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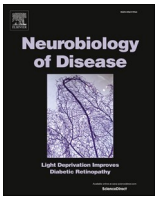

Non-invasive biomarkers for mild cognitive impairment and Alzheimer's disease

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ABSTRACT

Alzheimer's disease is the most common type of dementia in the elderly. It is a progressive degenerative disorder that may begin to develop up to 15 years before clinical symptoms appear. The identification of early biomarkers is crucial to enable a prompt diagnosis and to start effective interventions. In this work, we conducted a metabolomic study using proton Nuclear Magnetic Resonance (^{1}H NMR) spectroscopy in serum samples from patients with neuropathologically confirmed Alzheimer's disease (AD, $n = 51$), mild cognitive impairment (MCI, $n = 27$), and cognitively healthy controls (HC, $n = 50$) to search for metabolites that could be used as biomarkers. Patients and controls underwent yearly clinical follow-ups for up to six years. MCI group included samples from three subgroups of subjects with different disease progression rates. The first subgroup included subjects that remained clinically stable at the MCI stage during the period of study (stable MCI, S-MCI, $n = 9$). The second subgroup accounted for subjects which were diagnosed with MCI at the moment of blood extraction, but progressed to clinical dementia in subsequent years (MCI-to-dementia, MCI-D, $n = 14$). The last subgroup was composed of subjects that had been diagnosed as dementia for the first time at the moment of sample collection (incipient dementia, Incp-D, $n = 4$). Partial Least Square Discriminant Analysis (PLS-DA) models were developed. Three models were obtained, one to discriminate between AD and HC samples with high sensitivity (93.75%) and specificity (94.75%), another model to discriminate between AD and MCI samples (100% sensitivity and 82.35% specificity), and a last model to discriminate HC and MCI with lower sensitivity and specificity (67% and 50%). Differences within the MCI group were further studied in an attempt to determine those MCI subjects that could develop AD-type dementia in the future. The relative concentration of metabolites, and metabolic pathways were studied. Alterations in the pathways of alanine, aspartate and glutamate metabolism, pantothenate and CoA biosynthesis, and beta-alanine metabolism, were found when HC and MCI- D patients were compared. In contrast, no pathway was found disturbed in the comparison of S-MCI with HC groups. These results highlight the potential of ^{1}H NMR metabolomics to support the diagnosis of dementia in a less invasive way, and set a starting point for the study of potential biomarkers to identify MCI or HC subjects at risk of developing AD in the future.

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1. Introduction

Alzheimer's disease (AD) is the most common type of dementia in older adults (Olajide and Sarker, 2020). In 2020, over 55 million people worldwide were estimated to have dementia, a figure expected to rise to 78 million by 2030 (Gauthier et al., 2021). AD is a progressive neurodegenerative disorder characterized by the accumulation of extracellular plaques of ß-amyloid peptides and intracellular aggregation of tau protein, with the concomitant neuronal and synaptic loss (Lucey, 2020), resulting in the development of cognitive dysfunction and dementia. The pathophysiological alterations usually start between 10 and 15 years before clinical onset (Vignoli et al., 2020). The definitive diagnosis of Alzheimer's disease can only be made post-mortem (Wurtman, 2015). However, dementia of the Alzheimer's type is the clinical term to refer to dementia patients in which the Alzheimer's diagnosis is achieved based on the clinical symptoms *pre-mortem*. Age is the greatest risk factor for AD, and the incidence doubles every five years after the age of 65 (Lucey, 2020). Mild cognitive impairment (MCI) is a pathological condition characterized by the manifestation of a premature cognitive decline (Gauthier et al., 2006). Dementia-related MCI could be described as an intermediate state between normal aging and AD-type dementia. The prevalence of MCI in the population ranges between 15% and 20% in adults older than 60 years. MCI population is of great interest since their annual rate of progression to dementia is in the range of 8% to 15% (Duara et al., 2013). In this context, there is an increasing interest in identifying the subjects with MCI that will progress to dementia. Anticipating the progression of these MCI patients to dementia would be of interest to apply therapies in the early stages of the disease when these interventions may be more effective.

Nowadays, there is no treatment to restore the cognitive decline of AD patients. Very recently, anti-amyloid immunotherapies have been approved by the FDA, showing a rather modest clinical benefit by slightly slowing down disease progression at early phases of the disease, albeit amid some safety concerns (Couzin-Frankel, 2023; Larkin, 2023; Reardon, 2023). AD patients are identified according to their cognitive state, neuroimaging studies and biomarkers profile. Current clinical biomarkers for AD are variations in the cerebrospinal fluid (CSF) levels of tau proteins (total tau protein and phosphorylated tau protein) and β-amyloid 1–42 peptide (Aß42). AD patients show an increase in tau protein and a decrease in Aß42 levels compared to healthy subjects (Mattsson et al., 2009). Unfortunately, CSF extraction requires invasive procedures and specialised staff, and it is not exempt from risks in aged patients who usually present concomitant pathologies. Considering these factors, the identification of new biomarkers is urgently needed to implement non-invasive diagnostic techniques for AD, to stratify populations for clinical trials, and to find new therapeutic targets to prevent cognitive decline. AD has lately been related to a metabolic disease. In fact, it has been shown that a metabolic dysfunction of the brain could be a potential driver of AD (Zheng et al., 2019). These metabolic changes in the brain might be translated early on to other organs and biofluids. The observation of the global biochemical changes produced could provide information to reveal biomarkers related to AD and to further deepen our understanding of the molecular mechanism underlying cognitive decline and AD.

Accordingly, the study of the metabolic profile seems relevant in this context. Metabolomics is the study of the footprint of all metabolic pathways and chemical processes occurring in a living system, that is, the study of those metabolites present in a biological sample (Peng et al., 2015). Metabolomics is closer to the phenotype than any other -omics discipline, and informs about what has happened, and not about the possibility of something happening as in other -omics (D'Alessandro et al., 2012). The analysis of differential metabolites in a biological sample isolated from a healthy subject and a patient gives information about the biochemical processes occurring underneath the disease and gives an insight into new therapeutic approaches and the identification of biomarkers for the diagnosis of the disease. To perform metabolomic studies, nuclear magnetic resonance (NMR) spectroscopy together with mass spectrometry (MS) are the most predominant techniques. NMR spectroscopy allows the untargeted qualitative and quantitative analysis of a wide variety of biological samples (blood derivatives, urine, CSF, tissue, culture cells, and others) (Ćuperlović-Culf et al., 2010; Duarte et al., 2014; Fuss and Cheng, 2016; Kast et al., 2014). It is a non-destructive technique, thus enabling the performance of complementary assays in the same sample. The requirements to perform NMR spectroscopy metabolomic studies include straightforward sample processing procedures and small sample quantities. Moreover, there is a wide set of experiments for metabolite identification, and metabolite quantification is also possible with this technique. Remarkably, it is also a robust and very reproducible technique (Ward et al., 2010).

