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Serum High-Performance Liquid Chromatography and metabolomics identify disrupted homeostasis of D- and L-amino acids as biochemical signature in Parkinson's disease and frailty syndrome

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1 ABSTRACT

Recent studies showed increased concentrations of the glutamatergic NMDA receptor co-agonist D-serine and its precursor L-serine in the striatum of Parkinson's disease (PD) animal models, the caudate-putamen of human PD brains and the cerebrospinal fluid of *de novo* PD patients. Moreover, a systemic dysregulation of amino acids metabolism has been described in frailty, a common clinical syndrome of the elderly associated with high risk of disability, mortality and increased incidence of neurodegenerative diseases. Here, I investigated the serum amino acids profile in two independent cohorts composed by (i) PD patients and age-matched healthy subjects (PD cohort); (ii) elderly subjects without neurodegenerative diseases including frail, pre-frail and non-frail individuals (frailty cohort). Frailty was assessed with the Edmonton Frail scale (EFS), a multidimensional tool including nine health items, and with Fried criteria, as a measure of physical frailty.

Two complementary analytical approaches were adopted: (i) High-Performance Liquid Chromatography (HPLC) focused on the amino acids acting on glutamatergic transmission (L-glutamate, L-aspartate, glycine, D-serine, and their precursors L-glutamine, L-asparagine, and L-serine); (ii) untargeted ^{1}H-Nuclear Magnetic Resonance (^{1}H-NMR) metabolomics, to obtain an unbiased overview on the serum metabolite profile in PD and frailty. An additional targeted ultraperformance liquid chromatography-mass spectrometry (UPLC/MS) metabolomic analysis was performed in the PD cohort.

The HPLC study showed that D-serine and D-/Total serine ratio positively correlated with age and age at disease onset in PD patients, while there were no correlations with age in healthy controls (HC). Moreover, I found that higher Levodopa Equivalent Daily Dose (LEDD) correlated with lower levels of D-serine and the other excitatory amino acids. Following these results, the addition of LEDD as covariate in the analyses disclosed a selective significant increase of D-serine in PD compared to HC ($\Delta \approx 38\%$). Stratification by sex revealed that this effect was specific of female patients. Untargeted serum metabolomics revealed higher glutamine, serine, pyruvate and lower α-ketoglutarate levels in PD compared to HC, while UPLC/MS analysis showed (i) lower glutamate, tryptophan and kynurenine levels and (ii) higher cystathionine, glycine and threonine levels in PD than HC.

In the frailty cohort, HPLC analyses showed higher serum D-serine and D-/Total serine ratio as independent predictors of worse EFS score, but not of physical frailty. Furthermore, higher glycine levels and D-/Total serine correlated with worse cognition and depressive symptoms in the frail (but not in the non-frail) group. Untargeted metabolomics revealed distinct alterations of amino acids, lipids and energy metabolism in frail and pre-frail subjects. Notably, upregulated levels of the atypical amino acid betaine emerged as a biochemical fingerprint of pre-frail subjects.

Overall, these findings suggest that (1) serum D-serine and D-/Total serine may represent a valuable biochemical signature of PD; (2) higher serum D-serine levels correlate with worse multidimensional frailty severity; (3) increased serum glycine and D-/Total serine ratio could be associated with cognitive decline and depression in frail older populations; (iii) serum metabolomics represents a promising source of novel amino acids signatures in PD and frailty. Future longitudinal studies evaluating whether serum D- and L-amino acids may help in identifying novel biological subtypes of PD and frailty are warranted.

2 INTRODUCTION

The pursuit of healthy longevity represents a major challenge for modern societies. Despite intense research efforts, the biological and environmental factors determining the healthspan of older adults remain largely elusive [1]. Among the causes of disability and morbidity in the elderly, neurodegenerative diseases and frailty play a crucial role. Neurodegenerative disorders (mainly Alzheimer's disease, AD, and Parkinson's disease, PD) are indeed responsible for a wide proportion of Disability-Adjusted Life Years (DALYs) and mortality worldwide according to the Global Burden of Disease estimates [2]. Moreover, frailty was estimated to be responsible for 19% of total DALYs for those aged ≥70 years in 2017 [3].

Several physiopathological mechanisms underlying frailty and neurodegenerative diseases have been proposed, including inflammation, oxidative stress, mitochondrial dysfunction, protein aggregation or loss-of-function, polygenic inheritance and exposure to pollutants [4–6]. Moreover, increasing evidence suggests that altered homeostasis of amino acids metabolism may affect healthy and diseased aging trajectories. Sarcopenia, the age-related loss of muscle mass and strength, is associated with altered blood concentrations of certain amino acids, including branched-chain amino acids and other essential amino acids, as well as glutamate, aspartate and serine [7–9]. Among these, variations in the metabolism of L-serine might play a key role in the physiopathology of frail aging and neurodegeneration. Besides contributing to protein synthesis, L-serine is the precursor of important substrates for the de novo synthesis of glutathione, an essential antioxidant molecule known to prevent some of the pathogenetic events leading to frailty and neurodegeneration. Moreover, in the brain, L-serine is converted in D-serine, an essential co-agonist of glutamatergic N-methyl-D aspartate receptors (NMDARs), which regulates synaptic plasticity and functioning and, in turn, is involved in age-dependent neurophysiological and cognitive deficits [10]. Furthermore, altered striatal NMDAR-dependent glutamatergic transmission was demonstrated in several PD experimental models [11]. Altogether, these experimental indications let believe that regulation of serine metabolism could represent a biomarker of both frailty and neurodegeneration. In this study, I will investigate this hypothesis by evaluating the serum levels of a selected pool of amino acids known to be involved in glutamatergic neurotransmission in PD patients and frail older subjects. Next, I will explore the global metabolic background of PD and frailty through state-of-the-art serum metabolomics approaches. I decided to focus on PD and not on AD patients for two reasons: first, serum levels of D-serine were already investigated in patients with AD [12,13], but data in PD patients are scarce; second, I believe that PD phenotype better represents the pathological evolution of frailty, as both conditions are characterized by a "physical" component (bradykinesia, exhaustion,

sarcopenia) and a variable "cognitive" and "psychiatric" component (cognitive impairment, depression, apathy, anxiety, abulia) [14].

3 AIMS

Main objective of the present study is to investigate the serum amino acids and metabolomic profile of two common pathological conditions of the elderly, i.e. PD and frailty. The project was based on the availability of two independent and well-characterized cohorts of individuals including (i) sporadic PD patients and healthy controls with similar age ("PD cohort"); (ii) older subjects without neurodegenerative diseases encompassing the entire continuum from fitness to frailty ("frailty cohort"). Specifically, I addressed this research question through the following approaches:

1) Determination of the serum levels of a selected pool of D- and L-amino acids which are known to modulate the activation of glutamatergic NMDA receptors, including L-glutamate, L-aspartate, glycine, and D-serine, as well as their precursors L-glutamine, L-asparagine, and L-serine in both PD and frailty cohorts.
2) Untargeted (^{1}H-NMR) and targeted (UPLC/MS) serum metabolomics characterization of the PD cohort.
3) Untargeted serum metabolomics profiling of the frailty cohort.

This dissertation is divided in two main sections: the first dedicated to PD, the second to frailty. In each section, I will first provide a brief introduction of the clinical condition of interest and the current knowledge regarding blood amino acids profiling in PD (Chapter 4) and frailty (Chapter 5). Next, I will dive into the methods and results obtained in this study.

4 PARKINSON'S DISEASE COHORT

4.1 Introduction

4.1.1 Epidemiology

PD is an age-related disease, with incidence and prevalence increasing steadily with age [15]. However, the misconception that PD exclusively affects older people should be dismissed. The age of onset for almost 25% of affected individuals is younger than 65 years and for 5–10% is younger than 50 years. The global prevalence of PD is approximately 0.3% in the general population aged 40

and older, while incidence ranges from 8 to 18.6 per 100,000 person-years [15]. The global burden of PD - in terms of deaths and disability- has more than doubled in the past two decades [2]. Although PD affects both sexes, women show slightly lower incidence and higher age at onset [15].

4.1.2 Risk factors and pathogenesis

Three factors are relevant to the etiopathogenesis of PD: genetics, environment, and interactions thereof (Fig. 1) [16]. Genetic factors play a relevant role particularly when the age at symptoms onset is younger than 50 years [17]. To date, the most interest is focused on mutations in the genes *SNCA, LRRK2, PRKN, PINK1* and *GBA1*. While *GBA1* variants and mutations can be considered as a risk factor to develop PD, point mutations or rearrangements in the first four genes cause monogenetic forms of PD. The latter account for 1.4% of all PD cases and approximately 3% of young-onset PD [18]. *GBA1* encodes the lysosomal hydrolase glucocerebrosidase. In the homozygous state, *GBA1* mutations cause Gaucher disease, which leads to glucosylceramide accumulation in visceral organs and, in a minority of cases, the central nervous system (CNS) [19]. Heterozygous *GBA1* mutations are the most significant genetic risk factor for PD [20]; however, penetrance is only 10%–30% [21].

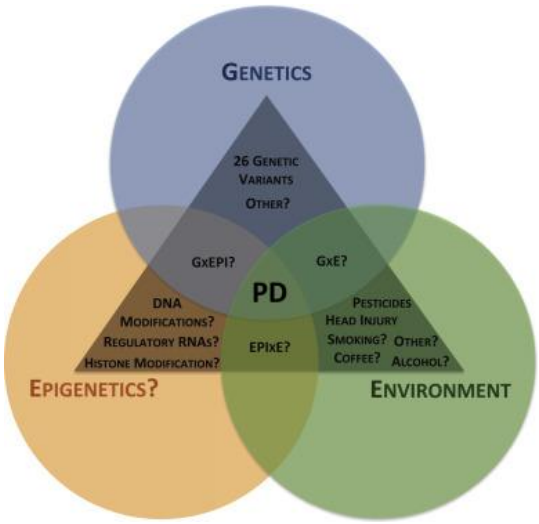

Figure 1. Schematic overview of the interplay of genetic, environmental and epigenetic domains possibly underlying PD pathophysiology. Each domain (i.e. genetics, environment/lifestyle, and epigenetics) is symbolized by a circle. The result of the action and interaction (indicated by the overlaps of circles) of these three domains is the development of PD, symbolized by the triangle. Question marks indicate fields with incomplete or missing knowledge. From: Lill CM. Mol Cell Probes. 2016 Dec;30(6):386–96.

PD is characterized by accelerated neuronal death of primarily dopaminergic neurons of the substantia nigra pars compacta (SNpc), but the neuropathology involves multiple other motor and non-motor circuits. Loss of SNpc dopaminergic neurons appears to result from the complex interplay of dysfunction of mitochondria, lysosomes or vesicle transport, synaptic transport issues, and neuroinflammation [22]. Pathologically, PD is defined by the accumulation of misfolded α-syn in Lewy bodies. A recent insight is that even in early disease stages, similar pathological changes can occur in multiple organs, including the skin, colon, and salivary glands, suggesting that PD is a multi-system disease [23]. Despite the aggregation of the pre-synaptic protein α-synuclein in basal ganglia and other brain regions has been so far considered "pathogenic" and "toxic", it should be acknowledged that (i) most brains from people without neurological symptoms present with Lewy bodies at autopsy; (ii) neither pathology nor distribution of α-synuclein aggregates correlate with disease severity or progression in PD [24]; (iii) low cerebrospinal fluid (CSF) α-synuclein levels

predict brain atrophy and clinical progression [25]. This evidence suggests that it may be not α-synuclein aggregation or its "gain of toxic function" to be actually detrimental, but the loss of monomeric α-synuclein, i.e. its loss-of-function, to drive PD pathogenesis [5].

4.1.3 Alteration of the glutamatergic signalling in PD

Along PD progression and dopaminergic treatment, glutamatergic signalling from the cortex to the striatum undergoes a significant modulation. In PD, the degeneration of nigrostriatal dopaminergic neurons leads to complex alterations of the basal ganglia pathways involving also impaired synaptic plasticity of striatal spiny projecting neurons (SPNs), the most represented neurons in the striatum [26]. Indeed, dopaminergic terminals from the SNpc and the glutamatergic input from the cortex converge onto SPNs dendritic spines and integrate to allow a proper motor behavior (Fig. 2).

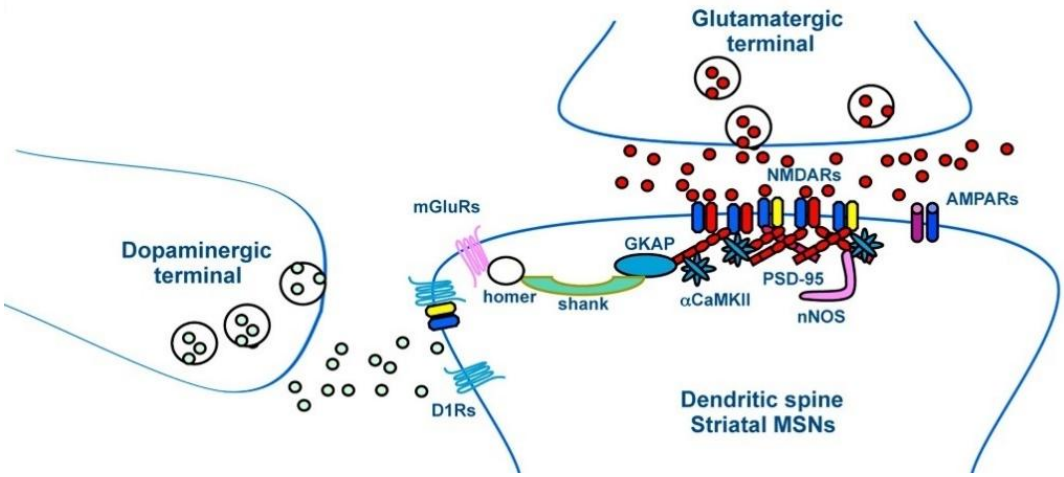

Figure 2. Schematic representation of the cortical glutamatergic and dopaminergic terminals converging on dendritic spine of the striatal SPNs in PD. MSNs, medium spiny neurons (currently named spiny projecting neurons). Adapted from: Mellone M, Gardoni F. Eur J Pharmacol. 2013 Nov;719(1–3):75–83.

Striatal dopamine depletion leads to changes in subcellular localization and activity of postsynaptic glutamate receptors, eventually causing loss of striatal plasticity [27]. Multiple studies mainly on 6-OHDA rodent and primate PD models indicate that different extent of dopaminergic denervation cause specific alterations of the glutamate receptor NMDAR [28]. NMDARs are heterotetrameric ionotropic receptors made up by combination of two or three different subunits (GluN1, GluN2 and GluN3). Their ability to mediate influx of Ca2+, a second messenger that elicits molecular and biochemical changes in the postsynaptic neurons, is the primary reason for their involvement in synaptic plasticity but also in excitotoxic neuronal death [26]. NMDAR activation depends on the

binding with its canonical agonist neurotransmitters, like L-glutamate and L-aspartate, but is also modulated by the binding of its obligatory co-agonists glycine and D-serine at glycine allosteric site (Fig. 3). Under physiological condition, D-serine is synthetized in neurons starting from L-serine by the enzyme serine racemase (SR), while its degradation occurs in astrocytes [29] through an oxidative deamination catalyzed by D-amino acid oxidase (DAAO) [30]. Thus, the D-/Total serine ratio represents a reliable index of D-serine metabolism [31].

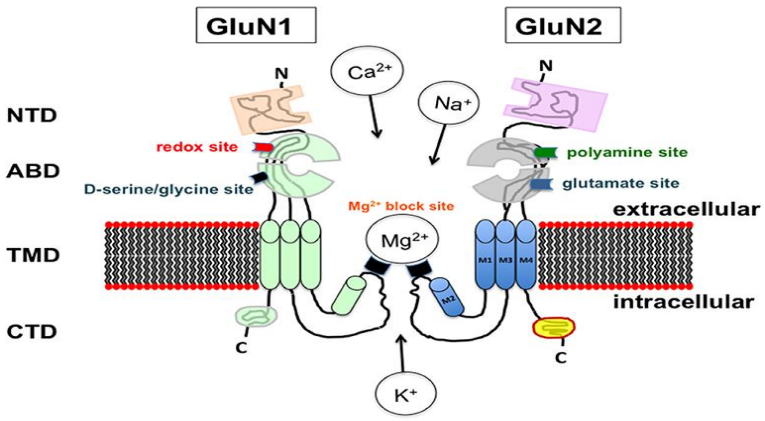

Figure 3. Schematic representation of the assembly and modular organization of NMDA receptor. The extracellular segment includes the N-terminal domain (NTD) and the agonist binding domain (ABD) where D-serine/glycine and glutamate bind to the GluN1 and GluN2 subunit respectively. The ion channel is localized in the transmembrane domain (TMD) that contains the site for the magnesium blockade while the C-terminal domain (CTD) is included in the intracellular segment. Adapted from: Billard J-M. Front. Mol. Biosci. 2018; 5:106.

4.1.4 D-serine involvement in PD

The involvement of D-serine as a key modulator of glutamatergic neurotransmission has recently gained attention in several neurological conditions, including AD, frontotemporal dementia (FTD), amyotrophic lateral sclerosis (ALS) and PD [32,33]. Specifically, in PD a few studies showed that the stimulation of NMDAR at glycine modulatory site promotes striatal dopaminergic reinnervation and can rescue motor dysfunction in different PD animal models [34–36]. In line with these pre-clinical findings, a preliminary randomized controlled clinical trial of adjuvant oral D-serine treatment in a small cohort of idiopathic PD patients showed improvement of both motor and non-motor symptoms compared to placebo, suggesting that D-serine may have neuroprotective effects in PD [33,37].

Despite several studies investigated CSF and blood levels of a wide spectrum of amino acids in PD (reviewed in [38]), data regarding D-serine levels in PD patients have been rarely documented [39]. More recently, Usiello and colleagues reported increased D-serine and L-serine levels in (i) the rostral putamen of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated monkeys [40], (ii) post-mortem striatum of human PD brains, and (iii) the CSF of *de novo* living PD patients compared to patients with other neurodegenerative diseases and neurological controls [41].

These findings collectively suggested that the homeostasis of serine enantiomers is disrupted in the caudate-putamen of PD patients, and paved the way for the investigation of D-serine and L-serine as putative PD biomarkers.

4.1.5 Blood and CSF amino acids as potential biomarkers of PD

A recent meta-analysis summarized the findings obtained by previous studies evaluating CSF and blood levels of L-amino acids in PD [38]. This work showed a significant decrease of CSF glutamate levels and a significant increase of CSF threonine and tyrosine levels in PD patients compared to healthy controls (HC). The meta-analysis of all studies performed on the serum or plasma showed decreased aspartate, serine, tryptophan and lysine and increased proline and homocysteine levels in PD patients compared to HC. Plasma glutamate and tyrosine levels were similar in PD patients and HC. No previous studies evaluated D-serine or other D-amino acids concentration in the blood of PD patients.

4.2 Methods

4.2.1 Participants

Consecutive patients with a clinical diagnosis of PD [42] with disease duration of at least one year and sustained dopaminergic treatment response were consecutively recruited at IRCCS Mondino Foundation, Pavia, Italy between January 2019 and December 2021. HC were selected among patients' caregivers and were subjected to a complete neurological examination and cognitive screening (Mini-Mental State Examination, MMSE) to exclude parkinsonian sign and cognitive impairment, respectively. This study was approved by the local ethics committee (protocol 20180097520, 09/11/2018) and was in conformity with the Helsinki Declaration. Written informed consent was obtained from all participants. All procedures were performed in compliance with relevant laws and institutional guidelines.

All PD patients underwent (i) brain magnetic resonance imaging in order to exclude prominent cortical/subcortical infarcts, cerebral small vessel disease or atypical signs (such as midbrain, cortical or cerebellar atrophy) indicating atypical parkinsonism; (ii) ^{123}I-FP-CIT SPECT imaging to confirm nigrostriatal dopaminergic degeneration. Each patient underwent a standardized neurological examination including the motor assessment with the Movement Disorders Society Unified Parkinson Disease Rating Scale part III (MDS-UPDRS-III), global cognition (MMSE) and PD-related QoL

measured with the 39-Item Parkinson's Disease Questionnaire (PDQ39) [43]. Levo-dopa equivalent daily dose (LEDD) was also calculated at baseline according to last proposed conversion factors [44]. Patients presenting with dementia according to current PD dementia criteria [45] were excluded from the study. The following exclusion criteria were also applied: (1) Hoehn and Yahr stage > 3 [46]; (2) atypical parkinsonism including corticobasal syndrome (CBS), progressive supranuclear palsy (PSP), multiple system atrophy (MSA), dementia with Lewy bodies (DLB); (3) any systemic condition potentially affecting serum amino acid levels, including kidney, liver, rheumatologic and neoplastic diseases, history of drug or alcohol abuse; (4) history of altered serum creatinine levels (> 1.2 mg/dl) or liver function parameters (aspartate transaminase or alanine transaminase > 50 U/l).

4.2.2 Collection and storage of serum samples

Blood sampling was performed after a 12-hour fasting and antiparkinsonian treatment washout period. Whole blood was collected by peripheral venipuncture into clot activator tubes and gently mixed. Sample was stored upright for 30 min at room temperature to allow blood to clot, and centrifuged at 2000 × g for 10 min at room temperature. Serum was aliquoted (0.5 ml) in polypropylene cryotubes and stored at –80 °C before usage.

4.2.3 HPLC analysis of amino acids content

Serum samples (100 µl) were mixed in a 1:10 dilution with HPLC-grade methanol (900 µl) and centrifuged at 13,000xg for 10 min; supernatants were dried and then suspended in 0.2 M trichloroacetic acid (TCA). TCA supernatants were then neutralized with 0.2 M NaOH and subjected to precolumn derivatization with o-phthaldialdehyde /N-acetyl-L-cysteine in 50% methanol. Amino acids derivatives were resolved on a UHPLC Nexera X3 system (Shimadzu) by using a Shim-pack GIST C18 3-µm reversed-phase column (Shimadzu, 4.0x150 mm) under isocratic conditions (0.1 M sodium acetate buffer, pH 6.2, 1% tetrahydrofuran, and 1 ml/min flow rate). A washing step in 0.1 M sodium acetate buffer, 3% tetrahydrofuran and 47% acetonitrile, was performed after every run. Identification and quantification of amino acids were based on retention times and peak areas, compared with those associated with external standards. The detected amino acids concentration was expressed as µM.

4.2.4 HPLC statistical analysis

Clinical and demographic characteristics were described using, as summary statistics, median and the interquartile range (IQR) or absolute and relative frequencies; while comparisons between PD patients and HC were evaluated using Mann Whitney U test for continuous variables and Chi-Square test for dichotomous variables. Comparison of serum amino acid levels between PD and HC was first performed using a two-way independent ANCOVA model with "diagnosis" and "sex" as between factors and "age" as covariate. Second, LEDD was added as covariate to the ANCOVA model to control for the effect of antiparkinsonian treatment on serum amino acid levels. The estimated group means of amino acid levels adjusted for the effect of age, sex and LEDD were then extracted from the ANCOVA model and reported in Table 5.

The correlation of serum amino acid concentration with age and age at PD onset was evaluated with Spearman's correlation test. Partial correlation analyses adjusted for the effect of potential confounders (age, sex, disease duration, LEDD) were adopted to test the correlation between serum amino acid levels and PD clinical features. The correlation between LEDD and disease duration was evaluated with Spearman's correlation test. Significance was set at $p < 0.05$ for all analyses. Data were analysed by using SPSS 26.0 software (IBM, Armonk, NY, USA).

4.2.5 Metabolomic serum analyses

4.2.5.1 Samples preparation

Blood sampling was performed after an overnight 12-h fasting and antiparkinsonian treatment washout period. Serum was collected according to Standard Operating Procedure (SOP)[47] to perform NMR-based metabolomic analysis. In order to remove excess proteins, the serum was filtered using Amicon Ultra-0.5 3000 MWCO pre-rinsed (washed 7 times) at 4 °C using a centrifuge (force 12,000 g). Before NMR spectroscopy measurements, the blood serum was aliquoted and stored at −80°C in Greiner cryogenic vials[47]. NMR samples were prepared by adding 250 µL of phosphate buffer to 250 µL of filtered sera, including 0.075 M Na_2HPO_4 x7 H_2O, 4% NaN_3, and H_2O. Trimethylsilyl propionic-2,2,3,3-d_4 acid, sodium salt (TSP 0.1% in D_2O) was used as an internal reference for the alignment and quantification of NMR signals; the mixture, homogenized by vortexing for 30 sec, was transferred to a 5 mm NMR tube (Bruker NMR tubes) before acquisition[47].

4.2.5.2 NMR data acquisition, processing and assignment

NMR experiments were acquired for all samples on a Bruker Ascend™ 600 MHz spectrometer equipped with a 5 mm triple resonance Z gradient TXI probe (Bruker Co, Rheinstetten, Germany) at 298 K. TopSpin, version 3.2 was used for the spectrometer control and data processing (Bruker Biospin). CPMG (Carr-Purcell–Meiboom–Gill) experiments were performed on serum samples and acquired using 20 ppm spectral width, 32 k data points, with f1 presaturation and T2 filter using D20 of 300 μsec, D1 of 4 sec. A weighted Fourier transform was applied to the time domain data with a line widening of 0.5 Hz followed by a manual step and baseline correction in preparation for targeted profiling analysis. Assignment of ^{1}H-NMR signals performed with Chenomix software[48] on 1D ^{1}H CPMG NMR spectra detected the presence of 45 metabolites (Fig. 2A in the Appendix). The quantification (concentrations in μM) was carried out using automated ASICS[49].

4.2.5.3 UPLC/MS methods

The concentrations of a panel of 44 amino acids and derivatives were measured on serum samples by UPLC/MS. The panel includes: 1-Methylhistidine, 3-Methylhistidine, 4-Hydroxyproline, α-Aminobutyric acid, β-Alanine, β-Aminobutyric acid, γ-Aminobutyric acid, Alanine, Allo-Isoleucine, Aminoadipic acid, Anserine, Arginine, Asparagine, Aspartic acid, Carnosine, Citrulline, Cystathionine, Cystine, Ethanolamine, Glutamic acid, Glutamine, Glycine, Glycil proline, Histidine, Homocitrulline, Homocysteine, Hydroxylysine, Isoleucine, Kynurenine, Leucine, Lysine, Methionine, Ornithine, Phenylalanine, Phosphoethanolamine, Proline, Sarcosine, Serine, Sulfocysteine, Taurine, Threonine, Tryptophan, Tyrosine, Valine. Briefly, 50 μL of the sample were added to 100 μL 10% (w/v) sulfosalicylic acid containing an internal standard mix (50 μM) (Cambridge Isotope Laboratories, Inc., Tewksbury, MA, USA). The mixture was centrifuged at 10,000 rpm for 15 min. 70 μL of borate buffer and 20 μL of AccQ Tag reagents (Waters Corporation, Milford, MA, USA) were added to 10 μL of the obtained supernatant and heated at 55 °C for 10 min. Next, samples were loaded onto a CORTECS UPLC C18 column 1.6 μm, 2.1 mm x 150 mm (Waters Corporation) for chromatographic separation (ACQUITY H-Class, Waters Corporation). Elution was accomplished at 0.5 mL/min flow-rate with a linear gradient (9 min) from 99:1 to 1:99 water 0.1% formic acid/acetonitrile 0.1% formic acid. Analytes were detected on an ACQUITY QDa single quadrupole mass spectrometer equipped with an electrospray source operating in positive mode (Waters Corporation).

The method includes a pre-column derivatization step using AccQ Tag, a reagent functionally specific for amino acids. It reacts with amino functional groups, improving ionization efficiency, enhancing chromatographic performance, and reducing matrix effects. This chemical modification, combined

with optimized liquid chromatography conditions, allows for the complete resolution of isobaric and isomeric compounds, such as leucine and isoleucine, enabling their confident quantification even in a Selected Ion Recording (SIR) mode using a single quadrupole MS system. This approach ensures excellent analytical sensitivity, specificity, and reproducibility.

