Plasma phospholipids identify antecedent memory impairment in older adults

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SUPPLEMENTARY INFORMATION

Nature Medicine: doi:10.1038/nm.3466

SUPPLEMENTARY TABLE 1. Subject Characteristics: Means and Standard Deviations

	N (M/F)	Age (years)	Education (years)
Normal Control (NC)			
Discovery Sample	53 (18/35)	81.55 (3.59)	15.68 (2.32)
Validation Sample	20 (9/11)	81.35 (3.25)	15.1 (2.49)
Total	**73 (27/46)**	**81.49 (3.48)**	**15.52 (2.36)**
Converter Baseline ($Converter_{pre}$)			
Discovery Sample	18 (8/10)	80.72 (2.99)	15.33 (3.14)
Validation Sample	10 (4/6)	79.3 (5.49)	14.5 (1.84)
Total	**28 (12/16)**	**80.21 (4.02)**	**15.04 (2.74)**
Converter After ($Converter_{post}$)			
Discovery Sample	18 (8/10)	82.22 (2.94)	15.33 (3.14)
Validation Sample	10 (4/6)	82.4 (5.52)	14.5 (1.84)
Total	**28 (12/16)**	**82.23 (3.95)**	**15.04 (2.74)**
Amnestic Mild Cognitive Impairment/Alzheimer's Disease (aMCI/AD)			
Discovery Sample	35 (10/25)	82.26 (4.75)	15.45 (2.19)
Validation Sample	11 (6/5)	80.0 (3.98)	16.0 (2.57)
Total	**46 (16/30)**	**81.72 (4.64)**	**15.59 (2.27)**

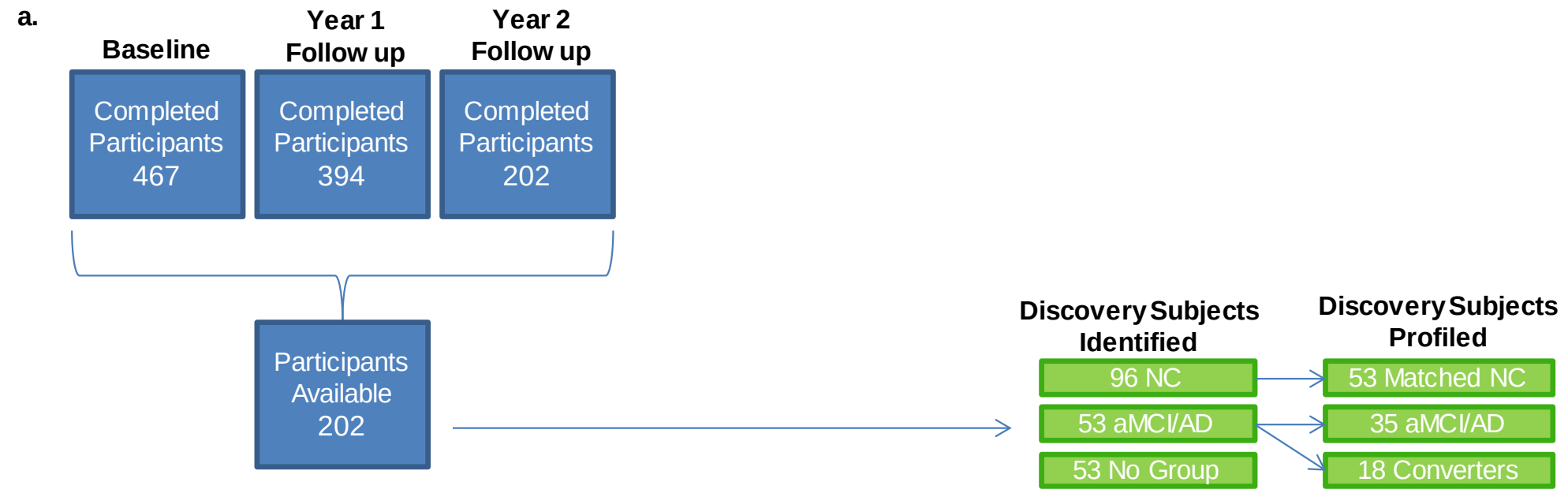
SUPPLEMENTARY TABLE 2. Cognitive Measures: Means and Standard Deviations

