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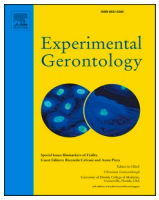

The association of circulating bioenergetic metabolites with healthy human aging

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ABSTRACT

Aging is an inevitable and gradual decline in several biological functions. Mitochondrial dysfunction is one of the most important hallmarks of aging. In this context, alterations in metabolites associated with mitochondrial dysfunction may serve as a significant biomarker. This study aimed to investigate the existence of a relationship between the key metabolites involved in bioenergetics metabolism and aging. 53 volunteers ranged 20–85 years participated in the study. We tested the association between different tricarboxylic acid (TCA) cycle metabolites, fatty acid metabolism, and amino acid metabolism with age, sex, body composition, and proxy markers of aging such as walking speed, grip strength and chair test. We found that lactic acid negatively correlated with age while several fatty acid metabolites, such as azelaic, sebacic, and linoleic acids, showed positive correlations with age ($p < 0.05$). Sex-specific trends, such as glycerol, and dodecanoic acid, were also observed for certain metabolites. Furthermore, citric acid levels were found to have a significant association with physical function and body composition measures. Participants with higher citric acid levels displayed improved performance in physical tests and favorable body composition indices. Additionally, fumaric acid and adipic acid showed positive correlations with fat-free body mass, while sebacic acid was negatively associated with measures of fat mass. These findings underscore the importance of understanding the role of circulating bioenergetics metabolites with age, sex variations, and their potential implications in body composition and physical performance.

1. Introduction

Aging is an inevitable and gradual decline in several biological functions, including cognitive abilities, immune function, and organ function, among others (Harman, 1981; Harman, 2001; Jin, 2010; López-Otín et al., 2023). Though a natural process, aging is associated with various chronic metabolic and degenerative diseases (Di Micco et al., 2021). Mitochondria are organelles present in all mammal cells. Its main function is Adenosine triphosphate (ATP) synthesis, involving pathways as the Tricarboxylic acid (TCA) cycle, also known as Krebs cycle, and β-oxidation of fatty acids in matrix, and multiple redox reactions, based on Coenzyme Q (CoQ) located in the inner mitochondrial membrane and as an essential part of the electron transport chain (ETC) (Alcázar-Fabra et al., 2016; Banerjee et al., 2022; Guarás et al., 2016; Martínez-Reyes et al., 2020). The decline in mitochondrial function is a hallmark of aging (Amorim et al., 2022; López-Otín et al., 2013; López-Otín et al., 2023), impairing cellular activities and compromising metabolic flexibility. This leads to weakness, frailty, and disability (Gonzalez-Freire et al., 2015; Harrington et al., 2023), impacting mitochondrial content and bioenergetic efficiency (Sharma et al., 2019;

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Tieland et al., 2018). Mitochondrial dysfunction also contributes to loss of skeletal muscle strength, decreased physical performance (Zane et al., 2017), and the development of skeletal muscle diseases and sarcopenia (Faitg et al., 2017; Hernández-Camacho et al., 2022; Migliavacca et al., 2019). Additionally, deregulated nutrient-sensing affects metabolic pathways that could lead to metabolic diseases such as diabetes (López-Otín et al., 2016). It is well-documented that during aging, impaired mitochondrial function leads to decreased ATP production and increased generation of reactive oxygen species (ROS) due to defects in ETC supercomplexes, causing damage to proteins, lipids, and DNA (Genova and Lenaz, 2014; Maldonado et al., 2023; Milenkovic et al., 2017; Rimal et al., 2023). Thus, a variety of pathomechanisms may arise due to the diversity of clinical phenotypes. Considering genetic background, mitochondrial homeostasis, compensation mechanisms, maternal effect, and possibly environmental factors, might determine mitochondrial homeostasis or adaptive mechanisms, thereby inducing a balanced mitochondrial function and contributing to the maintenance of mitochondrial cellular processes (Bennett et al., 2022). Glycolysis and β-oxidation are the primary pathways contributing to ATP production using glucose and fatty acids as fuels, providing acetyl CoA, which enters the TCA cycle (Choi et al., 2021). Excessive acetyl-CoA generated by oxidative metabolism pathway deficiencies is used in ketogenesis, providing ketone bodies such as acetoacetate, D-β-hydroxybutyrate, and acetone as auxiliary fuels to the TCA cycle in certain special and pathological situations (Kolb et al., 2021; Mitchell et al., 1995; Puchalska and Crawford, 2017). However, if these pathways are insufficient to meet the cell's energy needs, cellular damage and disease may ensue (Johnson et al., 2019; Lemos et al., 2023). The identification of predictive biomarkers includes those from metabolism circulating in the plasma with clinical purpose (Ubaida-Mohien et al., 2023). In this sense, we postulate that aging correlates with alterations in circulating metabolites from key bioenergetic pathways. To explore these potential changes and their relationship with aging, sex, and surrogate markers of the aging process (including walking speed, grip strength, and resting metabolic rate) we conducted targeted metabolomic profiling on serum samples from a well-characterized cohort representing a broad spectrum of healthy aging.

2. Material and methods

2.1. Study subjects

The research involved 53 participants aged between 20 and 85 who were part of an ongoing study called the Balearic Islands Study of Aging (BILSA). The BILSA study is a prospective open cohort study that began in January 2022 in the Mallorca Island area. These participants were healthy, active individuals without major diseases, except for controlled conditions such as hypertension, hypercholesterolemia, or diabetes. Participants with cognitive impairment, neuromuscular or muscular disorders, or a history of cancer in the past 10 years were excluded from the study. Additionally, only occasional smokers or former smokers were admitted. Participants were also instructed not to exercise 24 h before the study to prevent any increase in inflammatory markers in the blood that could affect the results.

The primary objective of the study was to examine the cross-sectional relationship between bioenergetic intermediate metabolites and aging, body composition, walking speed, and other physical tests (Supplemental Fig. 1). During the study, the participants underwent a comprehensive assessment lasting approximately 3 h, including physiological and psychological examinations and medical examinations carried out by specialized personnel at the Hospital Universitario de Son Espases. The study adhered to the principles of Good Clinical Practice and the ethical guidelines outlined in the Declarations of Helsinki. Ethical approval for the project (IB 4337/20 PI, 4 December 2020) was obtained from the ethics committee of the Balearic Islands. Prior to participation, the participants were fully informed about the study's objectives, procedures, and associated risks. Written informed consent was obtained from all participants.

