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Original Article

Early detection of Alzheimer's disease using small RNAs. Results from the EPAD cohort

Tobias Sikosek[a,1,*], Marco Heuvelman[a,1], Jagoda Mika[a], Mustafa Kahraman[a], Julia Jehn[a], Maurice Frank[a], Alberto Daniel-Moreno[a], Jessika Ceiler[a], Jasmin Skottke[a], Marta Sanchez-Delgado[a], Patrick Neubert[a], Christina Rudolf[a], Kaja Tikk[a], Rastislav Horos[a], Jeffrey L. Cummings[b], Josie Butchart[c], Craig Ritchie[d,e], Jean Manson[c], Bruno R. Steinkraus[a,*], the EPAD consortium

[a] *Hummingbird Diagnostics GmbH, Heidelberg, Germany*
[b] *Chambers-Grundy Center for Transformative Neuroscience, Department of Brain Health, Kirk Kerkorian School of Medicine, University of Nevada Las Vegas (UNLV), Las Vegas, Nevada, USA*
[c] *Edinburgh Dementia Prevention, Centre for Clinical Brain Sciences, University of Edinburgh, Edinburgh, Scotland, UK*
[d] *Scottish Brain Sciences, Edinburgh, Scotland, UK*
[e] *Department of Medicine, University of St Andrews, St Andrews, Scotland, UK*

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ABSTRACT

Background: Alzheimer's disease (AD) is the most common form of dementia, and early diagnosis is crucial to enable effective interventions. Currently, Alzheimer's disease is diagnosed through cognitive assessments, brain imaging and fluid biomarkers focused on determining amyloid (A) and, tau (T) protein levels as well as neurodegeneration (N) in the AT(N) framework. Prognostic biomarkers for predicting cognitive decline within the amyloid positive (Aβ+) individuals would further strengthen the framework.
Objectives: This study evaluated small RNAs as novel auxiliary biomarkers, independent of the AT(N) framework, either alone or in combination with established protein markers, for detecting the earliest cognitive decline in AD.
Design: The European Prevention of Alzheimer's Disease (EPAD) clinical trial platform is a prospective, multicenter study designed to investigate biomarkers for preclinical and prodromal AD.
Setting: Peripheral whole blood RNA sequencing was performed on participants across Europe with no cognitive impairment or very mild cognitive impairment (MCI), stratified by cerebrospinal fluid amyloid levels.
Participants: 1,913 participants, 50 years or older and free of dementia diagnosis at enrollment, were analyzed.
Intervention: (if any) Not applicable.
Measurements: Ultra-deep small RNA sequencing was performed on whole blood samples using a refined blocking protocol to eliminate highly abundant erythroid small RNAs, and thereby to open sequencing bandwidth for the discovery of less abundant biomarker RNAs. Biomarker RNAs were deconvolved into plasma or blood cell origin and analyzed for functional relevance. We define high and low amyloid groups based on a cutoff on the p-tau$_{181}$/A$\beta_{1\text{-}42}$ ratio as determined from cerebrospinal fluid.
Results: We identified a combination of small RNAs that predicted early cognitive decline (Clinical Dementia Rating of 0.5) with an area under the receiver-operator curve of ~0.7. Notably, when focusing on individuals with cognitive decline and high amyloid burden (Aβ+), the predictive accuracy improved to an AUC of 0.77. This performance could be extended to the entire cohort when combining blood RNA and CSF amyloid markers (AUC 0.76). We conducted bioinformatic analyses to interrogate the likely functional relevance of these small RNAs,

* Corresponding authors.
E-mail addresses: publication@hb-dx.com (T. Sikosek), publication@hb-dx.com (B.R. Steinkraus).
[1] These authors contributed equally

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uncovering several links to dementia-relevant pathways, including neuronal, cardiovascular, and inflammatory activities. Our findings also suggest that small nucleolar RNAs warrant further investigation as potential disease-relevant markers, in addition to microRNAs.

Conclusions: Integrating small RNA biomarkers with protein-based assays offers preliminary evidence for stratifying MCI, particularly within the amyloid positive continuum. Small nucleolar RNAs and microRNAs warrant further exploration as complementary diagnostic tools, and their use may enable more precise and effective interventions.

1. Introduction

Alzheimer's Disease (AD) is a progressive neurodegenerative disorder with no known cure, contributing to ~60% of global dementia cases [1]. AD prevalence rises sharply with age, affecting ~3% of individuals over 50 and 6% over 75, with higher incidence rates observed in females than males. The global economic burden was estimated to be $2.8 trillion USD in 2019 and is projected to increase to $8.5 trillion by 2040 [2], highlighting the urgent need for more effective diagnostic and therapeutic strategies [3].

The recent approval of three drugs (lecanemab, donanemab, and aducanumab) marks a milestone in AD research [4]. However, these therapies are limited to patients with cognitive symptoms, requiring expensive positron emission tomography (PET) or invasive cerebrospinal fluid (CSF) sampling to confirm amyloid burden. This approach limits the ability to intervene during the preclinical phase of AD, despite evidence that amyloid beta protein (Aβ) accumulation begins years before cognitive decline becomes apparent. Recent advances have led to the development of sensitive and accessible blood-based assays that detect AD-related protein markers — such as phosphorylated tau (p-tau), total tau (t-tau), and Aβ — offering substantial improvements in early screening efforts [3,5]. However, these assays may still benefit from auxiliary biomarkers to refine prognostic accuracy and better identify those most at risk of imminent cognitive decline. AD pathology is complex and multifactorial, involving amyloid plaques, vascular dysfunction, neuronal loss, and interactions with immune and inflammatory pathways [6]. Notably, a high amyloid burden alone does not reliably predict AD progression; many individuals with elevated amyloid remain cognitively intact [7]. Therefore, identifying complementary biomarkers that capture other aspects of AD biology outside the AT(N) (Amyloid, Tau, neurodegeneration) framework is crucial.

Among these, small RNAs (sRNAs), particularly microRNAs (miRNAs), are gaining attention for their potential use in early detection, patient stratification, treatment selection and disease monitoring [8–12]. miRNAs are short, non-coding RNA molecules derived from longer precursors that regulate gene expression and are implicated in AD pathology through various mechanisms, including the cleavage of amyloid precursor protein (APP) by β-secretases (BACE1) and γ-secretases [6,13]. BACE1, in particular, has drawn significant interest as a therapeutic target in AD research [14,15]. Furthermore, as molecular rheostats of diverse cellular and inflammatory processes, small RNAs offer a window into downstream effector mechanisms of disease pathology. As accessible readouts of key cellular and inflammatory processes, sRNAs can be reliably measured from blood samples using PCR and next-generation sequencing (NGS), making them attractive non-invasive biomarkers.

To explore the potential of sRNAs as biomarkers for AD, we analyzed whole blood samples from the EPAD (European Prevention of Alzheimer's Dementia) cohort, a pan-European project designed to investigate the preclinical and prodromal phases of AD. While this analysis was cross-sectional rather than longitudinal, the cohort's focus on early, pre-dementia stages of AD provides a unique opportunity to identify biomarkers that may reveal the earliest cognitive changes.

Using ultra-deep small RNA sequencing, with the implementation of blocking of highly abundant erythroid sRNAs, we identified biologically relevant sRNAs linked to AD and broader dementia pathways. These findings underscore the potential of combining sRNA and protein biomarkers to improve early diagnostic accuracy and patient stratification, paving the way for more accessible and effective interventions in the fight against AD [16].

2. Methods

2.1. Patient cohorts

All participants were recruited through the EPAD study [17,18]. The inclusion criteria were having a study partner (e.g. friend or relative) and being over the age of 50. For an exhaustive description see [19,20]. Participants were recruited from 31 sites in 10 countries throughout Europe. We used the final version of the EPAD LCS (longitudinal cohort study) [17].

2.2. Methodology EPAD data preparation

The EPAD dataset contains more than 2096 individuals with each subject having made between 1 to 5 visits [17]. Of these, 1934 individuals had available blood samples which were used in this study. During each visit, the subjects performed an array of cognitive tasks and some underwent MRI, CSF (cerebrospinal fluid) sampling, or blood donation. Not all subjects performed all tasks at all visits.

Since the EPAD study had a maximum of 4 visits (visits 1, 3, 4 and 5) where blood could be drawn, either one or multiple blood samples were received per subject. Not all subjects had blood drawn on the first visit. The earliest available blood was used for analysis. In some cases, CSF and blood were drawn on different visits.