Clinical/Cognitive Measures	Dependent Measure (Range)	Domain Assessed	Normal Control (n=73)	$Converter_{pre}$ (n=28)	aMCI/AD (n=74)
Multiple Assessment Inventory IADL Scale (MAI-IADL) Lawton MP. (1988) Instrumental Activities of Daily Living (IADL) scale: Original observer-rated version. Psychopharmacology Bulletin, 24, 785-7.	Total Score (0-27)	Functional capacities	26.51 (1.71)	26.65 (0.87)	24.82 (3.60)
Multifactorial Memory Questionnaire (MMQ) Troyer AK and Rich JB. (2002). Psychometric properties of a new metamemory questionnaire for older adults. Journal of Gerontology, 57(1), 19-27.	Total Score (0-228)	Memory complaints	130.32 (19.93)	139.71 (13.36)	121.01 (18.14)
Mini Mental State Examination (MMSE) Folstein, MF, Folstein, SE, and McHugh, PR. (1975). "Mini-mental state". Journal of Psychiatric Research, 12, 189-98**.**	Total Score (0-30)	Global cognitive ability	28.64 (1.30)	28.61 (2.49)	26.32 (2.87)
Geriatric Depression Scale-Short Form (GDS-SF) Sheikh JI and Yesavage JA. (1986). Geriatric Depression Scale (GDS): Recent evidence and development of a shorter version. Clinical Gerontologist, 5, 165-173.	Total Score (0-15)	Mood	1.47 (2.02)	1.32 (2.28)	1.97 (2.7)
Wechsler Memory Scale-III Forward Digit Span (WMS-III FDS) Wechsler D. Wechsler Memory Scale-III Manual. San Antonio, TX: The Psychological Corporation, 1997.	Span Length (0-9)	Attention	6.25 (1.05)	6.18 (0.95)	6.14 (1.13)
Trail Making Test- Part A (TMT-A) Reitan RM. (1958). Validity of the Trail Making Test as an indicator of organic brain damage. Perceptual and Motor Skills, 8, 271-6.	Completion time (1-300 sec)	Attention	36.69 (13.23)	46.14 (14.75)	55.26 (44.63)
Wechsler Memory Scale-III Backward Digit Span (WMS-III BDS) Wechsler D. Wechsler Memory Scale-III Manual. San Antonio, TX: The Psychological Corporation, 1997.	Span Length (0-8)	Executive ability	4.34 (0.9)	4.29 (0.76)	4.01 (0.91)
Trail Making Test- Part B (TMT-B) Reitan RM. (1958). Validity of the Trail Making Test as an indicator of organic brain damage. Perceptual and Motor Skills, 8, 271-6.	Completion Time (1-300 sec)	Executive ability	98.53 (41.30)	134.57 (63.89)	151.99 (69.82)
Category fluency (Animals) Borkowski J, Benton A, Spreen O. (1967). Word fluency and brain damage. Neuropsychologia, 5, 135-140	Animals named in 1-minute	Language	20.91 (4.72)	19.0 (5.24)	15.16 (5.03)

Boston Naming Test 60-Item version (BNT-60) Kaplan E, Goodglass H, and Weintraub S. (1983). Boston Naming Test. Philadelphia: Lea & Feibiger.	Total Correct (0-60)	Language	56.29 (3.19)	53.14 (7.96)	50.51 (9.46)
Rey Auditory Verbal Learning Test Learning (RAVLT Learning) Rey A. (1964). L'examen clinique en psychologie. Paris: Presses Universitaires de France.	Total words recalled over Trials 1-5 (0-75)	Verbal learning	43.43 (7.76)	37.0 (5.88)	27.08 (7.01)
Rey Auditory Verbal Learning Test Recall (RAVLT Retrieval) Rey A. (1964). L'examen clinique en psychologie. Paris: Presses Universitaires de France.	Words recalled at 20-minute delay (0-15)	Verbal retrieval	7.84 (2.48)	5.32 (2.59)	1.93 (1.64)
Rey Auditory Verbal Learning Test Retention (RAVLT Recognition) Rey A. (1964). L'examen clinique en psychologie. Paris: Presses Universitaires de France.	True pos. – false pos. (0-15)	Verbal retention	13.30 (1.57)	11.14 (2.24)	7.09 (3.15)
Hooper Visual Organization Test (HVOT) Hooper HE. Hooper Visual Organization Test (VOT) Los Angeles: Western Psychological Services; 1983.	Total score (0-30)	Visuo-perception	23.96 (3.05)	22.36 (3.72)	20.93 (4.51)

