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Plasma extracellular vesicles and phosphorylated tau 181 as early biomarkers of cognitive impairment in Alzheimer's dementia

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Abstract

Background Timely and accurate diagnosis of Alzheimer's disease (AD) in clinical practice is a great challenge, especially during early disease stages with subtle or mild symptoms of cognitive decline. Moreover, robust and more accessible blood-based screening tests for early diagnosis are needed. In this study, we investigated the core AD blood biomarkers — amyloid beta 42 ($A\beta_{42}$) and 40 ($A\beta_{40}$) peptides, phosphorylated tau 181 ($p\text{-}Tau_{181}$), neurofilament light chain (NfL), and total tau (t-Tau) — and extracellular vesicle (EVs) size and concentration in individuals characterized by different stages of cognitive decline to identify biochemical markers of dementia for early diagnosis.

Methods A total of $n=800$ human plasma samples were analyzed. Plasma levels of NfL, t-Tau, $p\text{-}Tau_{181}$, $A\beta_{42}$, $A\beta_{40}$ and plasma EVs were evaluated in $n=217$ elderly healthy subjects (CTRL), in individuals with subjective cognitive complaints (SCC, $n=48$), pre-mild cognitive impairment (pre-MCI, $n=58$) and mild cognitive impairment (MCI, $n=426$), and in $n=51$ probable AD dementia patients (AD-dem), using ultrasensitive Single Molecule

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Array technology (Simoa®) and nanoparticle tracking analysis (NTA). Logistic regression and Receiver Operating Characteristic (ROC) analyses were employed.

Results Plasma NfL displayed increased levels in AD-dem and MCI patients, while p-Tau$_{181}$, Aβ_{42}/Aβ_{40} ratio, Aβ_{42}/p-Tau$_{181}$ ratio, and EVs plasma levels were altered since the early stages of the pathology: in particular, p-Tau$_{181}$ levels increased as cognitive symptoms worsened, already in the SCC and pre-MCI groups compared to CTRL, while the ratio of EVs concentration and size (EVs ratio) was decreased in all groups compared to CTRL. Plasma p-Tau$_{181}$ best classified AD-dem patients from CTRL with an area under the curve (AUC) equal to 0.87, while EVs ratio best differentiated SCC from CTRL (AUC = 0.78). Combining p-Tau$_{181}$ and EVs ratio with Aβ_{42}/Aβ_{40} ratio and NfL, respectively, significantly improved the classification of pre-MCI and MCI from CTRL (AUC$_{comb}$ = 0.79 and AUC$_{comb}$ = 0.85). Combining biomarkers did not improve accuracy in discriminating MCI from SCC, pre-MCI and AD-dem.

Conclusions p-Tau$_{181}$ and EVs ratio are promising biomarkers for the identification of individuals at risk of degenerative dementia. Combining the core AD plasma biomarkers with EVs ratio can aid in diagnosing the early stages of AD dementia.

Keywords Alzheimer's dementia, Amyloid beta peptide, Early diagnosis, Extracellular vesicles, Mild cognitive impairment, Neurofilament light, Phosphorylated Tau 181, Plasma biomarkers, Preclinical stages, Ultrasensitive assays

Background

Alzheimer's disease (AD) is the most common cause of dementia and one of the major causes of disability and dependency among older people worldwide. AD is an irreversible and progressive neurodegenerative disorder characterized by the formation of intracellular amyloid beta (Aβ) peptide aggregates, the deposition of neurofibrillary tangles mainly composed of phosphorylated tau protein [1–5], and the progressive neuronal and synaptic damage, that leads to gradual loss of memory and cognitive abilities [6, 7]. The formation of Aβ and tau inclusions, a crucial event in AD onset and progression, starts years prior to the outbreak of clinical symptoms [8, 9].

Different disease stages characterize the course of AD: from the preclinical or presymptomatic stage in which individuals are cognitively normal, up to severe dementia due to AD, through a middle prodromal stage of mild cognitive impairment (MCI) [10, 11]. MCI is characterized by single or multiple impairments in cognition with "grossly" preserved functional abilities [12–14]. Compared to cognitively normal subjects, patients with MCI have a higher risk of conversion to dementia [15–17], but only 30–40% of them progress to dementia if followed-up for 3 to 5 years, while the remaining never become demented or even reverse to normal from the MCI stage [11, 18, 19].

Subjective cognitive complaints (SCC) are defined as self-reported concerns of persistent decline in cognitive functions, with or without objective cognitive deficits, and with performance on standardized cognitive tests within the normal range [20]. In 2014, the term "subjective cognitive decline" (SCD) provided a standardized terminology for indicating SCC [20]. Individuals with SCD and cognitively unimpaired individuals without SCD perform normally on cognitive tests and are, by definition, objectively unimpaired, thus, they cannot be objectively distinguished. However, in cognitively unimpaired individuals, SCC may represent the preclinical symptomatic manifestation of later cognitive impairment due to AD [21].

An intermediate stage between normal cognition and MCI, called "pre-MCI", has also been described [22, 23]. Pre-MCI subjects exhibit cognitive symptoms and mild functional impairments, although cognitive deficits are not confirmed by neuropsychological testing, resulting in either normal scores or scores that do not meet formal criteria for MCI [23]. Compared to cognitively normal individuals, pre-MCI subjects showed faster progression to MCI, but slower progression to dementia than MCI [23]. In addition, it has been reported that among subjects classified as pre-MCI by informant-based assessment alone who underwent autopsy — which accounted for 17% of all pre-MCI cases — 91% received a neuropathologic diagnosis of AD [22].

The identification of AD-like pathology relies on positron emission tomography (PET) and cerebrospinal fluid (CSF) biomarkers even in absence of clinical manifestations [24]. Aβ and tau protein levels in CSF can identify AD neuropathology already at presymptomatic and prodromal stages, and have the potential to predict cognitive decline even if the percentage of "positive" subjects without clinical manifestations is significant [25, 26]. Recently, the Alzheimer's Association Workgroup published the revised criteria for diagnosis and staging of AD [27] incorporating recent advances in biomarkers, with the objective of facilitating a connection between research endeavors and clinical practice. According to these criteria, the positivity of AD biomarkers is now considered mandatory to make an AD diagnosis in clinical setting both at MCI and dementia phase. However, such a view has been tempered down by the vast majority of experts who still consider the clinical and neuropsychological

diagnosis as the only one suitable [28, 29], and by the International Working Group (IWG) which continues to support the view of AD as a clinical and biological entity [30].

Considering the invasiveness and expensiveness of CSF collection and PET imaging, identifying robust and more accessible screening tests to be used in the initial disease stages where most treatment strategies are most effective is increasingly crucial [31, 32]. The advancements done in the context of ultrasensitive assays have made possible the detection in the blood of proteins at low concentration, thus improving the research on plasma AD biomarkers [33]. $A\beta_{42}$ peptide (or $A\beta_{42}/A\beta_{40}$ ratio), phosphorylated tau (p-Tau), neurofilament light chain (NfL), and total tau (t-Tau) are the core blood biomarkers of AD, which reflect Aβ pathology, tau pathology, and neuronal damage, respectively [34], and are the most promising biochemical markers for dementia diagnosis and/or prognosis [35–46]. In recent years, extracellular vesicles (EVs) (i.e., transfer vehicles between cells potentially carrying disease-associated proteins) have been conceptualized as a promising source of biomarkers. Recently, we investigated the role of EVs in AD and related dementias, specifically characterizing their concentration and size in human body fluids (i.e., blood and CSF). We reported that, in the CSF of AD patients, EVs parameters are related to CSF core biomarkers for AD, since a positive correlation was found between EVs concentration/size and $p\text{-}Tau_{181}/A\beta_{42}$ ratios [47]. Moreover, we provided evidence of alterations in plasma EVs size and concentration across distinct forms of dementia [48].

The aim of the present multicentric study promoted by the Italian IRCCS Network of Neuroscience and Neurorehabilitation (RIN) was to evaluate plasma levels of NfL, t-Tau, $p\text{-}Tau_{181}$, $A\beta_{42}$, $A\beta_{40}$, and plasma EVs in healthy subjects, in individuals with SCC, pre-MCI and MCI, and in probable AD dementia patients (AD-dem), to characterize individuals at risk of degenerative dementias who may benefit of "disease-modifying" treatments to slow or halt disease progression. Here, we propose plasma EVs as a novel and sensitive biomarker of early cognitive decline, with the potential to improve diagnosis during the presymptomatic and prodromal phases of AD dementia, especially when used alongside well-established AD blood biomarkers.