2.3. Cognitive assessment

The cognitive status of subjects was assessed using the Clinical Dementia Rating (CDR) global score [21,22]. Subjects with normal cognition (no signs of cognitive impairment) have a CDR of 0, whereas individuals with a CDR of 0.5 are considered to have very mild cognitive impairment and thus exhibit the earliest cognitive symptoms of AD. Only four individuals with CDR=1 (mild cognitive impairment) and no subjects with higher CDRs were enrolled.

As an alternative measure of cognition, the MMSE (Mini-Mental State Exam [23]) was also performed. In this test, individuals with normal cognition achieve scores close to the maximum of 30. Any significant decrease is considered a sign of cognitive decline.

2.4. CSF biomarkers

The following biomarkers from cerebrospinal fluid (CSF) of patients were assayed: amyloid beta 1-42 ($A\beta_{1\text{-}42}$ or Aβ 42), phosphorylated tau 181 ($p\text{-}tau_{181}$, or simply p-tau), and total tau (t-tau).

The CSF biomarker variables were binarized to determine amyloid status for classification tasks as reported previously [20,24,25]. While previous EPAD analyses defined amyloid positivity as Aβ 42 ($A\beta_{1\text{-}42}$) <1000 pg/ml, here the ratio of $p\text{-}tau_{181}$ / $A\beta_{1\text{-}42}$, was used with a threshold of >0.024. This threshold separated the population into a binary "amyloid status", i.e. low amyloid (Aβ-, negative) and high amyloid (Aβ+, positive) sub-groups. See Fig. S2 for an illustration of how the threshold cleanly separated the population along the $p\text{-}tau_{181}$ and $A\beta_{1\text{-}42}$ axes. The EPAD study did not include measurements of Aβ 40 ($A\beta_{1\text{-}40}$).

2.5. Genetic markers

Apolipoprotein E (APOE) genotyping was determined using TaqMan assays for rs7412 and rs429358. Genotyping of DNA, extracted from blood samples, took place on a ThermoFisher QuantStudio 12K Flex in the Genetics Core, Edinburgh Clinical Research Facility, University of Edinburgh. APOE e4 carriers were defined as having at least one e4 allele.

2.6. PAXgene collection and processing

Peripheral blood was drawn following the EPAD manual, which is available upon request. In general, blood was drawn in the mornings where possible. Fasting was not strictly required.

To generate deep sRNA profiles that capture both cell-free and immune cell-derived signals, we analyzed PAXgene RNA whole blood tubes. Blood samples (2.5 ml each) were collected following PAXgene (formerly PreAnalytiX, now Qiagen) guidelines. For long-term preservation, samples were stored at −80°C. RNA extraction was performed using the QIAsymphony PAXgene Blood RNA Kit on the QIAsymphony SP liquid handling station (Qiagen, Venlo, The Netherlands). For quality control, custom artificial spike-in sequences were added to resuspended pellets prior to extraction. Elution occurred at 72°C for 10 minutes in 200 μl of elution buffer, and RNA was aliquoted and stored at -80°C.

2.7. Sample inclusion criteria

Of a total of 3302 received blood samples, 2903 passed extraction and FragmentAnalyzer quality control (QC) when they resulted in a normal electropherogram.

1913 samples remained after excluding duplicate samples from the same individual and those missing CDR scores. 1742 samples were complete with matching CSF markers. Sample numbers used for calculating statistics or fitting machine learning (ML) models thus may differ depending on whether CSF markers were involved, as indicated in Table 2.

2.8. sRNA-Seq with blocking of highly abundant RNAs

For ligation, 200 ng of total RNA or up to 5 μl of RNA (if concentration was low) was combined with 0.25 μM of a 3'-adapter (IDT, Coralville, IO) and T4 RNA ligase 2 KQ (NEB, Ipswich, MA) for 1 hour at 28°C. Next, ligation with 0.125 μM of a 5'-adapter (IDT) was carried out with T4 RNA ligase 1 for 30 minutes at 28°C. Blocking oligos (IDT) were added directly to the ligation mix, which was then denatured at 75°C for 2 minutes and cooled to 25°C. Reverse transcription was performed with LunaScript (NEB, Ipswich, MA) and 0.08 μM of RT primer (IDT) for 1 hour at 50°C. RT clean-up was conducted using Mag-Bind beads (Omega Bio-Tek, Norcross, GA) at a 2.5 × sample-to-bead ratio, per the manufacturer's protocol. Library PCR was performed using the NEBNext ULTRA II Q5 master mix (NEB, Ipswich, MA) and dual index primers as previously described. PCR products were cleaned using Mag-bind beads with 0.9 × and 1.8 × ratios. DNA concentrations were assessed with the Quant-iT kit (ThermoFisher Scientific, Waltham, MA) on a VICTOR Nivo plate reader (Revvity, formerly PerkinElmer). A 15 µM multiplexed library pool was prepared, loaded onto a Novex 4%–12% PAGE gel (ThermoFisher Scientific, Waltham, MA), and fragments above 180 base pairs were excised. The library was eluted from the gel overnight at 37°C, purified with the DNA Clean and Concentrator Kit (Zymo Research, Freiburg, Germany), and sequenced using a dual-indexing approach on a NextSeq2000 (Illumina, San Diego, CA) as previously described. Further details are provided in [26] and supporting online material (Fig. S1).

2.9. Data preprocessing

FastQ files from the Illumina NextSeq 2000, generated through repeated measurements of each multiplexed pool (batch of samples processed together using dual indexing), were aggregated into a single FastQ file per pool. Reads of at least 18 nucleotides that appeared in at least three samples per sequencing pool and in at least 10% of all samples were UMI-corrected (to remove PCR artifacts) and converted into raw read count matrices by summing UMI-corrected counts of sRNA sequences. RNA expression was then calculated as: $Y_{ij} = log_2(1 + \frac{cX_{ij}}{M_i})$, where the values are log2-transformed, 1-shifted, library-size normalized reads per million (RPM), with ($c = 10^6$) for each patient sample i and RNA feature j.

2.10. Mitigation of confounder bias

Potential clinical confounders (expected to be correlated with disease and/or biomarkers) were age, sex, APOE status, and binary Amyloid-tau status. At this stage any samples with missing values or unclear group assignment were excluded, including those with rare APOE status, i.e. *e2/e2*. It has been established within the EPAD study that there is an interaction between amyloid, sex, and APOE [25].

These confounders were neutralized via propensity score matching (PSM) [27] (Table 3), where a logistic regression model is trained on the target variable using only confounders as independent variables. The numerical propensity score is used to match patients with similar confounder profiles in the negative (CDR normal) to those in the positive group (CDR impaired). Only matches with a propensity score difference smaller than 0.1 (PSM strength parameter) were considered. Since the chosen PSM strength parameter allows for minor mismatches, and to monitor any remaining confounder effects, the confounder variables were also included as model covariates. This practice ensures that no selected small RNA marker was used as a confounder proxy.

An additional confounder or batch variable, namely the EPAD clinical site, was not well suited for PSM matching due to the high number of clinics and heterogeneous sample distribution and class imbalances per clinic. Instead, as a test for clinic bias, models were trained with the binarized clinic identities (one-hot dummy encoding) given as model covariates in addition to small RNA expression. While disregarding the performance metrics from such models (which will be inflated by batch effects), any selected small RNAs can be assumed to be free of clinic bias, with the caveat of potentially removing too many features where clinic and disease signal coincide.

2.11. Machine learning

One hundred logistic regression classifiers were trained on a randomly chosen 75% of samples resulting from a given PSM matching, while the remaining 25% were used as test sets for performance evaluation. This process was repeated five times with different random shuffling of samples prior to PSM, to account for random sampling effects introduced by PSM itself.

A strict detection rate filter was applied to admit only small RNAs with non-zero (i.e. detected) expression values in at least 99% of samples (3161 sRNAs in total). This filter was used for the dual benefit of feature selection and avoidance of batch effects (which are often driven by noisy low-expression features). A strong L1 penalty was used to select a small number of predictive small-RNA features.

Small RNAs coinciding with blocking targets were excluded.

2.12. Importance ranking of small RNAs

Small RNAs with a non-zero weight assigned by L1-penalized logistic regression were mapped to the precursor (long) RNA of origin. Since each model was replicated 100 times, the number (percentage) of times a small RNA was selected could be used as an indicator of its importance. Precursor importance was calculated by averaging individual small RNA importance scores across five different PSM replicates using small RNAs only and summing averaged importance scores for all small RNAs from the same precursor, finally dividing the resulting values by 100.