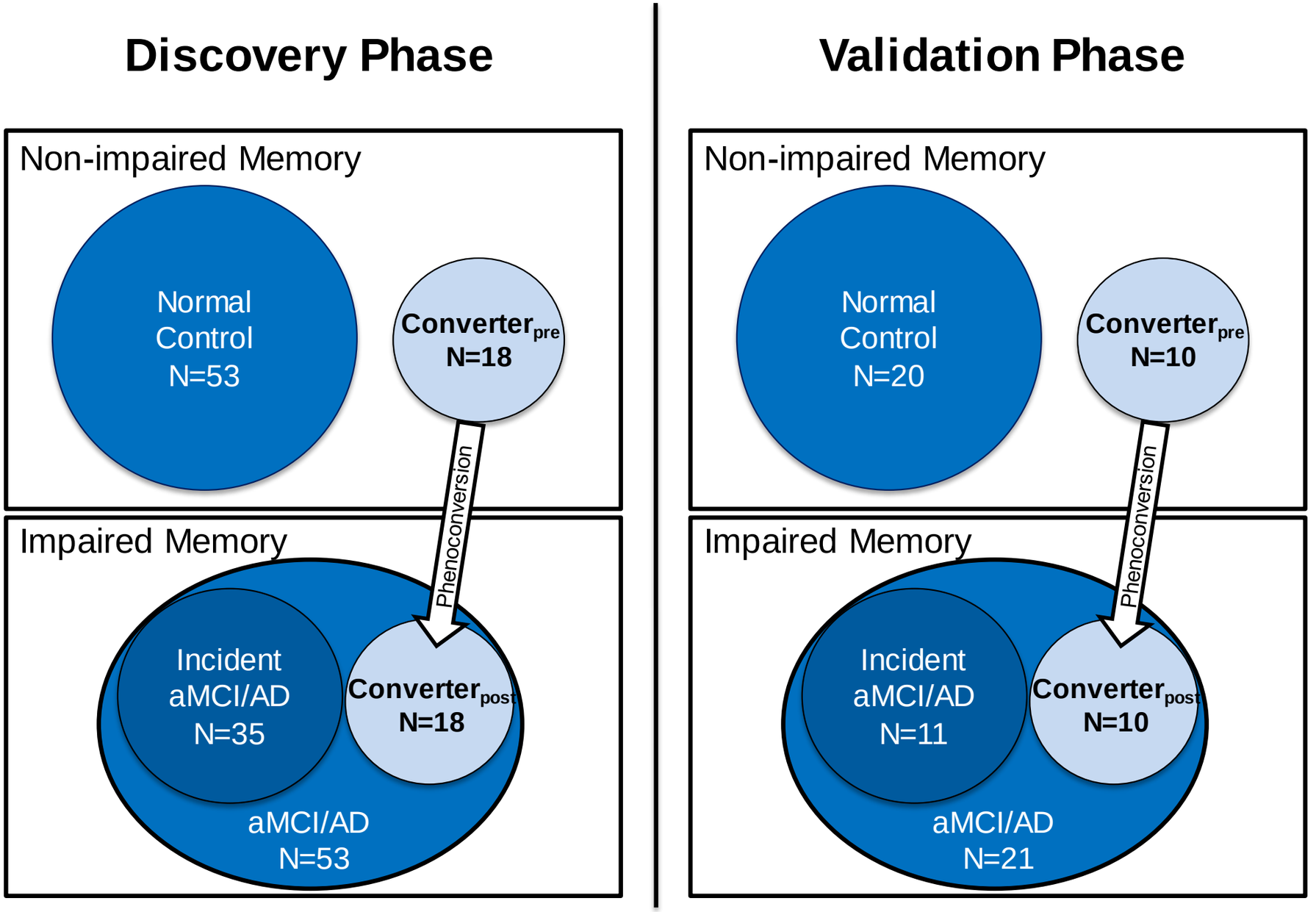
SUPPLEMENTARY TABLE 3. Composite Z-score Components

Attention (Z_{att})	**Executive (Z_{exe})**	**Language (Z_{lan})**	**Visuoperceptual (Z_{vis})**	**Memory (Z_{mem})**
Wechsler Memory Scale-III Forward Digit Span (WMS-III FDS)	Wechsler Memory Scale-III Backward Digit Span (WMS-III BDS)	1-min Category fluency (Animals)	Hooper Visual Organization Test (HVOT)	Rey Auditory Verbal Learning Test Learning (RAVLT Learning)
Trail Making Test- Part A (TMT-A)	Trail Making Test- Part B (TMT-B)	Boston Naming Test 60-Item version (BNT-60)		Rey Auditory Verbal Learning Test Retrieval (RAVLT Retrieval)
				Rey Auditory Verbal Learning Test Retention (RAVLT Recognition)



Supplementary Figure 1a. Study flow diagram. This figure shows the number of participants available for biomarker profiling at each phase of the study. The Discovery phase participants were selected in year 3 of the study and included only the 202 participants with three consecutive visits **(a)**. The 53 participants who did not meet criteria for aMCI, AD, or NC were classified No Group and not used in the analysis. The Validation selection took place at the end of year 5 of the study **(b)**. Here, all participants whose plasma was profiled in the Discovery phase were excluded from consideration. 295 participants with at least three consecutive visits were available for selection. For both Discovery and Validation phases, the cognitive data and blood sample from the last available visit was used. Due to rolling enrollment and drop outs during the course of the study the number of completed participants do not sum to the number of participants available.

Nature Medicine: doi:10.1038/nm.3466



Supplementary Figure 1b. Composition of participant groups. This figure shows the composition of the participant groups used in the Discovery and Validation phases of the study. The Discovery phase included 106 participants in two age-,sex-, and education-matched groups of 53 individuals. The 53 aMCI/AD participants consisted of 35 incident cases and 18 who phenoconverted from a non-impaired memory state at entry to the study. The smaller Validation Phase included 41 participants in two age-, sex-, and education-matched groups of 20 normal controls and 21 aMCI/AD individuals. The 21 aMCI/AD consisted of 11 incident cases and 10 who phenoconverted.

