Title: Evidence for unique small RNA modifications in Alzheimer's disease

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Abstract

Background: While significant advances have been made in uncovering the aetiology of Alzheimer's disease and related dementias at the genetic level, molecular events at the epigenetic level remain largely un-investigated. Emerging evidence indicates that small non-coding RNAs (sncRNAs) and their associated RNA modifications are important regulators of complex physiological and pathological processes, including aging, stress responses, and epigenetic inheritance. However, whether small RNAs and their modifications are altered in dementia is not known.

Methods: We performed LC-MS/MS–based, high-throughput small RNA modification assays on post-mortem samples of the prefrontal lobe of Alzheimer's disease (AD), vascular dementia+AD (VaD+AD), and control individuals.

Findings: We report altered small RNA modifications in the 15–25-nt fractions enriched for microRNAs, and 30–40-nt RNA fractions enriched for tRNA-derived small RNAs (tsRNAs), rRNA-derived small RNAs (rsRNAs), and YRNA-derived small RNAs (ysRNAs). Interestingly, most of these altered RNA modifications were unique to AD and were not identified in VaD+AD subjects. In addition, sequencing of small RNA in the 30–40-nt fraction from AD cortices revealed reductions in rsRNA-5S, tsRNA-Tyr, and tsRNA-Arg.

Interpretation: These data suggest that sncRNAs and their associated modifications are novel signals that may be linked to the pathogenesis and development of Alzheimer's Disease.

Funding: NIH grants (R01HL122770, R01HL091905, 1P20GM130459, R01HD092431, P50HD098593, GM103440), AHA grant (17IRG33370128), Sigmund Gestetner Foundation Fellowship to P Kehoe.

Keywords: Non-coding RNA, Alzheimer's disease, Prefrontal cortex, Epigenetics

60 **Research in Context**

61 *Evidence before this study*

62 Alzheimer's disease (AD) and vascular dementia (VaD) are marked by cognitive impairment
63 and neuropathologies caused by significant neuronal death. Associated gene mutations are rare
64 in subjects with dementia, and the aetiology of these diseases is still not completely understood.
65 Recent emerging evidence suggests that epigenetic changes are risk factors for the development
66 of dementia. However, studies assessing small RNA modifications—one of the features of
67 epigenetics—in dementia are lacking.

68 *Added value of this study*

69 We used high-throughput liquid chromatography-tandem mass spectrometry and small RNA
70 sequencing to profile small RNA modifications and the composition of small RNAs in post-
71 mortem samples of the prefrontal cortex of AD and control subjects. We detected and quantified
72 16 types of RNA modifications and identified distinct small non-coding RNAs and
73 modification signatures in AD subjects compared with controls.

74 *Implications of all the available evidence*

75 This study identified novel types and compositions of small RNA modifications in the
76 prefrontal cortex of AD patients compared with control subjects in post-mortem samples. The
77 cellular locations of these RNA modifications and whether they are drivers or outcomes of AD
78 are still not known. However, results from the present study might open new possibilities for
79 dissecting the dementia pathology.

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85 **Introduction**

86 Alzheimer's disease (AD) and severe vascular cognitive impairment, also known as vascular
87 dementia (VaD), are two of the most common types of dementia. They are characterized by
88 cognitive impairment and neuropathology caused by neurotoxic forms of amyloid beta peptide,
89 loss of synapses and neuronal function and, ultimately, significant neuronal loss. The aetiology
90 of these forms of dementia is still not fully understood.[1]

91

92 Recent studies have highlighted epigenetic changes associated with aging or acquired through
93 interactions with the environment as important risk factors for dementia, reflecting the fact that
94 causative gene mutations are rare and are only present in a very small percentage of dementia
95 patients. RNA modification is one of the most recently discovered epigenetic-mediated
96 mechanisms for regulating physiological and pathological processes. Small RNA modifications,
97 particularly those involving microRNAs (miRNAs) and transfer RNA-derived small RNAs
98 (tsRNAs), have attracted considerable recent attention for their potential clinical relevance and
99 possible use as diagnostic markers or therapeutic targets of diseases.[2-4] To date, evidence for
100 changes in small RNA modifications in dementia is lacking. Thus, to gain insights into possible
101 new mechanisms and identify potential biomarkers for dementia, we undertook an exploratory
102 study to profile small RNA expression and modification status in dementia.

103

104 In this report, we profiled small RNAs modifications in post-mortem samples of human brain
105 cortical tissue from patients with clinically and pathologically diagnosed AD, with or without
106 VaD, as well as control individuals. To simultaneously detect and quantify 16 types of RNA
107 modifications[5,6] in different small RNA fractions [15–25 nucleotides (nt), 30–40 nt] from AD
108 patients and control individuals, we used high-throughput liquid chromatography-tandem mass
109 spectrometry (LC-MS/MS). We also profiled the composition of small RNAs in AD and control

110 subjects using small RNA sequencing. These exploratory examinations revealed distinct
111 sncRNA expression and RNA modification signatures between AD and controls, suggesting
112 that these small RNAs are novel factors associated with the pathogenesis and/or progression of
113 AD.
114
115 **Materials and methods**
116 **Human subjects for RNA modification assays**
117 A total of 34 post-mortem brain prefrontal lobe cortex samples from AD patients (n=18),
118 VaD+AD patients (n=9), and control human subjects (n=7) were collected by post-mortem
119 autopsy, snap-frozen, and stored in liquid nitrogen. The time between biospecimen acquisition
120 and analysis ranged from 5 to 30 years, according to the Brain Bank database. Biospecimens
121 were shipped on dry ice. Information from clinical and pathology reports was obtained from
122 the brain bank database, developed as described.[7] On the basis of their clinical diagnosis,
123 subjects were categorized into three groups: no significant abnormalities, AD, and VaD. All
124 clinically diagnosed VaD subjects used in this study exhibited clear AD tau pathology and Aβ
125 plaques with a neuropathological report of AD. Thus, this group was termed VaD+AD for
126 purposes of distinguishing it from the AD group. Subjects were selected according to their
127 confirmed clinical and pathological diagnosis as AD or normal, and were obtained from the
128 Human Tissue Authority-licensed South West Dementia Brain Bank
129 http://www.bristol.ac.uk/translational-health-
130 sciences/research/neurosciences/research/dementia/swdbb/, accessed on March 24th, 2020),
131 University of Bristol, with tissue bank ethics approval from the South West–Central Bristol
132 Research Ethics Committee. Clinical and pathological data included patient history, diagnosis
133 and medications; pathological reports were available for retrospective analysis. Patients'
134 personal information has been de-identified. Data for this study was collected between 1989