2.13. Small RNA annotation

Our customized small RNA annotation strategy has been extensively described in [28]. Briefly, we generated a comprehensive reference database from sources that included miRbase [29], GtRNAdb [30], NCBI [31], snoDB [32], piRNA cluster database [33] and Ensembl [34]. Sequences were iteratively mapped against the database using bowtie [35], initially allowing zero mismatches, followed by subsequent rounds permitting up to 3 mismatches for previously unmapped reads. Small RNA sub-classes were defined as described previously [28].

2.14. sRNA co-expression analysis

The most important RNAs were taken from representative ML models (corresponding to row 6 in Table 2) as those selected in at least 50% of replicates. This set was expanded by identifying other RNAs with correlated expression above a Spearman coefficient of 0.7 and expression was averaged per precursor. A network was created by connecting highly correlated precursors. A correlation cut-off was chosen which formed a single connected network. This network contained almost all nodes (except one singular node), while also avoiding more connections than necessary.

All networks were visualized in Cytoscape [36].

2.15. Differential expression analysis

Differential expression was quantified at the precursor level by summing up read counts mapping to the same precursor, including the same RNAs from co-expression analysis. Log2 fold changes (FC) were calculated using the DESeq2 software [37]. A positive log2-FC indicates that a given RNA is more highly expressed in CDR-impaired individuals, whereas a negative value indicates higher expression in normal subjects.

2.16. miRNA gene targets and protein interaction network

A miRNA-target gene interaction network was generated using the web-based miRNET tool [38] (accessible under https://www.mirnet.ca/). The miRBase IDs of top-scoring miRNA precursors were queried against miRTarBase v9.0 [39] with the following parameters: organism: *Homo sapiens*, tissue type: unspecified, include protein-protein interactions from IMEx interactome [40]. The resulting network consisting of over 24 000 edges was scanned for the presence of AD-associated genes from KEGG [41] database (pathway no. 05010) as well as a set of manually curated AD-related genes based on literature reports. A subnetwork containing the abovementioned genes was created directly in miRNET [38] using the "extract currently highlighted modules" function. Selected genes from the resulting subnetwork were manually grouped by functional roles.

2.17. Deconvolution of cell type origin

Based on the data published in [42], small RNAs were deconvolved into their likely blood compartment origin including the plasma fraction and 10 different cell types. Fractions per precursor were calculated using a mean of fractions for individual small RNAs, each weighted by the ratio of given small RNA importance to the sum of importances of all small RNAs mapping to the same precursor.

3. Results

The EPAD participants were initially stratified into four cohorts based on cognitive status (CDR) and CSF-derived amyloid status (Table 1). Instead of the commonly used Aβ42/Aβ40, the alternative p-tau$_{181}$/Aβ42 ratio was used for binarizing amyloid status (in the absence of Aβ40 measurements; see Methods and Fig. S2). This metric had shown good performance in plasma and CSF [43]. Cohort 1 (n=1093) comprised cognitively normal individuals with low amyloid burden (Aβ-), whereas Cohort 2 (n=275) included cognitively normal individuals with high amyloid burden (Aβ+). Cohorts 3 (n=261) and 4 (n=284) included individuals with mild cognitive impairment (CDR $\geq$ 0.5) and low or high amyloid burden, respectively. Demographics (sex, APOE genotype, age) and clinical measurements (MMSE, p-tau$_{181}$, t-tau, A$\beta_{1\text{-}42}$) are summarized in Table 1. For the purpose of training predictors of mild cognitive impairment, cohorts 1 + 2, as well as 3 + 4 were combined into two groups, which were then confounder-matched (see below and Methods).

3.1. Amyloid status classification

Initial attempts to predict amyloid status solely from small RNA (sRNA) expression achieved a mean AUC of ~0.52 (Table 2; rows #1-5), suggesting negligible predictive power. Given that protein-based blood tests have recently become more accurate in detecting amyloid [44–46], as well as tau pathology [47–51], we did not pursue sRNA-based amyloid classification further.

3.2. Cognitive status classification

We next asked whether sRNAs might be more useful for predicting cognitive impairment – particularly mild cognitive decline which frequently precedes a formal AD diagnosis.

Cognitive status was determined by the Clinical Dementia Rating (CDR) scale [21,22], comparing CDR=0 (n=1368 before confounder matching, normal cognition; negative control group) with CDR≥0.5 (n=545 before confounder matching, impaired cognition; positive effect group). Only three participants had a CDR of 1, and none were higher, indicating predominantly mild cognitive impairment (MCI) in our dataset. MMSE scores confirmed the distinction between the two groups with a Cohen's d effect size of 0.841 and a t-test metric of T=-12 at a highly significant p-value of 1.64×10^{-29}. However, there may be resolution issues when matching MMSE scores to the CDR stages of 0 and 0.5 [52].

Models trained solely on sRNA features achieved a mean AUC of ~ 0.70 for differentiating CDR 0 from CDR $\geq$ 0.5, a marked improvement over a random baseline of 0.50 and over confounder-only baselines (~0.50), as shown in Fig. 1a and Table 2.

Stratifying participants by CSF-based amyloid status revealed lower predictive power (AUC ~0.64) among amyloid-negative individuals (Fig. 1b) but higher performance (AUC ~0.77) for amyloid-positive individuals (Fig. 1c). This suggests that the sRNA signal appears more closely linked to AD-related cognitive impairment than to other forms of cognitive impairment.

We used propensity score matching (PSM) prior to the discovery ML runs (discovery: offering a broad range of RNAs for selection) to minimize confounding (see Methods). The impact of confounders was very low, as indicated by virtually identical precursor importance and AUCs between models trained either without or with confounders (Table 2; rows #6-10 vs rows #11-15, respectively). The only deviation in precursor importance and AUC was observed when adding the clinic identities as additional input variables – with the aim of determining the theoretical potential of batch effects. Indeed, the highly inflated AUCs of around 0.84 (Table 2; rows #16-20) revealed a significant potential for batch effect due to class imbalances between clinics. The clinic itself can then become a predictor of CDR, diminishing the need for true RNA markers. Consequently, some RNA markers decreased in importance, while others remained relatively stable (most notably miR-186).

The observation that models trained without clinic identifiers performed at much lower AUCs, is an encouraging confirmation that those models were *not* exploiting batch effects. This is most likely due to the combination of strict detection rate filters removing very noisy features, and strong L1 penalty enforcing low feature count – in combination with

Table 1
Summary of subjects in the EPAD cohort, including subgroups based on cognitive and amyloid status.

Subgroups	Cohort 1 Low Amyloid burden, Normal cognition	Cohort 2 High Amyloid burden, normal cognition	Cohort 3 Low Amyloid burden, impaired cognition	Cohort 4 High Amyloid burden, impaired cognition
Sample size (n)	1093	275	261	284
sex				
f	634 (58%)	179 (65%)	137 (52%)	128 (45%)
m	459 (42%)	96 (35%)	124 (48%)	156 (55%)
APOE genotype				
e2/e2	5 (0%)	0 (0%)	1 (0%)	0 (0%)
e2/e3	108 (10%)	16 (6%)	30 (12%)	11 (4%)
e2/e4	27 (3%)	14 (5%)	5 (2%)	7 (3%)
e3/e3	617 (57%)	108 (40%)	154 (61%)	95 (34%)
e3/e4	304 (28%)	101 (38%)	61 (24%)	126 (45%)
e4/e4	15 (1%)	28 (10%)	3 (1%)	38 (14%)
Clinical dementia rating (CDR)				
0	1093 (100%)	275 (100%)	0 (0%)	0 (0%)
0.5	0 (0%)	0 (0%)	261 (100%)	280 (99%)
1	0 (0%)	0 (0%)	0 (0%)	4 (1%)
Binary amyloid status (p-tau$_{181}$ / A$\beta_{1\text{-}42}$)				
low	1093 (100%)	0 (0%)	261 (100%)	0 (0%)
high	0 (0%)	275 (100%)	0 (0%)	284 (100%)
age				
mean ± std	63.85 ± 7.06	67.84 ± 6.13	66.9 ± 7.71	70.33 ± 7.3
median (min, max)	64.0 (50, 85)	68.0 (50, 89)	67.0 (50, 88)	71.0 (50, 86)
CSF p-tau$_{181}$ levels (pg/ml)				
mean ± std	16.27 ± 5.7	28.55 ± 14.49	17.68 ± 6.47	32.95 ± 15.05
median (min, max)	15.33 (8, 51)	24.89 (8, 107)	16.52 (8, 43)	30.48 (8, 112)
CSF t-tau levels (pg/ml)				
mean ± std	195.47 ± 64.07	296.67 ± 124.43	213.79 ± 73.83	340.98 ± 135.35
median (min, max)	186.7 (80, 547)	271.5 (80, 848)	200.3 (87, 515)	325.7 (80, 947)
CSF A$\beta_{1\text{-}42}$ levels (pg/ml)				
mean ± std	1578.49 ± 710.87	716.51 ± 256.41	1623.4 ± 790.37	685.8 ± 248.88
median (min, max)	1448.0 (371, 7017)	685.6 (200, 1556)	1475.0 (533, 6828)	653.9 (210, 1478)
MMSE score				
mean ± std	28.88 ± 1.29	28.4 ± 1.63	28.14 ± 1.78	26.51 ± 2.95
median (min, max)	29.0 (19, 30)	29.0 (19, 30)	28.0 (21, 30)	27.0 (12, 30)
p-tau$_{181}$ / A$\beta_{1\text{-}42}$ ratio				
mean ± std	0.011 ± 0.004	0.043 ± 0.023	0.012 ± 0.004	0.052 ± 0.027
median (min, max)	0.01 (0.005, 0.024)	0.036 (0.024, 0.181)	0.01 (0.006, 0.024)	0.044 (0.024, 0.24)