Metabolomics, either using NMR spectroscopy or MS, has been extensively applied to the study of AD. Different biofluids have been studied to find AD biomarkers of disease or progression. CSF is in contact with nervous tissue, and it is supposed to be closer to the pathology, so it would be the best candidate to provide information on AD. Different metabolomic studies have been performed in CSF (Ibáñez et al., 2012; Jääskeläinen et al., 2020; Vignoli et al., 2020). Ibáñez et al., 2012 applied capillary electrophoresis-mass spectrometry (CE-MS) for the generation of models of AD progression, reaching 83% of accuracy in the discrimination of patients undergoing AD compared to MCI-AD patients and non-AD subjects. Metabolites such as choline, dimethylarginine, arginine, valine, proline, serine, histidine, creatine, carnitine, and suberylglycine were identified as potential AD progression biomarkers (Ibáñez et al., 2012). Jääskeläinen et al., compared the ability of classic CSF biomarkers (amyloid-ß 42, phosphorylated tau protein, and total tau) and metabolic profiles obtained by NMR spectroscopy for the classification of AD and healthy controls. The authors concluded that classic CSF biomarkers were better for classifying cognitive healthy controls (HC) vs. AD patients (AUC = 0.89), but metabolic subclasses by NMR spectroscopy were more effective for classifying MCI vs. AD samples (AUC = 0.68) (Jääskeläinen et al., 2020). Vignoli et al., 2020 used NMR spectroscopy to obtain the metabolomic profile of CSF samples, and generated discriminant models with 86.1% accuracy in discriminating between AD and HC and 70% accuracy for classifying AD vs. MCI. They found that acetate, valine, and 3-hydroxyisovalerate were altered in AD (Vignoli et al., 2020). Despite the interesting results obtained from CSF samples, this is an invasive technique that implies a lumbar puncture. Therefore, different biofluids that require less invasive procedures for sample collection, such as urine or blood, have been explored to determine AD biomarkers. Recently, studies in urine using NMR spectroscopy and UHPLC-MS together with metabolic quantitative trait loci (mQTL) were used to calculate models able to classify correctly 82.96% of cases MCI converting to AD, and 77.78% of stable MCI vs. controls. However, urine is separated from the brain not only by the blood-brain barrier, but also by glomerular filtration (Kurbatova et al., 2020). Blood fractions (serum, plasma) seem a compromise between less invasiveness and relation to the pathology. Olazarán et al., 2015 used UPLC-MS to determine metabolomic biomarkers for the diagnosis of AD using plasma as a biological sample in a set of 251 AD, HC and MCI subjects (Olazarán et al., 2015). In this study a panel of seven metabolites (glutamic acid, alanine, aspartic acid, 22:6n-3 DHA, deoxycholic acid, PE(36:4), SM (39:1)) was determined for the discrimination of AD from HC samples (AUC = 0.918) and MCI from HC (AUC = 0.826). Figuera et al. 2019 used NMR spectroscopy on serum to generate discriminative models able to classify AD and MCI samples from HC with AUC values of 0.61 and 0.71, respectively. In the study, the authors determined that the threonine-linked metabolic pathways were important in the pathological process.(Figueira et al., 2019) Graham et al. combined NMR and LC-MS to identify biomarkers able to discriminate AD and MCI from HC, obtaining models with sensitivity and specificity values ranging from 0.75 to 0.85 and 0.69–0.81, respectively. The authors concluded that the lipid metabolism was the most perturbed biochemical pathway in MCI and AD (Yilmaz et al., 2020).

Aimed with this background, herein, we present a metabolomic study by NMR spectroscopy of serum samples from HC, MCI and AD patients to determine biomarkers of disease and early biomarkers of progression.

2. Material and methods

2.1. Patient selection

Cognitively healthy, non-demented participants and subjects diagnosed with MCI ($n = 77$) were recruited from the Vallecas project, a single-center, multidisciplinary, observational, longitudinal study of a cohort of 1213 volunteers, aged 69–86 years and home-dwelling at baseline, recruited between 2011 and 2013 in Madrid, Spain, which is carried out in the Queen Sofia Foundation, funded by CIEN Foundation and Queen Sofia Foundation (Olazarán et al., 2015). Participants of the Vallecas project were cognitively healthy volunteers at baseline attending Queen Sofia Foundation Alzheimer Research Center. The AD group ($n = 51$) consisted of clinically diagnosed patients with moderate to severe AD that were institutionalized at the Queen Sofia Foundation Healthcare Center. Written informed consent was obtained from all participants or representatives according to the Declaration of Helsinki. Approval was obtained from the Research Ethics Committee of the Instituto de Salud Carlos III (CEI PEI 46_2011-v2015; CEI PI 78_2019).

Healthy controls ($n = 50$) and subjects included in the MCI group ($n = 27$) were followed up for six years to determine their clinical evolution and observe their possible progression to dementia and AD (Fig. 1). According to their clinical progression, subjects in the MCI group were classified into 3 subsets: i) first dementia diagnosis (incipient D, 4 subjects with a first diagnosis of dementia at the time of sample collection); ii) MCI diagnosis at sample collection were diagnosed with MCI but who, in the following one to three years, progressed to dementia (MCI-to-dementia, 14 patients), and MCI patients that in the follow-up, remained stable in MCI condition (stable MCI, 9 patients). Clinical and demographic data are provided in Table 1. The Fig. 1 represents schematically the study.

2.2. Cognitive assessment

Participants from the Vallecas project cohort were cognitively assessed by the well-known Mini Mental Scale Examination (MMSE) and the Clinical Dementia Rating (CDR) scales. To overcome the floor effect effect observed with the standard MMSE scale on moderate-to-severe AD population (Peavy et al., 1996), the AD group was cognitively assessed with the Severe MMSE (SMMSE) (Harrell et al., 2000). The fact that scoring in both scales give a range of values between 0 and 30 can be

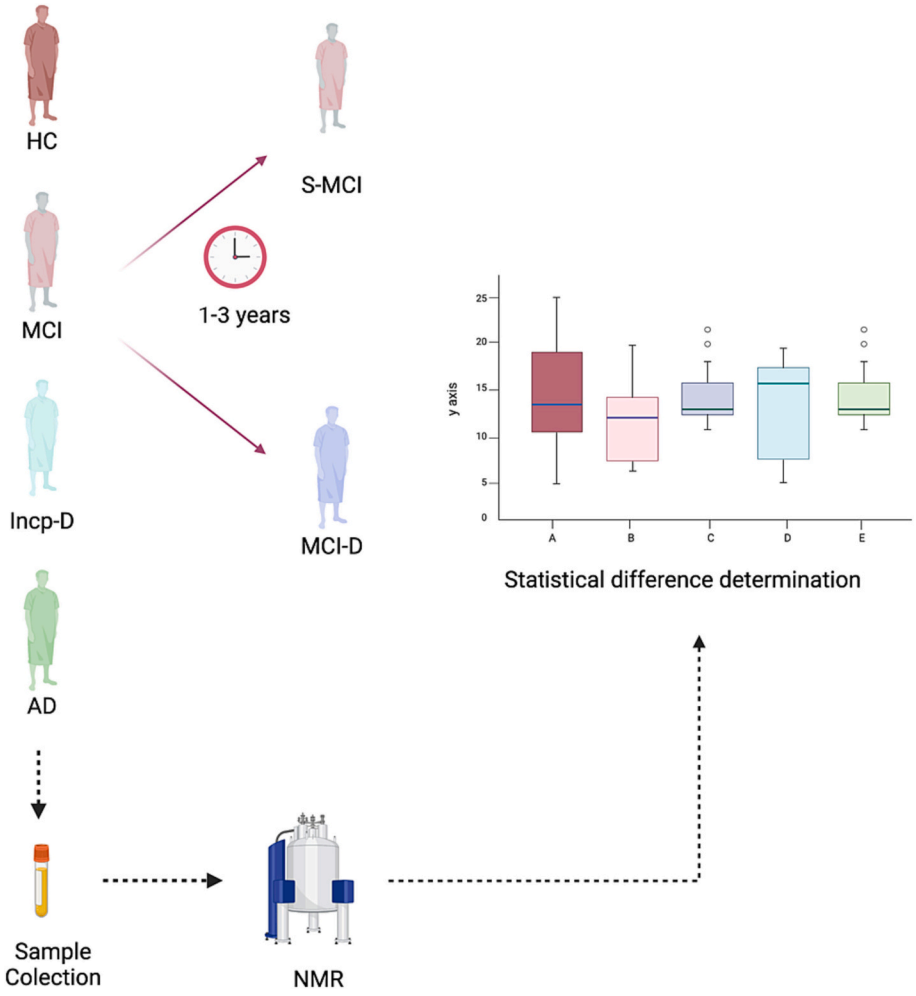

Fig. 1. Schematic representation of the analysed groups. Serum samples were collected from patients with different clinical diagnosis (HC, MCI, and AD). Within the MCI group, the clinical stage at sample collection and the progression in the follow up were diverse. Some MCI patients were in clinical condition close to dementia at sample collection, and were classified as Incp-D. In the follow-up time, some of the MCI patients progressed to a more evolved state of dementia (MCI-D) whereas for other MCI patients the clinical diagnosis remained the same (S-MCI). Taken this into consideration, statistical analyses were performed to these sets of NMR spectra to determine metabolomic differences between S-MCI and MCI-D, together with the other clinical groups: HC, Incp-D and AD.

