Inflammation and epigenetic age in Alzheimer's disease: do sex and APOE matter?

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ABSTRACT

Alzheimer's disease (AD) disproportionately affects females that carry the APOEε4 allele, and the progression from cognitively normal to mild cognitive impairment (MCI) and to AD may be sex-specific. The objective of this study was to investigate underlying physiological inflammatory and epigenetic mechanisms that may help explain why there are sex differences in AD and APOEε4 carriers. Inflammation is observed in AD and is related to aging, stress and neuroplasticity, and aging biomarkers also include changes in DNA methylation patterns which have been used to determine epigenetic age. Although studies are scarce, sex differences are noted in inflammation and in epigenetic aging. We investigated using the ADNI database the effect of sex and APOE genotype (non-carriers or carriers of 1 or 2 APOEε4 alleles) and sex and diagnosis (cognitively normal (CN), MCI, AD) on CSF and plasma markers of stress and inflammation, and blood epigenetic age. We found sex and APOE genotype interactions on CSF levels of IL-8 and IL-16 cytokines suggesting that the presence of APOEε4 alleles differentially affects male and female physiology. Furthermore, females had higher levels of plasma CRP and ICAM1 but lower levels of CSF ICAM1, IL-8, IL-16, and IgA than males, showing in part that results may differ by sample source (plasma and CSF). Carrying 1 or 2 of APOEε4 alleles and diagnosis (MCI and AD) decreased plasma CRP in both sexes. Neither sex, diagnosis, nor presence of APOEε4 alleles affected epigenetic age acceleration. Sex differences in inflammatory biomarkers support that the underlying physiological changes during aging differ by sex and tissue origin.

Keywords: Sex differences; Alzheimer's disease; APOE genotype; inflammation; cytokines; epigenetic age.

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disease characterized by severe cognitive decline (Alzheimer's Association, 2017). Modifiable risk factors associated with AD include stress (Caruso et al., 2018), sociocultural or lifestyle factors (e.g., education, marital status, exercise), and medical conditions (diabetes, obesity, and cardiovascular disease) (Baumgart et al., 2015; Nebel et al., 2018; Xu et al., 2015). Non-modifiable risk factors include age, biological sex, and APOE genotype (Riedel et al., 2016). In some studies, females show greater neuropathology (brain atrophy, neurofibrillary tangles) and cognitive decline than males, although this depends on whether females are already cognitively impaired (mild cognitive impairment (MCI) or AD) and on the presence of an APOEε4 allele, a well-known genetic risk factor of AD (Ardekani et al., 2016; Barnes et al., 2005; Holland et al., 2013; Hua et al., 2010; Irvine et al., 2012; Koran and Hohman, 2017; Lin et al., 2015; Wang et al., 2019). Previous studies using data from the Alzheimer's Disease Neuroimaging Initiative (ADNI) indicate that females with AD have greater brain atrophy rates and cognitive decline than males with AD (Holland et al., 2013; Hua et al., 2010; Lin et al., 2015). However, there is limited research into the role of sex and its interaction with APOE genotype in the possible mechanisms underlying AD. However, females with one APOEε4 allele present have a greater risk to develop AD at an earlier age (Neu et al., 2017), show greater cognitive decline and neuropathology associated with AD relative to males (Beydoun et al., 2012; Hohman et al., 2018; Koran et al., 2017), indicating that the APOE genotype interacts with sex on various factors related to AD. Understanding why females are at a higher risk and have a higher burden of the disease is important for the development of tailored treatments. Biomarkers are highly sought after to predict disease onset and progression and to understand the underlying mechanisms of diseases in order to develop or improve treatments.

Chronic inflammation is a hallmark of AD, as evidenced by increased expression of proinflammatory cytokines in the brains of AD patients (not analyzed by sex), which can exacerbate AD pathology (Heppner et al., 2015; Kinney et al., 2018; Swardfager et al., 2010). There is, however, increasing evidence that there are sex differences in immune responses in healthy adults with females mounting a stronger response compared to males after an acute challenge (Engler et al., 2016; Klein and Flanagan, 2016). In response to an endotoxin, females have higher levels of proinflammatory plasma cytokines (TNF-α and IL-6) while males have higher plasma levels of anti-inflammatory IL-10 (Engler et al., 2016; Wegner et al., 2017). In addition, sex differences in inflammation are associated with AD pathology (Walker et al., 2018) but not cognitive decline in normal aging (Walker et al., 2019). Specifically, higher systemic inflammation beginning in mid-life is associated with higher brain amyloid levels later in life in healthy males, but not females (Walker et al., 2018). To our knowledge, very few studies have stratified by sex and APOE genotype or sex and diagnosis of cognitive status on potential biomarkers of AD.

Sex differences in inflammatory biomarker systems may also differentially affect neuroplasticity (Dantzer, 2018; de Miranda et al., 2017), which is reduced in AD and correlates with cognitive decline (Apostolova et al., 2006; Petersen et al., 2000). In addition, peripheral cortisol, the main stress hormone in humans, is elevated in AD (Doecke et al., 2012) and is associated with higher amyloid levels in the brain (Toledo et al., 2012), a reduction in hippocampal volume, and cognitive impairment in older individuals (Lupien et al., 1998) that may depend on MCI status (Murphy et al., 2019). Peripheral cortisol is also associated with elevated pro-inflammatory cytokines (Engler et al., 2016; McEwen et al., 1997). However, it is not known how sex differences in markers of inflammation (e.g., cytokines, immunoglobulins,

CRP, intercellular adhesion molecule, ICAM1), and stress hormones (cortisol) may be related to sex differences in AD.

Aging biomarkers include epigenetic alterations that are associated with a variety of pathologies and adverse health conditions, including normal cognitive aging and neurodegenerative phenotypes such as AD (Hannum et al., 2013; Horvath, 2013; Levine et al., 2015; Yokoyama et al., 2017). Recently, molecular biomarkers of aging known as "epigenetic clocks" have been developed based on DNA methylation (DNAme) signatures (Hannum et al., 2013; Horvath, 2013). Epigenetic age or "DNAmAge" is a measure of the biological age of a sample (cell or tissue), and can be calculated across a range of tissues and time points, providing an accurate estimation of a sample's chronological age based on the presence or absence of methylation at the 5' carbon of cytosine in informative cytosine-guanine (CpG) dinucleotides throughout the human genome (Horvath, 2013). Positive deviations of epigenetic age from chronological age (positive epigenetic age acceleration) reflect more rapid biological aging and have been associated with numerous factors including smoking, obesity, Parkinson's disease, Trisomy 21, and cancer (Gale et al., 2018; Horvath, 2013; Horvath et al., 2015; Horvath and Ritz, 2015), while negative deviations of epigenetic age from chronological age (negative epigenetic age acceleration) have been associated with high life-expectancy populations and memory retention (Degerman et al., 2017; McEwen et al., 2017). In AD, epigenetic age acceleration of the frontal cortex was associated with amyloid load, neuritic plates, and cognitive decline (Levine et al., 2015). Within an individual, the DNA methylation profile in peripheral tissues is correlated with the epigenetic signature in the brain, likely due to identical genetic background affecting DNAme and common signatures of epigenetic aging (Braun et al., 2019), thus it is reasonable to hypothesize that epigenetic age acceleration may also be detectable in

peripheral tissues such as blood in AD participants. In healthy individuals, aging males exhibit more positive epigenetic age acceleration than females in blood, buccal tissue, and in multiple brain regions (Horvath et al., 2016). In AD, it is possible that the underlying sex-specific pathological mechanisms may be reflected in epigenetic age acceleration measures, and given the complexity of the sex-specific phenotypes of AD, this may be observed as either sex showing accelerated epigenetic age – for example, AD females could potentially have more positive epigenetic age acceleration than males.

Using the ADNI database, we examined sex differences in plasma and CSF physiological biomarkers (inflammation, stress, related) and blood epigenetic age, and how these may be affected by APOE genotype (non-carriers or carriers of 1 or 2 APOEε4 alleles), and dementia status (cognitively healthy (CN), MCI, AD). We tested the hypotheses that (1) females have higher levels of inflammation and stress hormones compared to males and these levels are disproportionately affected by the presence of APOEε4 alleles and AD diagnosis; and (2) females have more positive epigenetic age acceleration and this is exacerbated by APOEε4 carriership and AD diagnosis.

METHODS

ADNI database

Data used in the preparation of this article were obtained from the Alzheimer's Disease Neuroimaging Initiative (ADNI) database (adni.loni.usc.edu). The ADNI was launched in 2003 as a public-private partnership, led by Principal Investigator Michael W. Weiner, MD. The primary goal of ADNI has been to test whether serial magnetic resonance imaging (MRI), positron emission tomography (PET), other biological markers, and clinical and neuropsychological assessment can be combined to measure the progression of mild cognitive

impairment (MCI) and early Alzheimer's disease (AD). For up-to-date information, see www.adni-info.org. Data used in this article were downloaded on or before Jan 16, 2019. Inclusion and exclusion criteria of participants (Petersen et al., 2010; Aisen et al., 2010) and general procedures are detailed online (http://adni.loni.usc.edu/methods/documents/). Briefly, cognitively normal (CN) participants had normal memory function based on education-adjusted scores on the Wechsler Memory Scale Logical Memory II and a Clinical Dementia Rating (CDR) of 0. Amnestic late MCI (LMCI) participants had objective memory loss (measured by education-adjusted scores from Wechsler Memory Scale Logical Memory II), a CDR of 0.5, preserved daily activities, and absence of dementia. Early MCI (EMCI) participants had a milder episodic memory impairment compared to LMCI and were recruited using the Wechsler Memory Scale Logical Memory II scores (between ~0.5 and 1.5 SD below the mean of CN participants). All AD participants met NINCDS/ADRDA Alzheimer's Criteria and a CDR of 0.5 or 1.0.