135 and 2015. The Research Integrity Offices at the University of Nevada, Reno, and University of
136 Bristol have determined that this project complies with human research protection oversight by
137 the Institutional Review Board.
138
139 **Isolation of total RNA from the human prefrontal cortex**
140 Total RNA was isolated from 100 mg of human prefrontal tissues using the TRIzol reagent
141 (Thermo Fisher Scientific, Inc. Waltham, MA, USA), as described by the manufacturer. RNA
142 quantity was evaluated using a NanoDrop 2000 microvolume spectrophotometer (Thermo
143 Fisher). RNA samples were stored at -80°C until analysis. Researchers performing small RNA
144 sequencing and RNA modifications assays on RNA samples were blinded to diagnosis and
145 group-identifying information.
146
147 **Small RNA Sequencing**
148 Ten RNA samples from prefrontal lobe cortexes of control subjects and AD patients were
149 submitted to BGI (Cambridge, MA, USA) for sequencing of 15–40-nt small RNAs. The number
150 of clean reads varied from 30M to 40M across all samples. The Q20 score was greater than
151 99·5% for all samples. Raw sequencing data for each sample were processed and analysed using
152 our newly developed computational framework, *SPORTS1·0*[8]
153 (https://github.com/junchaoshi/sports1.0), which is designed to optimize the annotation and
154 quantification of non-canonical small RNAs (e.g., tsRNAs) in small RNA sequencing data.
155 Sequencing adapters were removed, after which reads with lengths outside of the defined range
156 and those with nucleotides other than ATUCG were discarded. Clean reads were mapped
157 against the precompiled human small RNA annotation database
158 (https://github.com/junchaoshi/sports1.0/blob/master/precompiled_annotation_database.md).
159 Small RNAs 15–25 and 30–40 nt in length were summarized separately.

160 **Small RNA purification and analysis of RNA modifications using LC-MS/MS**

161 Standardized ribonucleoside preparations, small RNA purification, and LC-MS/MS-based
162 analysis of RNA modifications in RNA samples were performed as previously described.[6]
163 Purified small RNAs (100–200 ng) from human brain samples were digested as input and
164 loaded onto a ThermoFisher Vantage Quadrupole mass spectrometer connected to a Thermo
165 Ultimate 3000 UHPLC system equipped with an electrospray ionization source. The MS system
166 was operated in positive ion mode using a multiple reaction monitoring (MRM) approach. LC-
167 MS/MS raw data were acquired using Xcalibur Workstation software and were processed using
168 Xcalibur QuanBrowser for quantification of modified ribonucleoside concentrations. The
169 percentage of each modified ribonucleoside was normalized to the total amount of quantified
170 ribonucleosides containing the same nucleobase, an approach that decreases/eliminates errors
171 caused by sample loading variation. For example, the percentage of m^5C = mole concentration
172 (m^5C)/mole concentration (m^5C + Cm + C + ac^4C). Fold-changes in RNA modifications
173 between different groups were calculated based on the percentage of modified ribonuclosides.
174 Brain RNAs 15–25 and 30–40 nucleotides (nt) in length were examined.

175

176 **Statistical analysis**

177 Data are presented as means ± SEM and were plotted using Prism8 software (GraphPad, La
178 Jolla, CA, USA). Differential expression analyses were performed using the *edgeR* tool (pmid:
179 19910308), controlling for age and sex. RNAs with a false discovery rate < 10% were deemed
180 to be differentially expressed. RNA modification levels between groups were compared using
181 a linear model that controls for age and sex. Unpaired *t* tests were used for comparisons of
182 participants' characteristics between groups. A *P*-value < 0·05 was considered statistically
183 significant.

184

185 **Results**

186 **Participant characteristics**

187 As summarized in Table 1, patients (AD and VaD+AD) and control individuals were similar in
188 terms of age. Almost all patients were White/European except for one patient whose race was
189 unknown. There were slightly more males (58·8%) than females (41·2%). Brain weight was
190 significantly lower in AD (P = 0·0099) and VaD+AD (P = 0·046) groups compared with the
191 control group. The post-mortem autopsy delay time was similar among groups.

192

193 **Altered small RNA modification profiles in the cerebral cortex of AD patients**

194 To systemically analyse small RNA expression and modification profiles, we collected the 15–
195 40-nt RNA fraction from the brain frontal lobe cortex for small RNA sequencing as we
196 previously described[5,6], and then performed bioinformatic analyses using our recently
197 developed software, *SPORTS1.0.*[8] In addition, 15–25-nt and 30–40-nt RNA fractions were
198 collected for high-throughput examination of RNA modification by LC-MS/MS (Figure 1).[5,9]
199 These analyses resulted in the identification and quantification of 16 types of RNA
200 modifications.

201

202 In the 15–25-nt RNA fraction from the cortex of AD brains (Figure 2A), we found an increase
203 in 2'-O-methylcytidine (Cm) and 7-methylguanosine (m^7G), and significant reductions in
204 N^2,N^2,7-trimethylguanosine ($m^{2,2,7}G$) and N^2,N^2-dimethylguanosine ($m^{2,2}G$) compared with
205 controls (Figure 2B–E). Interestingly, these changes were not present in the cortex of VaD+AD
206 patients' brains, and thus were unique to the brains' of AD-only subjects. Other RNA
207 modifications that were not significantly different among groups were also detected, as shown
208 in Supplemental Figure S1.

