

# Genetically identical twin-pair difference models support the amyloid cascade hypothesis

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## Abstract

The amyloid cascade hypothesis has strongly impacted the Alzheimer's disease research agenda and clinical trial designs over the past decades, but precisely how amyloid- $\beta$  pathology initiates the aggregation of neocortical tau remains unclear. We cannot exclude the possibility of a shared upstream process driving both amyloid- $\beta$  and tau in an independent manner instead of there being a causal relationship between amyloid- $\beta$  and tau. Here, we tested the premise that if a causal relationship exists, then exposure should be associated with outcome both at the individual-level as well as within identical twin-pairs, who are strongly matched on genetic, demographic and shared environmental background. Specifically, we tested associations between longitudinal amyloid- $\beta$  PET and cross-sectional tau-PET, neurodegeneration and cognitive decline using genetically identical twin-pair difference models, which provide the unique opportunity of ruling out genetic and shared environmental effects as potential confounders in an association.

We included 78 cognitively unimpaired identical twins with [<sup>18</sup>F]flutemetamol (amyloid- $\beta$ )-PET, [<sup>18</sup>F]flortaucipir (tau)-PET, MRI (hippocampal volume), and cognitive data (composite memory). Associations between each modality were tested at the individual-level using generalized estimating equation models, and within identical twin-pairs using within-pair difference models. Mediation analyses were performed to test for directionality in the associations as suggested by the amyloid cascade hypothesis.

At the individual-level, we observed moderate-to-strong associations between amyloid- $\beta$ , tau, neurodegeneration and cognition. The within-pair difference models replicated results observed at the individual-level with comparably strong effect sizes. Within-pair differences in amyloid- $\beta$  were strongly associated with within-pair differences in tau ( $\beta=0.68$ ,  $p<0.001$ ), and moderately associated with within-pair differences in hippocampal volume ( $\beta=-0.37$ ,  $p=0.03$ ) and memory functioning ( $\beta=-0.57$ ,  $p<0.001$ ). Within-pair differences in tau were moderately associated with within-pair differences in hippocampal volume ( $\beta=-0.53$ ,  $p<0.001$ ) and strongly associated with within-pair differences in memory functioning ( $\beta=-0.68$ ,  $p<0.001$ ). Mediation analyses showed that of the total twin-difference effect of amyloid- $\beta$  on memory functioning, the proportion mediated through pathways including tau and hippocampal volume was 69.9%, which was largely attributable to the pathway leading from amyloid- $\beta$  to tau to memory functioning (proportion mediated: 51.6%).

Our results indicate that associations between amyloid- $\beta$ , tau, neurodegeneration and cognition are unbiased by (genetic) confounding. Furthermore, effects of amyloid- $\beta$  on neurodegeneration and cognitive decline were fully mediated by tau. These novel findings in this unique sample of identical twins are compatible with the amyloid cascade hypothesis and thereby provide important new knowledge for clinical trial designs.

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**Running title:** Identical twin-difference models support the amyloid hypothesis

**Key words:** Alzheimer's disease, amyloid- $\beta$ , tau, neurodegeneration, cognition, identical twins

**Abbreviations:** A = amyloid- $\beta$ ; *APOE* = apolipoprotein E; BP<sub>ND</sub> = binding potential; BP<sub>ND</sub>/y = annual change in binding potential; C = cognition; GEE = generalized estimating equation; MMSE = mini mental state examination; mPACC = modified preclinical Alzheimer's cognitive composite; N = neurodegeneration; OFG = orbitofrontal gyrus; PCC = posterior cingulate cortex; ROI = region of interest; SPM = statistical parametric mapping; T = tau.

## Introduction

The amyloid cascade hypothesis has provided the leading framework for investigating and understanding Alzheimer's disease pathophysiology.<sup>1,2</sup> This hypothesis has been modified several times but the basic premise is that the deposition of amyloid- $\beta$  in the brain initiates a cascade of downstream events, which includes the aggregation of hyper-phosphorylated tau in the neocortex, synaptic dysfunction, neuronal loss and eventually cognitive impairment and dementia. *In vivo* support for this hypothesis has come from studies on participants with autosomal dominant mutations in genes involved in amyloid- $\beta$  metabolism leading to familial Alzheimer's disease,<sup>3,4</sup> as well as from prospective cohort studies on sporadic Alzheimer's disease, showing a temporal emergence of biomarker abnormality in line with the amyloid cascade sequence.<sup>5-7</sup> For example, whereas it is relatively common to observe amyloid- $\beta$  pathology in the absence of neocortical tau pathology, it is rare to observe neocortical tau pathology in the absence of amyloid- $\beta$  pathology.<sup>8</sup> However, although the amyloid cascade hypothesis has strongly impacted the research agenda and clinical trial designs over the past decades, precisely how amyloid- $\beta$  deposition initiates hyperphosphorylation of neocortical tau pathology remains unclear. The association between amyloid- $\beta$  and tau is further complicated by both a spatial and temporal paradox. Whereas amyloid- $\beta$  is often observed first in posterior cingulate and orbitofrontal regions approximately 20 years prior to symptom onset,<sup>9-11</sup> tau neurofibrillary tangles are first observed in the (trans)entorhinal cortex, and the spread of tau outside of the medial temporal lobe occurs in close proximity to symptom onset.<sup>12,13</sup> Therefore, we cannot exclude the possibility of a shared upstream process simultaneously driving amyloid- $\beta$  and tau in an independent (yet correlated) manner instead of there being a causal relationship between amyloid- $\beta$  and tau. Previous studies have suggested several biological pathways, including cholesterol metabolism, microglial immune activation, and apolipoprotein E (*APOE*), that may drive both amyloid- $\beta$  and tau pathology through shared but independent pathways.<sup>14</sup> Moreover, we have previously shown that genetically identical twins show substantial similarities in both amyloid- $\beta$  load and tau load,<sup>15,16</sup> and therefore some genetic factors that influence amyloid- $\beta$  may overlap with the genetic factors that also, independently, influence tau, which could potentially result in an association between amyloid- $\beta$  and tau that is actually confounded by genetic variation. Gaining a better understanding into whether the relationships between key pathological hallmarks of Alzheimer's disease may actually be causal (as suggested by the amyloid cascade hypothesis) is of utmost importance for clinical trial designs.