Serum amino acid concentrations were determined by comparison with values obtained from a standard curve for each amino acid (2.5–10–50–125–250–500 µmol/L for all analytes, and 5–20–100–250–500–1000 µmol/L for cysteine), using isotopically labeled internal standards for the majority of the amino acids, including isomeric species. The quantification was based on the ratio between the analyte signal and the corresponding internal standard signal (AA/IS), allowing for both accurate measurement and compensation for potential matrix effects.

The analytical process was monitored using amino acid controls (level 1 and level 2) manufactured by the MCA laboratory of the Queen Beatrix Hospital (The Netherlands).

Calibration curves and data processing were performed using the TargetLynx software (Waters Corporation).

4.2.5.4 ^{1}H-NMR and UPLC/MS statistical analysis

Before statistical and bioinformatic analyses, data obtained from mass spectrometry were processed to construct the final dataset. The following condition was applied for variable (amino acid) inclusion: variables that were not detected or had more than 50% missing values were excluded. Based on this threshold, 36 variables were selected, and the missing values were imputed using the PPCA method in Metaboanalyst 6.0.

The data matrices were normalised prior to the application of the biostatistical method. Specifically, a logarithmic transformation was applied to the NMR data matrix, and an autoscaling normalization was applied to UPLC/MS data. A univariate analysis was performed on both the LC-MS and NMR data matrices utilising a Robust Volcano plot determined using the FC, calculated as the ratio of PD/HC, with a threshold set at FC=1. Volcano plots were made using the FC= 1 and p-value < 0.1 as thresholds using MetaboAnalyst 6.0.

Multivariate classification model based on PLS–DA was built on both UPLC/MS and NMR data to define similarities and differences between the PD and HC groups. PLS-DA analysis was conducted using the mixOmics (6.30.0) R package. VIP scores > 1 were represented as dot plots generated using ggplot2, dplyr, and tidyr. The best number of components was calculated using a 10-fold cross-validation to minimize model's error. Model performance was validated using a 10-fold cross-

validation with 50 iterations. VIP scores were calculated to assess the importance of each variable to class separation.

The validation of the supervised models involved calculating the area under the curve and assessing the error rate through Balanced Error Rate (BER) and overall error (OVERALL) metrics computed on the first and second components using maximum, centroid, and Mahalanobis distance[50]. To provide an intuitive view of the data, we performed heatmaps using normalized data, average group concentration, Euclidian distance and Ward method[51]. Biomarker analysis was carried out by analysing the univariate ROC curve to calculate AUC and its 95% confidence intervals (500 bootstrap cycles methods)[52]. The analysis of the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways was carried out using Enrichment tool of MetaboAnalyst. Biochemical pathways with FDR-adjusted p-values below 0.05 and a hit value (i.e., the number of metabolites in the pathways) exceeding 1 were taken into account.

The correlation between the UPLC/MS metabolites showing VIP score > 1.0 and PD patients' clinical-demographic features was plotted as a correlation matrix showing Pearson's correlation coefficients and p-values obtained using the MATLAB Statistics and Machine Learning Toolbox (R2024a version, MathWorks Inc., Natick, MA, USA) and MATLAB corrcoef function (R2024b). Data were previously subjected to average normalization. Scatterplots of significative correlations were executed using MATLAB. Original and normalized (log – transformation) abundances were displayed.

4.3 Results

4.3.1 HPLC assessment of serum L- and D-amino acids concentration

Eighty-three consecutive PD patients and forty-one HC were enrolled in the study. Demographic and clinical features of participants are reported in Table 1. PD and HC were similar for age and global cognition (MMSE score), while PD group had a lower prevalence of females than HC.

	HC (n = 41)	PD (n = 83)	p
Age, years	71.4 (67.0-74.0)	72.0 (68.0-75.0)	0.339[a]
Female sex, n (%)	26 (63.4)	36 (43.4)	0.036[b]
Age at onset, years	-	66.0 (61.0-71.0)	-
Disease duration, years	-	6.0 (3.0-8.0)	-
MDS-UPDRS-III	-	24.0 (18.0-31.0)	-
LEDD, mg/day	-	550 (400-849)	-
MMSE	27.0 (25.9-30.0)	26.7 (25.3-30.0)	0.122[c]
PDQ-39 summary index	-	10.6 (6.8-20.4)	-

Table 1. Clinical and demographic features of PD and HC groups. Data are shown as median (IQR) for continuous variables and frequency (percentage) for categorical variables. Abbreviations: LEDD, Levodopa equivalent daily dose; MDS-UPDRS-III, Movement Disorders Society Unified Parkinson's Disease Rating Scale, part III; MMSE, Mini-mental State Examination; PDQ-39, Parkinson's disease Questionnaire 39. [a] Mann-Whitney U test; [b] Chi-square test; [c] two-way ANCOVA with group and sex as factors, age as covariate.

4.3.1.1 Basal serum serine enantiomers levels do not differ between PD patients and healthy controls

I first investigated whether the serum levels of amino acids acting on NMDAR neurotransmission are dysregulated in PD compared to HC. A representative HPLC chromatogram illustrating the peaks of the amino acids obtained from a serum sample is shown as Fig. 1A in the Appendix. Age- and sex-adjusted ANCOVA showed no between-group differences in D-serine, L-serine or any of the other amino acids level, except for a decrease of L-Glu in PD compared to HC (Table 2). Similarly, there were no differences in serum amino acid levels after stratification by sex of the two cohorts, except for a slight decrease of L-Glu in PD females compared to HC females and a mild increase of Gly in PD females compared to PD males (Table 2).

Considering the increased levels of D- and L-serine that I recently found in the post-mortem human PD brains and CSF of living PD patients, this finding implies that the concentration of serine enantiomers could be differently modulated between the CNS and periphery [40,41], and prompted us to investigate whether demographic and clinical factors may affect the serum levels of NMDAR-related amino acids.

	HC all (n = 41)	PD all (n = 83)	p[a]	HC females (n = 26)	HC males (n = 15)	PD females (n = 36)	PD males (n = 47)	p[b]	p[c]	p[d]	p[e]
L-aspartate (µM)	3.1 (1.8-4.2)	2.5 (1.6-3.7)	0.116	3.2 (2.1-4.8)	2.5 (1.3-4.2)	2.6 (1.9-3.6)	2.3 (1.4-3.6)	0.107	0.457	0.267	0.502
L-asparagine (µM)	19.4 (13.1-24.8)	18.1 (13.2-23.8)	0.822	13.5 (20.0-24.7)	17.8 (11.2-24.8)	16.8 (12.5-21.7)	19.7 (14.5-25.4)	0.209	0.550	0.803	0.108
Glycine (µM)	151.1 (114.7-205.1)	179.1 (134.8-223.1)	0.788	152.2 (120.3-251.0)	144.5 (103.7-198.1)	200.5 (165.9-246.8)	157.8 (121.5-196.9)	0.781	0.354	0.235	**0.036**
D-serine (µM)	1.2 (0.8-1.6)	1.3 (0.8-1.7)	0.856	1.1 (0.8-1.5)	1.3 (0.6-1.8)	1.2 (0.7-1.9)	1.5 (0.8-1.7)	0.919	0.946	0.988	0.694
L-serine (µM)	56.1 (43.8-71.5)	56.4 (40.4-67.6)	0.841	60.1 (45.1-75.4)	53.7 (34.6-59.8)	57.4 (44.2-66.7)	54.9 (39.2-71.9)	0.181	0.438	0.267	0.917
D-/Total serine (%)	2.0 (1.4-2.5)	2.3 (1.6-2.9)	0.861	1.8 (1.4-2.4)	2.3 (1.9-2.6)	1.9 (1.4-3.0)	2.4 (1.9-2.9)	0.511	0.754	0.441	0.850
L-glutamate (µM)	23.3 (13.3-30.4)	17.1 (14.1-26.2)	**0.020**	22.0 (12.8-29.8)	27.0 (13.5-34.6)	16.8 (13.2-22.1)	14.6 (17.7-28.7)	**0.048**	0.137	0.473	0.146
L-glutamine (µM)	255.8 (207.7-352.8)	259.7 (196.8-345.4)	0.719	255.4 (208.7-352.7)	262.8 (157.1-359.4)	233.1 (197.6-323.0)	277.9 (196.7-350.1)	0.293	0.741	0.890	0.230
L-glutamine/L-glutamate	11.5 (8.6-15.9)	13.3 (10.8-17.2)	0.093	13.2 (9.5-17.2)	9.7 (7.2-14.2)	13.9 (10.7-17.1)	13.3 (10.8-17.4)	0.743	0.085	0.480	0.729

Table 2. Serum levels of neuroactive amino acids in PD and HC considered (i) as whole groups and (ii) after stratifying by sex. Data are shown as median (IQR) of amino acids concentration. Significant p-values are shown in bold.

[a] PD (all) compared to HC (all). Two-way ANCOVA with diagnosis and sex as factors; age as covariate

[b] PD females compared to HC females. One-way ANCOVA with diagnosis as factor; age as covariate

[c] PD males compared to HC males. One-way ANCOVA with diagnosis as factor; age as covariate

[d] HC males compared to HC females. One-way ANCOVA with sex as factor and age as covariate

[e] PD males compared to PD females. One-way ANCOVA with sex as factor; age and disease duration as covariates

4.3.1.2 D-serine metabolism correlates with age and age at onset in PD

Results of all comparisons between serum amino acid levels and demographic and clinical features in the two groups are summarized in Table 3. Interestingly, D-serine and D-/Total serine positively correlated with age in PD patients, but not in HC. The sex stratification of PD cohort revealed that these findings were driven by female sex (D-serine: $r = 0.382$, $p = 0.021$; D-/Total serine: $r = 0.412$, $p = 0.013$), since no significant correlation between age and D-serine or D-/Total serine was found in PD males ($r = 0.231$, $p = 0.118$ and $r = 0.152$, $p = 0.306$, respectively). Moreover, no significant correlations with age was found for L-serine and all the other tested amino acids, in either HC or PD group (Fig. 5, Table 3).

Of note, D-serine and D-/Total serine, but not L-serine or other tested amino acids, correlated positively also with PD age at onset (Fig. 6). After sex stratification, I found that D-serine correlated with age at onset both in PD females and males, while D-/Total serine correlated with age at onset in PD females but not in males (Table 4).

Taken together, these results imply a specific relationship linking increased serum content of D-serine, but not L-serine or the other NMDAR-stimulating amino acids, with aging and age at onset in PD, and suggest that PD physiopathological mechanisms modulate peripheral D-serine levels in a sex- and age-dependent manner.

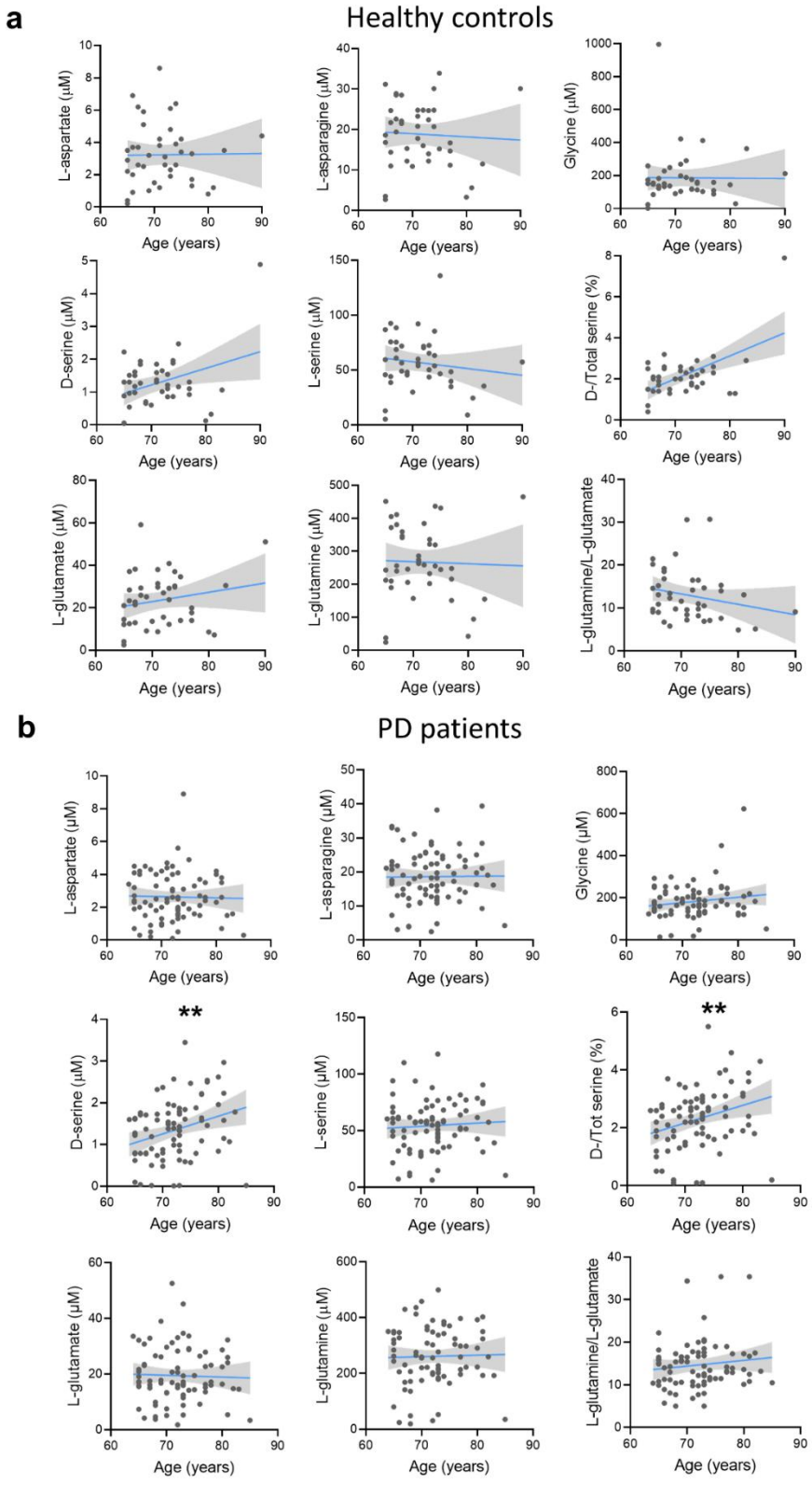

Figure 5. Scatterplots showing correlations between age and the serum concentration of the NMDAR-related amino acids in (a) HC and (b) PD groups. Blue lines and grey shadows represent the linear regression best fit line and its 95% CI, respectively. ** $p < 0.01$, Spearman's correlation test.

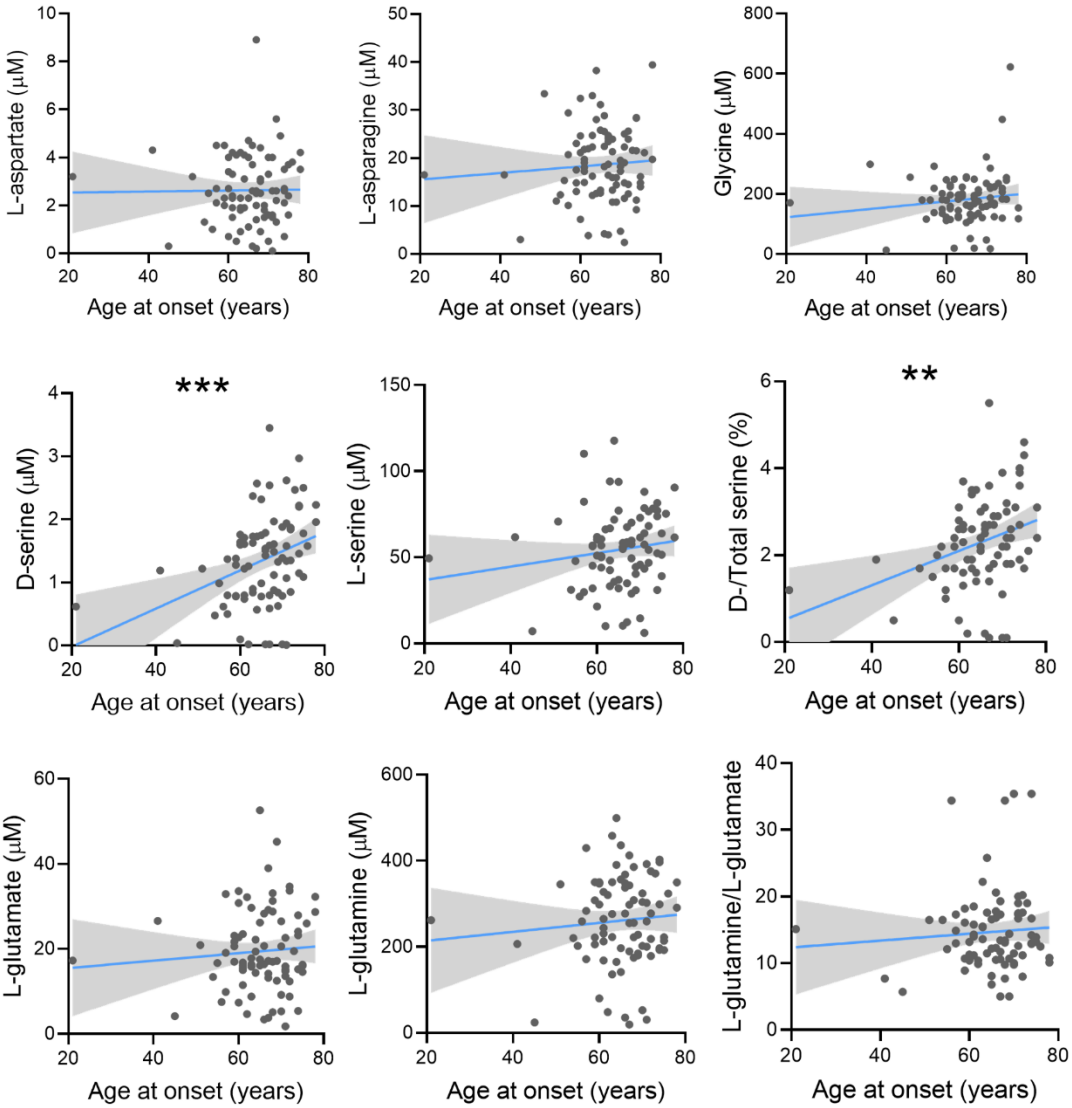

Figure 6. Scatterplots representing correlations between age at onset and serum neuroactive amino acid levels in PD group. **p < 0.01; ***p < 0.001, Spearman's correlation. Blue lines and grey shadows represent the linear regression best fit line and its 95% CI, respectively.

	Age				Age at onset		Disease duration		LEDD	
	PD		HC		PD		PD		PD	
	r	p[a]	r	p[a]	r	p[a]	r	p[b]	r	p[c]
L-aspartate (µM)	-0.023	0.835	0.083	0.607	0.013	0.906	-0.052	0.646	-0.259	**0.020**
L-asparagine (µM)	0.010	0.928	-0.057	0.723	0.017	0.882	-0.038	0.738	-0.254	**0.023**
Glycine (µM)	0.070	0.103	0.027	0.866	0.105	0.343	-0.107	0.341	-0.206	0.067
D-serine (µM)	0.313	**0.004**	0.079	0.624	0.379	**< 0.001**	-0.235	**0.034**	-0.248	**0.027**
L-serine (µM)	0.092	0.410	-0.156	0.329	0.195	0.077	-0.137	0.221	-0.199	0.077
D-/Total serine (%)	0.311	**0.004**	0.287	0.069	0.325	**0.003**	0.179	0.111	0.171	0.129
L-glutamate (µM)	-0.028	0.800	0.186	0.244	0.031	0.779	-0.093	0.410	-0.329	**0.003**
L-glutamine (µM)	0.020	0.855	-0.039	0.808	0.041	0.716	-0.055	0.623	-0.225	**0.045**
L-glutamine/L-glutamate	0.103	0.352	-0.265	0.094	0.042	0.705	0.014	0.899	0.126	0.266

Table 3. Correlations between age, age at PD onset, disease duration, LEDD and serum levels of neuroactive amino acids in the study cohorts. Significant p-values are shown in bold. Abbreviations: LEDD, Levodopa equivalent daily dose. [a] Spearman's correlation; [b] Age- and sex-adjusted partial correlation; [c] Age-, sex- and disease duration-adjusted partial correlation.

	Age at onset				LEDD			
	PD females (n = 36)		PD males (n = 47)		PD females (n = 36)		PD males (n = 47)	
	r	p[a]	r	p[a]	r	p[b]	r	p[b]
L-aspartate (µM)	0.037	0.830	-0.002	0.990	-0.466	**0.006**	-0.132	0.389
L-asparagine (µM)	0.036	0.836	-0.017	0.908	-0.422	**0.013**	-0.205	0.177
Glycine (µM)	0.189	0.269	0.126	0.400	-0.192	0.277	-0.240	0.112
D-serine (µM)	0.434	**0.008**	0.318	**0.029**	-0.380	**0.026**	0.213	0.160
L-serine (µM)	0.239	0.160	0.153	0.306	-0.416	**0.014**	-0.104	0.497
D-/Total serine (%)	0.414	**0.012**	0.194	0.192	-0.341	**0.048**	-0.174	0.254
L-glutamate (µM)	-0.036	0.836	0.049	0.743	-0.486	**0.004**	-0.244	0.107
L-glutamine (µM)	0.085	0.621	-0.047	0.753	-0.422	**0.013**	-0.204	0.179
L-glutamine/L-glutamate	0.159	0.354	-0.054	0.718	-0.014	0.936	0.092	0.546

Table 4. Correlations between age at disease onset, LEDD and serum neuro active amino acid levels in PD group stratified by sex. Significant p-values are shown in bold. Abbreviations: LEDD, levodopa equivalent daily dose; [a] Spearman's correlation; [b] Partial correlation adjusted for age and disease duration.

4.3.1.3 Serum levels of neuroactive amino acids negatively correlate with dopaminergic treatment

Since previous studies suggested that medications acting on dopamine receptors may affect the concentration of neuroactive amino acids in both the CNS and periphery [53,54], I investigated whether serum neuroactive amino acid levels could be influenced by dopaminergic treatment in PD patients. I found significant negative correlations between D-serine, L-aspartate, L-asparagine, L-glutamate, L-glutamine and the burden of daily antiparkinsonian treatment expressed as LEDD (Fig. 7 and Table 3). Again, this effect resulted to be mainly driven by female sex, since no significant correlation between any amino acid level and LEDD was found in PD males (Table 4). In the PD cohort, I also detected a negative correlation of D-serine, but not L-serine or the other measured amino acids, with disease duration (Table 3). This result, which at first glance may appear in contrast with the positive correlation between D-serine and age, is likely related to the increase of dopaminergic treatment dosage during the disease course. I verified this hypothesis testing the correlation between LEDD and disease duration, which resulted to be highly significant ($r = 0.551$, $p < 0.001$). Accordingly, the correlation between D-serine and disease duration became not significant upon addition of LEDD as covariate ($r = -0.132$; $p = 0.242$).

Overall, these findings indicate that higher dopaminergic treatment dosage is associated with lower serum levels of several neuroactive amino acids in PD female patients.

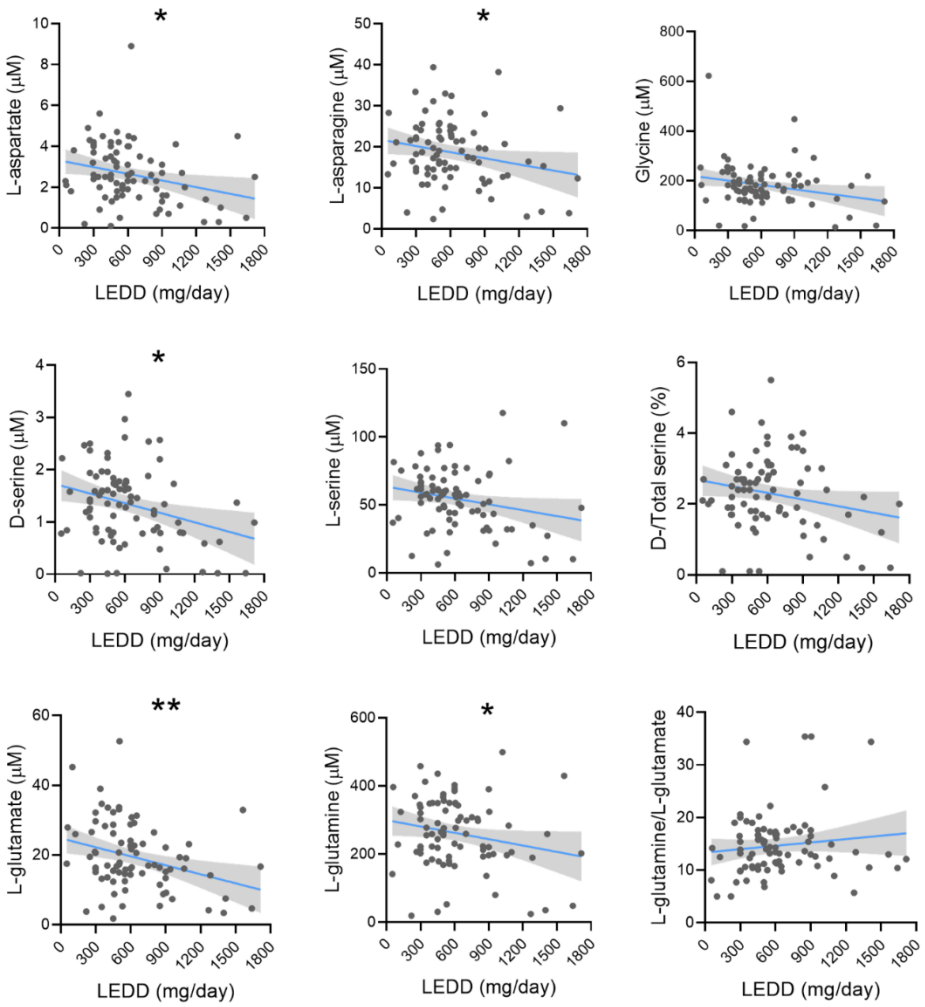

Figure 7. Scatterplots representing correlations between levodopa equivalent daily dose (LEDD) and serum neuroactive amino acid levels in PD group. *p < 0.05; **p < 0.01; age-, sex- and disease duration-adjusted partial correlations. Blue lines and grey shadows represent the linear regression best fit line and its 95% CI, respectively.

4.3.1.4 Serum glycine levels correlate with worse motor dysfunction in PD

I next investigated potential correlations between serum levels of NMDAR-related amino acids and clinical features of PD, including motor, cognitive and QoL domains. Results are summarized in Table 5. Upon adjusting for potential confounders, I disclosed a positive correlation between MDS-UPDRS-III score and Gly levels, while D-/Total serine levels showed a similar but non-significant trend. Regarding cognitive function, I did not find any correlation between serum serine enantiomers and MMSE score, in line with our recent findings showing no association between CSF D-serine levels and cognitive performance in *de novo* PD patients [41]. However, the analyses revealed a positive correlation between L-Gln and MMSE and an analogue but non-significant trend for L-Gln/L-Glu ratio. Finally, I failed to detect any significant correlation between serum serine enantiomers and the other neuroactive amino acid levels and PD-related QoL, including PDQ-39 summary index (Table 5) and the specific PDQ-39 sub-items (data not shown).

Overall, these findings suggest that in PD patients a dysregulation of serine/glycine metabolism is associated with worse motor dysfunction, while higher serum L-Gln correlates with better cognitive performance.