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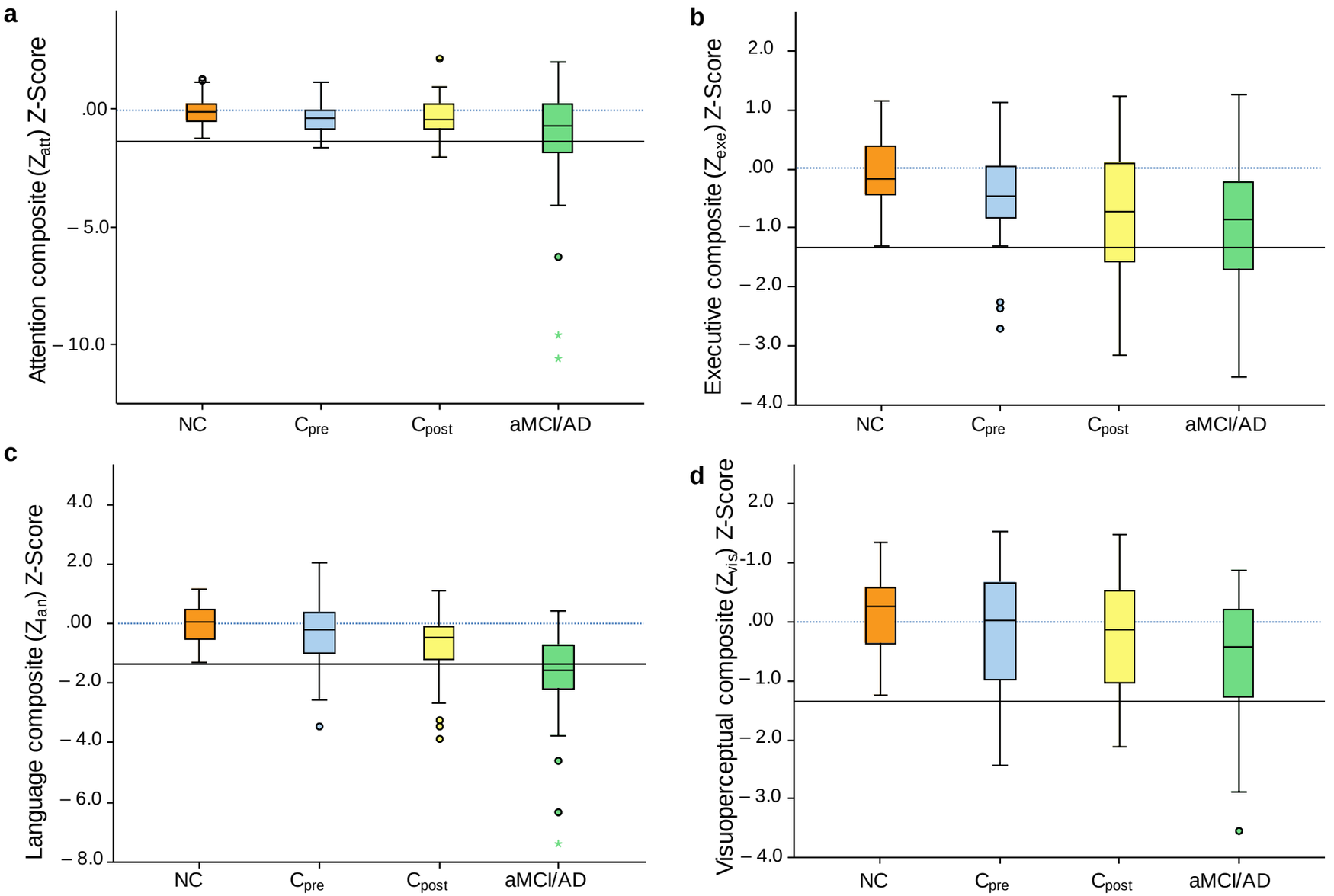
