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Plasma metabolomic profiles reveal sex-specific response to an oral glucose tolerance test in late middle-aged adults

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ABSTRACT

Sex is a key determinant of human phenotype, with males and females exhibiting distinct anthropometric and metabolic features that influence disease susceptibility. This study investigated sex-specific metabolic differences in late middle-aged adults without diagnosed metabolic diseases, both in the fasting state and during an oral glucose tolerance test (OGTT). Using data from the NutriTech project, we analyzed plasma metabolomic responses during the OGTT, along with detailed assessments of body composition and fasting clinical parameters. Females exhibited 28 % greater total adipose tissue, mainly subcutaneous, whereas males had more intra- abdominal fat and higher energy expenditure. Females showed elevated fasting levels of fatty acids— particularly very-long-chain fatty acids— leptin, and adiponectin. Males had slightly higher fasting glycemia (~ 5 %) and a more pronounced glycemic increase during the OGTT (17 %), along with elevated insulin levels. In both fasting and postprandial states, males showed higher circulating levels ($p < 0.05$) of aromatic and branched- chain amino acids (BCAA) and their catabolites. Conversely, females had higher sphingomyelins levels during fasting and throughout the OGTT, and increased postprandial levels of secondary bile acids ($p < 0.05$). These sex- specific metabolic features in late middle-aged adults may enhance our understanding of metabolic disease risk and support the development of more targeted prevention strategies.

Clinical trial registration number: NCT01684917

1. Introduction

More than 75 years ago, Vague described sex-specific patterns of fat deposition, reporting a lower incidence of cardiovascular diseases (CVD)-related mortality in females despite their having similar or even greater fat depots than males (Vague, 1947). Since then, the comprehension of sex-related metabolic differences has substantially advanced. New phenotyping tools have allowed the identification of a wide range of sex-related differences, including larger lean mass, higher haematocrit, higher levels of circulating branched-chain amino acids (BCAA), and lower levels of HDL-cholesterol (HDL-c) in males than in females. On the other hand, females exhibit better insulin sensitivity and reveal lower atherogenic lipid profile—characterized by higher HDL-c and lower levels of LDL-cholesterol (LDL-c) and triacylglycerols (TAG)—conferring enhanced metabolic health and protection against the development of metabolic derangements (Goossens et al., 2021; Kautzky-Willer et al., 2023).

With the use of omics approaches and advances in analytical platforms, additional phenotypic features are being discovered as sex-discriminant markers. Furthermore, the application of nutritional challenge tests—in which a standardized bolus of one or more nutrients is administered, followed by time-resolved biomarker analysis in the postprandial state—enables the detection of further metabolic differences between sexes (Wopereis, 2022). Despite the knowledge of sex-related differences, the health of males and females is still often assessed using the same tools and cut-offs, a practice that can compromise females' health, since male physiology is better studied, and females have often been underrepresented or excluded from preclinical and clinical research (Sugimoto et al., 2019). Some authors argue that most of the classic physiology studies were conducted primarily in Caucasian males, only extrapolating findings to females and other ethnic groups (Mauvais-Jarvis et al., 2020).

Metabolic differences between males and fertile females are particularly pronounced. Oestrogens participate in the regulation of intermediary metabolism and inflammation, thus reducing the risk of CVD in young females compared to age-matched males (Vogel et al., 2021). However, with aging and the onset of menopause in females and andropause in males, the reduction in circulating levels of sex hormones contributes to increased plasma levels of triacylglycerol (TAG) and LDL-c, reduced HDL-c, and impaired insulin sensitivity, predisposing females and males to CVD (Goossens et al., 2021; von Eckardstein and Wu, 2003). It remains largely unknown whether the metabolic differences discriminating young males and females persist into late middle age. Understanding these changes is crucial, considering the lifespan extension that has been attained in the last century and the need to improve understanding the physiological basis of aging and age-related metabolic dysfunctions.

The aim of our study was to investigate sex-specific metabolic differences in late middle-aged adults without diagnosed metabolic diseases. The analyses included variables

that reflect body composition and plasma metabolome in both the fasting state and during an oral glucose tolerance test (OGTT), which was used to assess metabolic responses to a glucose load. In a previous publication based on the same dataset, we identified two distinct metabotypes—comprising both sexes—based on glycaemic responses to the OGTT, enabling the selection of early bio- markers of insulin resistance (Fiamoncini et al., 2022). In the present study, we identified systemic and molecular differences distinguishing females and males in their sixth decade of life, confirming previously reported findings and revealing novel metabolic features that define sex-specific responses to a nutritional challenge.

2. Materials and methods

2.1. Ethical approval and study registration

This study was conducted as part of the NutriTech project and was carried out at the Imperial Clinical Research Facility, Hammersmith Hospital, Imperial College London. Ethical approval was granted by the Brent Research Ethics Committee (REC reference: 12/LO/0139), and the study was registered on ClinicalTrials.gov (identifier: NCT01684917).

2.2. Study design

The dataset analyzed in the present manuscript was obtained from the NutriTech trial, which included three nutritional challenge tests: an OGTT, a mixed-meal tolerance test (MMTT), and an MMTT combined with physical activity (MMTT+PA), undertaken before and after a weight-loss intervention. The current analysis is limited to data from the OGTT conducted prior to the intervention. Findings from the MMTT, MMTT+PA, and the effects of the weight-loss intervention have been reported elsewhere (Rundle et al., 2023).

Participants went through a health-screening recruitment visit at the research facility, which included assessments of anthropometric characteristics, blood pressure, electrocardiogram, and clinical chemistry markers such as glucose, insulin, glycated hemoglobin (HbA1c), plasma lipids, hematocrit, and liver and kidney function. Seventy-two late middle-aged adults (aged 50 – 65 years) were considered healthy, enrolled, and completed the study. From these participants, only those who were considered to be euglycaemic according to the cutoff of the American Diabetes Association (ADA) (fasting glycaemia < 5.6 mmol/L and 2-hour glycaemia during OGTT < 7.8 mmol/L) were included for the current study. Thus, the final dataset included a total of 32 females and 27 males (N = 59).

2.3. OGTT

Participants were instructed to abstain from alcohol consumption and physical exercise on the evening before the nutritional challenge test. The OGTT started at 09:00 a.m. following a 12-hour overnight fast. Immediately after baseline blood collection ($t = 0$) under fasting conditions, participants ingested 75 g of glucose dissolved in 250 mL of water. Successive blood sampling continued at 15, 30, 60, 90, 120 and 240 min after the intake of the glucose solution. The cannula placed into the antecubital vein was flushed with saline between each sampling, and the first 3 mL of blood drawn were discarded to avoid saline-diluted blood. Heparinized plasma and serum were separated by centrifugation at 1800 rpm and stored at -80°C until analysis. HOMA-IR was calculated as described in the original publication (Matthews et al., 1985).

2.4. Markers of intermediate metabolism and inflammation

Serum insulin concentration was assessed by radioimmunoassay using a human-specific RIA Kit (Millipore Corporation) accordingly to manufacturer's instructions. Serum glucose levels were measured by an enzymatic method using an Abbott Architect ci8200 analyzer. Plasma concentrations of peptide YY (PYY), glucagon, and glucagon-like peptide-1 (GLP-1) were also measured by radioimmunoassay. Serum concentrations of non-esterified fatty acids (NEFA), albumin, ammonia, urea, creatine, aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (GGTP), total cholesterol, HDL-c, LDL-c, TAG, and uric acid were measured using enzymatic methods according to manufacturer's instructions. Plasma leptin and adiponectin levels were assayed by sandwich enzyme-linked immunoassays (BioVendor, Czech Republic). Fasting plasma fatty acids were determined by gas chromatography with flame ionization detection (GC-FID). The levels of vitamins C and K2, lutein, retinol and beta-carotene were determined by HPLC-UV and HPLC-ECR-FLD.