	MDS-UPDRS-III		**MMSE**				**PDQ-39 summary index**	
	PD		PD		HC		PD	
	r	p[a]	r	p[b]	r	p[c]	r	p[b]
L-aspartate (µM)	0.070	0.124	0.129	0.255	-0.030	0.856	0.005	0.961
L-asparagine (µM)	0.110	0.334	0.187	0.097	-0.027	0.872	0.085	0.454
Glycine (µM)	0.241	**0.033**	0.150	0.186	-0.011	0.948	0.042	0.711
D-serine (µM)	0.109	0.338	0.162	0.152	-0.013	0.685	-0.034	0.764
L-serine (µM)	0.052	0.651	0.202	0.073	-0.067	0.685	0.119	0.294
D-/Total serine (%)	0.211	0.063	0.147	0.194	-0.007	0.967	-0.124	0.272
L-glutamate (µM)	0.124	0.275	0.044	0.696	-0.126	0.445	0.039	0.729
L-glutamine (µM)	0.069	0.547	0.228	**0.042**	-0.069	0.675	0.022	0.847
L-glutamine/L-glutamate	-0.038	0.736	0.201	0.074	0.046	0.780	-0.061	0.590

Table 5. Partial correlations between motor function, cognition, PD-related quality of life and serum levels of neuroactive amino acids in PD (MDS-UPDRS-III total score, MMSE, PDQ-39 summary index) and HC cohorts (MMSE). Significant p-values are shown in bold. Abbreviations: MMSE, Mini Mental State Examination; MDS-UPDRS-III, Movement Disorders Society Unified Parkinson's Disease Rating Scale, part III; PDQ-39, Parkinson's disease Questionnaire 39; r, partial correlation coefficient. [a] partial correlation adjusted for age, sex, disease duration and LEDD; [b] partial correlation adjusted for age, sex and disease duration; [c] partial correlation adjusted for age and sex.

4.3.1.5 Serum D-serine levels are selectively increased in female PD patients

Based on our observation that antiparkinsonian treatment affects the serum levels of D-serine and the other NMDAR-related excitatory amino acids in a dose-dependent manner, I performed a novel comparison of their serum levels between PD and HC upon adjusting the analyses for LEDD. Strikingly, I observed a statistically significant increase of D-serine in PD compared to HC (Δ% = 38.7%), while there were no overt differences in L-serine, D-/Total serine or the other neuroactive amino acid levels (Table 6).

I then conducted between-group comparisons of PD and HC cohorts upon stratification by sex, and found that the increase of D-serine levels observed in PD was mainly driven by female sex. Indeed, PD females showed significantly increased D-serine (Δ% = 68.7%) and D-/Total serine (Δ% = 64.2%)

levels compared to HC females, while no relevant differences were observed between PD and HC males (Table 6).

	HC all (n = 41)	PD all (n = 83)	p[a]	HC females (n = 26)	HC males (n = 15)	PD females (n = 36)	PD males (n = 47)	p[b]	p[c]
L-aspartate (µM)	2.7 (0.3)	2.9 (0.2)	0.724	2.7 (0.5)	2.6 (0.5)	3.3 (0.4)	2.6 (0.2)	0.363	0.984
L-asparagine (µM)	16.4 (1.6)	19.5 (1.0)	0.159	16.2 (1.9)	16.4 (2.7)	19.0 (1.5)	20.5 (1.3)	0.326	0.220
Glycine (µM)	155.2 (23.5)	194.8 (14.3)	0.206	188.1 (37.5)	121.3 (26.1)	215.8 (29.7)	175.6 (12.8)	0.628	0.091
D-serine (µM)	1.1 (0.1)	1.5 (0.1)	**0.038**	0.9 (0.2)	1.2 (0.2)	1.5 (0.1)	1.4 (0.1)	**0.031**	0.299
L-serine (µM)	48.9 (4.8)	57.2 (2.9)	0.192	51.6 (5.9)	45.2 (7.6)	59.8 (4.7)	56.7 (3.7)	0.364	0.217
D-/Total serine (%)	2.0 (0.2)	2.4 (0.1)	0.116	1.5 (0.2)	2.4 (0.3)	2.5 (0.2)	2.4 (0.2)	**0.012**	0.844
L-glutamate (µM)	20.3 (2.2)	21.0 (1.3)	0.792	17.7 (2.3)	22.7 (3.9)	20.4 (1.8)	22.2 (1.9)	0.446	0.925
L-glutamine (µM)	238.4 (22.0)	272.4 (13.5)	0.246	225.0 (27.2)	245.6 (35.3)	275.7 (21.5)	280.1 (17.4)	0.226	0.423
L-glutamine/L-glutamate	13.5 (1.3)	14.2 (0.8)	0.663	13.8 (1.5)	12.9 (2.1)	14.4 (1.2)	14.4 (1.0)	0.832	0.554

Table 6. Estimated serum levels of neuroactive amino acids in PD and HC extracted from ANCOVA models adjusted for the effect of LEDD. Data are shown as estimated mean (standard error) of amino acids concentration. Significant p-values are shown in bold. [a] PD (all) compared to HC (all). Two-way ANCOVA with diagnosis and sex as factors; age and LEDD as covariates; [b] PD females compared to HC females. One-way ANCOVA with diagnosis as factor; age and LEDD as covariates; [c] PD males compared to HC males. One-way ANCOVA with diagnosis as factor; age and LEDD as covariates.

4.3.2 Targeted and untargeted serum metabolomics

A total of 69 consecutive PD patients and 32 HC entered metabolomic analyses. The demographic and clinical features of the participants are reported in Table 7. PD and HC were comparable for age, sex distribution, and global cognition. PD group showed a lower Mini Nutritional Assessment (MNA) score, i.e. a slightly higher risk of malnutrition, compared to HC.

Study participants who entered ^{1}H-NMR and UPLC/MS analyses			
	HC (n = 32)	PD (n = 69)	p
Age, years	71.0 (67.2 – 74.0)	72.0 (68.0-74.5)	0.553[a]
Female sex, n (%)	17 (53.1)	30 (43.5)	0.490[b]
Age at onset, years	-	67.0 (61.0 – 74.0)	-
Disease duration, years	-	6.0 (3.0 – 8.0)	-
MDS-UPDRS-III	-	25.0 (19.5 – 31.5)	-
LEDD, mg/day	-	530 (390 – 729)	-
MMSE	27.1 (25.4 – 30.0)	27.0 (25.8 – 30.0)	0.260[c]
MNA	25.0 (23.8 - 26.5)	24.0 (21.0 – 25.5)	**0.005**[c]

Table 7. Clinical and demographic features of PD and HC groups enrolled in the metabolomics study. Data are shown as median (IQR) for continuous variables and sample size (percentage) for categorical variables. Abbreviations: LEDD, Levodopa equivalent daily dose; MDS-UPDRS-III, Movement Disorders Society Unified Parkinson's Disease Rating Scale, part III; MMSE, Mini-mental State Examination; MNA, Mini Nutritional Assessment; PDQ-39, Parkinson's disease Questionnaire 39. * MNA score was available for n = 30 HC and n = 67 PD. [a] Mann-Whitney U test; [b] Chi-square test; [c] two-way ANCOVA with group and sex as factors and age as covariate.

First, using ^{1}H-NMR analysis on the serum of PD and HC groups, we explored the metabolomic profile of the disease. Resonance assignment performed with CHENOMX software detected the presence of 45 metabolites (Fig. 1A in the Appendix). Metabolite concentrations for each sample were collected in data matrices. Univariate statistical approach was applied using a combined Fold change (FC) and T-test approach. Robust volcano plot analysis evidenced higher serum concentrations of serine, glutamine, creatinine, glycerophosphocholine and pyruvic acid and lower concentrations of 2-oxoglutarate (also named α-ketoglutarate) and acetoacetate in the blood of PD patients compared to HC (Fig. 8a).

The data matrix was analysed using the multivariate supervised partial least-squares discriminant analysis (PLS-DA) method [55,56]. PLS-DA diagrams indicate that the serum metabolomic profile of PD patients are significantly different from HC (Fig. 8b). To identify the molecules significantly responsible for metabolomic separation, we performed variable influence on projection (VIP) score analysis [57]. Accordingly, the metabolites characterised by a VIP score > 1 were considered good classifiers between the two clusters[58]. The VIP score graph (Fig. 8c) revealed that glycerophosphocholine (VIP: 1.81), N-acetylglycine (VIP: 1.62), creatinine (VIP: 1.33), serine (VIP: 1.33), leucine (VIP:1.31), pyroglutamate (VIP: 1.23), proline (VIP: 1.16), asparagine (VIP:1.04), tyrosine (VIP:1.02) and taurine (1.02) could discriminate the metabolomic profiles of PD patients and HC. Interestingly, VIP analysis showed that the metabolic profiles were also differentiated by several

key metabolites related to cellular bioenergetic processes, including 2-oxoglutarate (VIP: 2.04), pyruvic acid (VIP: 1.76), 2-hydroxybutyrate (VIP: 1.37), fructose (VIP: 1.27), glucose (VIP: 1.19) and glycerol (VIP:1.10). Heatmap analysis confirmed the results obtained by robust Volcano plot (Fig. 8d). Reduced concentrations of 2-oxoglutarate and acetoacetate along with an upregulation of creatinine, pyruvic acid, glycerophosphocholine and serine emerged as biochemical blood signatures in PD patients. Additionally, the Receiver Operating Characteristic (ROC) curve confirmed the involvement of 2-oxoglutarate as a mitochondrial-related biomarker in distinguishing PD patients from HC (AUC = 0.94) (Fig. 3A in the Appendix).

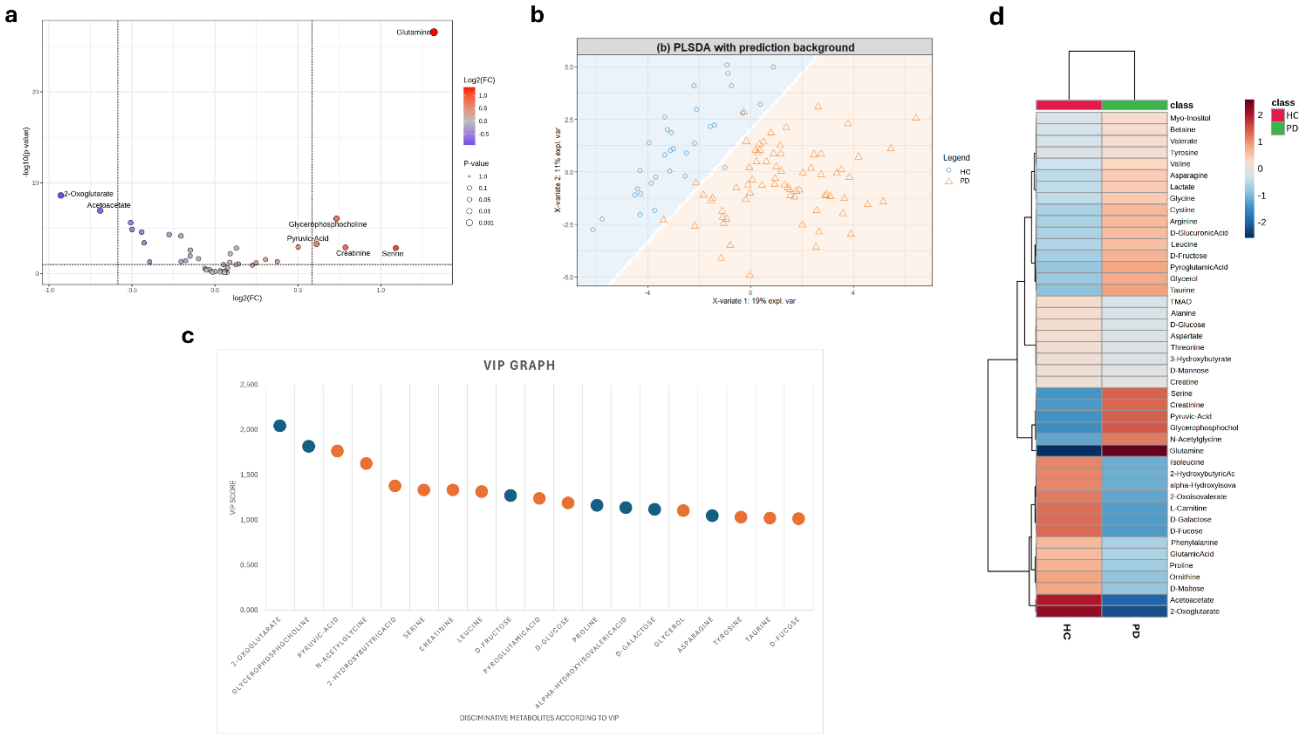

Figure 8. ^{1}H-NMR metabolomics results. **a** Volcano plot analysis of metabolic changes in PD patients and HC sera. Each point on the volcano plot was based on p-value and fold-change (FC) values, set at 0.05 and 2.0, respectively. Red points identify upregulated metabolites. **b** PLS-DA score scatter plots related to serum from PD patients (N = 69) and healthy controls (N = 32). The cluster analyses are reported in the Cartesian space described by the main components PC1:16.8% and PC2:11.4%. PLS-DA was evaluated using cross-validation (CV) analysis. CV tests performed according to the PLS-DA statistical protocol show a significant cluster separation (0.98 and 0.99 AUC values for PC1 and PC2; see Supplementary Figure 2 for additional details on PLS-DA model performance). **c** VIP score graphs of metabolites discriminating the two clusters. **d.** Heatmap of changed metabolites relative to ^{1}H-NMR analyses. The colour of each section corresponds to a concentration value of each metabolite calculated by a normalized concentration matrix (red, upregulated; blue, downregulated). The colour intensity represents the importance of each metabolite in separating the two clusters. Abbreviations: HC, healthy controls; PD, Parkinson's disease patients.

A subsequent enrichment analysis performed on NMR data revealed distinct metabolic pathways dysregulated in PD patients compared to HC, including (i) amino acid pathways, i.e. alanine metabolism, cysteine metabolism, phenylalanine and tyrosine metabolism, glycine and serine

metabolism, arginine and proline metabolism, glutamic acid metabolism, aspartate metabolism and tryptophan metabolism; (ii) amino acids catabolism and ammonia recycling, i.e. lysine degradation, valine, leucine and isoleucine degradation, urea cycle, and amino sugar metabolism; (iii) bioenergetic pathways, i.e. glucose-alanine cycle, Warburg effect and malate-aspartate shuttle (Fig. 4A in the Appendix).

Since several amino acids emerged among the best discriminating metabolites in ^{1}H-NMR analyses, we employed a targeted UPLC/MS approach to better evaluate the serum amino acids profile of PD patients. Of note, UPLC/MS boasts higher sensitivity and resolution compared to ^{1}H-NMR [59]. A panel consisting of 44 aminoacidic metabolites was included in UPLC/MS analysis (Fig. 5A in the Appendix). Figure 5A in the Appendix provides a summary of the common and different metabolites analysed with untargeted ^{1}H-NMR and targeted UPLC/MS approaches. Sixteen amino acids were identified in common between the two methodologies.

Univariate analysis on UPLC/MS data between the PD and HC groups highlighted that six amino acids were statistically relevant. Among these, Volcano plot indicates that threonine, glycine, and cystathionine displayed higher levels in PD compared to HC, whereas kynurenine, glutamic acid, and tryptophan were reduced in PD (Fig 9a). Based on the univariate analysis results, a PLS–DA classification model between PD and HC was built (Fig. 9b). A preliminary cross-validation (CV) was performed to determine the optimal number of components. Then, two components were selected for the final PLS-DA model, which was validated using CV. VIP score analysis, considering even in this case VIP score > 1, identified kynurenine (VIP: 2.17), glutamic acid (VIP: 2.03), tryptophan (VIP: 2.00), cystathionine (VIP: 1.74), threonine (VIP: 1.59), glycine (VIP: 1.47), aspartic acid (VIP: 1.28), arginine (VIP: 1.20), valine (VIP: 1.13), ornithine (VIP: 1.04), lysine (VIP: 1.04), and 4-hydroxyproline (VIP: 1.01) as major contributors in the classification (Fig 9c). Heatmap analysis confirmed the results obtained by Volcano plot (Fig. 9d).

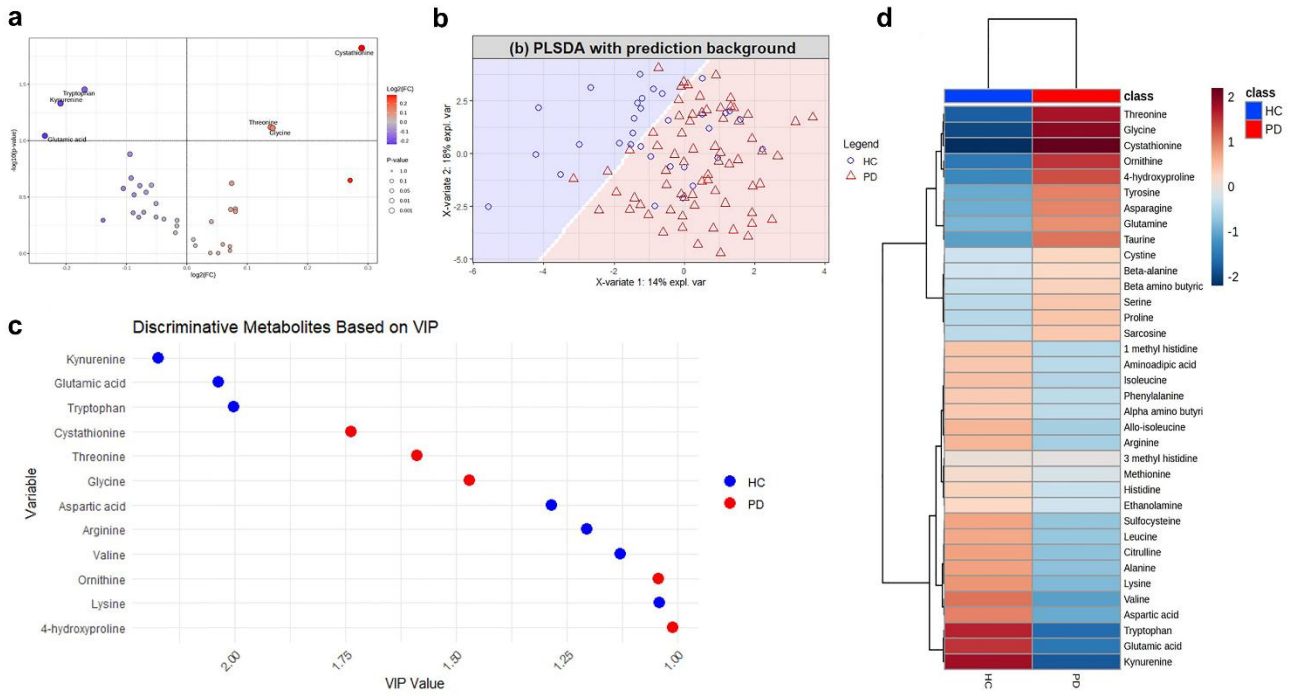

Figure 9. UPLC/MS analysis results **a.** Volcano plot analysis of metabolic changes in PD patients and HC sera. Each point on the volcano plot was based on p-value and fold-change (FC) values, set at 0.1 and 1 respectively. Red points identify upregulated metabolites, and blue points downregulated metabolites. **b** PLS-DA score scatter plots related to serum from PD patients (N = 69) and HC (N = 32). PLS-DA model was evaluated using ROC reporting an AUC value of 0.73 (pvalue: 0.0002) for the first component, and an AUC value of 0.79 (pvalue:3.83 e-6) for the second component. **c.** VIP scores, based on PLS – DA discriminant classification model, were calculated and filtered for a score > 1. Score values are displayed on the x – axis and VIPs on the y – axis. Colors reflect directions of the variables that discriminate the classes. **d.** Heatmap displaying metabolite abundance levels in the PD and HC groups.

An enrichment analysis conducted on UPLC/MS data identified three metabolic pathways that differentiated PD patients from HC: glutathione metabolism, porphyrin metabolism, and tryptophan metabolism (Fig. 7A in the Appendix).

Finally, we investigated whether the four metabolites that best discriminated PD from HC correlated with clinical-demographic features in PD patients. In line with our HPLC data on PD patients' serum, we found a positive correlation between glycine levels and motor impairment assessed with the MDS-UPDRS-III score. There were no other significant clinical-biochemical correlations (Figure 10).

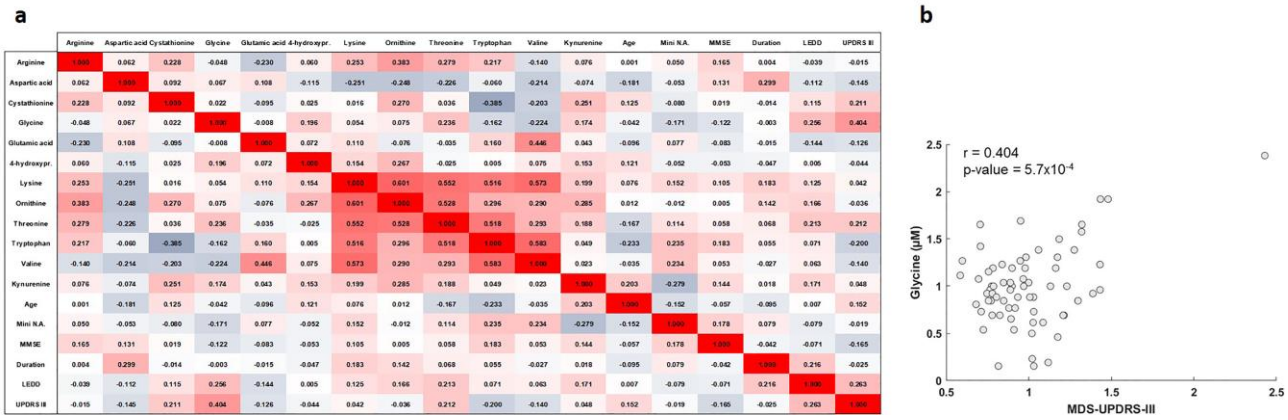

Figure 10. The Correlation Matrix shows correlations between VIPs and clinical measurements using Pearson's correlation coefficient (r). Correlation values range from -1 (strong negative correlation, blue cells) to 1 (strong positive correlation, red cells). The data have been previous subjected to average normalization. **b.** Scatterplot showing the positive correlation between serum glycine concentration and MDS-UPDRS-III score, with an r value of 0.404 and the corresponding p-value 5.7 $x10^{-4}$ for statistical significance. Abbreviations: LEDD, Levodopa Equivalent Daily Dose; Mini N.A., Mini Nutritional Assessment; MMSE, Mini Mental State Examination; MDS-UPDRS-III, Movement Disorder Society Unified Parkinson's Disease Rating Scale, part III.

4.4 Discussion

This study shed light on the serum D- and L-amino acid profile and metabolomic fingerprint of PD. Specifically, I found that the levels of the NMDA receptor co-agonist D-serine positively correlate with age at disease onset and are negatively modulated by dopaminergic treatment. Moreover, both targeted and untargeted serum metabolomics disclosed a complex dysregulation of amino acids metabolism in PD, mainly involving glycine-serine and glutamate pathways. These findings support previous evidence pointing out to glutamatergic dysfunction as a physiopathological hallmark of PD [11]. However, these neuroactive amino acids are also directly involved in the metabolism of several peripheral organs and systems, including liver, kidney, muscle, immune system and gut [60]. This means that the altered serum levels of these molecules observed in PD patients might mirror the systemic nature of PD [61], rather than brain-centered pathological mechanisms. In the following sections, I will discuss point-by-point the results obtained in this section of the study.

4.4.1 Serum D-serine concentration correlate with age at onset and dopaminergic treatment in Parkinson's disease

First, I investigated the serum levels of D-serine and the other amino acids acting on NMDAR neurotransmission in a well-characterized PD cohort compared to healthy controls. After adjusting for the effect of potential confounders, I found a selective increase of serum D-serine levels in PD

patients compared to HC, mainly driven by female sex. In the PD cohort, serum D-serine and D-/Total serine ratio positively correlated with age and age at disease onset, while the amount of dopaminergic treatment was found to negatively correlate with serum levels of all NMDAR-related amino acids. Finally, I observed a positive correlation between serum Gly and MDS-UPDRS-III score.

These findings suggests that the dysregulation of peripheral D-serine metabolism may represent a suitable biochemical signature of PD, and are in line with recent studies consistently showing increased D-serine levels in the rostral putamen of MPTP-treated monkeys [40], the *post-mortem* striatum of PD brains and the CSF of *de novo* living PD patients [41]. Of note, all these former studies also detected a parallel increase in L-ser content which I failed to detect in peripheral blood, suggesting a differential regulation of L-serine homeostasis between the CNS and periphery in PD. Consistent with this assumption, while in the CNS L-serine is synthesized by astrocytes through the phosphorylated pathway [10,62], the peripheral biosynthesis of this amino acid occurs mainly in the kidney and liver through the conversion of Gly to L-serine via the serine hydroxymethyltransferase (SHMT) pathway [10]. Moreover, blood levels of L-serine are directly influenced by the dietary intake and the catabolism of endogenous proteins [63]. The acknowledgment of the different metabolic pathways which regulate L-ser levels in the CNS and periphery is thus essential to reconcile the apparent divergent results observed between the present study and our previous works.

In a previous smaller study (including 9 drug-naïve PD, 13 L-dopa treated PD and 30 controls) no differences in serum D-serine levels between PD patients and controls were observed [39]. This divergence could relate to several factors, including differences in age, disease duration, sex and – above all – the limited number of patients included in the previous work, which did not allow the adjustment of statistical comparisons for the relevant variables affecting peripheral D-serine levels, including age, sex and LEDD.

Dysregulated D-serine levels have been recently implicated in the pathophysiology of a wide range of neurodegenerative diseases. Increased D-serine levels were observed in the spinal cord of the 93A-SOD1 mouse model of amyotrophic lateral sclerosis (ALS) and in patients with sporadic and familial ALS [64]; also, the genetic inactivation of D-amino acid oxidase (DAAO), the enzyme responsible for D-serine catabolism, caused lower motor neurons degeneration in mice [65]. Increased D-serine levels were also reported in the brain and CSF of patients affected by Alzheimer's disease (AD) [66,67] and frontotemporal dementia [68] compared to controls, while this was not confirmed in other studies [13,69]. Interestingly, recent works in AD patients detected elevated serum D-serine levels

which negatively correlated with cognitive function, supporting the hypothesis that peripheral D-serine may represent a potential biomarker for the progression of AD [12,70].

Despite these previous findings appear to implicate a putative detrimental effect of increased D-serine levels in ALS and AD, compelling evidence supports the hypothesis that NMDAR co-agonists may play a neuroprotective role in PD. Consistent with this assumption, treatment with D-cycloserine, a partial agonist at the glycine modulatory site of NMDAR, significantly improved motor and cognitive deficits and partially rescued neuroinflammation and neurodegeneration in different PD animal models [71–73]. Moreover, drug-induced increase of extracellular brain levels of Gly – which also act as an NMDAR co-agonist – induced the dopaminergic reinnervation of dorsal striatum in the 6-hydroxydopamine (6-OHDA)-injected mice [36], and improved dyskinesia, psychosis-like behaviour and motor impairment in parkinsonian mice and monkeys [34–36]. In keeping with this, a recent work highlighted the safety of 1-month exogenous D-serine treatment in the MPTP-treated mice [40]. These preclinical data are consistent with the findings of a pilot randomized controlled clinical trial showing that oral D-serine treatment, in add-on to standard PD therapy, improved both motor and non-motor symptoms in a small PD cohort [33,37]. In light of this evidence, I hypothesize that increased striatal, CSF and serum D-serine levels observed in PD may mirror the occurrence of an adaptive biochemical response to counteract the ongoing nigrostriatal pathway degeneration.

As a further indication of peripheral D-serine metabolism alteration in PD, I found a selective positive correlation between serum D-serine and D/Total serine with age in PD patients but not in HC, the latter finding being in line with previous investigations [74]. Interestingly, a recent study on AD showed similar findings, with serum D-serine and D-/Total serine ratio increasing with older age in AD patients but not in healthy controls [12]. Again, this effect was specific for D-serine, since no correlation between age and serum L-serine, L-Asp or D-Asp was observed in AD or HC groups. Despite no causal relationship can be drawn from correlation analyses, these findings suggest that aging affect peripheral D-serine homeostasis in different neurodegenerative conditions.