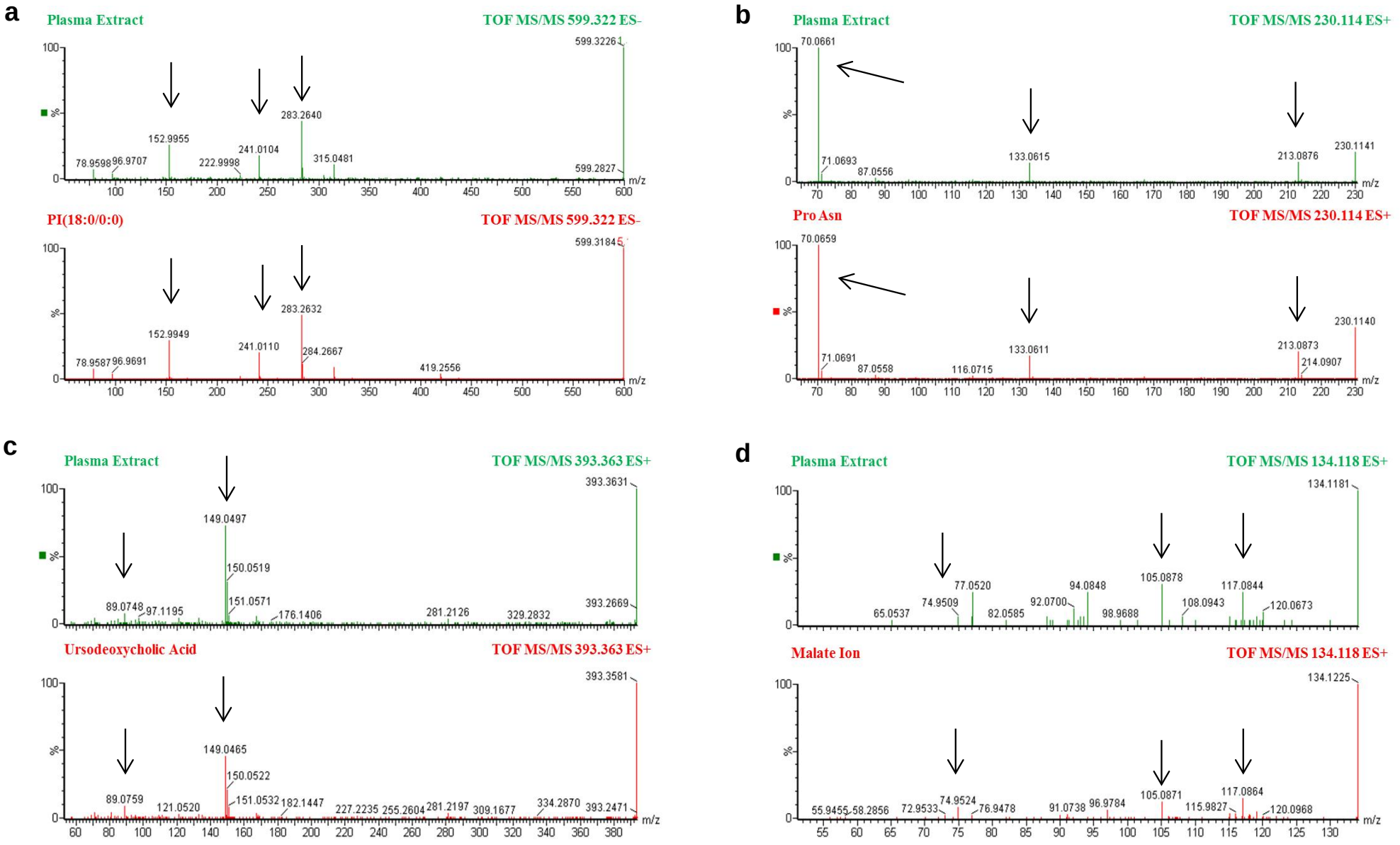
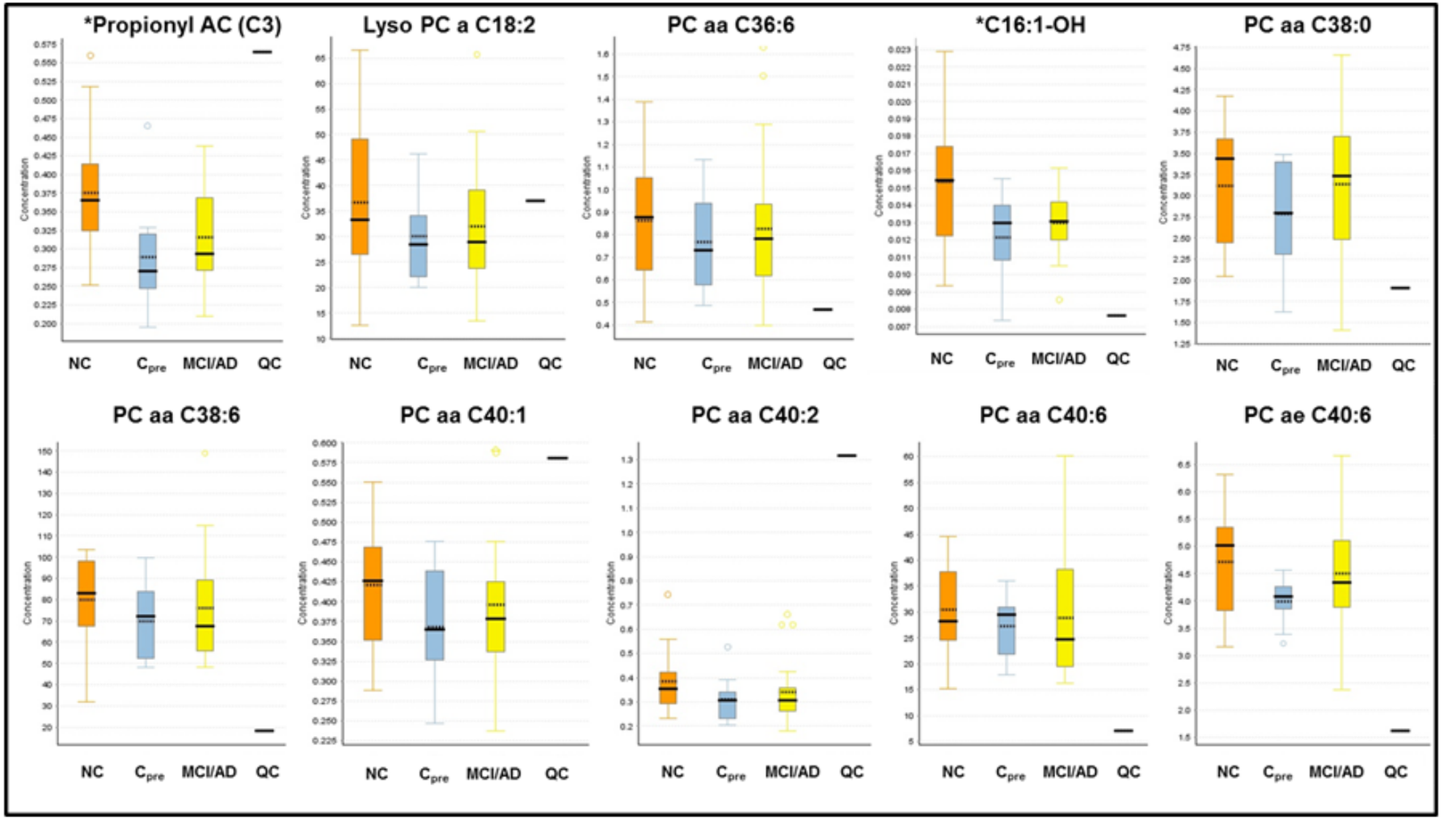