2.2. Physical performance test

The measurement of torque muscle strength was obtained by grip strength and the 5-times sit-to-stand test. Briefly, grip strength was assessed using a hand dynamometer (KERN MAP 80 K1, KERN, Germany), which was calibrated with known weights and adjusted for hand comfort and proper fit. Volunteers were instructed to keep their arms in a relaxed and stationary position. Three maximum grip measurements were taken, and the highest value recorded for each hand (Carson, 2018). During the 5-times sit-to-stand test, participants were seated with their back against the chair's backrest and performed five consecutive rises from the chair. They were instructed to rise without using their arms and without pausing between each repetition, aiming for the highest speed possible. Each stand was audibly counted to maintain the participant's orientation. The test concluded once the participant successfully achieved the standing position for the fifth repetition (Millor et al., 2013). The assessment of walking speed was conducted using the 4-m walking test at the usual and fast pace. Volunteers were instructed to wear comfortable clothing and suitable footwear for walking. Four meters was measured and marked to indicate the starting and ending points on the floor. Prior to the test, a demonstration was provided to familiarize the participants with the procedure. A trial walk was then performed by each participant. They were instructed to walk at their usual or fast pace across the 4-m distance on the floor. The timing commenced from the first foot movement and stopped when the participant's foot contacted the floor at the end of the walking course (Peel et al., 2012).

2.3. Measurements of body composition

We used a bioelectrical impedance analysis (BIA) device, InBody 770, (Korea's InBody Co. Ltd.) to determine body composition. Participants received instructions before the test to ensure accurate measurements. They were advised to refrain from wearing any metal objects or electronic medical devices, fast for at least 12 h, dress in light clothing, and avoid drinking any water for at least 2 h prior to the test. Participants stood barefoot on the BIA device, aligning themselves with the rear foot electrode. They were told to hold the hand electrodes while keeping their arms away from their sides. Participants remained in this position quietly until the test was over.

2.4. Measurement of serum metabolites

Following an overnight fasting period, blood samples were collected from the antecubital vein between 07:30 and 08:30 h. Participants were instructed to refrain from engaging in physical exercise or consuming any medication before the blood collection process. Within 4 h, the collected blood samples were subjected to centrifugation after prompt storage at 4 °C. Subsequently, the samples were divided into smaller aliquots and promptly frozen at a temperature of −80 °C. To homogenize the samples as much as possible and due to the sample quantity required for the assay, we decided to make pools of different patients within the same age and sex range. In the case of older patients, due to the small sample size obtainable from this population, we did not make pools and used one patient's entire sample for the analysis. Organic acid analysis was performed following the protocol described by Van Noolen et al. (Van Noolen et al., 2020). Briefly, samples were derivatized using the formation of trimethylsilylated derivatives and evaluated through gas chromatography/mass spectrometry (5975C Agilent Technologies, Madrid, Spain). The relative amount of each metabolite was determined by the signals (m/z) of the specific ions for every compound, corrected by the amount of the internal standard (undecanodioic ion 345). Compounds were identified by a mass spectrometer; a fragmentation

spectrum is obtained by electron impact (70 eV) of each compound (scanning mode) to verify the nature of the compound compared with the National Institute of Standards and Technology (NIST) library (Supplemental Table 1). Data are presented in the form of a total ion chromatogram (TIC). The mass spectrum coupled to the retention time allows for the reliable identification of the molecules.

2.5. Statistical analysis

Descriptive characteristics were presented as either means accompanied by the standard error of the mean (SEM) or standard deviation (SD), or as numbers and corresponding percentages. To assess differences among groups and sex a two-way ANOVA or 2 tailed *t*-test were carried out and post hoc test like Tukey's HSDO and the Holm–Bonferroni and false discovery rate methods for multiple