Besides the correlation with age, this is the first study to investigate the relationship between serum levels of NMDAR-related amino acids and age at PD onset. I found a strong positive correlation of serum D-serine, but not the other tested amino acids, with age at onset, suggesting a putative modulatory effect of D-serine on the age at onset of the disease. In light of the remarkable beneficial effect of NMDA-NR1 subunit stimulation in animal models [34–36] and PD patients [33,37], it can be hypothesized that higher D-serine levels may provide a neuroprotective effect, delaying the disease onset in PD patients. However, further studies are needed to confirm our hypothesis and to elucidate the related biological mechanisms.

I also found a positive correlation between serum Gly and MDS-UPDRS-III score in PD patients. In the kidney, liver and other peripheral organs, L-serine is rapidly interconverted with Gly in a single reaction catalysed by SHMT as part of the folate-mediated one-carbon metabolism [10]. Two isoforms of SHMT1 exist, the first located in the cytosol (SHMT1) and the second in the mitochondria (SHMT2), with SHMT2 being the predominant isoform in mammalians [75]. Interestingly, a recent work showed that SHMT2 pathway is upregulated in *PINK1/PRKN* fly PD model, while genetically induced overexpression of SHMT2 rescued both mitochondrial function and brain dopaminergic depletion in *PINK1* and *PRKN* mutants, suggesting that the activation of the one-carbon metabolism may play a neuroprotective role in PD [76]. Since mitochondrial dysfunction represents a pathogenic hallmark of PD [77], I can hypothesize that the observed increase in serum Gly levels with worsening motor dysfunction could be due to an adaptive upregulation of the SHMT2 pathway aimed at counteracting the concurrent mitochondrial dysfunction. However, it warrants further investigation to test whether one-carbon metabolism is dysregulated in idiopathic PD and confirm its putative relationship with serum Gly levels.

After adjusting for LEDD, I did not find any correlation between tested amino acids and other PD clinical features, including disease duration, cognition and QoL, in line with our previous data showing no association between NMDAR-related amino acids in the CSF and clinical features in *de novo* PD patients [41]. These results – along with the similar increase in striatal D- and L-serine content in the post-mortem striatum of PD brains with different neuropathological Braak stage [41] - suggest that central and peripheral changes of D-serine levels may be an early biochemical response related to PD physiopathology, and may thus not mirror the longitudinal clinical trajectories of PD symptoms.

The stratification by sex of HC and PD cohorts revealed that the increase of serum D-serine concentration in PD patients was mainly driven by female sex. Interestingly, recent investigations showed increased D-/Total serine ratio in the human post-mortem hippocampus and serum of AD female patients compared to HC females, males showed no differences [12,66]. Thus, D-serine metabolism may be dysregulated in different neurodegenerative diseases in a sex-specific manner. Increasing evidence revealed that sex hormones may play a role in the development and clinical progression of PD, with estrogens representing a putative neuroprotective agent against nigrostriatal degeneration [78]. Since estrogens regulate glutamatergic neurotransmission [79], it can be hypothesized that they may exert a modulatory effect on peripheral D-serine levels. However, further studies evaluating sex differences in D-serine metabolism in PD experimental models and patients are needed to elucidate this issue.

Despite D-serine has been mostly studied as a neuromodulator acting on NMDAR in the CNS, increasing evidence showed that D-serine metabolism is also affected by peripheral organs. Dietary intake may also affect serum D-serine levels, since common foods such as fermented products and seafood contains D-serine and other D-amino acids [80]. Moreover, a main source of D-amino acids in mammals is represented by the gut microbiota, which produces an entire set of these atypical amino acids [81]. A thorough evaluation of blood D-serine levels in healthy and disease populations should thus take into account the complex endogenous and exogenous origins of this D-amino acid, which may affect its peripheral concentration. However, previous investigations in mice showed that blood D-serine levels are not affected by serine racemase *knock-out* or *germ-free* conditions [82,83], implying the existence of a still unidentified enzyme able to synthesize D-serine. Two factors which appear instead to significantly affect blood D-serine are DAAO activity and renal parameters. Recent studies in mice showed a strong increase (>3x) in plasma D-serine levels following DAAO genetic inactivation [83], while plasma D-serine correlated positively with different markers of renal function, such as serum creatinine and cystatin C [74]. This last point is of particular interest, because previous epidemiological studies have demonstrated an increased incidence of PD in patients with chronic kidney disease [84], and the blood levels of D-serine correlated with markers of renal function in selected cohorts of patients with ischemic acute kidney injury [85,86], chronic kidney disease [87,88], kidney transplant donors and recipients [89] and middle-aged subjects without overt kidney diseases [74]. Since only subjects with normal serum creatinine values were enrolled in the present study, I exclude that our findings may be biased by differences in renal function among the study cohorts. However, future *ad hoc* studies should evaluate whether serum D-serine modulation in PD may relate to renal function.

I cannot exclude a possible contribution of CNS-derived D-serine in determining serum D-serine concentration. Previous studies suggested that the blood and CSF levels of amino acids, including serine and glycine, are significantly correlated in human subjects, indicating that the plasma levels of these amino acids reflect, to some extent, those in the central nervous system [90,91]. However, these studies evaluated the total amino acids level without chiral resolution of D- and L-enantiomers. Pre-clinical evidence showed that the systemic administration of D-serine increases its concentration in different brain regions and in the CSF of rodents [92–95], suggesting a slow diffusion of this NMDA receptor co-agonist through the blood-brain barrier. The blood-to-brain transport of L-serine is extremely low [96,97] and D-serine brain uptake is comparatively higher than that of L-serine [92]. Importantly, our previous studies revealed that D- and L-serine levels were increased in the rostral putamen of parkinsonian monkeys and in the striatum and CSF of PD patients, but unchanged in other brain regions, such as the subthalamic nucleus, globus pallidus, superior frontal gyrus [40,41], and

even reduced in the substantia nigra of MPTP-lesioned monkeys [39]. Overall, these findings indicate that the concentration of serine enantiomers is differently affected by PD physiopathology in different brain areas, thus hampering any inference between our previous findings and the results of the present work. Future studies evaluating simultaneously the CSF and blood levels of serine enantiomers in larger cohorts of PD patients are then warranted in order to disentangle this intriguing issue.

PD pharmacological treatment may also influence blood amino acid levels. To our knowledge, only one previous study evaluated the correlation between serum amino acid profile and dopaminergic treatment in PD patients, showing negative correlations between LEDD and alanine, arginine and phenylalanine, but not glutamate [98]. Here, I investigated for the first time the effects of antiparkinsonian treatment on serum levels of the NMDAR-related amino acids with chiral resolution of serine enantiomers. I found that dopaminergic treatment downregulates the serum levels of D-serine, L-serine, L-Glu, L-Asp and their precursors L-Gln and L-Asn in PD females but not in PD males. Interestingly, previous evidence from our group showed that, in the caudate putamen of female MPTP-lesioned monkeys, treatment with L-DOPA rescued the abnormally increased striatal concentrations of several NMDAR-stimulating amino acids, including D- and L-Asp and D- and L-serine [39]. Overall, these findings suggest that antiparkinsonian treatment downregulates D-serine and other excitatory amino acids acting on NMDAR both at the central and peripheral level in a sex-specific manner. This observation has potential clinical implications, since future clinical trial assessing the efficacy of putative drugs acting on NMDAR glycine site (e.g. D-serine) as add-on treatment in PD patients should take into account the interactions between concurrent dopaminergic treatment, sex and the experimental drugs pharmacokinetics.

4.4.2 Independent serum metabolomics approaches identify disrupted energy and amino acids metabolism in PD patients

A recent meta-analysis showed a considerable inconsistency among the findings obtained by 74 original PD-focused metabolomics studies, potentially attributable to the different biospecimens analysed, antiparkinsonian drugs-related effects, different disease stages, genetic background, ethnicity, diet, exercise level, and analytical platform employed[99]. This limited reproducibility highlights the need for deeply phenotyped cohorts to reckon with the several potential confounding factors that may bias the metabolomic profile of PD patients. Moreover, only one prior study used an integrated NMR and MS approach to characterize the plasma metabolomic profile of PD

patients[100]. Here, I attempted to untangle this matter adopting a dual approach based on NMR-based metabolomics and UPLC/MS analysis to dissect the serum metabolomic pathways dysregulations in a cohort of PD patients characterized with motor, cognitive, dopaminergic treatment and nutritional assessments. NMR and UPLC/MS analyse different metabolites and employ a different strategy — NMR being untargeted and UPLC/MS concentrating exclusively on amino acids (Fig. 6A), yet these two methods disclosed variations in metabolites belonging to shared cellular metabolic pathways. In particular, my findings indicate a complex dysregulation of serum amino acids and molecules associated with energy metabolism in PD patients. Consistently, NMR analysis revealed elevated levels of glutamine and serine, accompanied by decreased concentrations of the glutamic acid precursor α-ketoglutarate in PD patients, while UPLC/MS analysis indicated a reduction in serum glutamic acid levels (Figs. 8a, 9a). Notably, both ^{1}H-NMR and UPLC/MS revealed altered levels of amino acids closely related to each other. For instance, the lower glutamic acid levels emerged through UPLC/MS in PD patients (Fig. 9a) align well with the reduced α-ketoglutarate concentrations disclosed by NMR analyses (Fig. 8a), overall indicating the occurrence of mitochondrial-related bioenergy abnormalities in PD patients, as reported in previous studies[101]. Another example is represented by the higher serine and glycine concentrations observed in PD group through NMR and UPLC/MS, respectively. Since these two amino acids are interconverted through a single enzymatic reaction catalysed by serine hydroxymethyltransferase [10], increased serine levels may cause elevated glycine levels and vice versa. Overall, these metabolomic results confirm the homeostasis disruption of the amino acids acting on glutamatergic transmission in the physiopathological framework of PD [102], and furtherly supports previous data showing increased serine enantiomers concentration in the striatum of MPTP-lesioned monkeys [40], post-mortem human PD putamen[41] and in the CSF[41] and serum (see section 4.3.1.5) of PD patients.

The distinct blood metabolomic fingerprint of PD highlighted in this study is also in line with the findings of prior LC/MS[103–107] and NMR-based[108,109] studies and supports the idea that PD represents a multi-system clinical-pathological entity rather than a CNS-centred disease. Specifically, the remarkable amino acids dysregulation observed in this study is consistent with previous works reporting altered blood or CSF concentration of glutamic acid, glutamine, glycine and serine in PD patients compared to HC[38,99,108,110,111]. Notably, glutamate is involved in several physiological processes known to be altered in PD, including redox homeostasis, energy metabolism and neuroinflammation[112]. Along with glycine and cysteine, glutamic acid is a key constituent of the antioxidant tripeptide glutathione, and is thus required for maintaining the redox homeostasis. Altered serum glutamate levels may thus ultimately contribute to a dysfunctional

glutathione synthesis in PD[107,113,114]. Consistent with this, UPLC/MS data showed a reduction in glutamic acid levels, along with elevated concentrations of glycine, threonine, and cystathionine. This metabolic alteration nicely aligns with the results from UPLC/MS enrichment analysis, which identified glutathione metabolism as one of the most significantly affected pathways (Figure 6A), suggesting a disruption in the cellular antioxidant defense in PD. Since glutamic acid is a crucial precursor for glutathione synthesis, I argue that its depletion could impair the capacity to counteract oxidative stress, which is a hallmark of PD pathophysiology[115].

Our findings are largely consistent with the results of a previous meta-analysis investigating blood amino acids as potential biomarkers for PD [38]. For instance, blood aspartate, tryptophan and lysine levels were found to be reduced in PD patients compared to HC both in our work and in the aforementioned meta-analysis. The apparent discrepancy in blood serine levels (lower in PD than HC in Jiménez-Jiménez et al[38]; higher in PD in our work) may be related to several factors related to the study cohorts (e.g. different disease stage, antiparkinsonian treatment, ethnicity, diet or exercise level). Moreover, a recent metabolome-wide association study conducted on large U.S. PD and HC cohorts found increased serum serine in PD patients compared to controls[111], which is in line with our work.

On the other hand, the increased levels of glycine, cystathionine, and threonine, which are involved in one-carbon and sulfur amino acid metabolism, may reflect a compensatory response to limit oxidative damage or represent a harmful dysregulation in metabolic processes. The positive correlation observed between serum glycine concentration and MDS-UPDRS-III score supports a putative role for glycine as a peripheral marker of motor dysfunction in PD[110]. Overall, the observed data point to a complex interaction among amino acids, glutathione synthesis, oxidative stress, and the perturbation of key metabolic pathways in PD, highlighting the potential role of these disturbances in disease pathophysiology.

Furthermore, the concomitant downregulation in α-ketoglutarate levels observed in the serum of PD patients suggests a derangement of tricarboxylic acid cycle, which was already reported in other investigations[114,116–118]. This finding, along with (i) the abnormally higher pyruvate concentration; (ii) the perturbation of aerobic glycolysis and glucose-alanine cycle observed in the serum of PD patients, is consistent with the prominent alteration of energy metabolism which characterizes neurodegenerative diseases[101]. In addition to glutathione metabolism, glutamate is pivotal at the intersection of various metabolic pathways critical for energy metabolism, the processing of carbon skeletons (including the citric acid cycle and malate-aspartate shuttle), and ammonia recycling (via the urea cycle). Consistently, our untargeted NMR enrichment analysis

showed biochemical alterations in all these energy-related metabolic pathways (Fig. 10 and Fig. 4A).

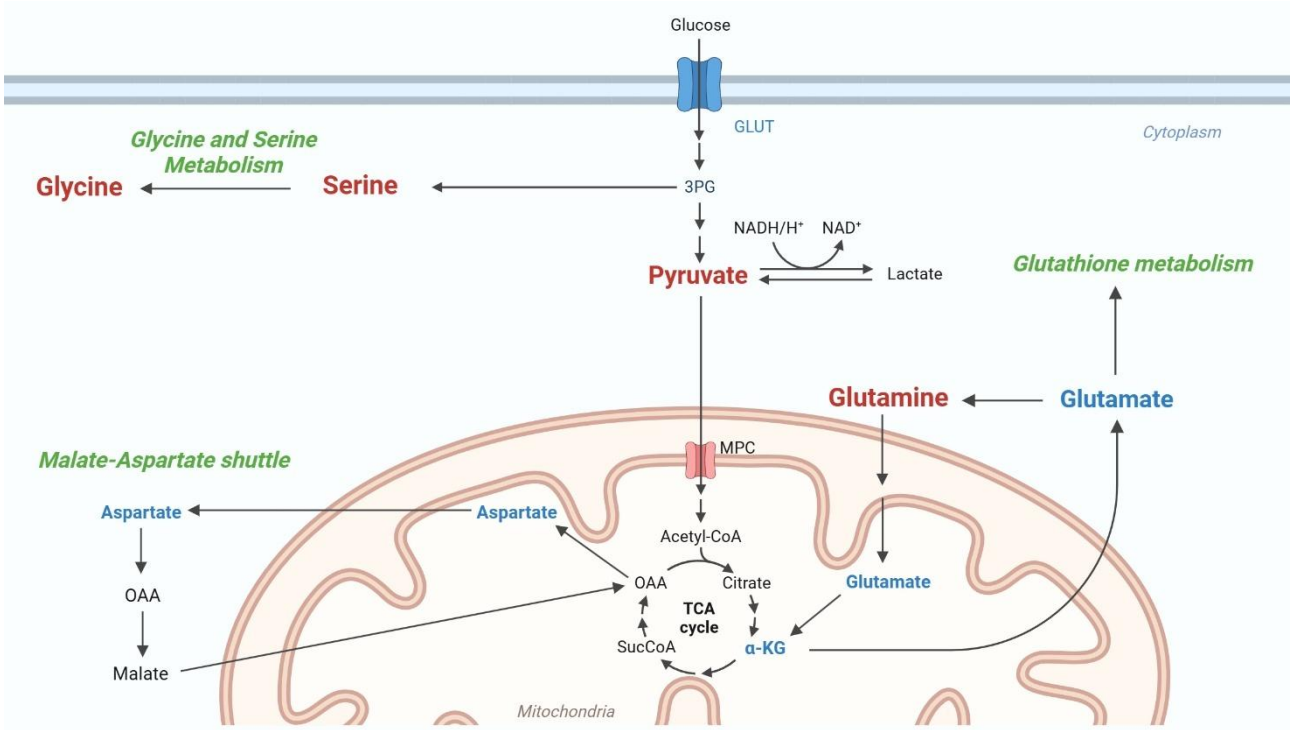

Figure 10. Cartoon depicting the main dysregulated metabolic pathways emerged from ^{1}H-NMR and UPLC/MS analyses on PD patients' serum. Upregulated and downregulated metabolites are shown in red and blue, respectively. Metabolic pathways over-represented in PD are labelled in green. Abbreviations: Acetyl-CoA, acetyl coenzyme A; α-KG, α-Ketoglutarate; GLUT, glucose transporter; MCT, Monocarboxylate transporter; MPC, Mitochondrial pyruvate carrier; OAA, Oxaloacetate; 3PG, 3-Phosphoglyceric acid; SucCoa, Succinyl-coenzyme A; TCA, Tricarboxylic Acid Cycle. Created with Biorender.com.

Finally, in PD patients I observed a decrease in both kynurenine and tryptophan levels, key metabolites in the tryptophan-kynurenine pathway, which is crucial for neuroprotection and immune modulation [119]. This reduction, combined with enrichment analysis revealing alterations in tryptophan metabolism, is consistent with growing experimental evidence showing a significant dysregulation of the tryptophan-kynurenine pathway in PD[120,121]. Kynurenine is involved in the biosynthesis of neuroprotective metabolites like kynurenic acid, which modulates glutamate activity and inflammation[119]. The concomitant depletion of tryptophan and kynurenine may indicate impaired neuroprotective mechanisms or increased oxidative stress, aligning with the altered tryptophan metabolism observed with both NMR and UPLC/MS (Figs. 3A, 6A).

Dietary regimen may also affect the blood metabolomic profile [99]. According to the MNA assessment, 34 (40.4%) of the PD patients enrolled in this study were at risk for malnutrition (i.e., MNA score < 23.5) versus only 5 (14.7%) HC. Although the impact of malnutrition on peripheral

metabolome has been poorly characterized in PD [122], differences in diet between the two groups may have partially contributed to the metabolomic signatures observed in this study. However, the lack of correlation between MNA score and the serum levels of the most discriminative metabolites identified through multivariate UPLC/MS analyses (Fig. 10a), as well as the overnight fasting preceding serum sampling, support a main contribution of PD physiopathology in determining the metabolic alterations observed in this study.

In conclusion, this section of the study highlights disrupted homeostasis of molecules related to glutamic acid, serine and energy metabolism as distinct serum signatures in PD patients. Analysis of the serum metabolome in populations at high risk of conversion to PD, such as subjects with idiopathic REM sleep behavior disorder or asymptomatic carriers of genetic risk variants, is warranted to assess its value as an early diagnostic biomarker.

4.4.3 Strengths and limitations

The strengths of this section focused on PD patients include: (i) the multimodal analysis of a wide spectrum of serum metabolites through three independent and complementary biochemical approaches, including (i) HPLC determination of a wide spectrum of NMDAR-related amino acids, (ii) untargeted ^{1}H-NMR and (iii) targeted UPLC/MS metabolomic analysis in the serum of a well phenotyped PD cohort and HC; (ii) the correlation analyses of serum NMDAR-stimulating amino acids with age at PD onset, antiparkinsonian treatment and PD clinical features, which had never been performed in former studies; (iii) the strict inclusion criteria for participants enrolment, which excluded subjects with inflammatory, neoplastic or other systemic diseases, thus increasing the confidence that the observed perturbation of peripheral metabolism is actually associated to PD pathophysiology.

However, I also acknowledge some limitations. First, PD patients were well into the disease course and all were taking oral antiparkinsonian drugs at the time of enrolment, thus limiting the inference of the results to *de novo* PD patients, a population suitable to receive putative neuroprotective interventions. Further studies are warranted to evaluate whether these findings can be extended to earlier disease stages, including drug-naïve PD patients and prodromal PD subjects. Second, CSF sampling was not included in the study design, thus preventing the correlation of serum levels of the amino acids and other metabolites with their relative content in the CSF or with biomarkers of neurodegeneration (e.g. α-synuclein, neurofilament light chain). Third, the assessment of biochemical parameters of kidney and liver function was not included in the study protocol, thus preventing the adjustment of the analyses for the serum levels of creatinine, aspartate transaminase

and alanine transaminase, which correlate with the blood levels of D-serine and several L-amino acids, respectively [74]. However, the history of any kidney or liver disease or altered parameters of renal and hepatic function was strictly considered as an exclusion criteria at the time of participants enrolment. I am thus confident that these findings are not biased by impaired peripheral organs function. Fourth, the unequal number of controls compared to PD patients may have underpowered the statistical analyses adopted. Fifth, disease stage may affect the blood amino acids profile in PD [135]. Unfortunately, the relatively small sample size of the PD cohort included in the present study did not allow us to reliably conduct metabolomic analyses stratified by disease stage. Future research studies conducted on larger PD and control populations with longitudinal and biochemical clinical follow-up are warranted to address this important issue. A further limitation of the present study is represented by the lack of common discriminant metabolites identified by ^{1}H-NMR and UPLC/MS. This apparent discrepancy may be partly due to the limited number of subjects enrolled in the study. Furthermore, the two quantification methods produce distinct matrices which, in the process of model construction, may result in variables with differing weights during clustering analyses. Further investigations are needed to evaluate the reproducibility of serum metabolomic profiles between NMR and UPLC/MS in PD and to validate the findings of this study.

5 FRAILTY COHORT

5.1 Introduction

5.1.1 Definition and clinical tools to measure frailty

Frailty is a complex clinical syndrome characterized by a progressive deterioration of physiological function of multiple organ systems, with consequent increased vulnerability to stressors and adverse health outcomes [123]. Frailty is common in elderly populations, with a prevalence in high-income countries ranging from 4 to 16 percent in people over 65 years of age and featuring a two-fold higher risk in women than in men [124–126].

Frailty is recognized as a main determinant of disability, institutionalization and mortality among older people. However, frailty also represents a dynamic condition which exists on a continuum from fit to frail, where a subject's status can change in either direction over time [123]. Previous longitudinal studies showed indeed that up to 57% of individuals experience at least one transition, which includes both worsening and improvement in frailty state [126,127]. This evidence suggests that the factors concurring to determine frailty may be targeted with preventive interventions especially in its early stage, i.e. pre-frailty, which holds better chances to revert to non-frail status compared to frailty [128,129].Heterogeneous frailty definitions and operational scales have been proposed, with large variations in their biological rationale and included components [130]. Among the most commonly adopted, Fried's frailty phenotype considers frailty as a biological syndrome and classifies individuals on the basis of five physical components, including weight loss, exhaustion, low physical activity, slow walking speed, and low grip strength [131]. A few years later, Rolfson and colleagues proposed the Edmonton Frail Scale (EFS), a brief and point-of-care frailty evaluation tool whose reliability is comparable to the most comprehensive geriatric assessment scales [132,133]. Among its nine items, the EFS includes an assessment of primary brain-related functions including cognition, mood and social support, whose impairment represents a key component of frailty and is associated with increased social isolation, disability and mortality [134,135] (Fig. 4).

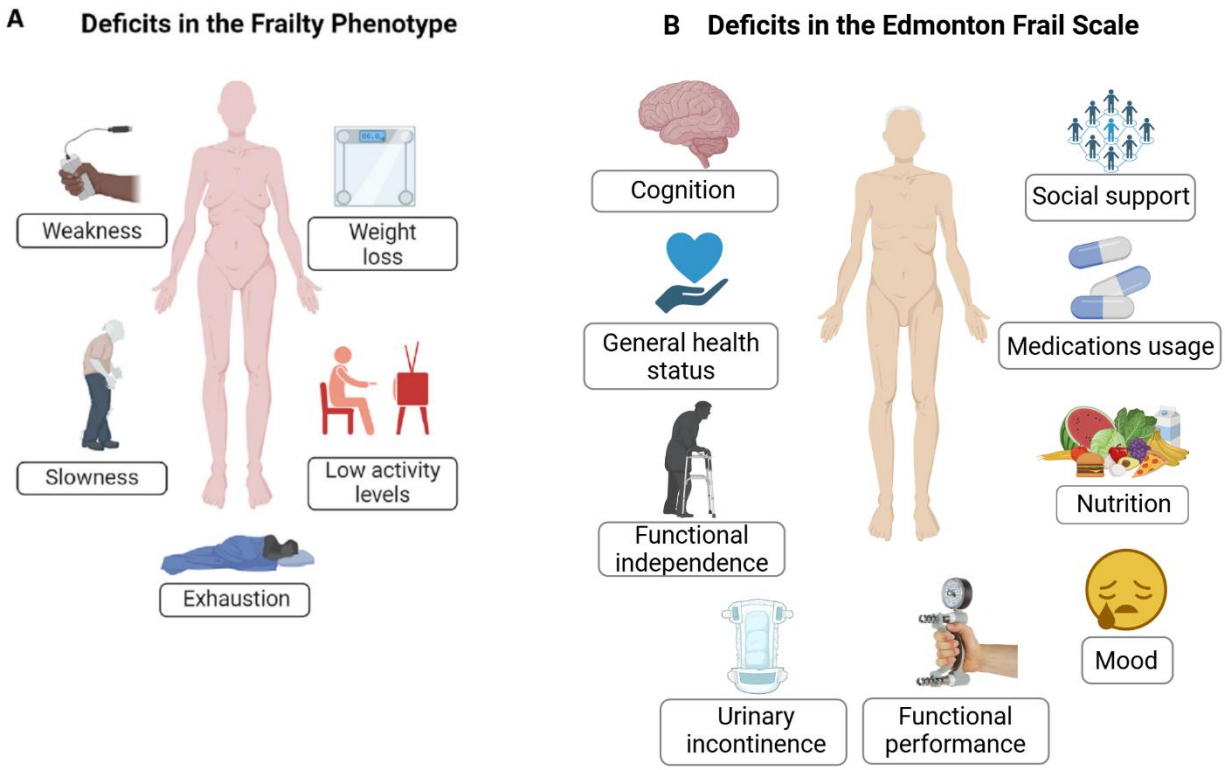

Figure 11. Items included in the Fried's frailty phenotype (A) and in the Edmonton Frail Scale (B). Created with Biorender.com.

5.1.2 Physiopathology of frailty and previous studies investigating blood metabolomic profile in frail populations

Despite the physiopathological mechanisms responsible for frailty still remain elusive, frailty prevalence and incidence have been linked to several defective physiological processes, including alterations in insulin resistance, energy-regulatory hormones, musculoskeletal system function and mitochondrial energy production, autonomic nervous system and systemic inflammation[4]. Consistent with the complex nature of frailty syndrome, several alterations involving multiple pathways and cellular processes in distinct organs have been disclosed by OMICS approaches[136]. In particular, recent metabolomics studies described biochemical alterations in frail subjects, including variations in anti-oxidant, inflammation, purine, urea cycle, kidney markers, tricarboxylic acid cycle and amino acids pathways [137–147]. The discovery of reliable biomarkers of frailty represents therefore a key milestone for identifying and monitoring the course of this syndrome along aging and, in turn, offering a possible therapeutic approach aimed at reverting frailty. However, previous OMICS results are inconsistent among independent studies [136] and, except for pro-inflammatory soluble cytokines, which are commonly increased in older frail subjects[147,148], a unified biochemical marker representative of this syndrome is currently lacking. Moreover, given the critical relevance of cognitive decline, and mood alterations reported in frailty[135,149,150], the

identification of a specific biochemical hallmark mirroring the progressive decay of brain functions before the occurrence of overt dementia represents an unmet clinical need.