Table 1
Sociodemographic and clinical data of participants in this study.

	Healthy controls (HC)	Mild Cognitive Impairment (MCI)			Alzheimer's disease (AD)
		Stable MCI (S-MCI)	MCI progressing to dementia (MCI-D)	Incipient Dementia (Incp D)	
Age, Mean (range)	77.2 (72–87)	80.2 (74–91)	78.9 (71–85)	80.2 (76–86)	82.35 (58–93)
Number (%)	50 (39.1)	14 (10.9)	9 (7)	4 (3.1)	51(39.8)
Sex, male/female	17/33	4/10	5/4	10/17	9/42
APOE4+ (%)	10 (20%)	2 (13%)	3 (37.5%)	2 (50%)	22 (43.1%)
MMSE* / SMMSE**	28.2 (24–30)*	25.9 (22–28)*	24.8 (19–30)*	21 (14–25)*	10.84 (0−30)**
CDR***	0 (0–0.5)	(0.5)	(0.5)	(1)	2.75 (1–3)

* MMSE = Mini Mental State Examination (0–30).
** SMMSE = Severe MMSE (0–30) (validated for Spanish-speaking population)(Díaz-Orueta et al., 2010).
*** CDR = Clinical Dementia Rating (0–3).

confusing, but scores from both scales should not be directly compared (Mougias et al., 2018). Thus, the CDR scale has also been included, which can be used with all groups studied (CDR = 0, cognitively unimpaired; CDR = 0,5, MCI; CDR = 1 mild dementia; CDR = 2, moderate dementia; CDR = 3, severe dementia).

AD-type dementia diagnosis was established according to the National Institute on Neurological Disorders and Stroke, and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) guidelines (McKhann et al., 2012). Forty three out of fifty one AD subjects had donated their brains and the AD diagnosis was neuropathologically confirmed post mortem. Participants with MCI were defined using criteria described by Petersen et al., 1999 (Petersen et al., 1999).

2.3. Sample preparation

Serum samples from healthy controls and MCI subjects from the Vallecas Project cohort as well as AD samples from the CIEN Foundation Brain Tissue Bank (BT-CIEN), were all collected in fasting conditions by venous punction. After clot removal, aliquots of 500 μL were preserved at −80 °C until the analysis.

Samples were prepared following the protocol described by Beckonert et al. (Beckonert et al., 2007). Briefly, before sample preparation, samples were thawed. Immediately, 400 μL of serum were introduced in 5 mm NMR tubes, and 200 μL of phosphate buffer (pH 7.4) were added. Phosphate buffer contained deuterated water (20% v/v) and sodium 2,2-dimethyl-2-silapentane-5-sulphonate (DSS) 1 mM as internal standard for chemical shift referencing.

2.4. NMR spectra acquisition and processing

Once the samples were prepared, NMR spectra were recorded in a Bruker Avance DRX 600 MHz spectrometer (Bruker GmbH, Rheinstetten, Germany) at *U26 NMR: Biomedical Applications II platform* from *Nanbiosis (Research Infrastructures & Services of CIBER-BBN)*. 1D ^{1}H NMR spectra were acquired for each sample using Carr-Purcell-Meiboom-Gill (cpmg) pulse sequence with water signal suppression and a total spin echo of 32 ms for each sample (interpulse delay between 180° pulses was 0.001 s, and the number of loops was 16). This pulse sequence reduces the contribution of signals from high molecular weighted molecules to the spectra, such as proteins or other macromolecules, owing to their short times of transverse relaxation (T_2). The temperature of the probe was set at 300 K (27 C). Together with the acquisition of ^{1}H cpmg spectra, 2D homonuclear (^{1}H-^{1}H TOCSY) and heteronuclear spectra were acquired (^{1}H-^{13}C HSQC) in a reduced set of samples to unequivocally identify and assign the signals in the spectra.

Once acquired, the 1D and 2D spectra were Fourier transformed and processed with *TopSpin 4.0.0* (Bruker BioSpin Corporation). For processing the 1D spectra an exponential line-broadening function of 0.5 Hz was applied followed by Fourier transformation. Phasing, baseline correction and chemical shift referencing to the trimethylsilyl signal of DSS at 0.0 ppm was also performed. For the processing of the 2D spectra the phase was corrected for rows and columns, and the chemical shift referenced to the trimethylsilyl signal of DSS at 0.0,0.0 ppm. The main signals in the spectra were assigned according to the data in the bibliography (Govindaraju et al., 2000; Martínez-Bisbal et al., 2004) and the Human Metabolome Data Base (HMDB) (Wishart et al., 2007).

After processing, meaningful signals in the cpmg spectra underwent deconvolution using *AMIX 4.0.2* software (Bruker BioSpin Corporation). The residual signals after water suppression in the area between 4.5 and 5.0 ppm, and those regions with chemical shifts lower than 0.5 ppm and higher than 8.5 ppm were excluded from the analysis. A total of 130 signals were selected in the 1D spectra and included for deconvolution (fig. S1). Then, a mixed Gaussian/Lorentzian variable function was applied for deconvolution of these signals. After deconvolution, integrals were obtained for all cpmg spectra, and were normalized to the sum of all integrals in each sample, resulting finally in a data set of 130 normalized integrals for each sample.

2.5. Multivariate statistical analysis

To determine differences between the serum metabolomic profiles of, HC, MCI and AD patients, multivariate statistical analyses were performed. For this purpose, the normalized data were fed into the software *PLS_Toolbox Solo 8.9* (Eigenvector Research, Inc., Manson, WA, USA).

Partial least squares-discriminant analyses (PLS-DA) were performed to generate predictive models able to classify the samples according to the clinical diagnosis, and using the information from the cpmg spectra deconvolution, i.e., based on its metabolic profile. Models were generated to discriminate HC vs. AD, and MCI vs. AD.

Before PLS-DA analyses the data were split in discovery (66% of the data was included for training and calculating the models) and validation subsets (33% of the data was used to apply the calculated model and to see the performance of the model). The split in 70% and 30% has been empirically proven to provide accurate models and results (Gholamy et al., 2018). Cross validation was used to determine the appropriated number of principal components.

The performance of multivariate statistic calculations is generally improved when the number of variables and samples is equilibrated (Adler and Yazhemsky, 2010). With this purpose, variable selection strategies are usually included previous to these analyses (Mehmood et al., 2012). Accordingly, in this study first, applying the knowledge on the signals in the spectra, only one representative peak from each metabolite was selected, thus reducing the number of variables from 130 to 48. Afterwards, variable selection in the calibration sets was performed selecting those variables with values of Variable Importance in Projection (VIP) higher than 0.8.