Statistical Methods: Inflammatory markers

We included all ADNI participants that had inflammatory markers measured in CSF (N = 279) and plasma (N = 527) listed in Table 1. Data included in our analyses were: demographics (age, years of education, and ethnicity), baseline diagnosis (cognitively normal, CN; late MCI, LMCI; or AD), and number of APOEε4 alleles. We collapsed APOE genotype into two groups: (1) participants carrying any ε4 alleles (homozygous ε4/ε4 and heterozygous ε4/-) and (2) participants with no ε4 risk alleles (-/-). Plasma and CSF samples from the ADNI study were collected in CN, LMCI and AD participants at baseline in the morning after an overnight fast. Processing, aliquoting and storage were performed according to the ADNI Biomarker Core

Laboratory Standard Operating Procedures. Inflammatory markers were measured using a commercially available multiplex proteomic panel (Human Discovery Multi-Analyte Profile; Luminex xMAP) developed by Rules-Based Medicine (Austin, TX), that measures a variety of markers including cytokines, metabolic markers, and growth factors. The protocols used to quantify plasma and CSF analytes are described in Craig-Schapiro et al. (2011) and Hu et al. (2012). We used the ADNI quality-controlled data for plasma and CSF provided by the ADNI Consortium. For plasma IL-16, we removed one outlier that was more than two times lower than the 25th percentile in the plasma data. Sensitivity analysis with the outlier present suggested that it was disproportionately influencing the results.

We compared all available data for each study variable between the sexes using the Wilcoxon rank sum test for continuous variables and Fisher's exact test for categorical variables. We used general linear models to determine the relationships between (1) sex and APOE genotype (non-carriers or carriers of 1 or 2 APOEε4 alleles) or (2) sex and baseline diagnosis as predictor variables, and biomarkers as dependent variables. Due to the limited sample size, we were not able to study sex, APOE genotype and baseline diagnosis in one model. All models included age and education as covariates. All models initially included an interaction between sex and presence of APOEε4 alleles or sex and baseline diagnosis; if this interaction was not significant, it was removed from the model to estimate the main effects of sex and APOE genotype or diagnosis. Significance was based on the likelihood ratio test, and all P-values for comparisons of sex and either APOE genotype or diagnosis for all outcomes combined were corrected for multiple testing using the Benjamini-Hochberg false discovery rate method with the family-wise error rate set to 0.05 (Benjamini and Hochberg, 1995). In total, three P-values per dependent variable were included in each set of models (interaction term and main effects of sex

and APOE or diagnosis) resulting in 27 P-values corrected in CSF (9 dependent variables) and 33 P-values corrected in plasma (11 dependent variables; Supplementary Tables S1 to S4) for each of the models (sex and APOE and sex and diagnosis). Significant interaction terms were followed up using pairwise simple-effects tests with Benjamini-Hochberg p-value correction. We report significance differences (adjusted $P<0.05$) and trends (adjusted $P<0.08$). All regression analyses were carried out in R v3.5.1 (R Core Team, 2018).

Statistical Methods: Epigenetic Age

We used DNAme data quantified with the Illumina Infinium HumanMethylationEPIC BeadChip array ("EPIC" array) for 1905 longitudinal blood samples from 640 unique ADNI participants with CN, EMCI, LMCI, and AD diagnosis (n=284 females, n= 356 males) (Vasanthakumar et al., 2017). DNAme IDAT files were read into R v3.5.1 (R Core Team, 2018) using the 'minfi' package, and annotated with the most recent version of the EPIC manifest, the Infinium MethylationEPIC v1.0 B4 Manifest File, (available from https://support.illumina.com/downloads.html) (Aryee et al., 2014; Fortin et al., 2017). We excluded 11 low quality samples from 9 unique participants from further analyses on the basis of having median methylated and unmethylated probe intensity <10.5 (Aryee et al., 2014; Fortin et al., 2017), the remaining samples were background normalized and dye-bias adjusted with normal exponential out-of-band ("noob") normalization (Triche et al., 2013). DNAme data were converted to beta values and the biological sex of all samples was confirmed by clustering samples on all array probes mapping to the X and Y chromosomes. Beta values were calibrated to Horvath's 21,368-probe training dataset using the calibrated beta-mixture quantile (BMIQ) normalization procedure suggested by Horvath, and epigenetic age was calculated using R code

modified for compatibility with the EPIC array using the 334/353 epigenetic clock probes present on the array from https://horvath.genetics.ucla.edu/ (Horvath, 2013; Teschendorff et al., 2013). Epigenetic age could have been calculated by imputing missing DNAme values at the 19 clock CpG sites not covered by the EPIC array based on the k-nearest neighbors method, but as we observed a high correlation between epigenetic age values calculated with the missing probes removed versus imputed with k=10 ($R=0.99$, $p<2.2e-16$), in agreement with previous reports (Fiorito et al., 2017; McEwen et al., 2018), we therefore chose to remove missing probes.

Prior to statistical analyses we removed all technical replicate samples. Epigenetic age acceleration was calculated as the residual of epigenetic age regressed on chronological age and technical batch covariates: the laboratory collection site at which blood samples were drawn and EPIC microarray chip and row. Intrinsic epigenetic age acceleration, a measure designed to be independent of age-related changes in whole blood cell-type proportions, was calculated as described in Chen et al. (2016) as the residual of epigenetic age regressed on chronological age, technical covariates of collection site, row, and chip, and the proportions of six blood cell types (CD8T, CD4T, NK, B cells, monocytes, and granulocytes) estimated from noob-normalized DNA methylation data with the Houseman algorithm (Houseman et al., 2012). ADNI is a longitudinal study, and participants contributed between 1-3 blood DNAme samples within the 2-year collection period. Because these data collected over two years fell within the median 3.6-year error of Horvath's epigenetic clock, longitudinal evaluation could not be meaningfully conducted. As such, for all epigenetic age measures reported and analyzed herein, we have calculated the average epigenetic age acceleration measure per participant from all available time points, and performed all statistical analyses on these mean values.

To determine if epigenetic age acceleration or intrinsic epigenetic age acceleration differed by sex or dementia diagnosis, we used unbalanced two-way ANOVA designs. APOEε4 genotype and CSF biomarker (amyloid beta, tau and p-tau) data were available from the ADNI repository for a smaller subset of participants with matched EPIC DNAme data, (n=533, see Table 1C). As above, due to the small size of this cohort, we collapsed APOE genotype into two groups: (1) non-carriers of ε4 risk alleles and (2) carriers of ε4 alleles. We subsequently used linear regression to test whether presence of an APOEε4 allele, tau, dementia diagnosis, or sex were significantly associated with epigenetic age acceleration. Amyloid beta and p-tau were not included in this model due to collinearity between amyloid beta, tau, and p-tau CSF concentrations (see Supplementary Table S5).

RESULTS

Demographic information

Table 1 gives a summary of the variables for the participants with: CSF biomarkers (Table 1A; N=279), whole blood EPIC DNAme data (Table 1B; N=640) and matched EPIC data and measured CSF biomarkers (Table 1C; N=533). Given the differences in sample sizes, we performed demographic analyses on the three datasets. In the biomarker dataset, females were significantly younger and had fewer years of education than males ($P<0.01$ and $P<0.0001$, respectively). Although there were no sex differences in distribution of APOEε4 alleles, the proportion of participants in each of the diagnosis categories was significantly different for females and males ($P=0.051$; Table 1A). For the data applicable to the participants with available EPIC DNAme data (Table 1B) and participants with matched EPIC DNAme data and CSF biomarkers (Table 1C), most of the demographics were similar to the biomarker dataset with the

exception that the proportion of participants in each of the diagnosis categories was not significantly different between females and males.

Sex and presence of APOEε4 alleles are associated with changes in inflammatory markers

Our first aim was to investigate whether sex and APOE genotype interact to influence inflammation using biomarkers, which we analysed separately in CSF and plasma (Supplementary Table S1 and S3, respectively). Caution should be noted as inflammatory signalling can differ depending on tissue examined (Bettcher et al., 2018; Setiawan et al., 2015).

For inflammatory markers measured in CSF, only IL-16 and IL-8 elicited a significant interaction between sex and APOE genotype for CSF IL-16 and IL-8 (P= 0.016 and P=0.035, respectively; Table 2). CSF IL-16 and IL-8 levels were significantly lower in females non-carriers of APOEε4 alleles compared to males (both P's<0.001), whereas levels were similar between the sexes in carriers of APOEε4 alleles (P's>0.9; Fig 1 A and B). Furthermore, in females with APOEε4 alleles, IL-16 was significantly higher than in non-APOEε4 female carriers (P=0.050), while a trend was observed in males (P=0.062). Whereas for IL-8, males with APOEε4 alleles had lower levels of IL-8 compared to males with no APOEε4 alleles (P=0.014) but there was no difference in females (P>0.3). Regardless of sex, CSF CRP levels were lower in individuals with 1 or 2 APOEε4 alleles compared to non-carriers (main effect of genotype: P=0.009; Table 2; Fig 1 C). There was a trend for an increase in IL-6 receptor levels in APOEε4 carriers regardless of sex compared to non APOEε4 carriers (main effect of genotype: P=0.071; Table 2). Lastly females had significantly lower CSF levels of IgA and ICAM1 and a trend for lower CSF cortisol levels compared to males (main effect of sex: P<0.001; P=0.009, and

P=0.070, respectively; Table 2). There were no other significant main or interaction effects on any other CSF biomarkers.

For biomarkers measured in plasma, there were no significant interactions between sex and APOE genotype (Supplementary Table S3). However, females had higher plasma CRP levels (main effect of sex: adjusted P=0.048; Fig 1 D) and ICAM1 (main effect of sex: P=0.051) compared to males and significantly lower levels of IL-18 (main effect of sex: P=0.001; Fig 1 E) and immunoglobulin E (IgE: main effect of sex: P<0.001; Fig 1 F) compared to males. Furthermore, plasma CRP decreased in carriers of APOEε4 alleles compared to non-carriers (main effect of genotype: P<0.001; Fig 1 D).