2.5. Magnetic resonance imaging (MRI)

The volumes and distribution of adipose tissue, as well as hepatic and muscular fat content, were assessed using MRI and proton magnetic resonance spectroscopy (MRS) on a 1.5 T Phillips multinuclear system (Thomas et al., 1998). Single voxel spectra ($2 \times 2 \times 2 \text{ cm}^3$; TE/TR = 135/1500 ms) were acquired from the liver using a PRESS sequence for measurement of intrahepatocellular lipid (IHCL). Spectra were also acquired from the soleus muscle of the calf to measure intra-myocellular lipid (IMCL). Whole-body adipose tissue was assessed using a rapid T1-weighted spin echo sequence. Participants were scanned from head to toes by acquiring 10 mm thick transverse images with 10 mm interslice gaps.

2.6. Energy expenditure and physical activity

Resting energy expenditure (REE) was assessed by indirect calorimetry (GEM, UK) following an overnight fast. Participants were asked to rest for 15 min before the measurement, performed according to a well-established method (Tan et al., 2013). A canopy was placed over the head and upper body during the 20-min data collection period. Resting metabolic rate (RMR) and respiratory quotient (RQ) were calculated. For the assessment of maximum oxygen uptake (VO₂max), subjects performed an incremental exercise on a cycle ergometer (Cosmed, Italy). The test started at a workload of 100 watts, with increments of 25 watts every 3 min until exhaustion. VO₂max, maximum aerobic power output, and maximum heart rate were recorded.

To assess the level of physical activity, participants wore an accelerometer (BodyMedia SenseWear, USA) on the non-dominant arm for seven consecutive days. Data were expressed as the average daily in metabolic equivalents (METs).

2.7. Mass spectrometry-based plasma metabolite profiling

In preparation for measurements with the different analytical platforms, plasma samples were randomized to minimize batch variation. Quality control plasma samples (Recipe Chemicals and Instruments, Munich, Germany) were included in each analytical batch to monitor for instrument drifting and technical issues.

Plasma concentrations of amino acids and biogenic amines (36), glycerophospholipids (90), sphingolipids (15) and total hexoses were quantified using the LC-MS/MS based AbsoluteIDQ® p180 Kit (Biocrates Life Sciences AG, Innsbruck, Austria). Additional acylcarnitines were quantified using an in-house method following deproteinization with methanol in the presence of deuterated standards, and subsequent butylation prior to analysis by LC-MS/MS using a Sciex 5500 MS (Sciex, USA) (Giesbertz et al., 2015). The twelve most abundant plasma bile acids (BA) were quantified using LC-MS/MS. Briefly, 10 µL of plasma were mixed with deuterated internal standards and subjected to methanolic extraction. After evaporation to dryness, samples were reconstituted in methanol:water (1:1, v/v) and injected for analysis.

For the GC-MS analysis, metabolites were extracted from 40 µL plasma using ice-cold methanol:water (8:1, v/v) at a ratio of 1:10 (sample:solvent). After centrifugation (13,200 rpm, 4 min, 4 °C), 200 µL of the supernatant was completely dried under vacuum. A two-step derivatization was performed using an autosampler (Agilent 7693, Agilent Technologies, Germany) by incubating the samples with methoxyamine hydrochloride (20 mg/mL in pyridine) at 45 °C for 30 min, followed by the addition of N-methyl-N-trimethylsilyl-tri-fluoroacetamide and a second incubation at 45 °C for 30 min. Samples were immediately subjected to GC-MS analysis (Agilent 6890 N GC coupled to an Agilent

5975 C inert XL - Agilent Technologies, Germany). The GC was equipped with a 30 m DB-35MS capillary column (Agilent J&W GC Column, Germany). Metabolites were eluted by a temperature gradient starting at 80 °C and rising by 11 °C/min to 325 °C, with a final 5 min hold at 325 °C. Metabolite identification and quantification were accomplished using the Metabolite Detector software (Hiller et al., 2009). Metabolites were identified according to their retention time and spectral similarity against entries in the Golm Metabolome Database.

2.8. Data analyses

A list of all metabolites and other variables included in the analyses is provided in Supplemental Table 1. A partial least squares discriminant analysis (PLS-DA) was performed to assess differences between males and females and identify discriminant variables. For variables quantified at all seven time points during the OGTT, each data point was treated as an independent variable. Considering these variables and those measured only under fasting conditions, the final model was built with 2086 variables. Among these, 596 (27 %) had a variable importance in projection (VIP) score > 1 and were selected for further analysis. From the variables (metabolites or hormones) assessed during the OGTT, only those with a VIP score > 1 at a minimum of two time points were considered relevant for the discrimination between males and females, and were further subjected to univariate analysis.

Depending on the nature of the comparisons, a Student's t-test or mixed-effects analyses were used to test differences between males and females. Multiple comparisons were examined using Tukey's post hoc test. Differences with $p < 0.05$ were considered significant. For multiple t-tests, the false discovery rate (FDR) approach was controlled using the two-stage step-up method of Benjamini, Krieger and Yekutieli, with desired FDR rate = 1 %. Shapiro-Wilk test was used to assess normality ($p > 0.05$). Outliers were detected and removed using the ROUT method. Pearson's multiple correlation analysis was applied to evaluate associations between variables, as most generally followed a normal distribution. GraphPad Prism Software (version 10) was used for statistical analyses and figure generation. PLS-DA was performed using SIMCA v.18 software (Umetrics, Sweden) after data scaling using unit variance. The quality of the model was judged by the goodness-of-fit parameter ($R^2 > 0.5$), the predictive ability parameter ($Q^2 > 0.4$), and cross-validation test.

3. Results

3.1. Population characteristics, clinical parameters, and discriminant variables

All overweight/obese individuals included in this analysis were considered healthy as assessed by a panel of clinical markers (Table 1) and euglycaemic status. There were no differences between males and females in terms of hepatic (albumin, GGTP and AST) and renal (eGFR) functions. However, males presented significantly higher diastolic blood pressure (DBP) compared to females ($p < 0.05$), while systolic blood pressure (SBP) did not differ significantly between sexes (Table 1).

Despite a 5 % higher fasting glucose concentration in males, there were no differences in insulin and HbA1c concentrations between males and females. Females had 11 % higher levels of HDL-c and total cholesterol. As expected, body weight, height, and waist circumference differed between male and female participants (Table 1). A PLS-DA model ($R^2 = 0.99$ and $Q^2 = 0.77$, and p -value from ANOVA of cross-validated residuals = 5.64×10^{-10}), built using all collected phenotypic data, confirmed sex-specific metabolic differences and identified key discriminant variables (Supplemental Figure 1).

Among the characteristics measured only in the fasting state, those related to the amount and distribution of adipose tissue (AT) were the most implicated in the differences between males and females. Females had more total adipose tissue (TAT), subcutaneous (SAT), abdominal subcutaneous (ASAT), and non-abdominal subcutaneous (NASAT) fat (Table 2). Males displayed a higher content of internal AT, with intra-abdominal AT (IAAT) approximately 100 % higher than that of females relative to TAT (Table 2). Females had higher plasma concentrations of beta-carotene and vitamin C, whereas males had higher levels of retinol (Table 2). In the fasting state, females also displayed elevated levels of fatty acids with very-long chain length, as well as gamma-linolenate (C18:3n6) and, to a lesser extent, palmitoleate (C16:1n7, $p = 0.06$). Compared to males, females had 72 % and 107 % higher plasma concentrations of adiponectin and leptin, respectively, probably reflecting their greater mass of SAT (Table 2). As expected, males showed higher REE, VO_{2max} and W_{max} (Table 2).

3.2. Postprandial kinetics of glucose, insulin, glucagon, triacylglycerol, and incretins

Despite minor differences in fasting glucose levels (slightly higher in males), there were considerable differences in glucose concentrations at 60 and 90 min after glucose ingestion, when the glycaemic response was higher in males than in females (Fig. 1A). Insulin concentrations were even more discrepant between the two groups during the OGTT, with males displaying higher values than females at 30, 60, 90 and 120 min (Fig. 1B). There were no differences in the HOMA-IR values (3.7 ± 0.2 in males compared to 3.0 ± 0.4 in females, $p = 0.08$) (Table 1). TAG concentrations were also higher in males during the OGTT, although not significantly different during fasting ($p = 0.096$) (Fig. 1C). While incretins were not identified as discriminating variables in the PLS-DA model, the glucose load stimulated incretin secretion, affecting both plasma PYY and GLP-1 levels. Plasma

levels of glucagon decreased during the OGTT (Fig. 1D-F). Despite no significant differences in the absolute plasma levels of these signalling molecules between the groups, females had a greater increase in PYY than males during the nutritional challenge.