After variable selection in the discovery sets, two PLS-DA models were obtained (to discriminate HC vs. AD, and MCI vs. AD, respectively). Cross validation (using venetian blinds) was used to determine the optimum number of latent variables for the model. After applying the

models to the validation sets, sensitivity, specificity and the area under the ROC curve (AUC) were calculated to determine the goodness of the models to discriminate between each set of samples. Once validated, to determine the robustness of the model and to test for over-fitting, permutation tests (200 iterations) were performed and pairwise Wilcoxon signed rank text (Wilcoxon test), pairwise signed rank test (Rank test) and randomization *t*-test (Rand t-test) probabilities were obtained in the self-prediction and in the cross-validated residuals.

2.6. Univariate statistical analysis

Mean comparison of the identified metabolites in HC vs. AD and MCI vs. AD was performed. For comparison of pairs t-test and Mann Whitney *U* test were used, depending on the results obtained in the normality test (Kolmogorov-Smirnov (Dementia) or Shapiro Wilks (HC and MCI)). *IBM SPSS Statistics* 25 version was used for univariate statistics. MCI-to-dementia, stable MCI and incipient D as defined previously were considered in the study of MCI set. ANOVA test and the post hoc Scheffé test were used for comparison between the different MCI subsets, and AD and HC groups. Boxplots of the above-mentioned groups of metabolites showing sequential changes according to the progression in the disease were performed using *R* (*RStudio 1.2.5001*).

2.7. Analysis of altered metabolic pathways in AD and MCI

To determine the potential metabolic pathways involved in the pathological processes, *Metaboanalyst* (Chong et al., 2018) was used. A concentration table made with the relative concentration of each metabolite as columns and samples as rows as used. The HMDB ID of each metabolite was used to include them in the pathway analysis, so those metabolites whose HMDB ID was not available were not included, such as fatty acids or unknown metabolites. The global test enrichment analysis selected for the topological analysis was Relative-betweenness centrality and the *Homo sapiens* library provided by metaboanalyst was used as reference metabolome. The enrichment method selected was global test. Once the analysis was obtained, the pathways with p value < 0.05 and impact factor > 0 were chosen as representative pathways.

3. Results and discussion

3.1. Metabolic profile of serum samples

The main signals in the spectra were assigned to enable the identification of potential biomarkers of AD-type dementia and progression in the discriminant models. Fig. 2 shows the serum spectrum of one of the samples with the assignment of the main peaks. For a better observation of the signals in the figure, spectrum has been split in two parts, aliphatic (Fig. 2.a) and aromatic part (Fig. 2.b). Reference spectra of MCI (Fig. 2.c) and HC (Fig. 2.d) are also shown for comparison.

The trimethylsilyl peak of DSS can be observed at 0.00 ppm as a singlet. All the assigned resonances are shown in Table S1 with the detail in the compound and in the functional group, and for each of them, the chemical shift, the multiplicity and J coupling. With this information, 27 compounds were assigned. Within the assigned compounds, there were 11 amino acids identified, 5 organic acids and 2 sugars, among other molecules, such as fatty acids or alcohols.

3.2. Multivariate analysis of the serum metabolomic profiles

A PLS-DA analysis was performed to generate a predictive model able to classify and differentiate between HC and AD serum samples. Three principal components were selected. From the first model generated with the calibration subset, variables with VIP > 0.8 were selected, resulting in a total of 21 variables. The R2 value was 0.7 and the Q2 was 0.59. This model was then applied to the validation set and 93.75% of sensitivity and 94.18% of specificity were obtained, with an AUC value of 0.9816 (Fig. 3.a). All the permutation tests performed proved the robustness of the model ($p < 0.05$).

The variables participating in this model (and potential biomarkers of the disease) are shown in the Table 2, having a greater impact in the model *n*-acetylglucosamine, CH_3- mixed lipoproteins, and *n*-acetylated compounds, pyruvate, lysine, threonine and glycine.

Following the same procedure, a PLS-DA was performed to generate a predictive model to discriminate between dementia of the AD type against MCI. The discrimination of these two sets of patients is of the most importance in this clinical context for early diagnosis. The model was generated as described before. In this case the model had a total of 18 variables and was made with 2 principal components with an R2 value of 0.7 and a Q2 value of 0.61 and was able to determine with a 100% of sensitivity and 82.35% of specificity (for the validation set) between AD and MCI with an AUC value of 0.9281 (Fig. 3.b). All the permutation tests performed proved the robustness of the model ($p < 0.05$). The variables participating in this model are shown in Table 2. The metabolites with a greater impact in the model are CH_3- mixed lipoproteins, *n*-acetylglucosamine, *n*-acetylated compound, lysine, pyruvate and CH_2- mixed lipoproteins. Finally, a third model for the discrimination of HC and MCI was performed. The model was made with 2 principal components. The classification and prediction results obtained by PLS-DA did not offered a good performance, yielding a specificity of the 50% and 67% of sensitivity (Fig. 3.c.). The AUC value was 0.556. The Q2 value was -0.1 and the R2 0.17. As reflected by the AUC and Q2 and R2 values, this model does not have capability for prediction.

The metabolites participating in this model are shown in Table 2. Given the clinical relevance of finding differences between this two groups, further analysis will be performed seeking for potential metabolic biomarkers of the differences between HC and MCI.

Previous NMR metabolomic studies in serum have been performed to obtain discriminative models for the identification of potential biomarkers of AD and MCI. Figuera et al. 2019, generated models able to classify MCI and HC samples with an AUC value of 0.6, similar to the results we obtained. They found that threonine, 2-hydroxybutyrate, glutamine, L-tyrosine, trimethylamine, isobutyrate and propylene glycol were important in the discriminative model. They as well generated models able to classify HC and AD samples with an AUC of 0.71. The metabolites that they found important for the discrimination of samples were threonine, aspartate, creatine, *N*,*N*-Dimethylglycine, L-alanine, acetic acid and acetoacetic acid (Figueira et al., 2019). Yilmaz et al., 2020, generated discriminative models for the identification of potential biomarkers of MCI and AD by NMR and LC-MS in plasma samples. They found that acetic acid and lyso-phosphatidilcholine (C16:1), ceramide (C18:2), sphingomiosine (C24:1) and sphingomiosine (C24:0) were the most important metabolites for the discrimination between HC and MCI (Yilmaz et al., 2020). Among the set of metabolites found in the work here presented, glutamine was also found important in the discrimination between MCI and HC, and threonine and creatine were found important in the discrimination between AD and HC, as had been previously reported in the bibliography. Regarding to the discrimination between MCI and AD, the metabolites reported were obtained by LC-MS in the model found in Yilmaz et al., 2020. It seems controversial the scarce coincidence of biomarkers found in the diverse studies. Nevertheless, even though NMR is a highly reproducible technique, there are several issues to address when comparing the results obtained from different metabolomic studies, including the way the samples have been collected and prepared, the patient inclusion and classification criteria, or the different ways for data processing as the integration, scaling and normalizing methods for NMR data.