209

210 In the 30–40-nt small RNA fraction from the cortex of AD brains (Figure 3A), we found
211 significantly higher levels of Cm, 2'-O-methyluridine (Um) and 7-methylguanosine (m^7G)
212 modifications, and reductions in 1-methylguanosine (m^1G), $m^{2,2,7}G$, and pseudouridine (psi or
213 Ψ) modifications compared with controls (Figure 3B–G). Among 30–40-nt small RNAs, we
214 found only one specific RNA modification, psi, that was changed in the VaD+AD brain cortex,
215 where this modification was reduced compared with controls (Figure 3G). Again, other RNA
216 modifications were detected in this 30–40-nt fraction that did not exhibit significant differences
217 among groups, as shown in Supplemental Figure S2.
218
219 **Altered 30–40-nt small RNA expression profiles in the cerebral cortex of AD patients**
220 To systemically analyse small RNA expression profiles, we collected the 15–40-nt fraction
221 from the brain frontal lobe cortex of AD patients and controls for small RNA sequencing, as
222 we previously described[5,6], and then performed bioinformatic analyses using our recently
223 developed software, *SPORTS1·0*[8]. Our RNA-seq analysis showed that the 15–25-nt RNA
224 population was predominantly miRNAs (Figure 4A) in AD and control samples, whereas the
225 expression profile of the small RNA population in the 30–40-nt fraction showed more dynamic
226 changes. Our RNA-seq data revealed three major subtypes in the 30–40-nt fraction, namely
227 tRNA-derived small RNAs (tsRNA), rRNA-derived small RNAs (rsRNAs) and Y RNA-
228 derived small RNAs (ysRNAs); other un-annotated RNAs were also found (Figure 4B).
229 Although there were trends toward an increase in tsRNAs and a reduction in ysRNAs in AD
230 patients, these differences did not reach statistical significance. A further analysis of specific
231 small RNA fragments in these samples revealed that the majority of the 15–25-nt fraction was
232 miRNAs (Figure 4A). We did not find significant differences in the specific small RNA
233 sequences in this fraction between AD and control samples (data not shown). Interestingly, in

234 the 30–40-nt fraction, we found a significant reduction in rRNA-5S, tsRNA-Tyr and tsRNA-
235 Arg fragments in AD patients compared with controls (Figure 4C–E).
236
237 **Discussion**
238 Emerging evidence shows that small RNAs, including miRNAs, tsRNAs and piwi-interacting
239 RNAs, harbour a diversity of RNA modifications.[10] The physiological and pathological
240 importance of these small, non-coding RNA modifications has only recently begun to emerge,
241 as highlighted by studies revealing their active involvement in stress responses, metabolism,
242 immunity, and epigenetic inheritance of environmentally acquired traits.[2] RNA modifications
243 have recently been detected in the nervous system, where they are involved in the regulation of
244 cortical differentiation, behaviour, and brain functions.[11,12] However, to our knowledge, there
245 have been no reports on the status of small RNA modifications in AD or VaD to date.
246
247 The current study showed intriguing differences in small RNA-modification signatures between
248 AD patients and control subjects, suggesting an association and possible contribution of such
249 modifications to the pathogenesis and/or progression of AD. Notably, most of the altered RNA
250 modifications were not observed in VaD+AD patients, with the exception of psi, as noted above,
251 providing molecular evidence for potential distinct signatures between these two patient groups.
252 In the last three decades, dementia research has demonstrated significant overlaps between AD
253 and VaD in terms of clinical symptoms, risk factors, and post-mortem brain autopsy findings.[13-
254 15] Therapeutically, drugs that enhance cholinergic activity are as effective in patients suffering
255 from VaD as they are in AD patients.[16] More recently, drugs that have traditionally been used
256 for cardiovascular diseases, especially renin-angiotensin system blockers, have shown benefits
257 not only in VaD patients, but also in AD patients.[17-19] However, the pathogenesis of AD, VaD,
258 and VaD+AD remains incompletely understood. We were surprised to find only one common

259 signature among RNA modifications in AD and VaD+AD patients that might contribute to
260 shared mechanisms of dementia pathology. Alternatively, these distinct molecular signatures
261 could suggest the existence of as-yet undiscovered differences in pathological processes
262 between these conditions, a possibility that might be harnessed for future condition-specific
263 treatments.
264
265 The 15–25-nt RNAs were predominantly miRNAs, whereas 30–40-nt RNAs were
266 predominantly tsRNAs, rsRNAs and ysRNAs, as demonstrated by small RNA sequencing. We
267 found changes in RNA-modification profiles in the AD brain cortex in both fractions of small
268 RNAs. While the importance of miRNA in the pathogenesis of AD is relatively well described[20],
269 the role of rsRNAs and ysRNAs in a pathophysiological context has only begun to emerge.[6,21,22]
270 The dynamic expression of these non-canonical small RNAs suggests the possibility of
271 unidentified biological functions that warrant further investigation[23,24], particularly in relation
272 to several risk factor genes that have been identified for AD in the last few decades.[25,26] This
273 situation is similar to that for tsRNAs, which have shown recently expanding functions.[4,27,28]
274 The novel RNA modifications that showed dynamic changes in AD patients included Cm, Um,
275 m^7G, m^1G, $m^{2,2,7}G$, and psi. The functions and identities of these small RNA modifications in
276 AD are not yet understood. We identified increases in 2'-O-methylation of cytidine (Cm),
277 guanosine (Gm) and uridine (Um), as well as m^7G, while observing a reduction in $m^{2,2,7}G$ in
278 the brain cortex of dementia patients, suggesting the potential importance of these RNA
279 modifications, either as compensatory responses (Cm, Gm, Um, m^7G)[29-31] or as actual causes
280 of pathogenesis ($m^{2,2,7}G$).[32,33]
281
282 tRNA-derived small RNAs are reported to contribute to multiple pathological processes,
283 including cancer, viral infection, and age-related neurodegenerative diseases.[2] In the

284 senescence-accelerated mouse prone 8 (SAMP8) model, which mimics age-related neuro-
285 disorders such as AD and Parkinson disease, tsRNAs, including tsRNA-Tyr and tsRNA-Arg,
286 in brain tissue are dysregulated compared with normal brain tissue. The targets of these
287 dysregulated tsRNAs are enriched in neurodevelopment pathways such as synapse formation.[34]
288 In addition, knockout of the RNA kinase, Clp1, in mice leads to progressive loss of motor
289 neuron function in association with the accumulation of Try-tRNA and Arg-tRNA small RNAs.
290 These tsRNAs sensitize motor neurons to oxidative stress-induced cell death, suggesting that
291 tsRNAs are involved in normal motor neuron functions and responses to oxidative stress.[35] A
292 similar phenomenon was reported in a human neurological disease cohort, and it was shown
293 that transfection of small RNAs from 5' Tyr-tRNA can protect CLP-mutant cells from oxidative
294 stress-induced cell death.[36] In our study, the decrease in 5'tsRNA-Tyr in AD subjects may
295 confer greater vulnerability to oxidative stress on neurons. Interestingly another report showed
296 that the expression of 5s rRNA and the oxidized level of 5S rRNA are dynamically modulated
297 in AD's subjects[37], findings that may be related to the biogenesis of rsRNAs and might explain
298 alterations of rsRNA-5s in the AD group in the current study.
299
300 Whether the changes in small RNAs and RNA modifications observed here represent drivers
301 or outcomes of AD remains unknown. The precise meaning of each of these changes in terms
302 of their specific functions awaits discovery. Future efforts to pinpoint the locations of these
303 modifications in each sncRNA population and identify enzymes involved in their regulation
304 would be invaluable[38,39] and will open new avenues for dissecting the nature of dementia
305 pathology.
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307
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Funding