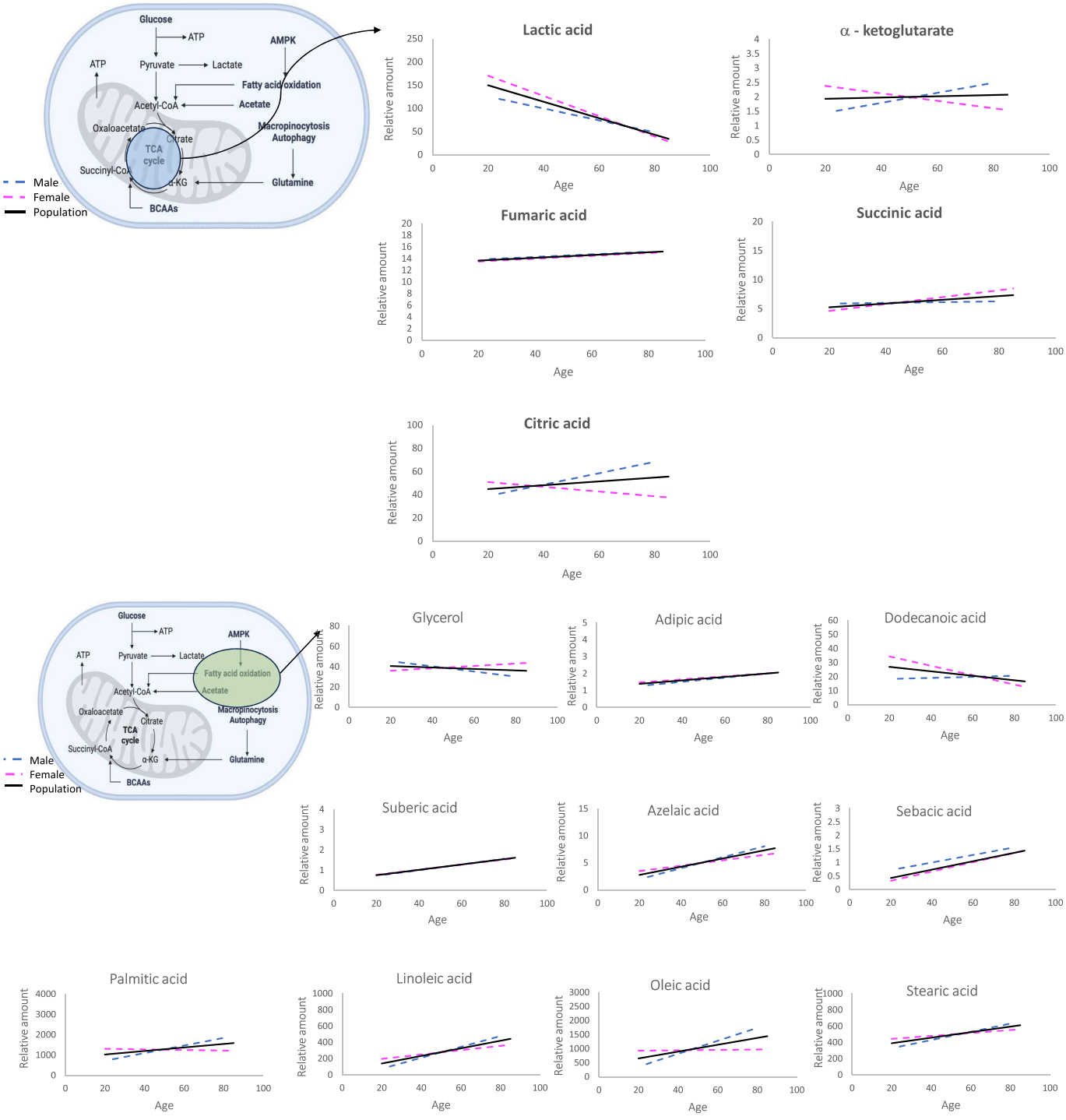

Fig. 1. Association between age, gender and the different metabolites measured in the study. A) TCA cycle metabolites, B) Fatty Acid metabolites; C) Other metabolites.
Relative amount of indicated metabolite analyzed by the specific targeted ion (*m/z* signal) normalized to that of internal standard undecanodioic acid (Y axis). These results were referred to each age group (X axis).

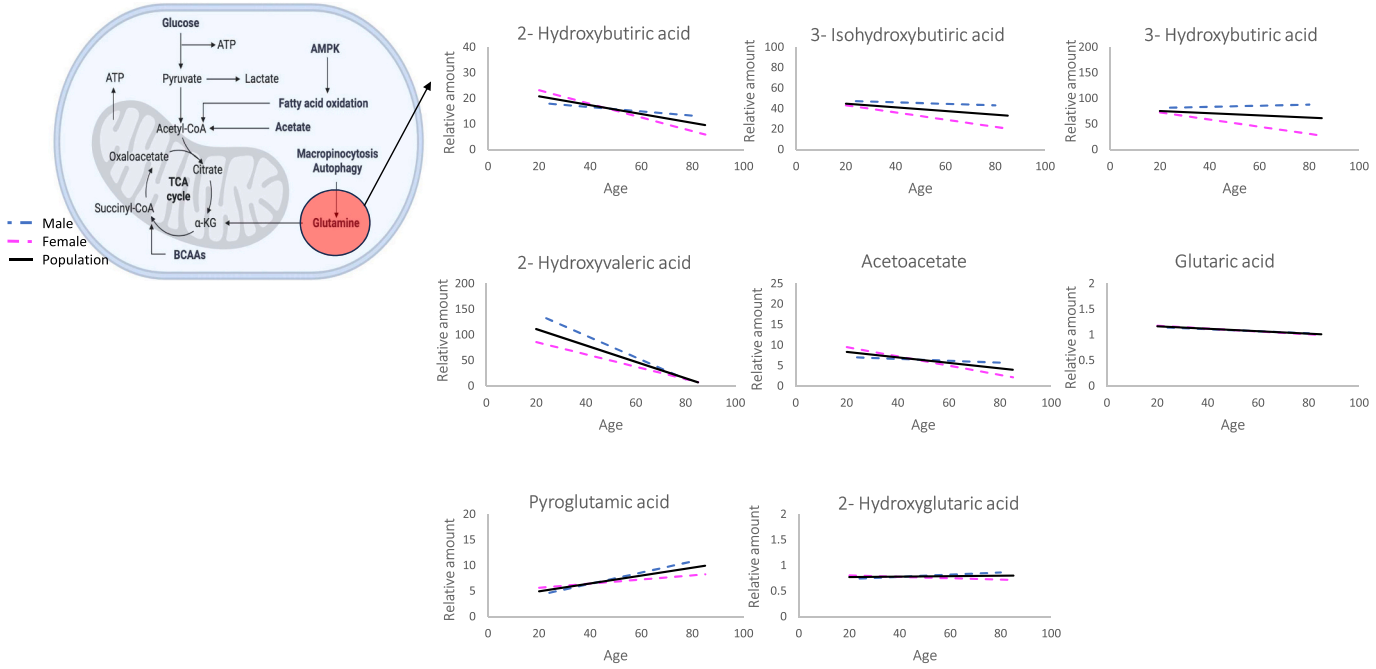

Fig. 1. (*continued*).

comparisons correction with maximum family-wise error rate at 0.05. To visualize the difference in means boxplots and 95 % confidence intervals plots were used. A p value <0.05 was considered significant. Correlations and linear regression models were used to estimate the association between metabolite levels and functional measures. Feature importance of each metabolite, using a Random Forest algorithm was used to predict age. Statistical analyses were performed using R Studio.

3. Results

3.1. Participants characteristics

Demographic and clinical characteristics of the study population are shown in Table 1. We analyze a total of 53 participants (49 % women). Since we measured the bioenergetics metabolites across age groups, we also present the baseline characteristics according to age and sex groups in Supplemental Table 2. The average age of the participants was 50.4 ± 3.04 years. Regarding body composition, men presented higher body mass index (BMI) ($p < 0.001$), were slightly taller and heavier than women ($p < 0.001$). As expected, men presented higher levels of muscle mass, fat-free mass and extracellular water ($p < 0.001$). The 50 kHz body phase angle, a proxy of muscle quality measured with InBody, was higher in men compared to women ($p < 0.001$). No changes in total cholesterol or low-density lipoproteins (LDL) were found among sex. Interestingly women presented higher levels of high-density lipoproteins (HDL) and lower levels of triglycerides compared to men ($p < 0.001$). In terms of physical performance, men were faster than women in the 4 m walking at fast velocity ($p = 0.003$) and had higher grip strength ($p < 0.001$).

