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Supplementary Materials for

Noninvasive characterization of Alzheimer's disease by circulating, cell-free messenger RNA next-generation sequencing

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Other Supplementary Material for this manuscript includes the following:

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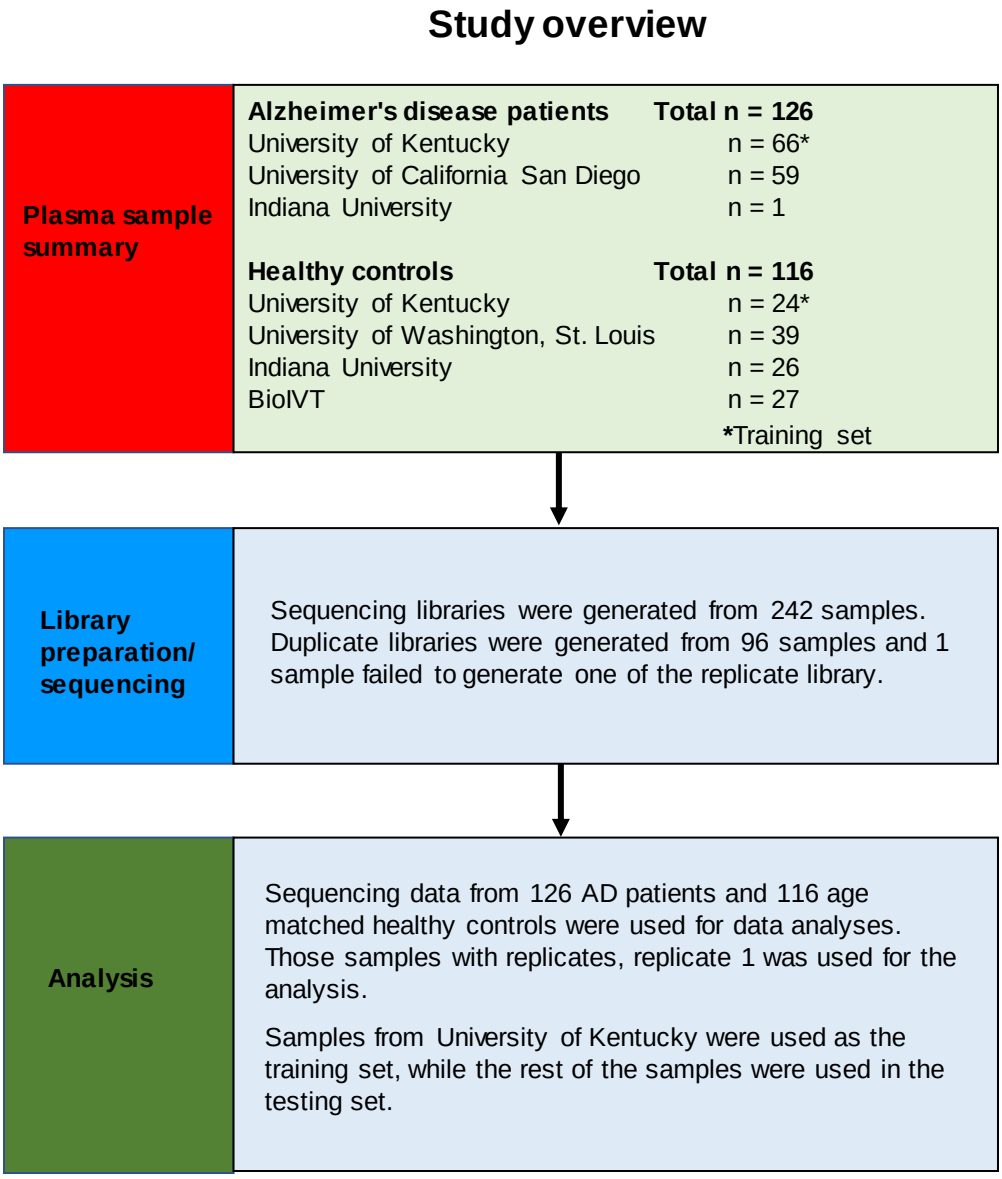
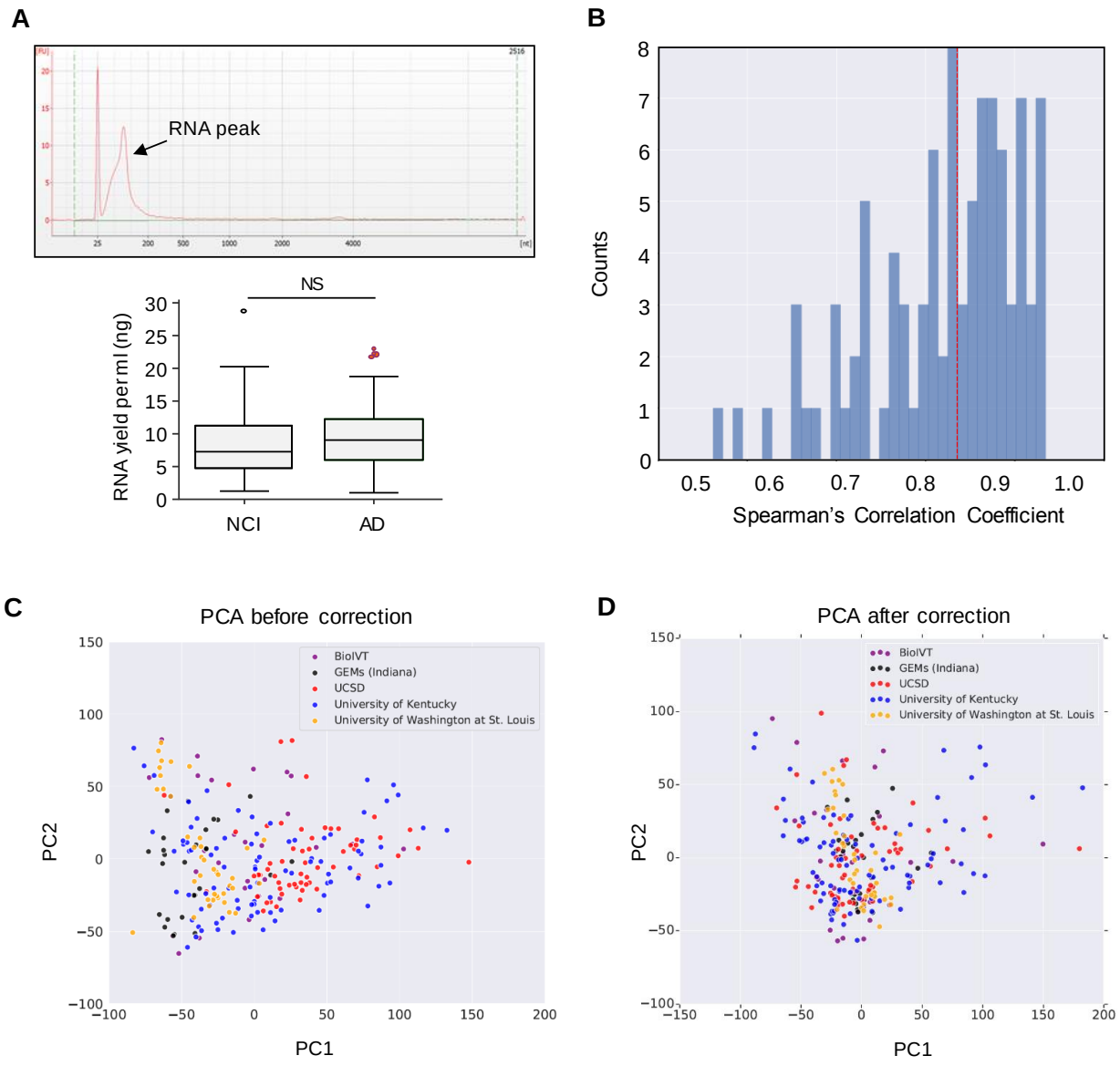
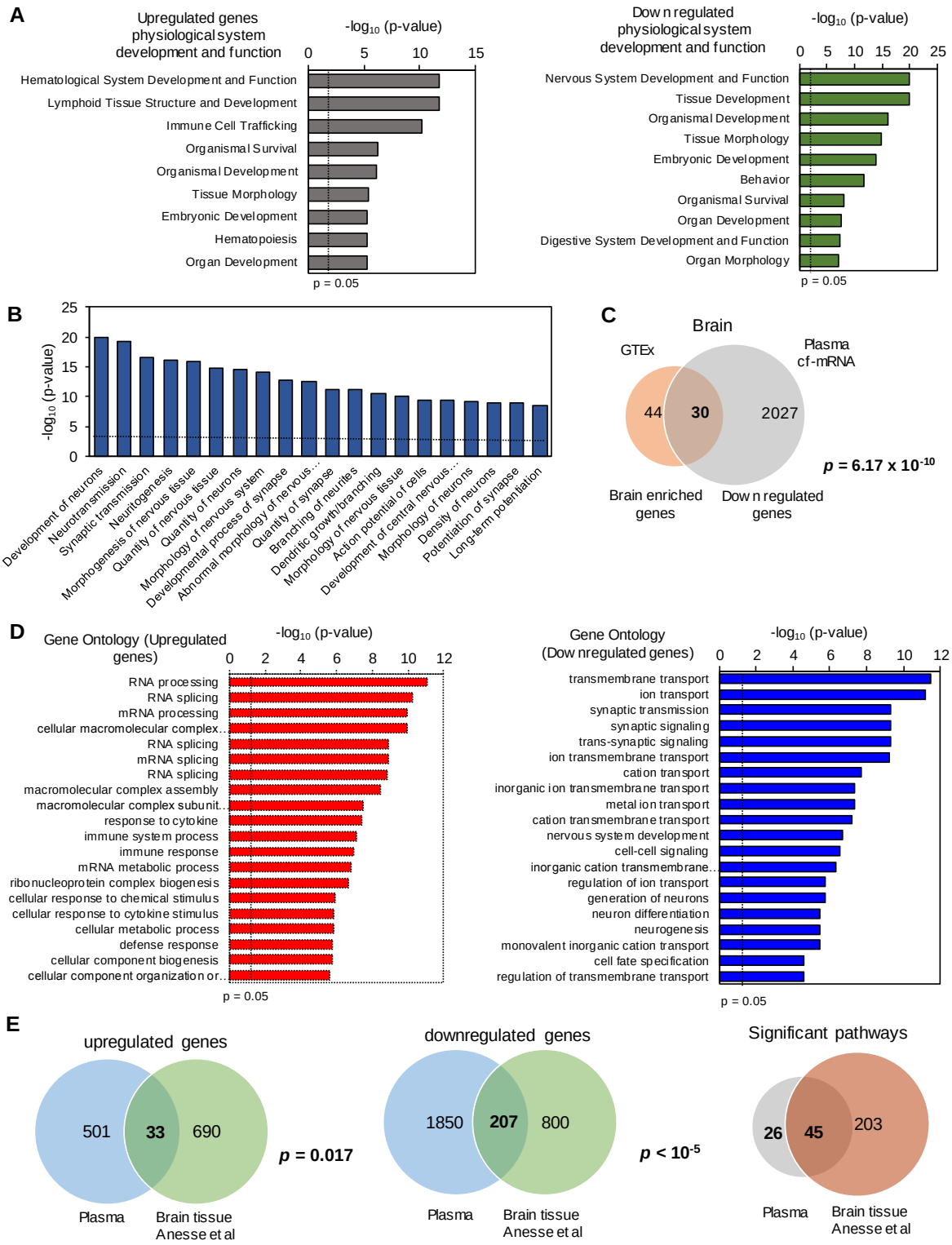