Methods

Participants

A total of $n = 800$ human plasma samples from $n = 51$ AD-dem, $n = 426$ MCI, $n = 58$ pre-MCI, $n = 48$ SCC, and $n = 217$ elderly subjects with normal cognitive function (range 50–86 years) and no reported cognitive complaints, as control group (CTRL), were analyzed in the present retrospective study. AD-dem, MCI, pre-MCI and SCC participants were enrolled within the framework of the AD-NET (NET-2011–02346784) and INTERCEPTOR studies and biological samples were made available for the present study, while CTRL subjects' biological samples were available at institutional biobanks/biorepositories of the centers involved in the study. All study groups were defined on clinical grounds with no biomarker information. Clinical diagnosis of AD dementia and MCI was made according to international guidelines [49, 50]. The study included individuals with a formal diagnosis of MCI according to the National Institute on Aging-Alzheimer's Association (NIA-AA) criteria [49], preserved independence in functional abilities, self- or informant-reported cognitive complaint and objective cognitive impairment. On a total of 426 MCI, $n = 212$ had a diagnosis of multiple domain MCI (mdMCI) and $n = 214$ were diagnosed as single domain MCI (sdMCI), according to [14, 49]. Pre-MCI were defined according to [22] modified criteria as follows: 1) self-experience of a persistent cognitive decline and either (a) equivalent scores (E.S.) = 1 on one or more neuropsychological tests with a Clinical Dementia Rating (CDR) scale = 0, or (b) CDR = 0.5; 2) no complaints of cognitive decline but CDR = 0.5. SCC subjects were defined as follows: 1) self-experience of persistent decline in cognitive capacity unrelated to an acute event; 2) E.S. ≥ 2 on all neuropsychological tests; and 3) CDR = 0 according to [20].

Demographic and clinical characteristics are reported in Additional file 1 (Table 1) and Additional file 2 (Supplementary Table 1). Participants signed an informed consent for blood collection and biobanking, as approved by the local Ethics Committees. The study protocol was approved by the local Ethics Committee "Comitato Etico IRCCS San Giovanni di Dio Fatebenefratelli" of the IRCCS Centro San Giovanni di Dio Fatebenefratelli, Brescia (Prot. N. 10/2022, Prot. N. 14/2023). The study received approval from the Ethics Committee of all participating centers.

Plasma sample collection and processing

Peripheral venous blood was collected under fasting conditions in ethylenediaminetetraacetic acid (EDTA) tubes and immediately centrifuged at 2,000 × g for 10 min to remove blood cells and prevent platelet activation and release of platelet-derived EVs. The supernatant plasma was transferred to new tubes and immediately stored in cryovials at temperatures below −75 °C until analyses. The time interval between blood collection and plasma freezing was kept minimal to preserve sample integrity. Biological samples and clinical data from the INTERCEPTOR and AD-NET studies were collected between December 2018 and December 2020, and from 2017 to 2020, respectively. Biological samples of CTRL subjects were selected from institutional biobanks/biorepositories

Table 1 Diagnostic accuracy and performance of the best predictors for diagnostic comparisons (ROC curve analysis)

	Best predictor	AUC (95% CI)	Cut-off	Sensitivity, % (95% CI)	Specificity, % (95% CI)
CTRL vs. SCC	EVs ratio	0.78 (0.71–0.85) ***	$< 1.14 \times 10^9$	75.0 (62.5–85.4)	71.9 (65.9–77.4)
CTRL vs. pre-MCI	$A\beta_{42}/A\beta_{40}$ ratio	0.71 (0.63–0.78) ***	> 0.054	53.5 (41.4–65.5)	77.4 (71.9–82.5)
CTRL vs. MCI	EVs ratio	0.81 (0.77–0.84) ***	$< 1.13 \times 10^9$	76.5 (72.5–80.3)	72.4 (66.4–78.3)
CTRL vs AD-dem	$p\text{-}Tau_{181}$	0.87 (0.82–0.92) ***	> 25.34 pg/mL	88.2 (78.4–96.1)	77.4 (71.4–82.9)
SCC vs. pre-MCI	-	-	-	-	-
SCC vs. MCI	NfL	0.59 (0.51–0.68) *	> 15.88 pg/mL	44.8 (40.0–49.5)	72.9 (60.4–85.4)
SCC vs. AD-dem	$A\beta_{42}/p\text{-}Tau_{181}$ ratio	0.72 (0.62–0.82) ***	< 0.24	58.8 (45.2–72.3)	70.8 (58.3–83.3)
pre-MCI vs. MCI	EVs ratio	0.66 (0.59–0.72) ***	$< 8.85 \times 10^8$	58.5 (54.0–63.1)	65.5 (53.5–77.6)
pre-MCI vs AD-dem	$A\beta_{42}/A\beta_{40}$ ratio	0.74 (0.64–0.83) ***	< 0.047	72.6 (60.8–84.3)	67.2 (55.2–79.3)
MCI vs. AD-dem	EVs ratio	0.69 (0.61–0.77) ***	$> 9.30 \times 10^8$	72.6 (60.8–84.3)	62.7 (60.0–67.6)

AUC Area under the curve, *CI* Confidence interval. Confidence intervals were estimated via bootstrapping ($n = 2000$). *$p < 0.050$, ***$p < 0.001$ for the logistic model used in the ROC analysis. The cut-offs were chosen according to Euclidean distance [51]

to ensure a comparable collection timeframe to that of patient samples. Biomarker analyses (AD biomarkers and EVs) on plasma samples were carried out between March 2022 and December 2023, using plasma samples that underwent a single thaw cycle upon receipt from biobanks/biorepositories.

Plasma biomarkers measurements

Plasma levels of NfL were measured using the commercially available Simoa® Human NF-light Advantage assay kit and Human NF-light V2 Advantage assay kit (Quanterix) on the automated Simoa® SR-X and HD-1 analyzer (Quanterix), following the manufacturer's instructions. Plasma levels of $p\text{-}Tau_{181}$ were measured using the commercially available Simoa® Human pTau-181 Advantage V2 assay kit and Simoa® Human pTau-181 Advantage V2.1 assay kit (Quanterix) on the automated Simoa® SR-X and HD-1 analyzer (Quanterix), following the manufacturer's instructions. Data transformation was applied to Simoa® p-Tau181 V2 data to align it with Simoa® p-Tau181 V2.1 data, according to the manufacturer's instructions. Plasma levels of $A\beta_{40}$, $A\beta_{42}$, and t-Tau were simultaneously measured using the commercially available Simoa® Human Neurology 3-Plex A Advantage assay kit (Quanterix, Lexington, MA, USA) on the automated Simoa® SR-X and HD-1 analyzer (Quanterix), following the manufacturer's instructions. Samples were thawed at room temperature (RT) for 60 min and centrifuged at 10,000 × g for 5 min prior to analyses, as suggested in the user protocol, to prevent any sample debris from interfering in measurement, then transferred to 96-well plates and diluted 1:4 with sample diluent, followed by a two-step digital immunoassay. Seven or eight calibrators and two quality controls were run in triplicate on each plate for each analyte. All samples were analyzed in duplicate.

Extracellular vesicles (EVs) isolation

Total plasma EVs isolation was performed with the Total Exosome Isolation Kit (from plasma) (InvitrogenTM, Waltham, MA, USA), following the manufacturer's instructions. Briefly, 125 µL of plasma were centrifuged at RT at 2000 × g for 20 min and at 10,000 × g for 20 min to remove cells and debris. The supernatant was then incubated for 10 min at RT with 62.5 µL (0.5 × plasma volume) of 0.2 µm-filtered 1X phosphate-buffered saline (PBS) and 37.5 µL (0.2 × total volume) of exosome precipitation reagent. After 10 min of incubation at RT, the suspension was centrifuged at 10,000 × g for 5 min. EVs pellets were finally resuspended in 100 µL of 0.2 µm-filtered 1X PBS and stored at + 4 °C until nanoparticle tracking analysis (NTA) was performed.

Standard operating procedures (SOPs) were applied to collect and process plasma samples. Thawed plasma samples from cases and controls underwent the same standardized pre-isolation processing, as described above, to remove residual cells and debris before EVs isolation. Additionally, EVs isolation was carried out in the same laboratory, with the same protocol and performed by the same trained operator, thereby minimizing technical variability across all samples. Moreover, to account for potential bias in EVs profiles, possible confounding factors such as body mass index (BMI) and metabolic and cardiovascular co-morbidities were evaluated in a subset of samples (Additional file 2, Supplementary Information 1 and 2).

Nanoparticle tracking analysis (NTA)

EVs resuspended in 1X PBS from patients and controls were analyzed with the NanoSight NS300 Instrument equipped with a 488 nm blue laser and a high sensitivity sCMOS camera (Malvern, Worcestershire, UK). Samples were diluted with 0.2 µm-filtered 1X PBS to obtain an optimal range of 20–150 particles/frame, a particle concentration range of $\sim 10^7$–10^9 particles/mL net of the dilution factor, a valid/total particles ratio of at least 1/5, and valid readings, according to the user manual recommendations. EVs samples from CTRL were acquired at dilutions ranging from 1:100 to 1:1600, while EVs samples

from SCC, pre-MCI, MCI, and AD-dem were acquired at dilutions ranging from 1:50 to 1:800. The same serial dilutions were applied to resuspended EVs samples. For each sample, 5 videos of 60 s were recorded and data were processed using NanoSight NTA Software 3.2 (Malvern, Worcestershire, UK). Optimized post-acquisition settings were kept constant between samples. Data collected were particle concentration (particles/mL), and average size (nm). Representative NTA curves showing the concentration (particles/mL) and size distribution of plasma EVs are reported in Supplementary Fig. 1 (see Additional file 2).

