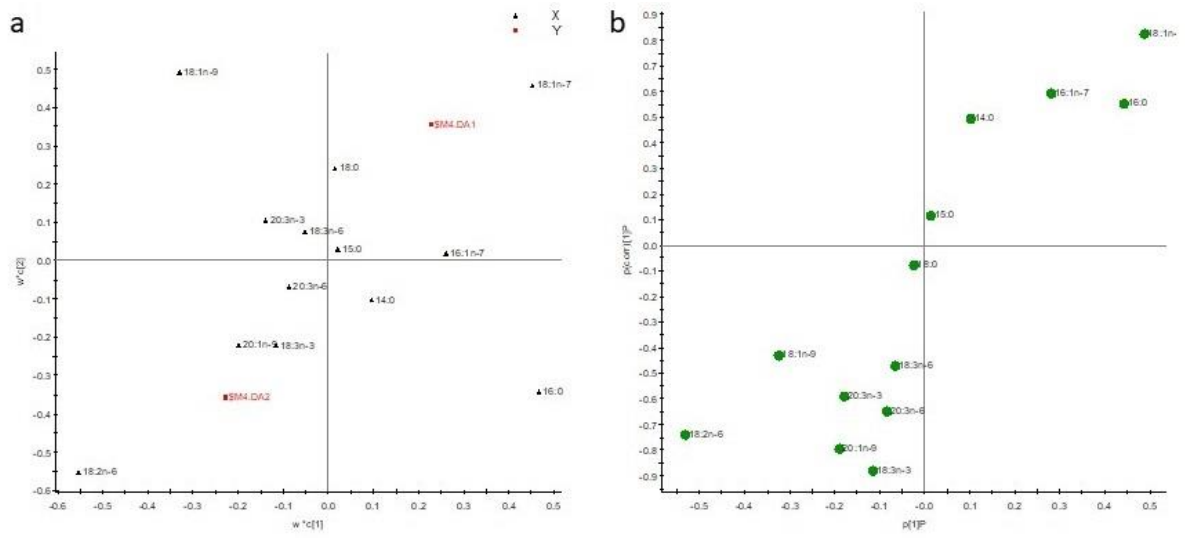


Fig. 44 PLS-DA loadings plot (a) and OPLS S-line (b) of liver TG composition of groups A and D.

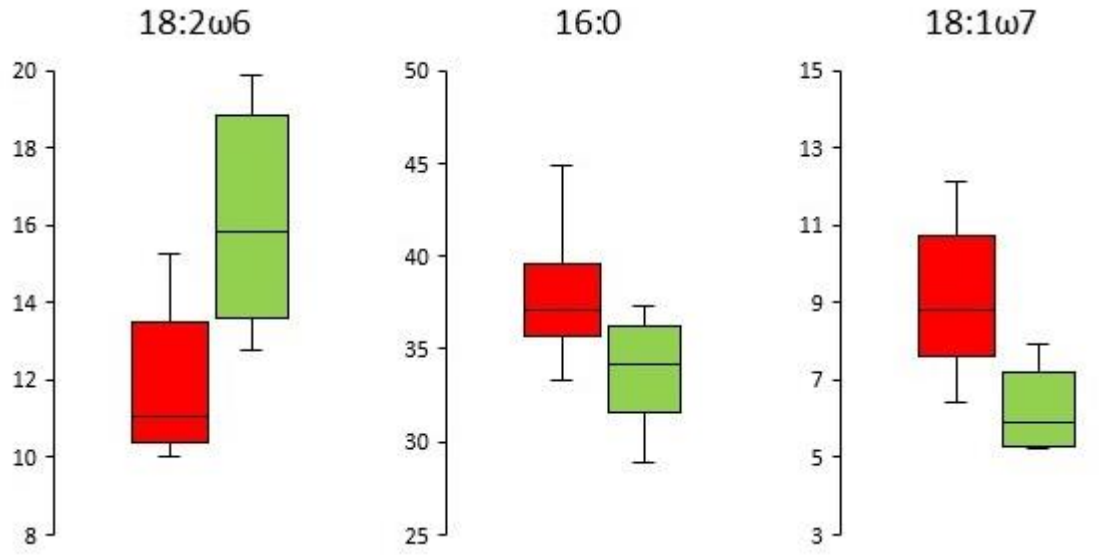


Fig. 45 Box plots of fatty acids contributing to the discrimination between groups A (red) and D (green). The vertical axis shows percentage in liver TGs. Each box represents the interquartile range, and the center line represents the median. Whiskers are extended to the most extreme data point that is no more than 1.5 times the interquartile range from the edge of the box (Tukey style).

Fig. 46 shows fatty acid concentrations and percent distributions in the form of heat maps according to Pareto scaling in the tissues and lipid categories in which discrimination between groups A (red) and D (green) was noticed by multivariate analysis. Fatty acids are sorted by similarity of changes. Obvious discriminations between groups are noticed in muscle PL (Fig. 46a) and liver TG concentrations (Fig. 46e). Moderate, but still visible, discrimination is

noticed in muscle PL fatty acid composition (Fig. 46b), liver PL concentration (Fig. 46c) and fatty acid composition (Fig. 46d) and liver TG fatty acid composition (Fig. 46f).

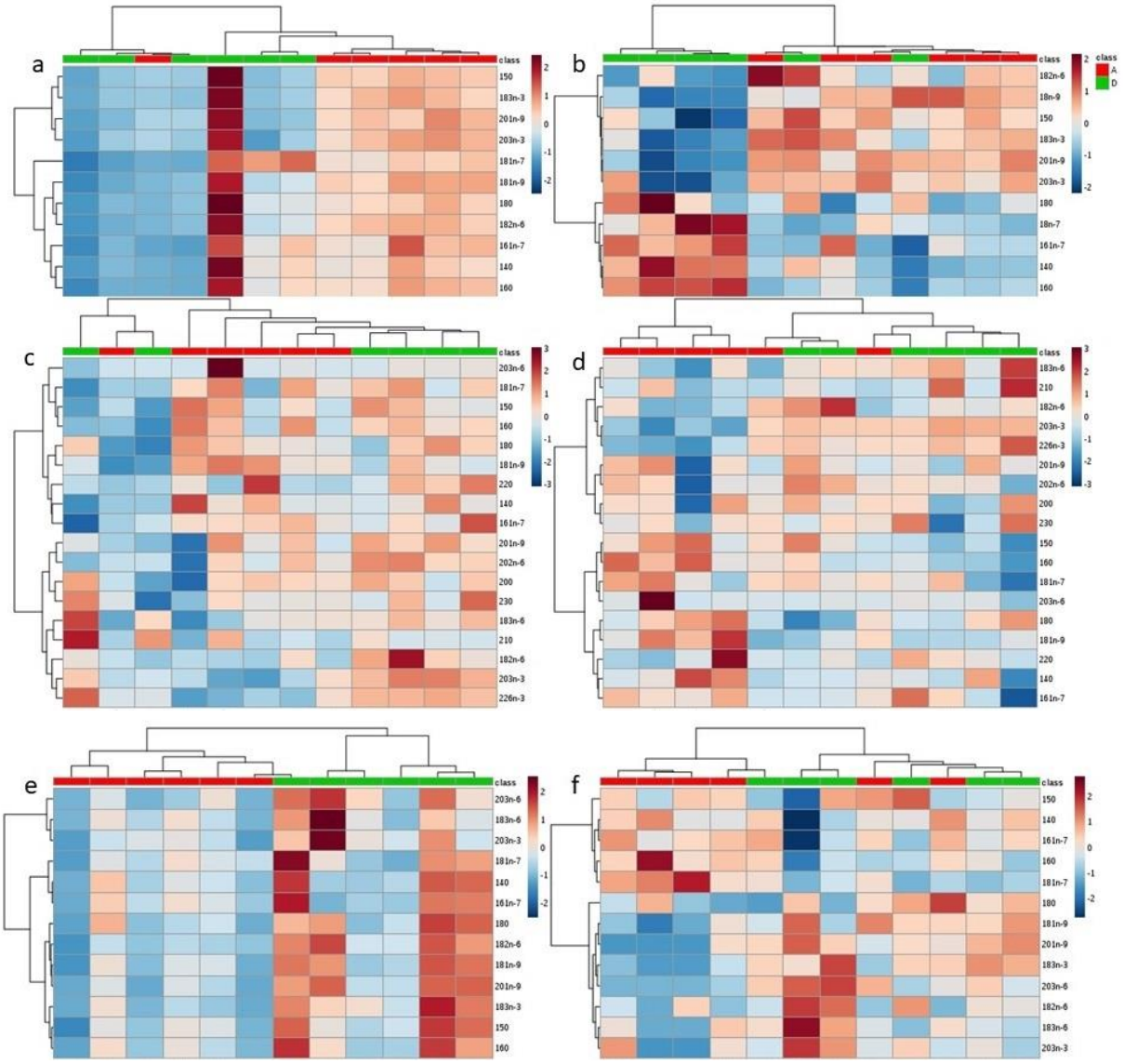


Fig. 46 Heat maps of muscle TG concentrations (a), muscle TG fatty acid composition (b), liver PL concentration (c), liver PL fatty acid composition (d), liver TG concentration (e) and liver TG fatty acid composition (f) of groups A (red) and D (green). Shades of red and blue show higher and lower values, respectively. Data are scaled by Pareto scaling, and motifs are ranked and sorted by Pearson's hierarchical clustering.

Univariate Analyses

The results of independent-sample *t* test analysis are presented below and the corresponding descriptive statistics in Tables 20 to 39.

Table 20 Acyl group content (μmol/g, mean ± SD) of quadriceps PL in groups A and D (at 22 months of age) and groups B and C (at 24 months of age).

Fatty acid	Group A	Group D	Group B	Group C
14:0	0.06 ± 0.04	0.03 ± 0.02	0.05 ± 0.02	0.04 ± 0.02
15:0	0.05 ± 0.01	0.05 ± 0.01	0.06 ± 0.01	0.06 ± 0.02
16:0	6.65 ± 0.58	5.83 ± 0.75	6.83 ± 0.41	6.88 ± 0.28
16:1ω7	0.15 ± 0.06	0.14 ± 0.03	0.15 ± 0.03	0.17 ± 0.04
18:0	3.70 ± 0.40	3.21 ± 0.29	3.85 ± 0.40	3.84 ± 0.44
18:1ω9	1.68 ± 0.47	1.46 ± 0.39	1.50 ± 0.19	1.72 ± 0.45
18:1ω7	0.83 ± 0.11	0.78 ± 0.16	0.82 ± 0.09	0.86 ± 0.07
18:2ω6	3.57 ± 1.03	3.10 ± 0.78	3.98 ± 0.63	4.00 ± 0.38
20:3ω6	0.12 ± 0.03	0.43 ± 0.49	0.13 ± 0.02	0.12 ± 0.03
20:3ω3	2.40 ± 0.44	1.96 ± 0.70	2.34 ± 0.57	2.58 ± 0.25
22:0	0.05 ± 0.01	0.30 ± 0.30	0.05 ± 0.02	0.06 ± 0.03
22:4ω6	0.10 ± 0.03	0.06 ± 0.04	0.08 ± 0.05	0.07 ± 0.02
22:6ω3	1.52 ± 0.55	1.61 ± 0.94	1.25 ± 0.24	1.35 ± 0.21
Total	20.87 ± 2.92	18.96 ± 2.47	21.75 ± 2.17	21.75 ± 1.24

Table 21 Acyl group categories (μmol/g, mean ± SD) and indices of quadriceps PL in groups A and D (at 22 months of age) and groups B and C (at 24 months of age).

Index	Group A	Group D	Group B	Group C
SFA	10.51 ± 1.00	9.42 ± 0.95	10.85 ± 0.71	10.88 ± 0.72
UFA	10.36 ± 2.02	9.54 ± 1.71	10.25 ± 1.57	10.88 ± 0.87
UFA/SFA	0.98 ± 0.11	1.01 ± 0.14	0.94 ± 0.10	1.00 ± 0.09
MUFA	2.66 ± 0.58	2.38 ± 0.53	2.48 ± 0.26	2.76 ± 0.45
PUFA	7.71 ± 1.66	7.17 ± 1.62	7.77 ± 1.37	8.12 ± 0.83
ω6	3.79 ± 1.05	3.60 ± 0.58	4.18 ± 0.67	4.19 ± 0.39
ω3	3.92 ± 0.80	3.57 ± 1.15	3.59 ± 0.80	3.93 ± 0.45
ω6/ω3	0.98 ± 0.21	1.09 ± 0.35	1.19 ± 0.20	1.07 ± 0.05

Table 22 Acyl group molar percentage distribution (mean ± SD) of quadriceps PL in groups A and D (at 22 months of age) and groups B and C (at 24 months of age).

Fatty acid	Group A	Group D	Group B	Group C
14:0	0.27 ± 0.18	0.15 ± 0.12	0.24 ± 0.07	0.18 ± 0.08
15:0	0.26 ± 0.03	0.26 ± 0.04	0.30 ± 0.03	0.27 ± 0.06
16:0	32.06 ± 2.01	30.85 ± 2.38	32.54 ± 2.22	31.65 ± 0.90
16:1ω7	0.68 ± 0.16	0.74 ± 0.10	0.71 ± 0.12	0.80 ± 0.17
18:0	17.79 ± 0.94	17.05 ± 1.20	18.30 ± 1.28	17.65 ± 1.79
18:1ω9	7.99 ± 1.66	7.69 ± 1.97	7.13 ± 0.57	7.88 ± 1.89
18:1ω7	4.01 ± 0.47	4.14 ± 0.89	3.93 ± 0.42	3.98 ± 0.26
18:2ω6	16.84 ± 2.69	16.39 ± 3.80	18.77 ± 1.34	18.40 ± 1.58
20:3ω6	0.56 ± 0.09	2.27 ± 2.52	0.60 ± 0.09	0.55 ± 0.10
20:3ω3	11.47 ± 0.97	10.29 ± 3.07	10.98 ± 1.91	11.88 ± 0.90
22:0	0.22 ± 0.03	1.62 ± 1.57	0.25 ± 0.10	0.29 ± 0.14
22:4ω6	0.48 ± 0.18	0.33 ± 0.19	0.35 ± 0.25	0.30 ± 0.08
22:6ω3	7.37 ± 3.03	8.22 ± 4.04	5.90 ± 0.62	6.19 ± 0.84

Table 23 Acyl group categories (%, mean ± SD) of quadriceps PL in groups A and D (at 22 months of age) and groups B and C (at 24 months of age).

Index	Group A	Group D	Group B	Group C
SFA	50.60 ± 2.83	49.93 ± 3.45	51.63 ± 2.78	50.03 ± 2.37
UFA	49.40 ± 2.83	50.07 ± 3.45	48.37 ± 2.78	49.97 ± 2.37
MUFA	12.68 ± 1.78	12.57 ± 2.68	11.76 ± 0.69	12.65 ± 1.75
PUFA	36.72 ± 3.76	37.50 ± 5.11	36.61 ± 3.01	37.33 ± 3.27
ω6	17.88 ± 2.61	18.99 ± 2.14	19.72 ± 1.44	19.26 ± 1.62
ω3	18.84 ± 3.30	18.51 ± 4.28	16.89 ± 2.45	18.07 ± 1.74

Table 24 Acyl group content (μmol/g, mean ± SD) of quadriceps TG in groups A and D (at 22 months of age) and groups B and C (at 24 months of age).

Fatty acid	Group A	Group D	Group B	Group C
14:0	1.84 ± 0.66	1.62 ± 1.44	0.96 ± 0.32	0.81 ± 0.37
15:0	0.73 ± 0.05	0.21 ± 0.09	0.32 ± 0.12	0.24 ± 0.11
16:0	40.38 ± 13.86	30.93 ± 25.23	18.70 ± 5.03	15.72 ± 5.89
16:1ω7	4.05 ± 1.70	2,95 ± 2.28	1.84 ± 0.31	1.70 ± 0.63
18:0	9.61 ± 3.11	6.41 ± 3.85	5.06 ± 2.11	3.80 ± 1.86
18:1ω9	62.06 ± 11.87	37.55 ± 34.13	24.32 ± 9.11	19.18 ± 8.96
18:1ω7	9.02 ± 1.15	7.91 ± 5.55	4.19 ± 0.86	3.87 ± 1.07
18:2ω6	34.18 ± 4.97	22.113 ± 21.79	13.48 ± 5.36	10.32 ± 5.54
18:3ω3	0.62 ± 0.24	0.15 ± 0.09	0.23 ± 0.07	0.16 ± 0.15
20:1ω9	2.96 ± 0.99	0.78 ± 0.57	1.08 ± 0.43	0.64 ± 0.60
20:3ω3	0.87 ± 0.28	0.27 ± 0.26	0.41 ± 0.06	0.45 ± 0.33
Total	166.32 ± 37.59	110.91 ± 93.40	70.58 ± 23.37	56.89 ± 25.38

Table 25 Acyl group categories (μmol/g, mean ± SD) and indices of quadriceps TG in groups A and D (at 22 months of age) and groups B and C (at 24 months of age).

Index	Group A	Group D	Group B	Group C
SFA	52.56 ± 17.51	39.17 ± 30.55	25.04 ± 7.54	20.58 ± 8.21
UFA	113.76 ± 20.24	71.75 ± 63.13	45.54 ± 15.92	36.31 ± 17.17
UFA/SFA	2.38 ± 0.83	1.76 ± 0.29	1.80 ± 0.15	1.73 ± 0.10
MUFA	78.09 ± 15.42	49.19 ± 41.20	31.43 ± 10.54	25.39 ± 11.19
PUFA	35.67 ± 5.47	22.55 ± 22.07	14.11 ± 5.47	10.93 ± 5.99
ω6	34.18 ± 4.97	22.13 ± 21.79	13.48 ± 5.36	10.32 ± 5.54
ω3	1.50 ± 0.51	0.42 ± 0.34	0.63 ± 0.12	0.61 ± 0.48
ω6/ω3	25.55 ± 10.20	62.41 ± 54.54	20.73 ± 4.48	15.55 ± 1.38

Table 26 Acyl group molar percentage distribution (mean ± SD) of quadriceps TG in groups A and D (at 22 months of age) and groups B and C (at 24 months of age).

Fatty acid	Group A	Group D	Group B	Group C
14:0	1.07 ± 0.22	1.45 ± 0.27	1.38 ± 0.16	1.43 ± 0.06
15:0	0.47 ± 0.14	0.25 ± 0.12	0.44 ± 0.03	0.42 ± 0.04
16:0	23.50 ± 4.40	28.12 ± 2.90	26.96 ± 1.78	28.17 ± 1.53
16:1ω7	2.33 ± 0.62	2.72 ± 0.59	2.73 ± 0.50	3.06 ± 0.32
18:0	5.63 ± 1.01	6.72 ± 1.39	7.05 ± 0.79	6.62 ± 0.26
18:1ω9	37.75 ± 3.03	33.01 ± 3.80	34.00 ± 2.63	33.52 ± 0.77
18:1ω7	5.58 ± 0.88	7.54 ± 2.16	6.18 ± 1.13	7.16 ± 1.26
18:2ω6	21.06 ± 3.02	19.02 ± 2.13	18.81 ± 1.19	17.69 ± 1.30
18:3ω3	0.36 ± 0.10	0.15 ± 0.11	0.32 ± 0.04	0.24 ± 0.14
20:1ω9	1.74 ± 0.35	0.76 ± 0.64	1.51 ± 0.22	0.96 ± 0.55
20:3ω3	0.51 ± 0.08	0.26 ± 0.24	0.61 ± 0.13	0.73 ± 0.36

Table 27 Acyl group categories (%, mean ± SD) of quadriceps TG in groups A and D (at 22 months of age) and groups B and C (at 24 months of age).

Index	Group A	Group D	Group B	Group C
SFA	30.66 ± 5.37	36.54 ± 3.67	35.82 ± 1.88	36.64 ± 1.33
UFA	69.34 ± 5.37	63.46 ± 3.67	64.18 ± 1.88	63.36 ± 1.33
MUFA	47.40 ± 3.16	44.03 ± 2.80	44.43 ± 1.75	44.71 ± 0.86
PUFA	21.94 ± 2.91	19.43 ± 2.09	19.75 ± 1.10	18.65 ± 1.65
ω6	21.06 ± 3.02	19.02 ± 2.13	18.81 ± 1.19	17.69 ± 1.30
ω3	0.87 ± 0.16	0.41 ± 0.34	0.93 ± 0.14	0.97 ± 0.49

Table 28 Acyl group content (μmol/g, mean ± SD) of liver PL in groups A and D (at 22 months of age) and groups B and C (at 24 months of age).

Fatty acid	Group A	Group D	Group B	Group C
14:0	0.20 ± 0.08	0.16 ± 0.08	0.12 ± 0.04	0.13 ± 0.03
15:0	0.14 ± 0.03	0.12 ± 0.04	0.13 ± 0.02	0.12 ± 0.02
16:0	12.65 ± 1.95	11.92 ± 1.89	12.25 ± 1.40	11.72 ± 0.79
16:1ω7	0.69 ± 0.11	0.63 ± 0.27	0.62 ± 0.15	0.68 ± 0.12
18:0	13.04 ± 1.46	12.94 ± 1.85	12.93 ± 1.23	12.48 ± 0.98
18:1ω9	2.80 ± 0.59	2.46 ± 0.38	2.84 ± 0.37	2.78 ± 0.39
18:1ω7	1.72 ± 0.26	1.65 ± 0.28	1.88 ± 0.43	1.98 ± 0.15
18:2ω6	3.93 ± 0.37	4.87 ± 1.07	4.84 ± 0.91	4.41 ± 0.36
18:3ω6	0.04 ± 0.03	0.09 ± 0.02	0.09 ± 0.02	0.09 ± 0.02
20:0	0.04 ± 0.01	0.04 ± 0.01	0.05 ± 0.01	0.04 ± 0.00
20:1ω9	0.06 ± 0.04	0.08 ± 0.02	0.08 ± 0.03	0.08 ± 0.02
20:2ω6	0.06 ± 0.03	0.07 ± 0.02	0.06 ± 0.03	0.07 ± 0.02
21:0	0.05 ± 0.04	0.10 ± 0.06	0.10 ± 0.10	0.06 ± 0.05
20:3ω6	0.45 ± 0.11	0.44 ± 0.14	0.33 ± 0.18	0.46 ± 0.13
20:3ω3	6.19 ± 0.98	8.39 ± 0.93	8.55 ± 1.19	8.93 ± 0.98
22:0	0.19 ± 0.02	0.23 ± 0.04	0.19 ± 0.04	0.18 ± 0.05
23:0	0.08 ± 0.02	0.09 ± 0.05	0.10 ± 0.01	0.08 ± 0.05
22:6ω3	1.90 ± 0.40	2.86 ± 0.36	2.78 ± 0.45	2.75 ± 0.50
Total	44.23 ± 3.77	47.14 ± 6.04	47.95 ± 4.77	47.03 ± 3.52

Table 29 Acyl group categories (μmol/g, mean ± SD) and indices of liver PL in groups A and D (at 22 months of age) and groups B and C (at 24 months of age).

Index	Group A	Group D	Group B	Group C
SFA	26.39 ± 3.30	25.60 ± 3.38	25.88 ± 2.41	24.80 ± 1.71
UFA	17.84 ± 1.15	21.54 ± 2.78	22.07 ± 2.77	22.23 ± 2.04
UFA/SFA	0.68 ± 0.09	0.84 ± 0.04	0.85 ± 0.08	0.90 ± 0.05
MUFA	5.27 ± 0.83	4.82 ± 0.74	5.41 ± 0.87	5.52 ± 0.52
PUFA	12.57 ± 1.28	16.72 ± 2.15	16.65 ± 2.18	16.71 ± 1.81
ω6	4.48 ± 0.41	5.47 ± 1.16	5.33 ± 1.01	5.03 ± 0.45
ω3	8.09 ± 1.25	11.25 ± 1.16	11.33 ± 1.57	11.68 ± 1.38
ω6/ω3	0.56 ± 0.10	0.48 ± 0.07	0.47 ± 0.09	0.43 ± 0.02

Table 30 Acyl group molar percentage distribution (mean ± SD) of liver PL in groups A and D (at 22 months of age) and groups B and C (at 24 months of age).

Fatty acid	Group A	Group D	Group B	Group C
14:0	0.45 ± 0.15	0.34 ± 0.14	0.26 ± 0.09	0.27 ± 0.07
15:0	0.31 ± 0.04	0.26 ± 0.06	0.27 ± 0.05	0.25 ± 0.05
16:0	28.47 ± 2.26	25.23 ± 1.74	25.53 ± 1.23	24.95 ± 1.41
16:1ω7	1.57 ± 0.19	1.33 ± 0.54	1.28 ± 0.27	1.46 ± 0.22
18:0	29.46 ± 1.75	27.47 ± 2.34	27.04 ± 1.92	26.54 ± 0.44
18:1ω9	6.30 ± 1.17	5.21 ± 0.31	5.93 ± 0.71	5.91 ± 0.78
18:1ω7	3.87 ± 0.39	3.52 ± 0.49	3.90 ± 0.67	4.22 ± 0.33
18:2ω6	8.93 ± 0.99	10.28 ± 1.31	10.07 ± 1.50	9.38 ± 0.27
18:3ω6	0.10 ± 0.06	0.19 ± 0.05	0.19 ± 0.05	0.18 ± 0.05
20:0	0.09 ± 0.02	0.09 ± 0.02	0.11 ± 0.03	0.09 ± 0.01
20:1ω9	0.15 ± 0.08	0.17 ± 0.04	0.16 ± 0.05	0.17 ± 0.03
20:2ω6	0.13 ± 0.07	0.16 ± 0.04	0.13 ± 0.06	0.14 ± 0.03
21:0	0.11 ± 0.09	0.23 ± 0.15	0.22 ± 0.22	0.12 ± 0.11
20:3ω6	1.01 ± 0.22	0.92 ± 0.24	0.68 ± 0.36	0.98 ± 0.24

20:3ω3	14.07 ± 2.49	17.85 ± 0.56	17.81 ± 1.52	18.95 ± 0.90
22:0	0.43 ± 0.04	0.48 ± 0.05	0.40 ± 0.08	0.39 ± 0.09
23:0	0.19 ± 0.04	0.19 ± 0.11	0.21 ± 0.02	0.17 ± 0.10
22:6ω3	4.36 ± 1.19	6.10 ± 0.78	5.79 ± 0.70	5.84 ± 0.88

Table 31 Acyl group categories (%, mean ± SD) of liver PL in groups A and D (at 22 months of age) and groups B and C (at 24 months of age).

Index	Group A	Group D	Group B	Group C
SFA	59.51 ± 3.02	54.29 ± 1.23	54.04 ± 2.35	52.77 ± 1.50
UFA	40.49 ± 3.02	45.71 ± 1.23	45.96 ± 2.35	47.23 ± 1.50
MUFA	11.89 ± 1.32	10.23 ± 0.80	11.27 ± 1.30	11.75 ± 0.97
PUFA	28.60 ± 3.97	35.49 ± 1.20	34.68 ± 1.94	35.47 ± 1.77
ω6	10.17 ± 1.05	11.54 ± 1.33	11.08 ± 1.60	10.68 ± 0.31
ω3	18.43 ± 3.51	23.94 ± 1.12	23.60 ± 1.99	24.79 ± 1.52

Table 32 Acyl group content (μmol/g, mean ± SD) of liver TG in groups A and D (at 22 months of age) and groups B and C (at 24 months of age).