a strong true biological signal from the RNA markers like miR-186 and others.

Finally, models only using clinical confounders (age, sex, APOE, amyloid status), i.e. using no RNAs, achieved no predictive performance under the same model parameters as before (AUC=0.5, see Table 2 rows #21-25), thus confirming the effectiveness of PSM (see also Table 3 for statistical tests before and after PSM).

3.3. Testing minimal combinations of sRNA and protein markers

When evaluating potential biomarkers, minimizing the number of markers can be beneficial, particularly when transitioning from high-throughput sequencing methods to lower-throughput analytical techniques such as PCR or digital PCR. Our analysis identified that a limited number of small RNAs contribute most significantly to predictive accuracy. Table 2 summarizes AUCs from models using either protein-based markers (CSF A$\beta_{1\text{-}42}$, p-tau, t-tau) or small RNAs, or a combination thereof. Individual RNA precursors or combinations of those were used, as indicated in the table.

The protein-only baseline is surpassed by all combinations with small RNAs, with models using as few as three different RNAs approaching peak performance. The addition of protein markers notably improves model performance within the low amyloid group, where RNA-only models show much lower performance compared to the high-amyloid group.

3.4. Biological relevance of putative AD markers

Machine learning models trained to predict CDR were analyzed for the most important features (sRNAs) selected for classification (see Methods). When aggregating results from multiple replicates, and then mapping small RNAs to their precursor RNA, a clear ranking of important sRNA classes emerged. miRNAs originating from several precursors such as mir-186, mir-363, mir-26a, and mir-22 are consistently selected as the most important features (see Fig. 2). Other classes, however, included tRNA, rRNA, snoRNA and mRNA fragments that contributed to classification.

We then analyzed the mixed-cell small RNA expression profiles to infer their probable cellular or plasma origins [42]. The heatmap presented in Fig. 2 illustrates these potential sources. Notably, the RNAs clustered into two distinct groups: one associated predominantly with immune cells (e.g. mir-26a, SNORD104, mir-150, 5S-rRNA, and mir-125a), and another group primarily linked to plasma and erythrocytes (e.g. mir-186, mir-363, tRNA-Glu and tRNA-Gly, 28S-rRNA, mir-22, mir-454, mir-423, and mir-1270).

3.5. Small RNA co-expression network and differential expression

The machine learning (ML) models developed from small RNA expression data were optimized to select a limited set of markers, thereby minimizing redundancy among highly correlated RNAs. However, to

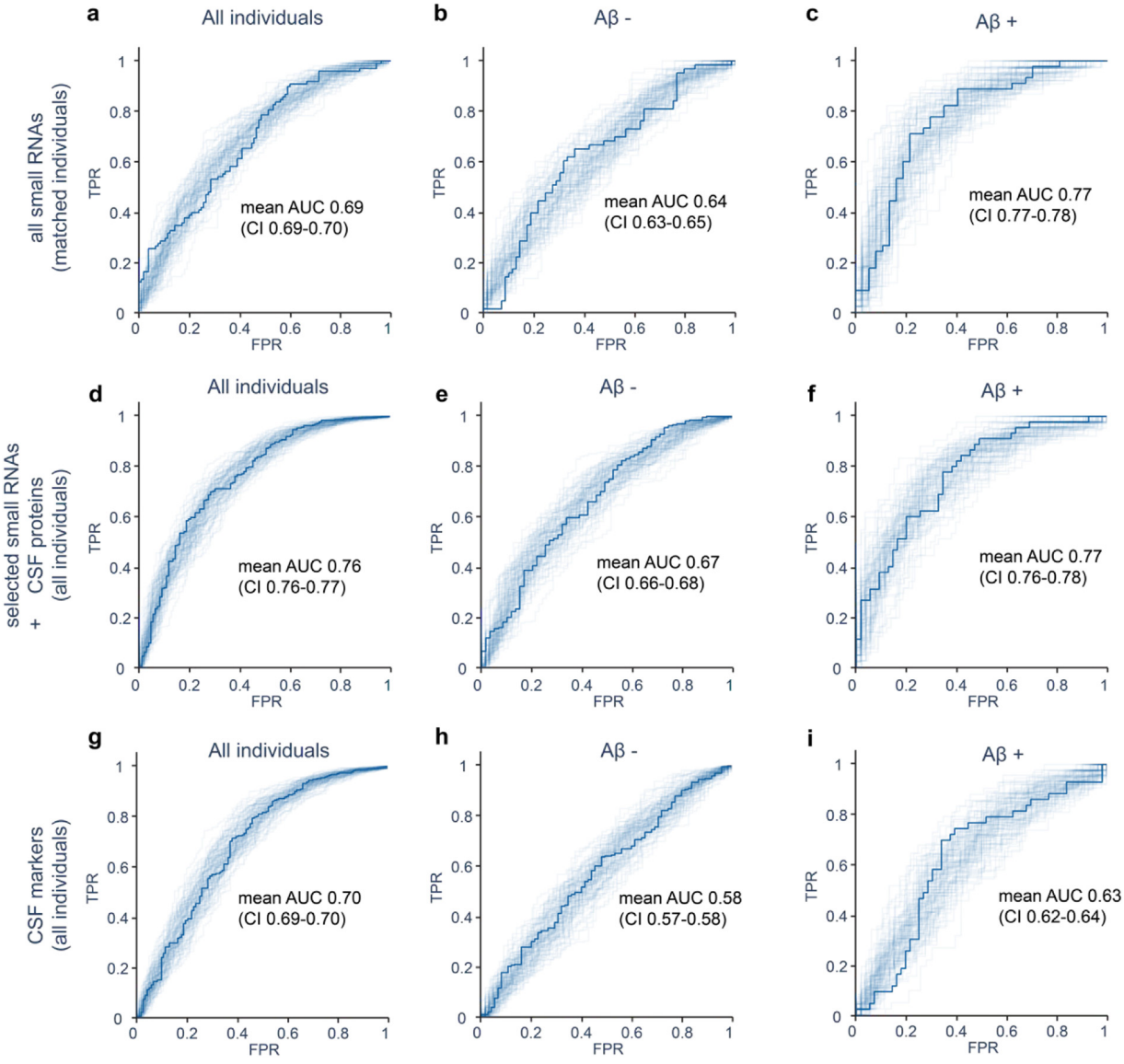

Fig. 1. Machine learning predictions of CDR cognitive impairment. ROC curves and AUCs were obtained for 100 randomly picked test set splits of patient samples. (a-c) Propensity score matching was applied to samples before splitting, admitting all small RNAs detected in at least 99% of samples for logistic regression machine learning. (b + e) shows ROC curves only for the low amyloid subset of patients, whereas (c + f) shows the high amyloid subset. (d-f) Only small RNAs from the mir-186, mir-363, and mir-150 precursors were admitted, combined with the CSF markers $A\beta_{1\text{-}42}$, p-tau, and t-tau.

better understand underlying biological interactions, it is useful to examine a broader set of correlated RNAs. Fig. 3a illustrates not only the key RNAs identified by ML models (highlighted with thick black boxes) but also includes additional RNAs with correlated (co-expressed) expression profiles, mapped to their shared precursor RNAs (network nodes). Lines (edges) between nodes represent significant correlations between precursor RNAs. Nodes are color-coded based on differential expression analysis, comparing cognitively normal to impaired groups independently from ML-derived importance. This network reveals three prominent clusters of strongly interconnected RNAs. Notably, one of these clusters (top center) primarily contains small nucleolar RNAs (snoRNAs) rather than the more common microRNAs. As anticipated, snoRNAs exhibit strong correlations with the 28S large ribosomal subunit, consistent with their known biological roles [53].