Supplementary Figure 2. Cognitive composite Z-Scores for non-mnemonic domains. These box and whisker plots depict the composite Z-scores of the combined discovery and validation samples for the (**a**) Attention (Z_{att}), (**b**) Executive (Z_{exe}), (**c**) Language (Z_{lan}),and (**d**) Visuoperceptual (Z_{vis}) domains. The performance of the Converter group after phenoconversion (C_{post}) is plotted for comparison. The blue line centered on 0 represents the median memory composite Z-score for the entire cohort of 525 participants. The black horizontal line represents the cut-off for impairment (-1.35 SD). Error bars represent s.e.m.



Supplementary Figure 3. Determination of chemical structures of metabolites in plasma extract by tandem mass spectrometry. The upper figure in each panel shows the unknown metabolite and lower panel shows the standard. Arrows indicate matching fragments in the metabolite and standard**. (a)** Metabolite with retention time 10.1 minutes and parent m/z of 599.32 identified as PI(18:0/0:0). (**b**) Metabolite with retention time of 2.5 minutes and parent m/z of 230.11 identified as Pro Asn. (**c**) Metabolite with retention time of 5.1 minutes and m/z of 393.363 identified as ursodeoxycholic acid. The glycine conjugate of ursodeoxycholic acid yields a parent m/z of 450 in the positive electrospray mode. (**d**) Metabolite with retention time of 0.6 minutes under the chromatographic conditions used and parent m/z of 134.118 in electrospray positive mode identified as Malate.



Supplementary Figure 4. Trend plots for the ten metabolite panel- Validation phase. This figure shows the results of the internal cross-validation for each of the ten metabolites using targeted quantitative mass spectrometry. The black solid and dotted lines in the boxplots represent median and mean abundance respectively, for the given group. The three groups depicted include NC (n=20), $Converter_{pre}$(n=10), and aMCI/AD(n=20). One of the aMCI/AD samples was not available for lipidomic analysis. The quantitative profiling data were subjected to the non-parametric Kruskal Wallis test using STAT pack module (Biocrates) for building a classifier based on differential abundance of metabolites in each group. Error bars are s.d. QC shows the scatter in the quality control samples. The p-values for analytes between groups were $p \leq 0.05$. The two metabolites with p-values less than 0.005 are indicated with an asterisk. Each Kruskal-Wallis test was followed by Mann-Whitney U Tests for post-hoc pair-wise comparisons (NC vs Cpre and NC vs aMCI/AD) Significance was adjusted for multiple comparisons using Bonferroni's method ($p<0.025$). The NC group had significantly higher levels of C16:1-OH, C3, PC aa C36:6, PC aa C40:2, PC aa C40:6, and PC ae C40:6 compared to the C_{pre} group and also higher levels of C16:1-OH, C3, lysoPC a C18:2, PC aa C38:6 and PC aa C40:6 compared to the aMCI/AD group.

SUPPLEMENTARY NOTE

Participants

All participants were community-dwelling, older adults from the greater Rochester, NY and Irvine, CA communities. Participants were recruited through local media (newspaper and television advertisements), senior organizations, and word of mouth. Inclusion criteria included age 70 or older, proficiency with written and spoken English and corrected vision and hearing necessary to complete the cognitive battery. Participants were excluded for the presence of known major psychiatric or neurological illness (including Alzheimer's disease or MCI, cortical stroke, epilepsy, and psychosis) at time of enrollment, current or recent (< 1 month) use of anticonvulsants, neuroleptics, HAART, antiemetics, and antipsychotics for any reason, and serious blood diseases including chronic abnormalities in complete blood count and anemia requiring therapy and/or transfusion. All participants gave written informed consent and all procedures in this study were approved by the University of Rochester, University of California Irvine, and Georgetown University Research Studies Review Boards. Participant characteristics can be found in Supplemental Table 1.

Sample Size Considerations

The signal intensity of the metabolites within similar groups was normally distributed with standard deviation of 1.5. If the true difference in the Converter_{pre} and NC groups' mean is 2 fold, we will have over 90% power to detect differential metabolites at an overall significance level of 5% with Bonferroni's adjustment using 30 subjects per group.