Fatty acid	Group A	Group D	Group B	Group C
14:0	0.30 ± 0.14	0.50 ± 0.28	0.33 ± 0.14	0.33 ± 0.21
15:0	0.11 ± 0.05	0.20 ± 0.07	0.14 ± 0.03	0.15 ± 0.10
16:0	8.49 ± 3.36	14.95 ± 6.66	9.93 ± 2.05	11.49 ± 3.60
16:1ω7	1.27 ± 0.57	1.91 ± 1.29	1.18 ± 0.44	1.67 ± 0.92
18:0	0.89 ± 0.49	1.65 ± 0.69	1.23 ± 0.48	1.71 ± 0.69
18:1ω9	6.95 ± 3.52	14.07 ± 5.84	10.19 ± 2.95	13.68 ± 8.02
18:1ω7	1.94 ± 0.52	2.76 ± 1.33	2.16 ± 0.63	2.98 ± 1.38
18:2ω6	2.66 ± 0.96	6.78 ± 2.27	5.09 ± 1.78	5.44 ± 3.02
18:3ω6	0.04 ± 0.03	0.09 ± 0.07	0.07 ± 0.02	0.09 ± 0.05
18:3ω3	0.03 ± 0.03	0.11 ± 0.05	0.06 ± 0.04	0.07 ± 0.07
20:1ω9	0.09 ± 0.10	0.36 ± 0.17	0.19 ± 0.20	0.25 ± 0.19

20:3ω6	0.02 ± 0.03	0.08 ± 0.04	0.04 ± 0.04	0.06 ± 0.05
20:3ω3	0.17 ± 0.14	0.45 ± 0.25	0.37 ± 0.15	0.37 ± 0.15
Total	22.96 ± 9.64	43.91 ± 17.74	30.97 ± 8.18	38.30 ± 17.48

Table 33 Acyl group categories (μmol/g, mean ± SD) and indices of liver TG in groups A and D (at 22 months of age) and groups B and C (at 24 months of age).

Index	Group A	Group D	Group B	Group C
SFA	9.80 ± 3.98	17.30 ± 7.56	11.63 ± 2.62	13.68 ± 4.15
UFA	13.16 ± 5.73	26.61 ± 10.41	19.34 ± 5.62	24.62 ± 13.41
UFA/SFA	1.32 ± 0.20	1.56 ± 0.21	1.64 ± 0.18	1.71 ± 0.38
MUFA	10.25 ± 4.63	19.09 ± 8.17	13.71 ± 3.84	18.58 ± 10.24
PUFA	2.91 ± 1.14	7.51 ± 2.57	5.64 ± 1.95	6.03 ± 3.23
ω6	2.72 ± 1.01	6.95 ± 2.35	5.21 ± 1.81	5.59 ± 3.09
ω3	0.20 ± 0.16	0.56 ± 0.26	0.43 ± 0.18	0.45 ± 0.20
ω6/ω3	10.92 ± 2.01	13.09 ± 3.52	12.85 ± 3.35	12.88 ± 3.95

Table 34 Acyl group molar percentage distribution (mean ± SD) of liver TG in groups A and D (at 22 months of age) and groups B and C (at 24 months of age).

Fatty acid	Group A	Group D	Group B	Group C
14:0	1.33 ± 0.15	1.12 ± 0.31	1.07 ± 0.30	0.84 ± 0.28
15:0	0.49 ± 0.04	0.47 ± 0.08	0.46 ± 0.08	0.38 ± 0.11
16:0	37.77 ± 3.81	33.82 ± 2.95	32.59 ± 2.68	31.25 ± 3.66
16:1ω7	5.46 ± 0.51	4.15 ± 1.64	3.89 ± 1.21	4.23 ± 0.78
18:0	3.83 ± 0.77	3.78 ± 0.67	3.89 ± 0.70	4.95 ± 2.55
18:1ω9	29.05 ± 4.05	31.77 ± 2.83	32.69 ± 1.87	34.40 ± 4.00
18:1ω7	9.06 ± 2.00	6.21 ± 1.06	6.99 ± 1.15	7.93 ± 2.61
18:2ω6	11.79 ± 1.95	16.11 ± 2.85	16.14 ± 2.18	13.85 ± 2.07
18:3ω6	0.13 ± 0.10	0.21 ± 0.15	0.25 ± 0.06	0.22 ± 0.13
18:3ω3	0.09 ± 0.08	0.25 ± 0.06	0.18 ± 0.10	0.16 ± 0.10

20:1ω9	0.27 ± 0.30	0.79 ± 0.17	0.55 ± 0.48	0.61 ± 0.31
20:3ω6	0.07 ± 0.09	0.19 ± 0.10	0.13 ± 0.09	0.16 ± 0.11
20:3ω3	0.65 ± 0.58	1.12 ± 0.55	1.19 ± 0.39	1.01 ± 0.32

Table 35 Acyl group categories (%, mean ± SD) of liver TG in groups A and D (at 22 months of age) and groups B and C (at 24 months of age).

Index	Group A	Group D	Group B	Group C
SFA	43.42 ± 4.09	39.19 ± 2.91	38.01 ± 2.63	37.42 ± 5.03
UFA	56.58 ± 4.09	60.81 ± 2.91	61.99 ± 2.63	62.58 ± 5.03
MUFA	43.84 ± 3.15	42.93 ± 2.30	44.12 ± 2.31	47.18 ± 3.88
PUFA	12.73 ± 1.93	17.88 ± 3.53	17.88 ± 2.54	15.40 ± 2.26
ω6	11.99 ± 1.89	16.51 ± 3.00	16.51 ± 2.22	14.22 ± 2.20
ω3	0.74 ± 0.62	1.37 ± 0.55	1.37 ± 0.45	1.17 ± 0.33

Table 36 Acyl group content (μmol/g, mean ± SD) of brain PL in groups A and D (at 22 months of age) and groups B and C (at 24 months of age)

Fatty acid	Group A	Group D	Group B	Group C
14:0	0.23 ± 0.12	0.20 ± 0.05	0.30 ± 0.11	0.23 ± 0.03
15:0	0.13 ± 0.02	0.14 ± 0.05	0.16 ± 0.03	0.14 ± 0.01
16:0	25.72 ± 2.76	24.44 ± 0.51	28.01 ± 2.37	28.94 ± 1.82
16:1ω7	0.42 ± 0.06	0.38 ± 0.05	0.49 ± 0.02	0.48 ± 0.02
18:0	26.66 ± 3.83	25.89 ± 1.05	28.91 ± 3.16	30.13 ± 2.31
18:1ω9	18.44 ± 2.80	17.36 ± 0.37	20.63 ± 2.09	20.67 ± 1.14
18:1ω7	5.36 ± 0.82	5.18 ± 0.32	6.01 ± 0.81	5.94 ± 0.40
18:2ω6	0.37 ± 0.09	0.38 ± 0.10	0.53 ± 0.08	0.50 ± 0.08
20:0	0.57 ± 0.15	0.53 ± 0.08	0.61 ± 0.07	0.62 ± 0.11
20:1ω9	1.72 ± 0.41	1.65 ± 0.19	1.97 ± 0.31	1.88 ± 0.20
20:3ω6	0.23 ± 0.06	0.25 ± 0.06	0.26 ± 0.07	0.25 ± 0.07
20:3ω3	5.79 ± 0.39	5.91 ± 0.69	6.13 ± 0.91	7.15 ± 0.46

20:5ω3	0.50 ± 0.09	0.50 ± 0.06	0.62 ± 0.09	0.60 ± 0.08
24:0	0.94 ± 0.14	0.94 ± 0.13	1.06 ± 0.08	1.13 ± 0.11
22:6ω3	6.03 ± 0.86	6.57 ± 1.16	6.72 ± 1.90	7.42 ± 1.15
Total	93.08 ± 11.34	90.34 ± 2.99	102.41 ± 10.49	106.07 ± 5.12

Table 37 Acyl group categories (μmol/g, mean ± SD) and indices of brain PL in groups A and D (at 22 months of age) and groups B and C (at 24 months of age).

Index	Group A	Group D	Group B	Group C
SFA	54.24 ± 6.86	52.14 ± 1.50	59.06 ± 5.11	61.19 ± 4.00
UFA	38.84 ± 4.55	38.20 ± 1.80	43.35 ± 5.58	44.89 ± 1.59
UFA/SFA	0.72 ± 0.02	0.73 ± 0.03	0.73 ± 0.04	0.74 ± 0.04
MUFA	25.94 ± 3.93	24.58 ± 0.76	29.09 ± 3.17	28.97 ± 1.33
PUFA	12.91 ± 1.10	13.62 ± 1.79	14.26 ± 2.75	15.92 ± 1.61
ω6	0.59 ± 0.11	0.63 ± 0.11	0.79 ± 0.11	0.75 ± 0.08
ω3	12.31 ± 1.11	12.99 ± 1.78	13.47 ± 2.73	15.17 ± 1.55
ω6/ω3	0.05 ± 0.01	0.05 ± 0.01	0.06 ± 0.01	0.05 ± 0.00

Table 38 Acyl group molar percentage distribution (mean ± SD) of brain PL in groups A and D (at 22 months of age) and groups B and C (at 24 months of age).

Fatty acid	Group A	Group D	Group B	Group C
14:0	0.24 ± 0.12	0.22 ± 0.06	0.30 ± 0.12	0.22 ± 0.03
15:0	0.14 ± 0.03	0.15 ± 0.05	0.16 ± 0.04	0.13 ± 0.01
16:0	27.68 ± 0.74	27.07 ± 0.55	27.42 ± 1.50	27.27 ± 0.60
16:1ω7	0.45 ± 0.04	0.43 ± 0.06	0.48 ± 0.06	0.46 ± 0.03
18:0	28.58 ± 0.76	28.66 ± 0.72	28.22 ± 1.04	28.38 ± 0.97
18:1ω9	19.79 ± 1.27	19.23 ± 0.61	20.16 ± 1.02	19.52 ± 1.46
18:1ω7	5.74 ± 0.32	5.73 ± 0.29	5.86 ± 0.32	5.59 ± 0.13
18:2ω6	0.40 ± 0.11	0.42 ± 0.11	0.52 ± 0.11	0.47 ± 0.07
20:0	0.60 ± 0.09	0.59 ± 0.08	0.60 ± 0.09	0.59 ± 0.11

20:1ω9	1.83 ± 0.23	1.83 ± 0.19	1.92 ± 0.21	1.78 ± 0.23
20:3ω6	0.24 ± 0.04	0.28 ± 0.06	0.26 ± 0.06	0.24 ± 0.06
20:3ω3	6.26 ± 0.47	6.54 ± 0.68	5.97 ± 0.49	6.75 ± 0.35
20:5ω3	0.53 ± 0.06	0.56 ± 0.06	0.61 ± 0.07	0.57 ± 0.09
24:0	1.01 ± 0.11	1.04 ± 0.13	1.04 ± 0.08	1.07 ± 0.11
22:6ω3	6.52 ± 0.94	7.26 ± 1.12	6.48 ± 1.23	6.98 ± 0.88

Table 39 Acyl group categories (%, mean ± SD) of brain PL in groups A and D (at 21 months of age) and groups B and C (at 24 months of age).

Index	Group A	Group D	Group B	Group C
SFA	58.24 ± 0.72	57.73 ± 0.88	57.74 ± 1.36	57.65 ± 1.28
UFA	41.76 ± 0.72	42.27 ± 0.88	42.26 ± 1.36	42.35 ± 1.28
MUFA	27.81 ± 1.43	27.22 ± 0.84	28.42 ± 1.48	27.35 ± 1.65
PUFA	13.95 ± 1.14	15.05 ± 1.66	13.84 ± 1.31	15.00 ± 1.16
ω6	0.64 ± 0.11	0.70 ± 0.10	0.78 ± 0.11	0.71 ± 0.05
ω3	13.31 ± 1.18	14.35 ± 1.67	13.06 ± 1.39	14.29 ± 1.14

No differences were found in the acyl group content or percentage distribution in muscle PLs between groups A and D. However, differences were noticed in muscle TGs. Specifically, the concentration of 15:0 was lower in group D (the group that exercised during the 2nd half) than in group A (the group that exercised during the 1st half, $p < 0.001$, ES = 7.14). Group D also had lower concentrations of 18:3ω3 ($p = 0.001$, ES = 2.59), 20:3ω3 ($p = 0.003$, ES = 2.22) and 20:1ω9 ($p = 0.001$, ES = 2.70). The percentage of 18:3ω3 was also lower in the same group ($p = 0.005$, ES = 2.00), as was the total content of ω3 fatty acids ($p = 0.002$, ES = 2.49).

Differences were also noticed in the acyl group content and distributions in both liver PLs and TGs. Specifically, in liver PLs, the contents of 20:3ω3 ($p = 0.003$, ES = 2.30) and 22:6ω3 ($p = 0.002$, ES = 2.52) were higher in group D than in group A. Also, the total content and percentage of ω3 FAs ($p = 0.001$, ES = 2.62 and $p = 0.004$, ES = 2.12, respectively) and PUFAs ($p = 0.00$, ES = 2.35 in both) was higher in group D. The total SFA content was lower ($p = 0.003$, ES = 0.24), whereas the total UFA content was higher in group D ($p = 0.003$, ES = 1.74). Finally, the UFA/SFA index was higher in group D ($p = 0.002$, ES = 2.30).

As far as the liver TGs are concerned, the content of 18:2ω6 (p = 0.005, ES = 2.36) and the content and percentage of 18:3ω3 (p = 0.004, ES = 1.94 and p = 0.003, ES = 2.26, respectively) were higher in group D than in group A. Also, the percentage of 20:1ω9 was higher in group D (p = 0.007, ES = 2.13). In support of the findings from the multivariate analysis, the percentages of 18:1ω7 (p = 0.012, ES = 1.78) and 18:2ω6 (p = 0.012, ES = 1.77) were found significantly different between groups, although they were excluded by the FDR correction. Finally, the total contents of ω6 FAs (p = 0.005, ES = 2.34) and PUFAs (p = 0.002, ES = 2.31) were higher in group D.

No differences were found in brain PLs; and unfortunately, 3 months of detraining resulted in no differences between groups B and C in any of the three tissues and their lipid categories.

DISCUSSION

In the present thesis the effects of long-term and lifelong moderate-intensity exercise on rat blood and urinary metabolites were investigated through a ^{1}H NMR- and an LC-MS-based approach, respectively. Moreover, the effects of acute exercise on rat urinary metabolites at a younger (3 months) and older (12 months) age were examined through LC-MS. Furthermore, we studied the effect of lifelong moderate-intensity exercise on insulin sensitivity of aged rats and when, after an acute bout of exercise, the effect wears off. Finally, the effects of long-term and lifelong exercise on the quadriceps, liver and brain fatty acid contents were investigated.

The relevance of this study lies in the increasing life expectancy observed over the last decades, which has resulted in increased prevalence of physical and mental diseases, such as CVD, inflammation, obesity, T2D and dementia, attributed largely to reduced physical activity levels due to the adoption of technologies that promote a sedentary lifestyle. Thus, documenting affordable or even cost-free ways of improving health is becoming increasingly important. One of these ways is exercise, and studies identifying its effects are essential for the development of new non-pharmaceutical strategies that will target these diseases.

The main strength of this study is that it monitored the interplay of aging and lifelong exercise on the same animals throughout most of their lifetime (18 out of about 24 months). The combination of a longitudinal design and a genetically homogeneous population eliminates much of the confounding effect of inter-individual variability and contributes to the validity of the findings.

Moderate-intensity exercise was chosen for this study in order to make the findings more applicable to the general population, especially when it comes to elderly individuals, since too strenuous interventions are almost impossible to apply to aged or frail adults. Studies have shown that moderate-intensity exercise is related to reduction in oxidative stress levels (Liguori et al. 2018) and may also confer improvements and positive adaptation of muscle function through activation of antioxidant enzymes and redox-regulated enzyme reactions (Navas-Enamorado et al. 2017). Other studies have also shown effects of mild- to moderate-intensity exercise on insulin sensitivity in young or aged humans and experimental animals (Frøsig & Richter 2009; Houmard et al. 2004).

By comparing a lifelong exercise group, two long-term exercise groups, and a group that did not exercise, we found that exercise training mainly affected body weight, food intake, and the

concentrations of several metabolites as well as fatty acids. However, the effects of aging were generally stronger than those of long-term and lifelong training. All effects will be discussed below.

Body Weight and Food Intake

Exercise seemed to play a beneficial role on body weight during the 2nd half of life, as evidenced by the finding that the animals which exercised during that period weighed less than those that did not. This can be explained by the finding of lower food consumption by the exercising animals despite higher energy expenditure. Thus, it appears that exercise had an anorexigenic effect during the 2nd half, which contrasts with its observed orexigenic effect during the 1st half. Interestingly, Garvey et al. (2015) also found that exercise (for 8 weeks) decreased body weight gain and food intake in older (16–17 months) rats, compared to non-exercising rats.

Insulin Sensitivity Test

Insulin sensitivity is associated with obesity and T2D. Physical exercise may, indeed, prevent or reduce the deleterious effects of T2D (Fernandes & Zanesco 2010). Studies in the past have shown that insulin sensitivity increases after acute, short-term or long-term exercise. Specifically, a 35% increase in middle-aged obese men 1 h after high-intensity acute exercise (Levinger et al. 2014) and a 36% increase in older men 60 h after the last session of long-term high-intensity exercise training (Kahn et al. 1990) have been reported.

In this study, moderate-intensity training did not alter insulin sensitivity. Thus, exercise does not appear to exert a chronic effect on insulin sensitivity in aged rats. This may seem to contradict the findings of other studies that claimed an effect of long-term exercise in either young or aged humans (Goodpaster et al. 2003; King et al. 1995). However, methodological differences may explain this contradiction, as those studies measured insulin sensitivity 1.5-3 days after the last exercise session, which raises the possibility that what they considered a training effect was actually a residual effect of the last exercise bout. In the present study, we also explored the relationship between insulin sensitivity and body weight, since Facchini et al. (2001) mention that differences in insulin-mediated glucose disposal are explained by differences in weight by 25% and by differences in habitual physical activity by another 25%. Nevertheless, we found no relationship between insulin sensitivity and weight, probably because the experimental animals did not suffer from any pathological conditions such as obesity or T2D.

Regarding the second aim of the insulin sensitivity study in this thesis (that of examining the

duration of the acute effect of exercise on insulin sensitivity), this has been investigated by others in a number of ways. It has been reported that the effect of a single exercise session in either young humans or rats can last from several hours (Goodyear & Kahn 1998; McConell et al. 2015) to a few days (Henriksson 1995; Newsom & Schenk 2014). Older research in humans showed that the training effect disappeared between 38 h and 10 days after the last exercise session of a training program (Heath et al. 1983). Hence, it is not surprising that a study in which measurements were conducted 72 h after the last exercise bout of a training program did not find a significant change in insulin sensitivity (Suskin et al. 2007). Another limitation of such research was that different groups of animals were used to examine how long the effect of exercise on insulin sensitivity lasts (Nagasawa et al. 1995), which may introduce inter-individual variability as a confounding factor. In contrast, in this study, we used the same animals to examine this question and found that the effect of exercise on insulin sensitivity wore off at between 3 and 5 days post-exercise.

The mechanisms regulating the increased insulin-stimulated glucose uptake in muscle for several days after acute exercise are not fully understood. Exercise may initiate several signalling cascades for glucose uptake in muscle, the most important being the PI3K signalling pathway (Leto & Saltiel 2012). Two adaptive responses that underlie the increase in insulin sensitivity are an upregulation of GLUT4 and increased GLUT4 translocation to the plasma membrane. AS160 and aPKC are likely involved in this translocation (Frøsig & Richter 2009). A review by Cartee (2015) notes that, although exercise can increase glucose uptake by stimulating the translocation of GLUT4 from the cell interior to the cell surface, “the specific insulin-stimulated GLUT4 trafficking steps that are influenced by prior exercise remain to be identified.” The author proposes the presence of “memory elements” for maintaining insulin sensitivity enhanced up to 48 hours post-exercise, one of which may be the sustained low muscle glycogen concentration. This finding is supported by research showing that increased insulin sensitivity is mainly observed in glycogen-depleted muscle due to a local contraction-induced mechanism (Frøsig & Richter 2009). However, more research needs to be done in order to establish such a relationship.

Effects of Acute Exercise on the Urinary Metabolome

Both acute exercise and aging elicited changes in about half of the identified metabolites, involved in different metabolic pathways. The results of both multivariate and univariate analyses are summarised in Fig. 47. This figure serves as a “bird’s eye view” of the combined metabolic effects of aging (colored metabolites) and exercise (circled metabolites). The effects of aging and acute exercise on urinary metabolites involved in major metabolic pathways will be discussed below.

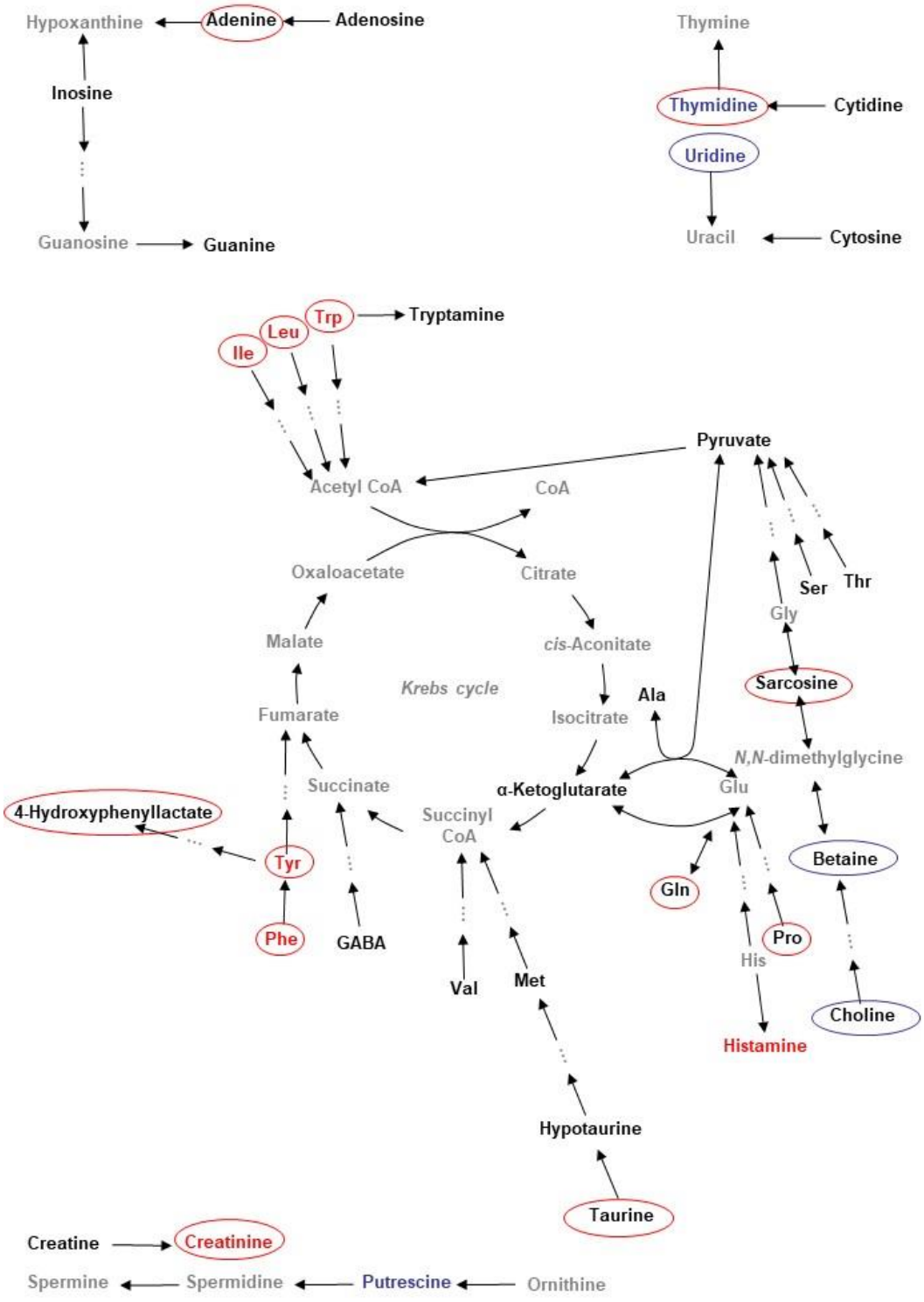


Fig. 47 Map of metabolic pathways with indication of urinary metabolites affected by age and/or acute exercise. Metabolites in red, blue, or black increased, decreased, or did not change with aging, respectively. Metabolites surrounded by red or blue ellipses increased or decreased with exercise, respectively. Metabolites in grey were not identified.

Amino Acids and Amino Acid Derivatives

Both aging and exercise seemed to have augmentative effects on amino acid concentrations. Specifically, exercise resulted in increases in two non-essential, glucogenic amino acids, that is, glutamine and proline, as well as in a non-protein amino acid, sarcosine. Glutamine is negatively associated with aging and is essential for the maintenance of skeletal muscle function and the prevention of sarcopenia (Dato et al. 2019; Houtkooper et al. 2011; Johnson et al. 2018). Glutamine was also found increased following high-intensity interval training in healthy men, which was attributed to higher protein degradation (Siopi et al. 2017). Ammonia is indeed found elevated during exercise and this increase is related to lactate concentration (Mougios 2020).

Sarcosine is an intermediate in the synthesis of glycine from choline, which was found decreased following acute exercise. Another intermediate in the same pathway, which was found decreased following exercise, is betaine. The previous findings may suggest that acute exercise resulted in increased flow of choline and betaine toward sarcosine. Unfortunately, glycine was not identified in the samples analysed by LC-MS and, therefore, it cannot be verified whether this resulted in increased glycine production as well. Past research, however, found decreased excretion of glycine following acute exercise, although its levels rose from 1 to 1.5 h post-exercise (Pechlivanis et al. 2015). Sarcosine, however, has also been identified as a potential biomarker of aging, since its values seem to decrease with age (Walters et al. 2018). It increased, however, in the blood of healthy male humans in response to long-term exercise (Felder et al. 2017). Therefore, the exercise-induced increase of sarcosine could suggest a protective role of exercise against aging. Betaine, which was found decreased following acute exercise, was also found decreased in the blood of male humans with metabolic syndrome following three different modes of exercise (high-intensity interval exercise, resistance exercise and continuous moderate-intensity exercise, Siopi et al. 2019). Choline was found decreased following acute exercise in the present study in both multivariate and univariate analyses. However, it was found increased in the urine of older men following long-term exercise (Sheedy et al. 2014). These contradictory findings could be the result of the difference between the effects of acute exercise and long-term training.

Exercise at 12 months resulted in increases in isoleucine-leucine, according to the multivariate analysis employed in the present study. Increased levels of isoleucine and leucine have been found in the blood of aged individuals (Houtkooper et al. 2011) and in individuals with low muscle quality (Moaddel et al. 2016). This increase may be attributed to impaired mitochondrial activity and transport of amino acids to and from the aged muscle. The rise of these two metabolites in the blood may have also been reflected in the urine, as is the case

in the present study. Although exercise is thought to promote BCAA catabolism (Daskalaki et al. 2015; Pechlivanis et al. 2010), in this study it appears that BCAA catabolism is diminished with age, even when exercise is involved. This may either suggest a larger effect of aging than that of exercise on BCAAs or it could imply that the exercise intensity used was not high enough to affect BCAA metabolism. A study by Sheedy et al. (2014) also showed increases in isoleucine and leucine in older men, although long-term training was involved.

Three aromatic amino acids, which serve as brain neurotransmitters, that is, phenylalanine, tryptophan and tyrosine, also increased due to both aging and exercise. Exercise also resulted in an increase in 4-hydroxyphenyllactate, a tyrosine metabolite. This last finding is in agreement with another study that also observed post-exercise increases in the hydroxy-derivatives of aromatic amino acids (Pechlivanis et al. 2010). The increases in the three amino acids, however, contradict the results of studies that show decreased concentrations with increasing age (Lustgarten & Fielding 2017; Refaey et al. 2017) and following acute exercise (Pechlivanis et al. 2010). Tryptophan is the only metabolite that has also been found increased in older men, although after long-term exercise (Sheedy et al. 2014). Histamine, synthesised from the aromatic amino acid histidine, was found increased with age only in the multivariate analysis, suggesting increased activity of histidine carboxylase, the enzyme that catalyses this reaction. However, no studies could be found with which to compare these results.

Carbohydrate and Lipid Metabolism

Mannitol, which decreased with age, is found in many foods, and its decrease is possibly the result of changes in either food intake or intestinal permeability. This finding is in agreement with Valentine et al. (2014), who also found decreased urinary mannitol in aged adults. Acetylcarnitine was found increased following acute exercise, in both multivariate and univariate analyses. Increased excretion of acetylcarnitine was also observed in female rats following 16 months of swim-training (Deda et al. 2017) and 4 h following high-intensity interval training in healthy male humans (Siopi et al. 2017). The latter was attributed to increased activity of carnitine *O*-acetyltransferase, which catalyses the conversion of acetylCoA and carnitine to acetylcarnitine and CoA. Short-duration, high-intensity exercise has also been shown to increase the acetylcarnitine content of both human and animal muscle in an older study (Hiatt et al. 1989). Acetylcarnitine is also formed when there is abundant acetyl CoA due to increased carbohydrate or lipid oxidation (Mougios 2020). Since both carbohydrate and lipid oxidation are the sources of energy during low- to moderate-intensity exercise, which was the case in the present study, the previous statement could justify the aforementioned findings.