Studying genetically identical twins provide the unique opportunity of ruling out genetic and shared environmental effects as potential confounders in an association, which typically is a concern for causal inferences in observational studies. The genetically identical within-pair difference design is a strong non-experimental case-control design in which individuals are strongly matched on genetic, demographic and shared environmental background. If a causal relationship exists, then we would expect exposure to be associated with outcome both at the individual level as well as within identical twin-pairs, in which potential shared confounding effects are excluded. Although the within-pair difference design cannot rule out all possible alternatives to prove causality, it allows testing associations beyond what is possible in typical cohort studies. Therefore, the primary aim of the current study was to utilize genetically identical within-pair difference models in order to investigate the relationship between amyloid- $\beta$  accumulation and aggregated tau **while** taking potential confounding by, amongst others, genetic factors into account. Secondary aims included to investigate associations with neurodegeneration and cognitive decline. To this end, we used longitudinal amyloid- $\beta$  PET and cross-sectional tau PET, MRI, and cognitive data from genetically identical twins with initially normal cognition. Inspired by previous twin studies,<sup>17</sup> we tested the following predictions generated by the amyloid cascade hypothesis:

First, if there is a causal relationship between amyloid- $\beta$  and tau, then the twin that shows higher levels of amyloid- $\beta$  should also have higher levels of tau compared to the genetically identical co-twin. Second, if changes in amyloid- $\beta$  are the driving force behind tau accumulation, then the twin that shows larger longitudinal changes in amyloid- $\beta$  should also have higher levels of tau compared to the genetically identical co-twin. We tested these **predictions** using within-pair difference models, in which within-pair differences in amyloid- $\beta$  were regressed on within-pair differences in tau. The resulting within-pair difference effect represents an association that is free of confounding due to factors that are shared between the two twins of the same pair, which includes genetic factors (the twins are genetically identical), but also shared demographic (e.g. sex, age) and shared environmental (e.g. growing up in the same environment) factors. Finally, if tau pathology mediates the associations between amyloid- $\beta$  pathology and neuronal loss and cognitive impairment (as predicted by the directionality in the amyloid cascade hypothesis) this should be supported by serial mediation models between within-pair differences in amyloid- $\beta$ , tau, neurodegeneration and cognitive decline.

# Materials and methods

## Participants

We included genetically identical twins from the on-going longitudinal Amsterdam sub-study of the EMIF-AD PreclinAD cohort.<sup>18</sup> At study entry, all participants were  $\geq 60$  years old and had normal cognition based on the performance on several neuropsychological tests.<sup>18</sup> Twin zygosity was confirmed by DNA analysis. Exclusion criteria included any significant neurologic, systemic or psychiatric disorder that could cause cognitive impairment. The study protocol for the total cohort (n=204) included an amyloid- $\beta$  PET scan at baseline and at 4-year follow-up. A subset of the cohort (n=80) was selected to additionally undergo tau PET at 4-year follow-up.<sup>16</sup> This subset included twin-pairs of whom both twins or either one of the twins were amyloid- $\beta$  positive or were classified into a high amyloid- $\beta$  stage,<sup>9</sup> twin-pairs that carried an *APOE*  $\epsilon 4$  allele, as well as age and sex matched amyloid- $\beta$  negative twin-pairs (details are described previously<sup>16</sup>). Structural MRI and cognitive data was also acquired at 4-year follow-up.

For the analyses in the current study, we included twins who completed amyloid- $\beta$  PET, tau PET, structural MRI, and neuropsychological assessment at the 4-year follow-up visit to enable cross-sectional associations between each modality. We therefore refer to the 4-year follow-up data as “cross-sectional data”. Participants with incomplete cross-sectional data (n=125) or participants with data of insufficient quality (n=1 of whom cross-sectional amyloid- $\beta$  PET was not suitable for quantification due to movement) were excluded, resulting in a total of 78 twins (37 genetically identical pairs and 4 singletons) included in the analyses. Twins’ amyloid- $\beta$  status (positive/negative) was defined by consensus visual read of cross-sectional [<sup>18</sup>F]flutemetamol PET according to manufacturer guidelines.

This study was approved by the Medical Ethics Review Committee of the VU University Medical Center (Amsterdam, The Netherlands). All participants provided written informed consent.