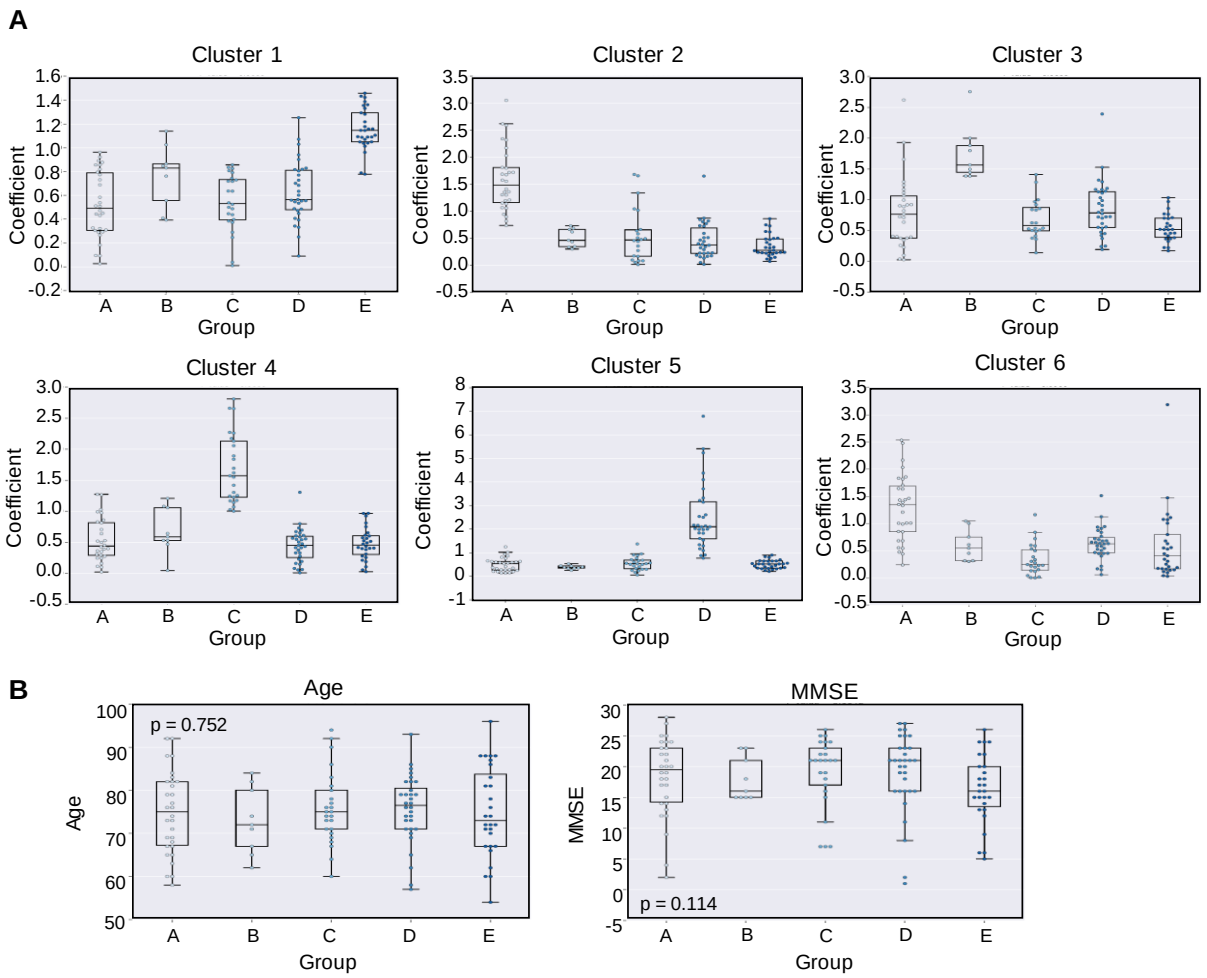
Supplementary Fig. 1: Study design and sample exclusion overview. Flow chart containing the overview of samples used in the study, summary of excluded samples and samples used in the final analysis.