Purine and Pyrimidine Metabolism

Urinary adenine was found increased following acute exercise in the present study. Adenine was also found increased in the urine of female humans 2 h after eccentric exercise (Jang et al. 2018). Uridine was found decreased due to both aging and acute exercise in the present study, whereas in another study it was found increased in blood following a single maximal exercise on bicycle ergometer (Dudzinska et al. 2013). The increase in blood uridine has been associated with increases in insulin and glucose concentrations and the development of insulin resistance (Daskalaki et al. 2014). There was an interesting fluctuation in thymidine, which was found decreased in the 12-month old rats but increased post exercise. This increase could be a favourable change, since it may imply that there is a lower loss of pyrimidine metabolites. However, no studies could be found to compare these results with.

Gut Microbiome Metabolism

TMAO has been associated with atherosclerosis, CVD and obesity (Wishart 2019). In the present study TMAO was found decreased both post-exercise and in the middle-aged rats. The decrease in the urinary excretion of TMAO could be attributed to exercise-induced decrease in intestinal blood circulation, resulting in its lower absorption. TMAO was also found decreased after exercise in other studies, and this decrease has been related to health benefits (Enea et al. 2010; Pechlivanis et al. 2015). In another study, however, it was found increased following high-intensity exercise, and this increase was attributed to food intake, specifically to consumption of seafood, which is known to increase TMAO concentration in urine (Wang et al. 2015). Contrary to the findings of the present study, increased excretion of TMAO was observed at older ages, possibly due to impairment in renal activity and compromised reabsorption (Psichogios et al. 2008). A progressive increase with age in TMAO was also seen in rats, and this change was attributed to the degradation of dietary choline by the gut microbiota, whose activity varies with aging (Wu et al. 2008). The findings of these studies could imply that at 12 months of age the rats of the present study had not reached the age at which aging-induced changes occur.

Other Metabolites

Creatinine was found increased in the urine of the middle-aged rats and post-exercise. Increased urinary creatinine was also found in rats from the 4th to the 18th month of age (Wu et al. 2008). The increase in creatinine excretion post-exercise is in agreement with Neal et al. (2013), who found higher concentrations of the same metabolite in humans following moderate-intensity exercise.

Methylamine excretion was found decreased both due to aging and following acute exercise. Methylamine also exhibited a progressive decrease from the 4th to the 24th month of age of rats (Wu et al. 2008). A study by Sheedy et al. (2014), however, found increased concentrations of the same metabolite after long-term exercise training in older men. Hence, the excretion of methylamine may depend on exercise duration and frequency. Methylamine subsequently produces formaldehyde, and both metabolites are considered cytotoxic, causing even kidney damage (Poortmans et al. 2005). Therefore, the exercise-induced reduction found in this study may imply a protective role against the accumulation of these two toxic metabolites, possibly caused by the ingestion of food rich in methylamine or formaldehyde.

Thiamine, or vitamin B_1, was found decreased after acute exercise and due to aging, in both multivariate and univariate analyses. Its excretion was not found altered with age in a previous study (Fukuwatari et al. 2008). However, more research states that thiamine deficiency is common among older adults, and it is associated with heart failure (Nazmi et al. 2014). Its exercise-induced decrease, however, may indicate its use in several metabolic pathways activated by exercise or its decreased absorption due to lowered intestinal blood circulation. Our finding is supported by a study in which, although urinary thiamine levels increased within 5 weeks of training, the levels in the exercising group were lower compared to those in the non-exercising group (Kim et al. 2015).

Taurine, which is abundant in muscle, was found increased following acute exercise. It was also found increased in the urine of athletes immediately after a marathon run (Cuisinier et al. 2001) and in blood following strenuous exercise (Chorell et al. 2012). Its blood concentration has been negatively associated with obesity (Brennan et al. 2018). The exercise-induced increase, however, could indicate increased oxidative stress or could be related to muscle damage (Chorell et al. 2012), which could not be the case in the present study, since moderate-intensity exercise was conducted.

Putrescine, a polyamine involved in many cell functions, was found decreased with aging in the multivariate analysis only. Declines with age were also observed in the blood of humans with low muscle quality (Moaddel et al. 2016). An older study did not find any changes in the excretion of putrescine with age; however, they found very large diurnal variations (Pöyhönen et al. 1990).

In summary, more than half of the findings were expected. However, there were some unexpected findings regarding the effects of exercise and aging on specific metabolites, such as the aromatic amino acids, suggesting that exercise intensity and duration were not sufficient to produce significant changes and that rats at 12 months had not reached the age at which

aging-induced alterations could be observed. However, as far as exercise is concerned, the specific protocol was deliberately chosen for pragmatical reasons that will be discussed below. Nevertheless, even moderate-intensity exercise seemed to result in increases in creatinine, a metabolite associated with muscle mass.

Effects of Long-Term and Lifelong Exercise on the Urinary Metabolome

Both exercise training and aging elicited changes in more than half of the identified metabolites, involved in different metabolic pathways. The results of both multivariate and univariate analyses are summarised in Fig. 48, presenting a dissection of the effects of training during each half of life. The figure also helps to identify some interesting opposite effects of training and non-training on betaine, α-ketoglutarate, thymidine and creatine during the 2nd half. The effects of training and aging on urinary metabolites involved in major metabolic pathways will be discussed below.

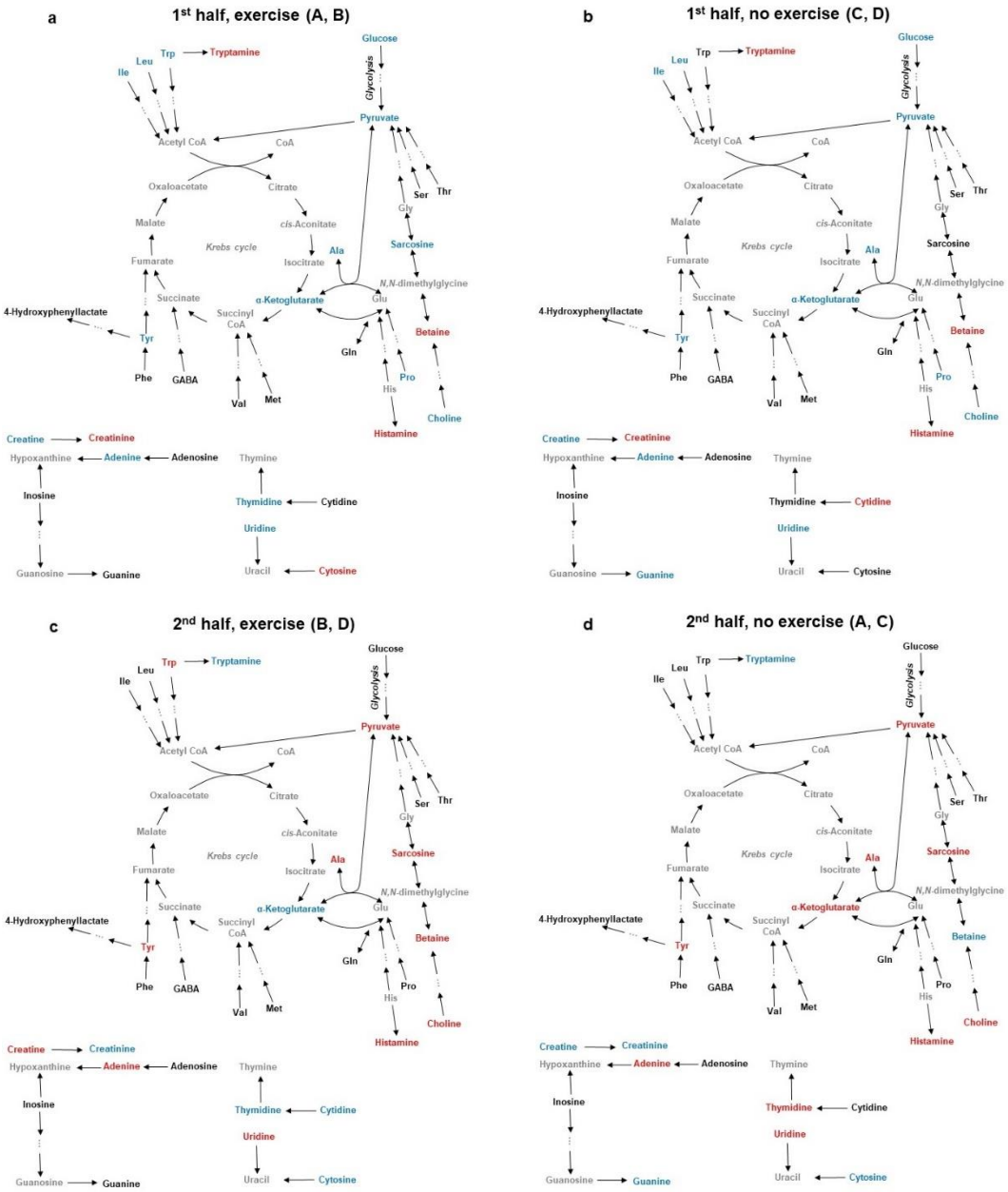


Fig. 48 Map of metabolic pathways with indication of urinary metabolites affected by age and/or exercise training in (a) the exercising groups during the 1st half, (b) the non-exercising groups during the 1st half, (c) the exercising groups during the 2nd half, and (d) the non-exercising groups during the 2nd half of life. Metabolites in red, blue, or black increased, decreased, or did not change, respectively. Metabolites in grey were not identified**.**

Amino Acids and Amino Acid Derivatives

Training seemed to increase amino acid concentrations, especially during the 2nd half, whereas aging during the 1st half and from 3 to 21 months seemed to decrease amino acid concentrations. Specifically, aging resulted in an expected decrease in two glucogenic amino acids, alanine and proline, from young age to midlife, while training resulted in an increase

from midlife to elderhood. Both alanine and proline are amino acids that have been negatively associated with aging (Houtkooper et al. 2011; Johnson et al. 2018).

Sarcosine was found increased due to aging, and exercise resulted in a decrease during the 1st half, although during the 2nd half the exercising groups exhibited a greater increase. Betaine increased from young age to midlife. From midlife to elderhood, training resulted in an increase, whereas no training resulted in a decrease. Sarcosine and betaine are intermediates in the synthesis of glycine from choline. Circulating betaine has been positively associated with physical activity (Xiao et al. 2016) and an increase has been reported after weight reduction (Kim et al. 2013). Choline decreased during the 1st half, with the exercising groups exhibiting a smaller decrease, and increased during the 2nd half. However, choline excretion has been found increased after 18 months of exercise training in older men (Sheedy et al. 2014).

Aging resulted in decreases in the urinary concentrations of two BCAAs, isoleucine and leucine. Although the effects of aging on BCAA metabolism are not clear, there are studies that have found decreased BCAAs in the blood of elderly subjects and have attributed this to reduced muscle activity (Chaleckis et al. 2016). Two aromatic amino acids, tryptophan and tyrosine, decreased from young age to midlife, whereas three aromatic amino acid derivatives, kynurenate, tryptamine and histamine, increased during the same period. These findings suggest increased aromatic amino acid catabolism. Moreover, tryptophan decreased more in the exercising groups than in the non-exercising ones. From midlife to elderhood, tyrosine showed an increase, whereas kynurenate and tryptamine showed a decrease. The decrease of kynurenate in the exercising groups was bigger than that in the non-exercising ones. Tryptophan did not show any changes due to aging during the same period, although an exercise-induced increase was noticed. Plasma concentrations of tryptophan and tyrosine are negatively associated with aging (Houtkooper et al. 2011; Johnson et al. 2018; Lustgarted & Fielding 2017; Rafeay et al. 2017). The concomitant increase in kynurenine, a precursor of kynurenate, has been associated with patients suffering from inflammatory diseases (Collino et al. 2013; Lee et al. 2017). In a study with older men, urinary tryptophan increased following 18 months of training. This increase was attributed to the increased serotonin production, for which tryptophan is a precursor, and was associated with exercise training (Sheedy et al. 2014). Following training, tryptophan levels increased, albeit in blood of aged mice, which was also the case in the present study (Lee et al. 2017). Increases in blood tryptophan and tryptophan metabolites have also been observed following both acute exercise and training and have been positively associated with VO_2max (Brennan et al.; Lewis et al. 2010; Lustgarten et al. 2013). Not much is known about the effects of exercise or training or aging

on histamine, which is synthesised from the aromatic amino acid histidine. However, as mentioned previously, its age-related increase (found only in the multivariate analysis) may suggest increased activity of histidine carboxylase. More research will be required to support this speculation.

Carbohydrate and Lipid Metabolism

The observed decrease in urinary glucose and pyruvate during the 1st half of life implies decreased flow of carbohydrates through glycolysis. By contrast, the increase in pyruvate during the 2nd half implies accelerated glycolysis. Other studies have also found an increase in glycolytic rate with aging (Feng et al. 2016; Mittendorfer & Klein 2001; Ravera et al. 2019). Mannitol decreased from young age to midlife, whereas from midlife to elderhood it increased. However, the levels at 21 months were lower than those at 3 months, suggesting an aging-related decrease. These fluctuations are possibly the results of either differences in food intake or impairment in intestinal permeability. This is, once more, supported by Valentine et al. (2014), who also found decreased urinary mannitol in aged adults.

Acetylcarnitine decreased from midlife to elderhood according to both multivariate and univariate analyses. A decrease in urinary acetylcarnitine concentration was also noticed in older individuals (Slupsky et al. 2007) and aged female rats (Deda et al. 2017). Since acetylcarnitine is formed when there is abundance of acetyl CoA (due to increased carbohydrate and lipid oxidation, Mougios 2020), its decrease may suggest a decrease in these two metabolic pathways with aging.

α-Ketoglutarate decreased from young age to midlife and from young age to elderhood, suggesting an age-related decrease. Decreased concentrations of several Krebs cycle metabolites in urine have been associated with aging and diminished mitochondrial function (Schnackenberg et al. 2007).

Purine and Pyrimidine Metabolism

Adenine decreased from young age to midlife and from young age to elderhood, although there was an increase from midlife to elderhood. No studies were found referring to the effects of aging and/or exercise on urinary purine concentrations. However, a study on plasma purine concentrations found that they increased with age, representing the depletion of the skeletal muscle adenine nucleotide pool (Zieliński et al. 2019). Guanine, another purine, was found decreased in the non-exercising groups during both the 1st and 2nd half.

Cytosine and cytidine increased from young age to midlife, while thymidine and uridine

decreased. From midlife to elderhood cytosine and cytidine decreased, while thymidine and uridine decreased. Pyrimidine metabolites are essential for the synthesis of DNA, RNA, lipids and carbohydrates, and their downregulation has been linked to aging and diseases, such as Alzheimer's and immunodeficiency (Wan et al. 2019). Cytosine also exhibited an exercise-related increase during the 1st half. Increases in pyrimidine nucleotides have been observed after both acute and prolonged exercise regimes (Daskalaki et al. 2015). Exercise also resulted in a decrease in thymidine during the 1st half, and only the non-exercising groups exhibited an increase in cytidine during the same period. During the 2nd half, the exercising groups exhibited a decrease in cytidine and thymidine, whereas the non-exercising groups showed an increase in thymidine.

Gut Microbiome Metabolism

From young age to midlife, dimethylamine and TMAO (two metabolites associated with gut microbiome metabolism) increased, whereas from midlife to elderhood they decreased. Exercise during the 2nd half accentuated the decrease in TMAO. This may be the result of increased absorption due to improved blood circulation in the gut. TMAO has been found decreased following exercise in other studies, and this decrease has been related to health benefits (Enea et al. 2010; Pechlivanis et al. 2015). Aging has been reported to cause higher concentrations of dimethylamine and TMAO, and this increase is speculated to result from impairments in renal function (Kochhar et al. 2006; Psichogios et al. 2008) and/or muscle composition (Lustgarten & Fielding 2017). Dimethylamine was also found elevated in patients with hypertension and cardiac dysfunction (Sheedy et al. 2014), and TMAO has been associated with reduced survival rates in conditions such as heart failure (Heaney et al. 2017). TMAO is also a product of choline metabolism; thus, the decrease in choline and the concomitant increase in TMAO during the 1st half may support the hypothesis of increased choline catabolism with aging, as mentioned above.

Other Metabolites

Creatine decreased from young age to midlife, whereas creatinine increased during the same period. This implies increased conversion of creatine to creatinine. During the 2nd half, however, no changes were seen in creatine, although creatinine showed a decrease. The excretion of creatinine has been reported to reflect muscle mass (Tosato et al. 2017). Likewise, in another study (Wu et al. 2008), urinary creatinine increased in rats from the 4th to the 18th month of age and then decreased up until the 24th month. The findings of the present study are in agreement with the aforementioned studies. Moreover, during the 2nd half, creatine increased in the exercising groups and decreased in the non-exercising ones. This finding may show a beneficial effect of exercise in protecting against the age-related loss of

muscle mass, known as sarcopenia. Aging and sarcopenia have been linked with a decline in muscle creatine, which is exacerbated with physical inactivity (Rawson & Venezia 2011).

Methylamine excretion decreased from midlife to elderhood in the present study. Urinary methylamine also decreased from the 4th to the 24th month (Wu et al. 2008) and from the 5th to 21st month of age in female rats (Deda et al. 2017). Thiamine and biotin, two B vitamins, decreased with aging. Specifically, thiamine decreased from young age to midlife, whereas biotin decreased from young age to elderhood. There are indeed alterations in nutrient availability due to aging, including altered vitamin uptake (Siddharth et al. 2017). Neither thiamine nor biotin excretion was found altered with age in an older study, with biotin specifically exhibiting an increase at a younger age and then remaining stable until elderhood (Fukuwatari et al. 2008). In another study, thiamine was found deficient among older adults and was associated with heart failure (Nazmi et al. 2014). During the 1st half, biotin was found decreased in the non-exercising groups. No studies could be found to compare this finding with; however, it may suggest that physical inactivity exacerbates the age-related impairment in vitamin availability.

Putrescine, a polyamine involved in many cell functions, decreased from young age to elderhood (according to the multivariate analysis only). Urinary putrescine did not exhibit changes with age in an older study, with the only alterations found depending on the time of day (Pöyhönen et al. 1990). Decreased putrescine levels with age have also been observed in the blood of subjects with low muscle quality (Moaddel et al. 2016).

In summary, training resulted in increases in urinary amino acids, especially during the 2nd half, whereas aging had the opposite effect. Aging was also associated with signs of increased glycolysis from midlife to elderhood. We found evidence for aging-related impairment in intestinal activity and permeability, as well as vitamin availability, with physical inactivity exacerbating these negative effects. A potentially important finding was the opposite changes of creatine during the 2nd half in the exercising and non-exercising groups (increase and decrease, respectively), suggestive of a protective effect of exercise against sarcopenia.

Effects of Long-Term and Lifelong Exercise on the Metabolome of Blood Lysates

Exercise and aging elicited changes in about half of the identified metabolites, involved in different metabolic pathways. The results of both multivariate and univariate analyses are summarised in Fig. 49, presenting a dissection of the effects of training during each half of life. The figure also helps to identify some interesting opposing effects of training and no training on alanine, tryptophan, glycine, succinate and lactate during the 1st half, as well as on tryptophan during the 2nd half. The effects of training and aging on blood metabolites involved

in main metabolic pathways will be discussed below.

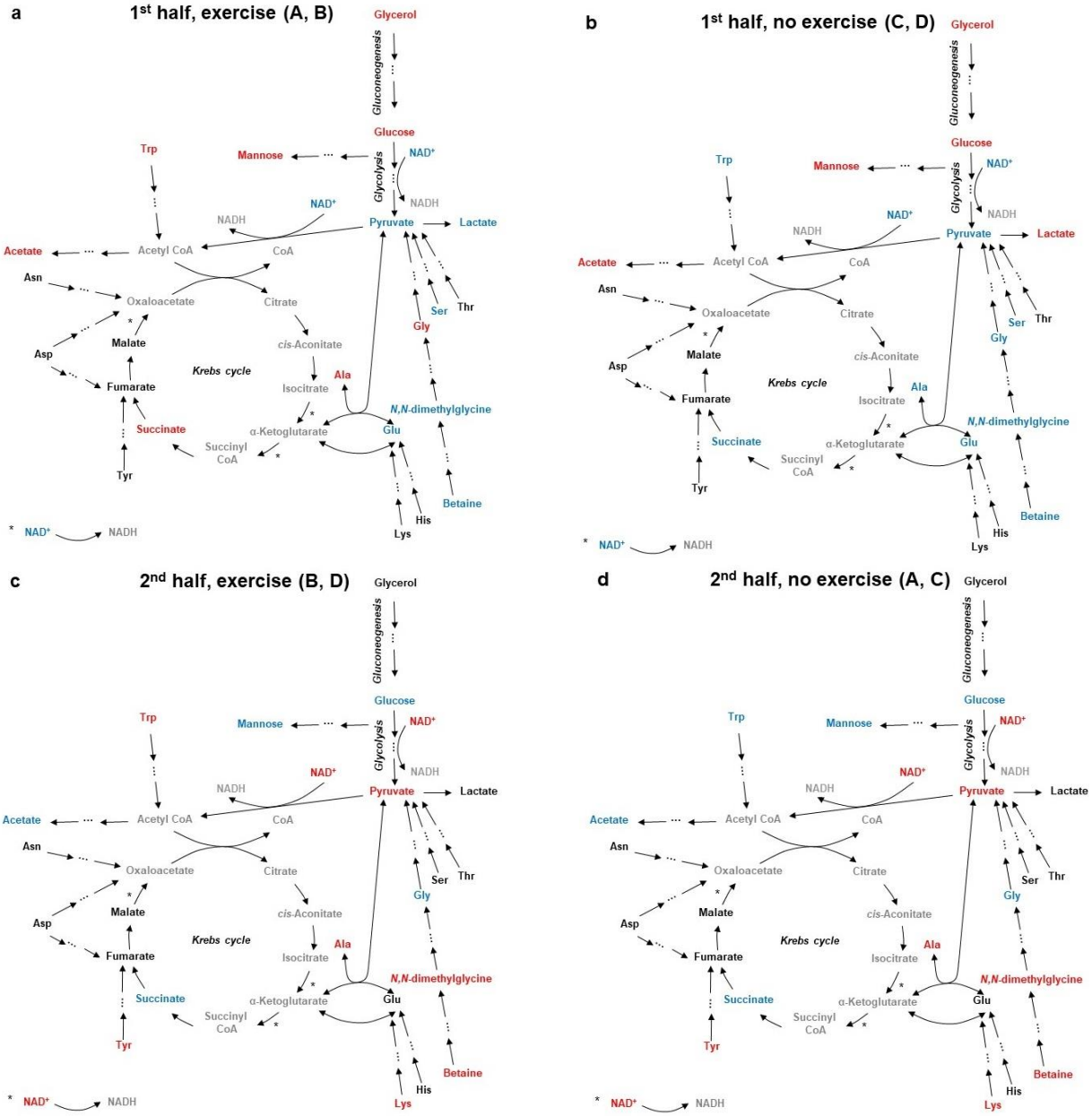


Fig. 49 Map of metabolic pathways with indication of blood metabolites affected by age and/or exercise training in (a) the exercising groups during the 1st half, (b) the non-exercising groups during the 1st half, (c) the exercising groups during the 2nd half, and (d) the non-exercising groups during the 2nd half of life. Metabolites in red, blue, or black increased, decreased, or did not change, respectively. Metabolites in grey were not identified.

Amino Acids

Exercise seemed to have anabolic effects on amino acid metabolism, especially during the 1st half, whereas aging during the same period seemed to have catabolic effects on amino acid metabolism. Specifically, training resulted in an expected increase of alanine and (according to multivariate analysis) glutamine up until midlife, as well as of tryptophan from young age to elderhood. Both alanine and glutamine have been found elevated due to long-term training (Daskalaki et al. 2014; Takeshita et al. 2012); in addition, they have also been found elevated due to acute exercise (Daskalaki et al. 2014; Lewis et al. 2010). The increases in alanine and glutamine can be attributed to their pivotal roles as recipients of amino groups from amino acid catabolism in extrahepatic tissues (including the working muscles) for transport, via the bloodstream, to the liver for disposal through the urea cycle (Mougios 2020). Regarding tryptophan (an essential amino acid), two possible explanations for its increase are enhanced protein degradation and reduced tryptophan degradation.

Aging resulted in an increase in one (histidine) and a decrease in four (aspartate, glutamate, glycine and serine) out of the seven glucogenic amino acids that were identified. Notably, serine was also identified as an important variable for the age-related discrimination of samples in the multivariate analysis. Decreases in blood glutamate and serine of aged rodents were also found by Houtkooper et al. (2011), who, in addition, noticed aging-induced increases in the ketogenic amino acid lysine, similarly to the present study.

Training during the 1st half of life reversed the reductive effect of aging on glycine, although training during the 2nd half had no such effect. Glycine has been found to drop years before the onset of prediabetes or T2D in humans and is considered a predictor of these conditions (Klein et al. 2016). Thus, exercise may mitigate an undesirable effect linking aging with the emergence of prediabetes and T2D. The fact that this beneficial effect was seen only during the 1st half of life points to a possible advantage of commencing regular exercise as earlier as possible in life.

Fuel Metabolism

The increase of glycerol during the 1st half and its higher values in the exercising groups at 12 months (as seen in the multivariate analysis) imply enhanced lipolysis up until midlife and due to exercise. Metabolite profiling platforms have previously reported increases in plasma concentrations of lipolytic products, with more fit individuals showing a greater increase compared to less fit ones (Lewis et al. 2010).

The parallel fluctuations of glucose and its isomeric mannose (that is, increase during the 1st

half of life and decrease during the 2nd half), in conjunction with the opposite fluctuations of pyruvate, point to enhanced glycolysis during the 2nd half. This agrees with Mittendorfer & Klein (2001), who argue that glucose may be preferred over fatty acids as a fuel in older individuals, presumably as a result of age-related changes in the respiratory capacity of skeletal muscle.

Similar to their effects on glycine, training and aging exerted opposite effects on succinate, a metabolite of the Krebs cycle: Training during the 1st half of life reversed the reductive effect of aging, and training during the 2nd half mitigated this effect. In support of the positive effect of training on succinate, Huffman et al. (2014) found that training increased only this Krebs cycle metabolite (not fumarate or malate, again in agreement with this study's findings) in human skeletal muscle. What is more, succinate exhibited the highest correlation with insulin sensitivity among the metabolites measured by Huffman et al. (2014). Based on these findings, it is suggested that training mitigated yet another undesirable effect of aging. This beneficial effect was stronger during the 1st, rather than the 2nd, half of life. Moreover, the exercise-induced increase in succinate and the concomitant decrease in lactate during the 1st half in the exercising groups suggest increased oxidative metabolism.

Redox Status

The expected decrease in NAD^+ with age, found through both multivariate and univariate analyses, agrees with the well-established notion that aging causes a decline in the NAD^+ content of many tissues (Chini et al. 2017; Fang et al. 2017). This is detrimental for ATP resynthesis, given the key role of NAD^+ in glycolysis, the citric acid cycle, and the electron transport chain (which feeds oxidative phosphorylation), as well as for signal transduction, given the fact that NAD^+ is a substrate for sirtuins. In fact, the decline in cellular NAD^+ content appears to be crucial for the development of metabolic dysfunction and age-related diseases (Chini et al. 2017; Fang et al. 2017). The training intervention employed in the present study did not affect the age-related decline in NAD^+, which was somewhat expected as, to the best of my knowledge, there are no studies demonstrating any training-induced changes in resting NAD^+ concentrations.

Small Aliphatic Carboxylic Acids

Acetate, formate, and isobutyrate displayed age-dependent decreases from young age to elderhood. Acetate, in particular, was also identified as an important variable for the discrimination between ages in the multivariate analysis, and both acetate and formate were identified as important variables for the discrimination between the exercising and non-

exercising groups at 12 months of age. However, no studies could be found to compare these results with. Shimazu et al. (2010) consider acetate an important source of acetyl-CoA in peripheral tissues under fasting conditions and speculate a role of acetate metabolism in aging. Due to the interconversion of acetate and acetyl-CoA and the multiple origins and functions of the latter, the same authors stress the central role of acetate in maintaining energy homeostasis. Acetate, formate, and isobutyrate are also involved in gut microbial metabolism. Thus, their decrease with age may indicate an effect on intestinal function, although more studies will be required to support this speculation.