3.3. Amino acid metabolism

Several amino acids and their catabolites were identified by the PLS-DA model as relevant for the discrimination between males and females. During the first two hours of the OGTT, the plasma levels of glutamate, isoleucine, leucine, methionine, phenylalanine, proline, tryptophan, and valine were higher in males than in females ($p < 0.05$). Similarly, levels of various acylcarnitines including propionyl-carnitine, isovaleryl-carnitine, methylmalonyl-carnitine, succinyl-carnitine, and glutaryl-carnitine, as well as creatinine, were also elevated in males than in females (Fig. 2A-N). On the other hand, plasma levels of glycine, taurine, and threonine were higher in females compared to males (Fig. 2O-Q). For many of these metabolites, sex-related differences observed in the fasting state persisted throughout most of the OGTT. Notably, concentrations of BCAA and phenylalanine decreased after the intake of glucose, followed by a rebound phase after 120 min, with no differences between sexes at 240 min (Fig. 2).

3.4. Bile acid postprandial kinetics

The PLS-DA model identified several BA as discriminant metabolites between males and females. Contrary to amino acids, fasting concentrations of BA were similar between sexes, whereas females displayed higher plasma concentrations of taurodeoxycholic acid (TDCA), tauroolithocholic acid (TLCA), as well as the sum of all taurine-conjugated BA after the first hour of the OGTT. The increase in the concentration of these BA was accompanied by a decrease in the fraction of cholic acid (CA) and chenodeoxycholic acid (CDCA) in females compared to males

3.5. Lipid catabolism

Fatty acid levels also exhibited differences between males and females. Fasting plasma levels of markers of lipolysis such as glycerol and NEFA, as well as higher levels of oleate and linoleate, were higher in females than males (Fig. 4). Similarly, markers of fatty acid oxidation and ketogenesis including hydroxy-butyryl-carnitine and 3-hydroxy-butyric acid were elevated in females. The concentrations of these metabolites decreased during the OGTT, reaching a nadir between 60 and 120 min after ingestion of the glucose. A rebound phase was observed between the second and fourth hours of the OGTT, during which metabolite levels gradually returned to fasting levels (Fig. 4). Plasma levels of NEFA

decreased more rapidly and reached lower values in females than in males. Concerning oleate and linoleate, plasma levels between the first and second hours of the OGTT were similar in both sexes, indicating a greater capacity in females to reduce the concentration of these metabolites postprandially, considering that these metabolites were found at higher levels in the fasting state in this group (Fig. 4C and D).

3.6. Plasma levels of sphingomyelins

Sphingomyelins were prominently represented among the metabolites discriminating males and females (Fig. 5). In some cases, such as for SM(OH)22:2, females displayed an average concentration 30 % higher than males (Fig. 5D). Other sphingomyelins, such as SM16:0, displayed smaller but significant differences between the groups, with higher plasma levels in females (Fig. 5F). The plasma levels of these metabolites remained stable during the OGTT, and the sex-specific differences observed in the fasting state persisted throughout the OGTT.

4. Discussion

Our integration of metabolomics platforms with multiple analytical methods provides insights into metabolic distinctions among late middle-aged adults, revealing a broad spectrum of metabolites with sex-specific alterations in circulating levels after an overnight fasting and in response to an OGTT. We observed that biomarkers associated with an unfavorable insulin response were exhibited at higher plasma levels in males, despite the careful selection of participants with no indication of dysglycemia. Whether these findings indicate that the males in this study are likely to become diabetic patients or if this is a natural phenotypic trait of males, remains debatable. A highlight of this study was the observation of elevated circulating levels of taurine-conjugated bile acids and sphingomyelins in females, which may serve as sex-specific biomarkers, as well as the confirmation of previously reported markers of sex-based metabolic dimorphism.

Despite minor differences in fasting glucose levels (~5 %) and similar insulin concentrations in the fasting state, males displayed higher glycaemia and insulinemia during the OGTT than females. This supports the notion that a nutritional challenge test like an OGTT, increases the sensitivity to detect metabolic differences not noticeable in the fasting state (Rundle et al., 2023). Conflicting findings have been reported regarding the relationship between sex and insulin resistance. Although some evidence suggests that females are more insulin resistant than males, possibly due to specific sex-linked gene expression and differences in metabolic control elements (Mittendorfer, 2005), in our study females displayed a smaller increase in both plasma glucose and insulin levels after the oral glucose load. In accordance with our findings, other studies have reported

that insulin sensitivity is higher in females than males, attributed to a more robust insulin and incretin response, although these metabolic advantages tend to disappear when the state of glucose tolerance deteriorates (Tramunt et al., 2020).

Sexual dimorphism in AT distribution contributes to sex differences in insulin sensitivity (Goossens et al., 2021). Oestrogen promotes fat accumulation in gluteo-femoral subcutaneous depots rather than in visceral adipose tissue (Ju and Yi, 2022). We report larger deposits of subcutaneous adipose tissue (SAT, ASAT, NASAT) in females (Table 2), whereas males had greater visceral adipose depots. Visceral AT differs from subcutaneous adipose depots in the type of adipocytes (reduced preadipocyte differentiating capacity and a higher proportion of large adipocytes), their endocrine function, lipolytic activity, as well as the response to insulin (Ibrahim, 2010). IAAT is characterized by a proinflammatory profile with a larger number of inflammatory cells, being more metabolically active and sensitive to lipolysis (greater capacity to release fatty acids, whereas SAT is more efficient at uptake of circulating fatty acids), and less sensitive to insulin as compared to SAT (Ibrahim, 2010). These IAAT features are responsible for adverse effects on insulin-mediated glucose disposal and lipid metabolism among individuals with large IAAT.

Plasma levels of both leptin and adiponectin were higher in females than in males. These sex-specific differences are likely influenced by variations in body composition, particularly the distribution of AT, which affects the production and secretion of adipokines. These alterations are partially mediated by estrogen receptors in adipocytes, which regulate adipose tissue expansion, inflammation, and fibrosis (Dearden et al., 2018). It has been reported that SAT secretes more leptin compared to IAAT, a process further amplified by insulin action (Russell et al., 1998). Our findings support previous evidence indicating that central adiposity is negatively related to the circulating adiponectin levels, being an important determinant linking intra-abdominal fat accumulation to insulin resistance (Cnop et al., 2003). On the other hand, femoral and gluteal subcutaneous AT depots have been positively associated with plasma adiponectin concentrations (White and Tchoukalova, 2014). Future studies incorporating time-resolved measurements of adipokines, as well as inflammatory and oxidative stress markers, during metabolic challenges such as the OGTT are warranted to further elucidate sex-specific regulatory mechanisms. In support of this, recent findings from our group demonstrated distinct postprandial inflammatory responses between postmenopausal and reproductive-age women (Donado-Pestana et al., 2025).

We found higher plasma NEFA levels in females both in the fasting state and at the end of the OGTT ($t = 240$ min). One can assume that because females have larger adipose depots, their plasma NEFA concentrations will be higher than in males. In addition, upper and lower body AT show different rates of lipolysis and fatty acid uptake (White and Tchoukalova, 2014), and a possible explanation for the higher fasting levels of NEFA in

females is the greater lipolytic activity in SAT (Shi et al., 2009). The increase in circulating insulin levels during the OGTT suppresses lipolysis in AT, leading to reduced plasma NEFA concentrations during the early phase of the OGTT (Frayn et al., 1995).