3.2. Metabolites levels and their association with age and sex

The mass-to-charge ratio values from spectrometry for bioenergetics metabolites across age and sex groups are included in Supplemental Table 3. Additionally, Table 2 presents the correlations of these metabolites with age, including the differentiation of these associations by sex. The interplay between age, sex, and the array of metabolites analyzed is graphically represented in Fig. 1. Supplemental Fig. 2 represents the pairwise differences in adjusted means (sex) for each significant metabolite across the different age groups.

Regarding TCA cycle metabolites (Fig. 1A), only lactic acid demonstrated a significant negative correlation with aging ($r = -0.568$, $p < 0.001$). Interestingly, there were no discernible differences between gender. For fatty acid metabolites (Fig. 1B), azelaic, sebacic, and linoleic acids all were positively correlated with age ($r = 0.629$, $p < 0.001$; $r = 0.876$, $p < 0.001$; $r = 0.516$, $p < 0.001$, respectively) and with similar pattern across gender. Conversely, some metabolites exhibited sex-specific trends with age. Specifically, in men, glycerol levels declined with age ($r = -0.381$, $p = 0.05$), while adipic, suberic, palmitic, and oleic acid levels increased ($r = 0.743$, $p < 0.001$; $r = 0.684$, $p < 0.001$; $r = 0.543$, $p = 0.003$; $r = 0.502$, $p < 0.001$, respectively). In contrast, only dodecanoic acid displayed a significant negative association with age in women ($r = -0.538$, $p = 0.005$). Among the other metabolites (Fig. 1C), 2-hydroxyvaleric acid showed a significant decline with age ($r = -0.724$, $p < 0.001$). In women, both 2-hydroxybutyric and glutaric acids decreased with age ($r = -0.460$, $p = 0.018$ and $r = -0.557$, $p = 0.003$, respectively). Conversely, in men, pyroglutamic acid levels increased with age ($r = 0.470$, $p = 0.013$).

Lastly, Fig. 2 shows the 15 most influential metabolites for predicting age (Fig. 2A) and sex (Fig. 2B) within our cohort. In this figure, the metabolites are ranked according to their feature importance in the Random Forest prediction model.

3.3. Metabolites and their association with body composition and physical function

Tables 3 and 4 present the correlations between physical tests and body composition and all the metabolites analyzed. Only citric acid levels exhibited a significant association with physical function. Specifically, participants with elevated citric acid levels demonstrated enhanced performance in the sit-to-stand test ($r = -0.310$, $p = 0.024$), walking speed test ($r = -0.310$, $p = 0.024$), and grip strength ($r = 0.359$, $p = 0.008$).

Elevated citric acid concentrations were inversely associated with the percentage of body fat mass, waist-to-hip ratio, and visceral fat area ($r = -0.480$, $p < 0.001$; $r = -0.360$, $p = 0.008$; $r = -0.518$, $p < 0.001$, respectively) and positively associated with body fat free mass ($r = 0.361$, $p = 0.008$). Other metabolites, including fumaric acid and adipic

Table 1
Baseline characteristics of the 53 participants included in the study.

	Total ($n = 53$)	Male ($n = 27$)	Female ($n = 26$)	*P*
Age (years)	50.4 (20–85) ± 3.04	51.5 (24–80) ± 3.04	49.3 (20–85) ± 3.04	0,619
Sex (female)	26 (49 %)	–	–	
Height (m)	1.70 (1.50–1.87) ± 0.12	1.77 (1.65–1.87) ± 0.01	1.62 (1.50–1.75) ± 0.01	<0,001*
Weight (kg)	69.3 (48.5–100) ± 1.86	78.91 (59.9–100) ± 2.15	59.32 (48.5–75.6) ± 1.38	<0,001*
Current smokers	3 (5.67 %)	–	3 (11.54 %)	0.069
Formers smokers	9 (16.98 %)	6 (22.22 %)	3 (11.54 %)	0.135
Comorbidities				
Hypertension	6 (11.32 %)	5 (18.52 %)	1 (3.85 %)	0.092
DM2	2 (3.77 %)	2 (7.40 %)	–	0.157
Hypercholesterolemia	8 (15.09 %)	6 (22.22 %)	2 (7.69 %)	0.140
Blood parameters				
Glucose (mg/dL)	87.30 (58–150) ± 2.01	88.59 (66–132) ± 2.54	85.96 (58–150) ± 3.18	0.520
HbA1c (mmol/mol)	35,10 (29–65) ± 0.68	35.70 (31–65) ± 1.20	34.46 (29–41) ± 0.59	0.365
Cholesterol (mg/dL)	176.47 (43–288) ± 6.45	183.56 (127–251) ± 5.67	169.12 (43–288) ± 11.72	0.267
LDL (mg/dL)	110.74 (55–171) ± 3.62	109.59 (55–163) ± 4.86	111.92 (71–171) ± 5.49	0.751
HDL (mg/dL)	64.36 (41–110) ± 1.96	57.37 (50–110) ± 2.30	71.61 (41–85) ± 2.54	<0.001*
Triglycerides (mg/dL)	73.45 (33–225) ± 4.89	83.22 (36–225) ± 8.16	63.30 (33–124) ± 4.64	0.041*
Ketones (mmol/L)	0.18 (0.1–0.9) ± 0.02	0.2 (0.1–0.9) ± 0.04	0.15 (0.1–0.3) ± 0.02	0.301
Body composition				
Body mass index (kg/m^2)	23.77 (17,26-30,61) ± 0.41	25.10 (19.78–30.61) ± 0.57	22.39 (17.26–28.11) ± 0.44	0.001*
BMR	1654.56 (908–2627) ± 52.11	1877.96 (1067–2627) ± 60.84	1422.58 (908–2017) ± 57.4	<0.001*
Body fatty mass (kg)	15.84 (5.8–31.5) ± 0.83	16.24 (5.8–31.5) ± 1.28	15.42 (7.4–27.10) ± 1.05	0.625
Soft lean mass (Kg)	50.64 (31.4–77.1) ± 1.67	59.12 (40.50–77.1) ± 1.65	41.83 (31.40–69.3) ± 1.70	<0.001*
Intracellular water (L)	24.49 (14.8–37.6) ± 0.8	28.66 (19.6–37.6) ± 0.82	20.16 (14.8–33.8) ± 0.74	<0.001*
Extracellular water (L)	14.83 (9.5–22.3) ± 0.46	17.31 (11.9–22.3) ± 0.45	12.25 (9.5–20) ± 0.41	<0.001*
Waist-Hip ratio	0.89 (0.77–1.09) ± 0.01	0.89 (0.77–1.08) ± 0.014	0.89 (0.79–1.09) ± 0.014	0.493
Visceral fat area	74.33 (22.2–160.3) ± 4.5	73.04 (22.2–156.8) ± 6.59	75.67 (27.70–160.3) ± 6.23	0.774
50 kHz body phase angle	5.56 (3.6–6.8) ± 0.10	5.88 (3.6–6.8) ± 0.14	5.23 (3.9–6.2) ± 0.12	0.001*
Fat free mass	54.17 (35–81.5) ± 1.75	62.68 (42.9–81.5) ± 1.75	45.32 (35–81.5) ± 1.75	<0.001*
Physical performances				
Chair test (s)	9.20 (5.3–20.66) ± 0.38	9.02 (5.59–20.66) ± 0.57	9.40 (5.30–15.93) ± 0.49	0.617
Walking speed (n) (s)	3.47 (2.23–4.43) ± 0.06	3.5 (2.76–4.43) ± 0.089	3.44 (2.23–4.06) ± 0.09	0.683
Walking speed (f) (s)	2.25 (1.75–3.28) ± 0.05	2.12 (1.75–3) ± 0.049	2.39 (1.9–3.28) ± 0.07	0.003*
Grip strength (Kg/cm)	30.66 (10.5–53.37) ± 1.43	38.61 (25.26–53.37) ± 1.26	22.39 (10.5–35.89) ± 1.29	<0.001*