Previous blood metabolomics studies identified a dysregulation in the homeostasis of glutamate pathway in frail individuals compared to controls[137,138,144–147]. Moreover, altered glutamate and aspartate metabolism has been associated in independent cohorts to sarcopenia[8,151,152], which represents one of the core features of the physical domain of frailty. In light of these findings, this study investigates by High-Performance Liquid Chromatography (HPLC) the serum concentrations of a pool of amino acids that collectively are known to modulate glutamatergic receptors activation (L-glutamate, L-aspartate, glycine, D-serine) or to represent the immediate precursors of these neuroactive molecules (L-glutamine, L-asparagine and L-serine) in a well-characterized cohort of elderly subjects encompassing the entire continuum from non-frail to frail condition. Noteworthy, in addition to their neuroactive role, these amino acids play critical roles in regulating various cellular pathways, including protein synthesis, tricarboxylic acid cycle, redox homeostasis, ammonium recycling, purine nucleotide cycle, folate and methionine cycles, and the synthesis of sphingolipids and phospholipids[153]. Consistently, these biomolecules have a vital relevance in orchestrating cognition, mood, energy homeostasis and immune system functions, as well as the metabolism of various peripheral organs, such as skeletal muscles, liver and kidney[4,149,154,155]. Given that the homeostasis of these systems and organs is severely affected in frail subjects, I investigated the relationship between the serum levels of these amino acids and frailty. Frailty status was assessed with (i) the EFS score, which I adopted as a reliable instrument mirroring the multidimensionality of frailty[132]; (ii) the Fried's phenotype, as a well-established tool to evaluate the physical domain of frailty[131]. I also took into account the effect of several comorbidities and health parameters representing key components of frailty and potentially impacting the blood levels of amino acids, including body mass index (BMI), visceral adipose tissue (VAT), sarcopenia, diabetes mellitus and cigarette smoking.

Finally, I will investigate the systemic metabolic framework of the same elderly cohort through untargeted serum metabolomics.

5.2 Methods

5.2.1 Participants

5.2.1.1 Enrolment and inclusion/exclusion criteria

Forty-five consecutive hospitalized subjects were recruited at the Physical Medicine and Rehabilitation Unit of Istituto Santa Margherita, Pavia, Italy, between February 2019 and August 2021. Eighty additional outpatients were recruited at the Endocrinology and Nutrition Unit of the same institute. The patients were included if (1) admitted for functional loss secondary to a non-disabling disease; (2) aged 65 years or older. The following exclusion criteria were applied: 1) any disease that could directly affect muscle strength (including neurological diseases, hip fractures or amputations); 2) dementia according to DSM-5 criteria[156]; 3) any systemic condition potentially affecting serum amino acid levels, including kidney, liver, rheumatologic and neoplastic diseases, history of drug or alcohol abuse; (4) history of altered serum creatinine levels (> 1.2 mg/dl) or liver function parameters (aspartate transaminase or alanine transaminase > 50 U/l).

Smoking status (current/former/never smoker) was assessed trough interview. The total number of drugs habitually taken by subjects was retrieved from medical records. This study was approved by the local ethics committee (protocol 20180097520, 09/11/2018) and was in conformity with the Helsinki Declaration. Written informed consent was obtained from all participants.

5.2.1.2 Cognitive and mood evaluation

Each subject underwent a standardized examination including evaluation of global cognition, performed through the Mini-Mental State Examination (MMSE) and Montreal Cognitive Assessment (MoCA)[157], and of depressive symptoms, measured with the Hamilton Depression Rating Scale (HAM-D)[158].

5.2.1.3 Quality of life

Quality of life was assessed through the Italian validation of the 36-Item Short Form Survey (SF-36)[159]. The arithmetic mean of the scores obtained in the nine scales of SF-36 was used as a global measure to compare the quality of life between non-frail and frail groups. I used the General Health scale score of SF-36 as a single frailty domain to be correlated with serum amino acids levels, since it is the SF-36 scale semantically closer to the General Health Status item of the EFS [132].

5.2.1.4 Sarcopenia and visceral adiposity

Body composition (fat mass (FM) and fat-free mass (FFM)) was evaluated using fan-beam dual-energy X-ray absorptiometry (DXA) (Lunar Prodigy DXA, GE Medical Systems). The *in vivo* coefficients of variation were 0.89% and 0.48% for FM and FFM, respectively. Skeletal Muscle Index (SMI) was calculated as the sum of fat-free soft tissue mass of arms and legs divided for height squared[160]. Visceral adipose tissue (VAT) volume was estimated using a constant correction factor (0.94 g/cm^3). The software automatically placed a quadrilateral box, representing the android region, outlined by the iliac crest and with a superior height equivalent to 20% of the distance from the top of the iliac crest to the base of the skull[161].

5.2.1.5 Functional performance and independence

Handgrip strength test was performed using a Jamar dynamometer adhering to the standardized protocol recommended by the American Society of Hand Therapists[162]. Handgrip measurement was assessed on the dominant hand and was considered "strong" or "weak" based on sex and body mass index (BMI)-adjusted cut-off scores, as previously described[131]. Basic Activities of Daily Living (BADL) and Independent Activities of Daily Living (IADL) were measured by interviewing the patients and caregivers[163].

5.2.1.6 Nutritional status

Nutritional status was evaluated with Mini Nutritional Assessment (MNA), which is composed of 18 items divided in four categories: anthropometric assessment, general state, dietary assessment and self-assessment. A score ≥ 24 points indicates a good nutritional status; a score between 17 and 23.5 points indicates risk of malnutrition, while a score ≤ 17 points indicates malnutrition[164].

5.2.1.7 Frailty

Frailty was separately evaluated with the EFS and the frailty phenotype. The EFS assesses nine frailty domains frailty (cognition, general health, functional independence, social support, medication usage, nutrition, mood, continence, functional performance)[132]. EFS score ranges from 0 to 17. Participants were classified as "non-frail" (EFS ≤ 5) or "frail" (EFS > 5) according to previously proposed cut-off [133]. Since only three subjects had an EFS score > 11 (used to define the "severe frail" category), I considered all the subjects with an EFS score > 5 as a single "frail" group.

The physical frailty phenotype contains 5 criteria, including weight loss, exhaustion, low physical activity, slow walking speed and low grip strength[131]. Participants who met 3 or more criteria

were defined “frail”, those who met 1 or 2 criteria were classified as “pre-frail” and those who met no criteria were defined “non-frail”.

5.2.2 Collection and storage of serum samples

Blood sampling was performed after a 12-hour fasting. Whole blood was collected by peripheral venipuncture into clot activator tubes and gently mixed. Sample was stored upright for 30 min at room temperature to allow blood to clot, and centrifuged at 2000 × g for 10 min at room temperature. Serum was aliquoted (0.5 ml) in polypropylene cryotubes and stored at –80 °C before usage.

5.2.3 HPLC analysis of amino acids content

Serum samples were analyzed following the same protocol adopted in the PD cohort (see paragraph 4.1.3).

5.2.4 HPLC Statistical analyses

Based on previous works assessing the serum levels of the same metabolites investigated in this study[12,13], I considered a sample size (n) equal to 125 (with at least n = 50 for both frail and non-frail groups) adapt to ensure adequate power and a medium effect size in between-group comparisons. Clinical and demographic characteristics were described using, as summary statistics, median and the interquartile range (IQR) or absolute and relative frequencies. The comparison of clinical-demographic features between non-frail and frail groups were performed with Mann-Whitney U test (for binary EFS-based stratification) or Kruskal-Wallis test (for the three frailty-phenotype categories) for continuous variables and Chi-square test for categorical variables. The normality of data distribution was checked with the Kolmogorov–Smirnov test. Due to non-normal distribution, the serum amino acid levels were $\log_{10}$-transformed and then compared between frail and non-frail groups using a four-way ANCOVA model with “frailty status”, “sex”, “type 2 diabetes” and “smoking” as factors and “age” and “BMI” as covariates. Levene’s test was used to check the equality of variances between groups.

The correlation of serum amino acid concentration with age was evaluated with Spearman’s correlation test. Partial correlation analyses adjusted for the effect of age and sex were adopted to test the correlation between serum amino acid levels and EFS score and the other clinical variables.

To assess the ability of serum amino acids levels to predict EFS score, I used multiple linear regression models including age, sex, the clinical predictors of EFS score[133] and the single amino acid concentrations as predictors and EFS score as dependent variable. To evaluate the ability of serum amino acids to predict the physical frailty phenotype[131] I adopted multinomial logistic regression models using age, sex and the single amino acid concentration as predictors and frailty category as dependent variable. For linear regression analyses, I verified that the residuals were normally distributed, there was no heteroscedasticity and no multicollinearity between the variables (variance inflation factor < 5). The latter was also evaluated in the logistic regression analyses. Significance was set at $p < 0.05$ for all analyses. All the statistical tests were two-sided. Data were analysed by using SPSS 26.0 software (IBM, Armonk, NY, USA).

5.2.5 ^{1}H-NMR serum analyses

NMR samples preparation and statistical analyses were conducted using the same protocol adopted for the PD cohort (see section 4.2.5).

5.3 Results

5.3.1 HPLC assessment of serum L- and D-amino acids concentration

5.3.1.1 Participants

One-hundred and twenty-five consecutive elderly subjects were enrolled in the study. The participants were stratified into non-frail (n = 74) and frail (n = 51) groups accordingly to EFS score [165]. Demographic and clinical features of study participants are reported in Table 8A. Frail subjects were older and showed higher females prevalence than non-frail participants. As expected, frail group showed worse performance in physical, sarcopenia, cognitive, nutritional, functional independence, and quality of life domains. Total medication count was higher in frail compared to non-frail group. The proportions of patients with type 2 diabetes mellitus and of current/former/never smokers were similar between non-frail and frail subjects.

MMSE and MoCA scores did not correlate with age in either non-frail ($r = 0.214$, $p = 0.075$ and $r = -0.013$, $p = 0.918$, respectively) or frail group ($r = -0.180$, $p = 0.220$ and $r = -0.236$, $p = 0.106$, respectively), indicating that the difference in MoCA score between non-frail and frail groups was not attributable to the older age of frail subjects.

A) Frail vs non-frail: clinical-demographic features					
	N	**Total**	**Non-frail**	**Frail**	**p**
Age, years	74 NF, 51 FR	74.0 (69.5-81.0)	72.0 (68.0-75.0)	81.0 (75.0-85.0)	**< 0.001**[a]
Female sex, n (%)	74 NF, 51 FR	95 (76.0)	51 (68.9)	44 (86.3)	**0.026**[b]
SPPB total score	74 NF, 51 FR	8.0 (5.0-10.0)	9.0 (8.0-10.0)	4.0 (3.0-7.0)	**< 0.001**[a]
Handgrip (kg)	74 NF, 51 FR	20.0 (16.0-26.0)	24.0 (20.0-32-0)	16.0 (12.0-20.0)	**< 0.001**[a]
SMI (kg/m^2)	74 NF, 51 FR	7.6 (7.1-8.6)	8.1 (7.1-8.9)	7.5 (6.9-8.1)	**0.012**[a]
MMSE	70 NF, 48 FR	27.1 (26.0-27.7)	27.2 (26.2-27.7)	27.1 (25.7-27.7)	0.374[a]
MoCA	70 NF, 48 FR	24.1 (21.5-26.1)	25.3 (23.4-26.7)	21.4 (19.7-25.1)	**< 0.001**[a]
MNA	74 NF, 50 FR	23.8 (20.6 – 25.5)	25.0 (23.5-26.0)	20.5 (18.5-23.1)	**< 0.001**[a]
BADL	72 NF, 46 FR	6.0 (6.0-6.0)	6.0 (6.0-6.0)	6.0 (5.0-6.0)	**0.001**[a]
IADL	72 NF, 46 FR	8.0 (6.0-8.0)	8.0 (8.0-8.0)	6.0 (4.0-8.0)	**< 0.001**[a]
HAM-D	72 NF, 46 FR	5.0 (2.0-10.0)	5.0 (2.0-9.8)	4.0 (2.0-12.0)	0.797[a]
SF-36 (mean score)	71 NF, 46 FR	66.8 (52.7-78.3)	73.7 (57.4-81.6)	61.9 (38.1-67.4)	**< 0.001**[a]
Number of drugs	72 NF, 49 FR	4.0 (2.5-8.0)	3.0 (2.0-5.0)	7.0 (5.0-11.5)	**< 0.001**[a]
Type 2 diabetes, n (%)	74 NF, 51 FR	21 (16.8)	10 (13.5)	11 (21.6)	0.236[b]
BMI (kg/m^2)	74 NF, 51 FR	27.7 (24.2-32.5)	27.9 (24.2-31.7)	27.6 (23.7-33.3)	0.752[a]
VAT (g)	73 NF, 51 FR	1035 (548-1557)	1049 (530-1652)	960 (555-1502)	0.463[a]
Current smokers, n (%)	74 NF, 51 FR	15 (12.0)	9 (12.2)	6 (11.8)	0.152[b]
Former smokers, n (%)	74 NF, 51 FR	22 (17.6)	9 (12.2)	13 (25.5)	
Never smokers, n (%)	74 NF, 51 FR	88 (70.4)	56 (75.7)	32 (62.7)	
EFS	74 NF, 51 FR	4.0 (2.0-7.0)	2.0 (1.0-4.0)	8.0 (6.0-9.0)	**< 0.001**[a]
B) Frail vs non-frail: serum amino acid levels					
	N	**Total (n = 125)**	**Non-frail (n = 74)**	**Frail (n = 51)**	**p**[c]
L-aspartate (µM)	74 NF, 51 FR	4.0 (3.0-5.6)	3.9 (3.0-5.5)	4.4 (3.1-6.5)	0.409
L-asparagine (µM)	74 NF, 51 FR	24.1 (19.8-34.3)	24.8 (20.8-28.0)	22.6 (19.2-28.8)	0.676
Glycine (µM)	74 NF, 51 FR	208.9 (174.0-288.2)	201.0 (161.8-268.3)	222.3 (185.6-400.4)	0.223
D-serine (µM)	74 NF, 51 FR	1.9 (1.6-2.3)	1.8 (1.5-2.1)	2.1 (1.7-2.6)	0.167
L-serine (µM)	74 NF, 51 FR	72.5 (60.0-88.9)	75.8 (62.3-89.5)	71.1 (54.5-85.1)	0.963
Glycine/L-serine	74 NF, 51 FR	2.9 (2.2-4.1)	2.6 (2.1-3.4)	3.2 (2.4-4.9)	0.270
D-/Total serine (%)	74 NF, 51 FR	2.5 (2.0-3.2)	2.3 (1.9-2.8)	2.8 (2.2-4.0)	0.181
L-glutamate (µM)	74 NF, 51 FR	26.7 (19.1-34.3)	25.3 (18.8-33.7)	28.3 (19.2-34.6)	0.299
L-glutamine (µM)	74 NF, 51 FR	323.0 (280.5-370.3)	325.3 (282.0-365.7)	316.5 (276.3-381.5)	0.456
L-glutamine/L-glutamate	74 NF, 51 FR	12.1 (9.8-16.7)	12.6 (10.1-16.6)	11.2 (9.8-17.4)	0.578

Table 8. (A) Clinical and demographic features of elderly cohort considered as a whole and after stratification by frailty status according to EFS. (B) Serum amino acid levels in elderly cohort considered as a whole and after stratification by frailty status. Data are shown as median (IQR) or absolute frequency (%) for continuous and categorical variables, respectively. The total number of non-frail (NF) and frail (FR) subjects for which data were

available is reported in the second column. [a] Mann-Whitney U test; [b] Chi Square test; [c] Four-way ANCOVA with frailty status, sex, diabetes and smoking as factors, age and BMI as covariates. The analysis was conducted on log-transformed amino acid concentrations to normalize the data distribution. Abbreviations: BADL, basic activities of daily living (preserved); BMI, body mass index; EFS, Edmonton Frailty Scale total score; HAM-D, Hamilton depression rating scale; IADL, instrumental activities of daily living (preserved); MMSE, mini-mental state examination; MNA, mini nutritional assessment; MoCA, Montreal Cognitive Assessment; SF-36, Short Form Health Survey 36 (SF-36 mean score was obtained by calculating the arithmetic mean of the scores relative to the 9 items of SF-36); SPPB, short physical performance battery; VAT, visceral adipose tissue.

5.3.1.2 Serum levels of D-serine and D-/Total serine ratio correlate with EFS score

I first investigated whether the serum levels of amino acids were different between frail and non-frail groups adjusting for the effect of the potential confounders. ANCOVA showed no between-group differences in D-serine, L-serine or any of the other amino acids level (Table 8B).

To further address this issue, I measured the partial correlation between the quantitative EFS score and the serum concentrations of amino acids, adjusting for age and sex. I found a significant mild positive correlation of EFS with serum D-serine ($r = 0.197$, $p = 0.032$) and D-/Total serine ratio ($r = 0.213$, $p = 0.020$), but not the other amino acids (Fig. 11).

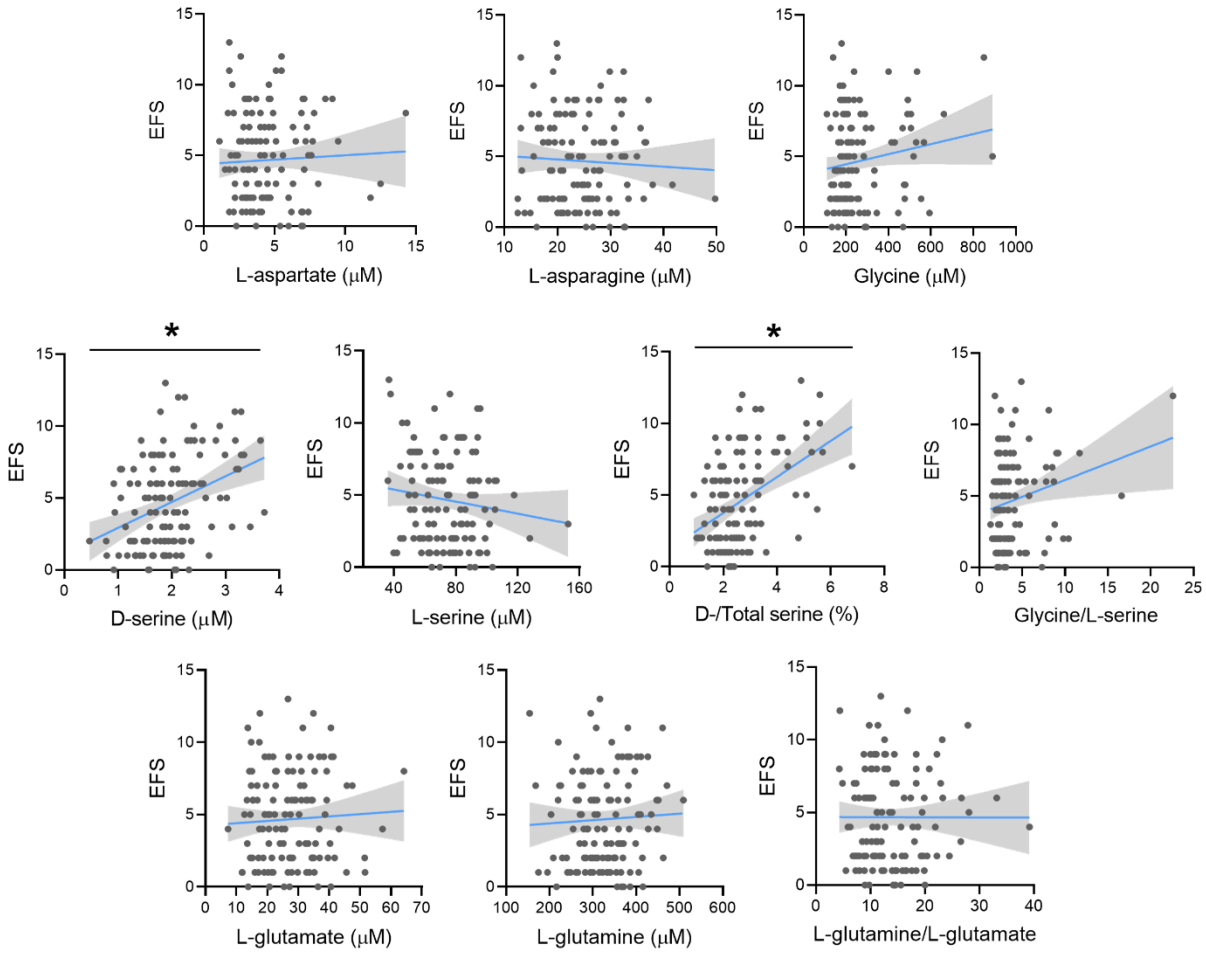

Figure 11. Correlations between the serum levels of amino acids and Edmonton Frailty Scale (EFS) total score in the whole elderly cohort. Blue lines and grey shadows represent the best fit line and its 95% CI, respectively. *p < 0.05, age- and sex-adjusted partial correlation.

5.3.1.3 Correlation of Serum levels of D-serine and D-/Total serine ratio with demographic and clinical features

I also investigated whether diabetes, obesity (BMI, VAT), sarcopenia (SMI) and cigarette smoking affected the serum concentration of amino acids. Diabetic subjects showed higher levels of L-asparagine, L-serine, L-glutamate, L-glutamine/L-glutamate ratio and lower glycine/L-serine ratio than non-diabetic participants (Table 9). After adjustment for age and sex, L-glutamate and L-Glutamine/L-Glutamate correlated with (i) BMI and VAT in both non-frail and frail participants; (ii) SMI only in the non-frail group (Table 10). Current and former smokers had reduced L-glutamine/L-glutamate ratio compared to never smokers (median [IQR]: never smokers, 13.0 [10.5-17.6]; former smokers, 10.2 [8.9-13.7]; current smokers, 9.9 [7.4-14.8]).

Serum D-serine correlated with age in the frail (r = 0.299, p = 0.033) but not in non-frail group, while D-/Total serine ratio correlated with age both in non-frail (r = 0.278, p = 0.017) and frail subjects (r = 0.415, p = 0.002) (Fig. 12).

	Non-diabetic (n = 104)	Diabetic (n = 21)	p[a]
L-aspartate (µM)	3.9 (2.9-5.2)	4.7 (3.6-7.3)	0.056
L-asparagine (µM)	23.4 (19.8-27.7)	31.1 (21.0-34.2)	**0.004**
Glycine (µM)	215.6 (173.9-313.6)	198.9 (176.2-225.2)	0.126
D-serine (µM)	1.9 (1.6-2.2)	1.8 (1.5-2.6)	0.828
L-serine (µM)	71.3 (59.0-85.6)	83.4 (60.8-94.8)	**0.015**
Glycine/L-serine	3.0 (2.3-4.4)	2.2 (2.0-3.3)	**0.010**
D-/Total serine (%)	2.5 (2.0-3.2)	2.5 (1.7-3.3)	0.104
L-glutamate (µM)	25.0 (17.8-31.8)	35.6 (27.8-40.0)	**0.003**
L-glutamine (µM)	317.7 (279.3-368.5)	337.1 (293.3-398.5)	0.217
L-glutamine/L-glutamate	12.7 (9.9-18.0)	10.2 (8.6-12.7)	**0.033**

Table 9. Serum amino acids levels in elderly cohort stratified in subjects with and without type 2 diabetes mellitus. Data are shown as median (IQR). [a] Two-way ANCOVA on log-transformed amino acids concentrations with diabetes and sex as factors, age as covariate.

	BMI				VAT				SMI			
	Non-frail		Frail		Non-frail		Frail		Non-frail		Frail	
	r	p	r	p	r	p	r	r	r	p	r	p
L-aspartate (µM)	0.120	0.316	0.003	0.984	0.145	0.229	0.136	0.352	0.068	0.573	-0.127	0.386
L-asparagine (µM)	-0.111	0.352	-0.048	0.743	-0.040	0.743	-0.050	0.732	0.001	0.995	0.094	0.521
Glycine (µM)	-0.055	0.645	-0.101	0.488	-0.025	0.839	-0.055	0.708	-0.054	0.649	-0.054	0.710
D-serine (µM)	0.066	0.584	-0.171	0.241	0.081	0.502	-0.027	0.852	0.076	0.527	-0.189	0.192
L-serine (µM)	-0.044	0.712	0.040	0.786	-0.060	0.621	0.140	0.336	0.044	0.714	-0.015	0.918
Glycine/L-serine	-0.022	0.855	-0.058	0.691	0.014	0.906	-0.057	0.696	-0.058	0.626	0.019	0.896
D-/Total serine (%)	0.091	0.448	-0.155	0.289	0.120	0.319	-0.142	0.331	0.054	0.650	-0.116	0.427
L-glutamate (µM)	0.288	**0.014**	0.268	0.063	0.261	**0.028**	0.430	**0.002**	0.328	**0.005**	0.022	0.879
L-glutamine (µM)	-0.123	-0.304	-0.263	0.068	-0.128	0.287	-0.135	0.355	0.092	0.442	-0.237	0.101
L-glutamine/L-glutamate	-0.290	**0.013**	**-0.385**	**0.006**	-0.322	**0.006**	-0.424	**0.002**	-0.233	**0.049**	-0.161	0.268

Table 10. Correlations between the serum levels of amino acids and BMI, VAT and SMI in the elderly cohort. Data were available for 74 non-frail and 51 frail (BMI, SMI) and 73 frail and 51 frail subjects (VAT). Abbreviations: BMI, body mass index; SMI, skeletal muscle index; VAT, visceral adipose tissue. p-values refer to age and sex-adjusted partial correlations.

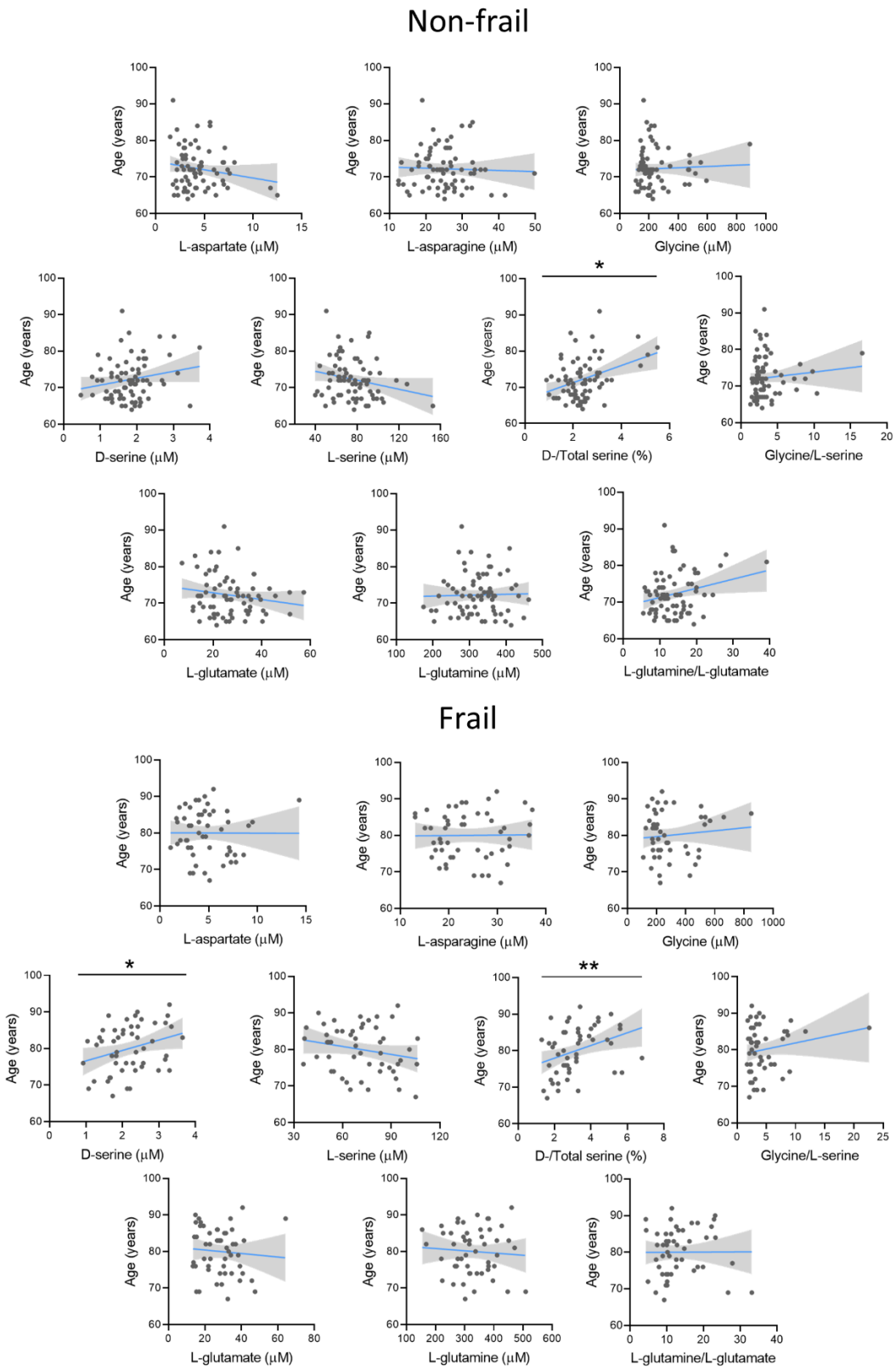

Figure 12. Correlations between the serum amino acids concentrations and age in elderly cohort stratified in frail and non-frail groups according to EFS. Blue lines and grey shadows represent the best fit line and its 95% CI, respectively. * $p < 0.05$; **$p < 0.01$, Spearman's correlation test.