Notably, insulin resistance has been associated with higher lipolytic rate in AT during both fasting and postprandial conditions (Costabile et al., 2011). Our female participants displayed higher NEFA concentrations at fasting than males, but a more pronounced decline in the plasma levels during the OGTT (83 % decline in comparison to 64 % decline in males), suggesting greater insulin sensitivity in this group.

During the first two hours of the OGTT, plasma levels of BCAA decreased markedly, with reductions of approximately 20 % for valine and up to 43 % for leucine, in parallel with rising plasma insulin levels. Similar decreases were observed for other amino acids, including proline and phenylalanine, while glycine and taurine concentrations remained unchanged following the glucose load. Insulin promotes intramuscular anabolic signalling and upregulates amino acid transporters (Drummond et al., 2010), leading to increased amino acid uptake and increased rate of protein synthesis in skeletal muscle, liver, and adipose tissues (Dimitriadis et al., 2011; Fujita et al., 2006; Weijzen et al., 2022). Beyond their function as substrates for protein synthesis, amino acids also contribute to critical intracellular metabolic processes such as nucleotide synthesis, redox balance, and ATP generation. In line with earlier observations (Costanzo et al., 2022; Krumsiek et al., 2015), amino acids were identified in our study as one of the most discriminative metabolites between females and males. For most amino acids and their degradation products, females exhibited lower plasma levels than males. A previous study has revealed a major sexual dimorphism in amino acid metabolism as a source of substrates for lipid synthesis (Torre et al., 2018). In female mice, the synthesis of lipids is maintained at the expense of amino acids, whereas in males, amino acids are preserved by restraining lipogenic and hepatic gluconeogenic pathways. The mechanism involves the estrogen receptor alpha, which can be transcriptionally activated by amino acids (Torre et al., 2018).

Post-menopausal females face an elevated risk of osteoporosis (Greco et al., 2019) and evidence links reduced plasma levels of BCAA in elderly females with reduced risk of osteoporosis (Panahi et al., 2022). In addition, elevated plasma BCAA and aromatic amino acids (AAA) levels (i.e. phenylalanine, tryptophan) have been linked to an increased risk of insulin resistance and diabetes (Gar et al., 2018; Wang et al., 2011). Recent pre-clinical data further suggest that diabetes is associated with higher circulating BCAA levels due to reduced BCAA degradation ability of the gut microbiota (Zheng et al., 2024). In our study, males exhibited higher plasma levels of both BCAA and AAA than females, which may partially explain the greater insulin resistance observed in males. In contrast to BCAA and AAA, we observed higher plasma levels of glycine, taurine, and threonine in females than males. Previous reports have demonstrated that

high concentrations of glycine and taurine are associated with a lower risk of diabetic complications, improved insulin sensitivity and glucose homeostasis (Adeva-Andany et al., 2018; Sak et al., 2019). Taurine appears to preserve insulin signalling by inhibiting the c-Jun N-terminal kinase (JNK) pathway, which is activated in response to oxidative stress (Wu et al., 2010). An inverse association between glycine and BCAA levels has been described, suggesting that the increased nitrogen load in tissues, resulting from elevated BCAA concentrations, is dissipated via the use of glutamate to convert pyruvate into alanine. The resulting decline in pyruvate can be compensated by glycine through enzymes involved in serine metabolism, ultimately leading to decreased glycine levels during the progression of insulin resistance (White et al., 2021).

During the OGTT, plasma sphingomyelin levels remained unaltered, and females displayed higher plasma sphingomyelin concentrations than males. These findings may shed light on the role of sphingolipids in neurodegenerative and cardiovascular diseases, which often display a sex bias (Albi and Alessenko, 2021; Jiang et al., 2000). A previous study with 472 participants from the Baltimore Longitudinal Study of Aging showed that higher plasma concentrations of sphingomyelins (C16:0, C24:1, and C26:1) were associated with decreased odds of abnormal HOMA-IR, suggesting a potential protective metabolic role (Semba et al., 2018). In accordance with our results, a cross-sectional study described higher plasma sphingomyelin levels in females than in males at 56–70 years of age (Mulwijk et al., 2021). These authors reported lower plasma levels of sphingolipids in females than males at young age (Mulwijk et al., 2021), indicating an age-dependent shift in sphingolipid metabolism. Whereas sphingomyelins are abundant in HDL particles (Martínez-Beamonte et al., 2013), the higher plasma sphingomyelin levels in females may be partially linked to their higher HDL cholesterol levels than in males (Table 1).

The kinetic pattern of plasma BA during the OGTT showed remarkable differences between males and females. Using the same dataset, we previously reported differences in postprandial levels of BA between males and females, in the OGTT as well as the MMTT (Fiamoncini et al., 2017). In our current analysis, BA differences were amongst the most striking discriminating parameters, reaching high VIP scores in the PLS-DA model. Higher levels of BA subtypes including taurine- and glycine-conjugated BA, were associated with females both at baseline and during the postprandial period, whereas cholic acid (particularly when considered as a proportion of total BA) had the opposite pattern and was more abundant in males. A cross-sectional study investigating determinants of the human blood metabolome reported higher plasma concentration of BA in males than in females, along with a reduction in plasma levels of BA with aging in males, but not in females (Frommherz et al., 2016). These sex-related differences may be attributed to sexual dimorphisms in the regulation and activity of enzymes involved in BA biosynthesis (Phelps et al., 2019).

Disruptions in BA metabolism may contribute to insulin resistance, impaired glycemic control, cholestasis, hepatic fibrosis, and hepatitis (Fuchs et al., 2024). Recent evidence revealed a discernible impact of pre/diabetes status on the BA metabolism, indicating that impaired glycemic control led to increased circulating BA levels, a shift toward a greater proportion of secondary BA, and an increase in the ratio of glycine to taurine-conjugated BA (Schlicht et al., 2025). Moreover, primary biliary cirrhosis, characterized by autoimmune destruction of bile ducts, portal infiltration, and fibrosis, is highly prevalent in middle-aged females. In contrast, primary sclerosing cholangitis, characterized by bile duct fibrosis and obstruction of bile flow, has a higher incidence in males (Chiang and Ferrell, 2020). In line with our findings, preclinical studies have demonstrated higher serum levels of total, primary, and secondary BA in female animals than in males. When analyzed by BA subtypes, tauro-conjugated BA including taurocholic, tauro- α -muricholic, tauro- β -muricholic, and tauro-chenodeoxycholic acids, were higher in female than male mice (Baars et al., 2018). Another study investigating dietary influence on BA homeostasis showed that female mice had higher plasma BA concentrations than males when fed a Western, low n-3 fatty acid, and high fructose diet, whereas a calorie-restricted diet closed the gap of the sex difference (Ma et al., 2021).

One strength of this study is the use of multiple analytical platforms, which allowed for a comprehensive metabolic phenotyping. Furthermore, the strict inclusion criteria of euglycaemic individuals based on ADA cutoffs (more stringent than WHO guidelines) was important to limit the bias of picking up insulin resistance rather than sex markers when comparing males and females. On the other hand, the relatively small sample size may have limited our ability to detect certain differences between groups. Therefore, the findings should be interpreted with caution and regarded as exploratory. While the absence of an independent validation set is understandable in the context of an exploratory study, it remains a limitation and is acknowledged accordingly. These results should be viewed as hypothesis-generating and require validation in larger, independent cohorts. Furthermore, although our statistical analysis adopted a strategy of mixed-effects models, alternative multivariate approaches that account for repeated measures could offer complementary insights and should be explored in future studies. Another limitation of our study is the lack of data on circulating sex hormone levels, which precludes the classification of menopausal or andropause status within the study population. Given the recognized influence of sex hormones on metabolic regulation, future research should include hormonal profiling to better elucidate their role in driving sex-related metabolic differences. Finally, one must consider that this study examined sex differences within a restricted age group; and therefore, the findings might not be directly translatable to differentiate females of reproductive age and age-matched males.