MicroRNAs were the most consistently impactful class, targeting genes involved in the functional categories of apoptosis, inflammation, neurotransmission, amyloid processing, and other AD-related pathways (Fig. 3b). Note that the homologous BACE2 is listed instead of BACE1 as a miR-186 target, as this was supported by miRNET (Methods). A table listing more detailed support for the given gene functions is provided in supporting online information (Table S1).

4. Discussion

4.1. Context and relevance of findings

This study provides the first comprehensive analysis of small RNA biomarkers within the EPAD cohort to classify cognitive impairment. The predictive performance, particularly among individuals with elevated amyloid levels, suggests potential utility for identifying subjects with early cognitive decline. Targeting at-risk populations (e.g., based on age or family history) and combining these with blood-based protein markers that serve as proxies for CSF-derived amyloid and tau, could significantly enhance early detection and facilitate timely neurological assessments.

4.2. Biological insights

Beyond prediction, our findings provide insights into molecular processes involved in mild cognitive decline. The small RNAs identified associate with critical molecular pathways previously linked to neurodegeneration, including neuronal development, apoptosis, inflammation, hypoxia, and amyloid metabolism. These associations highlight the potential biological relevance of small RNAs not only as biomarkers but also as active participants in Alzheimer's disease (AD) pathogenesis.

Interestingly, small RNA profiles correlated more closely with cognitive impairment rather than directly with amyloid burden. For example, miR-186, a robust predictor in our analysis, targets BACE1, a central enzyme in amyloid precursor protein processing. Despite this functional link, miR-186 levels did not correlate strongly with amyloid burden in this dataset (Pearson r=-8.12e-02; p-value=7.60e-04; n=1718), sug-

Table 2
Performance summary of machine learning models predicting CDR. AUCs are colored on blue gradient, except for the upper and lower performance baselines (clinic batch included or clinical confounders only, respectively); presence of non-RNA input data indicated in solid black; presence of RNA input data in solid gray, or alternatively for discovery, the RNA precursor importance is given as green horizontal bars. PSM=Propensity Score Matching.

#	prediction	discovery?	PSM replicate #	AUC	AUC high amyloid	AUC low amyloid	Sex	clinic identities (batch)	clinical confounders	all small RNA	Ab 1-42	p-tau	t-tau	mir-186	mir-363	mir-26a	mir-22	mir-150	SNORD104	sample size
1	amyloid	1	1	0.523	N/A	N/A	f, m	0	1	1	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	552
2	amyloid	1	2	0.54	N/A	N/A	f, m	0	1	1	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	558
3	amyloid	1	3	0.514	N/A	N/A	f, m	0	1	1	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	560
4	amyloid	1	4	0.529	N/A	N/A	f, m	0	1	1	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	558
5	amyloid	1	5	0.529	N/A	N/A	f, m	0	1	1	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	560
6	CDR	1	1	0.69	0.77	0.64	f, m	0	0	1	0	0	0	2.76	2.27	1.38	1.05	0.62	1.17	812
7	CDR	1	2	0.71	0.79	0.66	f, m	0	0	1	0	0	0	3.13	2.78	1.37	1.11	0.88	1.63	812
8	CDR	1	3	0.69	0.77	0.63	f, m	0	0	1	0	0	0	3.30	2.22	1.41	1.14	0.86	0.85	812
9	CDR	1	4	0.70	0.77	0.65	f, m	0	0	1	0	0	0	2.57	2.39	1.30	1.14	1.04	1.41	812
10	CDR	1	5	0.69	0.77	0.64	f, m	0	0	1	0	0	0	3.03	2.02	1.35	1.09	1.26	1.23	812
11	CDR	1	1	0.70	0.77	0.66	f, m	0	1	1	0	0	0	2.76	2.72	1.22	1.17	0.72	1.28	808
12	CDR	1	2	0.71	0.79	0.66	f, m	0	1	1	0	0	0	3.11	2.75	1.40	1.11	0.88	1.64	808
13	CDR	1	3	0.69	0.77	0.63	f, m	0	1	1	0	0	0	3.30	2.20	1.43	1.13	0.82	0.85	808
14	CDR	1	4	0.70	0.77	0.65	f, m	0	1	1	0	0	0	2.56	2.40	1.30	1.14	1.04	1.42	808
15	CDR	1	5	0.70	0.76	0.66	f, m	0	1	1	0	0	0	3.13	2.15	1.06	1.16	1.33	1.55	808
16	CDR	1	1	0.83	0.83	0.84	f, m	1	1	1	0	0	0	1.97	0.81	1.06	0.99	0.95	0.75	808
17	CDR	1	2	0.84	0.86	0.84	f, m	1	1	1	0	0	0	2.30	1.14	1.31	0.87	1.05	1.33	808
18	CDR	1	3	0.84	0.86	0.84	f, m	1	1	1	0	0	0	2.11	0.23	0.91	0.83	1.08	0.91	808
19	CDR	1	4	0.84	0.86	0.84	f, m	1	1	1	0	0	0	1.80	0.41	1.12	0.92	0.97	0.66	808
20	CDR	1	5	0.84	0.84	0.84	f, m	1	1	1	0	0	0	1.85	0.28	0.53	0.96	1.30	1.18	808
21	CDR	1	1	0.50	0.50	0.50	f,m	0	1	0	0	0	0	0	0	0	0	0	0	812
22	CDR	1	2	0.50	0.50	0.50	f, m	0	1	0	0	0	0	0	0	0	0	0	0	812
23	CDR	1	3	0.50	0.50	0.50	f, m	0	1	0	0	0	0	0	0	0	0	0	0	812
24	CDR	1	4	0.50	0.50	0.50	f, m	0	1	0	0	0	0	0	0	0	0	0	0	812
25	CDR	1	5	0.50	0.50	0.50	f, m	0	1	0	0	0	0	0	0	0	0	0	0	812
26	CDR	0	N/A	0.70	0.63	0.58	f, m	0	0	0	1	1	1	0	0	0	0	0	0	1742
27	CDR	0	N/A	0.61	0.70	0.58	f, m	0	0	0	0	0	0	1	0	0	0	0	0	1913
28	CDR	0	N/A	0.72	0.70	0.62	f, m	0	0	0	1	1	1	1	0	0	0	0	0	1742
29	CDR	0	N/A	0.60	0.61	0.60	f, m	0	0	0	0	0	0	0	1	0	0	0	0	1913
30	CDR	0	N/A	0.72	0.67	0.63	f, m	0	0	0	1	1	1	0	1	0	0	0	0	1742
31	CDR	0	N/A	0.61	0.66	0.59	f, m	0	0	0	0	0	0	0	0	1	0	0	0	1913
32	CDR	0	N/A	0.72	0.68	0.62	f, m	0	0	0	1	1	1	0	0	1	0	0	0	1742
33	CDR	0	N/A	0.62	0.66	0.59	f, m	0	0	0	0	0	0	0	0	0	1	0	0	1913
34	CDR	0	N/A	0.72	0.68	0.60	f, m	0	0	0	1	1	1	0	0	0	1	0	0	1742

(continued on next page)

Table 2 (*continued*)