Data are mean ± (SEM), range () or %. * Statistically significant ($P < 0.05$.

Table 2
Correlation between the different metabolites with age and gender.

	Total (n = 53)		Male (n = 27)		Female (n = 26)	
Metabolites	*r*	*p*	*r*	*p*	*r*	*p*
TCA Metabolites						
Lactic acid	**−0,568****	<0.001	**−0.432****	<0.001	**−0.731****	<0.001
Succinic acid	0.06	0.669	−0.074	0.713	0.253	0.212
Fumaric acid	0.171	0.222	0.143	0.477	0.148	0.472
α- Ketoglutarate	0.028	0.841	0.174	0.387	−0.208	0.309
Citric acid	0.055	0.695	**0.459****	0.016	−0.297	0.141
Fatty Acids						
Glycerol	−0.142	0.31	**−0.381****	0.05	0.284	0.16
Adipic acid	**0.353****	0.009	**0.743****	<0.001	0.112	0.585
Dodecanoic acid	**−0.339****	0.013	−0.24	0.228	**−0.538****	0.005
Suberic acid	**0.303****	0.0028	**0.684****	<0.001	0.066	0.748
Azelaic acid	**0.629****	<0.001	**0.840****	<0.001	**0.472****	0.015
Sebacic acid	**0.876****	<0.001	**0.899****	<0.001	**0.860****	<0.001
Palmitic acid	0.247	0.074	**0.543****	0.003	−0.039	0.852
Linoleic acid	**0.516****	<0.001	**0.592****	0.001	**0.412****	0.037
Oleic acid	**0.357****	0.009	**0.463****	0.015	0.152	0.459
Stearic acid	0.205	0.14	0.304	0.123	0.142	0.487
Aminoacids metabolism						
2-hydroxybutyric acid	−0.251	0.07	−0.049	0.808	**−0.460****	0.018
3-isohydroxybutyric acid	−0.063	0.652	−0.007	0.973	−0.211	0.3
3-hydroxybutyric acid	−0.041	0.773	−0.007	0.973	−0.193	0.346
2-hydroxyvaleric acid	**−0.724****	<0.001	**−0.832****	<0.001	**−0.711****	<0.001
Acetoacetate	−0.145	0.3	0.065	0.747	−0.311	0.121
Glutaric acid	**−0.306****	0.026	−0.202	0.313	**−0.557****	0.003
Pyroglutamic acid	0.101	0.472	**0.470****	0.013	−0.384	0.053
Hydroxyglutaric acid	0.156	0.265	0.176	0.379	0.082	0.692

Spearman and correlation by gender represented.
Bold and ** indicate significative results (P < 0.05).

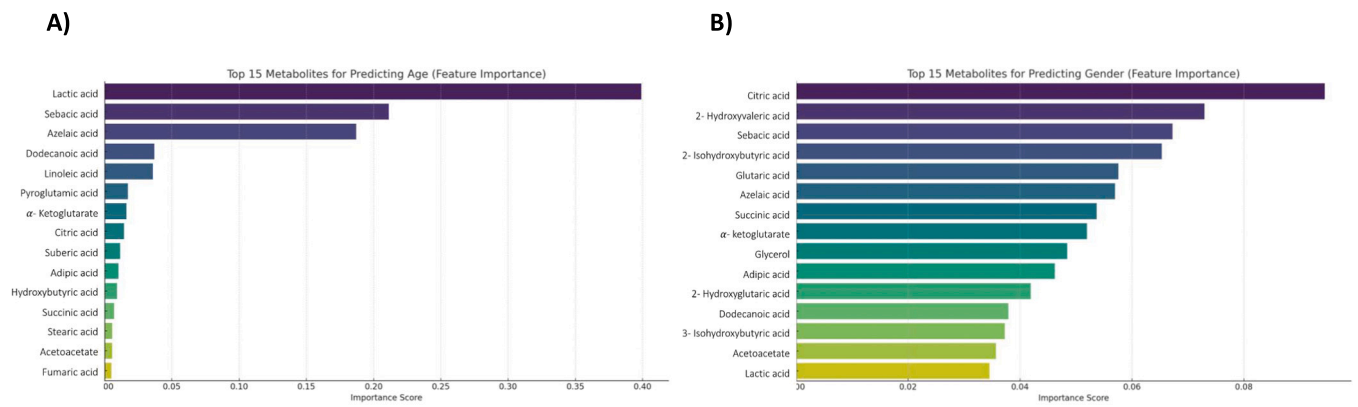

Fig. 2. Top 15 metabolites predictive of age and gender. This figure presents a bar plot showcasing the top 15 metabolites identified as significant predictors of age (A) and gender (B) in our cohort study. The metabolites are ranked based on their feature importance in the predictive model. Each bar represents a different metabolite, with the length of the bar indicating with the bar's length indicating the relative importance of that metabolite in determining age or gender.