This work was supported, in part, by grants from the National Institutes of Health (NIH/NHLBI) (R01HL122770, R01HL091905), NIH/NIGMS (1P20GM130459) and the American Heart Association National Center (17IRG33370128) to Y. Feng Earley, and from the NIH/NICHD to Q. Chen (R01HD092431) and T. Zhou (P50HD098593). Research reported in this publication used instruments from the Dr. Mick Hitchcock Proteomics Core supported by a grant from the NIH/NIGMS (GM103440) of the National Institutes of Health. University of Bristol Alumni. P Kehoe is supported by a Fellowship from the Sigmund Gestetner Foundation. The funders had no role in study design, data collection, analysis, and interpretation, as well as writing of the manuscript.

Declaration of competing interests

The authors declare no competing interests.

Data Availability

All data associated with this study are contained in the paper or Supplementary Materials. RNA sequencing data will be deposited in an online repository (Mendeley Data) upon acceptance of the manuscript.

Acknowledgements

We would like to thank Dr. David Quilici and Rebekah Woolsey from the Mick Hitchcock, Ph.D. Nevada Proteomics Center for technical support with the HPLC/MS instruments. We would especially like to thank Dr. Laura Palmer for tissue processing and facilitating access to clinical data from the South West Dementia Brain Bank at the University of Bristol, which is supported by grants from BRACE, ABBUK.

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335 **Supplemental Materials**

336 Supplementary material associated with this article can be found in the online version at doi:

337 10.6084/m9.figshare.12227393

338

339 **References:**

340 1. Panza F, Lozupone M, Watling M, Imbimbo BP. Do BACE inhibitor failures in
341 Alzheimer patients challenge the amyloid hypothesis of the disease? *Expert review of*
342 *neurotherapeutics* 2019; **19**(7): 599-602.
343 2. Zhang X, Cozen AE, Liu Y, Chen Q, Lowe TM. Small RNA Modifications: Integral to
344 Function and Disease. *Trends Mol Med* 2016; **22**(12): 1025-34.
345 3. Chen Q, Yan W, Duan E. Epigenetic inheritance of acquired traits through sperm RNAs
346 and sperm RNA modifications. *Nature reviews Genetics* 2016; **17**(12): 733-43.
347 4. Schimmel P. The emerging complexity of the tRNA world: mammalian tRNAs beyond
348 protein synthesis. *Nature reviews Molecular cell biology* 2018; **19**(1): 45-58.
349 5. Chen Q, Yan M, Cao Z, et al. Sperm tsRNAs contribute to intergenerational inheritance
350 of an acquired metabolic disorder. *Science* 2016; **351**(6271): 397-400.
351 6. Zhang Y, Zhang X, Shi J, et al. Dnmt2 mediates intergenerational transmission of
352 paternally acquired metabolic disorders through sperm small non-coding RNAs. *Nature cell*
353 *biology* 2018; **20**(5): 535-40.
354 7. Hyman BT, Phelps CH, Beach TG, et al. National Institute on Aging-Alzheimer's
355 Association guidelines for the neuropathologic assessment of Alzheimer's disease. *Alzheimer's*
356 *& dementia : the journal of the Alzheimer's Association* 2012; **8**(1): 1-13.
357 8. Shi J, Ko EA, Sanders KM, Chen Q, Zhou T. SPORTS1.0: A Tool for Annotating and
358 Profiling Non-coding RNAs Optimized for rRNA- and tRNA-derived Small RNAs. *Genomics*
359 *Proteomics Bioinformatics* 2018; **16**(2): 144-51.
360 9. Fang H, Deng M, Zhang L, et al. Role of (Pro)Renin Receptor in Albumin Overload-
361 Induced Nephropathy in Rats. *Am J Physiol Renal Physiol* 2018.
362 10. Roundtree IA, Evans ME, Pan T, He C. Dynamic RNA Modifications in Gene
363 Expression Regulation. *Cell* 2017; **169**(7): 1187-200.
364 11. Satterlee JS, Basanta-Sanchez M, Blanco S, et al. Novel RNA modifications in the
365 nervous system: form and function. *The Journal of neuroscience : the official journal of the*
366 *Society for Neuroscience* 2014; **34**(46): 15170-7.
367 12. Jung Y, Goldman D. Role of RNA modifications in brain and behavior. *Genes, brain,*
368 *and behavior* 2018; **17**(3): e12444.
369 13. Ravona-Springer R, Davidson M, Noy S. Is the distinction between Alzheimer's disease
370 and vascular dementia possible and relevant? *Dialogues in clinical neuroscience* 2003; **5**(1): 7-
371 15.
372 14. Mirra SS, Heyman A, McKeel D, et al. The Consortium to Establish a Registry for
373 Alzheimer's Disease (CERAD). *Neurology* 1991; **41**(4): 479.
374 15. Meyer JS, Xu G, Thornby J, Chowdhury MH, Quach M. Is mild cognitive impairment
375 prodromal for vascular dementia like Alzheimer's disease? *Stroke* 2002; **33**(8): 1981-5.
376 16. Erkinjuntti T, Kurz A, Gauthier S, Bullock R, Lilienfeld S, Damaraju CV. Efficacy of
377 galantamine in probable vascular dementia and Alzheimer's disease combined with
378 cerebrovascular disease: a randomised trial. *Lancet* 2002; **359**(9314): 1283-90.
379 17. Zhuang S, Wang H-F, Wang X, Li J, Xing C-M. The association of renin-angiotensin
380 system blockade use with the risks of cognitive impairment of aging and Alzheimer's disease:
381 A meta-analysis. *Journal of Clinical Neuroscience* 2016; **33**: 32-8.
382 18. Chou C-L, Yeh H-I. The Role of the Renin-Angiotensin System in Amyloid Metabolism
383 of Alzheimer's Disease. *Acta Cardiologica Sinica* 2014; **30**(2): 114-8.
384 19. Gebre AK, Altaye BM, Atey TM, Tuem KB, Berhe DF. Targeting Renin-Angiotensin
385 System Against Alzheimer's Disease. *Frontiers in pharmacology* 2018; **9**: 440.
386 20. Ayers D, Scerri C. Non-coding RNA influences in dementia. *Noncoding RNA Res* 2018;
387 **3**(4): 188-94.