5. Conclusions

Using multiple analytical methods, a comprehensive panel of plasma metabolites enabled the identification of a subset of markers both in the fasting state and during an OGTT, that distinguish individuals in late middle age in a sex-specific manner. A radar plot (Fig. 6) visually summarizes the key metabolic differences between males and females, delineating well-defined sex-specific metabolic phenotypes. Females exhibited elevated circulating levels of taurine-conjugated bile acids, sphingomyelins, and amino acids, including glycine, taurine, and threonine. Conversely, males showed elevated levels of branched-chain and aromatic amino acids, which are recognized as metabolites associated with an unfavorable insulin response. These findings contribute to the elucidation of sex-related differences in insulin resistance by confirming established metabolic signatures (e.g. BCCAs, AAA, NEFA, leptin), and identifying potentially novel targets, including taurine-conjugated bile acids and specific lipid subclasses such as sphingomyelins, in the context of aging.

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Table 1

Characterization of the study population. Anthropometric and clinical markers indicating the health status of the study population. p-value of Student's t-test is indicated for each comparison. n = 32 females and 27 males.

		Females		Males		p value
		Mean	SEM	Mean	SEM	
Age	years	58.84	0.75	59.00	0.84	0.89
Body weight	kg	76.52	1.84	90.56	2.36	< 0.0001
Height	m	1.63	0.01	1.76	0.01	< 0.0001
Waist circumference	cm	93.76	1.69	104.15	1.94	0.0001
BMI	kg/m ²	28.75	0.45	29.11	0.55	0.61
DBP	mmHg	75.09	1.64	80.06	1.46	0.03
SBP	mmHg	124.25	2.56	128.83	1.76	0.16
HR rest	bpm	68.56	1.90	67.19	1.91	0.61
Activity level	METs	1.51	0.05	1.55	0.04	0.53
HbA1c	mmol/ mmolHb	35.88	0.57	36.17	0.47	0.71
Fasting glucose	mmol/mL	4.88	0.08	5.11	0.07	0.04
Fasting insulin	mIU/L	13.67	0.88	16.29	1.62	0.14
HOMA-IR	arbitrary units	3.01	0.22	3.71	0.40	0.11
Cholesterol	mmol/mL	5.10	0.16	4.62	0.13	0.03
LDL-c	mmol/mL	2.82	0.15	2.42	0.12	0.05
HDL-c	mmol/mL	1.74	0.03	1.56	0.02	< 0.0001
Albumin	g/L	40.43	0.49	41.12	0.56	0.35
GGTP	UI/L	21.52	4.95	27.60	2.69	0.32
AST	UI/L	21.26	1.96	21.48	1.21	0.93
eGFR	mL/min	78.81	1.61	83.00	1.65	0.07
TSH	mIU/L	1.65	0.15	1.72	0.17	0.75
T4	pmol/L	13.17	0.25	13.37	0.26	0.57

BMI: body mass index; DBP: diastolic blood pressure; SBP: systolic blood pressure; HR rest: resting heart rate; HbA1c: glycated hemoglobin; HOMA-IR: Homeostasis model assessment of insulin resistance; LDL-c: LDL-cholesterol; HDL-c: HDL-cholesterol; GGTP: gamma-glutamyltranspeptidase; AST: aspartate aminotransferase; eGFR: estimated glomerular filtration rate; TSH: thyroid-stimulating hormone; T4: thyroxine.

Table 2

Discriminant variables measured in the fasting state. Variables received a VIP value > 1 in the PLS-DA model, a *p*-value < 0.05 in Student's *t*-test and that were measured exclusively in the fasting state. The last column presents the ratio of each variable between females and males. Highlighted rows in gray indicate variables with higher values in males. n = 32 females and 27 males. Adipose tissue was expressed in mass units (Kg), relative to total body weight (%BW) or relative to total body fat (%TBF).

		Females		Males		p value	q value	F/M
		Mean	SEM	Mean	SEM			
Body composition								
ASAT	kg	8.28	0.46	5.69	0.52	0.00040	0.00046	1.46
NASAT	kg	20.17	0.84	13.14	0.84	< 0.000001	< 0.000001	1.53
SAT	kg	28.44	1.26	18.83	1.33	0.00000	0.00001	1.51
TAT	kg	34.35	1.53	26.76	1.62	0.00124	0.00125	1.28
IAAT	kg	3.07	0.22	4.60	0.27	0.00004	0.00004	0.67
IAAT:ASAT	ratio	0.37	0.02	0.91	0.06	< 0.000001	< 0.000001	0.41
Internal	kg	5.91	0.34	7.93	0.42	0.00042	0.00033	0.75
Internal:subcutaneous	ratio	0.21	0.01	0.45	0.02	< 0.000001	< 0.000001	0.47
NAIAT	kg	2.84	0.15	3.32	0.20	0.05382	0.02356	0.86
Trunk:peripheral	ratio	0.49	0.01	0.63	0.02	0.000002	0.000002	0.78
Waist circumference	cm	93.76	1.70	104.15	1.94	0.00016	0.00013	0.90
Waist:Hip	ratio	0.86	0.01	0.97	0.01	< 0.000001	< 0.000001	0.88
Height	m	1.63	0.01	1.76	0.01	< 0.000001	< 0.000001	0.92
Body weight	kg	76.52	1.84	90.56	2.36	0.00001	0.00001	0.85
Vitamins/phytochemicals								
beta-carotene	µg/mL	0.50	0.06	0.33	0.04	0.02798	0.01696	1.54
Lutein	µg/mL	0.25	0.03	0.19	0.01	0.05617	0.02893	1.35
Vitamin C	µmol/L	71.25	3.65	57.51	3.67	0.01073	0.00887	1.24
Vitamin K2	ng/mL	2.43	0.18	1.94	0.13	0.03518	0.02063	1.25
Retinol	ng/mL	468.64	32.80	595.72	39.36	0.01586	0.00905	0.79
Fatty acids								
C18:3 n-6	µg/mL	12.83	0.86	10.17	0.95	0.04254	0.02417	1.26
C20:0	µg/mL	8.93	0.29	7.87	0.28	0.01211	0.00902	1.13
C20:4 n-6	µg/mL	182.06	5.48	159.59	6.77	0.01156	0.00902	1.14
C22:0	µg/mL	21.53	0.77	17.30	0.55	0.00007	0.00009	1.24
C23:0	µg/mL	10.98	0.46	8.42	0.33	0.00006	0.00009	1.30
C24:0	µg/mL	22.50	0.88	19.11	0.66	0.00402	0.00366	1.18
C24:1 n-9	µg/mL	40.84	1.14	36.11	1.18	0.00572	0.00495	1.13
C16:1,n-7	%	2.28	0.15	1.91	0.10	0.06179	0.03036	1.19
C18:3,n-6	%	0.41	0.02	0.33	0.02	0.01240	0.00902	1.25
C20:0	%	0.29	0.01	0.26	0.01	0.02018	0.01411	1.10
C20:4,n-6	%	5.97	0.21	5.39	0.21	0.05729	0.02893	1.11
C22:0	%	0.70	0.02	0.59	0.02	0.00159	0.00152	1.18
C23:0	%	0.36	0.01	0.29	0.01	0.00066	0.00070	1.23
C24:0	%	0.73	0.02	0.65	0.02	0.02119	0.01427	1.12
C16:0	%	20.84	0.27	21.78	0.40	0.04956	0.02246	0.96
C18:1 n9	%	19.19	0.33	20.30	0.49	0.05815	0.02463	0.95
C20:1n-9	%	0.14	0.00	0.16	0.01	0.00687	0.00430	0.88
Clinical data and signalling molecules								
Adiponectin	µg/mL	19.26	1.37	11.17	0.86	0.00001	0.00002	1.72
Leptin	ng/mL	19.24	1.31	9.29	1.10	< 0.000001	0.00000	2.07
Alkaline Phosphatase	IU/L	80.19	4.85	66.80	2.67	0.02752	0.01696	1.20
Cholesterol	mmol/L	5.10	0.16	4.62	0.13	0.02647	0.01696	1.10
HDL-chole	mmol/L	1.74	0.03	1.56	0.02	0.00006	0.00009	1.11
LDL-chole	mmol/L	2.82	0.15	2.42	0.12	0.04828	0.02660	1.16
DBP	mmHg	75.09	1.64	80.06	1.46	0.03036	0.01594	0.94
Uric acid	µmol/L	247.19	10.93	340.56	10.52	< 0.000001	< 0.000001	0.73
Energy expenditure								
VO2 max	mL/min/kg	20.00	0.78	25.40	1.71	0.000008	0.000010	0.79
Wmax/kg	Watt/kg	1.40	0.07	1.96	0.11	0.00009	0.00008	0.72
40 %VO2 max	mL/min	613.84	25.47	910.26	58.45	0.00001	0.00001	0.67
Wmax	Watt	110.94	4.62	173.15	7.77	< 0.000001	< 0.000001	0.64
REE	kcal	1178.80	34.37	1487.07	32.10	< 0.000001	< 0.000001	0.79