3.3. Mean comparison of metabolites

The relative concentration of metabolites in HC, AD and MCI was

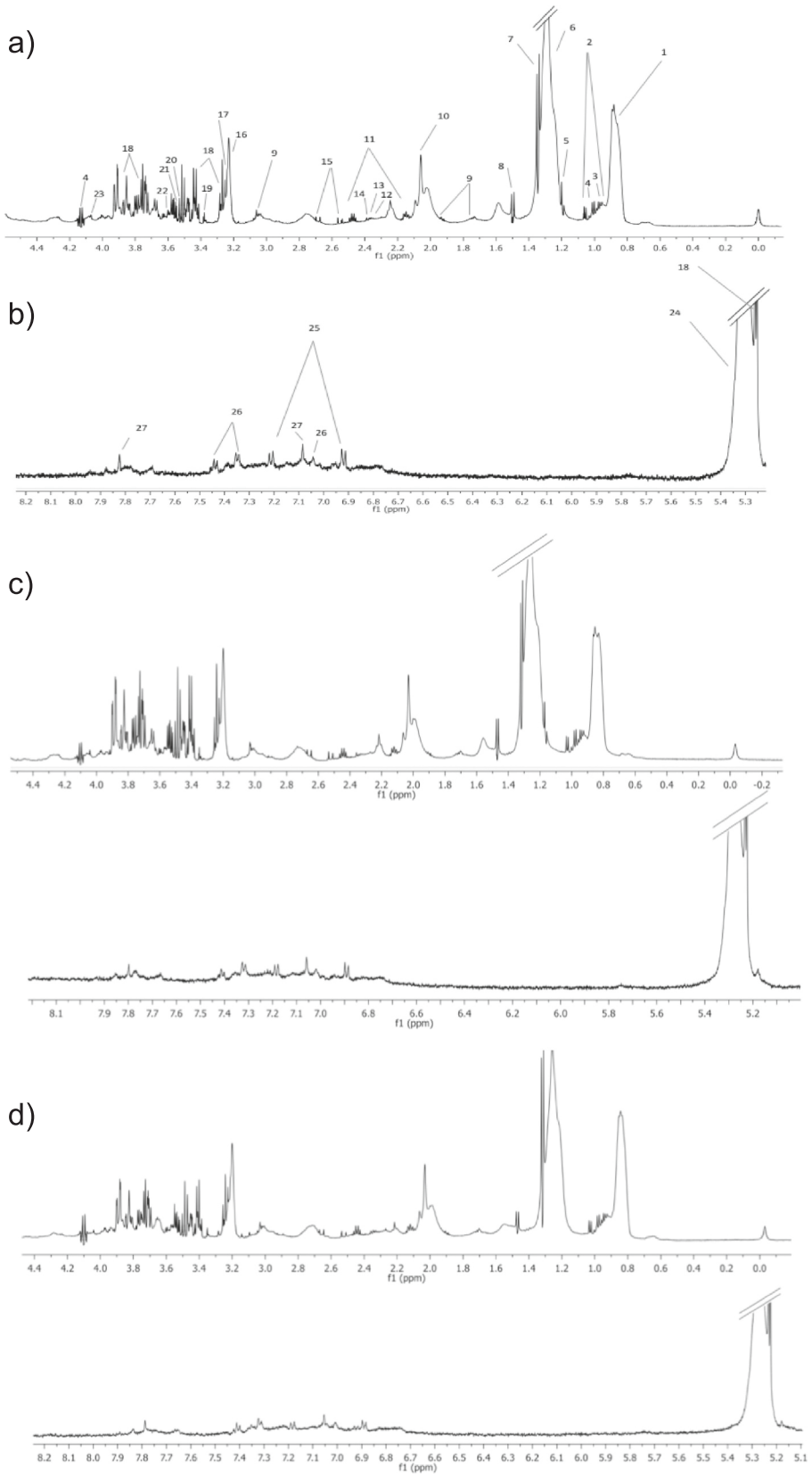

Fig. 2. Serum ^{1}H NMR spectrum of an AD patient a) Aliphatic region of the spectrum (δ_H 0–4.4 ppm). b) Aromatic region of the spectrum (δ_H 5.3–8.2 ppm). Due to a lower signal intensity in the aromatic region, the intensity was amplified 10 times in regard to the aliphatic region. The spectrum area corresponding the water suppression signal is not shown. c) Serum ^{1}H NMR spectrum of an MCI patient d) Serum ^{1}H NMR spectrum of a HC.

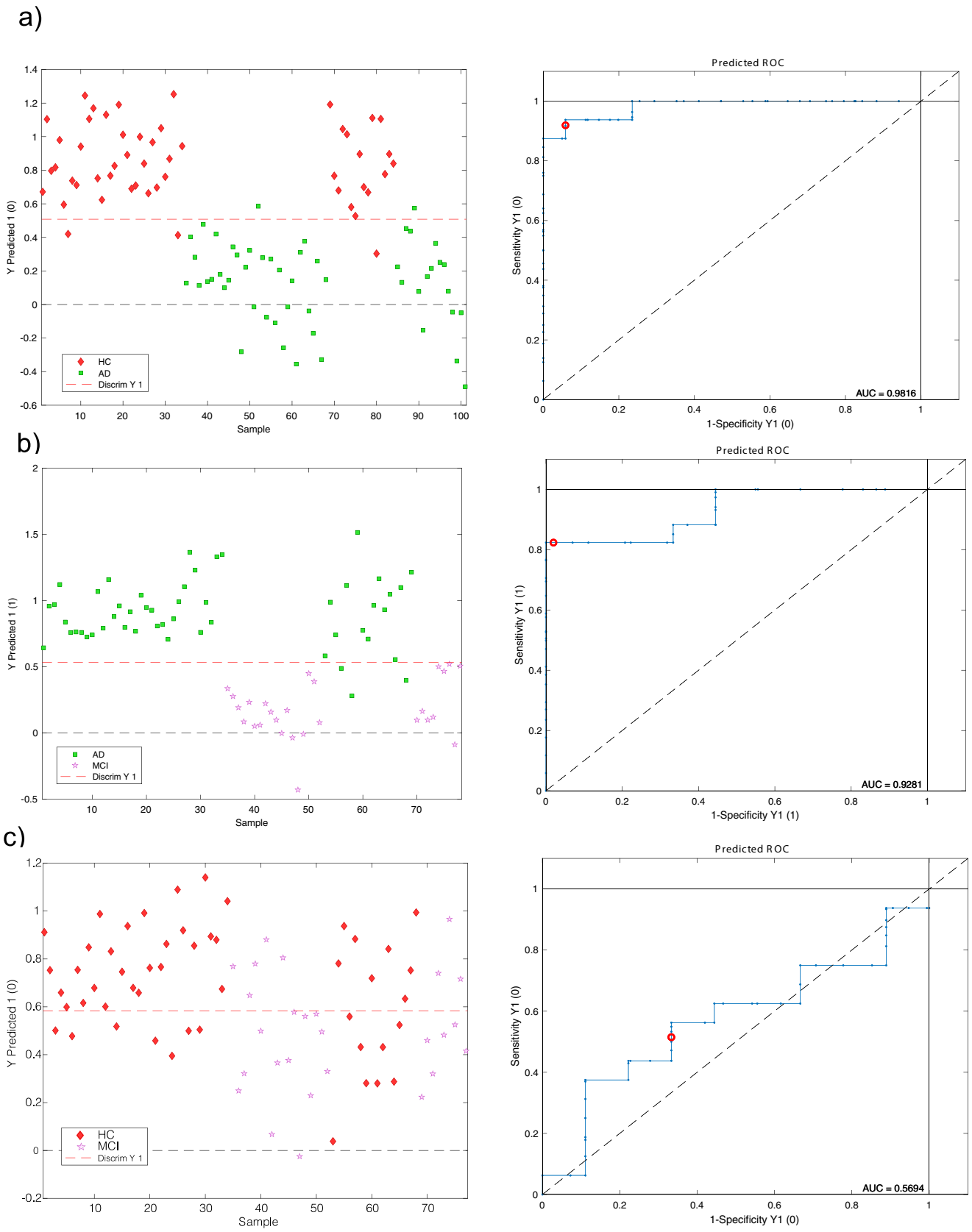

Fig. 3. PLS-DA scores and ROC curve for the classification of (a) HC vs. AD, (b) AD vs. MCI and (c) HC vs MCI. In the left side of the panel the prediction plot is shown, divided in the scores obtained in the calibration and validation subsets. In the right side of the panel, ROC curve is shown for each model, with an AUC value of (a) 0.9816, (b) 0.9281 and (c) 0.5694 for discrimination of HC vs. AD, AD vs. MCI and HC vs MCI.