Sex and baseline diagnosis are associated with changes in inflammatory markers

We next tested whether sex and baseline dementia status (CN, MCI, and AD) influenced CSF and plasma biomarkers of inflammation (Supplementary Table S2 and S4, respectively). There were no significant interactions between sex and diagnosis for any of the tested variables in CSF (Table 3 and Supplementary Table S2) or plasma (Supplementary Table S4). For CSF levels, females had significantly lower levels of IgA (main effect of sex: P<0.001) and ICAM1 (main effect of sex: P=0.026) and a trend for lower IL-16 levels (main effect of sex: P=0.055) compared to males (Table 3; Fig 2 A-C), but we did not observe any significant main effects of diagnosis for any CSF variable.

In plasma, we found that females had lower levels of IgE (main effect of sex: P<0.001) and IL-18 compared to males (main effect of sex: P=0.004) and trends for females to have higher levels of ICAM1 (main effect of sex: P=0.056) and CRP (main effect of sex: P=0.056; Fig. 2 D) compared to males. In addition, we found diagnosis significantly influenced plasma cortisol

(main effect of baseline diagnosis: P=0.01) with lower levels in LMCI compared to AD (P<0.001; Fig 2 E). We found trends for diagnosis to influence plasma IL-16, CRP and CD 40 levels (main effect of diagnosis: P=0.054, P=0.056; P=0.067). Plasma IL-16 (P's=0.006) and CRP (P=0.006 and P=0.02) levels were lower in LMCI and AD compared to CN (Fig 2 D and F). For plasma CD 40, levels were lower in LMCI compared to AD (P=0.01; Supplementary Table S4). In summary, although we detected associations between sex and diagnosis and various biomarkers, we did not find evidence of a sex and diagnosis interaction on any variables examined. Furthermore, the results for inflammatory markers in plasma did not always match results in CSF (Supplementary Tables S2 and S4).

Epigenetic age, sex, dementia diagnosis, and AD biomarkers

We investigated the hypothesis that sex and dementia diagnosis affect epigenetic age acceleration in blood samples of ADNI participants (Table 4). Epigenetic age acceleration was not associated with sex, dementia diagnosis (CN, EMCI, LMCI, and AD), nor the interaction of sex and diagnosis after multiple test correction (Fig 3). Intrinsic epigenetic age acceleration was also not significantly associated with participant sex, diagnosis, or their interaction term.

To assess the effect of sex and more broadly defined dementia-associated cognitive impairment on epigenetic age acceleration, we compared epigenetic age acceleration between participants with any form of clinically ascertained cognitive impairment (AD + LMCI + EMCI, N=423, proportion female 41%) and those without (CN, N=217, proportion female 50%). By two-way unbalanced ANOVA neither sex, dementia status, nor their interaction were significantly associated with epigenetic age acceleration after correction for multiple comparisons.

Matched biochemical data including APOEε4 genotype and CSF concentrations of amyloid beta, tau, and p-tau was available for a subset of participants with EPIC DNAme data (N=533). Based on the hypothesis that epigenetic age acceleration may be more strongly associated with concentrations of pathologically relevant compounds than with diagnosis, we assessed the impact of sex, APOEε4 genotype, and CSF tau concentration on epigenetic age acceleration and intrinsic epigenetic age acceleration with linear regression. None of these variables were significantly associated with epigenetic age acceleration (Table 5; results for intrinsic epigenetic age acceleration not shown).

In addition to dementia diagnosis for all participants, we also had access to two composite scores designed by ADNI collaborators to reflect (i) executive function and (ii) memory; these z-scores have been demonstrated to be independently predictive of the transition from MCI to a formal diagnosis of AD (Crane et al., 2012; Gibbons et al., 2012). By two-way unbalanced ANOVA models investigating the effect of sex and memory z-score on epigenetic age acceleration, neither sex (P=0.248), memory score (P=0.486), nor their interaction (P=0.227) were associated with epigenetic age acceleration. In a similar model, neither sex (P=0.260), executive function (P=0.105), or the interaction term of sex and executive function (P=0.153) were associated with epigenetic age acceleration.

DISCUSSION

In the present study using ADNI data from CN, MCI and AD participants we found interactions between sex and APOE genotype (but not between sex and diagnosis) on CSF and plasma levels of IL-8 and IL-16. CSF levels of IL-8 and IL-16 were lower in female APOEε4 non-carriers compared to males but similar levels were found between the sexes with 1 or 2

APOEε4 alleles. Regardless of sex, possessing 1 or 2 APOEε4 alleles was associated with decreased levels of CSF and plasma CRP. Sex differences were seen in inflammatory markers, regardless of diagnosis or genotype, as females had lower CSF cytokines (IL-16, IL-18), CSF ICAM1, CSF and plasma immunoglobulins (IgA, IgE) and plasma IL-18. However, tissue (CSF, plasma) mattered for results from certain inflammatory markers (ICAM1 and to a lesser extent CRP) as females had higher plasma CRP and ICAM1 compared to males, opposite to what was found in CSF, supporting previous studies suggesting differences in inflammatory signals between CSF and blood (Bettcher et al., 2018). Finally, we found no significant differences in epigenetic age acceleration by dementia diagnosis or sex in this cohort of samples. Sex and genotype differences in CSF and plasma inflammatory markers suggest differences in underlying physiology that may affect aging and the progression of AD and this should be considered in future studies. Researchers should be cautioned to use sex as a biological variable in all analyses.

Sex interacts with presence of APOEε4 alleles to affect levels of IL-8 and IL-16

In this study, we found that sex interacted with genotype to influence CSF IL-16 and IL-8. CSF IL-16 and IL-8 levels were lower in females with no APOEε4 alleles compared to males, but no sex differences in these cytokine levels were detected in participants with 1 or 2 ε4 alleles. Our results suggest that presence of APOEε4 alleles can modulate CSF (and potentially plasma) cytokine levels in a sex-dependent way. The APOE protein can regulate transcription in vitro (Theendakara et al., 2018) and APOE4, but not APOE3, increases levels of IL-6 and IL-8 in vitro (Theendakara et al., 2016). In the current study, we found that the sex differences in IL-16 and IL-8 levels disappeared with the presence of 1 or 2 APOEε4 alleles. One possibility is that the APOE4 protein regulates cytokine levels differently in males and females. IL-16 has been

implicated in AD (Rosa et al., 2006) and plasma IL-16 levels decreased with diagnosis (in males and females; current study) and AD severity (analysis without regard to sex; Motta et al., 2007). On the other hand, levels of IL-8 were not affected by diagnosis, consistent with a meta-analysis of cytokines in AD (Swardfager et al., 2010). It is unclear what the impact of regulation of CSF cytokine levels by sex and APOEε4 has on AD symptoms or pathology, however given that females with APOEε4 alleles are disproportionally affected by AD (Hohman et al., 2018; Neu et al., 2017), IL-16 and IL-8 levels are unlikely to be a mechanism for this effect as differences in sex by genotype were noticed in the absence not presence of APOEε4 alleles.

Females have higher CRP levels compared to males and CRP levels are lower in APOEε4 carriers

We found that plasma and CSF levels of CRP, a widely used inflammatory and cardiovascular marker (Koenig et al., 1999; Ridker et al., 1998), were independently affected by sex and APOE genotyoe. Females, regardless of diagnosis or APOEε4 alleles, had significantly higher plasma CRP relative to males, consistent with findings in healthy individuals (Khera et al., 2005). Higher levels of peripheral CRP may suggest higher systemic inflammation in females, which is associated with an increased risk in all-cause dementia (Koyama et al., 2013). Higher levels of serum CRP are also associated with higher levels of serum estradiol in postmenopausal healthy females (Eldridge et al., 2020) which suggests that sex differences in CRP levels may be partly due to sex differences in estradiol levels. In addition, we found that the presence of APOEε4 alleles decreased plasma and CSF CRP levels consistent with previous research in large population studies (Hubacek et al., 2010; Yun et al., 2015). In our study, we also found a trend for lower levels of plasma CRP with LMCI and AD compared to CN. Recent

meta-analyses did not find differences in peripheral levels of CRP in AD compared to control patients (Gong et al., 2016; Ng et al., 2018). However, in patients with mild and moderate dementia only, serum CRP levels were lower compared to the cognitively normal group (Gong et al., 2016). In healthy individuals, higher levels of plasma CRP in mid-life are associated with a higher amyloid burden later in life in males but not females (Walker et al., 2018). However, despite this finding, higher systemic inflammation (including CRP) was associated with greater cognitive decline later in life in both sexes in healthy individuals (Walker et al., 2019). It is possible that sex differences in inflammation earlier in life have long-term effects on the transition to MCI and/or AD.

Females have lower cytokine and immunoglobulin levels compared to males

We found some biomarkers that were affected by sex, but not diagnosis or presence of APOEε4 alleles. For example, females had lower CSF levels of ICAM1 compared to males, regardless of APOE genotype or diagnosis, but, although a trend, the opposite effect was seen in plasma. In contrast, in healthy adults (18-55 years old), serum levels of ICAM1 are lower in females compared to males (Ponthieux et al., 2003). ICAM1 is a type of adhesion molecule associated with microvascular endothelial activation (Zenaro et al., 2017) and plasma ICAM1 levels (but not CSF levels, Nielsen et al., 2007) were higher in patients with AD (Huang et al., 2015; Nielsen et al., 2007; Rentzos et al., 2004). Although in the present study we did not observe a significant effect of plasma ICAM1 with diagnosis, the unadjusted P-value was 0.063 with higher levels in LMCI and AD groups. It is intriguing that females have lower CSF levels of cytokines (IL-16, IL-18), ICAM1, and immunoglobulins (IgE and IgA) but higher plasma CRP and ICAM1 levels. Neuroinflammation is associated with AD but it can have both beneficial and

detrimental roles (Walters et al., 2016) as increased expression of pro-inflammatory cytokines contributes to neuronal loss, while anti-inflammatory effects contribute to amyloid beta clearance (Heneka et al., 2015). It is possible that males and females have varying levels of beneficial vs detrimental immune responses which can affect how the disease progresses in each of the sexes but it is also important to remember that CSF levels may not match levels in different tissues (blood) or regions of the brain.