21. Hizir Z, Bottini S, Grandjean V, Trabucchi M, Repetto E. RNY (YRNA)-derived small RNAs regulate cell death and inflammation in monocytes/macrophages. *Cell Death Dis* 2017; **8**(1): e2530.
22. Zhang Y, Shi J, Rassoulzadegan M, Tuorto F, Chen Q. Sperm RNA code programmes the metabolic health of offspring. *Nature reviews Endocrinology* 2019; **15**(8): 489-98.
23. Kowalski MP, Krude T. Functional roles of non-coding Y RNAs. *The international journal of biochemistry & cell biology* 2015; **66**: 20-9.
24. Kabeerdoss J, Sandhya P, Danda D. Y RNA derived small RNAs in Sjogren's syndrome: Candidate biomarkers? *International journal of rheumatic diseases* 2017; **20**(11): 1763-6.
25. Lambert JC, Ibrahim-Verbaas CA, Harold D, et al. Meta-analysis of 74,046 individuals identifies 11 new susceptibility loci for Alzheimer's disease. *Nature genetics* 2013; **45**(12): 1452-8.
26. Kunkle BW, Grenier-Boley B, Sims R, et al. Genetic meta-analysis of diagnosed Alzheimer's disease identifies new risk loci and implicates Abeta, tau, immunity and lipid processing. *Nature genetics* 2019; **51**(3): 414-30.
27. Oberbauer V, Schaefer MR. tRNA-Derived Small RNAs: Biogenesis, Modification, Function and Potential Impact on Human Disease Development. *Genes (Basel)* 2018; **9**(12).
28. Shi J, Zhang Y, Zhou T, Chen Q. tsRNAs: The Swiss Army Knife for Translational Regulation. *Trends Biochem Sci* 2019; **44**(3): 185-9.
29. Ji L, Chen X. Regulation of small RNA stability: methylation and beyond. *Cell research* 2012; **22**(4): 624-36.
30. Ren G, Xie M, Zhang S, Vinovskis C, Chen X, Yu B. Methylation protects microRNAs from an AGO1-associated activity that uridylates 5' RNA fragments generated by AGO1 cleavage. *Proc Natl Acad Sci U S A* 2014; **111**(17): 6365-70.
31. Lee M, Kim B, Kim VN. Emerging roles of RNA modification: m(6)A and U-tail. *Cell* 2014; **158**(5): 980-7.
32. Will CL, Luhrmann R. Spliceosomal UsnRNP biogenesis, structure and function. *Current opinion in cell biology* 2001; **13**(3): 290-301.
33. Hausmann S, Zheng S, Costanzo M, et al. Genetic and biochemical analysis of yeast and human cap trimethylguanosine synthase: functional overlap of 2,2,7-trimethylguanosine caps, small nuclear ribonucleoprotein components, pre-mRNA splicing factors, and RNA decay pathways. *J Biol Chem* 2008; **283**(46): 31706-18.
34. Zhang S, Li H, Zheng L, Li H, Feng C, Zhang W. Identification of functional tRNA-derived fragments in senescence-accelerated mouse prone 8 brain. *Aging (Albany NY)* 2019; **11**(22): 10485-98.
35. Hanada T, Weitzer S, Mair B, et al. CLP1 links tRNA metabolism to progressive motor-neuron loss. *Nature* 2013; **495**(7442): 474-80.
36. Schaffer AE, Eggens VR, Caglayan AO, et al. CLP1 founder mutation links tRNA splicing and maturation to cerebellar development and neurodegeneration. *Cell* 2014; **157**(3): 651-63.
37. Ding Q, Zhu H, Zhang B, Soriano A, Burns R, Markesbery WR. Increased 5S rRNA oxidation in Alzheimer's disease. *J Alzheimers Dis* 2012; **29**(1): 201-9.
38. Lyons SM, Fay MM, Ivanov P. The role of RNA modifications in the regulation of tRNA cleavage. *FEBS Lett* 2018; **592**(17): 2828-44.
39. Frye M, Harada BT, Behm M, He C. RNA modifications modulate gene expression during development. *Science* 2018; **361**(6409): 1346-9.

436 **Table 1.** Characteristics of study subjects

	No abnormalities (CONT)	Alzheimer disease (AD)	Alzheimer disease with Vascular dementia (VaD+AD)
Demographics			
Total patients (n)	7	18	9
Sex, male/female	5/2	9/9	6/3
Ethnicity			
White /unknown	6/1	18/0	9/0
Age (years)	85·4 ± 2·6	77·3 ± 2·6	79·6 ± 2·5
Brain			
Weight (g)	1286 ± 34·3	1130 ± 33 **	1180 ± 33·4*
PH	6·03 ± 0·08	6·15 ± 0·09	5·96 ± 0·08
Post-Mortem Delay Time	41·2 ± 4·9	36·9 ± 5·4	48·5 ± 7·6

437
438 Values are means ± SE, where appropriate. *$p < 0·05$, vs. control patients; unpaired *t*-test.
439

440

Figure legends:

Figure 1. Schematic illustration of LC-MS/MS for detection of RNA modifications. Total RNAs were extracted from brain tissue using the Trizol reagent and then urea-denatured-PAGE was performed to separate different RNA fragments. Targeted RNA fragments were cut according to their size, recovered from the gel, enzymatically treated to obtain single nucleosides, and then subjected to LC/MS-MS profiling. Raw mass spectrometry data were analysed using Xcalibur software, and the relative levels of different RNAs modifications were calculated.