Table 3
Physical test and metabolites correlations.

	Chair test		Walking Speed normal		Walking Speed fast		Grip strength	
Metabolites	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*
TCA Metabolites								
Lactic acid	−0.106	0.451	0.081	0.565	−0.109	0.438	0.047	0.74
Succinic acid	0.038	0.79	0.00	0.997	−0.061	0.664	0.058	0.679
Fumaric acid	−0.052	0.712	−0.084	0.551	−0.248	0.073	**0.333****	0.015
α- Ketoglutarate	−0.014	0.919	−0.056	0.69	−0.086	0.538	0.219	0.115
Citric acid	**−0.310****	0.024	−0.014	0.923	**−0.288****	0.036	**0.359****	0.008
Fatty Acids								
Glycerol	0.073	0.606	0.231	0.096	0.02	0.888	−0.242	0.081
Adipic acid	−0.019	0.895	0.134	0.339	−0.208	0.134	**0.342****	0.012
Dodecanoic acid	0.097	0.491	**−0.312****	0.023	−0.217	0.119	0.042	0.766
Suberic acid	0.002	0.991	0.117	0.404	−0.069	0.623	0.23	0.098
Azelaic acid	0.054	0.701	0.206	0.139	0.023	0.871	0.144	0.303
Sebacic acid	0.008	0.955	0.115	0.411	0.107	0.445	0.133	0.341
Palmitic acid	−0.002	0.99	−0.063	0.653	−0.051	0.715	−0.077	0.584
Linoleic acid	0.003	0.985	−0.002	0.986	0.00	0.998	−0.013	0.929
Oleic acid	−0.005	0.97	−0.156	0.263	−0.093	0.506	0.058	0.68
Stearic acid	0.099	0.482	−0.031	0.827	0.01	0.943	−0.208	0.135
Aminoacids Metabolism								
2-hydroxybutyric acid	−0.241	0.082	−0.14	0.316	−0.197	0.157	0.116	0.408
3-isohydroxybutyric acid	−0.18	0.197	−0.209	0.133	0.172	0.219	0.094	0.502
3-hydroxybutyric acid	−0.212	0.128	−0.204	0.143	−0.2	0.15	0.153	0.276
2-hydroxyvaleric acid	**−0.281****	0.041	−0.191	0.17	−0.198	0.156	0.028	0.84
Acetoacetate	−0.197	0.158	−0.051	0.719	−0.135	0.335	0.073	0.605
Glutaric acid	−0.188	0.178	−0.125	0.373	**−0.323****	0.018	0.228	0.101
Pyroglutamic acid	−0.152	0.276	0.035	0.804	−0.061	0.666	0.088	0.53
Hydroxyglutaric acid	−0.042	0.764	−0.141	0.314	−0.164	0.24	0.188	0.177

Spearman and correlation by physical test represented.
Bold and ** indicate significative results (P < 0.05).

acid, also exhibited positive correlations with fat-free body mass, with correlation coefficients of $r = 0.380$, $p = 0.005$ and $r = 0.374$, $p = 0.006$, respectively. Conversely, sebacic acid displayed a notable negative correlation with indicators of fat mass, such as body fat mass ($r = -0.330$, $p = 0.016$), waist-to-hip ratio ($r = -0.280$, $p = 0.045$), and visceral fat area ($r = -0.279$, $p = 0.043$).

4. Discussion

In this study we corroborate several established findings in the field of aging and metabolism. Previous research has highlighted that mitochondrial dysfunction, a prominent hallmark of aging, leads to alterations in metabolic pathways, including the β-oxidation of fatty acids and the TCA cycle affecting aerobic respiration (López-Otín et al., 2016; Ma and Li, 2015; Nguyen et al., 2013). Given the significance of mitochondrial dysfunction in aging, these deficiencies often result in the accumulation of intermediate metabolites in key pathways due to enzymatic malfunction, which then manifest in plasma levels. In this study, we have identified some age- and sex-dependent variations in metabolites across several human bioenergetic pathways, contributing to the identification of circulating metabolic biomarkers as required for clinical gerontology (Ubaida-Mohien et al., 2023). Lactic acid or lactate is generated by a range of cells within the body, such as muscle cells, red blood cells, and neurons. Lactate is produced from pyruvate by lactate dehydrogenase, and it is overloaded in different conditions such as hypoxia, defective ETC or excess of carbohydrates. It's often monitored in clinical settings to evaluate the oxygen status and to identify conditions like sepsis, shock, or mitochondrial diseases. Lactate is not a specific metabolite of TCA cycle, however, it is the first link in the chain, as it is the precursor of acetyl-CoA, which enter in the cycle to form citrate at the mitochondrial level (Gladden, 2004). In our study, the increase in plasma lactic acid levels at younger ages could be due to several factors

Table 4
Body composition and metabolites correlations.