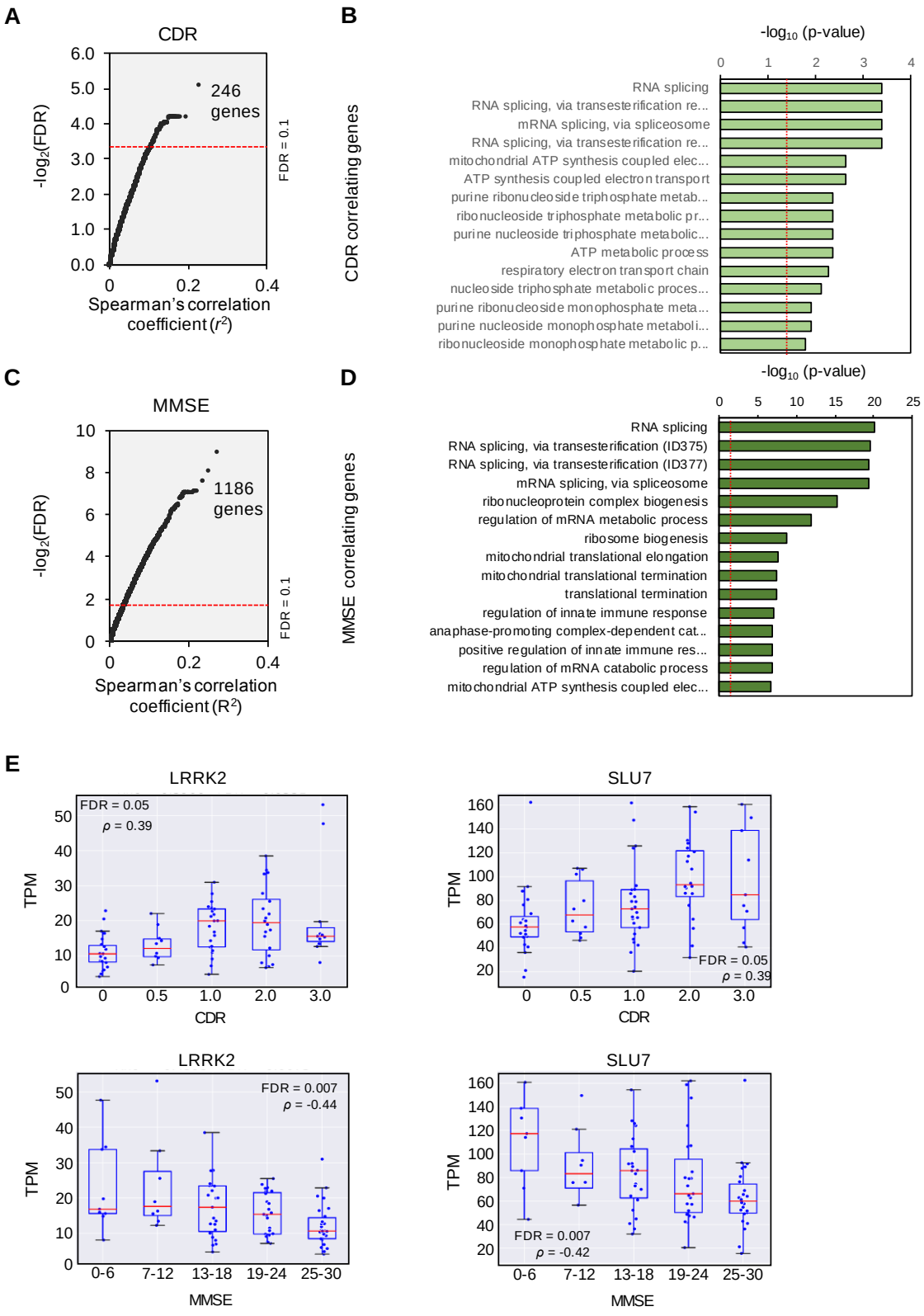
Supplementary Fig. 2: Technical performance and gene-expression profile sample distribution. (**A**) A typical Bioanalyzer profile of RNA extracted from plasma (top). Total RNA yields from AD and NCI plasma specimens (Welch test). (**B**) Histogram of Spearman's correlation coefficients between transcriptome correlations for 96 samples run in replicate. (**C**) Principal component analysis of all sequenced samples. Sample sources are represented in different colors. (**D**) Principal component analysis after site adjustment.