Operationalizing groups for biomarker profiling

The primary Memory outcome was based on the Rey Auditory Visual Learning Test (RAVLT). *Learning* was defined as the sum of the number of correct words recalled over the 5 learning trials; *Retrieval* was defined as the total number of correct words recalled from trial A7; and *Retention* was defined as the total number correct words recognized minus the number of false positives. Each of these three sub-scores (Learning, Retrieval, and Retention) was converted to an empirical *Z*-score by subtracting its sample mean and dividing by its sample SD. The three resulting positively correlated RAVLT-based *Z*-scores were then averaged to form the composite *Memory* score (Z_{mem}). So defined, Z_{mem} was approximately normal with mean 0, SD<1, and some negative skewness attributable to the fact that healthy participants often score at or near the upper bounds, especially for Retention.

The Attention (Z_{att}), Executive (Z_{exe}), and Language (Z_{lan}) scores were each defined as averages of empirical *Z*-score transforms of pairs of subscores, as follows. Z_{att}: (1) completion time (in seconds, truncated at 300s) for the Trail Making Test Part A and (2) the Wechsler Memory Scale-III Forward Digit Span; Z_{exe}: (1) completion time (in seconds, truncated at 300s) for the Trail Making Test Part B and (2) the Wechsler Memory Scale-III Backward Digit Span; Z_{lan}: (1) total score (out of 60) for the Boston Naming Test and (2) Category Fluency (Animals Named in 1 minute). The Visuoperceptual score (Z_{vis}) was simply the empirical Z-transform of the Total Score (out of 30) for the Hooper Visual Organization Test (HVOT), which itself was approximately normally distributed.

Standardization and Adjustment for Age, Gender, Education, and Visit

Linear Generalized Estimating Equation (GEE) models were used to model each of the five cognitive domains (Memory, Attention, Executive, Language, and Visuospatial) as a function of age, gender, education, and visit number, using a homoscedastic working independence covariance matrix. I.e., least squares was used to estimate all linear model coefficients based on the pooled data from all available visits at the Discovery phase and later at the Validation phase. Since Memory was the focal cognitive domain, the functional form for the covariates was selected to best model Memory, and exactly the same functional form was used for the other four cognitive domains. Given the evidence of nonlinearity, the effect of education was modeled using a continuous piecewise linear spline with knots at 12, 14, and 16 years of education. Memory increased with years of education between 5-12 years, decreased between 12-14 years, increased again between 14-16 years, and then nearly leveled off for 16-23 years. Age was modeled linearly, as there was insufficient evidence to support nonlinearity via piecewise linear spline knots or a quadratic component, yet Memory scores decreased linearly with age. Visit was modeled via an indicator for baseline visit 0, given that there was insufficient

evidence that subsequent visits differed from each other, yet there was evidence that subsequent visits had higher Memory scores compared with baseline visit 0. Gender was modeled via an indicator for males, who had lower memory scores than females. There was insufficient evidence to support any interactions. Residuals from each model were then robustly standardized to have median 0 and robust SD=1, where the robust SD = IQR/1.35, as 1.35 is the IQR (Inter-Quartile Range) of a standard normal distribution. The choice to use robust measures of location (median) and dispersion (IQR) was made to reduce the influence that cognitively impaired participants might have on the mean and SD, and in recognition of the slight negative skewness of the residuals. The robustly standardized residuals were then viewed as age-gender-education-visit-adjusted robust *Z*-scores for each of the five cognitive domains.

Defining aMCI/AD, Converters, and NC

For each subject, Z_{mem}(last), Z_{att}(last), Z_{exe}(last), Z_{lan}(last), and Z_{vis}(last) were defined as the age-gender-education-visit-adjusted robust Z-scores for the last available visit for each subject. We defined the aMCI/AD group to be those participants whose adjusted Z_{mem} was 1 IQR below the median at their last available visit, i.e. $Z_{mem}(\text{last}) \leq -1.35$. Converters were defined as that subset of the MCI/AD group whose adjusted Z_{mem} at baseline visit 0 was no more than 1 IQR below the median, i.e. $Z_{mem}(\text{visit=0}) > -1.35$ and $Z_{mem}(\text{last}) \leq -1.35$. Participants were classified as NC if they had central scores on all domains at both the first and last visits, i.e. only if they met all of the following six conditions: (i) $-1 < Z_{mem}(\text{last}) < 1$, (ii) $-1 < Z_{mem}(\text{visit=0}) < 1$, (iii) $Z_{min}(\text{last}) > -1.35$, (iv) $Z_{min}(\text{visit=0}) > -1.35$, (v) $Z_{max}(\text{last}) < 1.35$, and (vi) $Z_{max}(\text{visit=0}) < 1.35$, where Z_{max}(last) and Z_{max}(visit=0) denote the maximum of the five adjusted Z-scores at the last and first visits, respectively. Z_{mem} for normal participants had to be within 0.74 IQR (1 SD) of the median, rather than just 1 IQR (1.35 SD), in order to guarantee that they were > 0.25 IQR (0.35 SD) from aMCI/AD participants.