	Fat free mss		BFM		ICW		ECW		WHR		VFA		SLM		WEIGHT		HEIGHT		BMI	
Metabolites	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*
TCA Metabolites																				
Lactic acid	−0.052	0.71	0.138	0.326	−0.007	0.961	0.016	0.91	0.188	0.177	0.095	0.499	0.036	0.798	0.078	0.578	−0.067	0.633	0.184	0.188
Succinic acid	0.192	0.167	−0.074	0.599	0.006	0.964	0.005	0.97	0.023	0.872	−0.032	0.817	−0.051	0.717	−0.011	0.935	0.041	0.77	0.000	0.998
Fumaric acid	**0.38****	0.005	−0.194	0.164	0.203	0.145	0.194	0.164	−0.15	0.283	−0.0225	0.105	0.142	0.311	0.097	0.491	0.196	0.16	−0.026	0.851
α- Ketoglutarate	0.038	0.79	−0.165	0.239	0.137	0.33	0.153	0.273	−0.032	0.817	−0.128	0.361	0.174	0.213	0.146	0.297	0.12	0.392	0.05	0.721
Citric acid	**0.361****	0.008	**−0.48****	<0.001	0.237	0.087	0.251	0.07	**−0.36****	0.008	**−0.518****	<0.001	0.252	0.069	0.077	0.586	0.235	0.09	−0.099	0.48
Fatty Acids																				
Glycerol	−0.134	0.34	0.165	0.238	−0.111	0.428	−0.126	0.369	0.056	0.69	0.123	0.379	−0.089	0.528	−0.084	0.55	−0.118	0.4	0.019	0.893
Adipic acid	**0.374****	0.006	−0.231	0.096	**0.328****	0.017	**0.32****	0.019	−0.235	0.09	−0.264	0.056	**0.342****	0.012	0.175	0.21	**0.326****	0.017	−0.023	0.872
Dodecanoic acid	−0.105	0.452	0.049	0.726	−0.065	0.641	−0.062	0.66	0.235	0.09	0.095	0.499	−0.074	0.601	−0.031	0.826	−0.028	0.841	−0.048	0.731
Suberic acid	0.26	0.06	−0.062	0.659	0.237	0.088	0.231	0.096	−0.173	0.216	−0.098	0.486	0.253	0.068	0.151	0.279	**0.293****	0.033	−0.007	0.962
Azelaic acid	0.202	0.148	−0.114	0.417	0.161	0.25	0.169	0.227	−0.155	0.267	−0.092	0.513	0.15	0.284	0.08	0.571	0.224	0.106	−0.061	0.665
Sebacic acid	0.165	0.238	**−0.33****	0.016	0.065	0.645	0.096	0.494	**−0.28****	0.045	**−0.279****	0.043	0.028	0.843	−0.033	0.816	0.176	0.208	−0.218	0.117
Palmitic acid	−0.101	0.471	−0.023	0.868	−0.07	0.62	−0.031	0.824	0.09	0.519	−0.005	0.974	−0.102	0.469	−0.087	0.534	−0.036	0.79	−0.121	0.388
Linoleic acid	−0.012	0.929	−0.11	0.432	−0.026	0.856	0.018	0.9	−0.003	0.985	−0.082	0.562	−0.074	0.6	−0.08	0.568	0.042	0.763	−0.156	0.264
Oleic acid	0.004	0.976	−0.171	0.222	−0.015	0.914	0.014	0.92	−0.027	0.85	−0.168	0.228	−0.062	0.661	−0.089	0.525	0.019	0.895	−0.176	0.209
Stearic acid	−0.175	0.21	0.152	0.278	−0.19	0.172	−017	0.223	0.154	0.272	0.151	0.282	−0.24	0.083	−0.141	0.315	−0.105	0.452	−0.11	0.434
Aminoacids metabolism																				
2-hydroxybutyric acid	0.125	0.372	−0.048	0.732	0.09	0.522	0.103	0.461	0.000	0.997	−0.145	0.3	0.069	0.626	0.087	0.534	0.023	0.872	0.106	0.451
3-isohydroxybutyric acid	0.087	0.534	−0.026	0.854	0.047	0.741	0.051	0.716	0.017	0.906	−0.101	0.473	−0.018	0.901	0.06	0.671	0.02	0.889	0.07	0.617
3-hydroxybutyric acid	0.142	0.309	−0.057	0.686	0.092	0.513	0.096	0.493	−0.01	0.945	−0.138	0.323	0.027	0.846	0.083	0.0556	0.073	0.603	0.065	0.644
2-hydroxyvaleric acid	−0.034	0.811	−0.085	0.547	−0.039	0.782	−0.064	0.647	0.028	0.845	−0.082	0.561	−0.056	0.692	−0.036	0.8	−0.095	0.499	0.06	0.67
Acetoacetate	0.077	0.585	0.026	0.855	0.059	0.677	0.085	0.545	0.068	0.628	−0.048	0.735	0.03	0.832	0.086	0.539	0.047	0.738	0.094	0.502
Glutaric acid	0.245	0.077	−0.116	0.407	0.119	0.397	0.091	0.517	−0.13	0.354	−0.192	0.169	0.079	0.575	0.109	0.437	0.111	0.427	0.083	0.555
Pyroglutamic acid	0.089	0.524	−0.222	0.111	0.02	0.887	0.032	0.818	−0.193	0.166	−0.198	0.156	0.052	0.71	−0.015	0.917	0.064	0.65	−0.076	0.588
Hydroxyglutaric acid	0.213	0.126	−0.129	0.357	0.093	0.509	0.074	0.596	−0.066	0.64	−0.118	0.401	0.016	0.908	0.069	0.621	0.12	0.393	0.022	0.873

including, diet, physical activity and hormone levels among others. Younger individuals may have higher levels of physical activity, compared to aged groups, which could lead to increased production of lactate, although 24 h before the study the participants were referred to stop any kind of exercise. Interpreting these findings may require a comprehensive analysis taking into account various factors including the health status, lifestyle, and other physiological metrics of the individuals in the study group. The rise in lactate levels in the youngest could be also a result of decreased metabolic clearance of lactate, stemming from a metabolic shift from pyruvate to ketone oxidation in the younger group, due to a higher basal metabolic rate. This increase could also relate to changes in metabolic efficiency, alterations in glucose metabolism, or shifts in the balance between glycolysis and oxidative phosphorylation with age. Lactate during exercise is an indirect method to assess metabolic flexibility and oxidative capacity across individuals of widely different metabolic capabilities (San-Millán and Brooks, 2018). Circulating lactate has the highest turnover of circulating metabolites and provide energy to tissues except brain from dietary carbohydrates without carrying out glycolysis, contributing to glucose homeostasis regulation (Hui et al., 2017). It has been communicated an increase in lactate levels within the cerebrospinal fluid proportionally with age (Nakano et al., 2017).