## Amyloid- $\beta$ PET, tau PET and MR acquisition and processing

[<sup>18</sup>F]flutemetamol (amyloid- $\beta$ ) PET was performed on an Ingenuity TF PET/MRI (Philips Medical Systems, Best, The Netherlands) (time difference between longitudinal scans:  $4.1 \pm 0.4$  years). [<sup>18</sup>F]flortaucipir (tau) PET was performed on an Ingenuity TF PET/CT (Philips Medical Systems, Best, The Netherlands). Both [<sup>18</sup>F]flutemetamol and [<sup>18</sup>F]flortaucipir PET images

were acquired using a dynamic dual time-point acquisition protocol (0-30min and 90-110min post-injection for [<sup>18</sup>F]flutemetamol, and 0-30 min and 80-100 min post-injection for [<sup>18</sup>F]flortaucipir). Prior to both parts of the dynamic scan, for attenuation correction purposes, a T1-weighted gradient echo pulse MRI was acquired for [<sup>18</sup>F]flutemetamol, and a low-dose CT scan for [<sup>18</sup>F]flortaucipir. Details on [<sup>18</sup>F]flutemetamol and [<sup>18</sup>F]flortaucipir acquisition are described elsewhere.<sup>16,19</sup> For both tracers, we co-registered the two parts of the dynamic scan to each other using Vinci software.<sup>20</sup> Subsequently, 3D isotropic T1 weighted MR images were co-registered to the corresponding native-space PET images. Grey matter region-of-interests (ROIs) from the Hammers atlas<sup>21</sup> were automatically delineated on the co-registered MR images and superimposed on both [<sup>18</sup>F]flutemetamol and [<sup>18</sup>F]flortaucipir PET scans to extract time activity curves using PVElab. For [<sup>18</sup>F]flortaucipir, we additionally superimposed grey matter ROIs from the Svarer atlas<sup>22</sup> onto the PET scan, to extract time activity curves in the entorhinal cortex. Voxel-wise parametric images of binding potential (BP<sub>ND</sub>) were generated using SRTM2 for [<sup>18</sup>F]flutemetamol and using RPM for [<sup>18</sup>F]flortaucipir, validated in previous studies.<sup>20,23-26</sup> For both tracers, whole cerebellar grey matter was used as the reference region.

For [<sup>18</sup>F]flutemetamol PET, we additionally calculated (retrospective) annual change in BP<sub>ND</sub> ( $BP_{ND}/y = (BP_{ND[4y\text{-follow-up}] - BP_{ND[baseline]}) / \text{time difference in years}$ ) to test our hypothesis that longitudinal changes in amyloid- $\beta$  pathology are associated with tau pathology, neurodegeneration and cognitive decline. [<sup>18</sup>F]flutemetamol BP<sub>ND</sub>/y was missing for n=4 twins due to missing baseline [<sup>18</sup>F]flutemetamol PET.

For voxel-wise [<sup>18</sup>F]flortaucipir analyses, BP<sub>ND</sub> images were spatially normalized to Montreal Neurological Institute space (using the transformation matrixes derived from warping the co-registered T1 weighted MRI), followed by smoothing using an 8 mm isotropic Gaussian kernel, using Statistical Parametric Mapping (SPM) version 8 software (Wellcome Trust Center for Neuroimaging, University College London, UK) in line with our previous studies.<sup>27,28</sup> All warped images were visually checked for transformation errors.

Participants underwent three-dimensional T1 weighted MRI on a 3.0T Ingenuity TF PET/MR (Philips Medical Systems, Best, The Netherlands) with an 8-channel head coil. T1 weighted sequences were acquired using sagittal turbo field echo sequence (1.00 mm<sup>3</sup> isotropic voxels, TR/TE = 7.9 ms/4.5 ms, and flip angle = 8<sup>29</sup>). Cortical reconstruction and volumetric segmentation was performed with Freesurfer v7.1.1, which is documented and freely available online (<http://surfer.nmr.mgh.harvard.edu>). The technical details of these procedures are described previously.<sup>30</sup>

## **Amyloid- $\beta$ PET, tau PET and MRI regions-of-interest**

We computed two ROIs for each imaging modality (amyloid- $\beta$  PET, tau PET and MRI) based on previous literature: 1) a modality-specific early-AD ROI, and 2) a modality-specific AD-signature ROI.

For amyloid- $\beta$  PET, we created a volume-weighted average of  $BP_{ND}$  in the bilateral posterior cingulate cortex (PCC) and orbitofrontal gyrus (OFG) as the early amyloid- $\beta$  PET ROI.<sup>9,10</sup> A global ROI was created as the AD-signature amyloid- $\beta$  ROI, which was a volume-weighted average of  $BP_{ND}$  in the bilateral parahippocampal gyrus, ambient gyrus, superior temporal gyrus, middle temporal gyrus, inferior temporal gyrus, fusiform gyrus, insula, cinguli anterior, cinguli posterior, superior parietal gyrus, lateral parietal lobe, lateral occipital lobe, lingual gyrus, cuneus, middle frontal gyrus, gyrus rectus, orbitofrontal gyrus, inferior frontal gyrus, superior frontal gyrus.<sup>9,10</sup>