35	CDR	0	N/A	0.58	0.65	0.56	f, m	0	0	0	0	0	0	0	0	0	0	1	0	1913
36	CDR	0	N/A	0.70	0.66	0.58	f, m	0	0	0	1	1	1	0	0	0	0	1	0	1742
37	CDR	0	N/A	0.55	0.61	0.52	f, m	0	0	0	0	0	0	0	0	0	0	0	1	1913
38	CDR	0	N/A	0.70	0.64	0.58	f, m	0	0	0	1	1	1	0	0	0	0	0	1	1742
39	CDR	0	N/A	0.66	0.73	0.64	f, m	0	0	0	0	0	0	1	1	0	0	0	0	1913
40	CDR	0	N/A	0.75	0.74	0.66	f, m	0	0	0	1	1	1	1	1	0	0	0	0	1742
41	CDR	0	N/A	0.66	0.76	0.62	f, m	0	0	0	0	0	0	1	0	1	0	0	0	1913
42	CDR	0	N/A	0.75	0.76	0.66	f, m	0	0	0	1	1	1	1	0	1	0	0	0	1742
43	CDR	0	N/A	0.66	0.74	0.62	f, m	0	0	0	0	0	0	1	0	0	1	0	0	1913
44	CDR	0	N/A	0.74	0.73	0.64	f, m	0	0	0	1	1	1	1	0	0	1	0	0	1742
45	CDR	0	N/A	0.65	0.77	0.59	f, m	0	0	0	0	0	0	1	0	0	0	1	0	1913
46	CDR	0	N/A	0.73	0.74	0.62	f, m	0	0	0	1	1	1	1	0	0	0	1	0	1742
47	CDR	0	N/A	0.63	0.73	0.58	f, m	0	0	0	0	0	0	1	0	0	0	0	1	1913
48	CDR	0	N/A	0.72	0.71	0.61	f, m	0	0	0	1	1	1	1	0	0	0	0	1	1742
49	CDR	0	N/A	0.67	0.75	0.64	f, m	0	0	0	0	0	0	1	1	1	0	0	0	1913
50	CDR	0	N/A	0.76	0.75	0.67	f, m	0	0	0	1	1	1	1	1	1	0	0	0	1742
51	CDR	0	N/A	0.67	0.77	0.63	f, m	0	0	0	0	0	0	1	0	1	1	0	0	1913
52	CDR	0	N/A	0.76	0.76	0.66	f, m	0	0	0	1	1	1	1	0	1	1	0	0	1742
53	CDR	0	N/A	0.68	0.78	0.64	f, m	0	0	0	0	0	0	1	0	1	0	1	0	1913
54	CDR	0	N/A	0.76	0.77	0.66	f, m	0	0	0	1	1	1	1	0	1	0	1	0	1742
55	CDR	0	N/A	0.67	0.79	0.63	f, m	0	0	0	0	0	0	1	0	1	0	0	1	1913
56	CDR	0	N/A	0.76	0.77	0.66	f, m	0	0	0	1	1	1	1	0	1	0	0	1	1742
57	CDR	0	N/A	0.69	0.79	0.65	f, m	0	0	0	0	0	0	1	1	0	0	1	0	1913
58	CDR	0	N/A	0.76	0.77	0.67	f, m	0	0	0	1	1	1	1	1	0	0	1	0	1742
59	CDR	0	N/A	0.68	0.77	0.62	f, m	0	0	0	0	0	0	1	0	0	1	1	0	1913
60	CDR	0	N/A	0.74	0.76	0.64	f, m	0	0	0	1	1	1	1	0	0	1	1	0	1742
61	CDR	0	N/A	0.65	0.77	0.59	f, m	0	0	0	0	0	0	1	0	0	0	1	1	1913
62	CDR	0	N/A	0.73	0.74	0.62	f, m	0	0	0	1	1	1	1	0	0	0	1	1	1742
63	CDR	0	N/A	0.69	0.78	0.65	f, m	0	0	0	0	0	0	1	1	1	0	1	0	1913
64	CDR	0	N/A	0.76	0.77	0.67	f, m	0	0	0	1	1	1	1	1	1	0	1	0	1742
65	CDR	0	N/A	0.70	0.79	0.67	f, m	0	0	0	0	0	0	1	1	0	1	1	0	1913
66	CDR	0	N/A	0.76	0.78	0.68	f, m	0	0	0	1	1	1	1	1	0	1	1	0	1742
67	CDR	0	N/A	0.69	0.79	0.65	f, m	0	0	0	0	0	0	1	1	0	0	1	1	1913
68	CDR	0	N/A	0.76	0.77	0.67	f, m	0	0	0	1	1	1	1	1	0	0	1	1	1742
69	CDR	0	N/A	0.70	0.79	0.66	f, m	0	0	0	0	0	0	1	1	1	1	1	1	1913
70	CDR	0	N/A	0.76	0.78	0.68	f, m	0	0	0	1	1	1	1	1	1	1	1	1	1742
71	CDR	0	N/A	0.67	0.74	0.64	f, m	0	0	0	0	0	0	0	1	1	1	1	1	1913
72	CDR	0	N/A	0.75	0.74	0.67	f, m	0	0	0	1	1	1	0	1	1	1	1	1	1742
73	CDR	0	N/A	0.68	0.77	0.65	**m**	0	0	0	0	0	0	1	1	1	1	1	1	835
74	CDR	0	N/A	0.76	0.73	0.66	**m**	0	0	0	1	1	1	1	1	1	1	1	1	773
75	CDR	0	N/A	0.69	0.78	0.64	**f**	0	0	0	0	0	0	1	1	1	1	1	1	1076
76	CDR	0	N/A	0.75	0.80	0.67	**f**	0	0	0	1	1	1	1	1	1	1	1	1	969

gesting its role extends beyond amyloid metabolism. Additionally, miR-186 levels remained stable with increasing age (Pearson r=6.46e-02; p-value=4.96e-03; n=1888), contrasting previous observations in aging AD-model mice, where miR-186 declined with age [54]. This discrepancy underscores the complex and likely context-dependent regulation of miR-186's regulatory roles, necessitating further exploration in human and animal studies.

Pathways previously implicated in amyloid translocation across the blood-brain barrier (BBB), such as those mediated by LRP1 and APOE, were notably absent from our miRNA-target gene network. This absence might reflect differences in regulatory mechanisms captured by small RNAs versus proteins or suggest that these pathways operate independently of the cognitive decline detected by miRNA markers. This finding reinforces the concept that miRNAs complement protein biomarkers by