ASAT: abdominal subcutaneous adipose tissue; NASAT: non-abdominal subcutaneous adipose tissue; SAT: subcutaneous adipose tissue; TAT: total adipose tissue; IAAT: intra-abdominal adipose tissue; Internal: total internal AT was obtained by subdividing it into IAAT and NAIAT; NAIAT: non-abdominal internal AT; HDL-c: HDL- cholesterol; LDL-c: LDL-cholesterol; DBP: diastolic blood pressure; VO₂ max: maximal oxygen consumption; Wmax: weight-adjusted maximum power output; REE: resting energy expenditure.

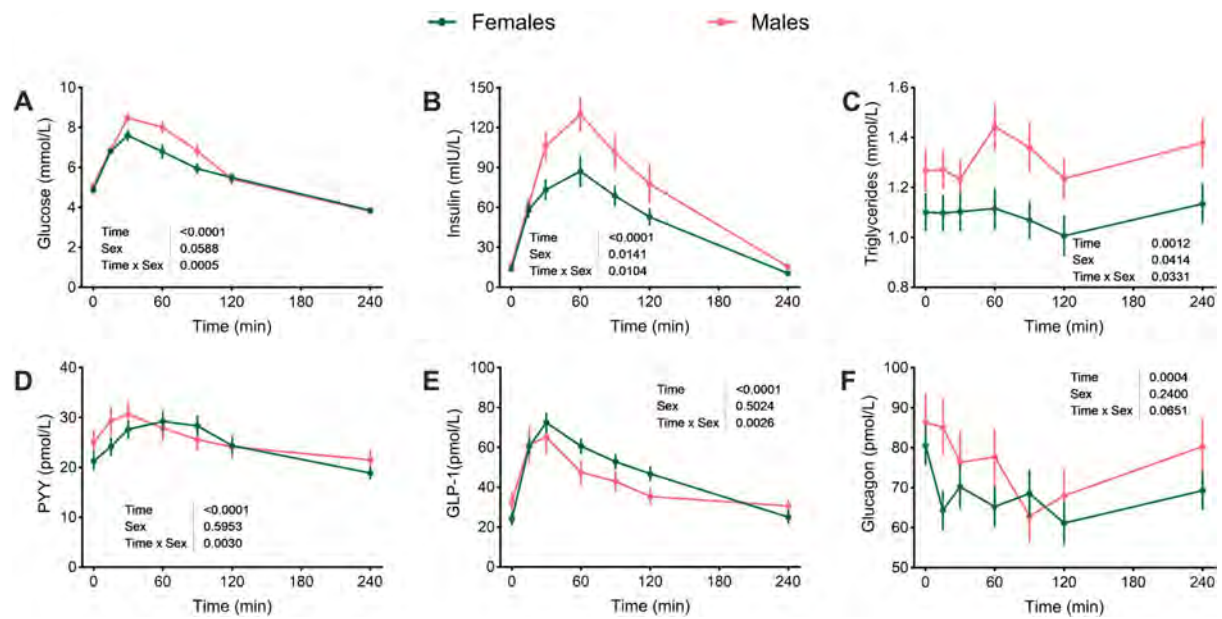


Fig. 1. Postprandial kinetics of glucose, insulin, glucagon, triacylglycerol, and incretins. Plasma levels of A) glucose, B) insulin, C) triacylglycerol, D) peptide YY (PYY), E) glucagon-like peptide-1 (GLP-1), and F) glucagon during the OGTT. n = 32 females and 27 males. Data represent means and standard error of mean. Results from mixed-effects analyses indicated in each graph.

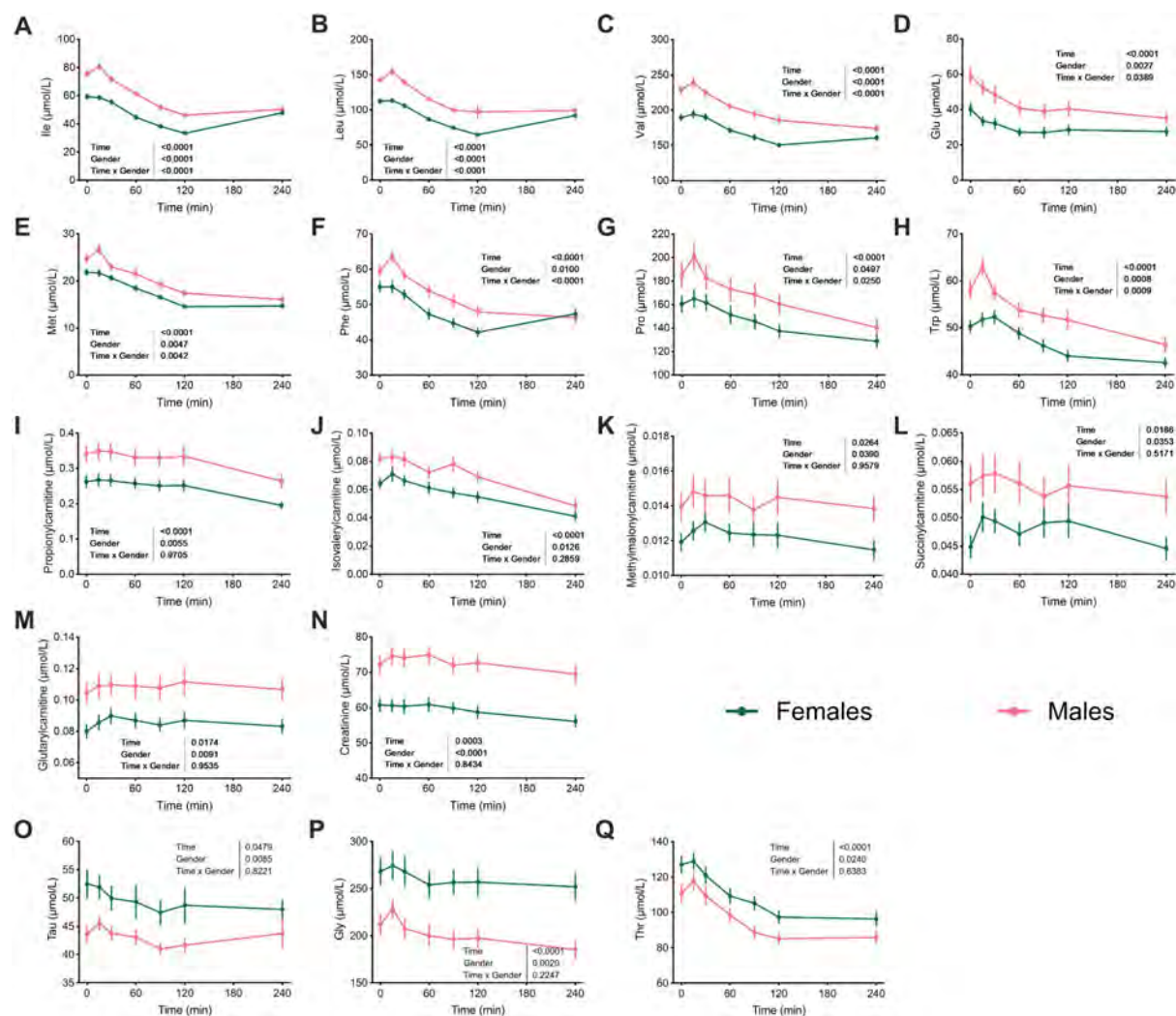


Fig. 2. Amino acid metabolism. A – I) Plasma concentrations of amino acids with higher concentrations in males. J – P) Plasma concentrations of amino acid metabolites (acylcarnitines and creatinine) with higher concentrations in males. Q – S) Plasma concentrations of amino acids with higher concentrations in females. $n = 32$ females and 27 males. Data represent means and standard error of mean. Results from mixed-effects analyses indicated in each graph.