5.3.1.4 Serum levels of D-serine and D-/Total serine ratio are independent predictors of frailty

Furthermore, to assess whether serum levels of D-serine and D-/Total serine ratio are independently associated with frailty, I performed multiple linear regression models using the quantitative EFS score as dependent variable and the known clinical predictors of EFS [133], added to the individual amino acids concentrations, as predictors. Interestingly, increased levels of D-serine and D-/Total serine ratio, but not the other amino acids, resulted to be independent predictors of EFS score, along with older age and worse nutritional status, handgrip, global cognition and higher number of drugs (Table 11). These findings highlight that an abnormally greater serum D-/Total serine ratio, used as an index of D-serine metabolism [31], along with blood D-serine concentrations, may represent a putative biochemical marker of frailty in elderly people.

Model 1: D-serineine and clinical features as predictors of EFS				
	β	**SE**	**Std β**	**p**
Constant	3.087	3.833		0.422
Age (years)	0.115	0.036	0.255	**0.002**
Male sex	0.214	0.649	0.029	0.742
MNA	-0.159	0.068	-0.167	**0.022**
Handgrip (kg)	-0.087	0.034	-0.260	**0.011**
BADL	0.095	0.269	0.031	0.723
IADL	-0.170	0.168	-0.101	0.313
HAM-D	0.021	0.030	0.042	0.477
MoCA	-0.144	0.063	-0.151	**0.025**
Number of drugs	0.170	0.056	0.215	**0.003**
D-serine (μM)	0.704	0.321	0.136	**0.031**
Model 2: D-/Total serine and clinical features as predictors of EFS				
	β	**SE**	**Std β**	**p**
Constant	4.635	3.822		0.228
Age (years)	.102	.038	.226	**0.008**
Male sex	.201	.645	.027	0.756
MNA	-.198	.069	-.208	**0.005**
Handgrip (kg)	-.083	.034	-.248	**0.015**
BADL	.065	.268	.021	0.809
IADL	-.125	.168	-.075	0.458
HAM-D	.017	.030	.033	0.574
MoCA	-.140	.063	-.147	**0.029**
Number of drugs	.167	.056	.212	**0.003**
D-/Total serine (%)	.553	.228	.162	**0.017**

Table 11. Multiple linear regression models for EFS prediction, including clinical variables and serum D-serine (model 1) or D-/Total serine ratio (model 2) as predictors. Complete clinical data were available for n = 110 subjects. Abbreviations: BADL, basic activities of daily living (preserved); EFS, Edmonton Frailty Scale; IADL, instrumental activities of daily living (preserved); HAM-D, Hamilton Depression Rating Scale; MNA, Mini Nutritional Assessment; MoCA, Montreal Cognitive Assessment; SE, standard error of β; Std β, standardized β coefficient.

5.3.1.5 Increased serum glycine/L-serine and D-/Total serine ratios correlate with worse global cognition in frail elderly subjects

Next, I investigated whether serum D-serine, D-/Total serine ratio and the other amino acids were associated with one or more of the frailty domains which concur to determine the EFS score. Notably, I found negative partial correlations between (i) glycine, glycine/L-serine ratio, D-/Total serine ratio and MMSE; (ii) glycine, glycine/L-serine ratio and MoCA score in the frail but not in the non-frail subjects (Fig. 13). The other amino acids did not correlate with cognitive measures (data not shown). Moreover, L-asparagine and L-glutamine correlated negatively with HAM-D score ($r = -0.403$, $p = 0.007$ and $r = -0.347$, $p = 0.021$, respectively), while glycine levels and glycine/L-serine ratio increased with worse depressive symptoms in frail but not in non-frail subjects ($r = 0.389$, $p = 0.009$ and $r = 0.526$, $p = < 0.001$, respectively). There were no significant correlations between the serum amino acids and the other frailty domains. Overall, these findings indicate that dysregulated blood glycine/L-serine and D-/Total serine ratios may represent metabolic biomarkers of cognitive impairment and depressive symptoms in frail older subjects.

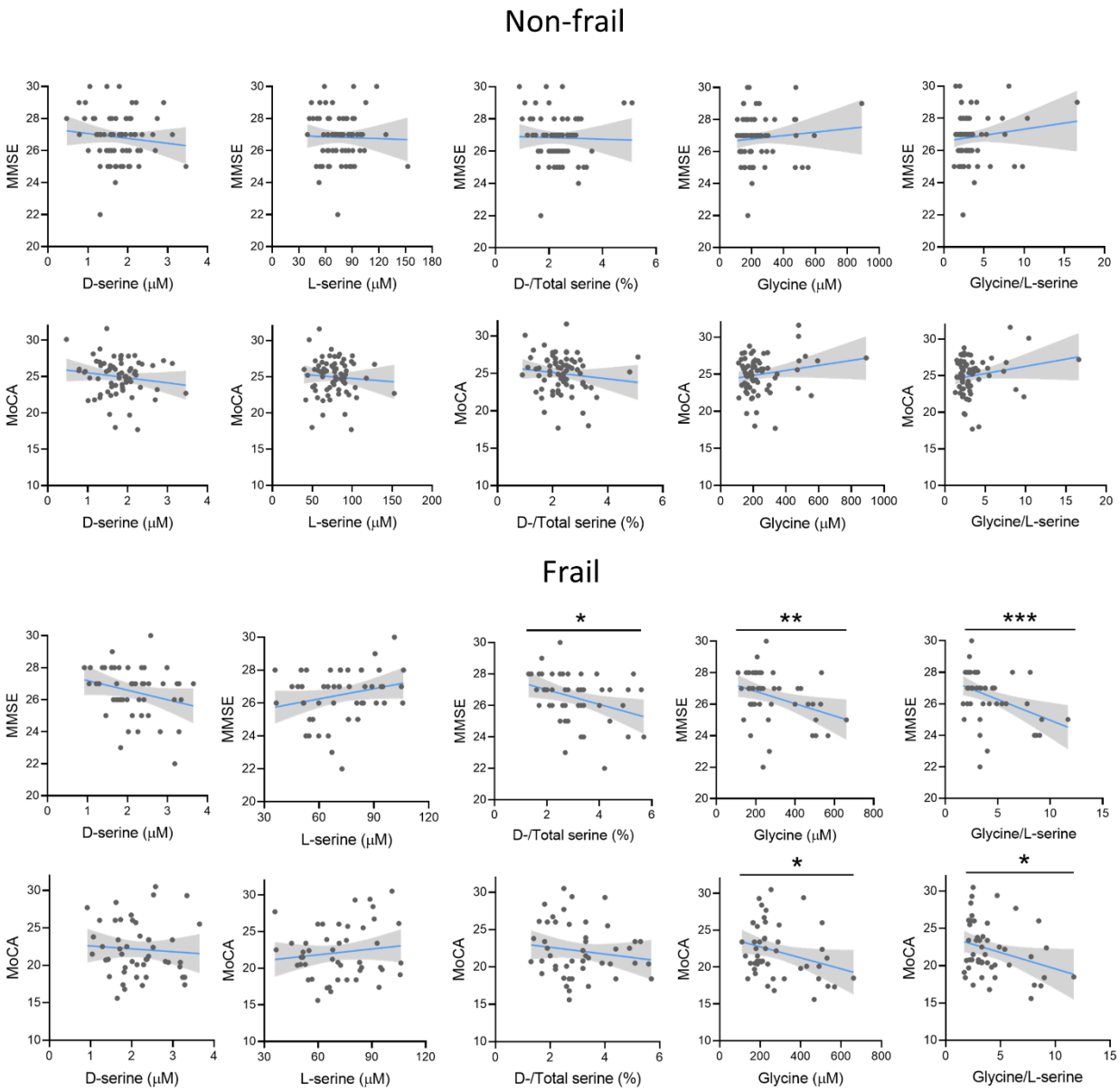

Figure 13. Correlations between the serum amino acids concentrations and measures of global cognition in elderly cohort stratified in frail and non-frail groups according to EFS. Blue lines and grey shadows represent the best fit line and its 95% CI, respectively. * $p < 0.05$; **$p < 0.01$, age and sex-adjusted partial correlations. Abbreviations: MMSE, Mini-Mental State Examination; MoCA, Montreal Cognitive Assessment.

5.3.1.6 Serum D-serine and D/Total serine do not correlate with physical frailty phenotype

To further evaluate the relationship between serum amino acids and frailty, I stratified the elderly cohort according to Fried's frailty phenotype [131]. Based on these criteria, 22 subjects were classified as non-frail, 51 as pre-frail and 52 as frail. After adjusting for the effect of potential confounders, there were no significant differences in the serum concentrations of the tested amino acids between the 3 groups (Table 12). To better assess whether the serum levels of these metabolites

may associate with physical frailty phenotype, I performed multinomial logistic regression models with Fried phenotype as dependent variable and age, sex and the individual amino acids concentrations as predictors. Remarkably, I found that neither the levels of D-serine (Table 13), nor those of the other amino acids (data not shown), were associated with physical frailty or pre-frailty status. Taken together, these findings suggest that the blood levels of D-serine and D-/Total serine ratio are not associated with the physical domain of frailty in elderly individuals.

	Non-frail (n = 22)	**Pre-frail (n = 51)**	**Frail (n = 52)**	**Total (n = 125)**	**p**
Age, years	68.0 (66.0-72.0)	72.0 (69.0-76.0)[d]	82.0 (76.0-85.0)[e,f]	74.0 (69.5-81.0)	**< 0.001[a]**
Female sex, n (%)	13 (59.1)	37 (72.5)	45 (86.5)	95 (76.0)	**0.031[b]**
L-aspartate (µM)	3.7 (3.1-5.2)	4.0 (3.0-6.3)	4.1 (2.9-5.5)	4.0 (3.0-5.6)	0.979[c]
L-asparagine (µM)	25.0 (20.5-27.8)	24.1 (20.0-27.8)	23.4 (19.1-29.6)	24.1 (19.8-28.4)	0.833[c]
Glycine (µM)	192.4 (144.8-229.0)	202.3 (163.7-287.0)	224.7 (186.1-330.0)	208.8 (174.0-288.1)	0.166[c]
D-serine (µM)	1.8 (1.5-2.1)	1.8 (1.4-2.1)	2.2 (1.7-2.6)	1.9 (1.5-2.3)	0.576[c]
L-serine (µM)	77.4 (64.0-89.4)	71.7 (62.0-89.4)	72.1 (53.4-84.8)	72.5 (59.9-88.9)	0.993[c]
Glycine/L-serine	2.3 (2.0-2.8)	2.8 (2.2-4.0)	3.3 (2.4-5.1)	2.9 (2.2-4.1)	0.234[c]
D-/Total serine (%)	2.3 (2.0-2.5)	2.4 (1.9-2.9)	2.8 (2.1-4.0)	2.5 (2.0-3.2)	0.633[c]
L-glutamate (µM)	25.3 (20.2-34.7)	27.0 (18.9-34.5)	26.7 (17.4-33.7)	26.7 (19.0-34.2)	0.970[c]
L-glutamine (µM)	345.4 (298.8-374.8)	315.1 (280.5-358.4)	317.6 (275.9-381.4)	323.0 (280.5-370.3)	0.771[c]
L-glutamine/L-glutamate	13.3 (10.3-16.5)	12.0 (9.2-16.4)	12.4 (9.9-18.2)	12.0 (9.8-16.7)	0.928[c]

Table 12. Demographic features and serum amino acid levels in elderly cohort stratified according to Fried's frailty phenotype. Data are shown as median (IQR) or absolute frequency (%) for continuous and categorical variables, respectively. [a] Kruskal-Wallis test; [b] Chi-square test; [c] Four-way ANCOVA with frailty status, sex, diabetes and smoking as factors, age and BMI as covariates. The analysis was conducted on log-transformed amino acid concentrations to normalize the data distribution and followed by post hoc tests with Bonferroni correction; [d] compared with non-frail, $p < 0.05$; [e] compared with non-frail, $p < 0.001$; [f] compared with pre-frail, $p < 0.001$.

Model A				
	β	**SE**	**p**	**OR (95%CI)**
Pre-frail				
Intercept	-15.813	5.351	0.003	
D-serine (μM)	0.360	0.525	0.493	1.4 (0.5-4.0)
Age (years)	0.221	0.076	**0.004**	1.2 (1.1-1.4)
Female sex	0.601	0.594	0.311	1.8 (0.6-5.8)
Frail				
Intercept	-31.930	6.135	< 0.001	
D-serine (μM)	.784	.604	0.194	2.1 (0.7-7.1)
Age (years)	.410	.085	**< 0.001**	1.5 (1.3-1.8)
Female sex	1.556	.787	0.048	4.7 (1.0-22.2)
Model B				
	β	**SE**	**p**	**OR (95%CI)**
Pre-frail				
Intercept	-15.341	5.308	0.004	
D-/Total serine (%)	0.076	0.401	0.851	1.0 (0.4-2.3)
Age (years)	0.221	0.078	**0.005**	1.2 (1.1-1.4)
Female sex	0.528	0.582	0.364	1.6 (0.5-5.3)
Frail				
Intercept	-30.913	6.084	< 0.001	
D-/Total serine (%)	0.295	0.426	0.490	1.3 (0.5-3.1)
Age (years)	0.407	0.087	**< 0.001**	1.5 (1.3-1.8)
Female sex	1.486	0.784	0.058	4.4 (0.9-20.5)

Table 13. Multinomial logistic regression models for frailty phenotype prediction according to Fried criteria including A) D-serine and B) D-/Total serine as predictor. Non-frail status was set as reference category.

5.3.1.7 The correlations between serum D-serine, D-/Total serine ratio and EFS are driven by female sex

The serum concentrations of amino acids were similar between females and males (Table 14). D-serine (females: rho = 0.315, p = 0.002; males: rho =0.416, p =0.022), D/Total serine (females: rho = 0.430, p < 0.001; males: rho = 0.619, p < 0.001) positively correlated with age in both sexes, while L-serine (rho= -0.370, p = 0.044) and glycine/L-serine (rho = 0.463, p = 0.010) selectively decreased with older age only in males. Consistent with these HPLC data, I found that the positive correlation between D-serine, D-/Total serine and EFS score observed in the whole cohort (Fig. 11) was mainly driven by female sex (D-serine: females, r = 0.240, p = 0.023; males, r = -0.040, p = 0.850; D-/Total serine: females, r = 0.273, p = 0.009; males, r = -0.240, p = 0.247). Multiple linear regression models adjusted for the clinical predictors of EFS showed that the levels of D-serine and D-/Total serine, but not the other amino acids, were independent predictors of EFS score in females (β = 0.989, p = 0.008 and β = 0.748, p = 0.007, respectively) but not in males (β = -0.800, p = 0.208 and β = -0.615, p = 0.172, respectively).

	Females (n = 95)	Males (n = 30)	p[a]
L-aspartate (µM)	4.1 (3.1-6.2)	3.6 (2.9-5.2)	0.307
L-asparagine (µM)	23.3 (19.3-27.9)	25.5 (21.2-31.4)	0.184
Glycine (µM)	215.9 (178.4-302.9)	196.2 (157.4-229.2)	0.123
D-serine (µM)	1.9 (1.5-2.3)	2.0 (1.6-2.4)	0.074
L-serine (µM)	73.7 (59.8-87.3)	72.2 (62.1-90.7)	0.980
Glycine/L-serine	3.0 (2.3-4.4)	2.4 (2.0-3.4)	0.380
D-/Total serine (%)	2.5 (2.0-3.2)	2.6 (2.0-3.3)	0.150
L-glutamate (µM)	26.7 (18.7-33.6)	25.2 (19.1-34.9)	0.523
L-glutamine (µM)	316.5 (276.4-365.0)	339.9 (292.9-399.7)	0.419
L-glutamine/L-glutamate	12.0 (9.9-16.4)	13.4 (9.2-18.5)	0.281

Table 14. Serum amino acid levels in elderly cohort after stratification by sex. Data are shown as median (IQR). [a] Three-way ANCOVA on log-transformed amino acid levels with sex, smoking and diabetes as factors, age and BMI as covariates.

5.3.1.8 *Increased serum glycine/L-serine and D-/Total serine ratios correlate with worse global cognition and quality of life in a sex-dependent manner*

I found a negative correlation between D-serine (r = -0.222, p = 0.035), D-/Total serine (r = -0.289, p = 0.006), glycine/L-serine (r = -0.299, p = 0.004) and MMSE score in females but not in males. Moreover, D-/Total serine negatively correlated with MoCA (r = -0.235, p = 0.025), SF-36 General Health (r = -0244, p = 0.021) and SPPB total score (r = -0.209, p = 0.043) in females but not in males. Finally, glycine and glycine/L-serine ratio increased with worse depressive symptoms (assessed through HAM-D score) in females but not in males (glycine, females: r = 0.216, p = 0.042; males: r = 0.013, p = 0.94; glycine/L-serine, females r = 0.334, p = 0.001; males: r = -0.008, p = 0.967.

5.3.2 ^{1}H-NMR untargeted metabolomics

Blood sera of a subgroup of 96 subjects classified as frail (n = 37), pre-frail (n = 20), and non-frail (n = 39) were studied for their metabolomic profile using NMR spectroscopy analysis, as previously reported [166]. ^{1}H resonance assignment detected the presence of 45 metabolites for each spectrum. Partial least-squares discriminant analysis (PLS-DA) score plots comparing frail, pre-frail, and non-frail groups (Fig. 14a) showed a clear separation of the three clusters with different metabolomic profiles reporting significant validation indexes (0.93 and 0.97 accuracy PC1 and PC2, respectively, with positive 0.78 and 0.79 Q2 indices). The main metabolites responsible for the metabolomic differences in the three groups were identified using VIP score analysis (Fig. 14b). In particular, increased malonate, leucine, and succinate characterised the blood metabolomic profile of frail

subjects, while betaine, histidine, serine, glutamate and ornithine were remarkably upregulated in the serum of the pre-frail group. Most importantly, the discriminatory power of betaine, glutamate, histidine and malonate was also confirmed in the comparison between frail and pre-frail participants (Fig. 14c-d).

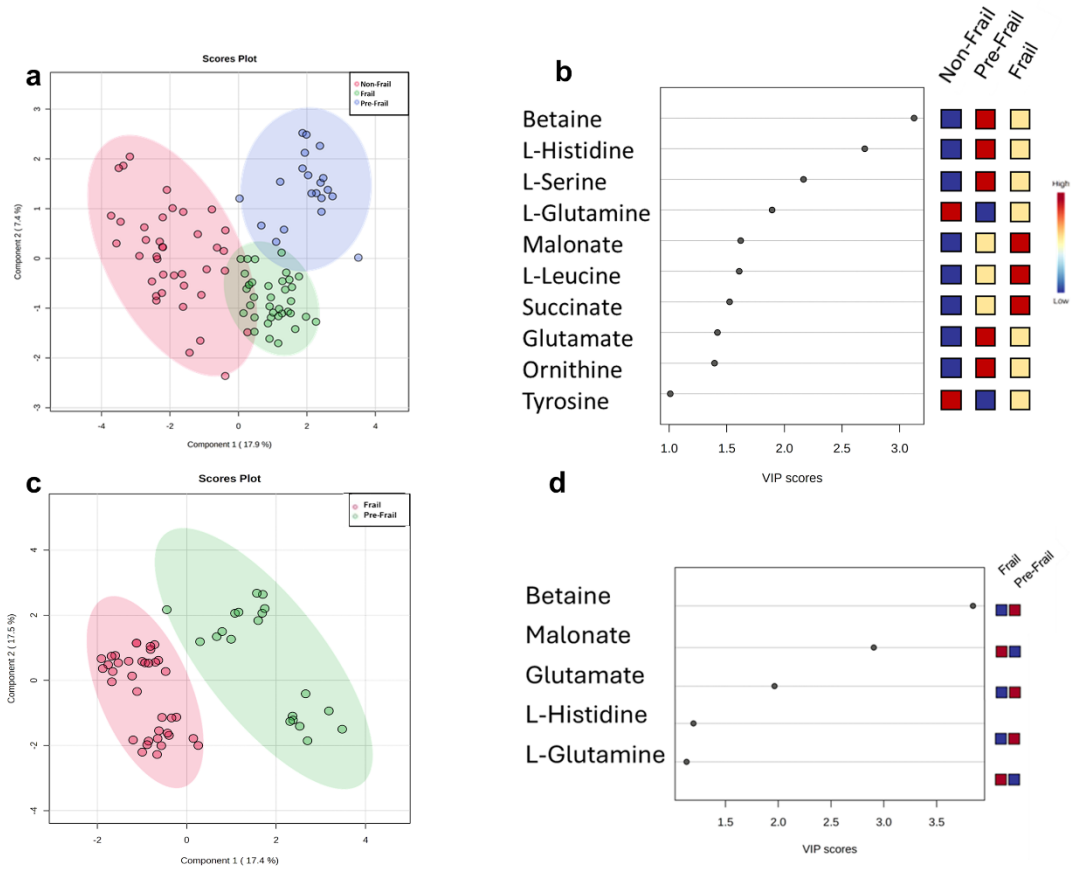

Figure 14. a. PLS-DA score scatter plots related to serum from pre-frail (n=20), frail (n=37) and non-frail participants (n=39). The cluster analyses are reported in the Cartesian space described by the main components PC1:17.9% and PC2:7.4%. Cross-validation (CV) tests performed according to the PLS-DA statistical protocol show a significant cluster separation (0.93 and 0.97 accuracy PC1 and PC2, respectively, with positive 0.78 and 0.79 Q2 indices). **b.** VIP score graphs of metabolites discriminating the three clusters. **c.** PLS-DA score scatter plots related to serum from pre-Frail (n=20), and frail subjects (n=37). The cluster analyses are reported in the Cartesian space described by the main components PC1:17.4% and PC2:17.5%. CV tests show a significant cluster separation (0.76 and 0.87 accuracy PC1 and PC2 respectively, with positive 0.69 and 0.75 Q2 indices). **d.** VIP score graphs of metabolites discriminating the two clusters.

Next, enrichment analyses were used to identify the distinct biochemical pathways modulated by the frailty metabotype. Pairwise comparisons revealed the significant enrichment of 19 and 28 pathways in pre-frail and frail participants, respectively, compared to non-frail subjects. The dysregulated pathways were mainly related to the metabolism of amino acids, ammonia recycling, energy

metabolism and lipids metabolism (Fig. 15a-b). The comparison between pre-frail and frail subjects showed six enriched pathways, mostly associated with amino acids metabolism (Fig. 15c). Interestingly, glycine-serine metabolism emerged as one of the most significantly enriched pathways in all three comparisons (Fig. 15a-c). In addition, to disclose potential metabolomic signatures specifically associated with pre-frail and frail phenotypes, the pathways revealed by the comparisons with the non-frail group were extracted and represented in a Venn diagram (Fig. 11d). Of note, betaine metabolism emerged as the exclusive dysregulated pathway of pre-frail compared to non-frail subjects. Conversely, frail patients showed a specific enrichment in 10 pathways related to lipid metabolism (ketone bodies, oxidation of branched-chain fatty acids, phosphatidylethanolamine biosynthesis, sphingolipids metabolism, butyrate metabolism and propanoate metabolism), energy and mitochondrial metabolism (carnitine metabolism and citric acid cycle), histidine metabolism and folate metabolism. Finally, frail and pre-frail groups revealed a common dysregulation of several amino acid pathways (Fig. 15d).

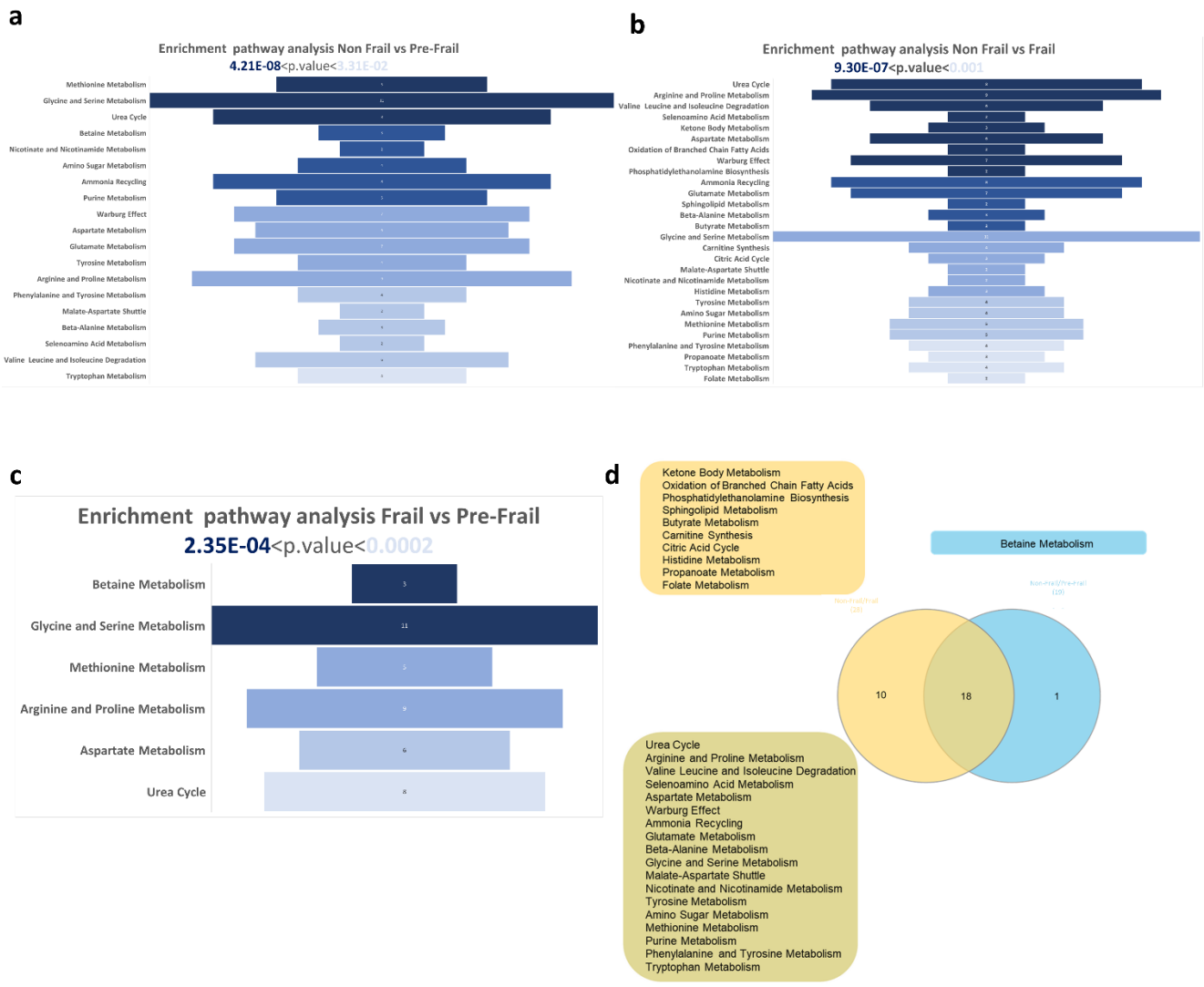

Figure 15. Enrichment pathway analysis performed comparing: **a** non-frail vs pre-frail, **b** non-frail vs frail and **c** frail vs pre-frail subjects. The discriminative pathways are ranked according to p-value and number of hits reported in the bars. **d** Venn diagram displaying the disrupted pathways emerged from the comparisons of frail and pre-frail subjects with non-frail controls. Blue box reports the unique pathway dysregulated in pre-frail but not in frail subjects; light yellow box reports the pathways enriched in frail but not in pre-frail subjects; dark yellow box reports the common pathways dysregulated in both frail and pre-frail participants.