Sex, AD and biochemical markers do not affect blood epigenetic age acceleration

We did not observe an association between either sex or diagnosis and epigenetic age acceleration or intrinsic epigenetic age acceleration. To our knowledge, no other study has similarly probed epigenetic age acceleration in a peripheral tissue in the presence of AD, or whether epigenetic age acceleration in AD is sex-specific.

This study was partially undertaken to investigate whether epigenetic age acceleration that has been associated with the AD brain is reflected in peripheral tissues. Levine et al. (Levine et al., 2015) have previously demonstrated increased epigenetic age acceleration in AD, however Levine's study was conducted on post-mortem prefrontal cortex tissue, and did not explicitly investigate the role of sex in epigenetic age acceleration (Levine et al., 2015). While brain-blood global DNA methylation profiles are reasonably correlated (r=0.86) (Braun et al., 2019), DNA methylation profiles of peripheral tissues are imperfect representatives of the brain, and do not recapitulate all epigenetic alterations with high fidelity. Thus, our findings do not contradict the finding of increased epigenetic age acceleration in the presence of AD in the prefrontal cortex, but suggest that accelerated epigenetic aging in AD is not a pan-tissue phenomenon. Our finding of a lack of significant association between AD, biological sex, and epigenetic age acceleration

in whole blood DNA methylation profiles could suggest a tissue-specific dysregulation of an epigenetic maintenance system, in which the brain epigenome is most strongly affected by AD (Levine et al., 2015). The phenotype of patients affected by AD and global gene expression patterns of the APOE protein, with high expression in brain, and low expression in whole blood (GTEx Project, 2018) further support this hypothesis.

Intriguingly, epigenetic age was observed to be lower on average than chronological age. Horvath's epigenetic clock was trained on DNAme data from older versions of the Illumina DNAme arrays with more limited genomic coverage; 19 of the 353 CpG sites required to calculate epigenetic age via Horvath's algorithm do not exist on the newer EPIC array. Two previous studies investigated the application of Horvath's epigenetic clock to EPIC data with conflicting results (Dhingra et al., 2019; McEwen et al., 2018), the largest issue being chronic underestimation of epigenetic age due to the positive linear regression coefficients associated with the missing probes (Dhingra et al., 2019). Both imputing and removing the missing probes from the array resulted in a chronic underprediction of epigenetic age with Horvath's clock suggesting that this is likely an artefact of the array platform and truncated probe-set rather than the method chosen to deal with missing values, although it is possible that an adjustment factor could be devised to more accurately apply Horvath's clock to EPIC data. In future explorations of epigenetic age with EPIC DNAme array data this should be considered, as there are other epigenetic age predictors available that have been trained on EPIC data such as the PhenoAge and GrimAge clocks, although these tools have limitations as well; for example, both PhenoAge and GrimAge were trained only on blood DNAme data, as compared to the original pan-tissue epigenetic clocks, and therefore may have limited applicability and relevance in other tissues (Levine et al., 2018; Lu et al., 2019).

Limitations

The ADNI cohort is not ethnically or socioeconomically diverse, being mostly composed of self-reported white (only 12 individuals were not-white) and highly educated individuals (average 15.69 years of education). As AD incidence, prevalence, and age of onset varies by ethnicity (Fitten et al., 2014; Mayeda et al., 2016; Steenland et al., 2016) and education (Sharp and Gatz, 2011), our conclusions may not apply to more ethnically and socially diverse populations. In addition to sex, it is possible the underlying mechanisms of AD are different depending on ethnicity. APOEε4 genotype is a well-known AD risk factor and in this cohort the proportion of participants in the different APOEε4 allele groups is correlated with diagnosis (Table S6). Future research with a larger cohort is required to test how sex, APOE genotype and diagnosis interact together in one model. Even in the larger 640-participant cohort, only 37 participants (5.78%) had an AD diagnosis, so statistical analyses were underpowered to detect subtle differences by diagnosis group, which may account for our lack of interaction between sex and diagnosis. Additionally, other pathologies in these participants, such as cancer, cardiovascular disease, smoking status, or obesity may have influenced inflammatory markers and epigenomes and limited our interpretations.

CONCLUSION

The current study provides evidence that sex and presence of APOEε4 alleles are associated with CSF levels of the inflammatory markers IL-16 and IL-8. We found sex differences indicating that females had lower cytokine and immunoglobulin levels but higher plasma CRP and ICAM1 levels compared to males, although the direction of the ICAM1 finding

was tissue-dependent. There were no significant associations between sex, APOEε4 alleles or diagnosis and epigenetic age. Together, our work suggests that that presence of APOEε4 alleles can affect cytokine levels differently in males and females and the underlying pathophysiology of aging and AD may be tissue- and sex-specific.

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CONFLICT OF INTEREST

The authors have no conflict of interest to report.

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FIGURE CAPTIONS

Figure 1. Marginal mean (±95% confidence interval) of **CSF levels** of A. IL-16 (pg/ml), B. IL-8 (pg/ml), C. C-reactive protein (CRP; µg/ml), and **plasma levels** of D. CRP (CRP; µg/ml), E. IL-18 (pg/ml), and F. IgE (ng/ml) in ADNI participants by sex and presence or absence of APOEε4 alleles (none, or 1 and 2 alleles).

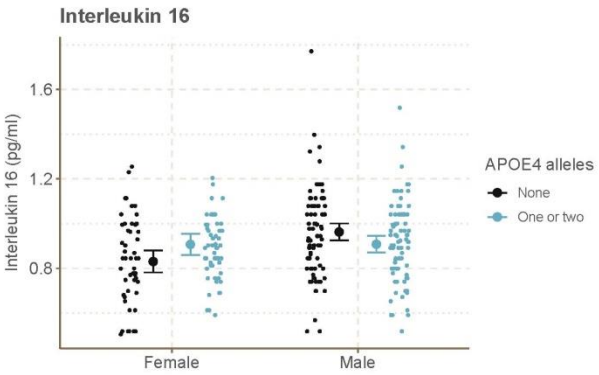
Figure 2. Marginal mean (± 95% confidence interval) of **CSF levels** of A. IL-16 (pg/ml), B. IgA (mg/ml), C. Intercellular adhesion molecule (ICAM1; ng/ml), and **plasma levels** of D. C-reactive protein (CRP; µg/ml), E. cortisol (ng/ml), and F. IL-16 in ADNI participants by sex and diagnosis (CN, cognitively normal; LMCI, late mild cognitive impairment; and AD, Alzheimer's disease).

Figure 3. Universal epigenetic age acceleration does not differ statistically significantly by participant sex or diagnosis (CN, EMCI, LMCI, AD) in this ADNI cohort. CN, cognitively normal; EMCI, early mild cognitive impairment; LMCI, late mild cognitive impairment; AD, Alzheimer's disease.

Figure 1

CSF

A



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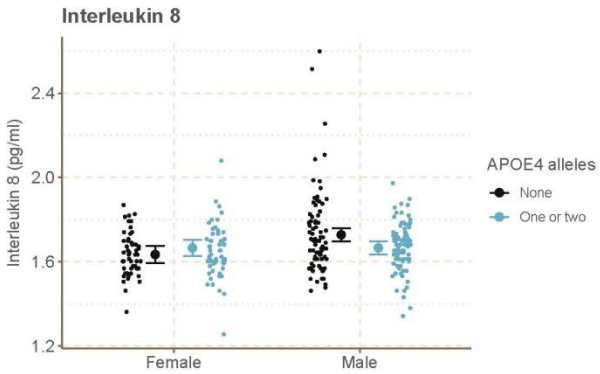

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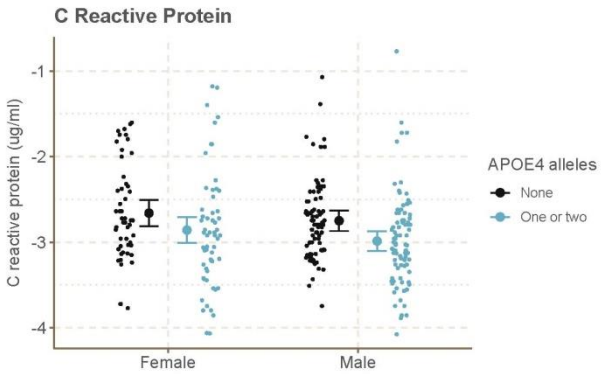
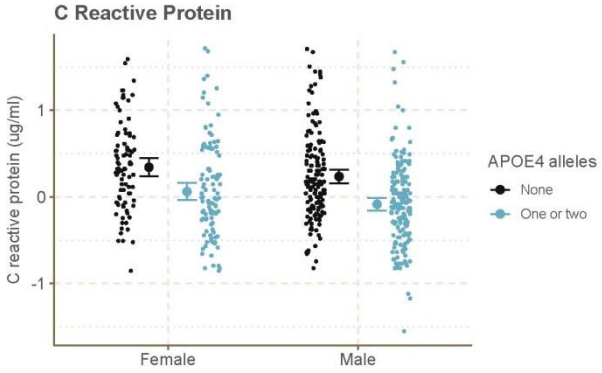


Plasma

D



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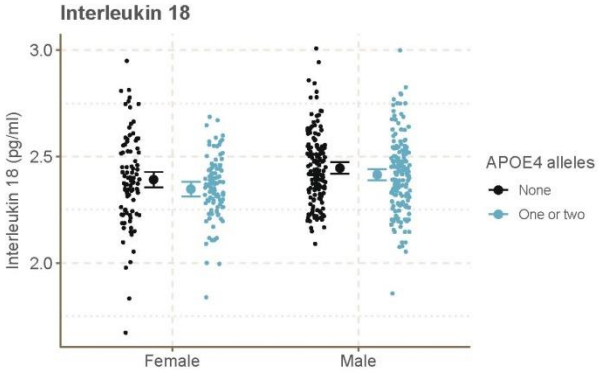