A
Upregulated genes physiological system development and function
-log10 (p-value)
Hematological System Development and Function
Lymphoid Tissue Structure and Development
Immune Cell Trafficking
Organismal Survival
Organismal Development
Tissue Morphology
Embryonic Development
Hematopoiesis
Organ Development
p = 0.05
Down regulated physiological system development and function
-log10 (p-value)
Nervous System Development and Function
Tissue Development
Organismal Development
Tissue Morphology
Embryonic Development
Behavior
Organismal Survival
Organ Development
Digestive System Development and Function
Organ Morphology
p = 0.05
B
-log10 (p-value)
Development of neurons
Neurotransmission
Synaptic transmission
Neuritogenesis
Morphogenesis of nervous tissue
Quantity of nervous tissue
Quantity of neurons
Morphology of nervous system
Developmental process of synapse
Abnormal morphology of nervous...
Quantity of synapse
Branching of neurites
Dendritic growth/branching
Morphology of nervous tissue
Action potential of cells
Development of central nervous...
Morphology of neurons
Density of neurons
Potentiation of synapse
Long-term potentiation
C
Brain
GTEx
Plasma cf-mRNA
44
30
2027
p = 6.17 x 10-10
Brain enriched genes
Down regulated genes
D
Gene Ontology (Upregulated genes)
-log10 (p-value)
RNA processing
RNA splicing
mRNA processing
cellular macromolecular complex...
RNA splicing
mRNA splicing
RNA splicing
macromolecular complex assembly
macromolecular complex subunit...
response to cytokine
immune system process
immune response
mRNA metabolic process
ribonucleoprotein complex biogenesis
cellular response to chemical stimulus
cellular response to cytokine stimulus
cellular metabolic process
defense response
cellular component biogenesis
cellular component organization or...
p = 0.05
Gene Ontology (Down regulated genes)
-log10 (p-value)
transmembrane transport
ion transport
synaptic transmission
synaptic signaling
trans-synaptic signaling
ion transmembrane transport
cation transport
inorganic ion transmembrane transport
metal ion transport
cation transmembrane transport
nervous system development
cell-cell signaling
inorganic cation transmembrane...
regulation of ion transport
generation of neurons
neuron differentiation
neurogenesis
monovalent inorganic cation transport
cell fate specification
regulation of transmembrane transport
p = 0.05
E
upregulated genes
501
33
690
p = 0.017
Plasma
Brain tissue Anesse et al
downregulated genes
1850
207
800
p < 10-5
Plasma
Brain tissue Anesse et al
Significant pathways
26
45
203
Plasma
Brain tissue Anesse et al