For tau PET, we created a volume-weighted average of  $BP_{ND}$  in the bilateral entorhinal cortex as the early tau ROI.<sup>13,16</sup> A temporal meta-ROI was created as the AD-signature tau ROI, which was a volume-weighted average of  $BP_{ND}$  in the bilateral entorhinal cortex, amygdala, parahippocampal gyrus, fusiform gyrus and middle and inferior temporal gyrus.<sup>16</sup>

For MRI, we calculated bilateral mean hippocampal volume (adjusted for intracranial volume) as an early neurodegeneration ROI. We calculated cortical thickness in a temporal meta-ROI as the AD-signature neurodegeneration ROI, which was an average of the bilateral entorhinal, inferior temporal, middle temporal, and fusiform cortices.<sup>31</sup>

## **Neuropsychological assessment**

We created two neuropsychological composite scores for capturing early cognitive decline: 1) a memory composite score, since this cognitive domain is often affected first in Alzheimer's disease, and 2) a modified Preclinical Alzheimer's Cognitive Composite (mPACC) score, which is designed to be sensitive to cognitive changes in the preclinical stage of Alzheimer's disease.<sup>32,33</sup> In the main text, we report on results with the composite memory score, whereas results for the mPACC are shown in the Supplement to have an alternate measure of cognitive functioning.

The memory composite score included the total immediate and delayed recall of the Dutch version of the Rey Auditory Verbal Learning Test,<sup>34,35</sup> the three and twenty minute recall of the Rey Complex Figure Test<sup>36</sup> and the delayed recall of names and occupations of the Face-



Name Associated Memory Exam.<sup>37</sup> The mPACC in the current study consisted of the Mini Mental State Examination (MMSE),<sup>38</sup> the delayed recall of the Dutch version of the Rey Auditory Verbal Learning Test, the Digit Symbol Substitution Test score,<sup>39</sup> the total score of the Face-Name Associated Memory Exam and the one-minute Animal Fluency test.<sup>40</sup> Missing data was imputed (for 2 participants, 1 cognitive test was missing) using predictive mean matching as method,<sup>41</sup> after which individual neuropsychological test scores were standardized using the mean and standard deviation from baseline neuropsychological test scores from the entire Amsterdam EMIF-AD PreclinAD cohort (n=204) and subsequently averaged into the composite scores.<sup>18</sup>

## Statistical analyses

We used R version 4.0.3 for statistical analyses unless specified otherwise. A  $p$ -value < 0.05 was considered significant.

First, we tested associations between amyloid- $\beta$  PET ( $BP_{ND}$  and  $BP_{ND}/y$ ), tau PET  $BP_{ND}$ , neurodegeneration and cognitive functioning at the individual level. We used generalized estimating equation (GEE) models between each modality, corrected for age and sex, and taking potential clustering within families into account. GEE models with cognitive functioning as the outcome measure additionally included years of education as a covariate. GEE models with amyloid- $\beta$  PET annual change ( $BP_{ND}/y$ ) as predictor were performed with and without correcting for initial amyloid- $\beta$  PET  $BP_{ND}$ . **Since our cohort consisted of a relatively large number of *APOE*  $\epsilon 4$  carriers, sensitivity analyses additionally correcting for *APOE*  $\epsilon 4$  status are shown in the Supplement.**

Subsequently, we tested associations between amyloid- $\beta$  PET ( $BP_{ND}$  and  $BP_{ND}/y$ ), tau PET  $BP_{ND}$ , neurodegeneration and cognitive functioning using genetically identical within-pair difference models, by regressing the within-pair difference of the predictor onto the within-pair difference of the outcome variable. The resulting effect represents an association that can be interpreted similarly to the GEE effect, but the twin-difference effect is free of confounding due to factors that are shared between the two twins of the same pair, which includes genetic factors (the twins are genetically identical), but also shared demographic (e.g. sex, age) and shared environmental (e.g. growing up in the same environment) factors. Age and sex were therefore not included as covariates in these analyses. Since twins of the same pair could differ on education, linear regression twin-difference models with within-pair difference in cognitive functioning as outcome measure were corrected for within-pair difference in years of

education. Linear regression twin-difference models with within-pair difference in amyloid- $\beta$  PET annual change ( $BP_{ND}/y$ ) as predictor were performed with and without correcting for within-pair difference in initial amyloid- $\beta$  PET  $BP_{ND}$ .

We further explored the association between amyloid- $\beta$  and tau in more detail, by examining the association between ROI-level (within-pair differences in) amyloid- $\beta$  PET ( $BP_{ND}$  and  $BP_{ND}/y$ ) and (within-pair differences in) voxel-wise tau PET  $BP_{ND}$  using linear regression models in SPM12. Analyses at the individual level were corrected for age and sex. Results are displayed at more liberal (i.e.  $p < 0.001$ , uncorrected) and more stringent ( $p < 0.05$ , family-wise error corrected) thresholds for visualization purposes.

Finally, following the cascade of events according to the amyloid cascade hypothesis, we tested the mediating effect of tau in the association between amyloid- $\beta$  and neurodegeneration, and the mediating effects of both tau and neurodegeneration in the association between amyloid- $\beta$  and cognitive decline (separate mediation models). These single mediation models were performed using the “Mediation” package in R. In a final serial mediation model, we tested a four-factor mediation model including direct and indirect pathways between amyloid- $\beta$ , tau, neurodegeneration and cognition, and using a bootstrap method (1000 iterations) for the mediation effects, using the “Lavaan” package in R.<sup>42</sup> All mediation analyses were performed on within-pair difference variables only.