F

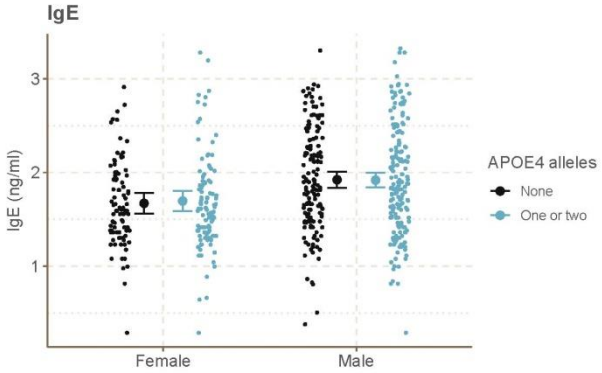
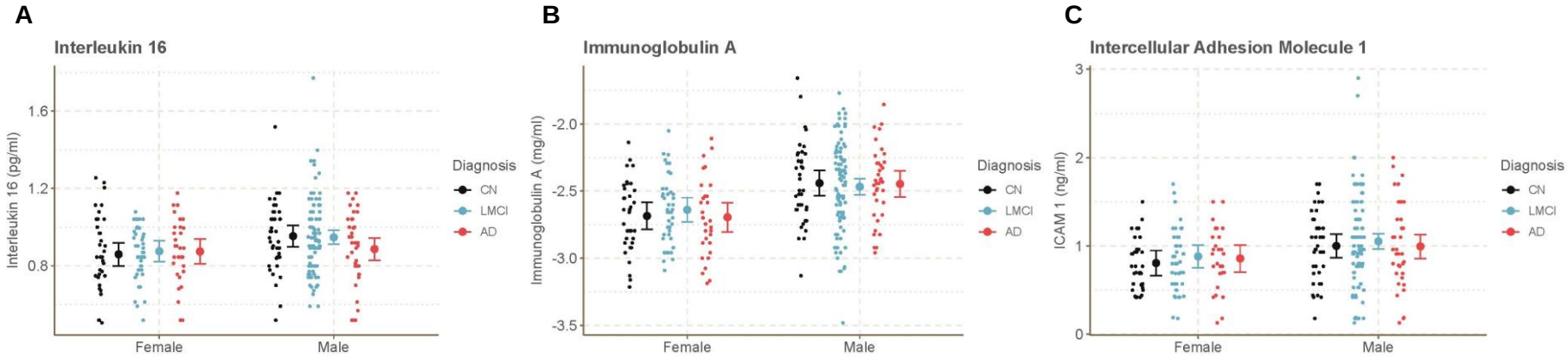


Figure 2

CSF



Plasma

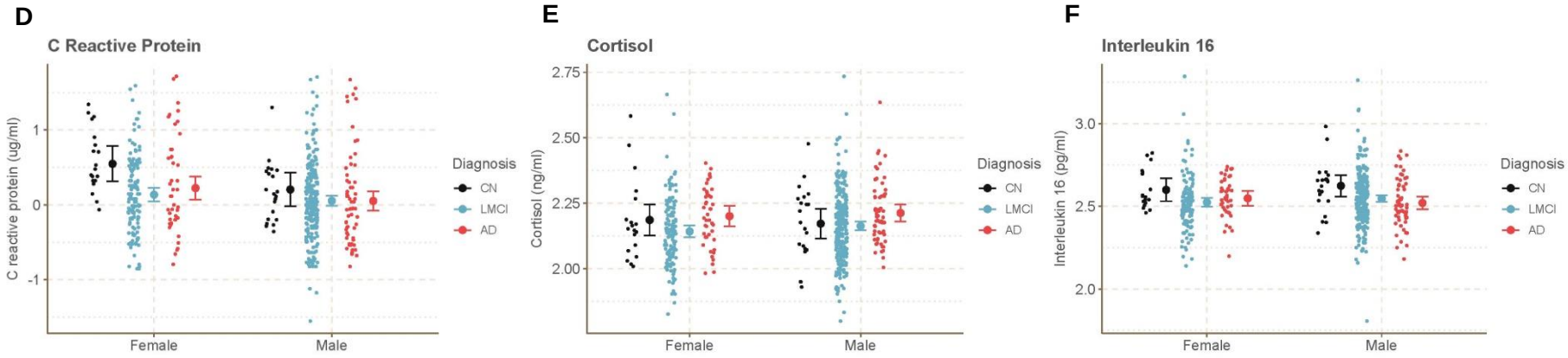


Figure 3

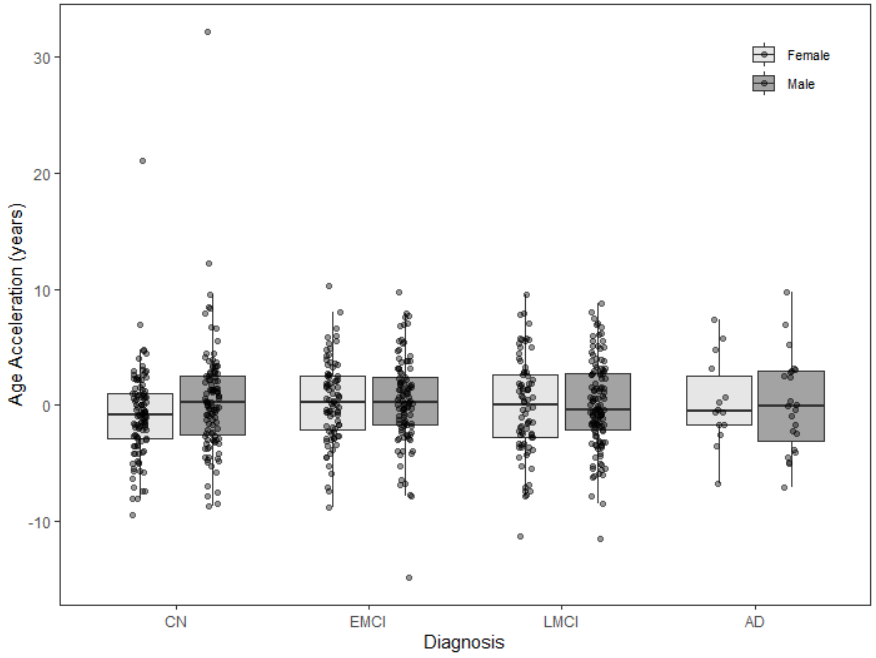

Female
Male
30
20
10
0
-10
Age Acceleration (years)
CN
EMCI
LMCI
AD
Diagnosis

Table 1. Demographic and clinical information for all ADNI participants subdivided by sex. A. Participants with measured biomarkers in cerebrospinal fluid (CSF), B. Participants with available whole blood Illumina HumanMethylationEPIC DNA methylation data, C. Participants with matched Illumina HumanMethylationEPIC DNA methylation array data and measured CSF biomarkers. In all three subdata sets, females were significantly younger and had fewer years of education than males. In data set A (but not B and C), more females (24.0 % compared to 21.8%) were diagnosed with AD, more females were cognitively normal (26.5% compared to 22.9%) and fewer females were diagnosed with late MCI compared to males (49.5% compared to 55.3%). In data set A, females had lower CSF cortisol, interleukin-3, interleukin 8, interleukin-16, immunoglobulin A, and intercellular adhesion molecule 1 compared to males. Empty cells indicate data not available or not analysed.