Raw concentration data (particles/mL) from NTA were normalized on the starting plasma volume to obtain EVs concentration in the human plasma sample. Normalized raw concentration data (EVs concentration, particles/mL) and average size (nm) of EVs were used to calculate EVs ratio, as follows [48]:

$$\text{EVs ratio} = \frac{\text{EVs concentration } (\frac{\text{particles}}{\text{mL}})}{\text{Average size (nm)}}$$

Statistical analyses

Normality assumption of continuous variables was evaluated with Shapiro–Wilk test. Mann–Whitney U test or Kruskal–Wallis test with Dunn's post-hoc tests were used for group comparisons of non-normally distributed continuous variables. Chi-square test was used to analyze differences in sex distribution among the study groups. Missing data (ranging from 1 to 6%) were addressed by imputing the median of the relative biomarker distribution. Generalized linear models, adjusted for age and sex, were employed to examine significant differences in biomarker levels among the four diagnostic groups (SCC, pre-MCI, MCI, and AD-dem), along with CTRL. A secondary analysis treated MCI diagnostic subgroups (sdMCI and mdMCI) as separate groups. Pairwise comparisons were adjusted using Tukey's method. Correlations were analyzed by Pearson's *r* or Spearman's *rho* coefficients based on their distribution. Partial correlations between plasma biomarkers and clinical-demographic variables were run correcting for age.

Biomarkers were standardized for subsequent logistic analysis. Simple logistic regression models assessed each biomarker's discriminatory power between diagnostic group pairs. Stepwise selection on a multiple logistic regression model, incorporating all biomarkers as predictors and a pair of diagnoses as the outcome, identified the most accurate biomarker combination discriminating between each pair of diagnoses. Bayesian information criterion (BIC), a metric akin to Akaike information criterion (AIC) but with a stricter penalty on parameter-rich models, guided model selection. Lower BIC values indicated better fit. Multicollinearity in the final model was tested using the variance inflation factor (VIF), considering that a VIF < 5 indicates no severe multicollinearity issues. Odds ratios (OR) (with 95% confidence intervals (CI)) were calculated for each biomarker in each final model. Diagnostic performance of biomarkers was assessed by area under the curve (AUC) obtained by receiver operating characteristic (ROC) analysis. ROC curves were generated for the best simple and multiple logistic models (selected base on BIC) for each diagnostic comparison. AUCs were computed for each model, with 95% CI derived through bootstrapping to ensure more robust estimates. Biomarker cut-offs maximizing sensitivity and specificity were identified using the Euclidean distance method [51]. As a secondary analysis, ROC curves were stratified by age (≤ 65 and > 65 years) for diagnostic comparisons where significant age differences were observed. The difference in classification performance between the simple and multiple logistic models, as well as between best combined predictor models including or excluding EVs ratio, was tested with the DeLong test. A two-sided p-value < 0.05 was considered significant.

All statistical analyses were conducted using IBM SPSS software (v.29.0.1.0) and R (version 4.3.2), and significance p-value was set at 0.05.

Results

Participant characteristics

The present retrospective study analyzed a total of 800 subjects, including $n = 51$ AD-dem, $n = 426$ MCI (of which $n = 214$ sdMCI and $n = 212$ mdMCI), $n = 58$ pre-MCI, $n = 48$ SCC, and $n = 217$ CTRL. Demographic and clinical characteristics of the study cohorts are reported in Additional file 1 (Table 1). Age was statistically different between the diagnostic groups ($p < 0.001$), in particular comparing MCI and AD-dem vs. CTRL (Kruskal–Wallis test followed by Dunn's *post-hoc* test, both $p_{adj} < 0.001$), and pre-MCI vs. MCI (Kruskal–Wallis test followed by Dunn's *post-hoc* test, $p_{adj} = 0.012$). No significant sex differences were observed. As expected, Mini-Mental State Examination (MMSE) scores differed among groups ($p < 0.001$).

Plasma biomarker levels across diagnostic groups

Levels of NfL, t-Tau, p-Tau$_{181}$, Aβ_{42}, Aβ_{40}, and concentration and size of EVs (reported as ratio between EVs concentration and size (EVs ratio)) were analyzed in plasma samples of AD-dem, MCI, pre-MCI, SCC, and CTRL subjects (Additional file 1: Table 1).

In line with published data [52, 53], NfL concentration was significantly increased in plasma of AD-dem patients compared to all other groups (AD-dem vs. CTRL $p_{adj} < 0.001$; AD-dem vs. SCC $p_{adj} = 0.005$; AD-dem

vs. pre-MCI $p_{adj} = 0.004$; AD-dem vs. MCI $p_{adj} = 0.009$). In addition, NfL levels were significantly higher in MCI patients compared to CTRL subjects ($p_{adj} = 0.001$) (Fig. 1a).

We observed a significant increase in p-Tau$_{181}$ plasma levels with the worsening of cognitive symptoms. Specifically, p-Tau$_{181}$ showed increased concentrations in all subject/patient groups compared to CTRL (SCC vs. CTRL $p_{adj} = 0.003$; pre-MCI vs. CTRL $p_{adj} = 0.008$; MCI and AD-dem vs. CTRL $p_{adj} < 0.001$), and higher levels in AD-dem compared to SCC, pre-MCI, and MCI ($p_{adj} = 0.039$, $p_{adj} = 0.006$, and $p_{adj} = 0.006$, respectively) (Fig. 1b). When p-Tau$_{181}$ was combined with Aβ$_{42}$ plasma levels [54], Aβ$_{42}$/p-Tau$_{181}$ ratio displayed a significant decrease only in pre-MCI, MCI, and AD-dem compared to CTRL ($p_{adj} = 0.002$, $p_{adj} < 0.001$, and $p_{adj} < 0.001$, respectively), and in AD-dem compared to SCC ($p_{adj} = 0.018$) (Fig. 1c). These data indicated that p-Tau$_{181}$ alone had a better capacity to discriminate SCC, pre-MCI, and MCI from CTRL and AD-dem along symptoms progression.

Aβ$_{42}$, Aβ$_{40}$, and t-Tau concentrations did not show significant differences between groups, except for Aβ$_{40}$ in pre-MCI vs. CTRL comparison ($p_{adj} < 0.001$), and Aβ$_{42}$ and Aβ$_{40}$ in MCI vs. CTRL ($p_{adj} < 0.001$) (Supplementary Fig. 2, see Additional file 2). When Aβ$_{42}$ plasma levels were combined with Aβ$_{40}$ levels [54], Aβ$_{42}$/Aβ$_{40}$ ratio exhibited a significant increase in SCC and pre-MCI vs. CTRL ($p_{adj} = 0.044$, and $p_{adj} < 0.001$, respectively), a decrease in MCI vs. pre-MCI ($p_{adj} = 0.028$), and a decrease in AD-dem compared to MCI, pre-MCI and SCC ($p_{adj} = 0.015$, $p_{adj} < 0.001$, and $p_{adj} = 0.038$, respectively). A difference was also observed in MCI vs. CTRL ($p_{adj} = 0.001$) (Fig. 1d).

The ratio between EVs concentration and size (EVs ratio) was lower in all subject/patient groups compared to CTRL (SCC, pre-MCI, MCI vs. CTRL $p_{adj} < 0.001$; AD-dem vs. CTRL $p_{adj} = 0.035$). Furthermore, EVs ratio slightly decreased in MCI vs. pre-MCI ($p_{adj} = 0.007$), and increased in AD-dem compared to SCC and MCI ($p_{adj} = 0.039$, and $p_{adj} < 0.001$, respectively) (Fig. 1e).

MCI patients were then stratified into sdMCI and mdMCI subgroups according to their clinical diagnosis. Clinical, demographic, and biological characteristics of stratified MCI are reported in Additional file 2 (Supplementary Table 1). NfL showed significantly higher plasma levels in mdMCI compared to CTRL ($p_{adj} < 0.001$), but not in sdMCI vs. CTRL, and significantly lower levels in sdMCI compared to AD-dem ($p_{adj} = 0.003$), but not in mdMCI vs. AD-dem, underlining the sensibility of plasma NfL to correlate with the neuronal damage (Supplementary Fig. 3, see Additional file 2). In sdMCI and mdMCI, p-Tau$_{181}$, Aβ$_{42}$/p-Tau$_{181}$ ratio, and EVs ratio exhibited the same significant differences of not-stratified MCI vs. CTRL and AD-dem. In addition, EVs ratio showed differences in pre-MCI vs. sdMCI only, and in sdMCI vs. mdMCI (see Supplementary Fig. 3, Additional file 2). Lastly, Aβ$_{42}$/Aβ$_{40}$ ratio showed significant lower levels only in mdMCI vs. pre-MCI, and in AD-dem and CTRL vs. sdMCI (Supplementary Fig. 3, see Additional file 2).