## Data availability

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

## Results

### Participant characteristics

Participant characteristics are shown in **Table 1**. In total, 78 twins with an average age of  $73.7 \pm 6.2$  years were included. The cohort consisted of 37 complete pairs and 4 singletons of which the co-twins were not included in the current study due to incomplete cross-sectional data. Of the 78 twins, 39 (50.0%) carried an *APOE*  $\epsilon 4$  allele and 30 (38.5%) were visually read as amyloid- $\beta$  PET positive.

## Associations between amyloid- $\beta$ and tau

**Table 2** shows all GEE and twin-difference estimates and  $p$ -values for both early-AD and AD-signature ROIs. In the text and figures, we report results for early-AD amyloid- $\beta$  PET (OFG/OCC), tau-PET (entorhinal cortex) and neurodegeneration (hippocampal volume) ROIs, but similar results were observed for AD-signature ROIs (**Table 2**).

We first tested the association between cross-sectional amyloid- $\beta$  PET  $BP_{ND}$  and tau PET  $BP_{ND}$ . At the individual level, higher amyloid- $\beta$  PET  $BP_{ND}$  was associated with higher tau PET  $BP_{ND}$  ( $\beta=0.64, p<0.001$ ). The twin-difference model replicated this. Across pairs, within-pair differences in amyloid- $\beta$  PET  $BP_{ND}$  were associated with within-pair differences in tau PET  $BP_{ND}$  ( $\beta=0.68, p<0.001$ ) (**Figure 1A**). We then tested this association using voxel-wise tau PET as the outcome measure. At the individual level, voxel-wise analyses showed that amyloid- $\beta$  PET  $BP_{ND}$  in the early-AD ROI was associated with tau PET  $BP_{ND}$  in medial and inferior lateral temporal brain regions. When regressing within-pair differences in early-AD ROI amyloid- $\beta$  PET  $BP_{ND}$  on within-pair differences in voxel-wise tau PET  $BP_{ND}$ , we observed an overlapping pattern, slightly extending into the parietal lobe (**Figure 1A**). Sensitivity analyses additionally correcting for *APOE*  $\epsilon 4$  status yielded similar results (**Supplementary Table 1**).

We then repeated the analyses using annual change in amyloid- $\beta$  PET  $BP_{ND}$  ( $BP_{ND}/y$ , corrected for initial  $BP_{ND}$ ) as predictor for tau PET  $BP_{ND}$  (**Figure 1B**). At the individual level, higher amyloid- $\beta$  PET  $BP_{ND}/y$  was associated with higher tau PET  $BP_{ND}$  ( $\beta=0.36, p<0.001$ ). The twin-difference model showed a similar result, with within-pair differences in amyloid- $\beta$  PET  $BP_{ND}/y$  being associated with within-pair differences in tau PET  $BP_{ND}$  ( $\beta=0.41, p=0.02$ ). We observed similar results without correcting for initial amyloid- $\beta$  PET  $BP_{ND}$  (**Supplementary Table 2**). Voxel-wise analyses showed effects of amyloid- $\beta$  PET  $BP_{ND}/y$  predominantly on medial temporal tau PET  $BP_{ND}$  lateralized to the left hemisphere at both the individual level and within twin-pairs (**Figure 1B**).

## Associations between amyloid- $\beta$ , tau and neurodegeneration

Next, we tested associations of amyloid- $\beta$  PET ( $BP_{ND}$  and  $BP_{ND}/y$ ) and tau PET  $BP_{ND}$  with neurodegeneration. In the text, we report results for early-AD ROIs, whereas a complete overview of results obtained for both early-AD and AD-signature ROIs are shown in **Table 2**. At the individual level, higher amyloid- $\beta$  PET  $BP_{ND}$  was associated with lower hippocampal volume ( $\beta=-0.19, p=0.02$ ), however amyloid- $\beta$  PET  $BP_{ND}/y$  (retrospective annual change) was

not ( $\beta=-0.03$ ,  $p=0.75$ ). Similarly, within-pair differences in cross-sectional amyloid- $\beta$  PET BP<sub>ND</sub>, but not annual change, were associated with within-pair differences in hippocampal volume ( $\beta=-0.37$ ,  $p=0.03$  and  $\beta=-0.04$ ,  $p=0.82$  respectively) (**Figure 2A**).

At the individual level, higher tau PET BP<sub>ND</sub> was associated with lower hippocampal volume ( $\beta=-0.22$ ,  $p=0.02$ ). In addition, within-pair differences in tau PET BP<sub>ND</sub> were associated with within-pair differences in hippocampal volume ( $\beta=-0.53$ ,  $p<0.001$ ) (**Figure 2B**).

We performed mediation analyses to test for the potential mediating effect of tau in the within-pair difference association between amyloid- $\beta$  and neurodegeneration. Mediation analyses were performed for early-AD ROIs and only on cross-sectional data, since twin-difference results for annual change in amyloid- $\beta$  PET BP<sub>ND</sub> were less pronounced and/or non-significant (**Table 2**). Upon testing whether the effect of within-pair differences in amyloid- $\beta$  PET BP<sub>ND</sub> on within-pair differences in hippocampal volume was mediated by within-pair differences in tau PET BP<sub>ND</sub> (A→T→N), we observed a full mediation effect of tau (proportion mediated: 91%,  $p<0.05$ ) (**Figure 2C, D**). We also tested whether the association between within-pair differences in tau PET BP<sub>ND</sub> and within-pair differences in hippocampal volume was mediated by within-pair differences in amyloid- $\beta$  PET BP<sub>ND</sub> (T→A→N), but we did not observe such a mediation effect (proportion mediated: 0.05%,  $p>0.05$ ) (**Supplementary Figure 1A**).