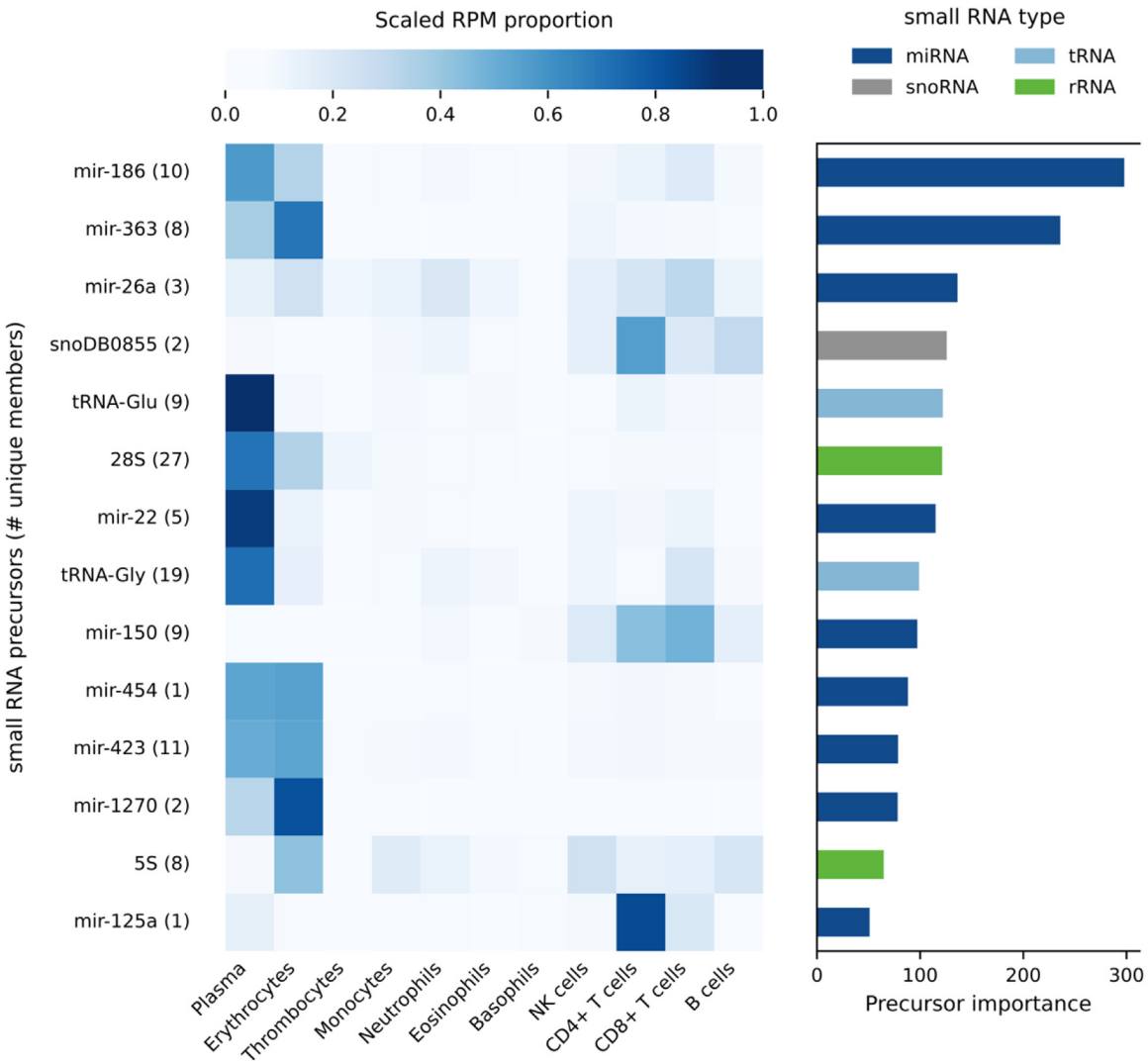

Fig. 2. Cell type deconvolution of most important small RNAs at precursor level.

capturing distinct aspects of neurodegeneration, offering a broader perspective on early disease progression.

A key question emerging from these findings pertains to the biological origin of the identified small RNAs. RNA preserved in PAXgene tubes originates from diverse blood components (e.g., red blood cells, platelets, leukocytes) and extracellular sources, such as plasma and exosomes, some of which may be brain derived. Given that BBB dysfunction is a hallmark of AD, increased BBB permeability may facilitate the translocation of CNS-derived RNAs into peripheral blood. Indeed, the enrichment of brain-related functions among the strongest predictive markers suggests at least partial CNS origins. Additionally, lymphocytes that traverse the BBB might contribute specific RNA signatures reflecting systemic interactions with brain tissue, highlighting the complex interplay between systemic and brain-derived factors in the context of cognitive impairment.

Our findings also shed light on vascular contributions to AD pathology [55,56]. Although advanced imaging techniques such as MRI and PET have demonstrated vascular involvement in AD [57,58], their high cost and limited accessibility restrict widespread clinical application. Small RNAs may bridge this gap by acting as accessible biomarkers reflecting vascular pathology.

We identified multiple small RNAs targeting critical vascular pathways. VEGFA (Vascular Endothelial Growth Factor A) and HIF1A (Hypoxia-Inducible Factor 1 Alpha) are among the targets of miR-186-5p. They are known vascular regulators and are implicated in neurodegeneration [59]. Furthermore, miR-363-3p targets NOTCH1 which is active in neurovascular aging and neurodegeneration [60,61]. Notably, NOTCH1 is processed by γ-secretase – a subunit of which (nicastrin, NCSTN) is also regulated by miR-186-5p [62]. Collectively, these interactions emphasize the potential importance of vascular mechanisms in early cognitive impairment and underscore the biomarker potential of small RNAs.

4.3. Limitations and future directions

While a core set of confounding variables has been adjusted for in this study, several additional factors may influence blood RNA levels and should be included in future studies where practically possible. These include different comorbidities, medications, fasting status, and other physiological conditions (e.g. renal conditions). While attempts were made to capture medication use in the EPAD study, the quality and completeness of these data were insufficient for robust analysis. While one previous study found a significance of fasting [63], others reported low short-term variability even when not controlling for fasting [64].

While the prognostic utility of small RNA markers is promising, several limitations must be acknowledged. Longitudinal studies tracking small RNA levels over time are required to delineate their trajectories across disease progression. Integrating additional protein biomarkers –

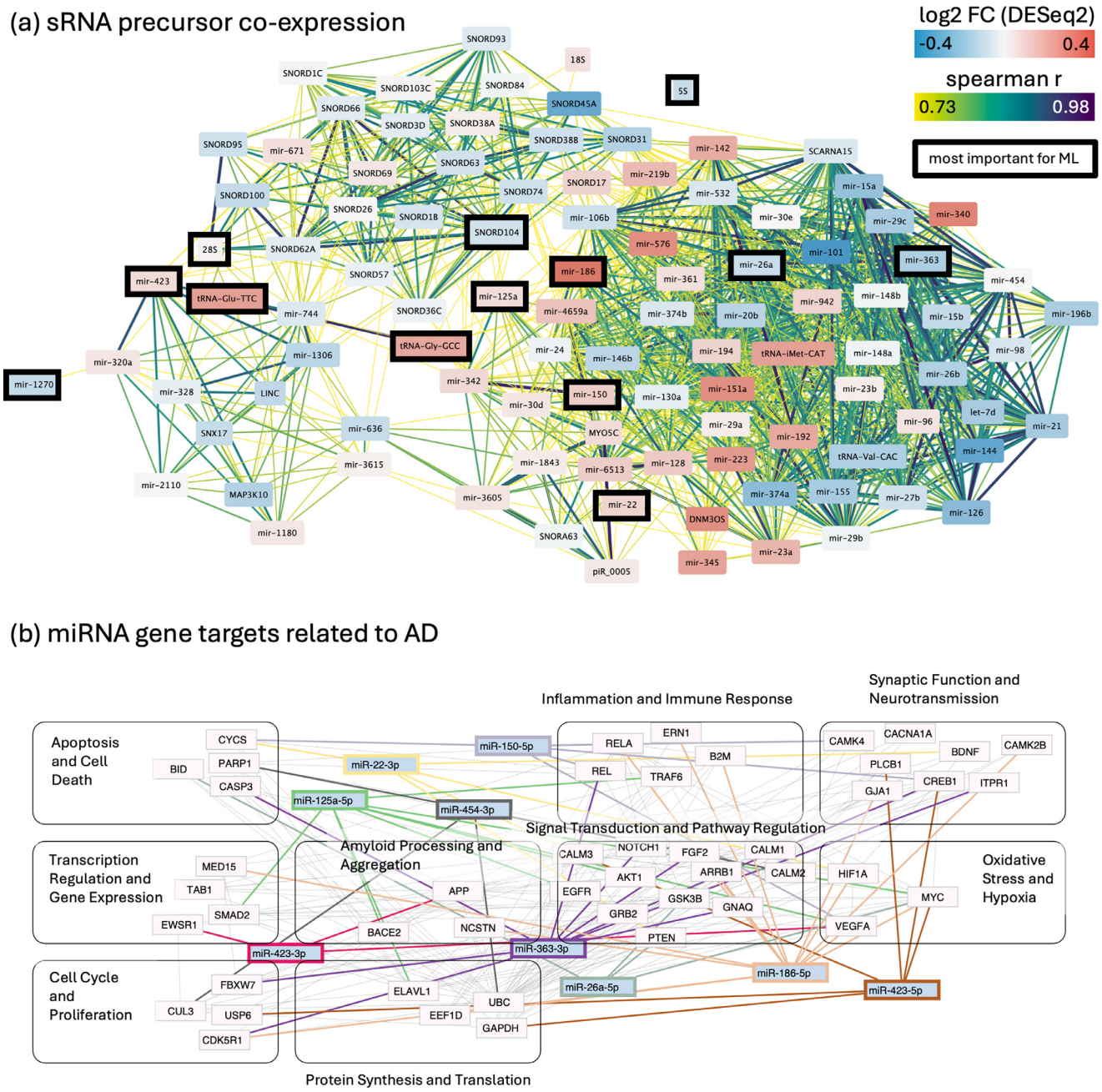

Fig. 3. Functional analysis of important small RNAs. (a) Co-expression network between small RNA classes (precursor level). Nodes are connected if the precursor expression vectors correlated with r>0.7 (r=Spearman rank correlation). Red boxes: higher expression in impaired individuals, blue boxes: higher in normal individuals. Thick box borders indicate most important precursors from ML models. (b) Functional categories of AD-related gene targets of most important miRNAs.

such as amyloid-β variant ratios (e.g., Aβ42/Aβ40) – may improve diagnostic precision and better contextualize small RNA findings.