Frequency matching aMCI/AD and NC on age, education, and sex

Comparing the distributions of age, education, and sex for participants classified as aMCI/AD and NC we observed that NC participants were younger. Given this, and given budget limitations for genetic profiling, we frequency matched NC participants to the aMCI/AD participants for each of the Discovery and Validation samples based on age, education, and sex. This was accomplished by stratifying the sample by four groups of age at the last visit (75-79, 80-85, 86-94, 95-100), three education groups (5-12, 13-18, 19-23), and two sexes (male, female), resulting in 24 age-education-sex strata. We randomly selected an equal number of NC participants to match the aMCI/AD participants per the strata for the Discovery sample and because one of the strata did not contain a sufficient number of NC participants, the Validation sample had one less NC subject than the aMCI/AD group.

Blood Collection, Shipment, and Specimen Processing Protocols

Collect Patient Vitals

1. Record date/time.
2. Collect and record height, weight, blood pressure, pulse and temperature.
3. Collect and record whether subject has had food/drink (except water) since midnight.
4. Record current medications/dosages.

Blood Draw

1. Draw 3 x 7 mL lavender top tubes and place on ice

Transfer Samples to Laboratory

1. Lavender top tubes should be shipped/transferred on blue ice packs or wet ice, but not frozen. Upon shipment arrival laboratory personnel will immediately process lavender top tube (see protocol below).

Shipment Protocol

Supplies:	*paper tape for tube/bag sealing*	*Styrofoam/sturdy outer box*
	absorbent material (paper towel)	*gel packs*
	bubble wrap bag	*packing tape*
	leakproof sealed bag	*"Exempt Human Specimen" labels*

1. Keep lavender tubes after blood draw on ice (NOT FROZEN) prior to shipment.
2. Remove 2 small gel packs from freezer approximately 11/2 hours before shipping and **thaw to refrigerator temperature (~34 degrees)**
3. Seal each tube at stopper with paper tape. Leave folded end tab on tape for easier removal
4. Wrap each tube individually with absorbent material and place in bubble wrap bag. Seal with tape. Place all wrapped tubes in leakproof sealed plastic bag
5. Place cool/thawed gel packs in bottom of styrofoam box (with outer corrugated carton.) Lay wrapped lavender tube bags on thawed gel packs. (Do not want lavender top tubes to freeze.) Fill all void space with paper to prevent product movement
6. Include a copy of collection form (in plastic) inside cooler. Tape styrofoam cooler box top closed with packing tape
7. Include copy of delivery information on top of styrofoam cooler. Close and securely seal outer box with pressure-sensitive plastic tape. Apply packing tape over all flaps and seams
8. Ship all samples on same day, via **FedEx "Priority Overnight"** for Next Day Morning Delivery

Supplies - Blood Draw and Shipping

Description	VWR Catalog Number and Price	
VWR Koolit Gel 8oz CS72	33500-585	$10.57
VWR Gel 16 oz. 6x6x1 CS36	33500-587	$6.94
Container Molded 8x6x6.75 PK 12 (shipping box w/cooler)	33500-404	$75.14
6x8 inch 3/16in bubble pouch CS250	80082-635	$41.65
TC 6x9 ziploc bag pk 1000	80094-734	$23.17

Description	Cardinal Catalog Number and Price	
7-ml Lavender Tube	B2991-52	$8.74 / 100
Sterile Gauze	GZ2208-2	$4.00 / 50
21 g butterfly w/ adapter	B3036-21	$38.39 / 50
23 g butterfly w/ adapter	B3036-20	$38.39 / 50
Vacutainer Holder for tubes	364815	$5.49 / bag
Latex Free Bandages	BF3403	$4.77 / 100
Alcohol prep pad	40000-110	$1.39 / 200
Micropore tape	7246S	$23.7 (12/box)
Specimen bag (w/ pouch)	49-96	$32 / 1000

Plasma Specimen Processing Protocol

1) Remove paperwork and set of specimen tubes (3 lavender top tubes per patient) from package. Recycle package and contents
2) Place lavender tubes in 15 ml centrifuge bucket and balance. Keep specimen sets together
3) Spin tubes at 2600 RPM (1500 x g) for 10 minutes at 20^{o}C (Program 2)
4) Remove tubes from centrifuge and place in Bio Hood by decontaminating with 70% EtOH
5) Remove the 50 ml tube caps for a **single specimen set** and place face down on underpad
6) Carefully remove the paper tape from the set of specimen lavender tubes corresponding to step 6. Next, remove purple caps by gently walking out the cap with Kimwipes. Place caps on stack of paper towels and save the Kimwipes

Nature Medicine: doi:10.1038/nm.3466

7) Collect plasma from each specimen tube with a 5 ml pipette (be careful not to disturb the buffy coat) and dispense into the 50 ml tube marked with a **P**. Recap P tube and place on ice.
8) Repeat steps 4 thru 7 as necessary for each specimen set
9) Remove P tube from ice and place in Bio Hood by decontaminating with 70% EtOH
10) Aliquot 25 µl of plasma onto a square of parafilm. Draw aliquot into a microcuvette by capillary action. Measure and record hemoglobin level using the HemoCue Photometer
11) Aliquot 750 µl of plasma across the 2 ml pre-labeled plasma tubes until all of the collection is dispensed.
12) Place plasma aliquot 2 ml pre-labeled tubes into freezer rack.
13) Store the Plasma aliquots at -80^{o}C.