Correlations between plasma biomarkers and clinical-demographic variables

To evaluate the relationship between plasma levels of NfL, t-Tau, p-Tau$_{181}$, Aβ$_{42}$/Aβ$_{40}$ ratio, Aβ$_{42}$/p-Tau$_{181}$ ratio, EVs ratio and clinical and demographic data, we carried out a correlation analysis. Age-NfL correlation analysis in the study cohorts (CTRL, SCC, pre-MCI, MCI, and AD-dem) revealed a significant positive correlation in each group (Spearman's $rho = 0.41$, $p < 0.001$ CTRL; $rho = 0.38$, $p = 0.008$ SCC; $rho = 0.47$, $p < 0.001$ pre-MCI; $rho = 0.27$, $p < 0.001$ MCI; $rho = 0.28$, $p = 0.043$ AD-dem). Aβ$_{42}$/p-Tau$_{181}$ ratio showed a positive correlation with age in the AD-dem group ($rho = 0.40$, $p = 0.004$), while we found a negative correlation between age and Aβ$_{42}$/Aβ$_{40}$ ratio in CTRL ($rho = -0.31$, $p < 0.001$). However, these results might reflect the age differences in our CTRL and AD-dem groups and, therefore, be limited to our cohorts.

Negative correlations were found between plasma NfL, p-Tau$_{181}$ concentrations and MMSE scores in the SCC group after age correction ($r = -0.33$, $p = 0.032$; $r = -0.33$, $p = 0.031$, respectively), and between p-Tau$_{181}$ levels, Aβ$_{42}$/Aβ$_{40}$ ratio, t-Tau and MMSE scores in AD-dem ($r = -0.30$, $p = 0.040$; $r = -0.35$, $p = 0.015$; $r = -0.31$, $p = 0.032$, age-corrected, respectively). No significant correlations were found between EVs ratio and MMSE scores or age in any of the diagnostic groups.

Next, we evaluated how the above-mentioned biomarkers correlated with each other. NfL and p-Tau$_{181}$ concentrations showed a positive correlation in MCI and AD-dem groups ($r = 0.25$, $p < 0.001$; $r = 0.44$, $p = 0.001$, adjusted for age, respectively), underlining the relationship between neuronal damage and tau pathology in the disease. Additionally, a negative correlation was found for Aβ$_{42}$/Aβ$_{40}$ ratio vs. EVs ratio in the SCC group after age correction ($r = -0.33$, $p = 0.021$). Although significant, weak correlations were found between EVs ratio and Aβ$_{42}$/Aβ$_{40}$ ratio in CTRL and MCI groups ($r = -0.17$, $p = 0.015$; $r = -0.15$, $p = 0.002$, respectively, after age correction), as well as between NfL, p-Tau$_{181}$, Aβ$_{42}$/p-Tau$_{181}$ ratio levels and EVs ratio in the MCI group ($r = 0.16$, $p < 0.001$; $r = -0.11$, $p = 0.023$; $r = 0.14$, $p = 0.005$, respectively, after age correction).

Scatter plots and heatmaps showing the correlations between plasma biomarkers and clinical-demographic variables are reported in Supplementary Fig. 4 and Supplementary Fig. 5, respectively (see Additional file 2).

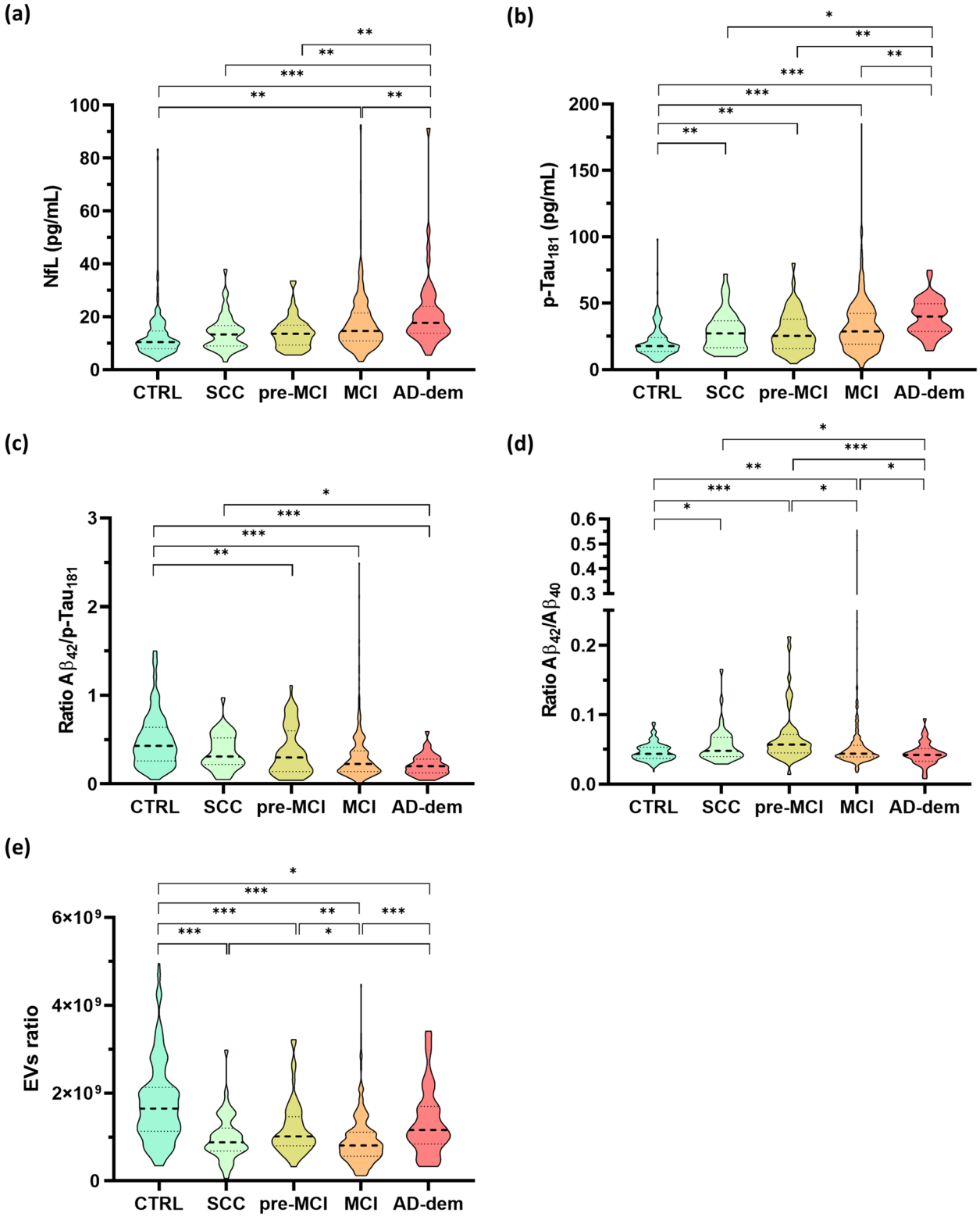

Fig. 1 Plasma biomarker concentrations in participants by clinical diagnosis. **a** NfL, (**b**) p-Tau$_{181}$, (**c**) ratio of Aβ$_{42}$/p-Tau$_{181}$, (**d**) ratio of Aβ$_{42}$/Aβ$_{40}$, and (**e**) EVs ratio (ratio between EVs concentration and size). CTRL, controls; SCC, subjective cognitive complaints; pre-MCI, pre-mild cognitive impairment; MCI, mild cognitive impairment; AD-dem, probable Alzheimer's disease dementia. Violin plots show raw data distribution with median (thick dashed black line) and interquartile range (25th and 75th percentile; thin dotted black line). *$p < 0.050$, **$p < 0.010$, ***$p < 0.001$ after multiple-comparisons with Tukey's post-hoc test, adjusted for age and sex (generalized linear model)

Logistic regression and diagnostic accuracy of plasma biomarkers

To investigate the association between plasma levels of NfL, t-Tau, p-Tau$_{181}$, Aβ_{42}/Aβ_{40} ratio, Aβ_{42}/p-Tau$_{181}$ ratio, EVs ratio and disease stages, we performed a logistic regression analysis. The best single biomarker and the best model combining two or more biomarkers able to discriminate between diagnoses was calculated for each diagnostic comparison. The classification accuracy of both single biomarkers and models was evaluated performing a ROC analysis. AUC, sensitivity, specificity, and cut-off values of the best single predictors for diagnostic comparisons are reported in Table 1. The predictor with the best AUC to differentiate SCC and MCI from CTRL was EVs ratio (AUC 0.78 [95% CI 0.71–0.85], $p < 0.001$; AUC 0.81 [95% CI 0.77–0.84], $p < 0.001$, respectively). p-Tau$_{181}$ and Aβ_{42}/p-Tau$_{181}$ ratio were the best predictors to differentiate AD-dem from CTRL and SCC, respectively (AUC 0.87 [95% CI 0.83–0.92], $p < 0.001$; AUC 0.72 [95% CI 0.62–0.82], $p < 0.001$, respectively), while Aβ_{42}/Aβ_{40} ratio was the predictor with the best AUC to differentiate pre-MCI from CTRL and AD-dem (AUC 0.71 [95% CI 0.63–0.78], $p < 0.001$; AUC 0.74 [95% CI 0.64–0.83], $p < 0.001$, respectively). EVs ratio was the best predictor in differentiating MCI from pre-MCI and AD-dem (AUC 0.66 [95% CI 0.59–0.72], $p < 0.001$; AUC 0.69 [95% CI 0.61–0.77], $p < 0.001$, respectively). NfL differentiated MCI from SCC, although with an AUC of only 0.59 ([95% CI 0.51–0.68], $p = 0.031$) (Table 1). Of note, no biomarkers were found able to discriminate pre-MCI from SCC.