Other Metabolites

The beneficial effect of exercise may be larger considering the evidence that it might have also protected against age-related loss of muscle mass, known as sarcopenia. This evidence comes from the findings that during the 1st half of life the exercising groups showed a higher increase in blood creatine than the non-exercising groups, resulting in the former ending up with an overall increase at 21 months, whereas the latter ended up with an overall decrease. A decline in the muscle creatine content with aging has been linked to sarcopenia, and physical inactivity may exacerbate this decline (Rawson & Venezia 2011). Thus, these data suggest a possible usefulness of the blood creatine concentration as an index of sarcopenia and its mitigation by regular exercise, although direct evidence for this through body composition assessment will be needed.

In summary, training resulted in anabolic effects on amino acid metabolism, especially during the 2nd half, whereas aging resulted in catabolic effects on amino acid metabolism during the same period. A notable result was the reversal of the reductive effect of aging on glycine by training, which may mitigate the development of prediabetes and T2D. Training was also associated with enhanced lipolysis and oxidative metabolism up until midlife, whereas aging was responsible for enhanced glycolysis during the 2nd half. These results are in agreement with the changes found in urinary metabolites, as stated above. An expected decrease in NAD^+ with age was also found, which may predispose for the emergence of metabolic dysfunction and age-related diseases; exercise did not seem to affect this age-related decline. Once more, aging seemed to affect metabolites involved in gut microbial metabolism, indicating impairments in intestinal function. Finally, an important finding was the potential usefulness of blood creatine concentration as an index of sarcopenia and on the beneficial effect that exercise may have by mitigating it.

Effects of Long-Term and Lifelong Exercise on Tissue Fatty Acid Content

Exercise during the 2nd half resulted in reduced levels of two saturated fatty acids, 15:0 in quadriceps phospholipids and 16:0 in liver triacylglycerols, as well as of total SFAs in liver phospholipids. No exercise-related information could be found about 15:0; however, 16:0, being the most common fatty acid in animals, has been studied extensively. Decreased concentration of 16:0 in plasma (Chorell et al. 2012) and muscle triacylglycerols (Petridou et al. 2005) has been found in fit individuals, following 1 h of cycling (Mougios et al. 2003) and after 6 weeks of training in middle-aged men (Shojaee-Moradie et al. 2007). The total SFA content of total blood phospholipids also decreased after 30 days of training (Corsetto et al. 2019). Age-related increases in plasma free SFAs and, specifically, 16:0 have been previously observed (Johnson et al. 2018; Pararasa et al. 2016) and have been associated with increased risk for T2D (Ma et al. 2015; Wishart 2019), inflammation (Corsetto et al. 2019), and cardiovascular disease (Kawanishi et al. 2018). Therefore, our findings suggest that exercise may have beneficial effects on health, since the exercising group during the 2nd half seemed to utilise more SFAs than the non-exercising one.

Moreover, exercise during the 2nd half resulted in a decrease in quadriceps triacylglycerol 20:1ω9 content, although the molar percentage of the same fatty acid increased in liver triacylglycerols. The concentration of 18:1ω7, however, decreased in liver triacylglycerols, according to the multivariate analysis. In muscle triacylglycerols, both 20:1ω9 and 18:1ω7 were previously found decreased in trained rats (Petridou et al. 2005), although muscle triacylglycerol 18:1ω7 was found increased following 4 weeks of training (Helge et al. 2001). Although 18:1ω7 increased in plasma (Djoussé et al. 2014) and brain phospholipids (McNamara et al. 2008) with age, it has been negatively associated with the risk of T2D (Ma et al. 2015; Weir et al. 2019), coronary heart disease (Djoussé et al. 2012) and heart failure (Djoussé et al. 2014). Age-related increases have also been observed in 20:1ω9 in brain phospholipids (McNamara et al. 2008) and retina phospholipids (Liu et al. 2010). The exercising groups seemed to utilise these two fatty acids more than the non-exercising ones, and their observed decrease might be beneficial, since both fatty acids are found increased with age.

As far as PUFAs are concerned, exercise during the 2nd half decreased the ω3 fatty acids in quadriceps triacylglycerols and increased the ω3 fatty acids in liver phospholipids. Specifically, 18:3ω3 and 20:3ω3 decreased in quadriceps triacylglycerols, and 18:3ω3, 20:3ω3 and 22:6ω3 increased in liver phospholipids. Decreased content of muscle triacylglycerol 18:3ω3 and increased content of liver phospholipid 18:3ω3 were also found previously in trained rats (Petridou et al. 2005). Increases in muscle 18:3ω3 were found with

aging (Houtkooper et al. 2011; Johnson et al. 2018), and its higher levels in blood have been associated with higher risk of sudden cardiac arrest (Lemaitre et al. 2009), hypertension (Tsukamoto & Sugawara 2017) and unhealthy aging (Lai et al. 2018). Moreover, increased levels of 22:6ω3 in serum have been associated with high fitness levels (Daskalaki et al. 2014) and were also found in plasma of fit individuals (Chorell et al. 2012). Increased levels of the same fatty acid were found in muscle phospholipids after 4 weeks of training (Helge et al. 2001). 22:6ω3, an important component of neuronal membranes, can confer long-term benefits on cardiovascular health and inflammation (Janssen & Kiliaan 2014). It has been found decreased with aging, and this has been linked with impaired cognitive function (Mohajeri et al. 2015; Thomas et al. 2015; Weiser et al. 2016). Therefore, both the decreased amount and percent of 18:3ω3 in quadriceps, as well as the increased amount of 22:6ω3 in the liver of the rats that had exercised during the 2nd half may indicate a beneficial effect of exercise on health.

18:2ω6 increased in liver triacylglycerols (according to both univariate and multivariate analyses). Increased levels of the same fatty acid have been observed in the plasma of female rats following exhaustive swimming exercise (Zhou et al. 2019), in human skeletal muscle phospholipids following four weeks of one-leg training (Helge et al. 2001), in the liver of rats following high-intensity endurance exercise (Starnes et al. 2017), and in the liver of trained rats compared to untrained ones (Petridou et al. 2005). 18:2ω6 has been associated with cardiovascular health (Harris et al. 2013) and the prevention of T2D (Wu et al. 2017), and it has been found to decrease with age by 3% each year (Harris et al. 2013). Hence, once more, exercise seemed to have desirable effects on the content of this essential fatty acid.

The increases in ω3 and ω6 fatty acids in liver phospholipids and triacylglycerols, respectively, may prove to be highly beneficial for health, especially in older adults, since they are needed for optimal function of the cardiovascular system, they play a vital structural and functional role in the central nervous system and are linked with cognitive performance and brain function (Denis et al. 2015; Proitsi et al. 2018; Úbeda et al. 2012). The total SFA content also decreased in blood phospholipids after 30 days of training (Corsetto et al. 2019). ω3 fatty acids have antioxidant (Tutino et al. 2018) and anti-inflammatory properties (Janssen & Kiliaan 2014), and ω6 fatty acids are associated with a lower risk of T2D (Wu et al. 2017). Muscle triacylglycerol ω3 fatty acids, though, decreased in the present study, although they were previously found increased in trained compared to untrained rats (Petridou et al. 2005). This may imply a short-term, rather than long-term, effect of exercise on muscle lipids, since the samples were collected 2,5 weeks after the last exercise session.

Total PUFAs and UFAs in both liver phospholipids and triacylglycerols were found increased

with training in the present study. These findings are in agreement with two studies that also found increased UFAs in liver in both animals and humans (Nikolaidis & Mougios 2004), and in both muscle phospholipids and triacylglycerols following 4 weeks of training (Helge et al. 2001). The groups that exercised during the 2nd half had a higher UFA to SFA ratio (by 23.5% compared to those that did not exercise) in liver phospholipids in the present study. The literature, however, does not show consistent results on this ratio. Following 4 weeks of training, increases were observed in plasma free fatty acids (Mougios et al. 2003; Nikolaidis & Mougios 2004), decreases in muscle triacylglycerols of trained compared to untrained rats (Petridou et al. 2005) and no changes in plasma free fatty acids 1 h and 2 h after high-intensity interval training or moderate-intensity continuous exercise (Peake et al. 2014).

In summary, long-term training induced changes mainly in phospholipid SFAs, triacylglycerol MUFAs, and both phospholipid and triacylglycerol PUFAs. In particular, training reduced the SFA and MUFA content, while increasing the PUFA content. The effect of exercise seemed to be highly beneficial, since, as discussed above, SFAs and MUFAs are mainly associated with metabolic diseases that have also been linked with aging, whereas PUFAs are essential for the normal function of the central nervous system and brain, as well as having antioxidant and anti-inflammatory properties.

Limitations and Delimitations

A limitation of this study is the rather small sample size, which was primarily dictated by the capacity of the available facilities regarding animal housing and daily training. Thus, the possibility exists that, with more animals in each group, more differences would reach the rather strict level of statistical significance (5% FDR with $\alpha = 0.05$) set in the present study. Nevertheless, metabolic studies with single-digit numbers of genetically homogeneous animals are quite common in the literature. Another limitation was the absence of a performance test to assess the effectiveness of training. Since such a test would have had to be training specific, it should have been swimming until exhaustion with the addition of weight. However, exhaustion under such circumstances is ascertained by near-drowning or even drowning, which was deemed unethical. Moreover, the necessity to cage rats in groups of three led us to obtain data of food intake from the average value of the three animals housed in each cage, rather than individually.

A delimitation of the study is that rats exercised during their early rest phase. Studies have shown that time of day is a major modifier of exercise capacity and is associated with fuel utilisation and metabolic pathway activation, particularly related to fatty acid oxidation and glycolysis (Ezagouri et al. 2019). This can be easily explained if one thinks of a typical daily

routine, in which early in the morning there is less spontaneous activity and food intake has just started, compared to later in the day when there has been more physical activity and more nutrients have been digested. Research has shown that exercise during the early active phase, which is usually during the first 3 hours after dark for rodents, exerts a robust metabolic response in skeletal muscle, including glycolysis, lipid oxidation and BCAA breakdown, resulting in higher utilisation of carbohydrates and ketone bodies together with lipid and amino acid degradation (Sato et al. 2019). On the other hand, exercise during the early rest phase, which is during the first 3 hours after light, stimulates the daily fluctuation of metabolites related to carbohydrate metabolism and energy expenditure rises significantly following a single bout of exercise (Sato et al. 2019). Therefore, since the physiology and behaviour of mammals exhibit daily oscillations that are driven by an endogenous circadian clock (Ezagouri et al. 2019), the results of the present study could have been different if exercise had been performed at a different time.

Another delimitation is that the animals lived at unregulated room temperature in order to mimic the seasonal fluctuation of indoor temperatures in temperate zones. In most studies, experimental animals are housed at temperatures of around 21^{o}C, which is below the range within which rodents maintain their basal metabolic rate (26-34^{o}C). Studies have shown that lower temperatures might cause chronic cold stress, which influences the ability of exercise to increase markers of mitochondrial biogenesis (McKie et al. 2019). The same study has found that mice bred and housed at room temperature (21^{o}C) had a 50% higher metabolic rate, had increased mass of certain organs (such as the liver, kidneys and heart), consumed more food, slept less, metabolised more lipids than carbohydrates and were mildly hypertensive, compared to mice housed at temperatures near thermoneutrality (30^{o}C). Moreover, mice housed at thermoneutrality ran more than mice housed at room temperature, which the authors attributed to their not being under thermal stress, as evidenced by increased serum corticosterone, a known marker of chronic stress in rodents, inversely correlated with average running distance (McKie et al. 2019).

Conclusions

In the present study, we investigated the effects of lifelong exercise and aging on the rat blood and urinary metabolome, as well as the effects of exercise on insulin sensitivity and the fatty acid content of rat tissues. Our main conclusions are the following:

- The impact of aging on the blood and urinary metabolomes was stronger than that of training.

- Regular moderate-intensity exercise during the 2nd half of life suppressed body weight gain and appetite.

- Regular exercise may have protected against diseases linked with aging such as sarcopenia and insulin resistance.

- Regular exercise increased the abundance of PUFAs, fatty acids with antioxidant and anti-inflammatory properties that are also essential for the normal function of the central nervous system and brain.

- Even moderate-intensity exercise had beneficial effects on health.

- The effect of exercise on insulin sensitivity was rather short-lived; therefore, maintaining enhanced insulin sensitivity requires regular exercise.

- Although commencing regular exercise as early as possible in life is vital, beginning at older ages may still be beneficial for health.

- Healthy lifestyles with habitual exercising may prevent or delay the occurrence of diseases related to aging and frailty.

Future Studies

Literature related to healthy aging and exercise is surprisingly scarce; therefore, more investigations on normal aging are needed. Future studies could address the question of whether regular exercise of higher intensity or volume has a higher impact on the metabolome and a longer-lasting effect on insulin sensitivity, although exercise tolerance for life might be an issue. Useful additions to future experiments might be examining the effects of exercise with caloric restriction or hypercaloric diet, more frequent sampling and sampling after the exercise sessions to examine the effects of aging and training on the post-exercise, as well as resting, metabolome. Other questions open to future research are the effects of inactivity on trained and sedentary animals, which can be addressed by housing trained and sedentary groups in either bigger or typical, smaller cages. Finally, further research is needed to examine why certain fatty acids are mobilised to a greater or lesser extent in different tissues during exercise.

REFERENCES

Antikainen H, Khayati K, Dobrowolski R (2017) Amino acid metabolites, mTORC1 and aging. *Aging* 9(7): 1641–1642. doi: 10.18632/aging.101266

Arbeev KG, Ukraintseva SV, Yashin AI (2016) Dynamics of biomarkers in relation to aging and mortality. *Mech Ageing Dev* 156: 42–54. doi: 10.1016/j.mad.2016.04.010

Asif Y, Wlodek ME, Black MJ, Russell AP, Soeding PF, Wadley GD (2018) Sustained cardiac programming by short-term juvenile exercise training in male rats. *J Physiol* 596(2): 163-180. doi: 10.1113/JP275339

Bae CR, Hasegawa K, Akieda-Asai S, Kawasaki Y, Senba K, Cha YS, Date Y (2014) Possible involvement of food texture in insulin resistance and energy metabolism in male rats. *J Endocrinol* 222(1): 61–72. doi: 10.1530/JOE-13-0553

Bai X (2018) Biomarkers of aging. In Z. Wang (Ed.), *Aging and Aging-Related Diseases,* Springer, Singapore, pp. 217–234. doi: 10.1007/978-981-13-1117-8_14

Bakun M, Senatorski G, Rubel T, Lukasik A (2014) Urine proteomes of healthy aging humans reveal extracellular matrix (ECM) alterations and immune system dysfunction. *Age* 36(1): 299–311. doi: 10.1007/s11357-013-9562-7

Beard JR, Officer A, de Carvalho IA, Sadana R, et al. (2016) The World report on ageing and health: a policy framework for healthy ageing. *Lancet* 387(10033): 2145–2154. doi: 10.1016/S0140-6736(15)00516-4

Benaki D, Mikros E (2018) NMR-based metabolic profiling procedures for biofluids and cell and tissue extracts. In: Theodoridis G, Gika H, Wilson I (eds) Metabolic Profiling. *Methods Mol Biol*, Humana Press, New York, 1738:117-131. doi: 10.1007/978-1-4939-7643-0_8

Benjamini Y, Hochberg Y (1995) Controlling the False Discovery Rate: a Practical and Powerful Approach to Multiple Testing. J R Stat Soc Series B 57(1): 289–300

Berry JD, Pandey A, Gao A, et al. (2013) Physical fitness and risk for heart failure and coronary artery disease. *Circ Heart Fail* 6(4): 627–634. doi: 10.1161/CIRCHEARTFAILURE.112.000054

Berton R, Conceição MS, Libardi CA, et al. (2017) Metabolic time-course response after resistance exercise: A metabolomics approach. *J Sports Sci* 35(12): 1211–1218. doi: 10.1080/02640414.2016.1218035

Bifari F, Nisoli E (2017) Branched-chain amino acids differently modulate catabolic and anabolic states in mammals: a pharmacological point of view. *Br J Pharmacol* 174(11): 1366–1377. doi: 10.1111/bph.13624

Biswas A, Oh PI, Faulkner GE, Bajaj RR, Silver MA, Mitchell MS, Alter DA (2015) Sedentary time and its association with risk for disease incidence, mortality, and hospitalization in adults. *Ann Intern Med* 162(2):123–133. doi: 10.7326/M14-1651

Blomstrand E, Saltin B (1999) Effect of muscle glycogen on glucose, lactate and amino acid metabolism during exercise and recovery in human subjects. *J Physiol* 514(1): 293–302. doi: 10.1111/j.1469-7793.1999.293af.x

Booth FW, Laye MJ, Roberts MD (2011) Lifetime sedentary living accelerates some aspects of secondary aging. *J Appl Physiol* 111(5):1497–1504. doi: 10.1152/japplphysiol.00420

Bradley NS, Heigenhauser GJ, Roy BD, et al. (2008) The acute effects of differential dietary fatty acids on human skeletal muscle pyruvate dehydrogenase activity. *J Appl Physiol* 104(1): 1–9. doi: 10.1152/japplphysiol.00636.2007

Brennan AM, Benson M, Morningstar J, et al. (2018) Plasma metabolite profiles in response to chronic exercise. *Med Sci Sports Exerc* 50(7): 1480–1486. doi: 10.1249/MSS.0000000000001594

Bürkle A, Moreno-Villanueva M, Bernhard J, et al. (2015) MARK-AGE biomarkers of ageing. *Mech Ageing Dev* 151: 2–12. doi: 10.1016/j.mad.2015.03.006

Caligiuri SPB, Parikh M, Stamenkovic A, Pierce GN, Aukema HM (2017) Dietary modulation of oxylipins in cardiovascular disease and aging. *Am J Physiol Heart Circ Physiol* 313(5): H903-H918. doi: 10.1152/ajpheart.00201.2017

Carta G, Murru E, Banni S, Manca C (2017) Palmitic acid: physiological role, metabolism and nutritional implications. *Front Physiol* 8:902. doi: 10.3389/fphys.2017.00902

Cartee GD (2015) Mechanisms for greater insulin-stimulated glucose uptake in normal and insulin-resistant skeletal muscle after acute exercise. *Am J Physiol Endocrinol Metab* 309(12): E949-E959. doi: 10.1152/ajpendo.00416.2015

Castro G, Fernanda M, Areias C, et al. (2013) Diet-induced obesity induces endoplasmic reticulum stress and insulin resistance in the amygdala of rats. *FEBS Open Bio* 3: 443–449. doi: 10.1016/j.fob.2013.09.002

Chaleckis R, Murakami I, Takada J, Kondoh H, Yanagida M (2016) Individual variability in human blood metabolites identifies age-related differences. *PNAS* 113(16): 4252–4259. doi: 10.1073/pnas.1603023113

Chini EN, Chini CCS, Tarrag MG (2017) NAD and the aging process: Role in life, death and everything in between. *Mol Cell Endocrinol* 455:62–74. doi: 10.1016/j.mce.2016.11.003

Chodzko-Zajko WJ, Proctor DN, Flatarone SMA, et al. (2009) American College of Sports Medicine position stand. Exercise and physical activity for older adults. *Med Sci Sports Exerc* 41(7): 1510-1530. doi: 10.1249/MSS.0b013e3181a0c95c

Chong J, Soufan O, Li C, Caraus L, Li S, Bourque G, Wishart DS, Xia J (2018) MetaboAnalyst 4.0: towards more transparent and integrative metabolomics analysis. *Nucleic Acids Res* 46(W1): W486-W494. doi: 10.1093/nar/gky310

Chorell E, Svensson B, Moritz T, Henrik A (2012) Physical fitness level is reflected by alterations in the human plasma metabolome. *Mol Biosyst* 8(4): 1187–1196. doi: 10.1039/c2mb05428k

Chou CH, Hwang CL, Wu Y (2012) Effect of exercise on physical function, daily living activities, and quality of life in the frail older adults: A Meta-Analysis. *Arch Phys Med Rehabil* 93(17): 237–244. doi: 10.1016/j.apmr.2011.08.042

Cohen J (1988) *Statistical power analysis for the behavioral sciences*. Vol. 2. Lawrence Earlbaum Associates Hillsdale, NJ

Collino S, Montoliu I, Martin J, et al. (2013) Metabolic signatures of extreme longevity in northern Italian centenarians reveal a complex remodeling of lipids, amino acids, and gut microbiota metabolism. *PLoS ONE* 8(3): 1–12. doi: 10.1371/journal.pone.0056564

Corsetto PA, Montorfano G, Klersy C, Massimino L, Infantino V, Iannello G, et al. (2019) Fatty acid profile and antioxidant status fingerprint in sarcopenic elderly patients: role of diet and exercise. *Nutrients* 11(11): 2569. doi: 10.3390/nu11112569

Crimmins E, Vasunilashorn S, Kim JK, Alley D (2018) Biomarkers related to aging in human populations. *Adv Clin Chem* 46: 161–216. doi: 10.1016/s0065-2423(08)00405-8

Cuisinier C, Ward RJ, Francaux M, Sturbois X, de Witte P (2001) Changes in plasma and urinary taurine and amino acids in runners immediately and 24 h after a marathon. *Amino Acids* 20(1): 13–23. doi: 10.1007/s007260170062

Daskalaki E, Blackburn G, Kalna G, Zhang T, Anthony N, Watson DG (2015) A study of the effects of exercise on the urinary metabolome using normalisation to individual metabolic output. *Metabolites* 5(1): 119–139. doi: 10.3390/metabo5010119

Daskalaki E, Easton C, Watson DG (2014) The application of metabolomic profiling to the effects of physical activity. *Curr Metabolomics* 2: 233–263. doi: 10.2174/2213235X03666150211000831

Dato S, Hoxha E, Crocco P, Iannone F, Passarino G, Rose G (2019) Amino acids and amino acid sensing: implication for aging and diseases. *Biogerontology* 20(1): 17–31. doi: 10.1007/s10522-018-9770-8

Deda O, Gika HG, Taitzoglou I, Raikos N, Theodoridis G (2017) Impact of exercise and aging on rat urine and blood metabolome. An LC-MS based metabolomics longitudinal study. *Metabolites* 7(1):1–15. doi: 10.3390/metabo7010010

Dishman RK, Berthoud H, Booth FW, et al. (2006) Neurobiology of exercise. *Obesity* 14(3): 345–356. doi: 10.1038/oby.2006.46

Djoussé L, Matsumoto C, Hanson NQ, Weir NL, Tsai M, Gaziano JM (2014) Plasma cis-vaccenic acid and risk of heart failure with antecedent coronary heart disease in male physicians. *Clin Nutr* 33(3): 478–482. doi: 10.1016/j.clnu.2013.07.001

Djoussé L, Matthan NR, Lichtenstein AH, Gaziano JM (2012) Red blood cell membrane concentration of cis-palmitoleic and cis-vaccenic acids and risk of coronary heart disease. *Am J Cardiol* 110(4): 539–544. doi: 10.1016/j.amjcard.2012.04.027

Dong Z, Sinha R, Richie JP Jr (2018) Disease prevention and delayed aging by dietary sulfur amino acid restriction: translational implications. *Ann NY Acad Sci* 1418(1): 1–12. doi: .org/10.1111/nyas.13584

Dudzinska W, Lubkowska A, Jakubowska K, Suska M, Skotnicka E (2013) Insulin resistance induced by maximal exercise correlates with a post-exercise increase in uridine concentration in the blood of healthy young men. *Physiol Res* 62(2): 163–170. PMID: 23234409

Dudzinska W, Suska M, Lubkowska A, Jakubowska K, Olszewska M, Safranow K, Chlubek D (2018) Comparison of human erythrocyte purine nucleotide metabolism and blood purine and pyrimidine degradation product concentrations before and after acute exercise in trained and sedentary subjects. *J Physiol Sci* 68(3): 293–305. doi: 10.1007/s12576-017-0536-x

Dupont J, Dedeyne L, Dalle S, Koppo K, Gielen E (2019) The role of omega-3 in the prevention and treatment of sarcopenia. *Aging Clin Exp Res* 31(6): 825–836. doi: 10.1007/s40520-019-01146-1

Duvivier BM, Schaper NC, Bremers MA, van Crombrugge G, Menheere PP, Kars M, Savelberg HH (2013) Minimal intensity physical activity (standing and walking) of longer duration improves insulin action and plasma lipids more than shorter periods of moderate to vigorous exercise (cycling) in sedentary subjects when energy expenditure is comparable. *PLoS One* 8(2): e55542. doi: 10.1371/journal.pone.0055542

Ellis DI, Dunn WB, Griffin JL, Allwood JW, Goodacre R (2007) Metabolic fingerprinting as a diagnostic tool. *Pharmacogenomics* 8(9): 1243–1266. doi: 10.2217/14622416.8.9.1243

Enea C, Seguin F, Petitpas-Mulliez J et al. (2010) (1)H NMR-based metabolomics approach for exploring urinary metabolome modifications after acute and chronic physical exercise. *Anal Bioanal Chem* 396(3): 1167–1176. doi: 10.1007/s00216-009-3289-4

Ezagouri S, Zwighaft Z, Sobel J, et al. (2019) Physiological and molecular dissection of daily variance in exercise capacity. *Cell Metab* 30(1): 1–14. doi: 10.1016/j.cmet.2019.03.012

Faber MJ, Bosscher RJ, Chin A Paw MJ, van Wieringen PC (2006) Effects of exercise programs on falls and mobility in frail and pre-frail older adults: a multicenter randomized controlled trial. *Arch Phys Med Rehabil* 87(7): 885–896. doi: 10.1016/j.apmr.2006.04.005

Facchini FS, Hua N, Abbasi F, Reaven GM (2001) Insulin resistance as a predictor of age-related diseases. *J Clin Endocr Metab* 86(8):3574-3578. doi: 10.1210/jcem.86.8.7763

Fallah N, Mitnitski A, Searle SD, et al. (2011) Transitions in frailty status in older adults in relation to mobility: a multistate modeling approach employing a deficit count. *J Am Geriatr Soc* 59(3): 524–529. doi: 10.1111/j.1532-5415.2011.03300.x

Fang EF, Hou Y, Demarest TG, Croteau DL, Mattson MP, Bohr VA (2017) NAD^+ in aging: molecular mechanisms and translational implications. *Trends Mol Med* 23(10):899–916. doi: 10.1016/j.molmed.2017.08.001

Felder TK, Ring-Dimitriou S, Auer S, et al. (2017) Specific circulating phospholipids, acylcarnitines, amino acids and biogenic amines are aerobic exercise markers. *J Sci Med Sport* 20(7): 700–705. doi: 10.1016/j.jsams.2016.11.011

Felig P, Wahren J (1971) Amino acid metabolism in exercising man. *J Clin Invest* 50(12): 2703–2714. doi: 10.1172/JCI106771

Feng Z, Hanson RW, Berger NA, Trubitsyn A (2016) Reprogramming of energy metabolism as a driver of aging. Oncotarget 7(13): 15410–15420. doi: 10.18632/oncotarget.7645

Fernandes FA, Zanesco A (2010) Early physical activity promotes lower prevalence of chronic diseases in adulthood. *Hypertens Res* 33(9):926-931. doi: 10.1038/hr.2010.106

Floegel A, Wientzek A, Bachlechner U, et al. (2014) Linking diet, physical activity, cardiorespiratory fitness and obesity to serum metabolite networks: findings from a population-based study. *Int J Obesity* 38(11): 1388–1396. doi: 10.1038/ijo.2014.39

Flores MB, Fernandes MF, Ropelle ER, et al. (2006) Exercise improves insulin and leptin sensitivity in hypothalamus of wistar rats. *Diabetes* 55(9): 2554–2561. doi: 10.2337/db05-1622

Forbes SC, Little JP, Candow DG (2012) Exercise and nutritional interventions for improving aging muscle health. *Endocrine* 42(1): 29–38. doi: 10.1007/s12020-012-9676-1

Freiberger E, Häberle L, Spirduso WW, Zijlstra GA (2012) Long-term effects of three multicomponent exercise interventions on physical performance and fall-related psychological outcomes in community-dwelling older adults: a randomized controlled trial. *J Am Geriatr Soc* 60(3): 437–446. doi: 10.1111/j.1532-5415.2011.03859.x