Figure 2. Small RNA (15–25 nt) modifications in the prefrontal lobe cortex of dementia patients. (a) Representative image showing purification of 15–25-nt small RNAs for the analysis of RNA modifications. (b–e) Increases in 2'-O-methylcytidine (Cm) and 7-methylguanosine (m^7G) modifications, and reductions in N^2,N^2,7-trimethylguanosine ($m^{2,2,7}G$) and N^2,N^2-dimethylguanosine ($m^{2,2}G$) modifications in the cortex of AD and VaD+AD patients compared with CONT subjects (n = 7 for CONT, n = 18 for AD, n = 9 for VaD+AD; *$P < 0{\cdot}05$ vs. CONT).

Figure 3. Small RNA (30–40 nt) modifications in the prefrontal lobe cortex of dementia patients. (a) Representative image showing purification of 30–40-nt small RNAs for the analysis of RNA modifications. (b–g) Increases in 2'-O-methylcytidine (Cm), 2'-O-methyluridine (Um) and 7-methylguanosine (m^7G) modifications, and reductions in 1-methylguanosine (m^1G), N^2,N^2-dimethylguanosine ($m^{2,2}G$) and pseudouridine (psi or Ψ) modifications in the cortex of AD and VaD+AD patients compared with CONT subjects (n = 7 for CONT, n = 27 for AD; * $P < 0{\cdot}05$ vs. CONT).

Figure 4. Reductions in 5S rRNA, tRNA-Tyr, and tRNA-Arg in prefrontal lobe cortex samples of AD patients, identified by small RNA sequencing. (a) Composition of small RNAs (15–25 nt). (b) Composition of small RNAs (34–40 nt). (c) 5s RNA, (d) tRNA-Tyr, (e) tRNA-Arg in the cortex of AD patients compared with CONT subjects (n = 4 for CONT, n = 6 for AD; *$P < 0{\cdot}05$ vs. CONT).

Figure 1.