## Associations between amyloid- $\beta$ , tau, neurodegeneration and cognitive functioning

Next, we tested associations of amyloid- $\beta$  PET (BP<sub>ND</sub> and BP<sub>ND</sub>/y), tau PET BP<sub>ND</sub> and neurodegeneration with cognitive functioning. **In the text, we report results for early-AD ROIs, whereas a complete overview of results obtained for both early-AD and AD-signature ROIs are shown in Table 2.** At the individual level, higher amyloid- $\beta$  PET BP<sub>ND</sub> and higher amyloid- $\beta$  PET BP<sub>ND</sub>/y were associated with lower composite memory scores ( $\beta=-0.47$ ,  $p<0.001$  and  $\beta=-0.25$ ,  $p=0.01$  respectively). Furthermore, higher tau PET BP<sub>ND</sub> and lower hippocampal volume were also associated with lower composite memory scores ( $\beta=-0.63$ ,  $p<0.001$  and  $\beta=0.48$ ,  $p<0.001$  respectively). Twin-difference models showed largely similar results. Within-pair differences in cross-sectional amyloid- $\beta$  PET BP<sub>ND</sub> ( $\beta=-0.57$ ,  $p<0.001$ ), tau PET BP<sub>ND</sub> ( $\beta=-0.68$ ,  $p<0.001$ ) and hippocampal volume ( $\beta=0.56$ ,  $p<0.001$ ) were all associated to within-pair differences in composite memory scores (**Figure 3A-C, Table 2**). However, we did not

observe a significant association between within-pair differences in amyloid- $\beta$  PET BP<sub>ND</sub>/y and within-pair differences in composite memory scores ( $\beta=-0.22$ ,  $p=0.22$ ). We performed sensitivity analyses with mPACC as a measure of cognitive functioning instead of composite memory and found very similar results for both GEE models and twin-difference models (results shown in **Supplementary Table 3**).

We then investigated whether within-pair differences in tau PET BP<sub>ND</sub> and within-pair differences in hippocampal volume mediated the association between within-pair differences in amyloid- $\beta$  PET BP<sub>ND</sub> and within-pair differences in composite memory scores ( $A \rightarrow T \rightarrow C$  and  $A \rightarrow N \rightarrow C$  respectively). In line with the previous mediation model, these analyses were only performed on cross-sectional data and for early-AD ROIs. The association between within-pair differences in amyloid- $\beta$  PET BP<sub>ND</sub> and within-pair differences in composite memory scores was fully mediated by within-pair differences in tau PET BP<sub>ND</sub> (proportion mediated: 70%,  $p<0.001$ ) and partially mediated by within-pair differences in hippocampal volume (proportion mediated: 27%;  $p<0.05$ ) (**Figure 3D-F**). We again tested the possibility of within-pair differences in amyloid- $\beta$  PET BP<sub>ND</sub> mediating the association between within-pair differences in tau PET BP<sub>ND</sub> and within-pair differences in composite memory scores ( $T \rightarrow A \rightarrow C$ ), but we did not observe such a mediation effect (proportion mediated: 14%,  $p>0.05$ ) (**Supplementary Figure 1B**).

## **Serial mediation model between amyloid- $\beta$ , tau, neurodegeneration and cognitive functioning**

Finally, to test the full amyloid cascade hypothesis, we tested a four-factor serial mediation model including pathways between amyloid- $\beta$ , tau, neurodegeneration and cognitive functioning. In line with the simpler mediation models above (**Figure 2** and **Figure 3**), this serial mediation model was performed on within-pair difference scores, cross-sectional data and early-AD ROIs. For the total effect of amyloid- $\beta$  (A) on memory functioning (C), we calculated the proportion that was mediated through the following pathways: pathway 1) via tau (T) ( $A \rightarrow T \rightarrow C$ , while taking pathways 2 and 3 into account); pathway 2) via hippocampal volume (N) ( $A \rightarrow N \rightarrow C$ , while taking pathways 1 and 3 into account); and pathway 3) via tau and subsequently hippocampal volume ( $A \rightarrow T \rightarrow N \rightarrow C$ , while taking pathways 1 and 2 into account). The total proportion mediated (pathways 1, 2 and 3 combined) was 69.9% ( $p<0.001$ ). Pathways 1, 2 and 3 separately indicated that this was largely attributable to pathway 1, which

had the largest mediating effect (51.6%,  $p=0.03$ ). Pathways 2 and 3 accounted for only 0.7% ( $p=0.83$ ) and 17.8% ( $p=0.26$ ) respectively. After accounting for the different mediation pathways, there was no direct effect anymore of amyloid- $\beta$  on memory functioning, amyloid- $\beta$  on hippocampal volume, and hippocampal volume on memory functioning (all  $p>0.05$ ). Sensitivity analyses with mPACC as a measure of cognitive functioning instead of composite memory showed highly similar results (**Supplementary Figure 2**).