Frøsig C, Richter EA (2009) Improved insulin sensitivity after exercise: focus on insulin signaling. *Obesity* 17(3): S15-S20. doi: 10.1038/oby.2009.383

Fukuwatari T, Wada H, Shibata K (2008) Age-related alterations of B-group vitamin contents in urine, blood and liver from rats. *J Nutr Sci Vitaminol* 54(5): 357–362. doi: 10.3177/jnsv.54.357

Gargiulo S, Gamba P, Testa G, Leonarduzzi G, Poli G (2016) The role of oxysterols in vascular ageing. *J Physiol* 594(8): 2095–2113. doi: 10.1113/JP271168

Garvey SM, Russ DW, Skelding MB, Dugle JE, Edens NK (2015) Molecular and metabolomic effects of voluntary running wheel activity on skeletal muscle in late middle-aged rats. *Physiol Rep* 3(2): 1–17. doi: 10.14814/phy2.12319

Gerber T, Borg ML, Hayes A, Stathis CG (2014) High-intensity intermittent cycling increases purine loss compared with workload-matched continuous moderate intensity cycling. *Eur J Appl Physiol* 114(7): 1513–1520. doi: 10.1007/s00421-014-2878-x

Gibala MJ (2001) Regulation of skeletal muscle amino acid metabolism during exercise. *Int J Sport Nutr Exerc Metab* 11(1): 87–108. doi: 10.1123/ijsnem.11.1.87

Goodpaster BH, Katsiaras A, Kelley DE (2003) Enhanced fat oxidation through physical activity is associated with improvements in insulin sensitivity in obesity. *Diabetes* 52(9):2191-2197. doi: 10.2337/diabetes.52.9.2191

Goodyear LJ, Kahn BB (1998) Exercise, glucose transport, and insulin sensitivity. *Annu Rev Med* 49(1):235-261. doi: 10.1146/annurev.med.49.1.235

Gries KJ, Raue U, Perkins RK, et al. (2018). Cardiovascular and skeletal muscle health with lifelong exercise. *J Appl Physiol* 125(5): 1636–1645. doi: 10.1152/japplphysiol.00174.2018

Hanlon P, Nicholl BI, Jani BD, Lee D, McQueenie R, Mair FS (2018) Frailty and pre-frailty in middle-aged and older adults and its association with multimorbidity and mortality: a prospective analysis of 493 737 UK Biobank participants. *Lancet. Public health* 3(7): e323–e332. doi: 10.1016/S2468-2667(18)30091-4

Hansen M, Kennedy BK (2016) Does longer lifespan mean longer healthspan? *Trends Cell Biol* 26(8): 565-568. doi: 10.1016/j.tcb.2016.05.002

Harridge SDR, Lazarus NR (2017) Physical activity, aging, and physiological function. *Physiology* 32(2): 152–161. doi: 10.1152/physiol.00029.2016

Harris WS, Pottala JV, Varvel SA, Borowski JJ, Ward JN, McConnell JP (2013) Erythrocyte omega-3 fatty acids increase and linoleic acid decreases with age: Observations from 160,000 patients. *Prostaglandins Leukot Essent Fatty Acids* 88(4): 257–263. doi: 10.1016/j.plefa.2012.12.004

Hayes LD, Grace FM, Sculthorpe N, et al. (2013) The effects of a formal exercise training programme on salivary hormone concentrations and body composition in previously sedentary aging men. *SpringerPlus* 2(1): 1–5. doi: 10.1186/2193-1801-2-18

Heaney LM, Deighton K, Suzuki T (2017) Non-targeted metabolomics in sport and exercise science. *J Sports Sci* 37(9): 959-967. doi: 10.1080/02640414.2017.1305122

Heath GW, Gavin JR, Hinderliter JM, Hagberg JM, Bloomfield SA, Holloszy JO (1983) Effects of exercise and lack of exercise on glucose tolerance and insulin sensitivity. *J Appl Physiol Respir Environ Exerc Physiol* 55(2):512-517. doi: 10.1152/jappl.1983.55.2.512

Helge JW, Wu BJ, Willer M, Daugaard JR, Storlien LH, Kiens B (2001) Training affects muscle phospholipid fatty acid composition in humans. *J Appl Physiol*, 90(2): 670–677. doi: 10.1152/jappl.2001.90.2.670

Hellsten Y (2016) Limitations of skeletal muscle oxygen supply in ageing. *J Physiol* 594(8): 2259-2260. doi: 10.1113/JP272062

Henriksson J (1995) Influence of exercise on insulin sensitivity. *J Cardiovasc Risk* 2(4):303-309. doi: 10.1177/174182679500200404

Hiatt WR, Regensteiner JG, Wolfel EE, Ruff L, Brass EP (1989) Carnitine and acylcarnitine metabolism during exercise in humans. Dependence on skeletal muscle metabolic state. *J Clin Invest* 84(4): 1167–1173. doi: 10.1172/JCI114281

Hood DA, Terjung RL (1990) Amino acid metabolism during exercise and following endurance training. *Sports Med* 9(1): 23–35. doi: 10.2165/00007256-199009010-00003

Houmard JA, Tanner CJ, Slentz CA, Duscha BD, McCartney JS, Kraus WE (2004) Effect of the volume and intensity of exercise training on insulin sensitivity. *J Appl Physiol* 96(1):101-106. doi: 10.1152/japplphysiol.00707.2003

Houtkooper RH, Argmann C, Houten SM, et al. (2011) The metabolic footprint of aging in mice. *Sci Rep* 1:134. doi: 10.1038/srep00134

Hubbard RE, Lang IA, Llewellyn DJ, Rockwood K (2010) Frailty, body mass index, and abdominal obesity in older people. *J Gerontology A Biol Sci Med Sci* 65(4): 377–381. doi: 10.1093/gerona/glp186

Huffman KM, Slentz CA, Bateman LA, et al. (2011). Exercise-induced changes in metabolic intermediates, hormones, and inflammatory markers associated with improvements in insulin sensitivity. *Diabetes Care* 34(1): 174–176. doi: 10.2337/dc10-0709

Huffman KM, Koves TR, Hubal MJ, et al. (2014) Metabolite signatures of exercise training in human skeletal muscle relate to mitochondrial remodelling and cardiometabolic fitness. *Diabetologia* 57(11):2282–2295. doi: 10.1007/s00125-014-3343-4

Jang HJ, Lee JD, Jeon HS, Kim AR, Kim S, Lee HS, Kim KB (2018) Metabolic profiling of eccentric exercise-induced muscle damage in human urine. *Toxicol Res* 34(3): 199–210. doi: 10.5487/TR.2018.34.3.199

Janssen CI, Kiliaan AJ (2014) Long-chain polyunsaturated fatty acids (LCPUFA) from genesis to senescence: the influence of LCPUFA on neural development, aging, and neurodegeneration. *Prog Lipid Res* 53: 1–17. doi: 10.1016/j.plipres.2013.10.002

Jefferis BJ, Whincup PH, Lennon LT, Papacosta O, Goya Wannamethee S (2014) Physical activity in older men: longitudinal associations with inflammatory and hemostatic biomarkers, n-terminal pro-brain natriretic peptide, and onset of coronary heart disease and mortality. *J Am Geriatr Soc* 62(4): 599–606. doi: 10.1111/jgs.12748

Johnson AA, Stolzing A (2019) The role of lipid metabolism in aging, lifespan regulation, and age-related disease. *Aging Cell* 18(6): e13048. doi: 10.1111/acel.13048

Johnson LC, Martens CR, Santos-Parker JR, et al. (2018) Amino acid and lipid associated plasma metabolomic patterns are related to healthspan indicators with ageing in humans. *Clin Sci* 132(16): 1765-1777. doi: 10.1042/CS20180409

Jones JH (2007) Resource book for the design of animal exercise protocols. *Am J Vet Res* 68(6): 583-583. doi: 10.2460/ajvr.68.6.583

Justice JN, Ferrucci L, Newman AB, et al. (2018). A framework for selection of blood-based biomarkers for geroscience-guided clinical trials: report from the TAME Biomarkers Workgroup. *GeroScience*, 40(5-6): 419–436. doi: 10.1007/s11357-018-0042-y

Kahn SE, Larson VG, Beard JC, Cain KC, Fellingham GW, Schwartz RS, et al. (1990) Effect of exercise on insulin action, glucose tolerance, and insulin secretion in aging*. Am J Physiol* 258(6 Pt1): E937-E943. doi: 10.1152/ajpendo.1990.258.6.E937

Kawanishi N, Kato Y, Yokozeki K, Sawada S, Sakurai R, Fujiwara Y, et al. (2018) Effects of aging on serum levels of lipid molecular species as determined by lipidomics analysis in Japanese men and women. *Lipids Health Dis* 17(1): 135. doi: 10.1186/s12944-018-0785-6

Kim MJ, Yang HJ, Kim JH, Ahn CW, Lee JH, Kim KS, Kwon DY (2013) Obesity-related metabolomic analysis of human subjects in black soybean peptide intervention study by ultraperformance liquid chromatography and quadrupole-time-of-flight mass spectrometry. *J Obesity* (9492): 874981. doi: 10.1155/2013/874981

Kim S, Cheon HS, Song JC, Yun SM, Park SI, Jeon JP (2014) Aging-related changes in mouse serum glycerophospholipid profiles. *Osong Public Health Res Perspect* 5(6): 345–350. doi: 10.1016/j.phrp.2014.10.002

Kim YN, Choi JY, Cho YO (2015) Regular moderate exercise training can alter the urinary excretion of thiamin and riboflavin. *Nutr Res Pract* 9(1): 43–48. doi: 10.4162/nrp.2015.9.1.43

King DS, Baldus PJ, Sharp RL, Kesl LD, Feltmeyer TL, Riddle MS (1995) Time course for exercise-induced alterations in insulin action and glucose tolerance in middle-aged people. *J Appl Physiol* 78(1):17-22. doi: 10.1152/jappl.1995.78.1.17

Klein MS, Shearer J (2016) Metabolomics and type 2 diabetes: translating basic research into clinical application. *J Diabetes Res* 2016:3898502. doi: 10.1155/2016/3898502

Kochhar S, Jacobs DM, Ramadan Z, Berruex F, Fuerholz A, Fay LB (2006) Probing gender-specific metabolism differences in humans by nuclear magnetic resonance-based metabonomics. *Anal Biochem* 352(2): 274–281. doi: 10.1016/j.ab.2006.02.033

Krug S, Kastenmüller G, Stückler F, et al. (2012) The dynamic range of the human metabolome revealed by challenges. *FASEB J* 26(6):2607-2619. doi: 10.1096/fj.11-198093

Kuhl J, Moritz T, Wagner H, et al. (2008) Metabolomics as a tool to evaluate exercise-induced improvements in insulin sensitivity. *Metabolomics* 4(3): 273–282. doi: 10.1007/s11306-008-0118-2

Kujala UM, Mäkinen V, Heinonen I, et al. (2013) Long-term leisure-time physical activity and serum metabolome. *Circulation* 127(3): 340–348. doi: 10.1161/CIRCULATIONAHA.112.105551

Lai HT, de Oliveira Otto MC, Lemaitre RN, McKnight B, Song X, King IB, et al. (2018) Serial circulating omega 3 polyunsaturated fatty acids and healthy ageing among older adults in the Cardiovascular Health Study: prospective cohort study. *BMJ* 363: k4067. doi: 10.1136/bmj.k4067

Lara B, Salinero JJ, Gutiérrez J, et al. (2016) Influence of endurance running on calcaneal bone stiffness in male and female runners. *Eur J Appl Physiol* 116(2): 327–333. doi: 10.1007/s00421-015-3285-7

Lauretani F, Bandinelli S, Bartali B, Cherubini A, Iorio AD, Blé A, et al. (2007) Omega-6 and omega-3 fatty acids predict accelerated decline of peripheral nerve function in older persons. *Eur J Neurol* 14(7): 801–808. doi: 10.1111/j.1468-1331.2007.01860

Lazarus NR, Lord JM, Harridge SDR (2018) The relationships and interactions between age, exercise and physiological function. *J Physiol* 597(5): 1299-1309. doi: 10.1113/JP277071

Lee K, Jung K, Cho J, et al. (2017) High-fat diet and voluntary chronic aerobic exercise recover altered levels of aging-related tryptophan metabolites along the kynurenine pathway. *Exp Neurobiol* 26(3): 132–140. doi: 10.5607/en.2017.26.3.132

Leeuwenburgh C, Hansen PA, Holloszy JO, Heinecke JW (1999) Oxidized amino acids in the urine of aging rats: potential markers for assessing oxidative stress in vivo. *Am J Physiol* 276(1): R128–R135. doi: 10.1152/ajpregu.1999.276.1.R128

Lehmann R, Zhao X, Weigert C, et al. (2010) Medium chain acylcarnitines dominate the metabolite pattern in humans under moderate intensity exercise and support lipid oxidation. *PLoS ONE* 5(7): e11519. doi: 10.1371/journal.pone.0011519

Lemaitre RN, King IB, Sotoodehnia N, Rea TD, Trivellore ER, Rice KM, et al. (2009). *Metabolism* 58(4): 534-540. doi: 10.1016/j.metabol.2008.11.013

Leto D, Saltiel AR (2012) Regulation of glucose transport by insulin: traffic control of GLUT4. *Mol Cell Biol* 13(6):383-396. doi: 10.1038/nrm3351

Levine ME, Lu AT, Quach A, et al. (2018) An epigenetic biomarker of aging for lifespan and healthspan. *Aging* 10(4): 573–591. doi: 10.18632/aging.101414

Levinger I, Jerums G, Stepto NK, Parker L, Serpiello FR, McConell GK et al. (2014) The effect of acute exercise on undercarboxylated osteocalcin and insulin sensitivity in obese men. *J Bone Miner Res* 29(12):2571-2576. doi: 10.1002/jbmr.2285

Lewis G, Farrell L, Wood MJ, et al. (2010) Metabolic signatures of exercise in human plasma. *Sci Transl Med* 2(33): 33-37. doi: 10.1126/scitranslmed.3001006

Liguori I, Russo G, Curcio F, et al. (2018) Oxidative stress, aging, and diseases. *Clin Interv Aging* 13: 757–772. doi: 10.2147/CIA.S158513

Liu W, Liu Y, Yang Y, et al. (2018) Metabolic biomarkers of aging and aging-related diseases in Chinese middle-aged and elderly men. *J Nutr Health Aging* 22(10): 1189-1197. doi: 10.1007/s12603-018-1062-0

Lopez-Otin C, Blasco MA, Partridge L, Serrano M, Kroemer G (2013) The hallmarks of aging. *Cell* 153(6): 1194–1217. doi: 10.1016/j.cell.2013.05.039

Lustgarten MS, Fielding RA (2017) Metabolites related to renal function, immune activation, and carbamylation are associated with muscle composition in older adults. *Exp Gerontol* 100: 1–10. doi: 10.1016/j.exger.2017.10.003

Lustgarten MS, Price LL, Logvinenko T, et al. (2013). Identification of serum analytes and metabolites associated with aerobic capacity. *Eur J Appl Physiol* 113(5): 1311–1320. doi: 10.1007/s00421-012-2555-x

Lyudinina AY, Ivankova GE, Bojko ER (2018) Priority use of medium-chain fatty acids during high-intensity exercise in cross-country skiers. *J Int Soc Sports Nutr* 15(1): 57. doi: 1186/s12970-018-0265-4

Ma W, Wu JH, Wand Q, Lemaitre RN, Mukamai KJ, Djoussé L, et al. (2015). Prospective association of fatty acids in the de novo lipogenesis pathway with risk of type 2 diabetes: the Cardiovascular Health Study. *Am J Clin Nutr* 101(1): 153–163. doi: 10.3945/ajcn.114.092601

McConell GK, Kaur G, Falcão-Tebas F, Hong YH, Gatford KL (2015) Acute exercise increases insulin sensitivity in adult sheep: a new preclinical model. *Am J Physiol Regul Integr Comp Physiol* 308(6): R500-R506. doi: 10.1152/ajpregu.00466.2014

McKie GL, Medak KD, Knuth CM, et al. (2019) Housing temperature affects the acute and chronic metabolic adaptations to exercise in mice. *J Physiol* 597(2): 1–20. doi: 10.1113/JP278221

McNamara RK, Liu Y, Jandacek R, Rider T, Tso P (2008) The aging human orbitofrontal cortex: decreasing polyunsaturated fatty acid composition and associated increases in lipogenic gene expression and stearoyl-CoA desaturase activity. *Prostaglandins Leukot Essent Fatty Acids* 78(4-5): 293–304. doi: 10.1016/j.plefa.2008.04.001

Mittendorfer B, Klein S (2001) Effect of aging on glucose and lipid metabolism during endurance exercise. *Int J Sport Nutr Exerc Metab* 11: S86-S91. doi: 10.1123/ijsnem.11.s1.s86

Moaddel R, Fabbri E, Khadeer MA, et al. (2016) Plasma biomarkers of poor muscle quality in older men and women from the Baltimore longitudinal study of aging. *J Gerontol A Biol Sci Med Sci* 71(10): 1266–1272. doi: 10.1093/gerona/glw046

Mohajeri MH, Troesch B, Weber P (2015) Inadequate supply of vitamins and DHA in the elderly: implications for brain aging and Alzheimer-type dementia. *Nutrition* 31(2): 261–275. doi: 10.1016/j.nut.2014.06.016

Morris C, Grada CO, Ryan M, et al. (2013) The relationship between aerobic fitness level and metabolic profiles in healthy adults. *Mol Nutr Food Res* 57(7): 1246–1254. doi: 10.1002/mnfr.201200629

Motshakeri M, Ebrahimi M, Goh YM, Matanjun P, Mohamed S (2013). Sargassum polycystum reduces hyperglycaemia, dyslipidaemia and oxidative stress via increasing insulin sensitivity in a rat model of type 2 diabetes. *J Sci Food Agric* 93(7): 1772–1778. doi: 10.1002/jsfa.5971

Mougios V (2020) Exercise biochemistry. Human Kinetics, Champaign, IL

Mougios V, Petridou A (2012) Analysis of lipid profiles in skeletal muscles. In: J. DiMario (Ed.), *Methods in Molecular Biology* 798: 325–355. doi: 10.1007/978-1-61779-343-1

Mougios V, Ring S, Petridou A, Nikolaidis MG (2003) Duration of coffee- and exercise-induced changes in the fatty acid profile of human serum. *J Appl Physiol* 94:476-484. doi: 10.1152/japplphysiol.00624.2002

Mougios V, Kouidi E, Kyparos A, Deligiannis A (1998) Effect of exercise on the proportion of unsaturated fatty acids in serum of untrained middle-aged individuals. *Br J Sports Med* 32(1): 58-62. doi: 10.1136/bjsm.32.1.58

Mougios V, Kotzamanidis C, Koursari C, Atsopardis S (1995) Exercise-induced changes in the concentration of individual fatty acids and triacylglycerols of human plasma. *Metabolism* 44(5): 681-688. doi: 10.1016/0026-0495(95)90129-9

Muhsen AA, Burleigh M, Daskalaki E, Zhang T, Easton C, Watson DG (2016) Metabolomic profiling of submaximal exercise at a standardised relative intensity in healthy adults. *Metabolites* 6(1): 1–16. doi: 10.3390/metabo6010009

Mukherjee K, Edgett BA, Burrows HW, et al. (2014) Whole blood transcriptomics and urinary metabolomics to define adaptive biochemical pathways of high-intensity exercise in 50-60-year-old masters athletes. *PLoS ONE* 9(3): e92031. doi: 10.1371/journal.pone.0092031

Nagasawa J, Muraoka I, Sato Y (1995) Long-lasting effect of training on insulin responsiveness in the rat. *Int J Sports Med* 16(2):91-93. doi: 10.1055/s-2007-972971

Navas-Enamorado I, Bernier M, Brea-Calvo G, de Cabo R (2017) Influence of anaerobic and aerobic exercise on age-related pathways in skeletal muscle. *Ageing Res Rev* 37: 39–52. doi: 10.1016/j.arr.2017.04.005

Nazmi A, Weatherall M, Wilkins B, Robinson GM (2014) Thiamin concentration in geriatric hospitalized patients using frusemide. *J Nutr Gerontol Geriatr* 33(1): 37–41. doi: 10.1080/21551197.2013.875501

Neal CM, Hunter AM, Brennan L, et al. (2013) Six weeks of a polarized training-intensity distribution leads to greater physiological and performance adaptations than a threshold model in trained cyclists. *J Appl Physiol* 114(4): 461–471. doi: 10.1152/japplphysiol.00652.2012

Netzer M, Weinberger KM, Handler M, et al. (2011). Profiling the human response to physical exercise: a computational strategy for the identification and kinetic analysis of metabolic biomarkers. *J Clin Bioinforma* 1(1): 34. doi: 10.1186/2043-9113-1-34

Newsom,SA, Schenk S (2014) Interaction between lipid availability, endurance exercise and insulin sensitivity. *Med Sport Sci* 60:62-70. doi: 10.1159/000357336

Nieman DC, Gillitt ND, Henson DA, et al. (2012) Bananas as an Energy Source during Exercise: A Metabolomics Approach. *PLoS ONE* 7(5): e37479. doi: 10.1371/journal.pone.0037479

Nieman DC, Gillitt ND, Knab AM, Shanely RA, Pappan KL, Jin F, Lila MA (2013) Influence of a polyphenol-enriched protein powder on exercise-induced inflammation and oxidative stress in athletes: a randomized trial using a metabolomics approach. *PLoS ONE* 8(8): e72215. doi: 10.1371/journal.pone.0072215

Nieman DC, Shanely RA, Gillitt ND, Pappan KL, Lila MA (2013) Serum metabolic signatures induced by a three-day intensified exercise period persist after 14 h of recovery in runners. *J Proteome Res* 12(10): 4577-4584. doi: 10.1021/pr400717j

Nieman DC, Shanely RA, Luo B, Meaney MP, Dew DA, Pappan KL (2014) Metabolomics approach to assessing plasma 13- and 9-hydroxy-octadecadienoic acid and linoleic acid metabolite responses to 75-km cycling. *Am J Physiol Regul Integr Comp Physiol* 307(1): R68–R74. doi: 10.1152/ajpregu.00092.2014

Nikolaidis MG, Petridou A, Mougios V (2006) Comparison of the phospholipid and triacylglycerol fatty acid profile of rat serum, skeletal muscle and heart. *Physiol Res* 55: 259-265. PMID: 16083311

Nikolaidis MG, Petridou A, Matsakas A, Schulz T, Michna H, Mougios V (2004) Effect of chronic wheel running on the farry acid composition of phospholipids and triacylglycerols in rat serum, skeletal muscle and heart. *Acta Physiol Scand* 181:199-208. doi: 10.1111/j.1365-201X.2004.01277.x

Nikolaidis MG, Mougios V (2004) Effects of exercise on the fatty-acid composition of blood and tissue lipids. *Sports Med* 34(15): 1051-1076. doi: 10.2165/00007256-200434150-00004

OECD, EU (2012) Health at a glance: Europe 2012. *OECD Publishing*. doi: 10.1787/9789264183896-en

OECD, EU (2018) Health at a glance: Europe 2018: state of health in the EU cycle. *OECD Publishing*, Paris. doi: 10.1787/health_glance_eur-2018-en

Palacios G, Pedrero-Chamizo R, Palacios N, et al. (2015) Biomarkers of physical activity and exercise. *Nutr Hosp* 31(3): 237–244. doi: 10.3305/nh.2015.31.sup3.8771

Pararasa C, Ikwuobe J, Shigdar S, Boukouvalas A, Nabney IT, Brown JE, et al. (2016) Age-associated changes in long-chain fatty acid profile during healthy aging promote pro-inflammatory monocyte polarization via PPARγ. *Aging* Cell 15(1): 128–139. doi: 10.1111/acel.12416

Pasini E, Corsetti G, Aquilani R, Romano C, Picca A, Calvani R, Dioguardi FS (2018) Protein-amino acid metabolism disarrangements: the hidden enemy of chronic age-related conditions. *Nutrients* 10(4): 1–11. doi: 10.3390/nu10040391

Peake JM, Tan SJ, Markworth JF, Broadbent JA, Skinner TL, Cameron-Smith D (2014) Metabolic and hormonal responses to isoenergetic high-intensity interval exercise and continuous moderate-intensity exercise. *Am J Physiol Endocrinol Metab* 307(7): E539–E522. doi: 10.1152/ajpendo.00276.2014

Pechlivanis A, Kostidis S, Saraslanidis P, et al. (2010) (1)H NMR-based metabonomic investigation of the effect of two different exercise sessions on the metabolic fingerprint of human urine. *J Proteome Res* 9(12): 6405–6416. doi: 10.1021/pr100684t

Pechlivanis A, Kostidis S, Saraslanidis P, et al. (2013) (1)H NMR study on the short- and long-term impact of two training programs of sprint running on the metabolic fingerprint of human serum. *J Proteome Res* 12(1): 470–480. doi: 10.1021/pr300846x

Pechlivanis A, Papaioannou KG, Tsalis G, Saraslanidis P, Mougios V, Theodoridis GA (2015) Monitoring the response of the human urinary metabolome to brief maximal exercise by a combination of RP-UPLC-MS and 1 H NMR spectroscopy. *J Proteome Res* 14(11): 4610-4622. doi: 10.1021/acs.jproteome.5b00470

Petibois C, Déléris G. (2003) Effects of short- and long-term detraining on the metabolic response to endurance exercise. *Int J Sports Med* 24(5): 320–325. doi: 10.1055/s-2003-40708

Petridou A, Lazaridou D, Mougios V (2015) Lipidemic profile of athletes and non-athletes with similar body fat. Int *J Sport Nutr Exerc Metab* 15(4):425-432. doi: 10.1123/ijsnem.15.4.425

Pohjanen E, Thysell E, Eklund C, et al. (2007) A multivariate screening strategy for investigating metabolic effects of strenuous physical exercise in human serum. *J Proteome Res* 6(6): 2113–2120. doi: 10.1021/pr070007g

Pollock RD, Carter S, Velloso CP, Duggal NA, Lord JM, Lazarus NR, Harridge SD (2015) An investigation into the relationship between age and physiological function in highly active older adults. *J Physiol* 593(3): 657–680. doi: 10.1113/jphysiol.2014.282863

Poortmans JR, Kumps A, Duez P, Fofonka A, Carpentier A, Francaux M (2005) Effect of oral creatine supplementation on urinary methylamine, formaldehyde and formate. *Med Sci Sports Exerc* 37(10): 1717–1720. doi: 10.1249/01.mss.0000176398.64189.e6

Pöyhönen MJ, Uusitalo UM, Kari A, Takala JA, Alakuijala LA, Eloranta TO (1990) Urinary excretion of polyamines: importance of circadian rhythm, age, sex, menstrual cycle, weight, and creatinine excretion. *Am J Clin Nutr* 52(4): 746–751. doi: 10.1093/ajcn/52.4.746

Proitsi P, Kuh D, Wong A, Maddock J, Bendayan R, Wulaningsih W, Hardy R, Richards M (2018) Lifetime cognition and late midlife blood metabolites: findings from a British birth cohort. *Transl Psychiatry* 8(1): 203–214. doi: 10.1038/s41398-018-0253-0

Psihogios NG, Gazi IF, Elisaf MS, Seferiadis KI, Bairaktari ET (2008) Gender-related and age-related urinalysis of healthy subjects by NMR-based metabonomics. *NMR Biomed* 21(3): 195–207. doi: 10.1002/nbm.1176

Quijano C, Trujillo M, Castro L, Trostchansky A (2016) Redox biology interplay between oxidant species and energy metabolism. *Redox Biol* 8: 28–42. doi: 10.1016/j.redox.2015.11.010

Ravera S, Podestà M, Sabatini F, Dagnino M, Cilloni D, Fiorini S, et al. (2019) Discrete changes in glucose metabolism define aging. *Sci Rep* 9:10347. doi: 10.1038/s41598-019-46749-w

Rawson ES, Venezia AC (2011) Use of creatine in the elderly and evidence for effects on cognitive function in young and old. *Amino Acids* 40(5): 1349–1362. doi: 10.1007/s00726-011-0855-9

Rea IM (2017) Towards ageing well: Use it or lose it: Exercise, epigenetics and cognition. *Biogerontology* 18(4): 679-697. doi: 10.1007/s10522-017-9719-3