Table 2
Variables participating in the models sorted by VIP value.

AD vs HC		AD vs MCI		HC vs MCI	
Variable	VIP S.	Variable	VIP S.	Variable	VIP S.
N-acetylglucosamine	1.54	CH_2 m.l. *(1.20 ppm)	1.67	Leucine	1.57
CH_2 m.l. (1.20 ppm)	1.52	*N*-acetylglucosamine	1.56	Choline	1.39
N-acetyled comp.	1.34	*N*-acetyled comp.	1.31	Valine	1.27
Pyruvate	1.30	Lysine	1.27	Pyruvate	1.25
Lysine	1.10	Pyruvate	1.15	Creatinine	1.22
Threonine	1.09	CH_3 m.l. (0.83 ppm)	1.13	*N*-acetyled comp.	1.02
Glycine	1.08	Phenylalanine	0.99	Lysine	1.02
Ethanol	0.93	Ethanol	0.99	Arginine	1.01
CH_3 m.l. (0.85 ppm)	0.91	Tyrosine	0.87	Glutamine	1.00
Choline	0.90	Unk (3.86)	0.87	Glycerol	0.94
CH_3 m.l. (0.83 ppm)	0.90	Citrate	0.82	Alanine	0.84
Valine	0.90	Choline	0.79	Acetyl Choline	0.83
CH_3 m.l. (0.87 ppm)	0.89	Glycine	0.68	Isoleucine	0.83
Creatine	0.87	Lactate	0.66	Threonine	0.77
Phenylalanine	0.84	Creatine	0.65	CH_3 m.l. (0.81 ppm)	0.71
Glycerol	0.81	Unk (7.01 ppm)	0.63	CH_3 m.l. (0.83 ppm)	0.70
Unk (7.01 ppm)	0.73	Acetone	0.44	Ethanol	0.66
Acetylcholine	0.68	Glycerol	0.41	Lactate	0.66
Methanol	0.67				
CH_3 m.l. * (0.88 ppm)	0.61				
Acetone	0.55				

* m.l. means mixed lipoproteins.

compared. The 48 signals, used for the multivariate statistics were here analysed, including identified metabolites, unknown peaks, and different peaks from fatty acids and lipoproteins. Significant differences were identified in most of the metabolites analysed in both comparison HC vs. AD and AD vs. MCI, highlighting the wide impact that the disease has in the cellular metabolism (Table 3).

Creatine, ethanol, threonine, glycine, methanol, lysine, *n*-acetylglucosamine, alanine, CH_2 mixed lipoproteins, valine, CH_3 mixed lipoproteins, phenylalanine, acetylcholine, choline, pyruvate, acetone, glycerol, isoleucine, *N*-acetyled compounds and two unassigned signals (7.01 ppm and 3.86 ppm) showed statistical differences in its relative mean concentrations between AD and HC. In the comparison of AD vs. MCI, statistical differences ($p < 0.05$) were found in lactate, creatine, ethanol, glycine, lysine, *n*-acetylglucosamine, CH_3 mixed lipoproteins, CH_2 mixed lipoproteins, phenylalanine, pyruvate and choline. At this point, it deserves to be noted that most of the variables in the PLS-DA models showed significant differences in their means for both comparisons, HC vs. AD and MCI vs. AD (with exception of valine in both comparisons, as well as the unknown compound at 7.01 ppm in MCI vs. HC).

For the comparison between HC and MCI we found significant differences in the relative concentration of lactate and threonine. The levels of lactate in cerebrospinal fluid have been associated to disease severity in other neurological diseases (Albanese et al., 2016). However, other studies, also performed in CSF observed the same behaviour as here is presented in serum, showing an increase in the concentration of lactate between HC and AD that is not significant, whereas a higher and statistically significant increase is observed in the serum samples of MCI patients when compared to HC and AD (Zebhauser et al., 2022). It is

Table 3
Mean comparison of relative concentration of metabolites with significant differences between HC and AD and/or MCI and AD.

Metabolites	[Metab]rel x 10^3			p value		
	HC	MCI	AD	HC vs AD	MCI vs AD	HC vs MCI
Unk (7.01)	1.030	0.959	0.763	0.007	0.069	0.578
Lactate	1.181	1.460	1.249	0.740	0.038	0.025
Creatine	3.221	3.350	4.256	0.000	0.008	0.669
Unk (3.86)	11.850	13.511	7.235	0.002	0.000	0.370
Threonine	1.838	2.031	2.108	0.002	0.797	0.023
Glycine	3.524	3.625	4.221	0.001	0.025	0.685
Methanol	1.483	1.505	2.003	0.011	0.079	0.468
Lysine	4.856	4.499	6.101	0.000	0.000	0.211
N-acetylglucosamine	35.144	34.781	45.484	0.000	0.000	0.741
Alanine	4.860	5.169	5.767	0.012	0.224	0.179
CH_2 m.l.* (0.852)	44.679	41.141	52.991	0.000	0.000	0.795
Phenylalanine	0.487	0.476	0.744	0.000	0.001	0.866
Acetylcholine	26.860	22.151	21.353	0.006	0.805	0.660
Choline	33.989	32.328	28.069	0.000	0.000	0.146
Pyruvate	2.429	2.521	3.360	0.000	0.000	0.551
Acetone	7.827	7.803	8.973	0.038	0.098	0.940
Isoleucine	4.577	4.560	4.261	0.048	0.655	0.781
Ethanol	3.527	3.680	3.429	0.000	0.000	0.423
Glycerol	4.631	4.883	2.594	0.000	0.000	0.417
N-acetyled compound	12.271	12.608	15.963	0.000	0.000	0.527
CH_3 m.l.* (1.204)	45.991	46.874	25.969	0.000	0.000	0.814
CH_2 m.l.* (0.865)	16.242	14.679	23.375	0.004	0.000	0.240
CH_2 m.l.* (0.832)	60.757	53.507	65.815	0.248	0.031	0.130
CH_2 m.l.* (0.813)	37.802	33.615	35.517	0.034	0.152	0.823

* m.l. means mixed lipoproteins.

interesting to observe that this association between higher lactate levels and earlier stages of dementia is also found in serum, which can be obtained following minimally invasive procedures compared to those needed to obtain CSF. Zebhauser et al. suggested that the increase of lactate levels in CSF in MCI could be produced by the activation of microglia (Zebhauser et al., 2022).

3.4. Metabolic changes between MCI patients with different progression rates

Attention should be drawn to MCI that is a diverse group, and a clinically relevant issue would be to be able to discriminate, previous to the manifestation of overt clinical symptoms, whether a patient with MCI in the near future will progress to dementia or, on the contrary, if this patient will remain in MCI condition. For that purpose, the MCI patient's progression along the follow up period (between 1 and 3 years) was considered. According to their clinical progression, the MCI patients were classified in MCI-to-dementia (MCI-*D*), stable MCI (S-MCI) and incipient dementia (Incp-D).

When the MCI patients were projected onto the HC vs AD model, they were not classified according to the progression of cognitive impairment (Fig. S2), that is why a different strategy was followed seeking for differences that could be found within the MCI group, and could be related to the progression of the disease.