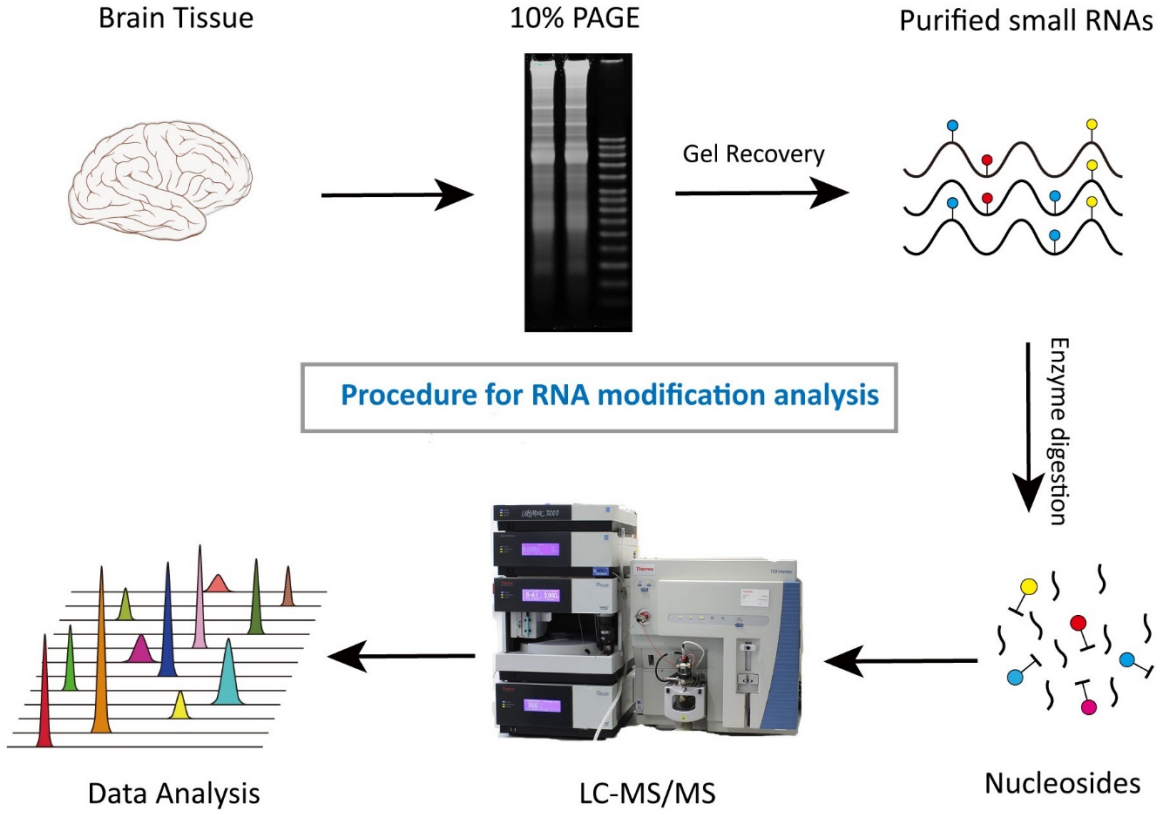
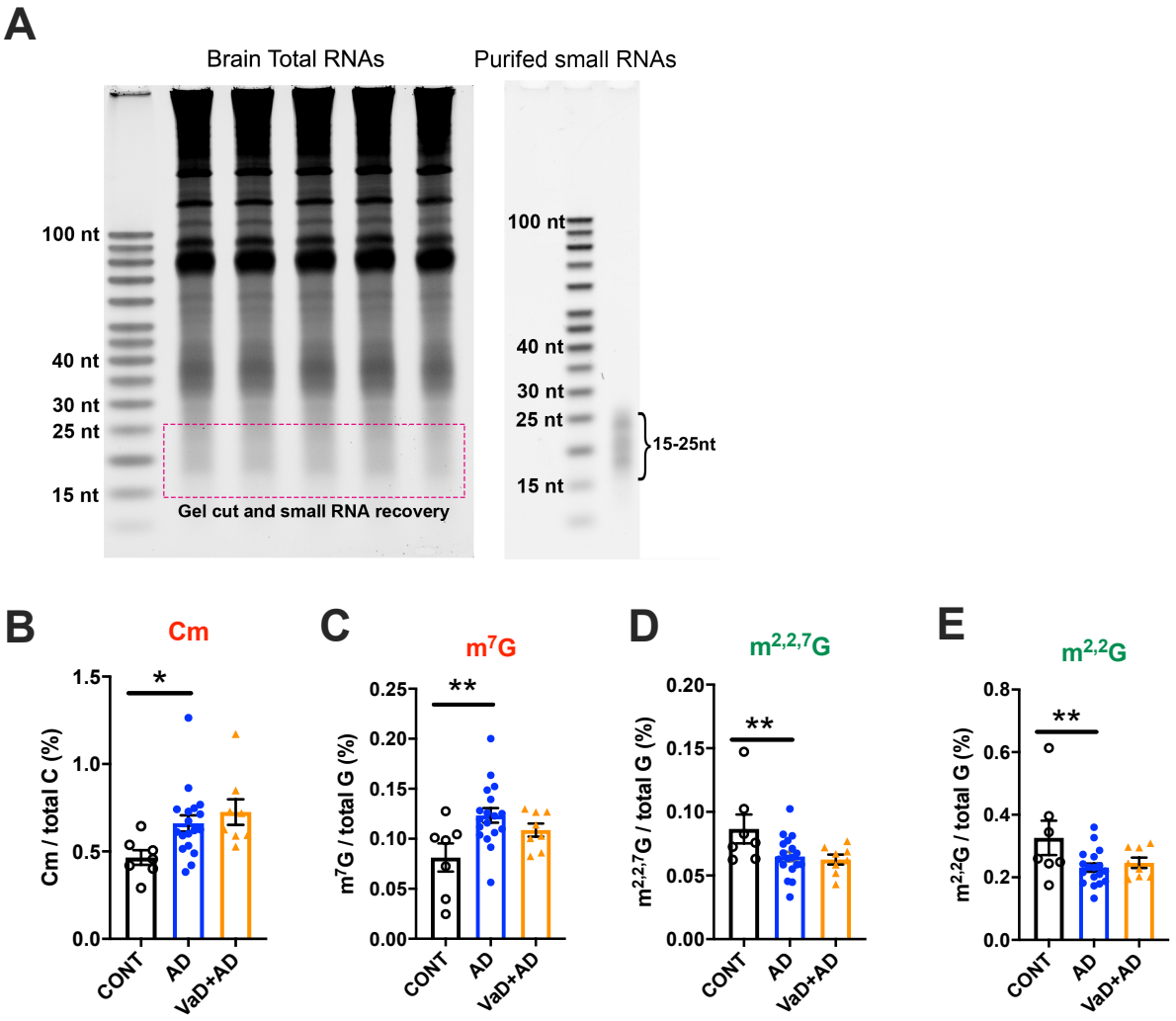
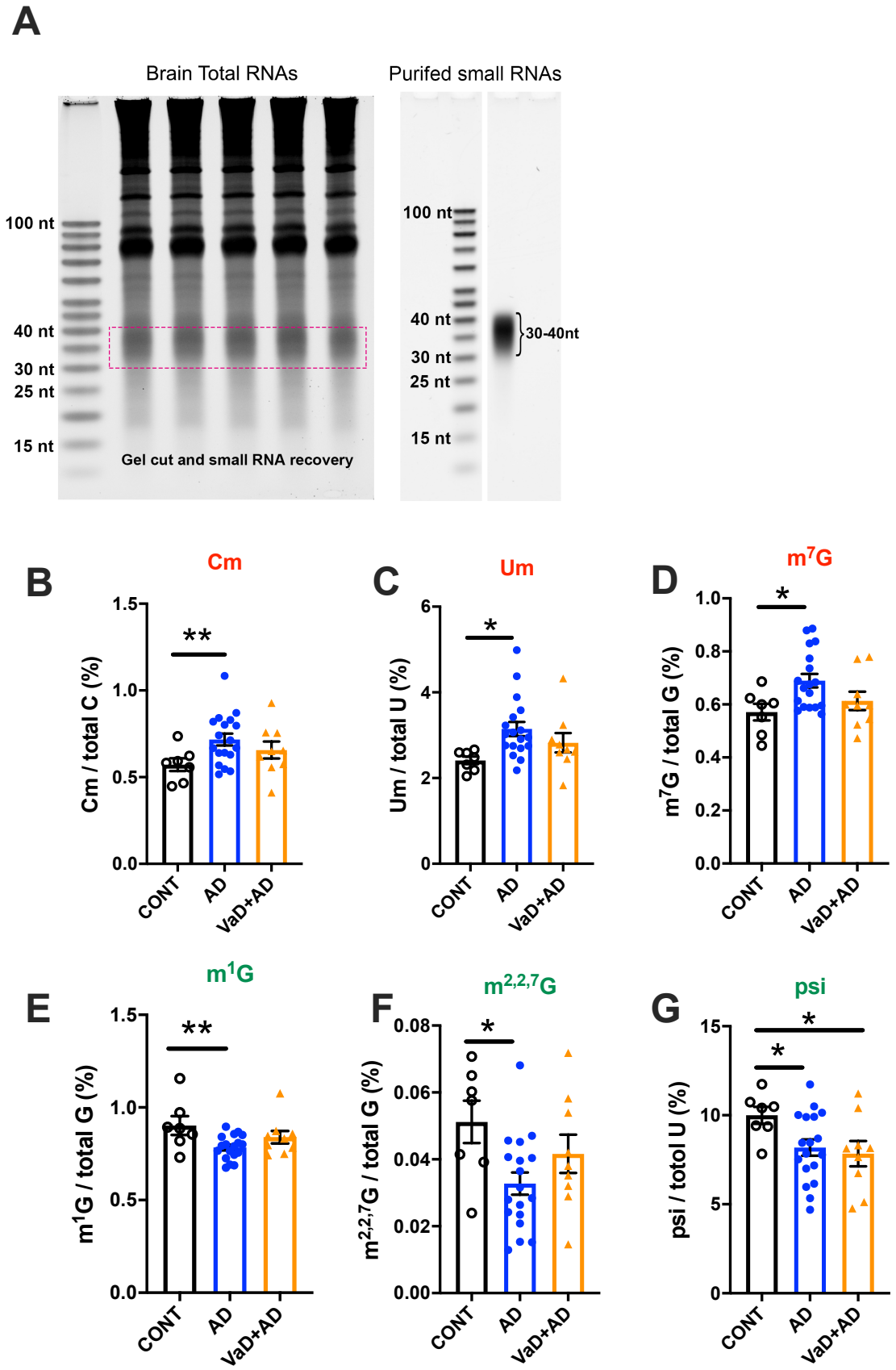

Brain Tissue
10% PAGE
Purified small RNAs
Gel Recovery
Procedure for RNA modification analysis
Enzyme digestion
Data Analysis
LC-MS/MS
Nucleosides

Figure 2.