Refaey ME, McGee-Lawrence ME, Fulzele S, et al. (2017) Kynurenine, a tryptophan metabolite that accumulates with age, induces bone loss. *J Bone Miner Res* 32(11): 2182–2193. doi: 10.1002/jbmr.3224

Rennie MJ, Tipton KD (2000) Protein and amino acid metabolism during and after exercise and the effects of nutrition. *Annu Rev Nutr* 20: 457–483. doi: 10.1146/annurev.nutr.20.1.457

Richardson JR, Pipkin JA, O'Dell LE, Nazarian A (2014) Insulin resistant rats display enhanced rewarding effects of nicotine. *Drug Alcohol Depend* 140: 205-207. doi: 10.1016/j.drugalcdep.2014.03.028

Sakaguchi CA, Nieman DC, Signini EF, Abreu RM, Catai AM (2019) Metabolomics-based studies assessing exercise-induced alterations of the human metabolome: a systematic review. *Metabolites* 9(8): 1–15. doi: 10.3390/metabo9080164

Salminen A, Kauppinen A, Hiltunen M, Kaarniranta K (2014) Krebs cycle intermediates regulate DNA and histone methylation: Epigenetic impact on the aging process. *Ageing Res Rev* 16: 45–65. doi: 10.1016/j.arr.2014.05.004

Sampson DL, Broadbent JA, Parker AW, Upton Z, Parker TJ (2014) Urinary biomarkers of physical activity: candidates and clinical utility. *Expert Rev Proteomics* 11(1): 91–106. doi: 10.1586/14789450.2014.859527

Sato S, Basse AL, Schönke M, et al. (2019). Time of exercise specifies the impact on muscle metabolic pathways and systemic energy homeostasis. *Cell Metab* 30(1): 92-110. doi: 10.1016/j.cmet.2019.03.013

Schnackenberg LK, Sun J, Espandiari P, Holland RD, Hanig J, Beger RD (2007) Metabonomics evaluations of age-related changes in the urinary compositions of male Sprague Dawley rats and effects of data normalization methods on statistical and quantitative analysis. *Bioinformatics* 8(7): S3. doi: 0.1186/1471-2105-8-S7-S3

Seals DR, Justice JN, Larocca TJ (2016) Physiological geroscience: targeting function to increase healthspan and achieve optimal longevity. *J Physiol* 594(8): 2001–2024. doi: 10.1113/jphysiol.2014.282665

Sebastiani P, Thyagarajan B, Sun F, Schupf N, Newman AB, Montano M, Perls TT (2017) Biomarker signatures of aging. *Aging Cell* 16(2): 329–338. doi: 10.1111/acel.12557

Sheedy JR, Gooley PR, Nahid A, et al. (2014) 1H-NMR analysis of the human urinary metabolome in response to an 18-month multi-component exercise program and calcium–vitamin-D3 supplementation in older men. *Appl Physiol Nutr Metab* 39(11): 1294–1304. doi: 10.1139/apnm-2014-0060

Shimazu T, Hirschey MD, Huang JY, Ho LTY, Verdin E (2010) Acetate metabolism and aging: An emerging connection. *Mech Ageing Dev* 131(7–8):511–516. doi: 10.1016/j.mad.2010.05.001

Shojaee-Moradie F, Baynes KC, Pentecost C, Bell JD, Thomas EL, Jackson NC, et al. (2007) Exercise training reduces fatty acid availability and improves the insulin sensitivity of glucose metabolism. *Diabetologia* 50(2): 404–413. doi: 10.1007/s00125-006-0498-7

Shoveller AK, McKnight LM, Wood KM, Cant JP (2018) Lessons from animal nutritionists: dietary amino acid requirement studies and considerations for healthy aging studies. *Ann NY Acad Sci* 1418(1): 20–30. doi: 10.1111/nyas.13546

Siddharth J, Chakrabarti A, Pannérec A, et al. (2017) Aging and sarcopenia associate with specific interactions between gut microbes, serum biomarkers and host physiology in rats. *Aging* 9(7): 1698–1714. doi: 10.18632/aging.101262

Siopi A, Deda O, Manou V, et al. (2017) Effects of different exercise modes on the urinary metabolic fingerprint of men with and without metabolic syndrome. *Metabolites* 7(5):1-15. doi: 10.3390/metabo7010005

Siopi A, Deda O, Manou V, et al. (2019) Comparison of the serum metabolic fingerprint of different exercise modes in men with and without metabolic syndrome. *Metabolites* 9(116):1–17. doi: 10.3390/metabo9060116

Slupsky CM, Rankin KN, Wagner J, et al. (2007) Investigations of the effects of gender, diurnal variation, and age in human urinary metabolomic profiles. *Anal Chem* 79(18): 6995–7004. doi: 10.1021/ac0708588

Solon-Biet SM, Cogger VC, Pulpitel T, et al. (2019) Branched chain amino acids impact health and lifespan indirectly via amino acid balance and appetite control. *Nat Metab* 1(5): 532–545. doi: 10.1038/s42255-019-0059-2

Sparks LM (2017) Exercise training response heterogeneity: physiological and molecular insights. *Diabetologia* 60(12): 2329–2336. doi: 10.1007/s00125-017-4461-6

Starnes JW, Parry TL, O'Neal SK, et al. (2017) Exercise-induced alterations in skeletal muscle, heart, liver, and serum metabolome identified by non-targeted metabolomics analysis. *Metabolites* 7(3): 1–14. doi: 10.3390/metabo7030040

Stella AB, Cappellari GC, Barazzoni R, Zanetti M (2018) Update on the impact of omega 3 fatty acids on inflammation, insulin resistance and sarcopenia: a review. *Int J Mol Sci* 19(1): 218. doi: 10.3390/ijms19010218

Suskin NG, Heigenhauser G, Afzal R, Finegood D, Gerstein HC, McKelvie RS (2007) The effects of exercise training on insulin resistance in patients with coronary artery disease. *Eur J Cardiovasc Prev Rehabil* 14(6):803-808. doi: 10.1097/HJR.0b013e3282eea540

Takeshita H, Horiuchi M, Izumo K, et al. (2012) Long-term voluntary exercise, representing habitual exercise, lowers visceral fat and alters plasma amino acid levels in mice. *Environ Health Prev Med* 17(4):275–284. doi: 10.1007/s12199-011-0249-3

Tarnopolky LJ, MacDougall JD, Atkinson SA, Tarnopolsky MA, Sutton JR (1990) Gender differences in substrate for endurance exercise. *J Appl Physiol* 68(1): 302–308. doi: 10.1152/jappl.1990.68.1.302

Thysell E, Chorell E, Svensson MB, Jonsson P, Antti H (2012) Validated and predictive processing of gas chromatography-mass spectrometry based metabolomics data for large scale screening studies, diagnostics and metabolite pattern verification. *Metabolites* 2(4): 796–817. doi: 10.3390/metabo2040796

Thomas J, Thomas CJ, Radcliffe J, Itsiopoulos C (2015) Omega-3 fatty acids in early prevention of inflammatory neurodegenerative disease: a focus on Alzheimer' s disease. *Biomed Res Int* 2015: 172801. doi: 10.1155/2015/172801

Timmerman KL, Volpi E (2008) Amino acid metabolism and regulatory effects in aging. *Curr Opin Clin Nutr Metab Care* 11(1): 45–49. doi: 10.1097/MCO.0b013e3282f2a592

Tosato M, Marzetti E, Cesari M, Savera G, Miller RR, Bernabei R, Landi F, Calvani R (2017) Measurement of muscle mass in sarcopenia: from imaging to biochemical markers. *Aging Clin Exp Res* 29(1): 19–27. doi: 10.1007/s40520-016-0717-0

Tsukamoto I, Sugawara S (2018) Low levels of linoleic acid and α-linolenic acid and high levels of arachidonic acid in plasma phospholipids are associated with hypertension. *Biomed Rep* 8: 69–76. doi: 10.3892/br.2017.1015

Tutino V, de Nunzio V, Caruso MG, Bonfiglio C, Franco I, Mirizzi A, et al. (2018) Aerobib physical activity and low glycemic diet reduce the AA/EPA ratio in red blood cell membranes of patients with NAFLD. *Nutrients* 10(9): E1299. doi: 10.3390/nu10091299

Valentini L, Ramminger S, Haas V, Postrach E, Werich M, Fischer A, et al. (2014) Small intestinal permeability in older adults. *Physiol Rep 2*(4): e00281. doi: 10.1002/phy2.281

Viña J, Rodriguez-Mañas L, Salvador-Pascual A, Tarazona-Santabalbina FJ, Gomez-Cabrera MC (2016) Exercise: The lifelong supplement for healthy ageing and slowing down the onset of frailty. *J Physiol* 594(8): 1989–1999. doi: 10.1113/JP270536

Virgiliou C, Sampsonidis I, Gika HG, Raikos N, Theodoridis GA (2015) Development and validation of a HILIC-MS/MS multitargeted method for metabolomics applications. *Electrophoresis* 36(18): 2215–2225. doi: 10.1002/elps.201500208

de Vries NM, van Ravensberg CD, Hobbelen JS, Olde Rikkert MG, Staal JB, Nijhuis-van der Sanden MW (2012) Effects of physical exercise therapy on mobility, physical functioning, physical activity and quality of life in community-dwelling older adults with impaired mobility, physical disability and/or multi-morbidity: A meta-analysis. *Ageing Res Rev* 11(1): 136–149. doi: 10.1016/j.arr.2011.11.002

Wagenmakers AJ (1998) Muscle amino acid metabolism at rest and during exercise: role in human physiology and metabolism. *Exerc Sport Sci Rev* 26: 287–314. PMID: 9696993

Wang F, Han J, He Q, Geng Z, Deng Z, Qiao D (2015) Applying ^{1}H NMR spectroscopy to detect changes in the urinary metabolite levels of Chinese half-pipe snowboarders after different exercises. *J Anal Methods Chem* 1-9. doi: 10.1155/2015/315217

Walters RO, Arias E, Diaz A, Burgos ES, Guan F, Tiano S, et al. (2018) Sarcosine is uniquely modulated by aging and dietary restriction in rodents and humans. *Cell Rep* 25(3): 663-676. doi: 10.1016/j.celrep.2018.09.065

Wan QL, Meng X, Fu X, Chen B, Yang J, Yang H, Zhou Q (2019) Intermediate metabolites of the pyrimidine metabolism pathway extend the lifespan of C. *elegans* through regulating reproductive signals. *Aging* 11(12): 3993–4010. doi: 10.18632/aging.102033

Weir NL, Steffen BT, Guan W, Johnson LM, Djousse L, Mukamal KJ, Tsai MY (2019) Circulating omega-7 fatty acids are differentially related to metabolic dysfunction and incident type II diabetes: the multi-ethnic study of atherosclerosis (MESA). *Diabetes Metab* 1–7. doi: 10.1016/j.diabet.2019.10.005

Weiser MJ, Butt CM, Mohajeri MH (2016) Docosahexaenoic acid and cognition throughout the lifespan. *Nutrients* 8(2): 99. doi: 10.3390/nu8020099

Wishart DS (2019) Metabolomics for investigating physiological and pathophysiological processes. *Physiol Rev* 99(4): 1819–1875. doi: 10.1152/physrev.00035.2018

Wishart DS, Feunang YD, Marcu A, Guo AC, Liang K, et al. (2018) HMDB 4.0: the human metabolome database for 2018. *Nucleic Acids Res* 46(D1): D608-D617. doi: 10.1093/nar/gkx1089

Wu B, Yan S, Lin Z, Wang Q, Yang Y, Yang G, Shen Z, Zhang W (2008) Metabonomic study on ageing: NMR-based investigation into rat urinary metabolites and the effect of the total flavone of Epimedium. *Mol Biosyst* 4(8): 855-861. doi: 10.1039/b800923f

Wu J, Gao Y (2015) Physiological conditions can be reflected in human urine proteome and metabolome. *Expert Rev Proteomics* 12(6): 623–636. doi: 10.1586/14789450.2015.1094380

Wu JHY, Marklund M, Imamura F, Tintle N, Ardisson Korat AV, de Goefe J, et al. (2017) Omega-6 fatty acid biomarkers and incident type 3 diabetes: pooled analysis of individual-level data for 39740 adults from 20 prospective cohort studies. *Lancet Diabetes Endocrinol* 5(12): 965–974. doi: 10.1016/S2213-8587(17)30307-8

Xiao Q, Moore SC, Keadle SK, et al. (2016) Objectively measured physical activity and plasma metabolomics in the Shanghai Physical Activity Study. *Int J Epidemiol* 45(5): 1433–1444. doi: 10.1093/ije/dyw033

Young VR (1990) Protein and amino acid metabolism with reference to aging and the elderly. *Prog Clin Biol Res* 325:279-300. PMID: 2405423

Zhang A, Sun H, Wu X, Wang X (2012) Urine metabolomics. *Clinica Chimica Acta* 414: 65–69. doi: 10.1016/j.cca.2012.08.016

Zhou W, Zeng G, Lyu C, Kou F, Zhang S, Wei H (2019) The effect of exhaustive exercise on plasma metabolic profiles of male and female rats. *J Sports Sci Med* 18(2): 253–263. PMID: 31191095

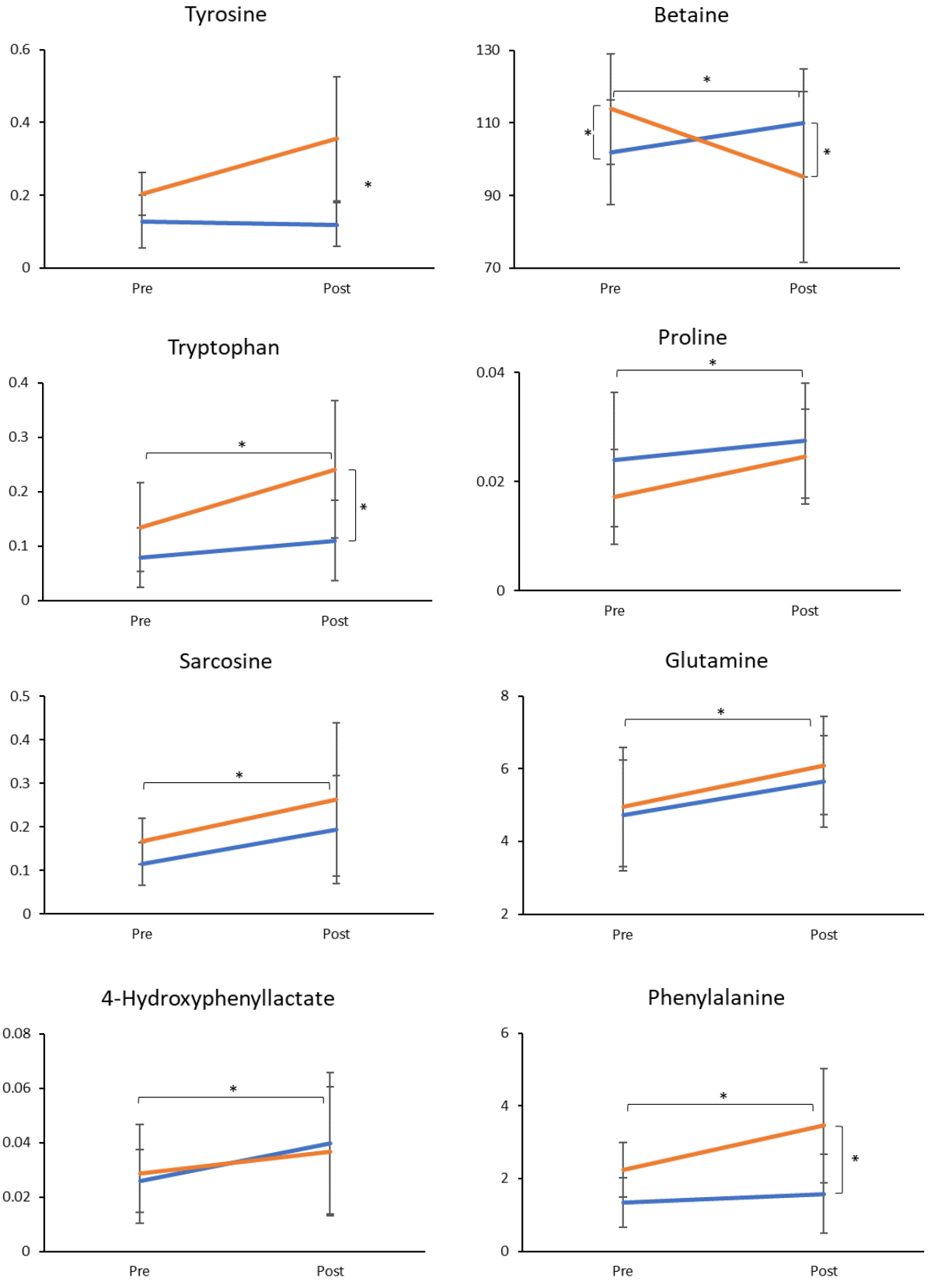
Zielínski J, Krasinska B, Kusy K (2013) Hypoxanthine as a predictor of performance in highly trained athletes. *Int J Sports Med* 34(12): 1079–1086. doi: 10.1055/s-0033-1337947

Zielínski J, Kusy K (2012) Training-induced adaptation in purine metabolism in high-level sprinters vs. triathletes. *J Appl Physiol* 112(4): 542–551. doi: 10.1152/japplphysiol.01292.2011

Zielínski J, Kusy K, Rychlewski T (2011) Effect of training load structure on purine metabolism in middle-distance runners. *Med Sci Sports Exerc* 46(9): 1798–1808. doi: 10.1249/MSS.ObO13e318215d10b

Zieliński J, Slominska EM, Król-Zielińska M, Krasiński Z Kusy K (2019) Purine metabolism in sprint- vs endurance-trained athletes aged 20–90 years. *Sci Rep* 9: 1–10. doi: 10.1038/s41598-019-48633-z

APPENDIX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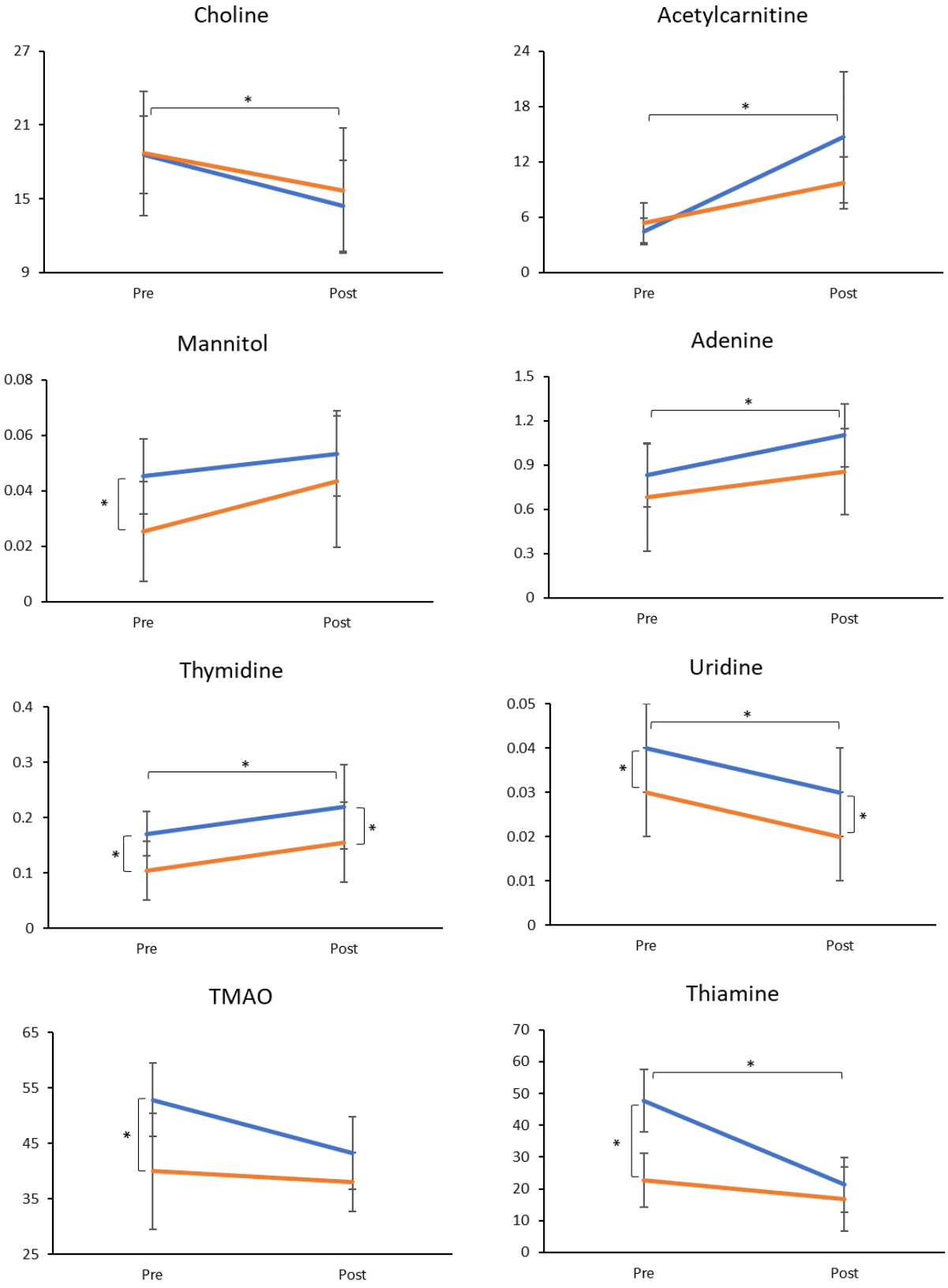
0.6
0.4
0.2
0
Pre
Post
*
Betaine
130
110
90
70
Pre
Post
*
*
*
Tryptophan
0.4
0.3
0.2
0.1
0
Pre
Post
*
*
Proline
0.04
0.02
0
Pre
Post
*
Sarcosine
0.5
0.4
0.3
0.2
0.1
0
Pre
Post
*
Glutamine
8
6
4
2
Pre
Post
*
4-Hydroxyphenyllactate
0.08
0.06
0.04
0.02
0
Pre
Post
*
Phenylalanine
6
4
2
0
Pre
Post
*
*


Choline
27
21
15
9
*
Pre
Post
Acetylcarnitine
24
18
12
6
0
*
Pre
Post
Mannitol
0.08
0.06
0.04
0.02
0
*
Pre
Post
Adenine
1.5
1.2
0.9
0.6
0.3
0
*
Pre
Post
Thymidine
0.4
0.3
0.2
0.1
0
*
*
*
Pre
Post
Uridine
0.05
0.04
0.03
0.02
0.01
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*
*
*
Pre
Post
TMAO
65
55
45
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Pre
Post
Thiamine
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40
30
20
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0
*
*
Pre
Post

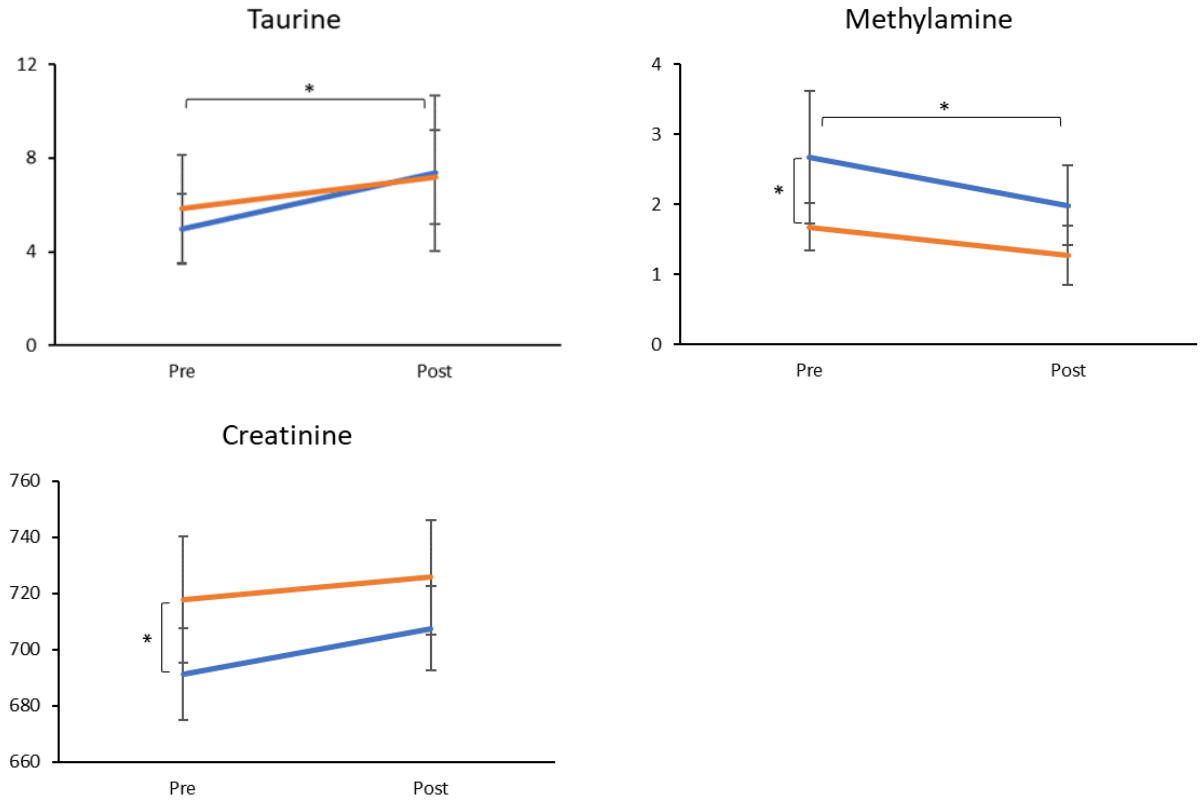
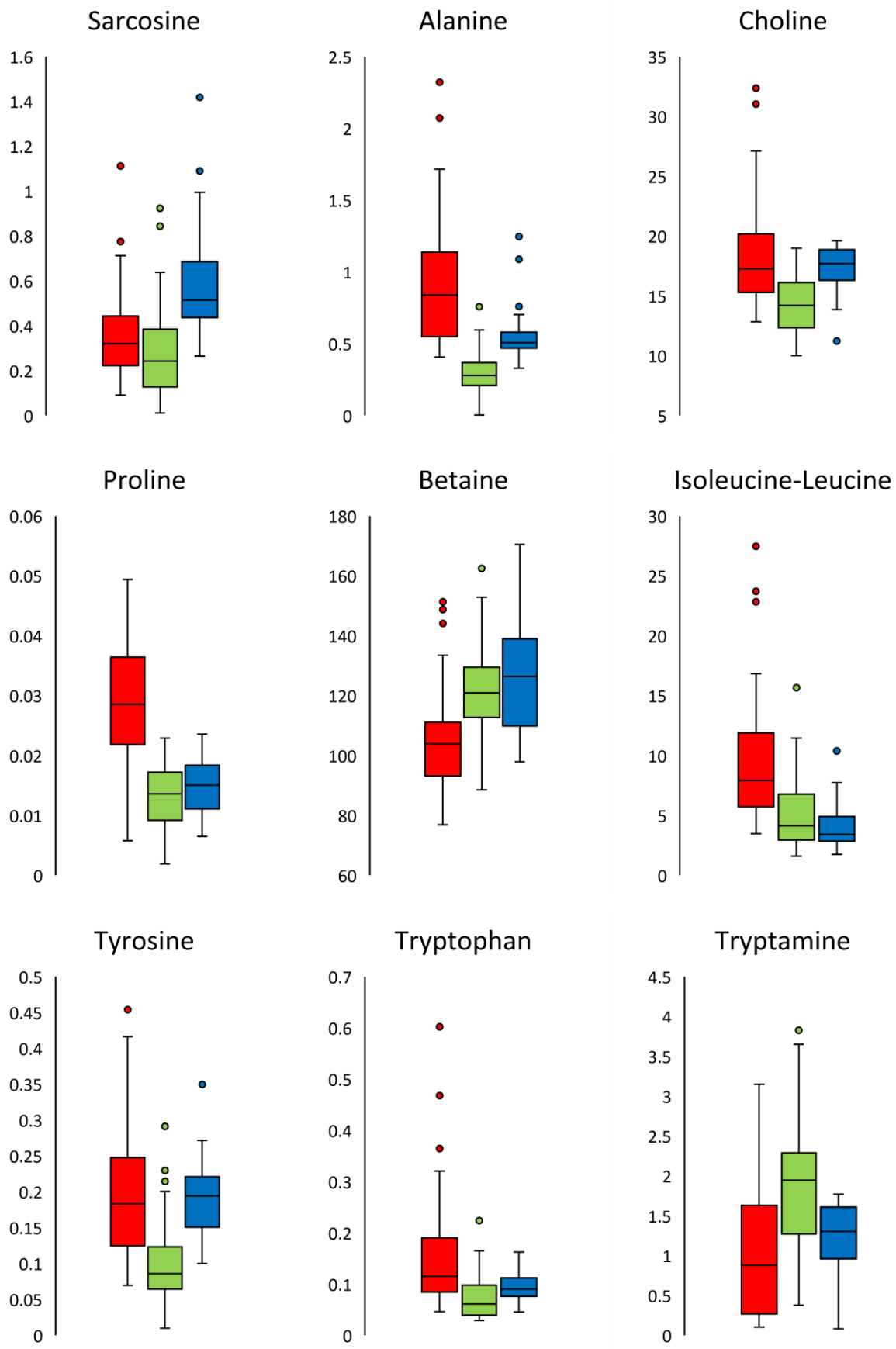
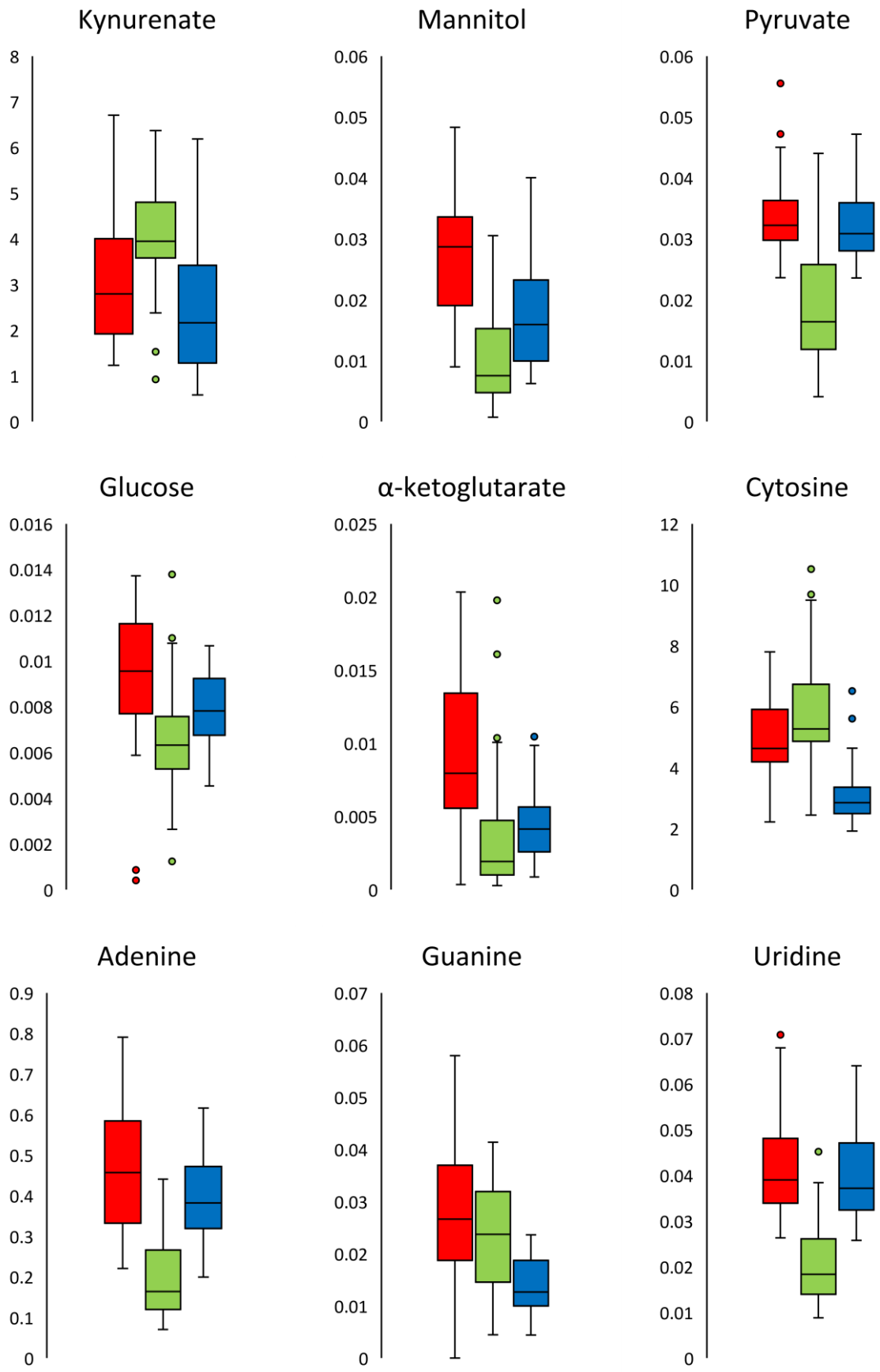