With that purpose of identifying metabolic changes between the different clinical evolution in MCI subgroups in comparison to HC and AD patients, boxplots of the different metabolites were represented, ordered according to the clinical severity of each subset of patients, HC, S-MCI, MCI-D, Incp-D and AD-type dementia (Fig. 1).

A progressive increase in the concentration of phenylalanine, lysine, pyruvate, and CH_2 mixed lipoproteins, and a progressive decrease in the concentration of choline, was observed (Fig. 4).

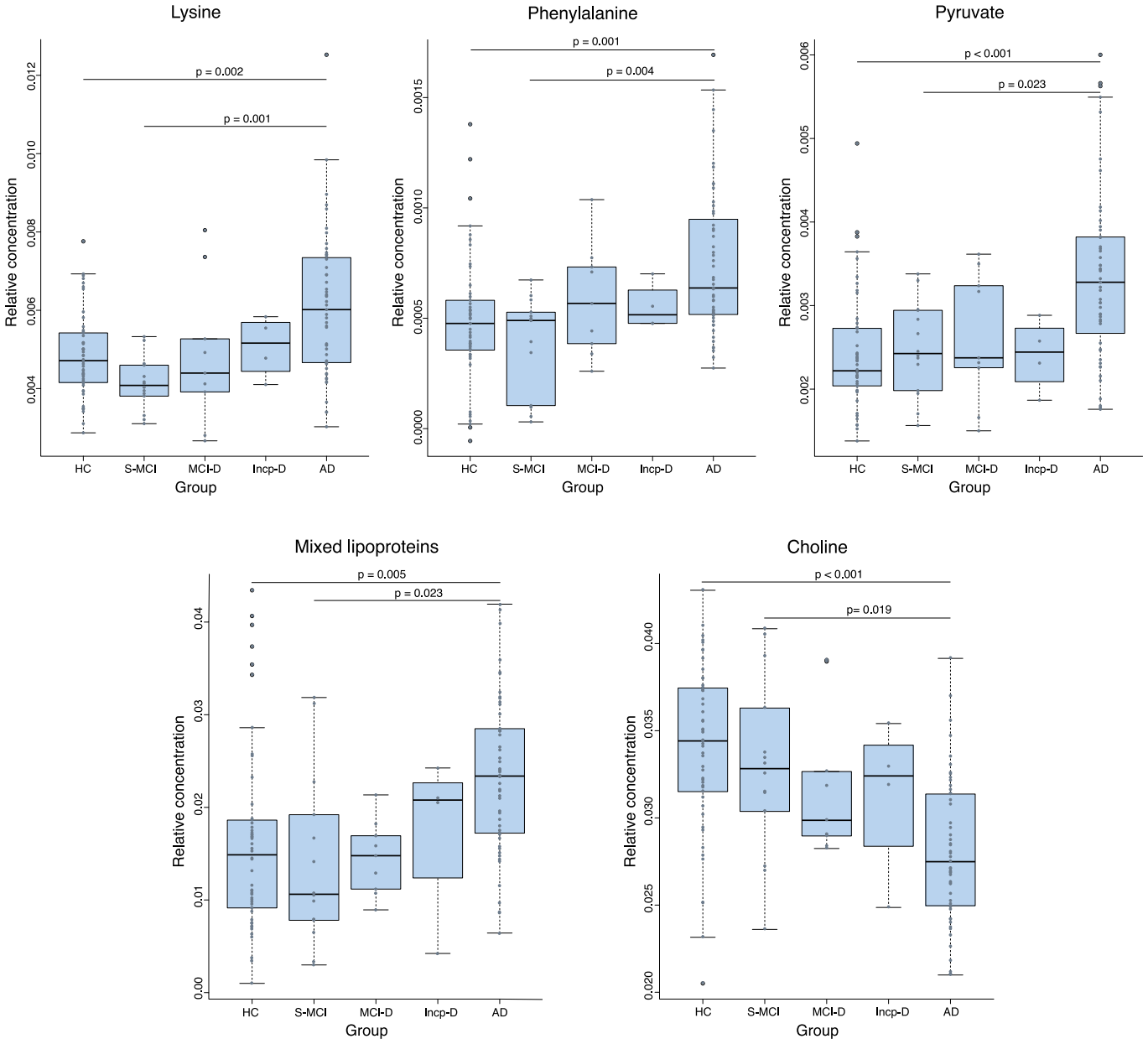

Fig. 4. Relative concentration of metabolites with a progressive trend between the groups of study. The median (horizontal bar) of lysine, phenylalanine, pyruvate, CH_2 mixed lipoproteins and choline relative concentration for the different groups (HC, S-MCI, MCI-D, Incp-D and AD) is depicted. The p value of groups with means significantly different is shown.

Statistical value of these differences was assessed by ANOVA test, and afterwards by Scheffé test to determine the difference between each group. The Scheffé test is appropriated to be used when the size of the groups is small or the samples size between groups is unbalanced as it is in our case. Differences between S-MCI and AD patients were found ($p < 0.05$), but these differences were not significant between MCI-D and AD patients. These results show that at the time of sample collection the concentration of these metabolites in MCI patients that later evolved to dementia (MCI-D) had already values closer to AD than those from MCI patients who did not evolve (S-MCI). In that way, these metabolites could be postulated as potential predictive biomarkers. An interval value of these metabolites could be determined to discriminate whether MCI samples would evolve to AD or other advanced states of dementia before the clinical symptoms were developed.

In a parallel analysis, the metabolic pathways that could be altered in the development of dementia were explored. The different groups of