Our findings also reveal some discrepancies, particularly in the patterns of citric acid levels. Citrate, an essential intermediate of TCA cycle, which is also involved in lipid and amino acid metabolism, has been related with longevity (Li et al., 2022). Unlike prior studies that found citrate levels to increase uniformly with age regardless of sex (Silaidos et al., 2018; Sol et al., 2023), our data indicated an increase only in males. An increase in citric acid levels with aging might suggest changes in the TCA cycle's efficiency with age. This could be a compensatory mechanism, where the body tries to maintain energy production in the face of declining mitochondrial efficiency. Alternatively, it could reflect a change in the metabolic balance or in substrate availability for the TCA cycle. This discrepancy suggests that sex-specific metabolic differences may influence age-related changes in the TCA cycle, a novel observation that could prompt further investigation into sex-based metabolic profiling in aging.

Furthermore, the association between higher citric acid levels and better physical performance could indicate that citric acid supports better muscle function and energy availability. This supports the vital role of the TCA cycle in aerobic metabolism, which is crucial for physical performance. This is corroborated by literature highlighting the role of TCA cycle metabolites as humoral mediators of exercise adaptation process (Maurer et al., 2021). A positive association between citric acid levels and fat-free mass (which largely represents muscle) is consistent with the idea that muscle tissue, being metabolically active, has higher TCA cycle activity (Martínez-Reyes and Chandel, 2020). Thus, individuals with more muscle might have higher citric acid levels. It is also well-known the significant relationship between body fat mass and visceral fat area (Albulescu and Iliescu, 2014; Kwon and Han, 2019). Excess fat, especially visceral fat, is associated with metabolic disturbances, insulin resistance, and inflammation. It's possible that higher citric acid levels are protective against fat accumulation or that metabolic disturbances associated with higher fat mass might lead to reduced TCA cycle efficiency. Also, higher levels of citric acid could suggest more active cellular metabolism, potentially leading to more efficient use of fat stores for energy and thus lower body fat mass (Muroyama et al., 2003). Citric acid might influence insulin sensitivity; therefore, improved insulin sensitivity can lead to better regulation of glucose and lipid metabolism, potentially reducing fat storage (Yadikar et al., 2022). Some studies suggest that citric acid or its derivatives may play a role in appetite regulation and satiety. If higher citric acid levels are associated with reduced appetite or increased satiety, this could lead to lower overall caloric intake and consequently lower body fat (Muroyama et al., 2003; Ohia et al., 2002).

Alpha-ketoglutarate (AKG) has gained interest for its potential role in aging and age-related conditions (Bayliak and Lushchak, 2021). AKG is a main point in the TCA cycle because its presence determines the citric acid cycle ratio (Wu et al., 2016). In our study, we observed no association with aging or differences among sexes in this metabolite, while some studies have shown that supplementation with AKG prolongs lifespan in rodents (Asadi Shahmirzadi et al., 2020). Research on mice has shown that AKG, particularly when delivered as a calcium salt (CaAKG), can extend lifespan and compress morbidity, underscoring a connection between metabolism and aging (Rhoads and Anderson, 2020). Lastly, our study did not reveal significant variations in levels of other TCA cycle intermediates, such as succinate and fumarate, across different age groups or between both genders. In the current study, we evidenced that the hydroxybutyrate and ketonic derivates metabolites (2-hydroxybutyric acid, 3-isohydroxybutyric acid and 3-hydroxybutyric acid) end products decreased with aging in both genders, supporting the notion of poorer mitochondrial TCA and β-oxidation as we age. The enzyme glycerol kinase is the responsible of convert glycerol into glycerol-3-phosphate and then be used for the synthesis of triglycerides, phospholipids and to be stored in adipose tissue (Lebeck and Brock, 2021). It has been shown that glycerol decreased in response to mitochondrial dysfunction in aging, leading to a lower glycolysis activity decreasing glyceraldehyde-3 phosphate requirements (Westbrook et al., 2022). Most fatty acids in our investigation followed the pattern of most of the investigation carried out to date (Ma and Li, 2015), becoming more deficient the different metabolic routes that involve fatty acids and therefore lead to their accumulation (Ma and Li, 2015). Interestingly, we found that fatty acids follow a sex dimorphism pattern as we age. Some of these differences could be related to diet habits. Additionally, research on this specific topic may be limited, so more studies would be needed to establish a clear age-related pattern if one exists.

In summary, our study highlights several strengths that contribute significantly to the field of gerontology. First, metabolic profiling by GC–MS is a well-established and robust technique in our field. Additionally, we employed triplicate injections for each sample to ensure data reliability, further enhancing the robustness of our results. Furthermore, we controlled confounding variables, such as physical activity and diet, immediately prior to sample collection, enhancing the accuracy of our findings in linking metabolic alterations specifically to aging and sex. However, our study also presents certain limitations. The cross-sectional design restricts our ability to infer causality or track metabolic changes over time, suggesting a need for longitudinal studies to confirm these findings. Additionally, the generalizability of our results may be limited due to the demographic composition of our sample, which may not adequately represent broader population diversity. Our study's findings have yet to undergo external validation to establish their robustness across different populations. Lastly, while our research proposes potential mechanisms for the observed metabolic changes, it does not experimentally verify these mechanisms, indicating a gap that future research could aim to fill by integrating molecular and cellular analyses to elucidate the biological processes involved.

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CRediT authorship contribution statement

C. Navas-Enamorado: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Conceptualization. **X. Capo:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Formal analysis, Data curation, Conceptualization. **A.M. Galmes-Panades:** Writing – review & editing, Project administration, Investigation, Data curation. **A. Ortega-Moral:** Writing – review & editing, Supervision, Investigation. **A. Sánchez-Polo:** Writing – review & editing, Validation, Investigation, Data curation. **L. Masmiquel:** Writing – review & editing, Writing – original draft, Resources, Methodology, Investigation, Data curation. **M. Torrens-Mas:** Writing – review & editing, Writing – original draft, Supervision, Project

administration, Methodology, Funding acquisition, Formal analysis, Conceptualization. **P. Navas:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Investigation, Funding acquisition, Formal analysis, Conceptualization. **M. Gonzalez-Freire:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare no competing financial interests.

Data availability

Data will be made available on request.

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