	A				**B**				**C**			
		Sex				**Sex**				**Sex**		
	Total No. 279	**Female No. 109**	**Male No. 170**	**P-value**	**Total No. 640**	**Female No. 284**	**Male No. 356**	**P-value**	**Total No. 533**	**Female No. 243**	**Male No. 290**	**P-value**
Age												
Mean (SD)	75.15 (±6.86)	73.75 (±6.69)	76.04 (±6.83)	0.007	75.63 (±7.68)	74.78 (±8.03)	76.31 (±7.32)	<0.0001	75.01 (±7.61)	74.31 (±8.10)	75.61 (±7.11)	0.0019
Education (years)												
Mean (SD)	15.69 (±2.95)	14.68 (±2.74)	16.34 (±2.90)	< 0.0001	16.21 (±2.70)	15.53 (±2.59)	16.75 (±2.68)	<0.0001	16.24 (±2.64)	15.57 (±2.49)	16.83 (±2.64)	< 0.0001
Ethnicity												
White	267 (95.70%)	103 (94.50%)	164 (96.47%)	0.55	627 (97.97%)	279 (98.23 %)	348 (97.75%)	0.78	521 (97.75 %)	238 (97.94 %)	283 (97.94 %)	0.99
Not White[ł]	12 (4.30%)	6 (5.50%)	6 (3.53%)		13 (2.03%)	5 (1.76%)	8 (2.25%)		12 (2.25 %)	5 (2.06 %)	7 (2.41 %)	
Baseline diagnosis												
CN	74 (26.5%)	35 (32.1%)	39 (22.9%)	0.051	217 (33.9%)	109 (38.38%)	108 (30.34%)	0.11	171 (32.08 %)	88 (36.21 %)	83 (28.62 %)	0.19
EMCI					186 (29.06%)	83 (29.23%)	103 (28.93%)		173 (32.46 %)	79 (32.51 %)	94 (32.41 %)	
LMCI	138 (49.5%)	44 (40.4%)	94 (55.3%)		200 (31.25%)	78 (27.46%)	122 (34.27%)		155 (29.08 %)	94 (38.68 %)	92 (31.72 %)	
AD	67 (24.0%)	30 (27.5%)	37 (21.8%)		37 (5.78%)	14 (4.23 %)	23 (6.46%)		34 (6.38 %)	13 (5.35 %)	21 (7.24 %)	
APOEε4 allele number												
0	134 (48.03%)	51 (46.79%)	83 (48.82%)	0.78	369 (57.66 %)	169 (59.51%)	200 (56.18%)	0.37	313 (58.72 %)	146 (60.08 %)	167 (57.59 %)	0.45
1	109 (39.07%)	42 (38.53%)	67 (39.41%)		220 (34.38%)	97 (34.15%)	123 (34.55%)		173 (32.46%)	80 (32.92 %)	93 (32.07 %)	
2	36 (12.90%)	16 (14.68%)	20 (11.76%)		51 (7.97%)	18 (6.34%)	33 (9.27%)		47 (8.82%)	17 (7.00%)	30 (10.34 %)	
Cortisol (ng/mL)												
Mean (SD)	16.05 (±6.04)	14.92 (±6.01)	16.78 (±5.96)	0.008								
C reactive protein (ug/mL)												
Mean (SD)	-2.83 (±0.56)	-2.77 (±0.64)	-2.87 (±0.51)	0.23								
CD40 antigen (ng/mL)												
Mean (SD)	-0.65 (±0.12)	-0.66 (±0.10)	-0.64 (±0.14)	0.12								
Interleukin 16 (pg/mL)												
Mean (SD)	0.91 (±0.18)	0.87 (±0.17)	0.94 (±0.19)	0.004								
Interleukin 3 (ng/mL)												
Mean (SD)	-2.22 (±0.32)	-2.28 (±0.29)	-2.17 (±0.34)	0.001								
Interleukin 6 receptor (ng/mL)												
Mean (SD)	-0.01 (±0.15)	-0.02 (±0.14)	-0.00 (±0.15)	0.30								
Interleukin 8 (pg/mL)												
Mean (SD)	1.68 (±0.15)	1.64 (±0.11)	1.70 (±0.16)	0.001								
Intercellular adhesion molecule 1 (ng/mL)												
Mean (SD)	0.96 (±0.44)	0.83 (±0.33)	1.04 (±0.48)	0.0001								
Immunoglobulin A (mg/mL)												
Mean (SD)	-2.54 (±0.31)	-2.68 (±0.26)	-2.45 (±0.31)	< 0.0001								
Amyloid Beta												
Mean (SD)									1040.98 (±454.72)	1055.50 (±449.23)	1028.35 (±459.36)	0.18
Tau												
Mean (SD)									289.80 (±124.68)	300.90 (±139.07)	280.13 (±109.82)	0.072
PTau												
Mean (SD)									27.47 (±13.65)	28.25 (±15.08)	26.78 (±12.24)	0.36

P-values are from Wilcoxon rank sum tests for continuous variables and Fisher's exact tests for categorical variables. łIncludes self-reported Black, Asian, American Indian/Alaskan, and >1 ethnicity.

Table 2. Linear regression results for models with sex and APOE status. P-values are for overall tests and are FDR-adjusted. Only shown are the models with significant associations (adjusted P<0.05) and trends (adjusted P<0.08). All model summaries are available in Supplementary Table S1.

	Cortisol Cortisol ng ml			**C Reactive Protein ug/ml**			**Interleukin 16 pg/ml**			**Interleukin 6 receptor ng/ml**			**Interleukin 8 pg/ml**			**Immunoglobulin A mg/ml**			**Intercellular Adhesion Molecule ng/ml**		
Predictors	*Estimates (CI)*	*p*	*adjusted p*	*Estimates (CI)*	*p*	*adjusted p*	*Estimates (CI)*	*p*	*adjusted p*	*Estimates (CI)*	*p*	*adjusted p*	*Estimates (CI)*	*p*	*adjusted p*	*Estimates (CI)*	*p*	*adjusted p*	*Estimates (CI)*	*p*	*adjusted p*
(Intercept)	1.43 (-7.00 – 9.87)			-3.03 (-3.84 – -2.23)			0.42 (0.15 – 0.68)			-0.27 (-0.48 – -0.06)			1.33 (1.11 – 1.54)			-2.91 (-3.35 – -2.48)			-0.23 (-0.83 – 0.38)		
AGE (years)	0.22 (0.12 – 0.32)			0.01 (-0.00 – 0.02)			0.01 (0.00 – 0.01)			0.00 (0.00 – 0.01)			0.00 (0.00 – 0.01)			0.00 (-0.00 – 0.01)			0.01 (0.01 – 0.02)		
EDUCATION (years)	-0.21 (-0.45 – 0.03)			-0.01 (-0.03 – 0.02)			-0.01 (-0.01 – 0.00)			-0.00 (-0.01 – 0.00)			0.00 (-0.00 – 0.01)			0.00 (-0.01 – 0.01)			-0.00 (-0.02 – 0.02)		
Male (ref = Female)	1.72 (0.25 – 3.19)	0.022	**0.07**	-0.11 (-0.25 – 0.03)	0.126	0.22	0.13 (0.07 – 0.19)			0.02 (-0.02 – 0.05)	0.372	0.5	0.09 (0.04 – 0.14)	<0.001		0.21 (0.14 – 0.29)	<0.001	**<0.001**	0.18 (0.07 – 0.28)	0.001	**0.009**
APOE4 1 or 2 alleles (ref = 0 alleles)	0.82 (-0.55 – 2.19)	0.241	0.35	-0.22 (-0.35 – -0.09)	0.001	**0.009**	0.08 (0.01 – 0.14)			0.04 (0.00 – 0.07)	0.025	**0.071**	0.03 (-0.02 – 0.09)	0.261		-0.00 (-0.07 – 0.07)	0.898	0.97	0.08 (-0.02 – 0.18)	0.128	0.22
Interaction: Male by 1 or 2 alleles							-0.13 (-0.22 – -0.05)	0.003	**0.016**				-0.09 (-0.16 – -0.02)	**0.008**	**0.035**						
Observations	279			279			279			279			279			279			279		
R^2 / adjusted R^2	0.095 / 0.082			0.055 / 0.042			0.122 / 0.106			0.046 / 0.032			0.092 / 0.076			0.123 / 0.111			0.105 / 0.092		

Table 3. Linear regression results for models with sex and baseline diagnosis. Only shown are the models with significant associations. P-values are for overall tests and are FDR-adjusted. All model summaries are available in Supplementary Table S2.

	Interleukin 16 pg/ml			**Immunoglobulin A mg/ml**			**Intercellular Adhesion Molecule ng/ml**		
Predictors	*Estimates (CI)*	*p*	*adjusted p*	*Estimates (CI)*	*p*	*adjusted p*	*Estimates (CI)*	*p*	*adjusted p*
(Intercept)	0.51 (0.26 – 0.77)			-2.92 (-3.35 – -2.48)			-0.19 (-0.80 – 0.42)		
AGE (years)	0.01 (0.00 – 0.01)			0.00 (-0.00 – 0.01)			0.01 (0.01 – 0.02)		
EDUCATION (years)	-0.01 (-0.01 – 0.00)			0.00 (-0.01 – 0.01)			-0.00 (-0.02 – 0.02)		
Male (ref = Female)	0.06 (0.02 – 0.11)	0.007	**0.055**	0.21 (0.14 – 0.29)	<0.001	**<0.001**	0.17 (0.06 – 0.28)	0.002	**0.026**
Diagnosis (ref = CN)		0.4	0.61		0.99	0.99		0.54	0.67
LMCI	0.01 (-0.05 – 0.06)			0.00 (-0.08 – 0.09)			0.06 (-0.06 – 0.18)		
AD	-0.03 (-0.09 – 0.03)			-0.01 (-0.11 – 0.09)			0.02 (-0.12 – 0.16)		
Observations	279			279			279		
R^2 / adjusted R^2	0.099 / 0.082			0.124 / 0.107			0.101 / 0.085		

Table 4. Results of epigenetic age and epigenetic age acceleration calculation for all DNAme analyses, for both the larger DNAme cohort and the subset of samples with matched CSF biomarker data.

	DNAme Cohort				DNAme & CSF Biomarker Data Cohort			
		Sex				Sex		
	Total No. 640	**Female No. 284**	**Male No. 356**	**P-value**	**Total No. 533**	**Female No. 243**	**Male No. 290**	**P-value**
Age								
Mean (SD)	75.63 (±7.68)	74.78 (±8.03)	76.31 (±7.32)	<0.0001	75.01 (±7.61)	74.31 (±8.10)	75.61 (±7.11)	0.0019
Epigenetic Age (years)								
Mean (SD)	69.92 (±8.06)	67.45 (±8.15)	70.11 (±7.79)	<0.0001	68.47 (±8.17)	67.05 (±8.33)	69.72 (±7.82)	<0.0001
Epigenetic Age Acceleration (years)								
Mean (SD)	0.025 (±4.22)	-0.14 (±4.16)	0.16 (±4.26)	0.1	0.027 (±4.30)	-0.18 (±4.23)	0.20 (4.35)	0.057
Intrinsic Age Acceleration (years)								
Mean (SD)	0.026 (±4.11)	-0.19 (±4.06)	0.20 (±4.15)	0.019	0.020 (±4.18)	-0.25 (±4.14)	0.26 (±4.21)	0.021

P-values are from Wilcoxon rank sum tests for continuous variables and Fisher's exact tests for categorical variables

Table 5. Linear model for assessment of relationship of biochemical concentrations and presence of APOE ε4 risk allele on universal epigenetic age acceleration. Intrinsic epigenetic age acceleration linear model not shown.

	Age Acceleration & CSF Biomarkers		
Predictors	*Estimates*	*CI*	*adjusted p*
(Intercept)	-0.61	-1.67 – 0.46	0.61
Male (ref = Female)	0.6	-0.11 – 1.31	0.34
Diagnosis (ref = CN)			
EMCI	0.71	-0.17 – 1.60	0.39
LMCI	0.4	-0.55 – 1.36	0.64
AD	0.53	-1.07 – 2.14	0.64
APOE risk allele			
1 or 2 alleles (ref = 0 alleles)	-0.24	-1.01 – 1.31	0.813
CSF Tau	0.0001	-0.0077 – 0.023	0.95
Observations	533		
R^2 / adjusted R^2	0.0108/-0.000438		

Supplemental File

Inflammation and epigenetic age in Alzheimer's disease: do sex and APOE matter?