Fig. A1 Graphical representations of changes in urinary metabolites between 3 (blue) and 12 (brown) months of age, pre- and post-exercise. The vertical axis shows peak area in arbitrary units. *$p < 0.05$.


Sarcosine
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Alanine
2.5
2
1.5
1
0.5
0
Choline
35
30
25
20
15
10
5
Proline
0.06
0.05
0.04
0.03
0.02
0.01
0
Betaine
180
160
140
120
100
80
60
Isoleucine-Leucine
30
25
20
15
10
5
0
Tyrosine
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Tryptophan
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Tryptamine
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0


Kynurenate
8
7
6
5
4
3
2
1
0
Mannitol
0.06
0.05
0.04
0.03
0.02
0.01
0
Pyruvate
0.06
0.05
0.04
0.03
0.02
0.01
0
Glucose
0.016
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
α-ketoglutarate
0.025
0.02
0.015
0.01
0.005
0
Cytosine
12
10
8
6
4
2
0
Adenine
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Guanine
0.07
0.06
0.05
0.04
0.03
0.02
0.01
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Uridine
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0

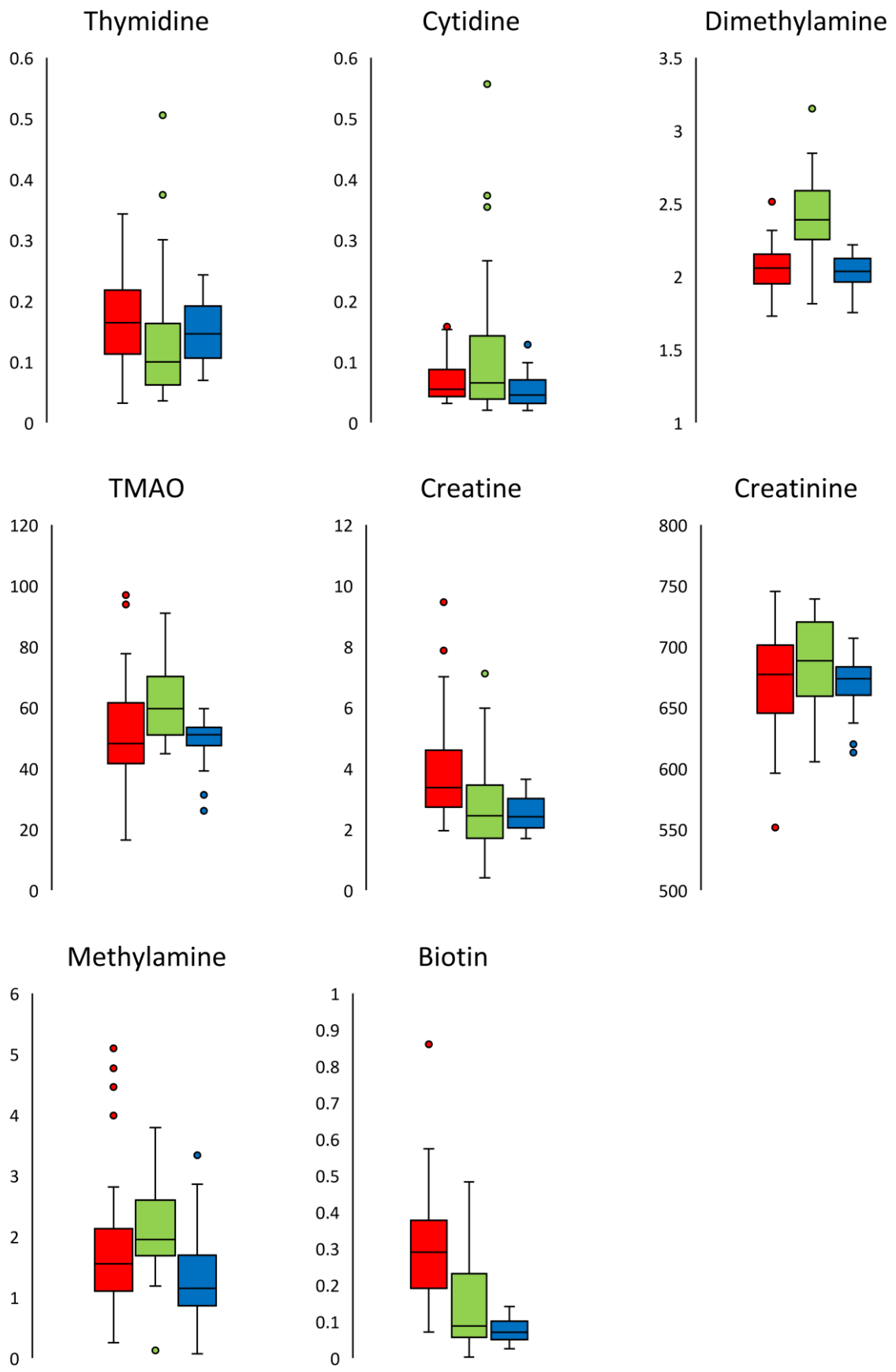
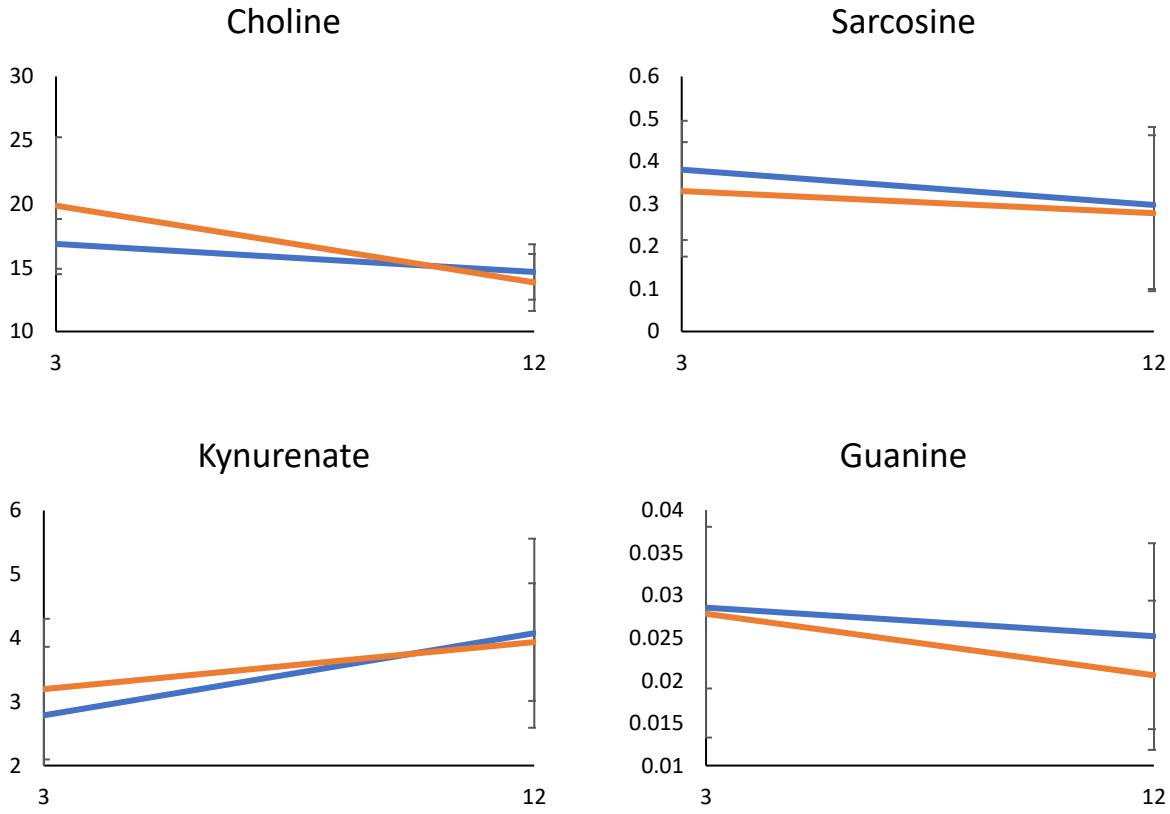

Thymidine
0.6
0.5
0.4
0.3
0.2
0.1
0
Cytidine
0.6
0.5
0.4
0.3
0.2
0.1
0
Dimethylamine
3.5
3
2.5
2
1.5
1
TMAO
120
100
80
60
40
20
0
Creatine
12
10
8
6
4
2
0
Creatinine
800
750
700
650
600
550
500
Methylamine
6
5
4
3
2
1
0
Biotin
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0

Fig. A2 Box plots of changes in urinary metabolites between 3 (red), 12 (green) and 21 (blue) months of age. The vertical axis shows peak area in arbitrary units. Each box represents the interquartile range, and the center line represents the median. Whiskers are extended to the most extreme data point that is no more than 1.5 times the interquartile range from the edge of the box (Tukey style). Dots represent outliers.



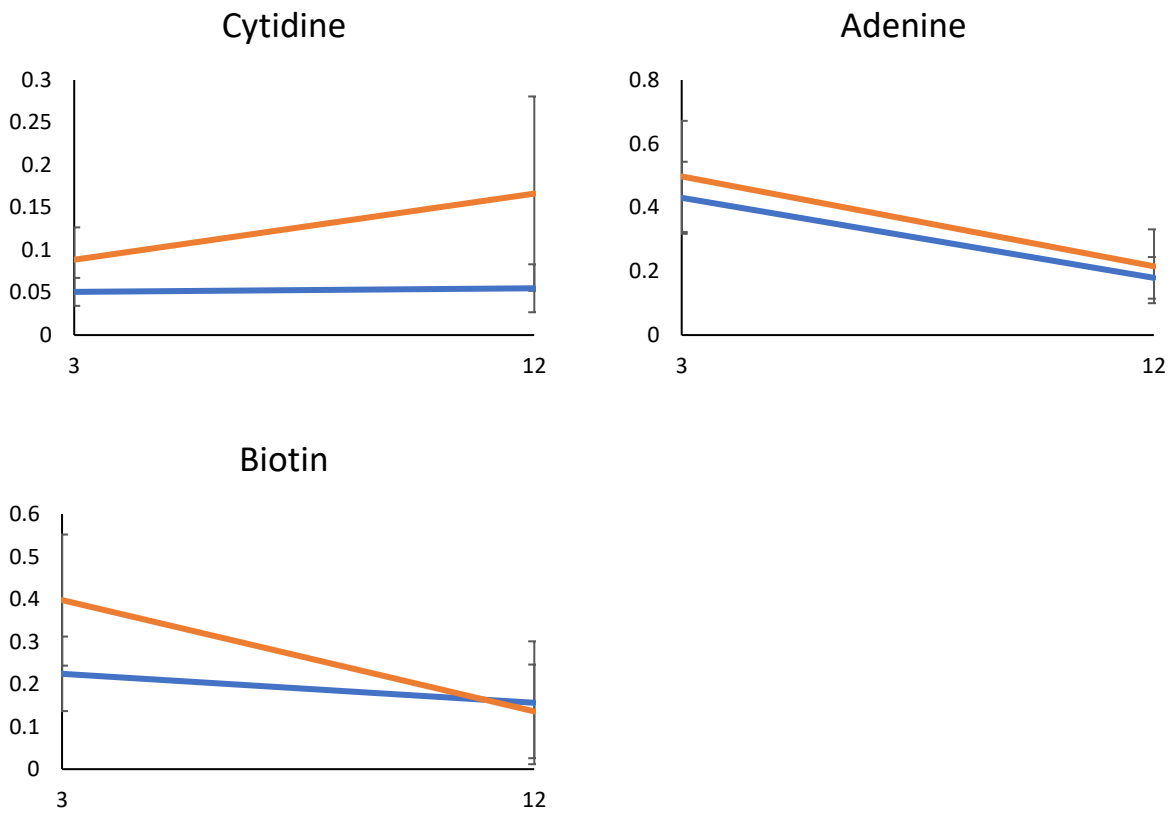
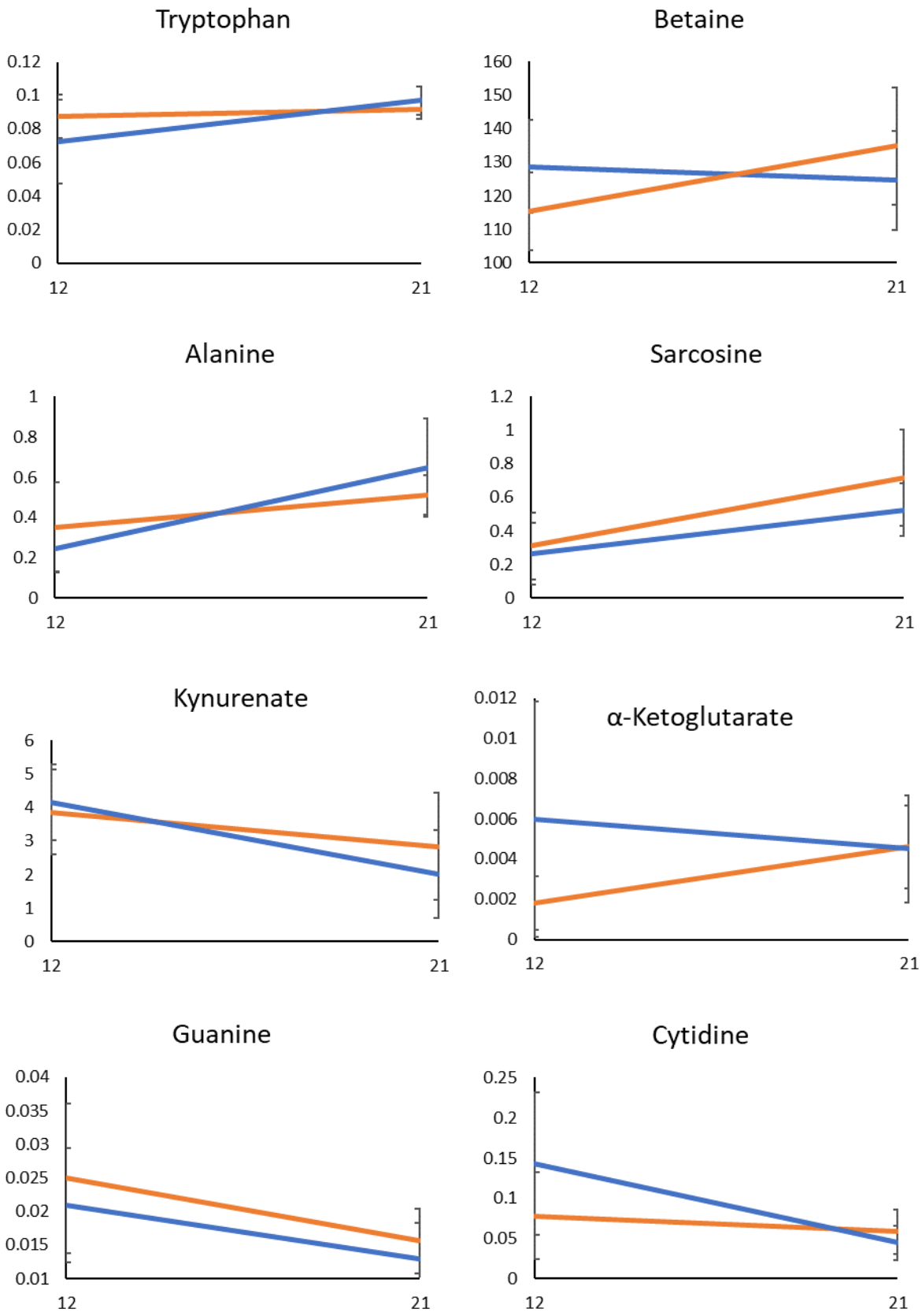


Fig. A3 Graphical representations of changes in urinary metabolites between the exercising groups (A and B, blue) and the non-exercising groups (C and D, brown) during the 1st half of life. The vertical axis shows peak area in arbitrary units.


Tryptophan
0.12
0.1
0.08
0.06
0.04
0.02
0
12
21
Betaine
160
150
140
130
120
110
100
12
21
Alanine
1
0.8
0.6
0.4
0.2
0
12
21
Sarcosine
1.2
1
0.8
0.6
0.4
0.2
0
12
21
Kynurenate
6
5
4
3
2
1
0
12
21
α-Ketoglutarate
0.012
0.01
0.008
0.006
0.004
0.002
0
12
21
Guanine
0.04
0.035
0.03
0.025
0.02
0.015
0.01
12
21
Cytidine
0.25
0.2
0.15
0.1
0.05
0
12
21

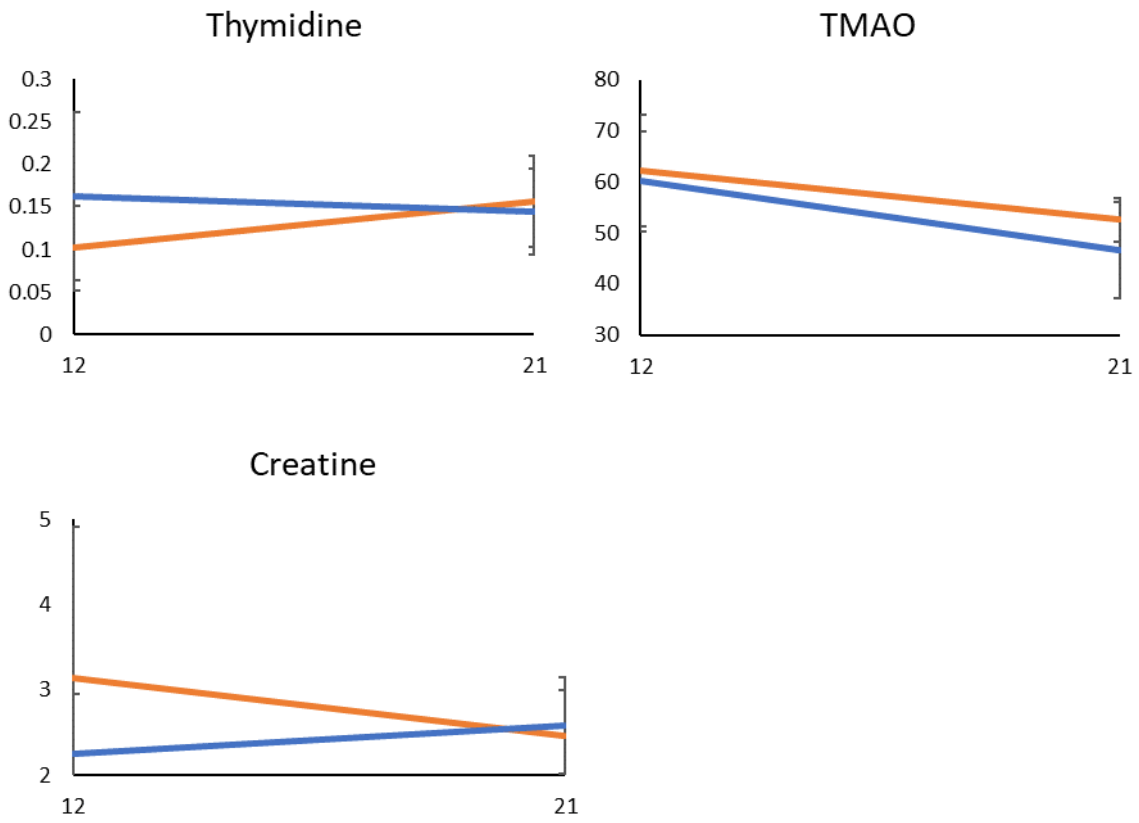
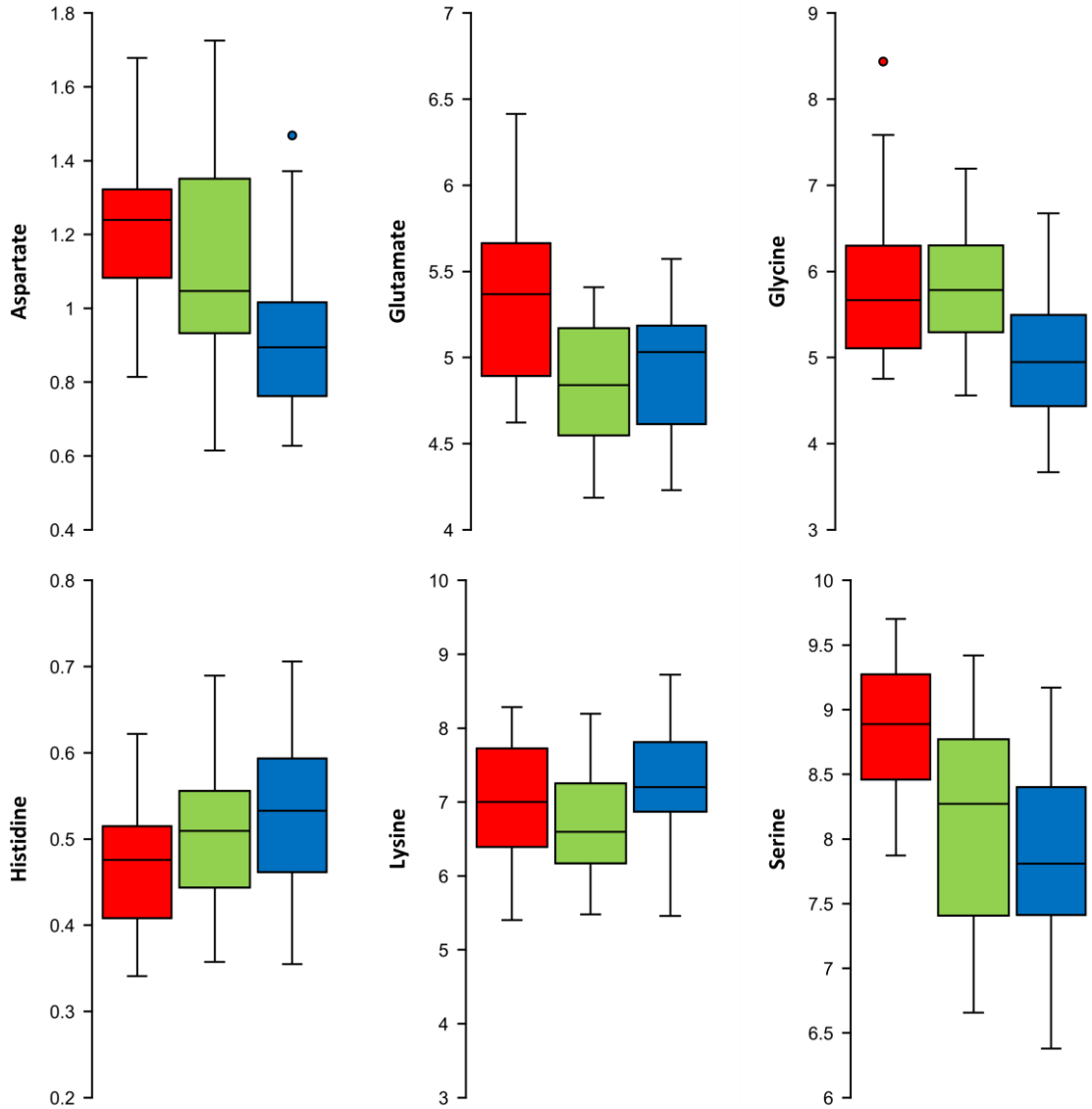
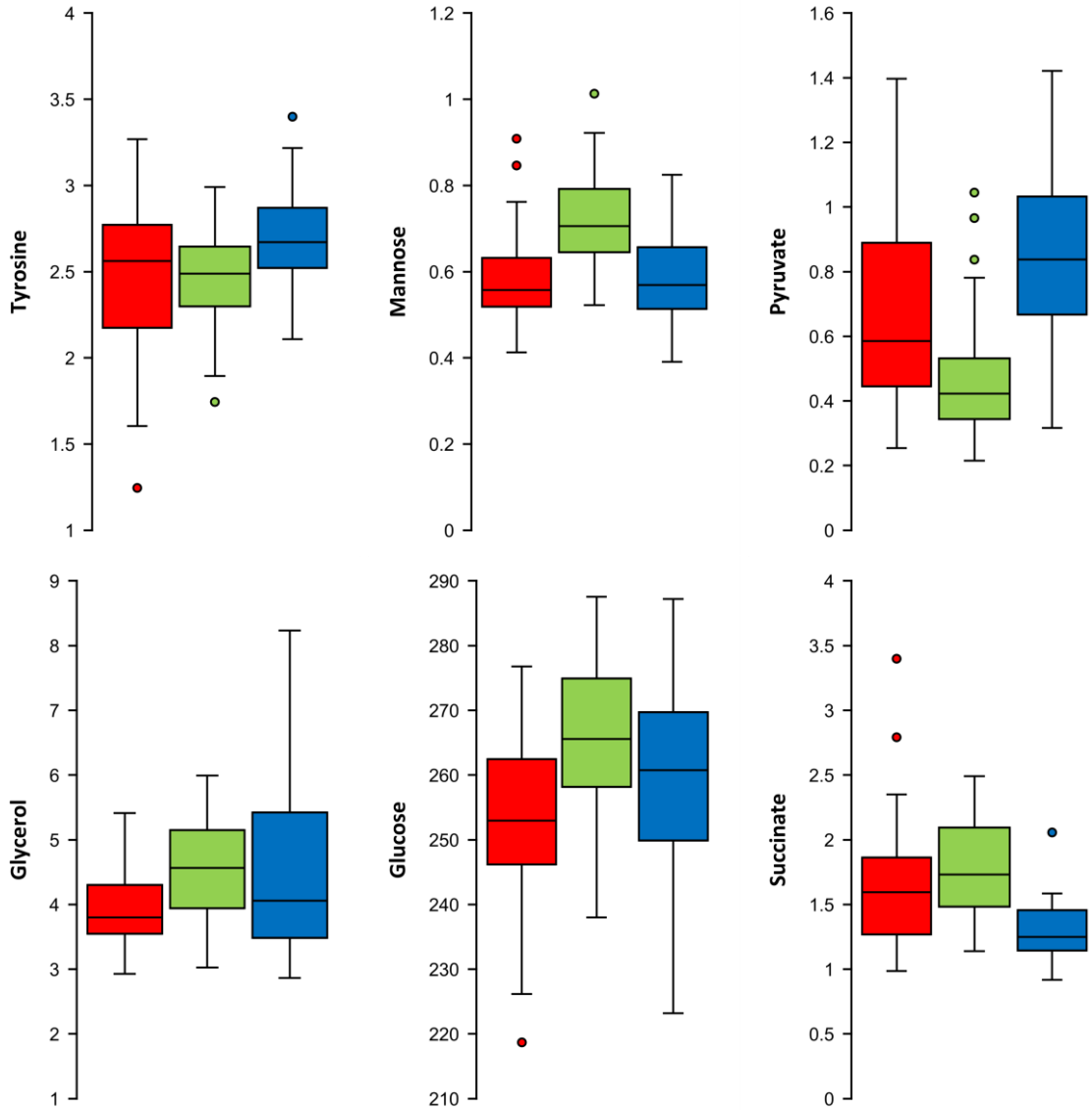


Fig. A4 Graphical representations of changes in the urinary metabolites between the exercising groups (B and D, blue) and the non-exercising groups (A and C, brown) during the 2nd half of life. The vertical axis shows peak area in arbitrary units.