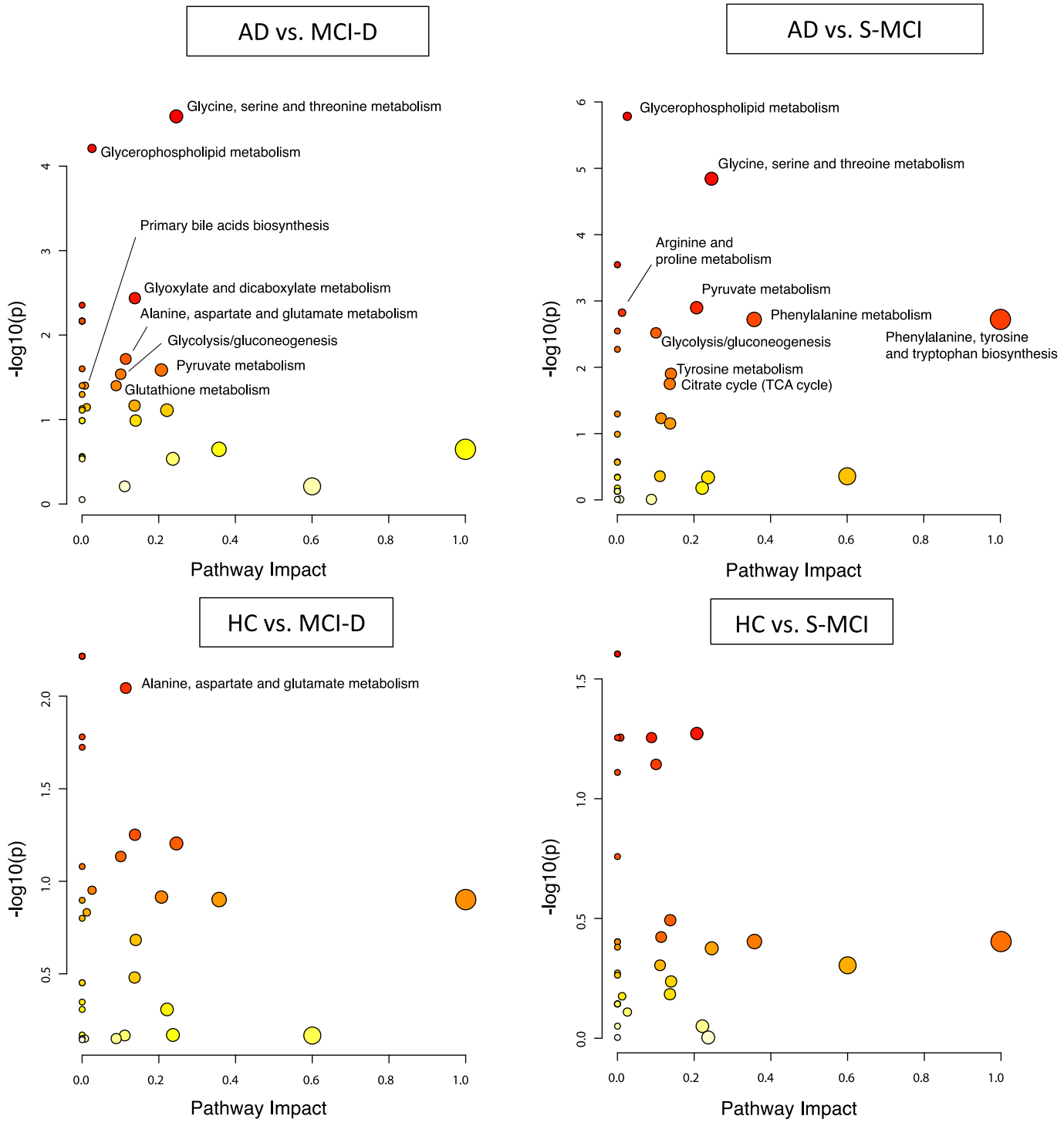

Fig. 5. Metabolic pathways altered in AD and MCI subsets. Only the significant metabolic pathways are labelled (p-value <0.05, impact >0). Colour and size of the circles indicate the p-value and impact index, respectively: $-\log_{10}(p)$ is represented from higher values (red) to lower values (yellow) and the pathway impact is reflected in the size of the circles from smaller circles (lower impact) to bigger circles (higher impact). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

patients, including the MCI subgroups, were compared to find metabolic pathways affected in the different conditions of the disease in the MCI and dementia context.

There were 14 metabolic pathways altered between dementia and HC (not shown), 8 of them were as well altered between AD and MCI-D / Incp-D, whereas 9 pathways were altered between AD and S-MCI (Fig. 5). Following a logic similar to the one presented before, a higher number of metabolic pathways were altered in the comparison between AD and S-MCI than when AD samples were compared with those samples that were closer, or later evolve, to dementia (MCI-D / Incp-D) (Fig. 5). Remarkably, four pathways did not show significant differences between AD and MCI-D / Incp-D, but showed significant differences between AD and S-MCI: phenylalanine, tyrosine and tryptophan biosynthesis, phenylalanine metabolism, tyrosine metabolism, and TCA cycle. These results suggest that these four pathways and the involved metabolites should be further studied in an attempt to determine which MCI patients are at higher risk of developing AD. Phenylalanine, tyrosine, acetoacetate, citrate and pyruvate are metabolites involved in the routes above mentioned. Phenylalanine and pyruvate, as described before, have shown a progressive increase in its concentration from HC to AD passing though the different MCI subgroups (Fig. 4), and both metabolites have been demonstrated to be important in the discrimination between dementia and HC and between dementia and MCI according to the PLS-DA models (Table 2). The disturbance of some of these pathways and metabolites, and its related biological processes has been previously reported.

Current knowledge about Alzheimer's disease points out the alterations in the metabolism glucose (Yan et al., 2020), highlighting the dysfunction of glycolysis (Hipkiss, 2019), as well as successive processes involved in the energetic metabolism, such as the decreased functioning of the pyruvate dehydrogenase complex (Sorbi et al., 1983). In this work, an increment in the concentration of pyruvate already in MCI patients has been observed, being higher the increment in MCI-D patients than in S-MCI. In this context, it is also remarkable the differences found in the TCA cycle between dementia patients and HC and dementia patients and stable MCI, but not between AD and MCI-D / Incp-D. The TCA cycle is the main pathway of glucose oxidation in the brain, and a diminution in isocitrate dehydrogenase and α-ketoglutarate dehydrogenase complex has been previously described (Bubber et al., 2005). Phenylalanine metabolism has also been previously reported to be modified in dementia serum samples(Sun et al., 2020), and brain tissue (Liu et al., 2021).

On the other hand, the comparison of HC and stable MCI pathways did not show any relevant difference, whereas the alanine, aspartate and glutamate metabolism, was altered in the comparison of HC group and MCI-to-dementia/incipient dementia (Fig. 5). Glutamine, citrate, pyruvate, and alanine are the metabolites involved in this pathway. The implications of pyruvate and associated pathways has already been addressed in this work. However, other studies have involved some of these metabolites with dementia. For example, alterations in the levels of circulating glutamine (Adams, 2020) and impairments in the glutamate/glutamine cycle (Robinson, 2000) has been previously related to the development of dementia. This impairment of the glutamate/glutamine cycle are related with changes in mood, behaviour and memory loss among others, all of them are alterations observed in AD patients (Robinson, 2000). All together these results show that there are changes in the MCI group that could be further analysed in order to have an earlier predictive diagnosis of those patients that would evolve to more advanced stages of dementia. To confer clinical applicability potential to the analysis performed by NMR spectroscopy, threshold values and metabolic signatures should be defined and stablished for a molecular diagnosis and prognosis of AD. To do that, a study with a big enough cohort should be developed ensuring that the clinical criteria and analysis process are standardized.

4. Conclusions

In this study the differences between serum samples from HC, AD patients and MCI patients with different levels and progressions have been analysed by ^{1}H NMR spectroscopy. Two predictive models have been generated to discriminate between AD and HC samples and AD and MCI samples, with high levels of sensitivity and specificity (93.75% and 94.75% for discrimination of AD and HC, and 100 and 82.35% for AD and MCI), according to the metabolic information in the NMR spectra. These models could be of use as a non-invasive tool to support dementia diagnosis. Furthermore, significant differences between AD and HC have been found in the relative concentration of most of the analysed metabolites, highlighting the impact that the cellular metabolism has in dementia. Moreover, significant differences were also found in 12 metabolites when comparing MCI and dementia serum samples. Furthermore, differences within the MCI group in agreement with the clinical evolution have been found, which would allow to find biomarkers that could help to determine which MCI patients would progress to dementia. An increase in phenylalanine, lysine and pyruvate and a progressive decrease in the concentration of choline, can be observed in the progression to dementia. On the other hand, alanine, aspartate and glutamate metabolism; pantothenate and CoA biosynthesis; and beta-alanine metabolism have been found altered when comparing HC and MCI-D whereas no pathway was altered between HC and S-MCI, which has allowed us to determine some differences in the metabolism of the different kind of patients inside the MCI group. What is described here could be a starting point to explore how the development of MCI to dementia could be effectively predicted by the study of serum using NMR spectroscopy. Future projects include the follow-up of the patients here studied, and the increment of the number of patients to confirm the results.

CRediT authorship contribution statement

Marina Botello-Marabotto: Investigation, Formal analysis, Writing – original draft. **M. Carmen Martínez-Bisbal:** Methodology, Investigation, Writing – review & editing. **Miguel Calero:** Methodology, Resources, Writing – review & editing. **Andrea Bernardos:** Investigation, Writing – review & editing. **Ana B. Pastor:** Resources, Writing – review & editing. **Miguel Medina:** Conceptualization, Funding acquisition, Writing – review & editing. **Ramón Martínez-Máñez:** Conceptualization, Funding acquisition, Writing – review & editing.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nbd.2023.106312.

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