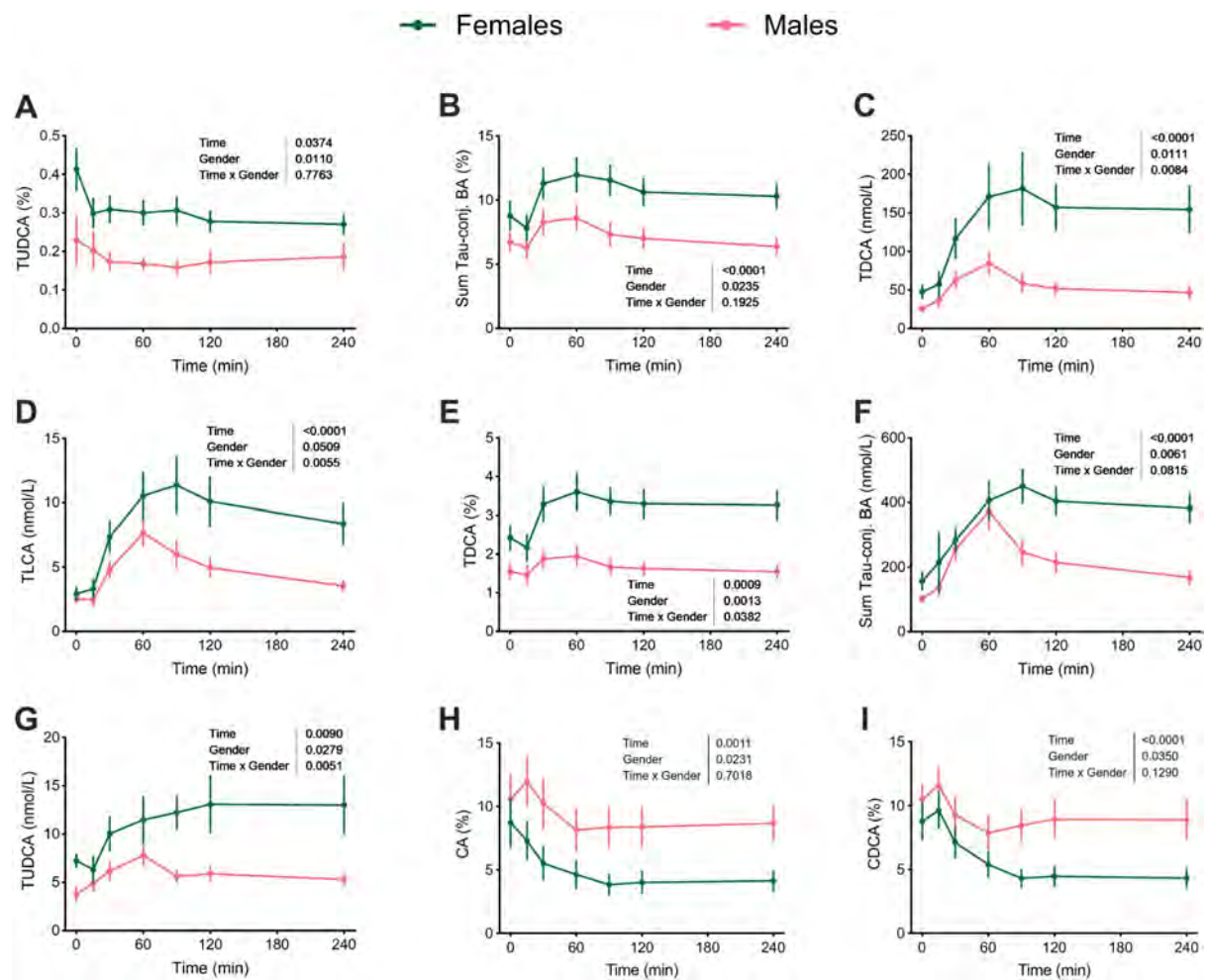


Fig. 3. Bile acid postprandial kinetics. A – G) Plasma concentrations of bile acids with higher concentration in females. H – I) Bile acids proportionally more abundant in the plasma of males. $n = 32$ females and 27 males. Data represent means and standard error of mean. Results from mixed-effects analyses indicated in each graph. CA = cholic acid; CDCA = chenodeoxycholic acid; TCDCA = taurochenodeoxycholic acid; TDCA = taurodeoxycholic acid; TLCA = tauroolithocholic acid; TUDCA = tauroursodeoxycholic acid; Tau-conj = bile acids conjugated to taurine.

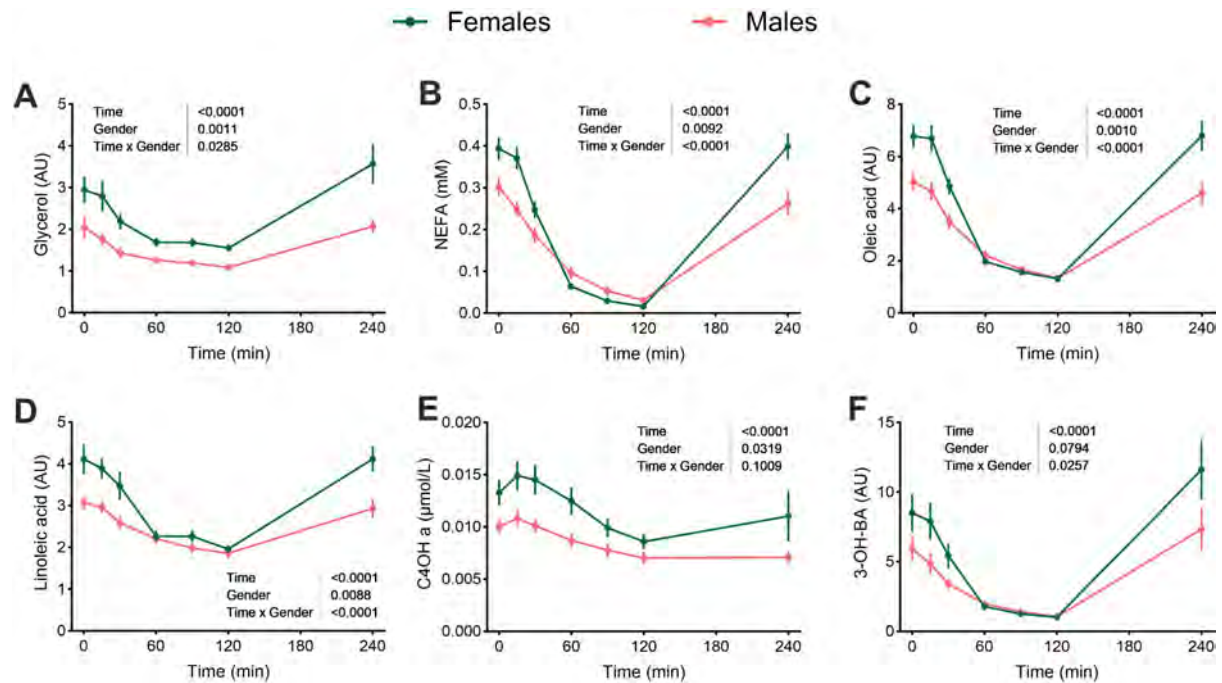


Fig. 4. Markers of lipid catabolism. A – D) Plasma levels of glycerol, non-esterified fatty acids, oleate, and linoleate represent markers of lipolysis. E) Plasma concentrations of hydroxy-butyrylcarnitine (C4-OH) and F) Plasma concentrations of 3-hydroxy-butyric acid are markers of fatty acid β -oxidation/ ketogenesis. $n = 32$ females and 27 males. Data represent means and standard error of mean. Results from mixed-effects analyses indicated in each graph. AU = arbitrary units from GC-MS qualitative measurements.