Next, univariate analysis using robust volcano plot, which showed (i) increased serine, histidine, glutamate, ornithine, and betaine and (ii) decreased glutamine, aspartate, phenylalanine, tyrosine, alanine, valine and formate levels in pre-frail subjects compared to non-frail subjects (Fig. 16a). Moreover, robust volcano plot analysis revealed (i) decreased levels of the amino acids aspartate, valine, phenylalanine, tyrosine and (ii) increased malonate, arginine and serine levels in frail subjects compared to non-frail controls (Fig. 16b). Finally, I observed higher concentrations of glutamate, betaine, and histidine and lower concentrations of arginine and malonate in the sera of pre-frail subjects compared to the frail group (Fig. 16c).

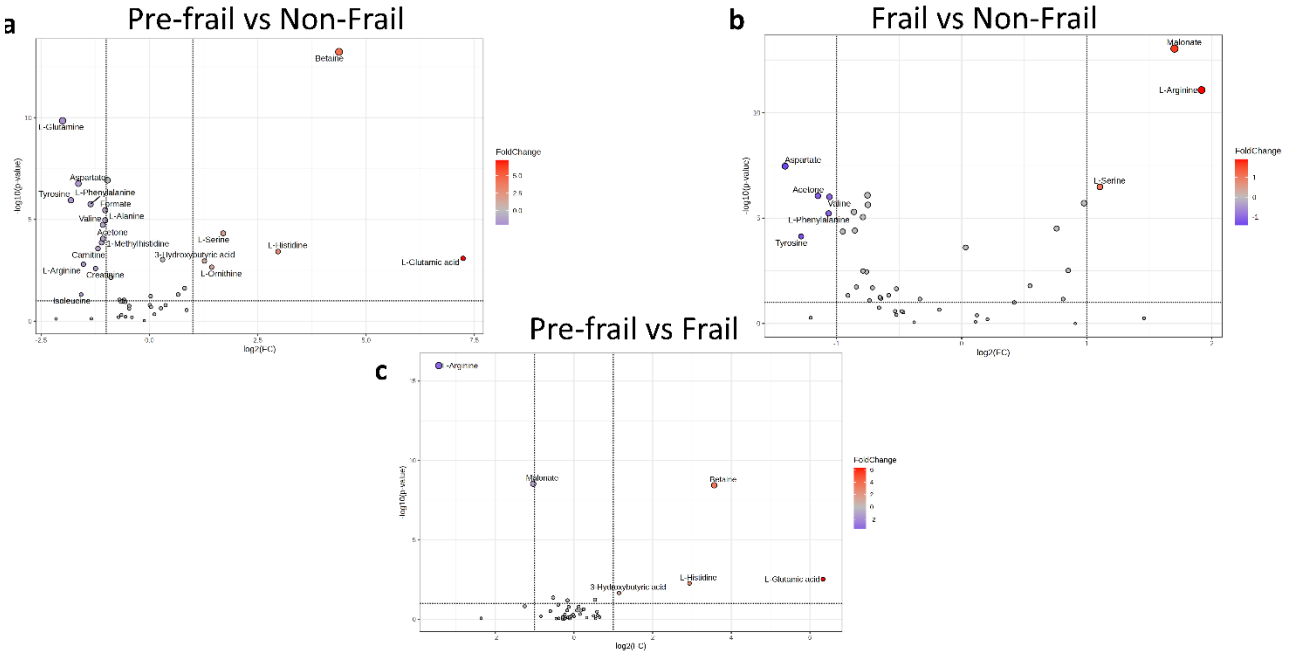

Figure 16. a-c Volcano plot analyses of metabolic changes in pre-frail vs non-frail, frail vs non-frail and pre-frail vs frail subjects' serum. Each point on the volcano plot was based on p- and fold-change values, set at 0.05 and 1.0, respectively. Red and blue circles identify upregulated and downregulated metabolites, respectively. Variations are expressed as follows: panels a-b are graphed as a function of pathological group; panel c is graphed as a function of pre-frail group.

Given the potential role of sex in modulating frailty-related phenotypes[167] and serum metabolomic profile[168], robust volcano plots between sexes were carried out. Interestingly, my data showed that the increase in betaine and glutamate were characteristic of the pre-frail females when compared to

non-frail females (Fig. 17a). Furthermore, the comparison between pre-frail and frail females revealed the influence of sex in modulating betaine and serine blood levels, which were specifically upregulated in pre-frail females (Fig. 17c). Conversely, frail and pre-frail males showed a peculiar up-regulation of malonate compared to non-frail males (Fig. 18a-b). The discriminating role of malonate in prefrail males is further confirmed when compared its occurrence with frail males (Fig. 18c).

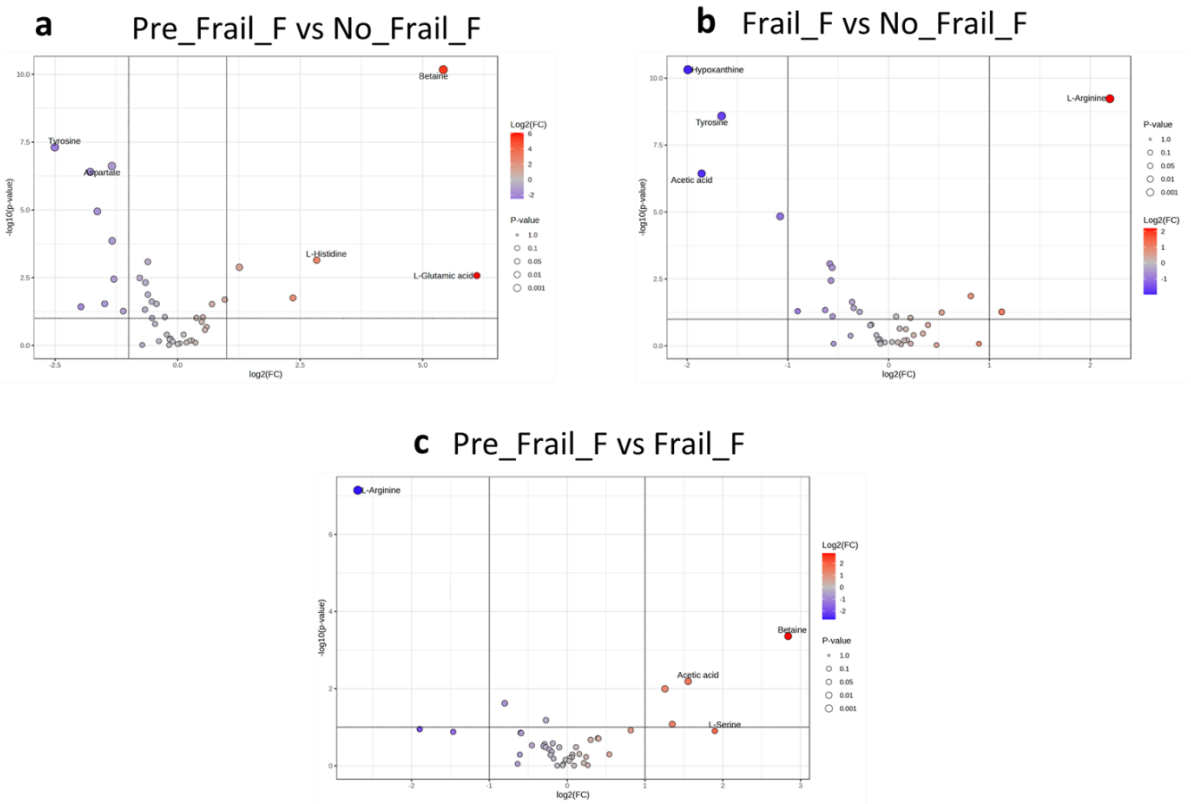

Figure 17. Volcano plot analyses of metabolic changes in pre-frail vs non-frail, frail vs non-frail and pre-frail vs frail female subjects' serum. Each point on the volcano plot was based on p- and fold-change values, set at 0.05 and 1.0, respectively. Red and blue circles identify upregulated and downregulated metabolites, respectively. Variations are expressed as follows: panels **a-b** are graphed as a function of pathological groups; panel **c** is graphed as a function of pre-frail group.

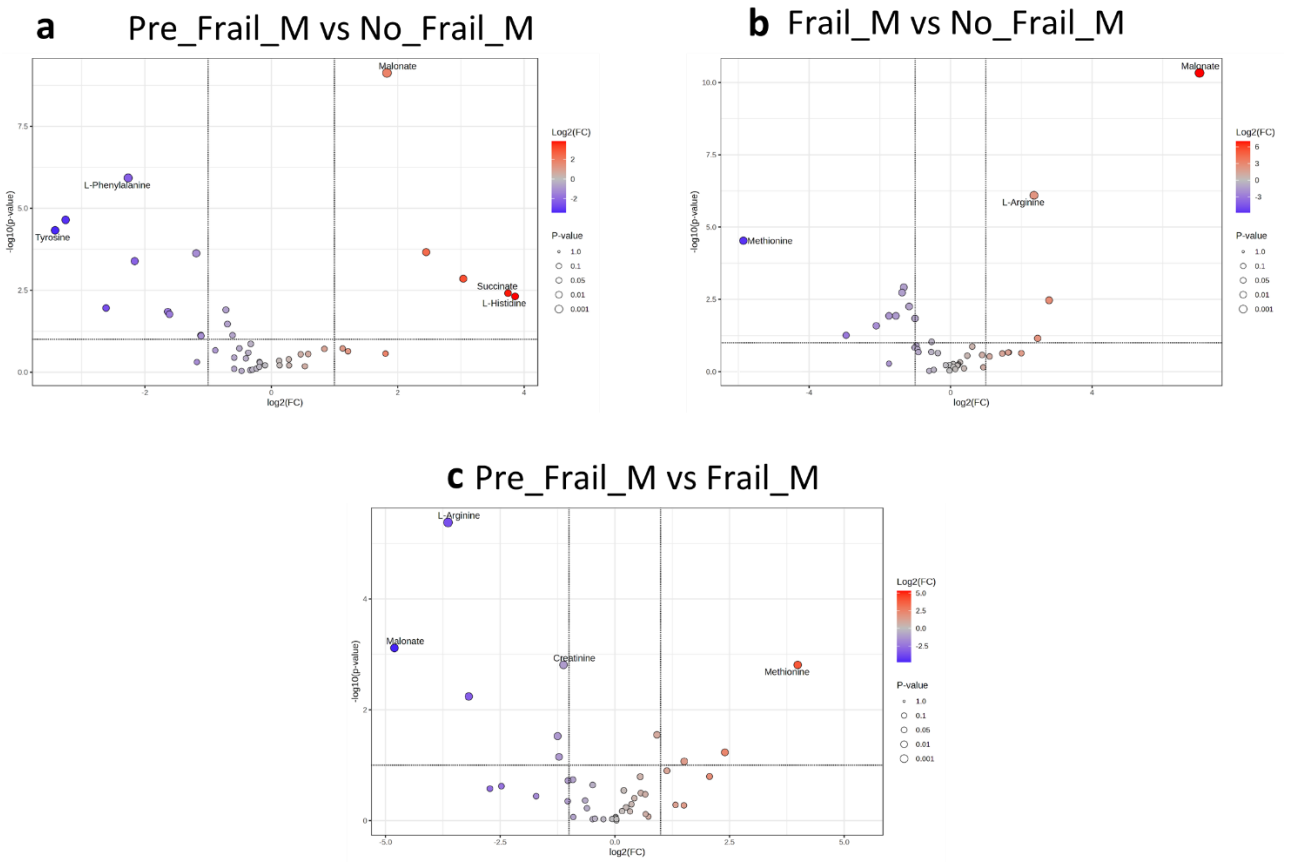

Figure 18. Volcano plot analyses of metabolic changes in pre-frail vs non-frail, frail vs non-frail and pre-frail vs frail male subjects' serum. Each point on the volcano plot was based on p- and fold-change values, set at 0.05 and 1.0, respectively. Red and blue circles identify upregulated and downregulated metabolites, respectively. Variations are expressed as follows: panels **a-b** are graphed as a function of pathological groups; panel **c** is graphed as a function of pre-frail group.

5.4 Discussion

This section of the study investigated the serum D- and L-amino acid levels and metabolomic profile of a common pathological conditions of the elderly, i.e. clinical frailty. D-serine and D-/Total serine ratio emerged as independent predictors of multidimensional frailty measured with the EFS [165], but not of physical frailty phenotype as defined by Fried *et al* [131]. Furthermore, higher levels of glycine, glycine/L-serine and D-/Total serine were associated with worse cognition and depressive symptoms in the frail group. Untargeted metabolomics revealed a disruption in the homeostasis of amino acids, lipids, and energy metabolism in frail and pre-frail subjects, with glycine-serine metabolism emerging among the most significant discriminating pathways. Finally, upregulated serum betaine levels discriminated pre-frail individuals from both non-frail and frail subjects. Overall, these findings suggest that changes in peripheral amino acids homeostasis may represent a novel

biochemical correlate of frailty. Here below follows a detailed discussion of the findings emerged by HPLC and metabolomic investigations.

5.4.1 Serum dysregulation of serine and glycine metabolism predics cognitive decline in frail elderly subjects

Compelling studies have shown that changes in the CSF and blood levels of amino acids acting on the glutamatergic NMDAR represent a neurochemical signature in various neuropathologies. These include psychiatric conditions such as schizophrenia[31,169], major depression[170], and a wide spectrum of neurological diseases, including AD [12,13,67], frontotemporal dementia[68], PD [39–41], amyotrophic lateral sclerosis [64,171], mild cognitive impairment [172,173], multiple sclerosis [112,174] and traumatic brain injury [175]. Surprisingly, no investigation so far specifically addressed the relationship between these neuroactive molecules and frailty phenotypes, including those related to cognitive decline and depression. Here, I sought to fill this gap by investigating the endogenous levels of D-serine, glycine and the other amino acids acting on glutamatergic neurotransmission in a well-characterized cohort of older subjects encompassing the entire continuum existing between fit and frail aging. Overall, our biochemical determinations suggest that disrupted systemic D-serine homeostasis may represent a potential predictive biomarker of frailty, while increased serum glycine/L-serine and D-/Total serine ratios could be associated with cognitive decline and depression in frail elderly individuals.

Previous blood metabolomics studies identified several metabolites associated with frailty, belonging to redox homeostasis, inflammation, amino acids, purine metabolism, urea and tricarboxylic acid cycles and sugar metabolism pathways [136]. Among the amino acids identified as dysregulated, glutamate metabolism was found to be affected in frail compared to non-frail subjects [138,144–147]. In light of this finding, and given the close relationship linking frailty with cognitive decline [135,149,176], I investigated whether the serum levels of amino acids acting on glutamatergic NMDAR and their precursors could predict frailty status, and specifically its cognitive domain, in elderly adults. Interestingly, I found that serum D-serine is an independent predictor of the EFS score. D-serine is synthesized by serine racemase (SR) [177] starting from its L-enantiomer and then degraded through D-amino acid oxidase (DAO) activity[178,179] . Once released in the forebrain, D-serine act as an obligatory co-agonist at the glycine modulatory site on GluN1 subunit of NMDAR, a ionotropic glutamatergic receptor playing a key role in sensorimotor gating, synaptic plasticity and cognitive functions [180]. Despite a few reports suggested that circulating blood D-serine

concentrations slightly decrease [180] or remain unchanged[12,74] during healthy aging, recent studies found a positive correlation between serum D-serine and age in patients affected by AD [12] and PD (presented here).

Our observations showing that D-serine and D-/Total serine ratio significantly increase with aging in frail but not in non-frail controls suggests that a dysregulation of blood D-serine homeostasis may represent a common ageing-related metabolic variation across different neuropathologies.

While EFS was conceived to evaluate frailty through a multidimensional approach, the Fried's frailty phenotype is a widely used tool able to capture the physical domain of frailty[131]. Notably, I failed to find any association between D-serine or the other amino acids levels and frailty phenotype. Therefore, I argue that D-serine may not mirror all the components of frailty syndrome, but could instead represent a biochemical fingerprint of its cognitive domain. Consistent with this view, D-serine has recently been proposed as an early gender-related biomarker of AD since its serum concentrations correlated with cognitive deterioration in female patients[12,67]. However, other Authors failed to confirm significant changes of CSF and blood D-serine levels in the whole AD clinical spectrum[13,69]. Interestingly, a recent clinical-pathological study showed that Aβ and tau brain deposition and frailty have a synergistic impact in determining the onset of dementia[176]. This finding, considered together with (i) the previous studies linking increased D-serine with AD-related pathology and cognitive decline[12,66,67] and (ii) the ability of D-serine to diffuse across the blood-brain barrier[94], suggests that blood levels of this D-amino acid could be adopted as a metabolic marker to identify older adults at higher risk of conversion to dementia.

Notably, the stratification of our elderly cohort by sex disclosed that the correlation between serum D-serine, EFS score and global cognition was mainly driven by females. In agreement with this view, recent investigations showed increased D-/Total serine ratio in the human post-mortem hippocampus and serum of AD female patients compared to healthy females[12,66]. Similarly, I found a significant increase of serum D-serine in PD females, but not in male patients, compared to HC. These findings suggest that a dysregulation of blood D-serine may reflect the occurrence of different neuropathologies in a sex-dependent manner. Considering the neuroprotective role played by estrogens and the compelling evidence that estrogens loss after menopause can accelerate the effect of aging on cognitive functions[181], I speculate that the link between increased systemic D-serine levels and cognitive decline may be mediated, at least in part, by the reduced estrogens levels which characterize females aging. However, further studies on larger elderly cohorts are needed to address this outstanding issue.

Remarkably, I also found that higher serum glycine concentrations and glycine/L-serine ratio correlated with worse cognitive function and depressive symptoms in the frail but not in the non-frail group. Similarly to D-serine, glycine binds the GluN1 subunit of NMDAR and acts as a major obligatory co-agonist[182]. However, in other central nervous system (CNS) regions, glycine also regulates inhibitory neurotransmission via glycine receptors (GlyR) [183]. Considering that a dysfunctional glycinergic transmission has been implicated in the physiopathology of cognitive decline and depression [182,183], the correlation between blood glycine levels, cognitive performance and depressive symptoms may mirror an altered metabolism of this amino acid in the CNS of frail subjects.

Despite these biochemical data are highly intriguing, the findings of the present study should be interpreted cautiously, bearing in mind that (i) the assessment of AD and other dementia-related biomarkers was not included in this study, thus preventing any inference linking the serum amino acid levels and the presence of concomitant neurodegenerative diseases; (ii) frailty is characterized by a decline in the function of multiple organ systems, which may directly influence the serum concentration of D-serine, glycine and the other amino acids. Indeed, recent studies showed that blood D-serine levels correlate positively with biochemical renal parameters[74,87,89,184], while various L-amino acids correlated with metabolic parameters such as liver enzymes, lipids and blood glucose[74]. Moreover, blood glycine levels may be affected by physical exercise[185], regional adiposity[186] and bone mineral density[187].

Dietary intake and D-amino acids produced by the gut microbiota may also affect serine enantiomers metabolism[80,81]. Gut commensal bacteria represent indeed a main source of several D-amino acids in mammals, including D-serine, and the impact of gut microbiota metabolism on blood levels of D-amino acids, peripheral organs and the gut-brain axis function is currently a hot research topic[188]. For instance, D-glutamate synthesized by gut bacteria has been proposed to influence the NMDAR neurotransmission and cognitive function in AD patients[188]. This result support the hypothesis that gut microbiota plays a role in modulating the gut-brain axis through D-amino acids metabolism, and could therefore be a potential target of intervention in neurological and neuropsychiatric diseases[188,189]. Further studies correlating the peripheral D-serine levels with the composition of gut microbiota in elderly frail individuals with and without neurological disorders are warranted.

Interestingly, studies in animal models showed that D-serine is detectable in multiple organs, including heart, pancreas, spleen, liver, kidney, lung and muscles[82,190], and glutamatergic receptors play relevant functions in the modulation of physiological processes in several peripheral tissues[191]. Of note, recent studies showed that SR and NMDAR are highly expressed in human

pancreatic islet β cells[192], and systemic D-serine administration modulates insulin secretion in a dose-dependent manner[193,194]. Despite I found similar serum D-serine levels between diabetic and non-diabetic subjects, L-serine and L-glutamate were increased in diabetic compared to non-diabetic group, consistently with previous blood metabolomics evidence[145,146]. In line with other studies[195], I also observed a positive correlation between serum L-glutamate concentration, BMI and visceral adiposity in both non-frail and frail participants. Although the biological mechanisms responsible for this association are still unclear, considering that glutamate signalling modulates the immune system[196] and that increased VAT promotes systemic inflammation[197], elevated blood L-glutamate levels could represent a metabolic signature underpinning the abnormal increase in oxidative stress and inflammation associated with obesity. Concurrently, my data suggest a correlation between serum L-glutamate concentration and SMI. This is in line with previous investigations showing that glutamate is crucial in maintaining the homeostasis of energy metabolism in skeletal muscle[198]. Surprisingly, this relationship was observed in the non-frail but not in the frail group, suggesting that different biological pathways may modulate the maintenance of skeletal muscle mass across healthy and frail aging. However, these results may be affected by the very low prevalence of subjects with SMI scores below the proposed cut-off to define sarcopenia[160] and therefore require validation in larger cohorts.

In the kidney, liver and other peripheral organs, L-serine is rapidly interconverted with glycine in a single reaction catalyzed by serine hydroxymethyltransferase (SHMT) as part of the folate-mediated one-carbon metabolism[10]. This direct relationship makes the serum glycine/L-serine ratio a reliable index of serine-glycine interconversion[54]. Consequently, the positive correlation of serum glycine/L-serine and D-/Total serine ratios with cognitive dysfunction and depressive symptoms observed in the frail group indicates that disturbed serine-glycine metabolism emerge as a peripheral proxy of brain functions decline in frail elderly populations.

Besides its neuroactive role, glycine primarily influences anti-oxidative reactions and immune system[10]. In agreement with this knowledge, glycine has been used to prevent tissue injury, enhance anti-oxidative capacity, improve immunity, and treat metabolic disorders in obesity, diabetes and various inflammatory diseases[199]. Thus, consistently with the reported “geroprotective” effects of glycine, I cannot rule out that the negative correlation of this amino acid and the glycine/L-serine ratio with cognitive function in frail older individuals might represent an adaptive mechanism aimed at counteracting the systemic inflammation and metabolic dysfunctions that characterize frailty syndrome [4,148], rather than being causally linked to cognitive impairment.

In the same way, I argue that systemic D-serine metabolism variation in frailty may represent a biochemical adaptation to CNS and multi-system deteriorations. In line with this idea, D-serine supplementation or treatment with DAO inhibitors significantly improved cognitive functions in animal models of aging[200], as well as in healthy subjects, PD and schizophrenia patients[37,201–203].

Given that (i) altered CNS and peripheral homeostasis of the NMDAR-stimulating amino acids have been observed in multiple brain pathologies[103] and non-neurological disorders[67]; (ii) these amino acids are directly involved in a plethora of neuronal processes and metabolic pathways, I cannot assume that changes in their concentrations observed in this study are exclusive of frailty syndrome. Conversely, serine enantiomers and glycine metabolism variation may represent a common biochemical marker of brain dysfunctions across a broad spectrum of neurological and non-neurological conditions.

These findings have practical implications for future clinical research. First, this study paves the way for further investigations evaluating the levels of the two enzymes implicated in glycine – L-serine and L-serine – D-serine interconversion (SHMT and DAO, respectively) as putative markers of brain function in frail elderly cohorts. In line with this, previous evidence showed that blood DAO levels increase during cognitive progression in AD[172] and amnestic mild cognitive impairment[204,205]. Moreover, single nucleotide polymorphisms in the *DAOA* gene, which encodes for the DAO activator protein G72, have been associated with schizophrenia[206] and autism spectrum disorder[207]. On the other hand, SHMT may play neuroprotective roles in AD[208] and PD[76]. Second, our study lays the foundation for future metabolomics investigations aimed at comprehensively assessing the whole serine-glycine and other amino acids metabolism in frailty. Third, the use of D-/Total-serine and glycine/L-serine ratios instead of the single amino acids concentrations makes these indexes easily comparable between different analytical techniques, thus simplifying the translation of our approach to other research laboratories.

5.4.2 ^{1}H-NMR metabolomics identifies disrupted betaine and glycine-serine metabolism as serum signature of pre-frailty

Previous studies investigating blood metabolomics in frail subjects showed heterogeneous and often inconsistent results (reviewed in Shekarchian et al [209]). This inconsistency is likely attributable to differences in the clinical tools used to classify frailty, analytical platforms employed, comorbidities, ethnicity, exercise levels and diet regimens. However, few classes of metabolites often emerged as

dysregulated in frail populations, including antioxidants, amino acids [136] and mitochondria-related [210] molecules. Here, I attempted to shed light on this matter by evaluating the serum metabolome in a well-characterized elderly cohort whose clinical and serum amino acid profiles were already known. Our present findings confirm a complex dysregulation of amino acids, lipids and energy metabolism-related metabolites in frail and pre-frail patients, with glycine-serine metabolism emerging among the most significant discriminating pathways. Strikingly, glycine-serine metabolism was the pathway featuring the highest number of metabolite hits altered in both frail and pre-frail subjects (n = 11, Fig. 2a-c). Multivariate and univariate analyses further supported this result by showing upregulated serine levels in frail and pre-frail compared to non-frail subjects, nicely mirroring my HPLC findings. Moreover, I highlighted a remarkable upregulation of glutamate and downregulated glutamine levels as biochemical signatures specifically associated with pre-frailty. These findings are in line with previous metabolomics studies showing dysregulated glycine-serine metabolism [211–214] and increased blood glutamate levels [144–147,213,215,216] in frail and pre-frail subjects compared to non-frail controls. Interestingly, increased serum aspartate concentration was reported in frail compared to non-frail individuals [144,146,147,213,214], while I found downregulated aspartate levels in both pre-frail and frail patients. Beyond differences in study design, this discrepancy may be due to the lower proportion of sarcopenic patients in our pre-frail and frail groups (5%; see Table 1) compared to previous studies (100% in frail group [144,146], 13% and 50% in pre-frail and frail groups, respectively [213]). Since high blood aspartate levels were previously associated with sarcopenic trait [8,152], our findings suggest that serum aspartate may be differentially modulated among frail subjects with and without sarcopenia. Overall, these results entirely support my HPLC findings, further confirming a role of amino acids acting on glutamatergic transmission as putative biochemical signature of frail aging. In this regard, besides their pivotal neuroactive role, these biomolecules are directly involved in the metabolism of several peripheral organs, including the liver, kidney, skeletal muscle and immune system [10,217]. Therefore, the disrupted amino acids homeostasis highlighted in this and previous studies most likely signals a decline in functioning across multiple organs and physiological systems that characterizes frailty, rather than a specific fingerprint of brain health. In addition, frail and pre-frail subjects showed dysregulated levels of several metabolites related to lipids, urea cycle and mitochondrial metabolism, including the tricarboxylic acid cycle. These observations are consistent with previous studies [136,209,210,218] and, in turn, strengthen the hypothesis that energy metabolism and mitochondrial dysfunction play a key etiopathogenetic role in frailty [219].