Future research should integrate miRNA and protein analyses from both blood and CSF to investigate marker migration and identify synergies for diagnostics. Specifically, p-tau$_{217}$ has been found as a potent blood marker for AD and future studies should investigate the potential benefits of adding small RNA markers for early detection of MCI and AD.

A comparison of RNA capture methods, such as PAXgene versus serum sampling tubes, is essential for standardizing findings. A preliminary comparison with a previous PAXgene-based study in a smaller cohort with more advanced cognitive decline, indicates some agreement with the miRNAs found here, but notably failed to detect miR-186 [65]. The targeted depletion of highly abundant RNAs in the present study potentially contributed to the discovery of novel markers, emphasizing the value of advanced RNA profiling techniques.

Exploring CNS-derived RNAs in the bloodstream—via exosomes, immune cells, or BBB disruption—remains a critical area for further investigation. Expanding resources like the miR-Blood atlas [42] to encompass neurodegeneration could accelerate the understanding of small RNA migration mechanisms. Additionally, deconvoluting small RNA contributions from immune cells in the blood could enhance our understanding of systemic immune responses in neurodegeneration.

Lastly, although our machine learning approach was rigorous, the lack of an independent validation cohort limits external generalizability. Future studies incorporating diverse external populations and validation datasets will be essential to confirm the clinical utility and robustness of these biomarkers.

Table 3
Effect of propensity score matching (PSM) on confounder variables in normal and impaired CDR. The upper table contains samples without PSM whereas the lower table has samples with PSM. Chi-square testing was applied to categorical variables and a t-test was performed on the continuous variable age.

confounder	impaired	normal	p-value
APOE status			7.65E-05
e2/e2	1 (0%)	5 (0%)	
e2/e3	41 (8%)	124 (9%)	
e2/e4	12 (2%)	41 (3%)	
e3/e3	249 (47%)	725 (54%)	
e3/e4	187 (35%)	405 (30%)	
e4/e4	41 (8%)	43 (3%)	
Sex			2.13E-05
Female	265 (49%)	813 (59%)	
Male	280 (51%)	555 (41%)	
Amyloid status			1.68E-45
Negative	261 (55%)	1094 (86%)	
Positive	216 (45%)	171 (14%)	
Age			7.64E-25
mean ± std	68.69 ± 7.68	64.66 ± 7.07	

confounder (PSM)	impaired (PSM)	normal (PSM)	p-value
APOE status			0.61
e2/e2	0 (0%)	0 (0%)	
e2/e3	33 (8%)	40 (10%)	
e2/e4	10 (2%)	13 (3%)	
e3/e3	194 (48%)	201 (50%)	
e3/e4	138 (34%)	129 (32%)	
e4/e4	31 (8%)	23 (6%)	
Sex			0.04
Female	205 (50%)	235 (58%)	
Male	201 (50%)	171 (42%)	
Amyloid status			1
Negative	252 (62%)	252 (62%)	
Positive	154 (38%)	154 (38%)	
Age			0.94
mean ± std	67.69 ± 7.45	67.72 ± 6.78	

Additionally, variability in cognitive impairment assessments may introduce diagnostic bias, underscoring the potential advantage of employing modern standardized cognitive evaluation tools in future RNA biomarker studies [66].

4.4. Beyond miRNAs: Other RNA Classes

While miRNAs are the dominant predictive markers in our dataset, other RNA classes merit exploration. tRNA and rRNA fragments, traditionally associated with protein translation, may exert regulatory functions that remain underexplored [67,68]. snoRNAs, exemplified by SNORD104—known to methylate PARP1 in cancer contexts—also suggest potential involvement in AD pathology through epigenetic regulation [69]. Thus, future studies should routinely incorporate analyses of diverse RNA classes to capture a broader spectrum of biologically relevant signals.

4.5. Aging, Comorbidities, and Cross-Disease Insights

The intersection of aging, AD, and other age-related diseases (e.g. cancer, cardiovascular disease) underscores the shared mechanisms and complicates biomarker specificity. Small RNAs targeting key regulators like BACE1 or poly (ADP-ribose) polymerase 1 (PARP1) likely influence multiple aging-related pathologies [70–72]. Broader comparative analyses that span multiple disease states are necessary to delineate shared versus disease-specific pathways. The paradoxical pattern of miR-186, elevated in AD yet declining with age in mouse models, underscores the importance of investigating small RNAs in varied biological contexts to clarify their disease-specific versus aging-associated roles.

Given the heterogenous nature of cognitive decline, broadening biomarker panels beyond amyloid and tau proteins will be crucial, especially for cases involving atypical clinical presentations or complex comorbidities [73].

4.6. Conclusion

Our study demonstrates the potential of small RNA biomarkers for the early detection of cognitive decline, especially among amyloid positive individuals within the EPAD cohort. As RNA profiling methodologies evolve and our understanding of RNA biology advances, these markers could become components of practical, non-invasive diagnostic tools, enabling earlier interventions and improved patient outcomes. Ultimately, miR-186 and related small RNAs may offer valuable insights into the broader molecular landscape of neurodegeneration beyond traditional amyloid and tau-centric frameworks as they regulate vascular and inflammatory pathways, among others, that play a crucial role in MCI and AD.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Tobias Sikosek (TS), Jagoda Mika (JM), Mustafa Kahraman (MK), Julia Jehn (JJ), Alberto Daniel-Moreno (ADM), Jessika Ceiler (JC), Jasmin Skottke (JS), Marta Sanchez-Delgado (MSD), Patrick Neubert (PN), Christina Rudolf (CRu), Kaja Tikk (KT), Rastislav Horos (RH), and Bruno Steinkraus (BS) are current or previous employees of Hummingbird Diagnostics. Marco Heuvelman (MH) and Maurice Frank (MF) are paid consultants for Hummingbird Diagnostics.

Jeffrey L. Cummings (JLC) has provided consultation to Acadia, Acumen, ALZpath, Annovis, Aprinoia, Artery, Axsome, Biogen, Biohaven, BioXcel, Bristol-Myers Squibb, Cervomed, Eisai, Fosun, GAP Foundation, Green Valley, Hummingbird Diagnostics, IGC, Janssen, Kinoxis, Lighthouse, Lilly, Lundbeck, LSP/eqt, Mangrove Therapeutics, Merck, MoCA Cognition, New Amsterdam, Novo Nordisk, NSC Therapeutics, Optoceutics, Otsuka, Oxford Brain Diagnostics, Praxis, Prothena, ReMYND, Roche, Scottish Brain Sciences, Signant Health, Simcere, Sinaptica, T-Neuro, TrueBinding, and Vaxxinity pharmaceutical, assessment, and investment companies. JLC is also a member of the editorial board of the *Journal of Prevention of Alzheimer's Disease*.

Craig Ritchie (CR) is the Founder, CEO, and majority shareholder of Scottish Brain Sciences, which has received compensation for study-related activities from AC Immune SA. CR has received consulting fees from Biogen, Eisai, MSD, Actinogen, Roche, Eli Lilly, and Novo Nordisk, as well as honoraria for lectures from Roche, Eisai, and Eli Lilly. CR is also a member of the advisory board for Novo Nordisk.

If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Tobias Sikosek: Writing – original draft, Visualization, Supervision, Software, Project administration, Methodology, Investigation, Formal analysis. **Marco Heuvelman:** Writing – review & editing, Investigation, Formal analysis. **Jagoda Mika:** Writing – review & editing, Visualization, Formal analysis. **Mustafa Kahraman:** Methodology, Data curation. **Julia Jehn:** Resources, Methodology. **Maurice Frank:** Software, Methodology. **Alberto Daniel-Moreno:** Methodology. **Jessika Ceiler:** Methodology. **Jasmin Skottke:** Methodology. **Marta Sanchez-Delgado:** Methodology. **Patrick Neubert:** Resources, Data curation. **Christina Rudolf:** Resources, Data curation. **Kaja Tikk:** Supervision, Resources, Data curation. **Rastislav Horos:** Supervision, Resources, Methodology. **Jeffrey L. Cummings:** Resources, Writing – review & editing. **Josie Butchart:** Project administration. **Craig Ritchie:** Writing

– review & editing, Resources, Project administration, Conceptualization. **Jean Manson:** Resources, Project administration, Conceptualization, Writing – review & editing. **Bruno R. Steinkraus:** Writing – review & editing, Supervision, Investigation, Funding acquisition, Conceptualization, Project administration, Methodology.

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During the preparation of this work the author(s) used ChatGPT in order to improve readability and assist in literature search. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the published article.

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Supplementary materials

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