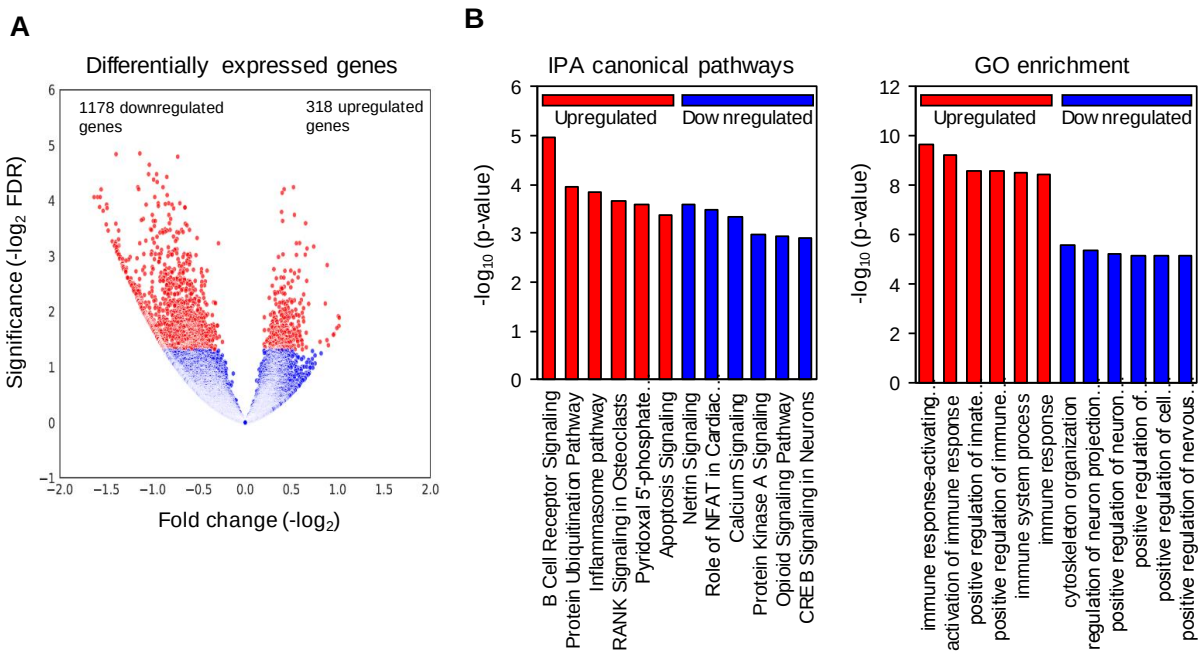
Supplementary Fig. 3 Biological processes and signaling pathways dysregulated in cf-mRNA of AD patients are associated with AD. (**A**) Most enriched biological processes determined by IPA analysis for genes that are upregulated in cf-mRNA of AD patients (left) and downregulated in cf-mRNA of AD patients (right). (**B**) Most significant subcategories within Nervous system development and function (IPA) for genes that are downregulated in cf-mRNA of AD as input. (**C**) Overlap between GTEx defined brain-enriched genes and downregulated genes in cf-mRNA of AD. P-values shows comparison between number of overlapped genes versus expected number. (**D**) Most prominent biological processes determined by Gene Ontology for genes that are upregulated in cf-mRNA of AD (left). Most prominent biological processes determined by Gene Ontology for genes that are downregulated in cf-mRNA of AD (right). (**E**) Overlap between genes that are upregulated (left) and downregulated (center) in cf-mRNA of AD patients and genes found to be upregulated/downregulated in the brain tissue of AD patients by Anesse et al. Overlap of signaling pathways identified in this study (grey) and by Anesse et al. (red) (tissue data obtained from Annese et al. 2018) (right).