Despite several investigations explored the peripheral metabolomic profile of frailty, data focused on pre-frailty are scarce. Here, I identify a metabolomic signature able to distinguish pre-frail from frail

and non-frail individuals. Specifically, betaine metabolism emerged as the distinctive pathway exclusively enriched in pre-frail subjects. Betaine, or trimethyl glycine, is an amino acid derivative that can be endogenously synthesized by the oxidation of choline and exogenously absorbed with diet [220]. Betaine serves as an alternative methyl donor in the methionine cycle, where it transfers a methyl group to homocysteine to synthesize methionine and dimethyl glycine. Dimethyl glycine is then catabolized to glycine and serine through multiple enzymatic reactions in the mitochondria of kidney and liver [220]. Remarkably, dysregulated methionine metabolism in both pre-frail and frail groups and elevated plasma homocysteine levels were previously associated with frailty in older adults [221]. Moreover, preclinical studies suggested that betaine supplementation counteracts oxidative stress and inflammation [222], which have been proposed as key physiopathological mechanisms of frailty [148]. Although increased betaine levels could result from the mitochondrial dysfunction that characterizes frailty [210] or be secondary to the elevated serine levels observed in pre-frail subjects (Fig. 14), I speculate that the higher serum betaine concentration mainly found in the pre-frail females represents an adaptive mechanism to counteract an abnormal increase in homocysteine levels and protect against oxidative stress and inflammation. This protective metabolic mechanism may subsequently be lost during the transition from pre-frailty to overt frailty.

5.4.3 Strengths and limitations

The strengths of this section focused on frailty include (i) the novelty of investigating NMDAR-related amino acids and their precursors in the serum of a well-characterized elderly cohort, including the entire clinical spectrum existing from fit to frail condition; (ii) the adoption of two independent analytical approaches, including HPLC and untargeted ^{1}H-NMR metabolomics (iii) the adjustment of statistical analyses with multiple potential confounding factors which may affect the serum amino acids concentration, such as diabetes, body composition and cigarette smoking [223]; (iv) the assessment of frailty with two different but complementary tools [131,132].

However, I recognize some limitations. First, the cross-sectional design and the clinical-biochemical correlations observed in the present study did not allow to define the cause-effect relationship between the serum changes in amino acids levels and the clinical phenotypes. Future longitudinal studies on larger elderly cohorts adopting a multidimensional approach, including the measurement of blood biomarkers mirroring brain (e.g. neurofilament light chain, Aβ42, phosphorylated tau [224]) and peripheral organs damage, as well as inflammation, are warranted to elucidate this issue. Second, the sex ratio was unbalanced with a higher prevalence of females, potentially biasing the analyses conducted after stratifying the cohort by sex. Third, the assessment of biochemical parameters of

kidney and liver function was not included in the study protocol, thus preventing the adjustment of the analyses for the serum levels of creatinine, aspartate transaminase and alanine transaminase, which correlate with the blood levels of D-serine and several L-amino acids, respectively [74]. However, the history of any kidney or liver disease or altered parameters of renal and hepatic function was strictly considered as exclusion criteria at the time of participants enrolment. Due to the relatively limited sample size of the included cohort, this exploratory study requires further validation in independent larger cohorts of pre-frail and frail individuals. However, my findings have practical implications for future clinical research aimed at evaluating amino acids and betaine metabolism as a potential source of suitable blood biomarkers and targeted interventions to counteract frailty in its early stages.

6 CONCLUSIONS

This study provides original evidence of dysregulated serum D- and L-amino acids metabolism in PD and frailty, suggesting their possible involvement in the physiopathology of these two common clinical syndromes of the elderly.

After adjusting for the effect of dopaminergic treatment, I found increased D-serine levels in PD patients compared to HC, suggesting that this neuroactive amino acid may represent a biochemical signature of PD. The positive correlation between serum D-serine and age at PD onset supports the hypothesis that D-serine may play a neuroprotective role in PD by delaying the onset of the disease. Moreover, untargeted and targeted serum metabolomics highlighted disrupted homeostasis of molecules related to glutamic acid, serine and energy metabolism as distinct serum fingerprints in PD patients. Analysis of the serum metabolome in populations at high risk of conversion to PD, such as subjects with idiopathic REM sleep behavior disorder or asymptomatic carriers of genetic risk variants, is warranted to assess its value as an early diagnostic biomarker.

In the frailty cohort, I found that increased serum D-/Total serine and glycine/L-serine ratios mirror worse cognitive decline and depressive symptoms in frail older subjects. The observation that D-serine correlates with frailty scores and global cognition in females but not in males suggest that this effect may also be modulated by sex-related biological factors. Furthermore, untargeted ^{1}H-NMR metabolomics revealed disrupted homeostasis of amino acids, lipids, and energy metabolism-related molecules in frail and pre-frail patients, with glycine-serine metabolism emerging among the most significant discriminating pathways. Finally, upregulated betaine levels specifically identified pre-frail subjects from non-frail and frail groups.

Interestingly, D-serine and glycine levels correlated with several demographic and clinical measures in both PD and frailty cohort, while glycine-serine metabolism emerged as the pathway featuring the highest number of metabolite hits altered in these two clinical syndromes. These observations show that both PD and frailty represent systemic conditions that directly affect the energy and amino acids metabolism within the brain and peripheral organ-systems. Notably, previous clinical and epidemiological evidence showed that frailty increases the risk of developing PD and *viceversa* [225–227]. Consistently, this study identifies a specific biochemical background shared between PD and frailty.

Unfortunately, the original study design and the relatively small sample size of the two cohorts included in this study did not allow to investigate whether the evaluation of serum amino acids and metabolome may help in the identification of novel biological clusters of patients affected by PD or frailty, i.e. disease subtypes sharing a similar biological background and thus candidate to receive targeted experimental therapies. Indeed, it is likely that these two pathological conditions represent the clinical expression of a very broad (probably infinite) spectrum of underlying biological and molecular alterations [228–230]. Further studies on larger cohorts of PD patients and frail subjects integrating multidimensional omics approaches (e.g. genomics, transcriptomics, proteomics and metabolomics) are thus warranted to evaluate whether the biochemical signatures emerged in this study may be integrated into novel frameworks aimed at enabling precision medicine strategies for PD and frailty. However, the findings of the present study open the way to new lines of research aimed at evaluating the diagnostic and prognostic value of D-serine and the other amino acids here identified in PD and frailty. The inverse correlation between serum D-serine and age at PD onset, combined with previous preclinical [36] and clinical [37] evidence of a putative beneficial effect of NMDAR co-agonist site stimulation in PD, paves the way to novel studies evaluating D-serine supplementation as a possible novel treatment for PD (https://www.cuimc.columbia.edu/news/can-experimental-drug-rewire-brain-treat-parkinsons).

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8 APPENDIX

8.1 Figures

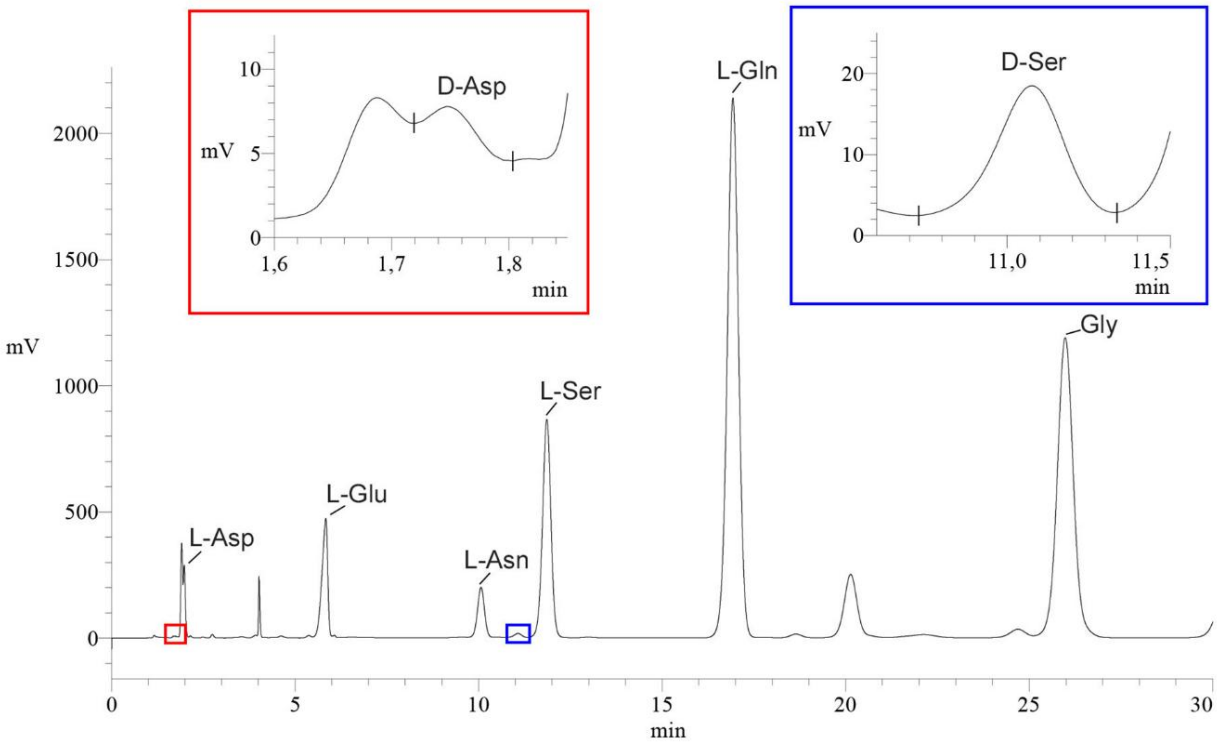

Figure 1A. Representative HPLC chromatogram illustrating L-aspartate, L-asparagine, glycine, D-serine, L-serine, L-glutamate and L-glutamine peaks obtained from a serum sample.

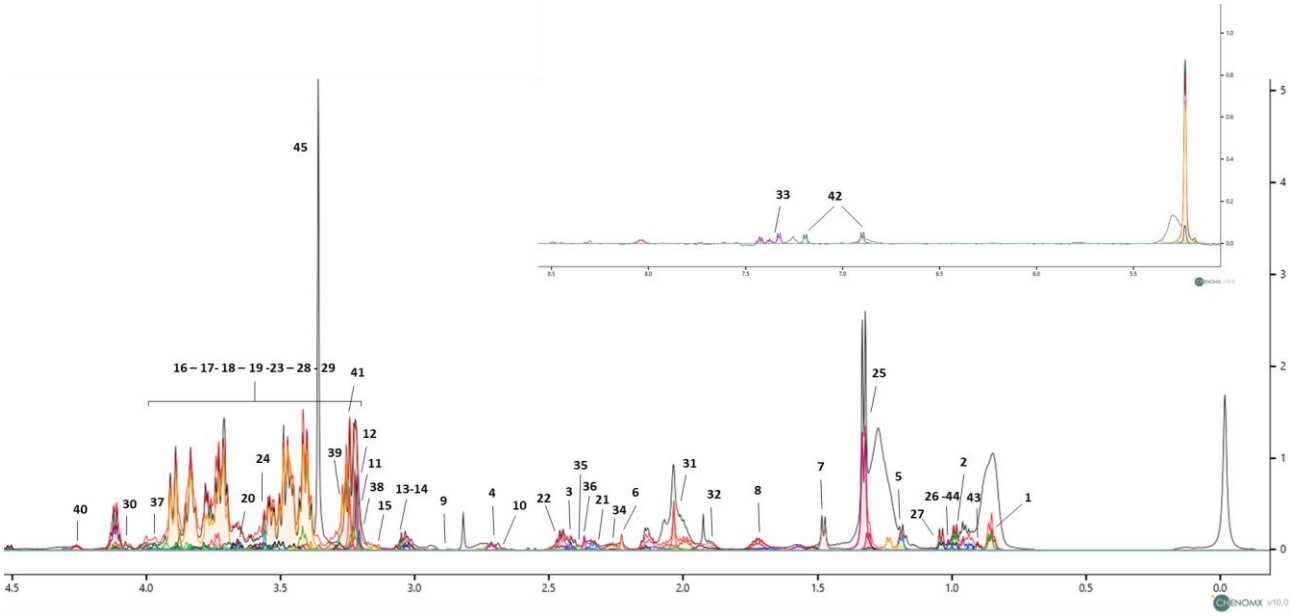

Figure 2A. Representative 1D ^{1}H CPMG spectrum of PD patient serum. The spectrum was acquired at 600 MHz and dT = 298 K. Fourty-four metabolites were identified and annotated as follows: 1: 2-Hydroxybutyrate; 2: 2-Hydroxyisovalerate; 3: 2-Oxoglutarate; 4: 2- Oxoisovalerate; 5: 3-Hydroxybutyrate; 6: Acetoacetate; 7: Alanine ; 8: Arginine; 9: Asparagine; 10:Aspartate; 11: Betaine; 12: Carnitine; 13: Creatine; 14: Creatinine; 15: Cystine; 16: Fructose; 17: Fucose; 18: Galactose; 19: Glucose; 20: Glucuronate; 21: L-Glutamic acid 22; L-Glutamine; 23:Glycerol; 24: Glycine; 25: Lactate; 26: Leucine; 27: Isoleucine; 28: Maltose; 29: Mannose; 30: Myo-inositol; 31: N-acetylGlycine; 32: Ornithine; 33: Phenylalanine; 34: Proline; 35: Pyroglutamate; 36: Pyruvate; 37:Serine; 38: Glycero-3-phophocholine; 39: Taurine; 40: Threonine;41: TMAO; 42: Tyrosine; 43: Valeric acid; 44: Valine; 45:Methanol.

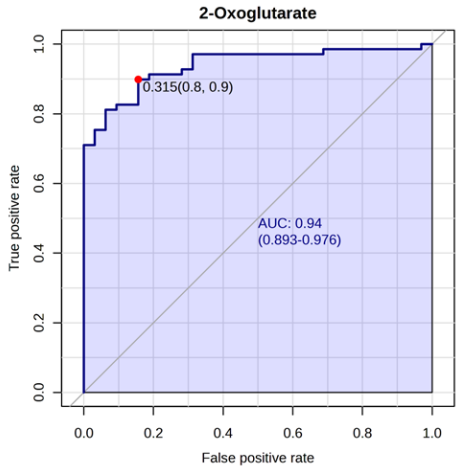

Figure 3A. Receiver operating characteristic (ROC) curves related to 2-oxoglutarate serum concentration. The Cartesian space is described by x-axis: false positive rate and y-axis: true positive rate. ROC curve has two components, the empirical ROC curve that is obtained by joining the points represented by the sensitivity and the specificity for the different cutpoints and the chance diagonal represented by the 45-degree line drawn through the coordinates (0,0) and (1,1).

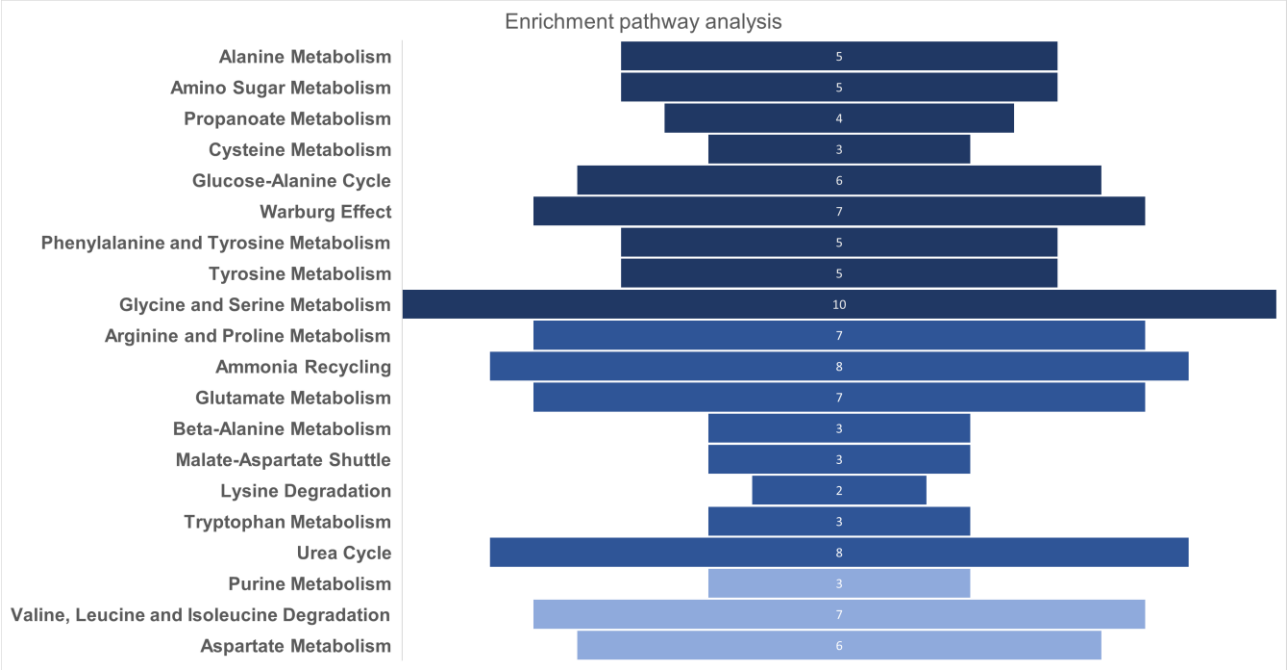

Figure 4A. Enrichment pathways analysis performed on PD and HC metabolites identified by 1H-NMR using Metaboanalyst 6.0; the discriminative pathways are ranked according to p-value and number of hits reported in the bars.

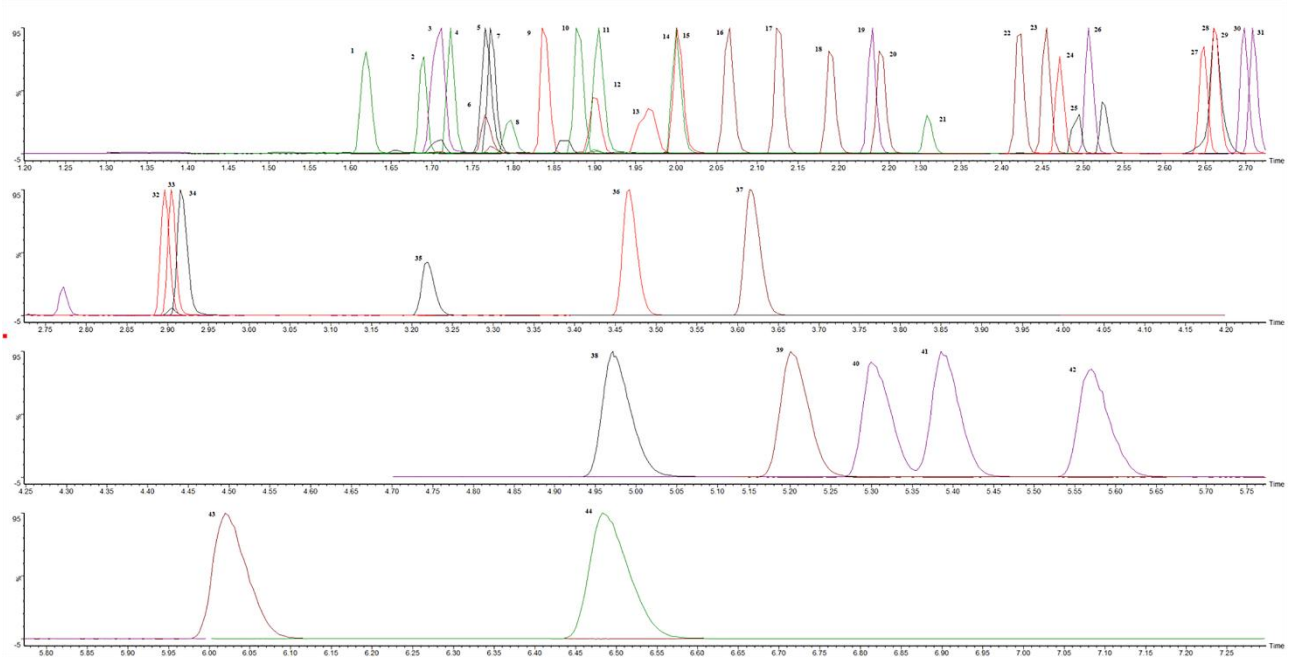

Figure 5A. Representative MS Total Ion Current (TIC) of PD patients' serum. The spectrum was acquired in Selected Ion Recording (SIR), and forty-four amino acids were quantified. Peak annotation as follows: 1: histidine and histidine-IS; 2: 3-methyl-histidine; 3: hydroxyproline; 4: 1-methyl-histidine; 5: asparagine and asparagine-IS; 6: arginine and arginine-IS; 7: carnosine; 8: phosphoethanolamine; 9: anserine; 10: taurine; 11:serine and serine-IS; 12: glutamine and glutamine-IS; 13: sulfocysteine; 14: ethanolamine; 15: glycine and glycine-IS; 16: aspartic acid and aspartic acid-IS; 17: citrulline; 18: glutamic acid and glutamic acid-IS; 19: sarcosine; 20: β-alanine; 21: threonine and threonine-IS; 22: homocitrulline; 23: alanine and alanine-IS; 24: γ-aminobutyric acid; 25: hydroxylysine; 26: aminoadipic acid; 27: proline and proline-IS; 28: glycil proline; 29: β-aminobutyric acid; 30: ornithine; 31: cystathionine; 32: α-aminobutyric acid; 33: cystine and cystine-IS; 34: lysine and lysine-IS; 35: tyrosine and tyrosine-IS; 36: methionine and methionine-IS; 37: valine and valine-IS; 38: homocystine; 39: kynurenine; 40: isoleucine and isoleucine-IS; 41: allo-isoleucine; 42: leucine and leucine-IS; 43: phenylalanine and phenylalanine-IS; 44: tryptophan and tryptophan-IS.

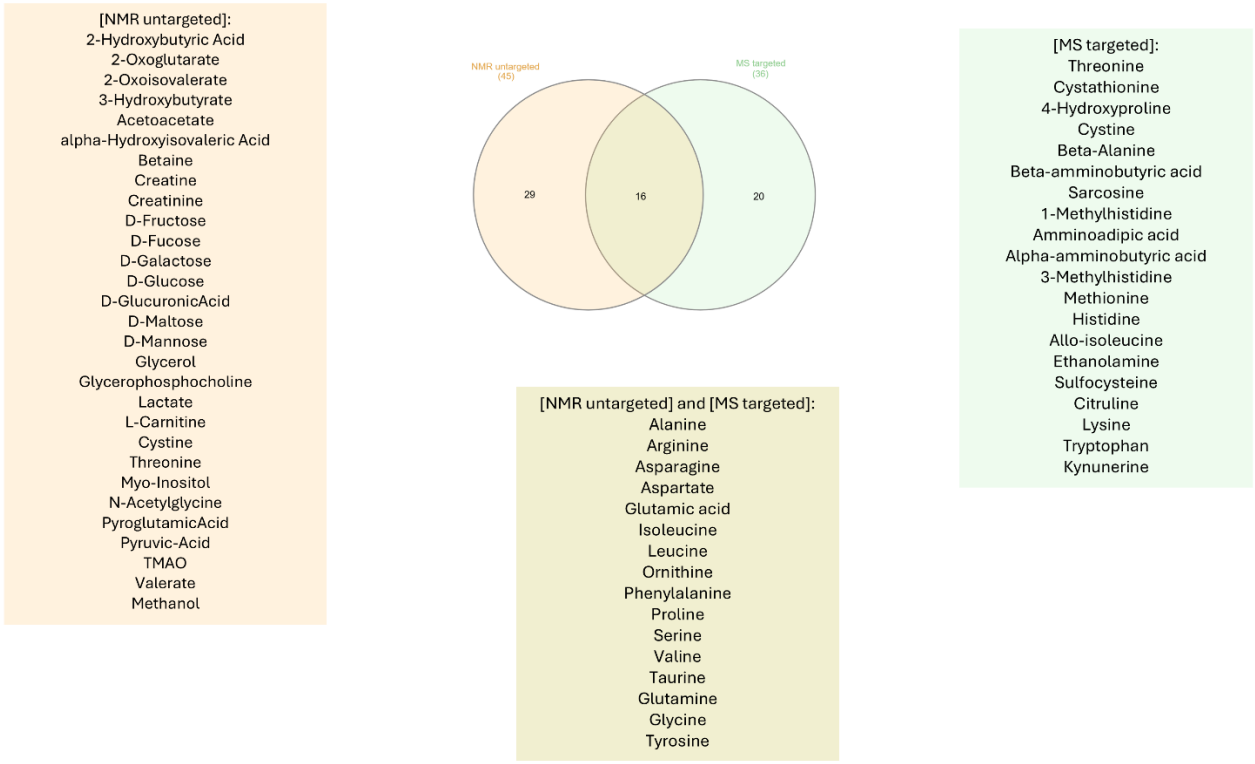

Figure 6A. Venn diagrams showing the representative metabolites examined using untargeted NMR metabolomics in conjunction with a targeted UPLC/MS approach. The two employed methods have 16 amino acid metabolites in common.

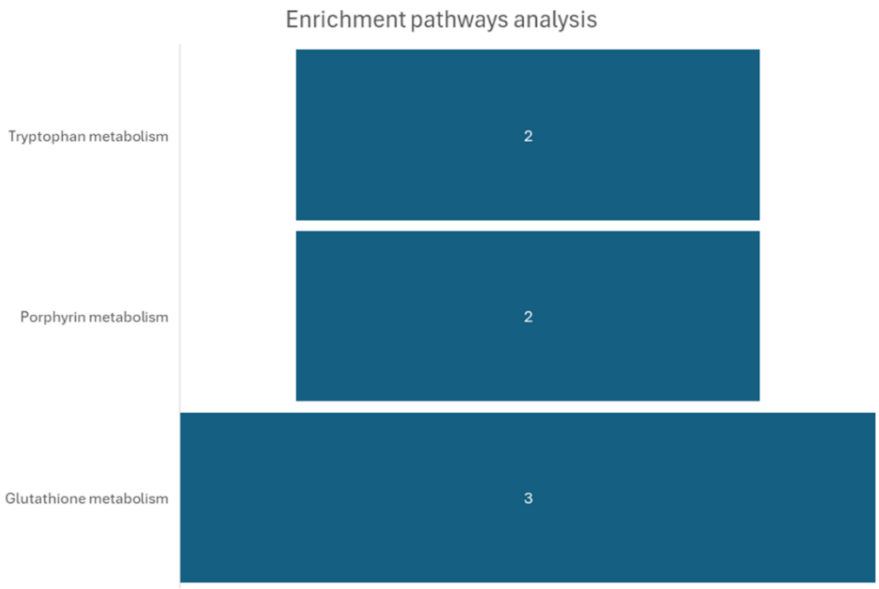

Figure 7A. Enrichment pathways analysis based on UPLC/MS data; the discriminative pathways between PD and HC are ranked according to p-value and number of hits reported in the bars. The hits represent the matched metabolites from the user-uploaded data; p-value describes the pathways' statistical significance index. In particular, Tryptophan metabolism (pvalue 1.12 e-2), Porphyrin metabolism (pvalue 3.33 e-2), and Glutathione metabolism (3.43 e-2).

8.2 List of publications related to this Doctoral thesis

1. Imarisio A*, Yahyavi I*, Avenali M, Di Maio A, Buongarzone G, Galandra C, et al. Blood D-serine levels correlate with aging and dopaminergic treatment in Parkinson's disease. Neurobiol Dis 2024;192:106413. https://doi.org/10.1016/j.nbd.2024.106413.
2. Imarisio A*, Yahyavi I*, Gasparri C, Hassan A, Avenali M, Di Maio A, et al. Serum dysregulation of serine and glycine metabolism as predictive biomarker for cognitive decline in frail elderly subjects. Transl Psychiatry 2024;14:281. https://doi.org/10.1038/s41398-024-02991-z.
3. Marino C*, Imarisio A*, Gasparri C, Napolitano E, Di Maio A, Avenali M, et al. 1H-NMR-based metabolomics identifies disrupted betaine metabolism as distinct serum signature of pre-frailty. Npj Aging 2025;11:26. https://doi.org/10.1038/s41514-025-00218-z.
4. Gervasoni J*, Marino C*, Imarisio A*, Santucci L, Napolitano E, Nuzzo T, et al. Independent serum metabolomics approaches identify disrupted glutamic acid and serine metabolism in Parkinson's disease patients. NPJ Parkinsons Dis 2025;11:274. https://doi.org/10.1038/s41531-025-01126-5.

*Joint first authorship.