## Discussion

In this study, we used amyloid- $\beta$  PET, tau PET, MRI, and cognitive data from cognitively unimpaired genetically identical twins to test hypotheses of causality, as suggested by the amyloid cascade hypothesis, beyond typical group-analyses. Using genetically identical twin-difference designs, we observed that associations between amyloid- $\beta$ , tau, neurodegeneration, and cognition are minimally affected by (genetic) confounding. This is **congruent with the causal predictions generated by the amyloid cascade hypothesis**. Sequential mediation analyses revealed that within-pair difference effects of amyloid- $\beta$  on neurodegeneration, and within-pair difference effects of amyloid- $\beta$  on cognitive functioning, were fully mediated by tau. This underscores the important mediating role of tau pathology in the pathway leading from amyloid- $\beta$  to cognitive decline, even in very early disease stages when tau load (at least when measured with PET) is still relatively low. This is of specific interest for clinical trials, which are increasingly focused on preclinical stages of the disease.

One of the most important findings of this study is the robustness of the associations between amyloid- $\beta$ , tau, neurodegeneration, and cognitive decline. In our study, the twin that had more amyloid- $\beta$  pathology compared to the genetically identical co-twin, also had more tau pathology, lower hippocampal volume, and worse performance on neuropsychological tests as underlined by the within-pair difference models. The effect sizes of the regression of amyloid- $\beta$  on tau, neurodegeneration and cognition seen at the individual level were highly comparable to the effect sizes seen in the within-pair differences regression. This means that the associations that have been reported in the population at large between these parameters<sup>6-8,43-47</sup> can be considered to be unbiased by genetic confounding. In addition to eliminating genetic confounding, many other forms of confounding are eliminated including confounding by shared demographic and shared environmental factors. These important new findings in this unique sample of identical twins **further increases the possibility of true causal relationships** between amyloid- $\beta$ , tau, neurodegeneration, and cognitive decline.

Our primary focus was the relationship between amyloid- $\beta$  and tau. The twin-difference models replicated the GEE models, indicating that both the association between cross-sectional amyloid- $\beta$  and tau, and the association between annual change in amyloid- $\beta$  and tau are robust and minimally affected by (genetic) confounding. Moreover, the strong similarities in voxel-wise results at the individual level and within twin-pairs particularly support a relationship between amyloid- $\beta$  deposition (in a region that is affected by amyloid- $\beta$  early in the disease<sup>9,10</sup>) and the accumulation of tau in medial temporal and inferolateral temporal regions. These regions correspond to what has been observed in previous non-twin studies.<sup>46,48,49</sup> It is, however, important to recognize that although within-pair difference models are more powerful compared to analyses at the individual level, we cannot rule out all possible alternatives to prove causality in this association. **For example, although identical twins are strongly matched on genetic and shared environmental factors, they are not matched on non-shared environmental factors, and confounding by non-shared environmental factors can therefore not be excluded.**<sup>50</sup> Furthermore, based on the current study, we cannot make any inferences on the exact mechanism underlying the **link** between amyloid- $\beta$  and tau, and the spatial and temporal paradox between the two pathologies remains of interest. Previous studies have suggested that amyloid- $\beta$  may lead to neuronal hyperactivity,<sup>51</sup> which in turn may amplify the secretion and spread of tau.<sup>52,53</sup> Although there thus seems to be a deterministic association between amyloid- $\beta$  and tau (if a twin had more amyloid- $\beta$  compared to the identical co-twin, that twin also had more tau), the fact that twins of the same pair could show differences in both amyloid- $\beta$  and tau also indicates that the timing of the onset of pathology could be probabilistic.<sup>54</sup> It is likely a complex interplay between genes and environment that determines if and when pathology will develop.

While twin-difference models can exclude (genetic) confounding, additional use of mediation models allows further tests of directionality in the associations. We therefore performed multiple single mediation models, and one serial mediation model combining all modalities, to test the sequence of events as described by the amyloid cascade hypothesis. Our single mediation models using twin-difference scores as input variables (since these variables are free of confounding due to factors that twins share) showed that tau fully mediated the associations between amyloid- $\beta$  and hippocampal volume, and between amyloid- $\beta$  and cognitive decline. Importantly, amyloid- $\beta$  did not mediate the associations between tau and hippocampal volume or between tau and cognitive decline, for which only a direct effect of tau was observed. These results are compatible with a pathway leading from amyloid- $\beta$  to tau, as suggested by the amyloid cascade hypothesis.<sup>1</sup> In a single mediation model, we observed a



partial mediation effect of hippocampal volume in the association between amyloid- $\beta$  and cognitive decline. However, in the serial mediation model – which also took mediation pathways of tau into account – the proportion mediated by the pathway from amyloid- $\beta$  to hippocampal volume to cognitive decline was only 0.7%. In contrast, the proportion mediated directly through tau was 51.6%, and the proportion mediated through tau and subsequently hippocampal volume loss was 17.8%. These results corroborate to a wide variety of previous studies suggesting that in order to slow or halt cognitive decline (and neurodegeneration), one must need to prevent or slow tau accumulation – even in very early stages of the disease when tau load is still relatively low.<sup>6,55-58</sup>