Since age was statistically different between MCI and AD-dem vs. CTRL, and pre-MCI vs. MCI, we performed ROC analyses stratified by age for these comparisons, applying a cut-off value of 65 years (see Additional file 2). EVs ratio remained the best predictor to differentiate MCI from CTRL, with an equal AUC for age ≤ 65 years or > 65 years, (AUC 0.79 [95% CI 0.72–0.85], $p < 0.001$; AUC 0.79 [95% CI 0.74–0.84], $p < 0.001$, respectively). p-Tau$_{181}$ remained the best predictor in differentiating AD-dem from CTRL after age stratification, with an AUC equal to 0.92 ([95% CI 0.87–0.98], $p < 0.001$]) for age ≤ 65 years and an AUC equal to 0.82 ([95% CI 0.74–0.89], $p < 0.001$) for age > 65 years, although the ≤ 65 years subgroup is small to draw robust conclusions ($n = 12$). Aβ_{42}/Aβ_{40} ratio and EVs ratio differentiated MCI from pre-MCI when stratified by age ≤ 65 years (Aβ_{42}/Aβ_{40} ratio AUC 0.66 [95% CI 0.53–0.77], $p < 0.050$) or > 65 years (EVs ratio AUC 0.69 [95% CI 0.62–0.77], $p < 0.010$) (Additional file 2, Supplementary Table 2).

Multiple-biomarker logistic regression models showed a significantly greater AUC of Aβ_{42}/Aβ_{40} ratio combined with EVs ratio and p-Tau$_{181}$ than Aβ_{42}/Aβ_{40} ratio alone in differentiating pre-MCI from CTRL (AUC_{comb} 0.79 vs. $AUC_{A\beta42/A\beta40\ ratio}$ 0.71, $p = 0.005$) (Fig. 2a), and a significant increase in AUC of EVs ratio combined with NfL and p-Tau$_{181}$ in differentiating MCI from CTRL (AUC_{comb} 0.85 vs. $AUC_{EVs\ ratio}$ 0.81, $p = 0.002$) (Fig. 2b). In addition, Aβ_{42}/p-Tau$_{181}$ ratio combined with Aβ_{42}/Aβ_{40} ratio, EVs ratio, and NfL better differentiated AD-dem from SCC (AUC_{comb} 0.85 vs. $AUC_{A\beta42/p\text{-}Tau181\ ratio}$ 0.72, $p = 0.004$) (Fig. 2c), while Aβ_{42}/Aβ_{40} ratio combined with Aβ_{42}/p-Tau$_{181}$ ratio and NfL showed higher AUC in differentiating AD-dem from pre-MCI (AUC_{comb} 0.83 vs. $AUC_{A\beta42/A\beta40\ ratio}$ 0.74, $p = 0.022$) (Fig. 2d) (see Tables 1 and 2).

No significant differences were found between the AUCs obtained with the single- and multiple-biomarker model for SCC vs. CTRL, AD-dem vs. CTRL, MCI vs. pre-MCI, and AD-dem vs. MCI comparisons ($p = 0.199$, $p = 0.888$, $p = 0.484$, $p = 0.162$, respectively) (see Tables 1 and 2). No biomarker combination was found capable of discriminating pre-MCI from SCC. Moreover, no biomarker combination performed better than NfL in distinguishing MCI from SCC.

Among the age-stratified comparisons, only the MCI vs. CTRL subgroup for individuals aged ≤ 65 years showed a significant improvement in classification performance when using ROC curves generated from the combined multivariate logistic model (EVs ratio combined with p-Tau$_{181}$) compared to those from the single predictor (AUC_{comb} 0.83 vs. $AUC_{EVs\ ratio}$ 0.79, $p = 0.035$). Age-stratified ROC analyses are presented in Supplementary Tables 2 and 3, as well as Supplementary Fig. 6 (see Additional file 2).

Finally, we evaluated the contribution of EVs ratio in the multiple-biomarker models for those diagnostic comparisons where the logistic regression combined models significantly differed from the single-biomarker model (i.e., CTRL vs. pre-MCI, CTRL vs. MCI, SCC vs. AD-dem, and pre-MCI vs. AD-dem) (see Fig. 2). ROC comparisons between models including EVs ratio ($_{comb}$) or excluding EVs ratio ($_{EVs\ ratio\ excl.}$) indicated that the inclusion of EVs ratio significantly improved the classification performance of pre-MCI vs. CTRL (AUC_{comb} 0.79 [95% CI 0.72–0.85] vs. $AUC_{EVs\ ratio\ excl.}$ 0.73 [95% CI 0.66–0.81], $p = 0.018$) and MCI vs. CTRL comparisons (AUC_{comb} 0.85 [95% CI 0.81–0.88] vs. $AUC_{EVs\ ratio\ excl.}$ 0.75 [95% CI 0.71–0.79], $p < 0.001$), as illustrated in Fig. 3a and Fig. 3b, respectively. The inclusion of EVs ratio also significantly improved the diagnostic performance of MCI vs. CTRL in individuals aged ≤ 65 years (AUC_{comb} 0.83 [95% CI 0.77–0.88] vs. $AUC_{EVs\ ratio\ excl.}$ 0.71 [95% CI 0.63–0.79], $p = 0.003$). In contrast, the exclusion of EVs ratio from the model did not significantly affect the diagnostic performance of Aβ_{42}/p-Tau$_{181}$ ratio combined with Aβ_{42}/Aβ_{40} ratio and NfL in distinguishing SCC from AD-dem (AUC_{comb} 0.85 [95% CI 0.78–0.92] vs. $AUC_{EVs\ ratio\ excl.}$ 0.83 [95% CI 0.75–0.91], $p = 0.173$). Moreover, excluding

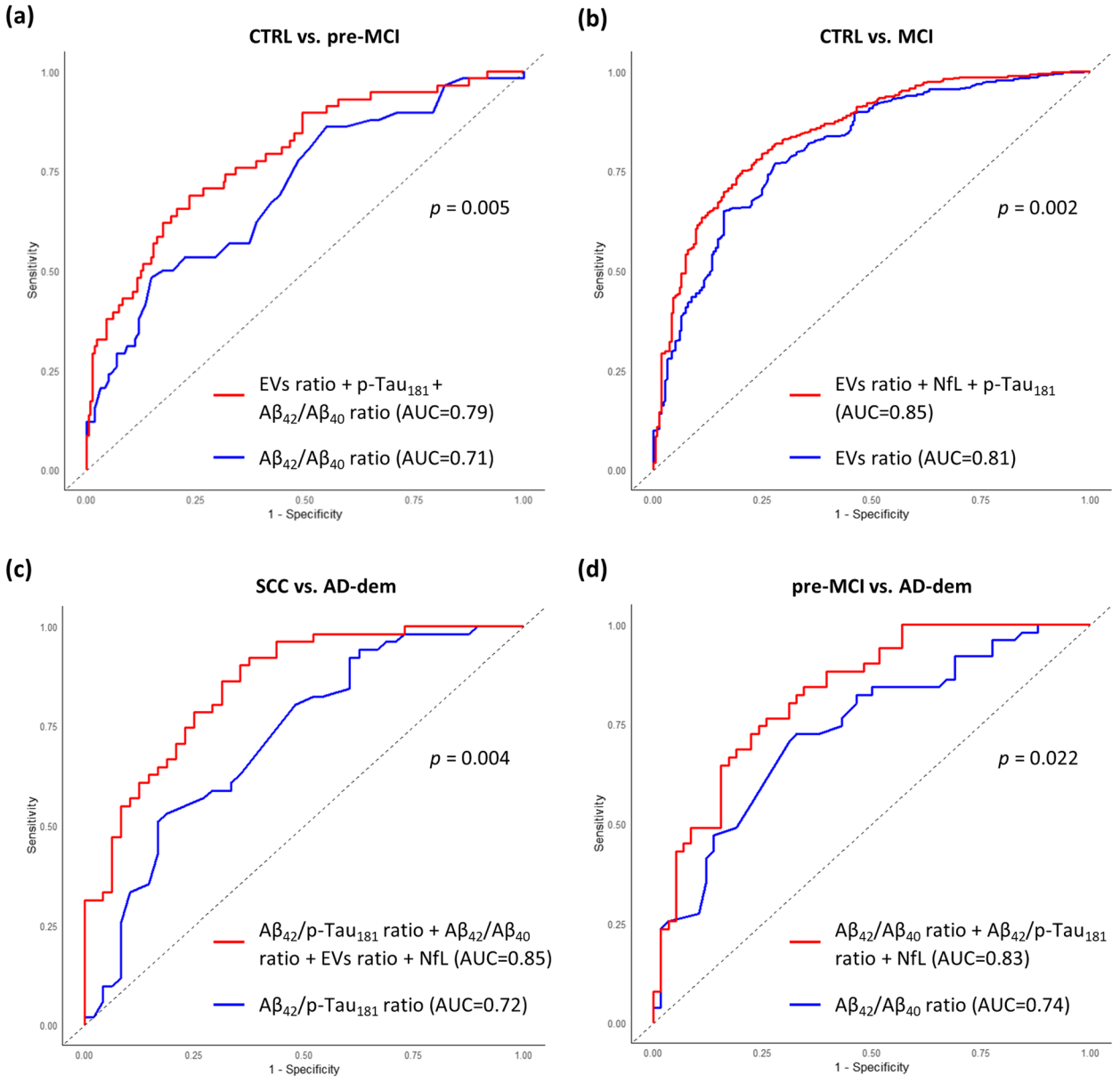

Fig. 2 ROC curves showing the improvement in classification accuracy of combined predictors of multiple logistic models compared to single predictors. Only significant AUC comparisons are shown. ROC curves of single and combined predictors are depicted in blue and red, respectively. **a** CTRL vs. pre-MCI, (**b**) CTRL vs. MCI, (**c**) SCC vs. AD-dem, (**d**) pre-MCI vs. AD-dem

EVs ratio did not change the best combined predictors for the pre-MCI vs. AD-dem comparison. These results highlighted the added advantage of using EVs ratio in multiple-biomarker models.