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Table S1. Linear regression results for all variables investigated by sex and APOE status. Markers in CSF

	Cortisol Cortisol ng ml			**C Reactive Protein ug/ml**			**CD 40 antigen ng/ml**			**Interleukin 16 pg/ml**			**Interleukin 3 ng/ml**		
Predictors	*Estimates (CI)*	*p*	*adjusted p*	*Estimates (CI)*	*p*	*adjusted p*	*Estimates (CI)*	*p*	*adjusted p*	*Estimates (CI)*	*p*	*adjusted p*	*Estimates (CI)*	*p*	*adjusted p*
(Intercept)	1.43 (-7.00 – 9.87)			-3.03 (-3.84 – -2.23)			-1.10 (-1.27 – -0.93)			0.42 (0.15 – 0.68)			-3.03 (-3.49 – -2.57)		
AGE (years)	0.22 (0.12 – 0.32)			0.01 (-0.00 – 0.02)			0.01 (0.00 – 0.01)			0.01 (0.00 – 0.01)			0.01 (0.00 – 0.02)		
EDUCATION (years)	-0.21 (-0.45 – 0.03)			-0.01 (-0.03 – 0.02)			-0.00 (-0.01 – 0.00)			-0.01 (-0.01 – 0.00)			0.00 (-0.01 – 0.01)		
Male (ref = Female)	1.72 (0.25 – 3.19)	0.022	**0.07**	-0.11 (-0.25 – 0.03)	0.126	0.22	0.01 (-0.02 – 0.04)	0.432	0.55	0.13 (0.07 – 0.19)			0.08 (-0.00 – 0.16)	0.057	0.15
APOE4 1 or 2 alleles (ref = 0 alleles)	0.82 (-0.55 – 2.19)	0.241	0.35	-0.22 (-0.35 – -0.09)	0.001	**0.009**	0.01 (-0.02 – 0.04)	0.59	0.72	0.08 (0.01 – 0.14)			-0.04 (-0.12 – 0.03)	0.262	0.37
Interaction: Male by 1 or 2 alleles										-0.13 (-0.22 – -0.05)	0.003	**0.016**			
Observations	279			279			279			279			279		
R^2 / adjusted R^2	0.095 / 0.082			0.055 / 0.042			0.125 / 0.112			0.122 / 0.106			0.076 / 0.063		

Table S1. Continued

	Interleukin 6 receptor ng/ml			**Interleukin 8 pg/ml**			**Immunoglobulin A mg/ml**			**Intercellular Adhesion Molecule ng/ml**		
Predictors	*Estimates (CI)*	*p*	*adjusted p*	*Estimates (CI)*	*p*	*adjusted p*	*Estimates (CI)*	*p*	*adjusted p*	*Estimates (CI)*	*p*	*adjusted p*
(Intercept)	-0.27 (-0.48 – -0.06)			1.33 (1.11 – 1.54)			-2.91 (-3.35 – -2.48)			-0.23 (-0.83 – 0.38)		
AGE (years)	0.00 (0.00 – 0.01)			0.00 (0.00 – 0.01)			0.00 (-0.00 – 0.01)			0.01 (0.01 – 0.02)		
EDUCATION (years)	-0.00 (-0.01 – 0.00)			0.00 (-0.00 – 0.01)			0.00 (-0.01 – 0.01)			-0.00 (-0.02 – 0.02)		
Male (ref = Female)	0.02 (-0.02 – 0.05)	0.372	0.5	0.09 (0.04 – 0.14)	<0.001		0.21 (0.14 – 0.29)	<0.001	**<0.001**	0.18 (0.07 – 0.28)	0.001	**0.009**
APOE4 1 or 2 alleles (ref = 0 alleles)	0.04 (0.00 – 0.07)	0.025	**0.071**	0.03 (-0.02 – 0.09)	0.261		-0.00 (-0.07 – 0.07)	0.898	0.97	0.08 (-0.02 – 0.18)	0.128	0.22
Interaction: Male by 1 or 2 alleles				-0.09 (-0.16 – -0.02)	0.008	**0.035**						
Observations	279			279			279			279		
R^2 / adjusted R^2	0.046 / 0.032			0.092 / 0.076			0.123 / 0.111			0.105 / 0.092		

Table S2. Linear regression results for all variables investigated by sex and baseline diagnosis. Markers in CSF

	Cortisol Cortisol ng ml			**C Reactive Protein ug/ml**			**CD 40 antigen ng/ml**			**Interleukin 16 pg/ml**			**Interleukin 3 ng/ml**		
Predictors	*Estimates (CI)*	*p*	*adjusted p*	*Estimates (CI)*	*p*	*adjusted p*	*Estimates (CI)*	*p*	*adjusted p*	*Estimates (CI)*	*p*	*adjusted p*	*Estimates (CI)*	*p*	*adjusted p*
(Intercept)	1.49 (-6.96 – 9.95)			-3.12 (-3.94 – -2.30)			-1.08 (-1.25 – -0.91)			0.51 (0.26 – 0.77)			-3.03 (-3.49 – -2.57)		
AGE (years)	0.22 (0.12 – 0.32)			0.01 (-0.00 – 0.02)			0.01 (0.00 – 0.01)			0.01 (0.00 – 0.01)			0.01 (0.00 – 0.02)		
EDUCATION (years)	-0.23 (-0.47 – 0.01)			-0.00 (-0.03 – 0.02)			-0.00 (-0.01 – 0.00)			-0.01 (-0.01 – 0.00)			0.00 (-0.01 – 0.01)		
Male (ref = Female)	1.55 (0.07 – 3.04)	0.041	0.18	-0.10 (-0.24 – 0.05)	0.19	0.42	0.01 (-0.02 – 0.04)	0.51	0.65	0.06 (0.02 – 0.11)	0.007	**0.055**	0.08 (-0.00 – 0.16)	0.052	0.18
Diagnosis (ref = CN)		0.24	0.49		0.15	0.36		0.067	0.2		0.4	0.61		0.5	0.65
LMCI	1.23 (-0.43 – 2.88)			-0.15 (-0.31 – 0.01)			0.01 (-0.03 – 0.04)			0.01 (-0.05 – 0.06)			-0.04 (-0.13 – 0.05)		
AD	0.10 (-1.83 – 2.02)			-0.15 (-0.34 – 0.03)			-0.03 (-0.07 – 0.00)			-0.03 (-0.09 – 0.03)			-0.06 (-0.17 – 0.04)		
Observations	279			279			279			279			279		
R^2 / adjusted R^2	0.100 / 0.084			0.030 / 0.012			0.141 / 0.125			0.099 / 0.082			0.077 / 0.060		

Table S2. Continued

	Interleukin 6 receptor ng/ml			**Interleukin 8 pg/ml**			**Immunoglobulin A mg/ml**			**Intercellular Adhesion Molecule ng/ml**		
Predictors	*Estimates (CI)*	*p*	*adjusted p*	*Estimates (CI)*	*p*	*adjusted p*	*Estimates (CI)*	*p*	*adjusted p*	*Estimates (CI)*	*p*	*adjusted p*
(Intercept)	-0.22 (-0.43 – -0.01)			1.35 (1.14 – 1.56)			-2.92 (-3.35 – -2.48)			-0.19 (-0.80 – 0.42)		
AGE (years)	0.00 (0.00 – 0.01)			0.00 (0.00 – 0.01)			0.00 (-0.00 – 0.01)			0.01 (0.01 – 0.02)		
EDUCATION (years)	-0.00 (-0.01 – 0.00)			0.00 (-0.00 – 0.01)			0.00 (-0.01 – 0.01)			-0.00 (-0.02 – 0.02)		
Male (ref = Female)	0.02 (-0.02 – 0.05)	0.42	0.61	0.04 (0.01 – 0.08)	0.019	0.12	0.21 (0.14 – 0.29)	<0.001	**<0.001**	0.17 (0.06 – 0.28)	0.002	**0.026**
Diagnosis (ref = CN)		0.46	0.65		0.85	0.96		0.99	0.99		0.54	0.67
LMCI	0.01 (-0.04 – 0.05)			0.01 (-0.03 – 0.05)			0.00 (-0.08 – 0.09)			0.06 (-0.06 – 0.18)		
AD	-0.02 (-0.07 – 0.03)			0.01 (-0.03 – 0.06)			-0.01 (-0.11 – 0.09)			0.02 (-0.12 – 0.16)		
Observations	279			279			279			279		
R^2 / adjusted R^2	0.034 / 0.016			0.062 / 0.045			0.124 / 0.107			0.101 / 0.085		

Table S3. Linear regression results for all plasma variables investigated by sex and APOE genotype