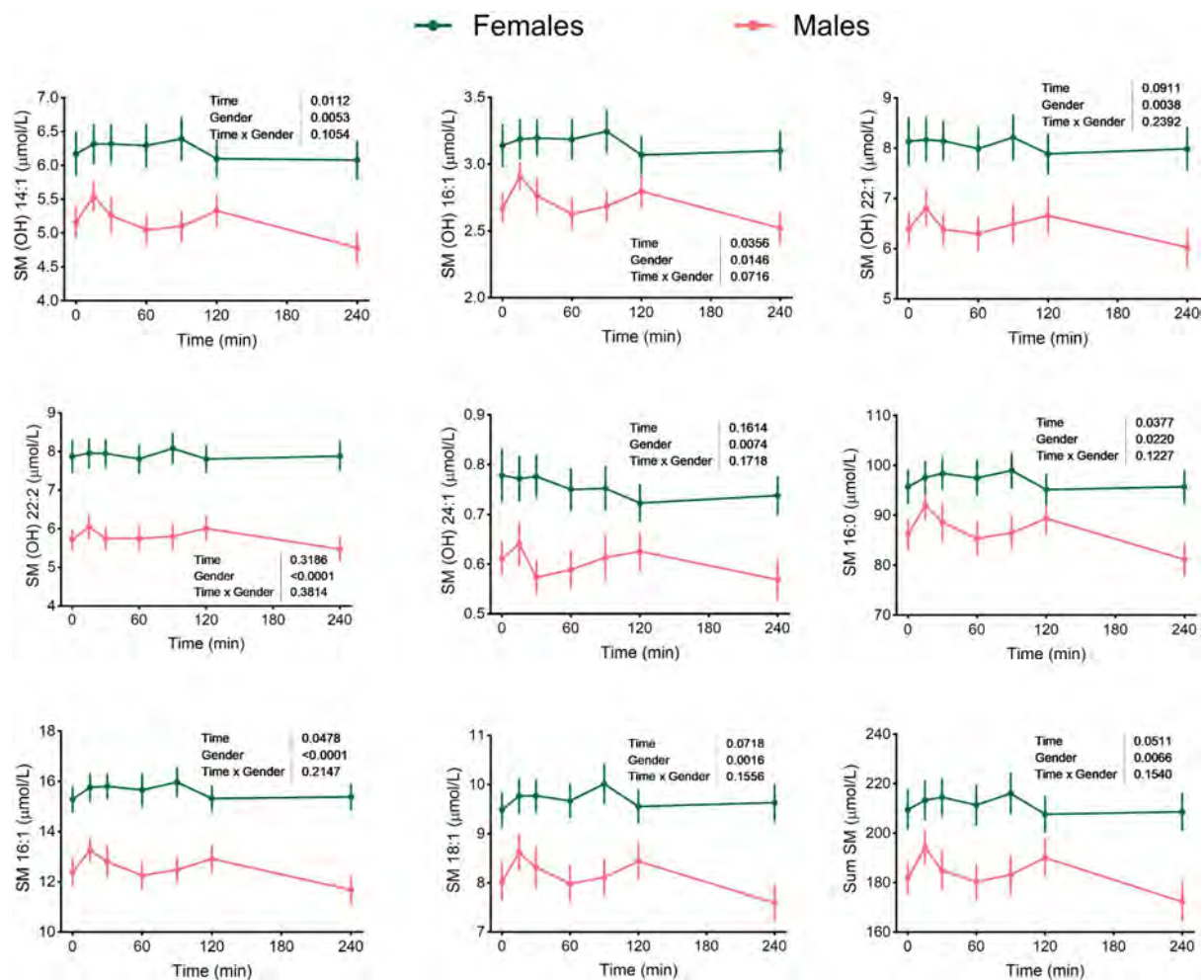


Fig. 5. Plasma levels of sphingomyelins during an OGTT. A - I) Some sphingomyelins plasma concentrations were higher in females. n = 32 females and 27 males. Data represent means and standard error of mean. Results from mixed-effects analyses indicated in each graph.

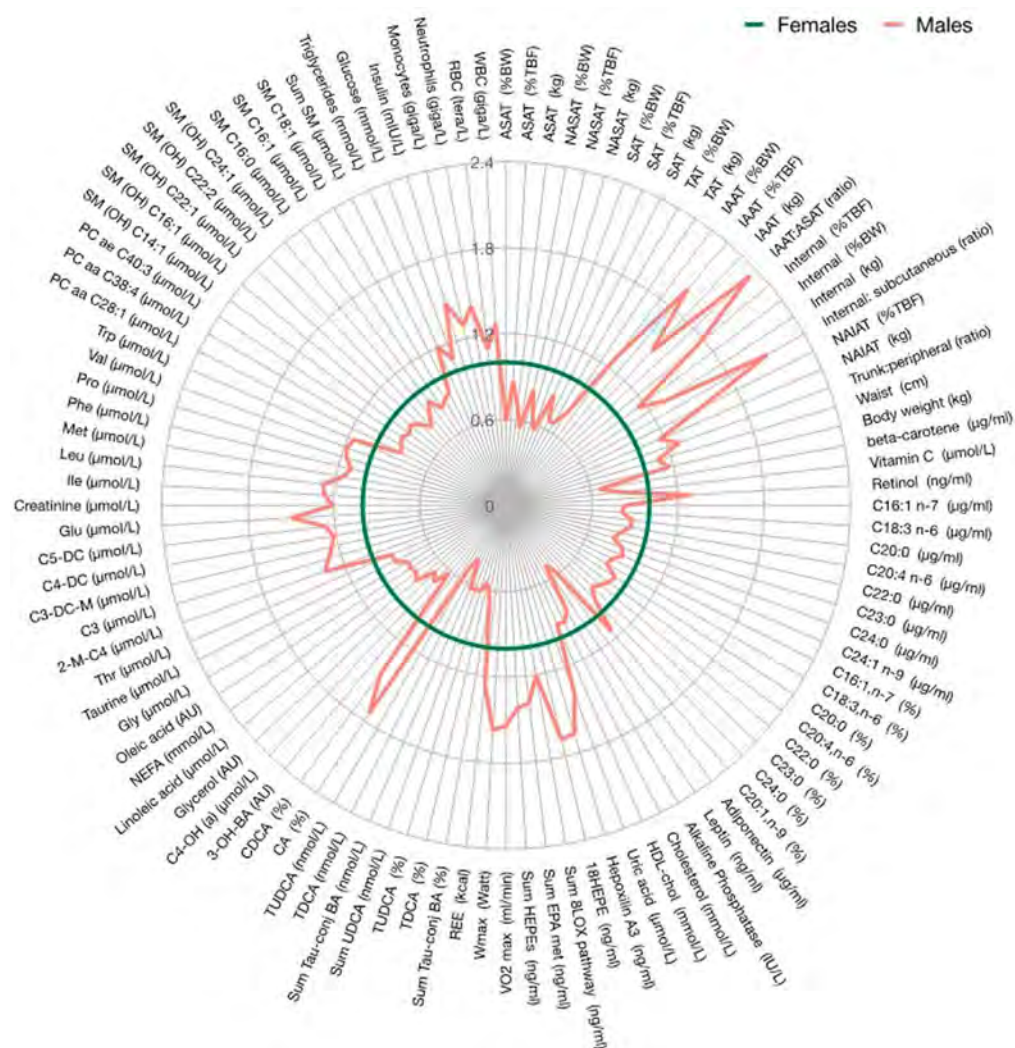


Fig. 6. Radar plot illustrating sex-specific differences in late middle-aged adults. The plot displays variables identified as discriminative between females and males by the PLS-DA model. Data are presented as standardized z-scores.