Discussion

Timely and accurate diagnosis of AD in clinical practice is a great challenge, especially during early disease stages with subtle or mild symptoms of cognitive decline [55]. In the last years, huge efforts have been made in the context of blood-based biomarkers to identify robust and more accessible screening tests to be used in the initial disease stages. Significant steps forward have been attained thanks to the development of ultrasensitive assays that enabled the detection of proteins in the blood at very low concentrations [33].

Taking advantage of these technologies, in the present work we aimed at evaluating plasma levels of different biomarkers in individuals characterized by different stages of cognitive decline and at identifying biochemical markers of dementia for early diagnosis. In particular, we evaluated plasma concentrations of NfL, t-Tau, p-Tau$_{181}$, Aβ$_{42}$, and Aβ$_{40}$ in patients with mild and severe cognitive decline (MCI and AD-dem) and in subjects

Table 2 Diagnostic accuracy and classification performance of combined predictors in multiple logistic regression models

	Predictors in the model	BIC	AUC_{comb} (95% CI)	*p*-Value
CTRL vs. SCC	EVs ratio $A\beta_{42}$/p-Tau_{181} ratio	217.41	0.80 (0.73–0.86)	0.199 [§]
CTRL vs. pre-MCI	EVs ratio p-Tau_{181} $A\beta_{42}/A\beta_{40}$ ratio	248.12	0.79 (0.72–0.85)	**0.005**[§]
CTRL vs. MCI	EVs ratio NfL p-Tau_{181}	610.21	0.85 (0.81–0.88)	**0.002**[§]
CTRL vs. AD-dem	$A\beta_{42}$/p-Tau_{181} ratio EVs ratio NfL	193.15	0.88 (0.83–0.92)	0.888 [§]
SCC vs. AD-dem	$A\beta_{42}$/p-Tau_{181} ratio $A\beta_{42}/A\beta_{40}$ ratio EVs ratio NfL	114.29	0.85 (0.78–0.92)	**0.004**[§]
pre-MCI vs. MCI	EVs ratio NfL	351.15	0.68 (0.62–0.73)	0.484 [§]
pre-MCI vs AD-dem	$A\beta_{42}/A\beta_{40}$ ratio $A\beta_{42}$/p-Tau_{181} ratio NfL	125.04	0.83 (0.75–0.90)	**0.022**[§]
MCI vs. AD-dem	$A\beta_{42}$/p-Tau_{181} ratio EVs ratio	311.73	0.73 (0.66–0.79)	0.162 [§]

BIC Bayesian information criterion, AUC_{comb} Area under the curve of combined predictors, *CI* Confidence interval. Confidence intervals were estimated via bootstrapping ($n = 2000$). The selection of the best logistic regression models was guided by the lowest BIC index and by no multicollinearity issues (VIF < 5)

[§]DeLong test comparing AUCs of single best predictors and multiple logistic models for the same diagnostic comparison. Unreported comparisons yielded results consistent with those presented in Table 1

In bold are reported the p-values of significant AUC comparisons. See Supplementary Table 4 (Additional file 2) for odds ratios (OR) and standard deviations (SD)

in asymptomatic and prodromic phases (SCC and pre-MCI), including elderly healthy individuals. Moreover, we investigated plasma EVs as a novel biomarker for dementia recently described by our group [48].

Our results showed that distinct biomarkers were able to distinguish different stages of cognitive decline. NfL concentrations showed a marked increase in AD-dem patients compared to all groups and in MCI compared to CTRL, particularly in the mdMCI condition, showing an increasing trend that reflects the sensitivity of plasma NfL to correlate with the ongoing neuronal degeneration, although in our cohorts NfL was not effective in discriminating individuals in the presymptomatic and prodromal stages [56, 57] (Fig. 1a and Supplementary Fig. 3, see main text and Additional file 2). Accordingly, previous studies reported significantly higher plasma NfL levels in AD patients compared to MCI [52, 53] and in AD and MCI compared to CTRL [53], while studies on familial AD showed increased NfL levels already about 15 years before symptoms onset in presymptomatic AD [58–60]. We observed positive correlations between age and plasma NfL in all groups, consistent with previous studies reporting the age-dependent increase of NfL [53, 61, 62] and the importance of age-related cut-offs [43]. Simrén and colleagues established reference values for plasma NfL in healthy individuals ranging 5–90 years [62]: in line with these results, in our CTRL cohort we identified plasma NfL cut-off values of 18.77 pg/mL, 22.58 pg/mL, 33.79 pg/mL for age categories 50–60, 61–70 and > 70, respectively.

However, NfL is a sensitive but not-specific marker of neuroaxonal damage, increased in different neurological disorders irrespective of the underlying cause [43, 63, 64]. On the contrary, plasma phosphorylated tau has demonstrated specificity to AD versus non-AD neurodegenerative diseases, also showing significant correlations with tau and Aβ brain pathology [37, 39, 65–67]. Our results demonstrated that p-Tau_{181} plasma levels increased as cognitive symptoms worsened, resulting in a significant increase already in SCC and pre-MCI and reaching the highest concentration in AD-dem patients (Fig. 1b). Many studies have reported higher levels of p-Tau_{181} in AD and MCI compared to cognitively unimpaired individuals [66, 68], also showing a gradual increase along the AD progression [37, 65, 69, 70]. Yet, few studies have investigated the role of plasma p-Tau_{181} in subjects reporting memory complaints and found lower levels of p-Tau_{181} compared to MCI and AD [71–74]. In our cohorts, we found that p-Tau_{181} alone could not differentiate between the preclinical and prodromal stages of AD dementia, showing similar plasmatic concentrations in pre-MCI and MCI, and also in SCC. Additionally, we found positive correlations between plasmatic p-Tau_{181} and NfL in MCI and AD-dem, further confirming that p-Tau_{181} levels are related to neuroaxonal damage [75]. To the best of our knowledge, no previous study has provided, within a singular investigation, an exhaustive analysis of the plasma concentrations of p-Tau_{181} across the stages of SCC and pre-MCI that precede MCI, together with elderly healthy individuals. A recent study by Planche and colleagues [76] analyzed blood biomarkers for AD in a large cohort of SCC and MCI patients and found p-Tau_{181} to be the best blood predictor of AD and mixed dementia at 5-year follow-up. In contrast, in the study by Perna et al. [77], p-Tau_{181} did not predict conversion to dementia in SCC, but it was associated with an increased risk of depression. Our results suggest the potentiality of p-Tau_{181} to predict cognitive changes in the early stages of the disease.

The $A\beta_{42}/A\beta_{40}$ ratio is a validated CSF biomarker for estimating brain amyloidosis [78], with a superior correlation with amyloid PET compared to individual biomarkers [54]. Many studies have reported lower plasma $A\beta_{42}$ levels and $A\beta_{42}/A\beta_{40}$ ratio in preclinical, prodromal, and AD dementia [79–83], and that lower levels of $A\beta_{42}$ and $A\beta_{42}/A\beta_{40}$ ratio increase the risk of AD