Supplementary Fig. 4. cf-mRNA based subtyping of patients according to their molecular characteristics. (**A**) Cluster values for each of the 5 AD patient subcategories. (**B**) Age (left) and MMSE (right) distribution among 5 patient groups identified in (**A**). ANOVA analysis-Tukey's post-hoc test.


A
CDR
246 genes
FDR = 0.1
-log2(FDR)
Spearman's correlation coefficient (r2)
B
CDR correlating genes
-log10 (p-value)
RNA splicing
RNA splicing, via transesterification re...
mRNA splicing, via spliceosome
RNA splicing, via transesterification re...
mitochondrial ATP synthesis coupled elec...
ATP synthesis coupled electron transport
purine ribonucleoside triphosphate metab...
ribonucleoside triphosphate metabolic pr...
purine nucleoside triphosphate metabolic...
ATP metabolic process
respiratory electron transport chain
nucleoside triphosphate metabolic proces...
purine ribonucleoside monophosphate meta...
purine nucleoside monophosphate metaboli...
ribonucleoside monophosphate metabolic p...
C
MMSE
1186 genes
FDR = 0.1
-log2(FDR)
Spearman's correlation coefficient (R2)
D
MMSE correlating genes
-log10 (p-value)
RNA splicing
RNA splicing, via transesterification (ID375)
RNA splicing, via transesterification (ID377)
mRNA splicing, via spliceosome
ribonucleoprotein complex biogenesis
regulation of mRNA metabolic process
ribosome biogenesis
mitochondrial translational elongation
mitochondrial translational termination
translational termination
regulation of innate immune response
anaphase-promoting complex-dependent cat...
positive regulation of innate immune res...
regulation of mRNA catabolic process
mitochondrial ATP synthesis coupled elec...
E
LRRK2
FDR = 0.05
ρ = 0.39
TPM
CDR
SLU7
FDR = 0.05
ρ = 0.39
TPM
CDR
LRRK2
FDR = 0.007
ρ = -0.44
TPM
0-6
7-12
13-18
19-24
25-30
MMSE
SLU7
FDR = 0.007
ρ = -0.42
TPM
0-6
7-12
13-18
19-24
25-30
MMSE