A
Brain Total RNAs
Purifed small RNAs
100 nt
40 nt
30 nt
25 nt
15 nt
Gel cut and small RNA recovery
100 nt
40 nt
30 nt
25 nt
15 nt
15-25nt
B
Cm
Cm / total C (%)
1.5
1.0
0.5
0.0
*
CONT
AD
VaD+AD
C
m7G
m7G / total G (%)
0.25
0.20
0.15
0.10
0.05
0.00
**
CONT
AD
VaD+AD
D
m2,2,7G
m2,2,7G / total G (%)
0.20
0.15
0.10
0.05
0.00
**
CONT
AD
VaD+AD
E
m2,2G
m2,2G / total G (%)
0.8
0.6
0.4
0.2
0.0
**
CONT
AD
VaD+AD

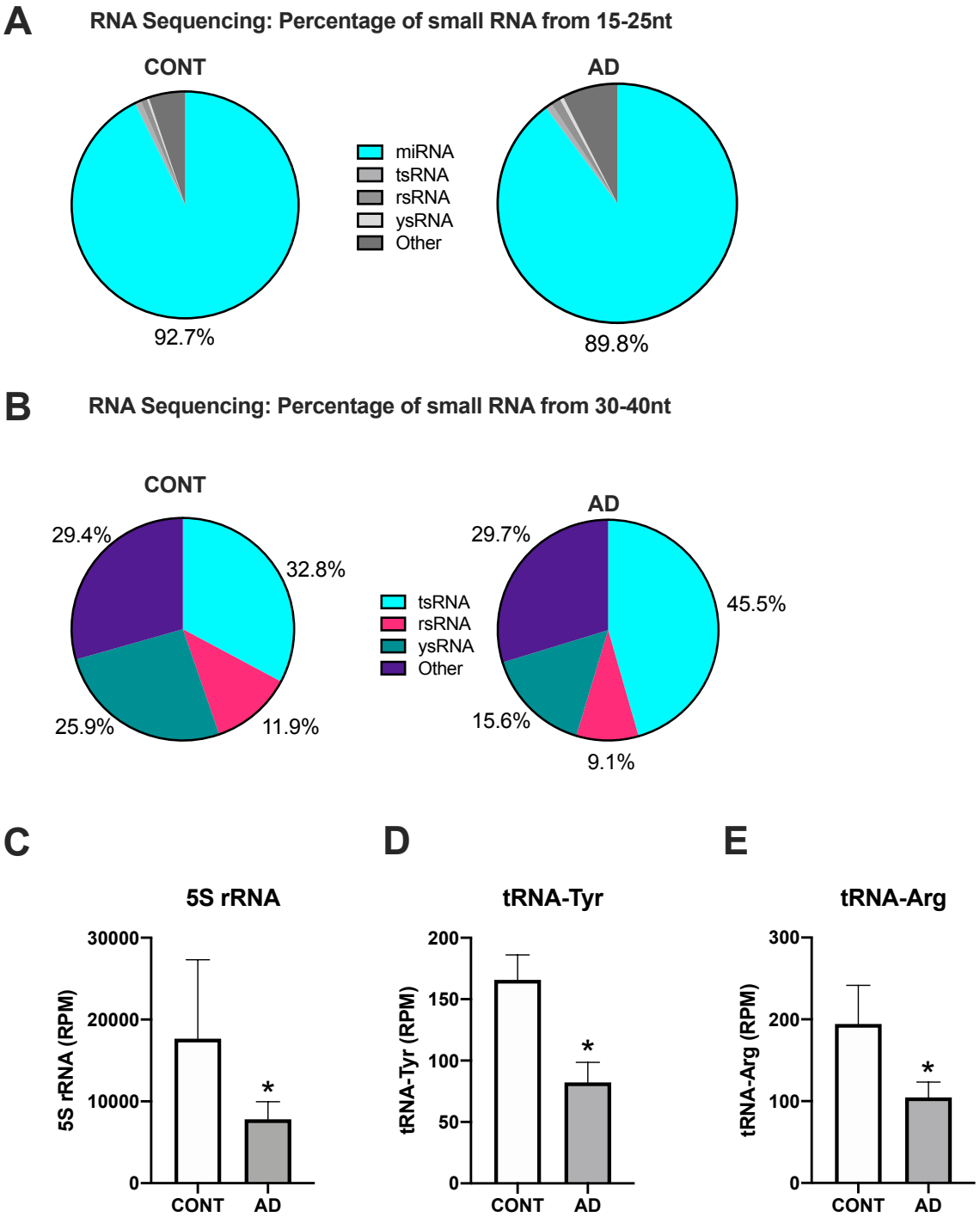
485

Figure 3.


A
Brain Total RNAs
Purifed small RNAs
100 nt
40 nt
30 nt
25 nt
15 nt
Gel cut and small RNA recovery
30-40nt
B
Cm
Cm / total C (%)
C
Um
Um / total U (%)
D
m7G
m7G / total G (%)
E
m1G
m1G / total G (%)
F
m2,2,7G
m2,2,7G / total G (%)
G
psi
psi / totol U (%)
CONT
AD
VaD+AD

486

Figure 4.


A
RNA Sequencing: Percentage of small RNA from 15-25nt
CONT
AD
miRNA
tsRNA
rsRNA
ysRNA
Other
92.7%
89.8%
B
RNA Sequencing: Percentage of small RNA from 30-40nt
CONT
AD
29.4%
32.8%
25.9%
11.9%
29.7%
45.5%
15.6%
9.1%
tsRNA
rsRNA
ysRNA
Other
C
5S rRNA
5S rRNA (RPM)
30000
20000
10000
0
CONT
AD
D
tRNA-Tyr
tRNA-Tyr (RPM)
200
150
100
50
0
CONT
AD
E
tRNA-Arg
tRNA-Arg (RPM)
300
200
100
0
CONT
AD