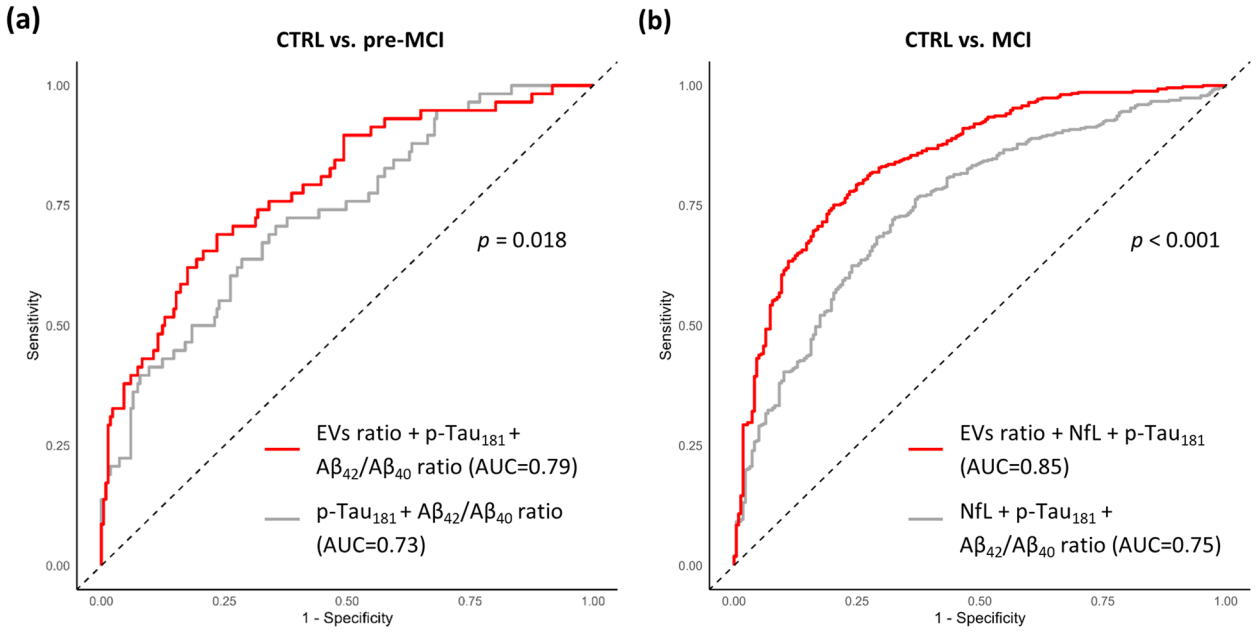

Fig. 3 ROC curve comparisons of the best biomarker combination in multiple logistic models, including or excluding EVs ratio. Only significant AUC comparisons are shown. ROC curves of models including EVs ratio ($_{comb}$) are depicted in red, while ROC curves of models excluding EVs ratio ($_{EVs\ ratio\ excl.}$) are depicted in gray. Panel (**a**) shows the CTRL vs. pre-MCI comparison, where the AUC of the model including EVs ratio, p-Tau$_{181}$, and Aβ$_{42}$/Aβ$_{40}$ ratio is significantly greater ($p = 0.018$) than the AUC of the model including p-Tau$_{181}$ and Aβ$_{42}$/Aβ$_{40}$ ratio, but not EVs ratio. Panel (**b**) shows the significantly improved performance ($p < 0.001$) of the model including EVs ratio, NfL, and p-Tau$_{181}$ compared to the model including NfL, p-Tau$_{181}$, and also Aβ$_{42}$/Aβ$_{40}$ ratio, but not EVs ratio, for the CTRL vs. MCI comparison. *P*-values estimated by DeLong test comparing the AUCs of the best predictor combination, including or excluding EVs ratio, for the same diagnostic comparison

dementia [82, 84–86]. Our results showed an increasing Aβ$_{42}$/Aβ$_{40}$ ratio in SCC and pre-MCI groups compared to CTRL, with the highest peak in pre-MCI, followed by a decrease in MCI and AD-dem to values similar to those observed in CTRL (Fig. 1d). Interestingly, Planche and colleagues showed higher levels of blood Aβ$_{42}$/Aβ$_{40}$ ratio in Aβ-negative SCC and MCI patients compared to Aβ-positive patients [76]. Thus, we could speculate that there might be a prevalence of Aβ-negative subjects in our SCC and pre-MCI groups. The trend of Aβ$_{42}$/Aβ$_{40}$ ratio could be mainly ascribed to Aβ$_{40}$ plasma concentration that showed lower, although not significant, levels in the SCC group, and in pre-MCI and MCI groups compared to CTRL. This is a peculiar trend for plasmatic Aβ$_{40}$, which has been described as not influenced in the AD progression [87, 88]. In addition, in MCI we observed significant lower levels of Aβ$_{42}$ compared to CTRL (Supplementary Fig. 2, Additional file 2). In fact, Aβ$_{42}$ concentrations showed a decreasing trend from pre-MCI to MCI and AD-dem, in line with the concept of a progressive accumulation of Aβ deposits in the brain and with reduced Aβ$_{42}$ CSF levels in MCI and AD patients [88–90]. To explain the observed reduction of Aβ$_{40}$ levels in pre-MCI and MCI, we might speculate that, in the early phases of cognitive decline, alterations in the metabolism of plasma Aβ can occur as a mechanism trying to reduce the deposition of Aβ and to improve cognitive function [91]. Conversely, several factors that might affect plasma Aβ and related to Aβ clearance may have contributed to biases in measurements [92, 93]. Limitations of Aβ$_{42}$/Aβ$_{40}$ ratio in blood and lack of robustness of the test have also been well reported [36, 83, 94–100].

EVs are a new concept in the biomarker field, representing natural carriers of bioactive molecules between cells, with a wide range of regulatory functions [101]. Literature data suggest an interdependence between EVs production and the endosomal pathway in the brain [102]. We previously provided evidence of an alteration in EVs release that is common across the three major neurodegenerative dementias, AD, dementia with Lewy bodies (DLB), and frontotemporal dementia (FTD), and described the ratio between concentration and size of EVs as a promising marker for dementia [48]. In the present work, we extended the study of EVs at the preclinical and prodromal stages of AD dementia, confirming the significant decrease of this biomarker in AD-dem patients. Interestingly, we observed a significant reduction of EVs ratio in all groups compared to CTRL (−47%, −38%, −51%, −30% for SCC, pre-MCI, MCI and AD-dem, respectively), providing evidence of an alteration in EVs production since the early stages of the pathology and underlining the sensitivity of EVs ratio in detecting early signs of disease (Fig. 1e). Additionally, we found a decrease of EVs ratio in MCI compared to pre-MCI.

However, the decrease of EVs ratio does not follow a linear pattern from CTRL to SCC, pre-MCI, MCI, and AD-dem. This pattern may reflect the biomarker's ability to detect the presence of the disease, even in its early stages, but not to explain disease progression, which is influenced by multiple underlying biological processes that require further exploration. Moreover, the statistical differences between SCC, pre-MCI, MCI, and AD-dem might be biased by the relatively small number of subjects in the SCC, pre-MCI, and AD-dem groups compared to CTRL and MCI groups. Previous studies investigating EVs in the plasma of AD and MCI patients reported no significant differences in the number of total plasma EVs and their size in comparison to healthy controls [103–109]. An increase in EVs concentration [110–112] and size [111] in AD compared to CTRL has also been described. However, the small number of samples analyzed in most of these studies, the characteristics of the subjects included, and the differences in the experimental conditions and procedures might justify these discrepancies. Lastly, the use of EVs as biomarkers for differentiating MCI, clinically confirmed dementias, and controls has been assessed by several studies and reviewed in a recent diagnostic accuracy meta-analysis by Taha [113].

Interestingly, the disease stage is associated with a reduction in EVs concentration and increased size in blood, whereas in CSF it is linked to an increased concentration and reduced size of vesicles [47]. Moreover, in CSF of AD patients we reported a positive correlation between EVs concentration/size ratio and AD core biomarkers (i.e., p-Tau_{181}/$A\beta_{42}$ ratio) [47]. Neuronal inflammation may compromise the blood–brain barrier, allowing the transport of EVs from the periphery to the brain, as demonstrated for alpha-synuclein in Parkinson's disease [114]. Based on this mechanism, we could speculate that the reduction of plasma EVs observed in this study could be linked to the transfer of EVs from the periphery to the brain, and that this event may occur in the early stages of the disease. Nevertheless, since AD dementia is a multifactorial disease affecting both the central nervous system and the periphery, we cannot rule out the possibility of altered EVs biogenesis or release also from peripheral tissues.

At present, plasma biomarkers taken individually are not sufficient to predict or make a clear diagnosis of AD. In this study, we assessed the classification accuracy of plasma NfL, t-Tau, p-Tau_{181}, $A\beta_{42}/A\beta_{40}$ ratio, $A\beta_{42}$/p-Tau_{181} ratio and EVs ratio, taken individually or in combination, in discriminating between disease stages. Among these biomarkers, we found that p-Tau_{181} classifies AD-dem patients as compared to healthy subjects with high sensitivity, specificity and great accuracy (88.2% and 77.9%, respectively; cut-off 25.34 pg/mL; AUC = 0.87), while EVs ratio is more suitable to discriminate SCC from CTRL, with 75.0% sensitivity, 71.9% specificity and good accuracy (AUC = 0.78; cut-off 1.14×10^9). p-Tau_{181} and EVs ratio perform significantly better in the classification of pre-MCI and MCI from CTRL when combined with $A\beta_{42}/A\beta_{40}$ ratio and NfL, respectively, with a good to great accuracy (AUC_{comb} = 0.79 and AUC_{comb} = 0.85, respectively). The classification accuracy of pre-MCI and MCI vs. CTRL of these combined biomarkers significantly decreases when EVs ratio is not included ($AUC_{EVs\ ratio\ excl.}$ = 0.73, p = 0.018; $AUC_{EVs\ ratio\ excl.}$ = 0.75, $p < 0.001$, respectively). Moreover, p-Tau_{181} and EVs ratio also perform significantly better in the classification of MCI from CTRL after stratification by age (AUC_{comb} = 0.83 for age ≤ 65 years). Great accuracy is also shown by the combination of $A\beta_{42}$/p-Tau_{181} ratio, NfL and $A\beta_{42}/A\beta_{40}$ ratio in classifying AD-dem from pre-MCI (AUC_{comb} = 0.83), and by $A\beta_{42}$/p-Tau_{181} ratio, NfL, $A\beta_{42}/A\beta_{40}$ ratio and EVs ratio in classifying AD-dem from SCC (AUC_{comb} = 0.85). In this case, $A\beta_{42}$/p-Tau_{181} ratio, NfL, and $A\beta_{42}/A\beta_{40}$ ratio without EVs ratio perform equally well in classifying AD-dem from SCC ($AUC_{EVs\ ratio\ excl.}$ = 0.83, p = 0.173). Altogether, EVs ratio proved to be the most effective biomarker in distinguishing SCC from CTRL and to significantly improve the diagnostic accuracy of pre-MCI and MCI vs. CTRL when combined with well-established biomarkers. This may enable a more accurate diagnosis in the early disease stages, especially in differentiating the presymptomatic and prodromal stages of dementia from healthy individuals. Furthermore, we did not find any biomarkers capable of distinguishing SCC from pre-MCI. Actually, SCC and pre-MCI represent heterogeneous populations in terms of clinical presentation and imaging profiling, with different possible underlying conditions [115].