Supplementary Fig. 5. Identification of genes in cf-mRNA that correlate with severity of cognitive impairment. (**A**) Plot between FDR (represented as -log) and Spearman's correlation coefficient between CDR score and TPM of genes. Red dotted line represents FDR = 0.1. (**B**) Top canonical pathways identified in GO pathway analysis using 246 genes that correlated with CDR scores. Red dotted line represents p = 0.05. (**C**) Plot between FDR (represented as -log) and Spearman's correlation coefficient for MMSE score vs TPM of genes. Red dotted line represents FDR = 0.1. (**D**) top canonical pathways identified in GO pathway analysis using 1186genes that correlates with MMSE scores. Red dotted line represents p = 0.05. (**E**) The expression of LRRK2 (left) and SLU7 (right) as a function of CDR (top) and MMSE (bottom) scores.



Supplementary Fig 6. Differentially expressed genes in circulation of early stage AD patients (**A**) Volcano plot showing genes dysregulated in circulation of AD patients with CDR $\leq$ 1 compared to control individuals. (**B**) Top IPA canonical pathways (left) and GO enrichment terms (right) identified using genes identified in (**A**) (upregulated genes in red, downregulated genes in blue).

Supplementary Table 1: Overall patient characteristics

Variable		AD	NCI
Patient number		125	116
Age	Average (± SEM)	74.5 ± 1.0	75.6 ± 0.7
Sex	Female (%)	74 (59%)	67 (58%)
	Male (%)	51 (41%)	49 (42%)
Cognitive impairment test	MMSE (patient number)	125	62
	CDR (patient number)	66	76

Supplementary Table 2: Cohort characteristics

Variable		AD			NCI			
		University of Kentucky	UC San Diego	Indiana University	University of Kentucky	University of Washington, St Louis	Indiana University	BioIVT
Patient number		66	59	1	24	39	26	27
Sex	Female	37	37	1	14	23	14	15
	Male	29	22	0	10	16	12	12
Age (average ± SEM)	Overall	77.2 ± 1.5	74.2 ± 1.1	84	83.9 ± 1.4	72.3 ± 0.7	80.2 ± 0.6	73.2 ± 1.9
	Female	77.2 ± 2.1	74.2 ± 1.4	84	84 ± 1.9	72.3 ± 0.9	80.2 ± 0.9	74.2 ± 2.8
	Male	76.5 ± 2.3	74.6 ± 1.6	-	83.7 ± 2.4	72.1 ± 1.1	80.3 ± 1.0	72.0 ± 2.5
MMSE (average ± SEM)	Overall	19.0 ± 1.0	20.5 ± 0.6	NA	26.5 ± 1.1	29.4 ± 0.1	-	-
	Female	19.0 ± 1.4	20.5 ± 0.7	NA	26.4 ± 1.4	29.4 ± 0.1	-	-
	Male	18.8 ± 1.6	20.6 ± 1.6	-	26.3 ± 1.9	29.4 ± 0.2	-	-
CDR	Overall	1.16 ± 0.12	NA	0.5	0	0	-	-
	Female	1.16 ± 0.16	NA	0.5	0	0	-	-
	Male	1.16 ± 0.18	NA	-	0	0	-	-

Supplementary File 1: AD v. healthy controls: Complete list of differentially expressed genes and associated pathway analyses.

Supplementary File 2: Complete list of genes in each AD cluster and associated pathway analyses.

Supplementary File 3: Genes that are correlated with cognitive impairment scores (CDR and MMSE) and associated pathway analyses.

Supplementary File 4: Genes that are used for the AD diagnostic classifier and associated pathways.