	Cortisol Cortisol ng ml			**C Reactive Protein ug/ml**			**CD 40 antigen ng/ml**			**Interleukin 16 pg/ml**			**Interleukin 3 ng/ml**		
Predictors	*Estimates (CI)*	*p*	*adjusted p*	*Estimates (CI)*	*p*	*adjusted p*	*Estimates (CI)*	*p*	*adjusted p*	*Estimates (CI)*	*p*	*adjusted p*	*Estimates (CI)*	*p*	*adjusted p*
(Intercept)	2.06 (1.92 – 2.19)			0.46 (-0.05 – 0.97)			-0.52 (-0.65 – -0.39)			2.42 (2.27 – 2.58)			-1.86 (-2.16 – -1.57)		
AGE (years)	0.00 (-0.00 – 0.00)			0.00 (-0.00 – 0.01)			0.01 (0.00 – 0.01)			0.00 (0.00 – 0.00)			0.00 (-0.00 – 0.00)		
EDUCATION (years)	-0.00 (-0.00 – 0.00)			-0.03 (-0.04 – -0.01)			-0.00 (-0.01 – 0.00)			-0.01 (-0.01 – -0.00)			0.01 (0.00 – 0.02)		
Male (ref = Female)	0.01 (-0.01 – 0.04)	0.26	0.57	-0.13 (-0.22 – -0.04)	0.006	**0.048**	-0.04 (-0.07 – -0.01)			0.01 (-0.02 – 0.04)	0.50	0.79	-0.02 (-0.07 – 0.04)	0.57	0.8
APOE4 1 or 2 alleles (ref = 0 alleles)	0.02 (-0.00 – 0.04)	0.11	0.29	-0.31 (-0.39 – -0.22)	<0.001	**<0.001**	-0.03 (-0.07 – 0.00)			-0.01 (-0.04 – 0.01)	0.31	0.66	0.02 (-0.03 – 0.07)	0.43	0.67
Interaction: Male by 1 or 2 alleles							0.05 (0.01 – 0.10)	**0.022**	0.11						
Observations	527			526			527			527			527		
R^2 / adjusted R^2	0.013 / 0.005			0.123 / 0.117			0.126 / 0.118			0.042 / 0.033			0.011 / 0.004		

Table S3. Continued

	Interleukin 6 receptor ng/ml			**Interleukin 8 pg/ml**			**Immunoglobulin A mg/ml**			**Intercellular Adhesion Molecule ng/ml**			**Interleukin 18 pg/ml**			**Immunoglobulin E ng/ml**		
Predictors	*Estimates (CI)*	*p*	*adjusted p*	*Estimates (CI)*	*p*	*adjusted p*	*Estimates (CI)*	*p*	*adjusted p*	*Estimates (CI)*	*p*	*adjusted p*	*Estimates (CI)*	*p*	*adjusted p*	*Estimates (CI)*	*p*	*adjusted p*
(Intercept)	1.43 (1.29 – 1.57)			0.79 (0.60 – 0.99)			0.48 (0.25 – 0.71)			1.89 (1.75 – 2.04)			2.42 (2.24 – 2.59)			1.42 (0.88 – 1.97)		
AGE (years)	0.00 (-0.00 – 0.00)			0.00 (0.00 – 0.01)			0.00 (-0.00 – 0.00)			0.00 (0.00 – 0.00)			-0.00 (-0.00 – 0.00)			0.00 (-0.00 – 0.01)		
EDUCATION (years)	-0.00 (-0.01 – 0.00)			0.00 (-0.00 – 0.01)			-0.00 (-0.01 – 0.01)			0.00 (-0.00 – 0.00)			-0.00 (-0.01 – 0.00)			0.01 (-0.01 – 0.02)		
Male (ref = Female)	-0.02 (-0.05 – -0.00)	0.049	0.14	-0.05 (-0.10 – 0.00)			0.02 (-0.02 – 0.06)	0.371	0.67	-0.04 (-0.06 – -0.01)	0.008	**0.051**	0.06 (0.03 – 0.09)	<0.001	**0.001**	0.24 (0.14 – 0.33)	<0.001	**<0.001**
APOE4 1 or 2 alleles (ref = 0 alleles)	0.00 (-0.02 – 0.03)	0.70	0.84	-0.08 (-0.13 – -0.02)			-0.02 (-0.06 – 0.02)	0.387	0.67	0.01 (-0.02 – 0.03)	0.586	0.80	-0.04 (-0.07 – -0.01)	0.019	0.10	0.01 (-0.09 – 0.10)	0.91	0.94
Interaction: Male by 1 or 2 alleles				0.07 (0.00 – 0.14)	0.046	0.14												
Observations	527			527			527			527			527			527		
R^2 / adjusted R^2	0.011 / 0.004			0.036 / 0.026			0.019 / 0.012			0.008 / 0.000			0.038 / 0.031			0.052 / 0.045		

Table S4. Linear regression results for all plasma variables investigated by sex and baseline diagnosis.

	Cortisol Cortisol ng ml			**C Reactive Protein ug/ml**			**CD 40 antigen ng/ml**			**Interleukin 16 pg/ml**			**Interleukin 3 ng/ml**		
Predictors	*Estimates (CI)*	*p*	*adjusted p*	*Estimates (CI)*	*p*	*adjusted p*	*Estimates (CI)*	*p*	*adjusted p*	*Estimates (CI)*	*p*	*adjusted p*	*Estimates (CI)*	*p*	*adjusted p*
(Intercept)	2.09 (1.95 – 2.22)			0.33 (-0.21 – 0.88)			-0.55 (-0.68 – -0.42)			2.48 (2.31 – 2.65)			-1.78 (-2.08 – -1.47)		
AGE (years)	0.00 (-0.00 – 0.00)			0.01 (0.00 – 0.01)			0.01 (0.00 – 0.01)			0.00 (0.00 – 0.00)			0.00 (-0.00 – 0.00)		
EDUCATION (years)	-0.00 (-0.00 – 0.00)			-0.02 (-0.04 – -0.01)			-0.00 (-0.00 – 0.00)			-0.01 (-0.01 – -0.00)			0.01 (0.00 – 0.02)		
Male (ref = Female)	0.02 (-0.01 – 0.04)	0.18	0.44	-0.12 (-0.21 – -0.03)	0.012	**0.056**	-0.01 (-0.03 – 0.01)	0.38	0.59	0.01 (-0.02 – 0.04)	0.39	0.57	-0.02 (-0.07 – 0.04)	0.56	0.75
Diagnosis (ref = CN)		0.0009	**0.010**		0.007	**0.056**		0.016	**0.067**		0.009	**0.054**		0.044	0.17
LMCI	-0.02 (-0.07 – 0.02)			-0.27 (-0.44 – -0.10)			-0.02 (-0.06 – 0.02)			-0.08 (-0.13 – -0.03)			-0.04 (-0.13 – 0.06)		
AD	0.03 (-0.02 – 0.08)			-0.24 (-0.43 – -0.05)			0.02 (-0.02 – 0.07)			-0.08 (-0.14 – -0.02)			-0.11 (-0.21 – -0.00)		
Observations	527			526			527			526			527		
R^2 / adjusted R^2	0.034 / 0.024			0.061 / 0.052			0.131 / 0.123			0.048 / 0.039			0.022 / 0.013		

Table S4. Continued

	Interleukin 6 receptor ng/ml			**Interleukin 8 pg/ml**			**Immunoglobulin A mg/ml**			**Intercellular Adhesion Molecule ng/ml**			**Interleukin 18 pg/ml**			**Immunoglobulin E ng/ml**		
Predictors	*Estimates (CI)*	*p*	*adjusted p*	*Estimates (CI)*	*p*	*adjusted p*	*Estimates (CI)*	*p*	*adjusted p*	*Estimates (CI)*	*p*	*adjusted p*	*Estimates (CI)*	*p*	*adjusted p*	*Estimates (CI)*	*p*	*adjusted p*
(Intercept)	1.47 (1.33 – 1.61)			0.73 (0.54 – 0.93)			0.43 (0.20 – 0.67)			1.90 (1.75 – 2.05)			2.33 (2.15 – 2.51)			1.44 (0.89 – 2.00)		
AGE (years)	0.00 (-0.00 – 0.00)			0.00 (0.00 – 0.01)			0.00 (-0.00 – 0.00)			0.00 (-0.00 – 0.00)			0.00 (-0.00 – 0.00)			0.00 (-0.00 – 0.01)		
EDUCATION (years)	-0.00 (-0.01 – 0.00)			0.00 (-0.00 – 0.01)			-0.00 (-0.01 – 0.01)			0.00 (-0.00 – 0.01)			-0.00 (-0.01 – 0.00)			0.01 (-0.01 – 0.02)		
Male (ref = Female)	-0.02 (-0.05 – 0.00)	0.063	0.19	-0.01 (-0.04 – 0.03)	0.65	0.79	0.02 (-0.02 – 0.06)	0.42	0.59	-0.03 (-0.06 – -0.01)	0.011	**0.056**	0.06 (0.03 – 0.09)	<0.001	**0.004**	0.24 (0.14 – 0.33)	<0.001	**<0.001**
Diagnosis (ref = CN)		0.34	0.56		0.11	0.30		0.72	0.82		0.063	0.19		0.32	0.56		0.88	0.94
LMCI	-0.03 (-0.08 – 0.01)			-0.04 (-0.10 – 0.02)			0.03 (-0.05 – 0.10)			-0.01 (-0.06 – 0.03)			0.04 (-0.01 – 0.10)			-0.00 (-0.18 – 0.17)		
AD	-0.03 (-0.08 – 0.02)			-0.00 (-0.07 – 0.07)			0.02 (-0.07 – 0.10)			0.02 (-0.03 – 0.08)			0.03 (-0.03 – 0.10)			-0.03 (-0.23 – 0.16)		
Observations	527			527			527			527			527			527		
R^2 / adjusted R^2	0.015 / 0.006			0.029 / 0.020			0.008 / -0.002			0.029 / 0.019			0.032 / 0.023			0.053 / 0.043		

Table S5. Correlation matrix of biochemical variables for those ADNI samples with available DNAme and biochemical data (n=533). Correlation coefficients are Spearman's rho.

	Amyloid Beta	**p value**	**Tau**	**p value**	**pTau**	**p value**
Amyloid Beta	1.00	<0.0001	-0.18	<0.0001	-0.27	<0.0001
Tau	-0.18	<0.0001	1.00	<0.0001	0.98	<0.0001
pTau	-0.27	<0.0001	0.98	<0.0001	1.00	<0.0001

Table S6. Contingency table for the distribution of the APOEε4 alleles (0 or 1 and 2 ε4 alleles) in each of the diagnosis groups (CN, late MCI and AD).

	CN	LMCI	AD
0	55 (74.32%)	60 (43.48%)	19(28.36%)
1 and 2	19 (25.68%)	78 (56.52%)	48 (71.64%)