Limitations

We acknowledge that EVs isolation was carried out using a single methodology (i.e., Total Exosome Isolation Kit) that relies on a polymer-based precipitation. This approach has certain limitations, including the inability to distinguish between small and large vesicles, the co-isolation of non-EV particles (e.g., lipoproteins), protein aggregates, and other extracellular contaminants, as well as the interference with downstream omics analysis [116, 117]. However, this method was chosen for its reproducibility, ease of use, high yield of recovery from relative low sample amount, low processing speed and cost, preservation of the structural and functional integrity of EVs, high-throughput scalability, and suitability for handling a relatively large number of samples in parallel, which was critical for the scope of our study. Several studies compared EVs isolated from different matrices (e.g., plasma, serum, and culture supernatant) using commonly employed methods based on different principles,

including the Total Exosome Isolation Kit, to assess differences in yield, size, purity, quality, and suitability of the isolated EVs for downstream applications. While precipitation-based methods require optimization due to the presence of contaminants and potentially toxic chemical impurities [118], they are valid alternatives to the gold standard ultracentrifugation, even when sample volumes are limited, such as in clinical or diagnostic studies [119–121].

We acknowledge that the relatively small number of subjects in the SCC, pre-MCI and AD-dem groups compared to MCI and CTRL, and the lack of stratification of the patients according to CSF, imaging, and genetic biomarkers may represent limitations of this study. Further studies in larger sample populations are desirable in order to consolidate these results and to interpret them on the basis of the underlying pathology. In addition, our proposed plasma biomarker (i.e., EVs ratio) should require further validation in other independent cohorts to assess the robustness, generalizability, and clinical applicability of our findings. Furthermore, longitudinal studies on patients undergoing clinical follow-up would be of valuable interest to identify predictive markers of conversion to dementia by exploring blood biomarkers in those individuals who progress to AD dementia.

On the other hand, to the best of our knowledge, no previous investigations gave a comprehensive analysis of plasmatic concentrations of NfL, p-Tau_{181}, $A\beta_{42}/A\beta_{40}$ ratio, and EVs concentration and size across the stages that may precede AD dementia.

Conclusions

Taken together, our findings demonstrate that p-Tau_{181} and EVs ratio are promising biomarkers for the identification of individuals at risk of degenerative dementia. This is due to the specificity of p-Tau_{181} in tracking disease progression and the sensitivity of EVs ratio to cognitive decline. Moreover, our results indicate that, when used individually, the core AD plasma biomarkers are not sufficient to accurately distinguish the early stages of AD dementia, supporting the combined use of multiple biomarkers, including EVs ratio, to aid in diagnosis.

Abbreviations

AD	Alzheimer's disease
AD-dem	Probable AD dementia
AUC	Area under the curve
AUCcomb	Area under the curve of combined predictors
Aβ	Amyloid beta
BIC	Bayesian information criterion
BMI	Body mass index
CDR	Clinical dementia rating
CI	Confidence interval
CSF	Cerebrospinal fluid
CTRL	Controls
DLB	Dementia with Lewy bodies
E.S.	Equivalent scores
EVs	Extracellular vesicles
FTD	Frontotemporal dementia
MCI	Mild cognitive impairment
mdMCI	Multiple domain mild cognitive impairment
MMSE	Mini-Mental state examination
NfL	Neurofilament light chain
NTA	Nanoparticles tracking analysis
OR	Odds ratio
PBS	Phosphate-buffered saline
PET	Positron emission tomography
p-Tau_{181}	Phosphorylated Tau 181
ROC	Receiver operating characteristic
SCC	Subjective cognitive complaints
SCD	Subjective cognite decline
SD	Standard deviation
sdMCI	Single domain mild cognitive impairment
Simoa	Single molecule array
t-Tau	Total Tau
VIF	Variance inflation factor

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13195-025-01897-2.

Additional file 1: Table 1. Clinical, demographic, and biological characteristics of the subjects involved in the study.

Additional file 2: Supplementary Table 1. Clinical, demographic, and biological characteristics of MCI patients stratified into single and multiple domain MCI. Supplementary Fig. 1. Representative NTA curves of plasma EVs samples. Supplementary Fig. 2. Plasma concentrations of $A\beta_{42}$, $A\beta_{40}$, and t-Tau in controls and participants. Supplementary Fig. 3. Plasma concentrations of NfL, p-Tau_{181}, ratio of $A\beta_{42}$/p-Tau_{181}, ratio of $A\beta_{42}/A\beta_{40}$, and EVs ratio in CTRL, SCC, pre-MCI, sdMCI and mdMCI, and AD-dem subjects. Supplementary Fig 4. Scatter plots showing correlation analyses with regression line. Supplementary Fig. 5. Heatmap representation of the correlation matrices between plasma biomarkers and clinical-demographic variables. Supplementary Table 2. Age-stratified diagnostic accuracy of the best predictors for diagnostic comparisons with significant age differences. Supplementary Table 3. Age-stratified diagnostic accuracy and classification performance of combined predictors in multiple logistic regression models for diagnostic comparisons with significant age differences. Supplementary Fig. 6. Age-stratified ROC curves comparing combined and single predictors in CTRL versus MCI. Supplementary Table 4. Odds ratios and standard deviations of predictors in multiple logistic regression models. Supplementary Information 1. Correlation analyses of EVs ratio with body mass index (BMI). Supplementary Information 2. Influence of co-morbidities on EVs ratio levels.

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Conceptualization and project administration, RL, FT, EMV, GF and RG; methodology, AG, RL, FT, EMV, GF and RG; formal analysis, VB, DC and AG; investigation, VB, DC, ER, ERZ, GG, SB (Simone Baiardi), AM, FV (Federico Verde) and BN; resources, FAC, FM (Fabio Moda), LB (Luca Battistini), RF, FV (Federico Verde), BN, CAE, MLM, ADF, PMR, CM (Camillo Marra), NV, AR, DP, PS, MC, SC, NC, FV (Fabrizio Vecchio), PT, FP, GBF, CM (Cristina Muscio), RL, PP, FT, EMV, GF and RG; Data curation: VB, DC, PMR, CM (Camillo Marra), NV, AR, DP, PS, MC, SC, NC, FV (Fabrizio Vecchio), PT, FP, GBF, CM (Cristina Muscio), RL, FT, EMV, GF and RG; writing—original draft preparation, visualization and figures conception, VB, DC, AG and RG; writing—review and editing, VB, DC, AG, ER, FAC, FM (Fabio Moda), ERZ, GG, LB (Luca Battistini), SB (Simone Baiardi), AM, RF, FV (Federico Verde), BN, CAE, MLM, AI, FRG, CF, ADF, LB (Leonardo Biscetti), MS, SB (Silvia Berra), FM (Francesca Miraglia), PMR, CM (Camillo Marra), NV, AR, DP, PS, MC, SC, NC, FV (Fabrizio Vecchio), PT, FP, GBF, CM (Cristina Muscio), RL, PP, FT, EMV, GF and RG; supervision, FM (Fabio Moda), LB (Luca Battistini), RF, FV (Federico Verde), BN, MLM, ADF, RL, PP, FT, EMV, GF and RG; funding acquisition, RL and GF. All authors have read and agreed to the published version of the manuscript.

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Data availability

The dataset generated during the current study is available in the Zenodo Data Repository at the following https://doi.org/10.5281/zenodo.14967249 [122]. (dataset creation date: March 4, 2025; dataset update: September 25, 2025).

Declarations

Ethics approval and consent to participate

Participants signed an informed consent for blood collection and biobanking, as approved by the local Ethics Committees. The study protocol was approved by the local Ethics Committee "Comitato Etico IRCCS San Giovanni di Dio Fatebenefratelli" of the IRCCS Centro San Giovanni di Dio Fatebenefratelli, Brescia (Prot. N. 10/2022, date of approval: 2 March 2022; Prot. N. 14/2023, date of approval: 1 March 2023). The study received approval from the Ethics Committee of all participating centers. The study was conducted in accordance with the Declaration of Helsinki.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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