Aspartate
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
Glutamate
7
6.5
6
5.5
5
4.5
4
Glycine
9
8
7
6
5
4
3
Histidine
0.8
0.7
0.6
0.5
0.4
0.3
0.2
Lysine
10
9
8
7
6
5
4
3
Serine
10
9.5
9
8.5
8
7.5
7
6.5
6


Tyrosine
4
3.5
3
2.5
2
1.5
1
Mannose
1.2
1
0.8
0.6
0.4
0.2
0
Pyruvate
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Glycerol
9
8
7
6
5
4
3
2
1
Glucose
290
280
270
260
250
240
230
220
210
Succinate
4
3.5
3
2.5
2
1.5
1
0.5
0

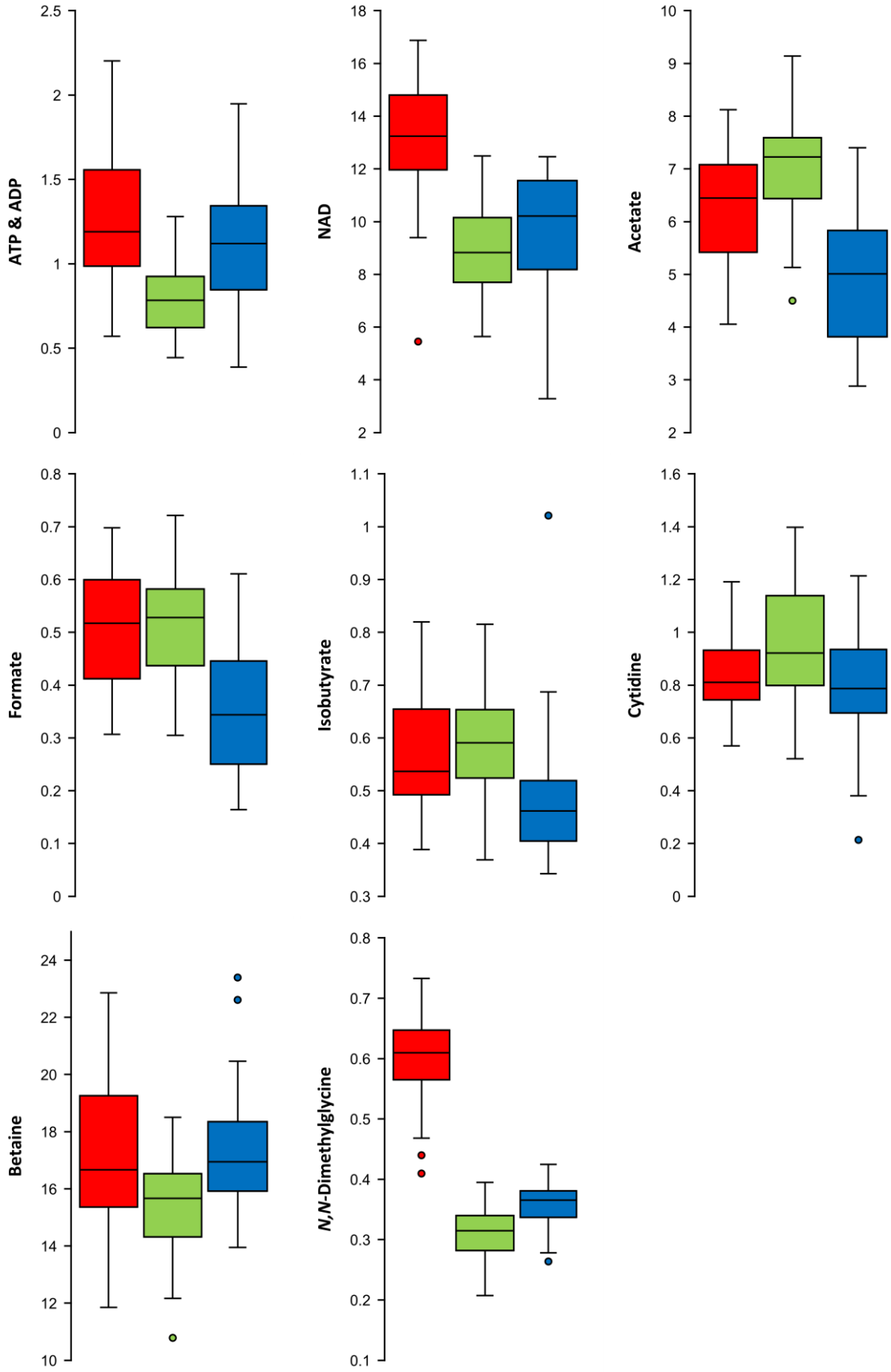

ATP & ADP
NAD
Acetate
Formate
Isobutyrate
Cytidine
Betaine
N,N-Dimethylglycine

Fig. A5 Box plots of changes in the blood metabolites between 3 (red), 12 (green) and 21 (blue) months of age. The vertical axis shows peak area in arbitrary units. Each box represents the interquartile range, and the center line represents the median. Whiskers are extended to the most extreme data point that is no more than 1.5 times the interquartile range from the edge of the box (Tukey style). Dots represent outliers.

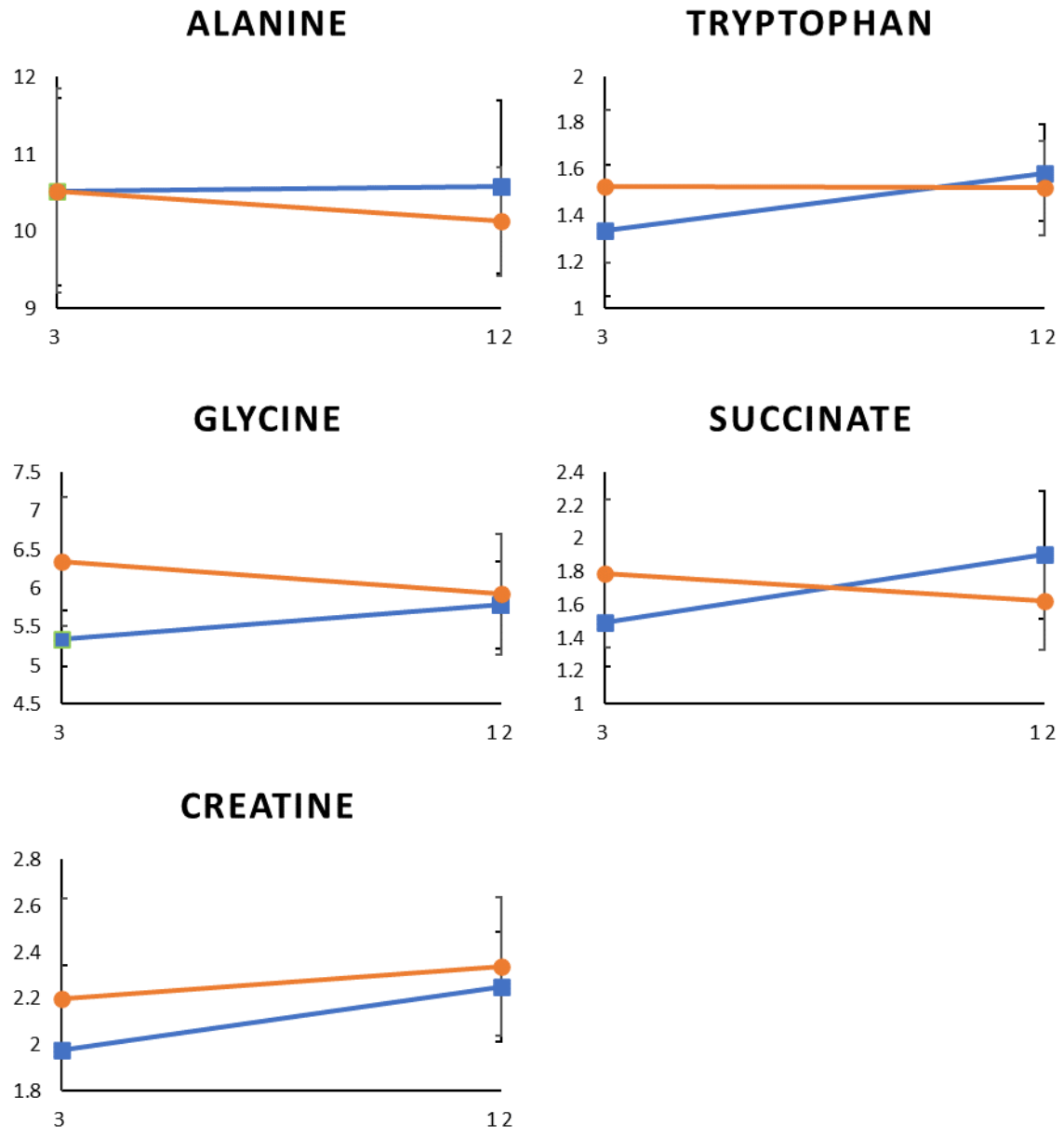


Fig. A6 Graphical representations of changes in blood metabolites between the exercising groups (A and B, blue) and the non-exercising groups (C and D, brown) during the 1st half of life. The vertical axis shows peak area in arbitrary units.

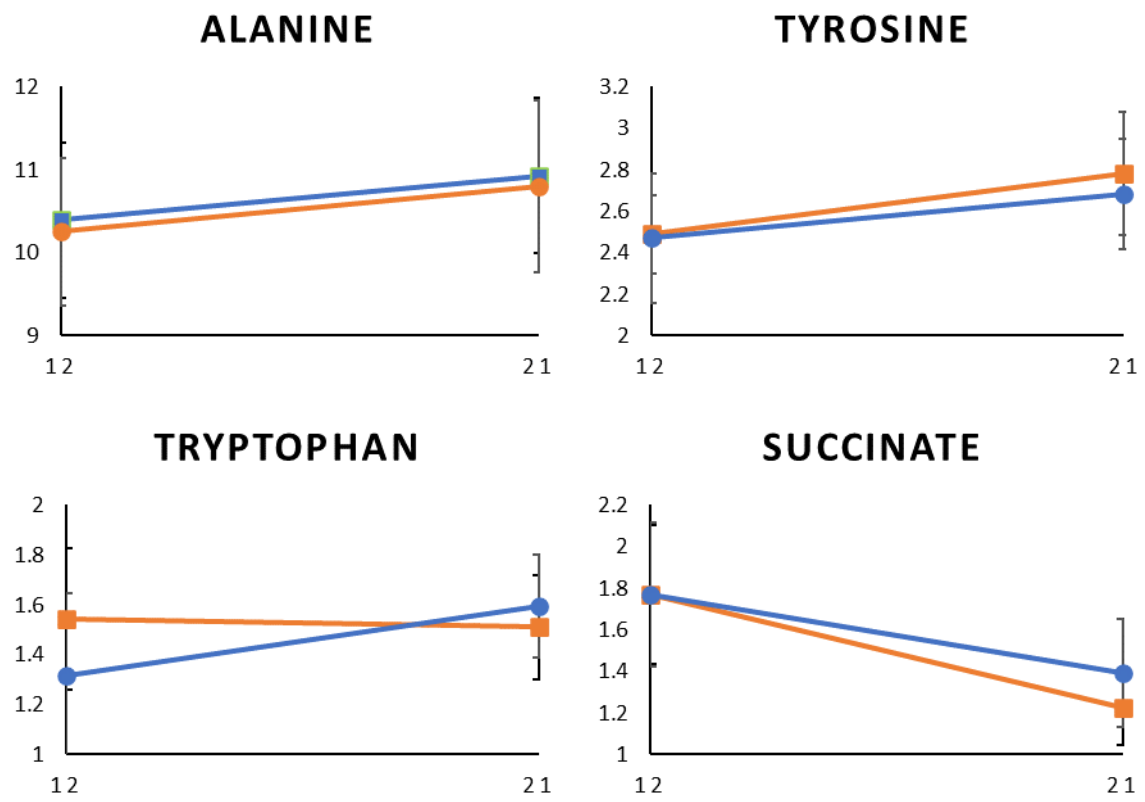


Fig. A7 Graphical representations of changes in blood metabolites between the exercising groups (B and D, blue) and the non-exercising groups (A and C, brown) during the 2nd half of life. The vertical axis shows peak area in arbitrary units.

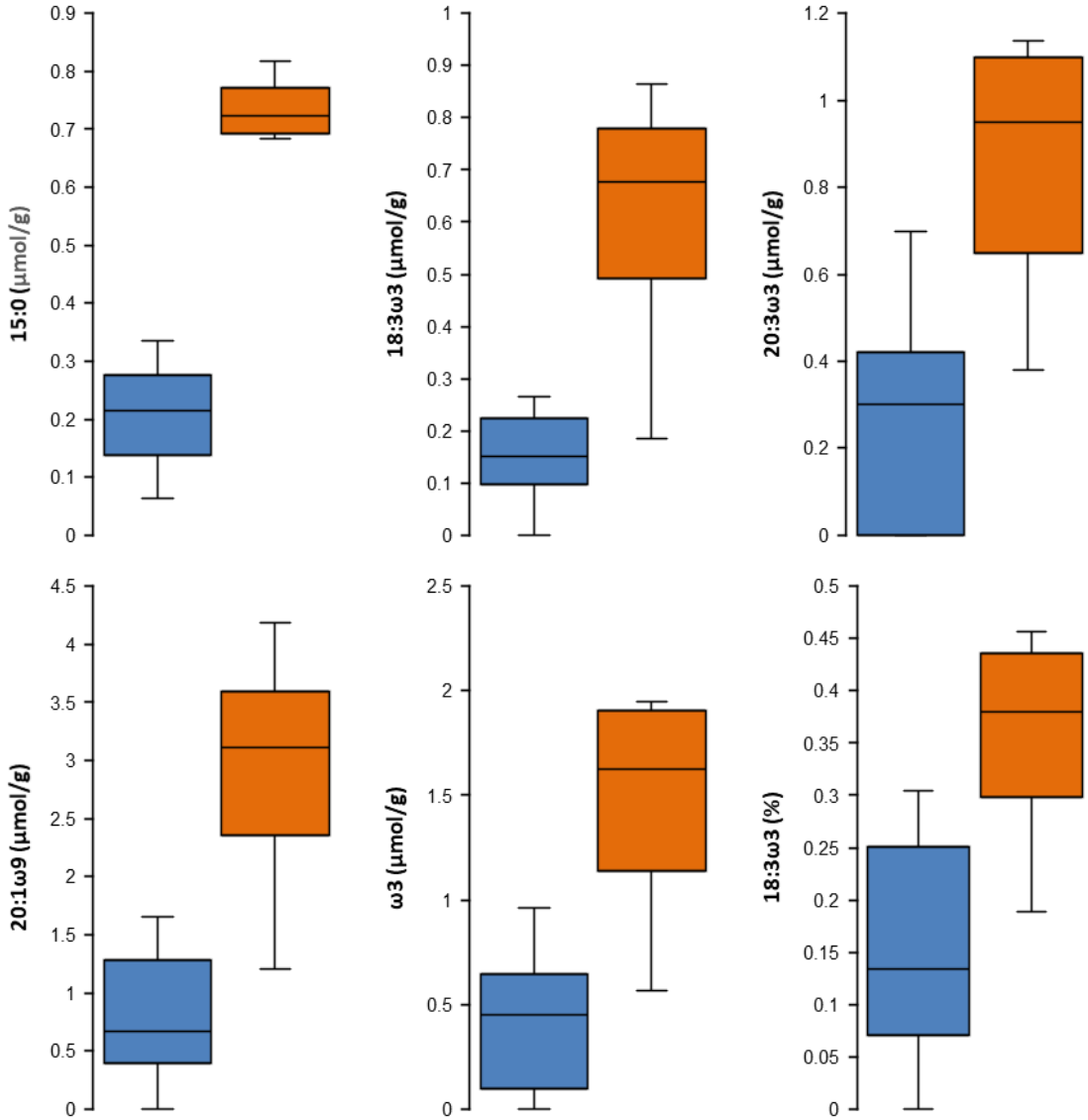


Fig. A8 Box plots of changes in quadriceps TG fatty acids between the exercising (D, blue) and the non-exercising (A, brown) group during the 2nd half of life. Each box represents the interquartile range, and the center line represents the median. Whiskers are extended to the most extreme data point that is no more than 1.5 times the interquartile range from the edge of the box (Tukey style). Dots represent outliers.

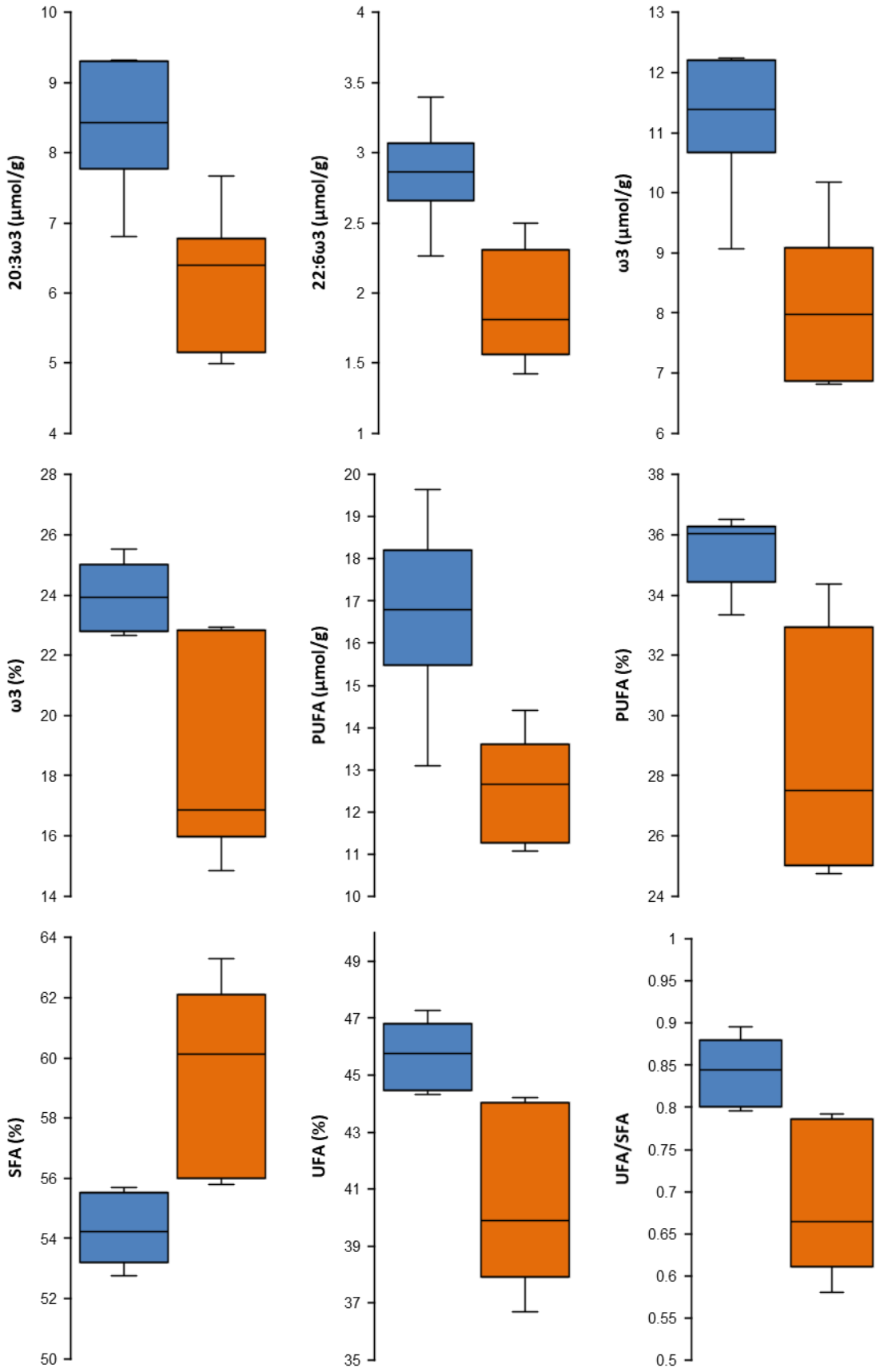


Fig. A9 Box plots of changes in liver PL fatty acids between the exercising (D, blue) and the non-exercising (A, brown) group during the 2nd half of life. Each box represents the

interquartile range, and the center line represents the median. Whiskers are extended to the most extreme data point that is no more than 1.5 times the interquartile range from the edge of the box (Tukey style). Dots represent outliers.

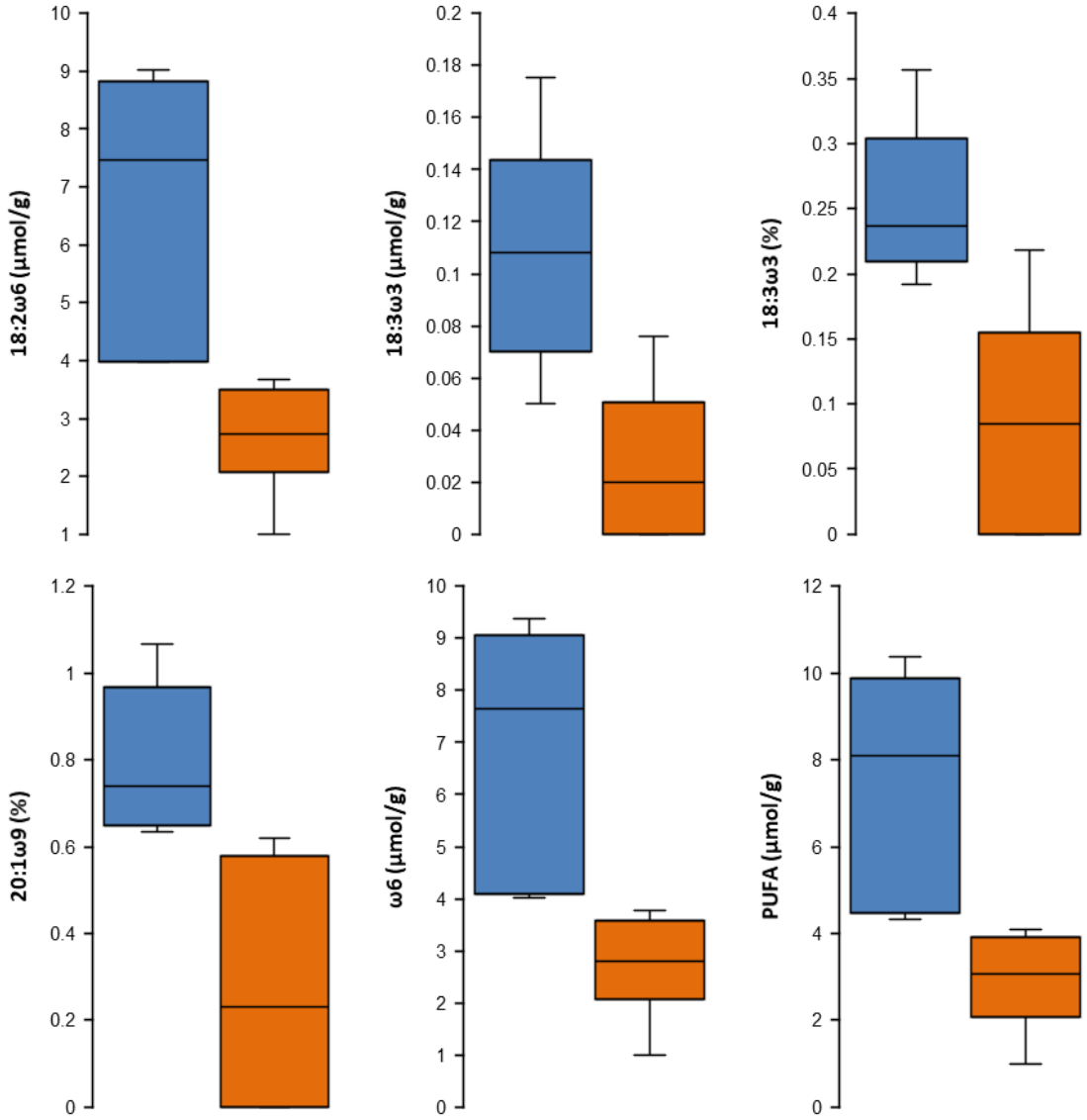


Fig. A10 Box plots of changes in liver TG fatty acids between the exercising (D, blue) and the non-exercising (A, brown) group during the 2nd half. Each box represents the interquartile range, and the center line represents the median. Whiskers are extended to the most extreme data point that is no more than 1.5 times the interquartile range from the edge of the box (Tukey style). Dots represent outliers.