Strengths of the study include the unique study population, the availability of multimodal imaging and the use of longitudinal amyloid- $\beta$  PET data. This study also had some limitations. The main limitation of this study is the relatively small sample size. Since twin-difference models can only be performed on complete pairs with complete data, we had to exclude a substantial number of pairs from the analyses. Some results (e.g. the serial mediation model) may have been affected by limited power. Furthermore, this cohort consisted of a relatively large percentage of *APOE* e4 carriers and amyloid- $\beta$  positive unimpaired individuals, which may limit generalizability to the general population. In addition, our [<sup>18</sup>F]flutemetamol PET scans were acquired on a PET/MR system, which has been shown to have less optimal attenuation correction methods compared to PET/CT systems. Finally, a general limitation when studying preclinical Alzheimer's disease pathology is that the overall levels of pathology are relatively low.

## Conclusion

The associations between amyloid- $\beta$ , tau, neurodegeneration and cognition are robust and minimally affected by (genetic) confounding. These novel findings in this unique sample of identical twins are compatible with predictions generated by the amyloid cascade hypothesis and thereby provide important new knowledge for clinical trial designs.

## Acknowledgements

We kindly thank all participating twins for their dedication. Research of Amsterdam Alzheimer Center is part of the Neurodegeneration program of Amsterdam Neuroscience. The Amsterdam



Alzheimer Center is supported by Alzheimer Nederland and Stichting VUmc funds. [<sup>18</sup>F]Flortaucipir PET scans were made possible by Avid Radiopharmaceuticals Inc.

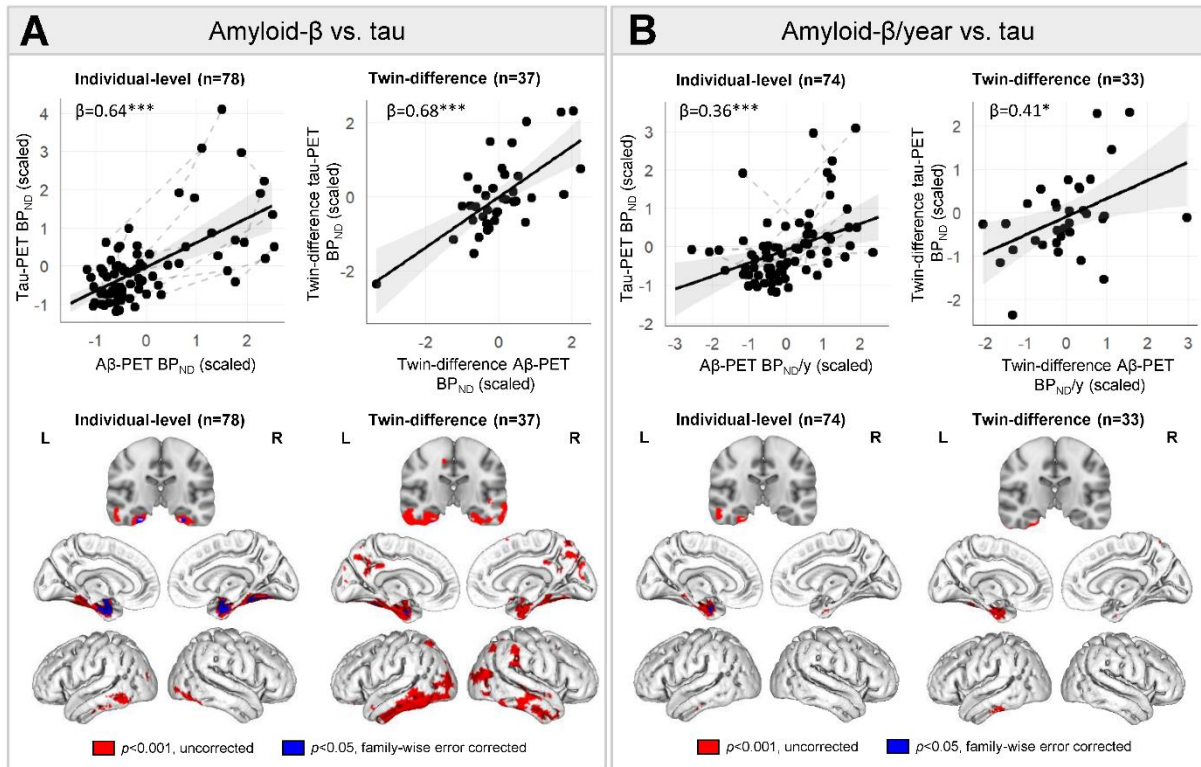
## **Funding**

This study was made possible by the EU/European Federation of Pharmaceutical Industries and Associations Innovative Medicines Initiative Joint Undertaking (EMIF grant 115372), the European Grand Prix for Research of the Foundation on Alzheimer Disease, Dioraphte, Alzheimer Nederland and ZonMW Memorabel. This project has received funding from the Innovative Medicines Initiative 2 Joint Undertaking (AMYPAD) under grant agreement No 115952. This Joint Undertaking receives support from the European Union's Horizon 2020 research and innovation program and EFPIA. This communication reflects the views of the authors and neither IMI nor the European Union and EFPIA are liable for any use that may be made of the information contained herein. M.K. has received research support from Alzheimer Nederland (WE.03-2021-16).

## **Competing interests**

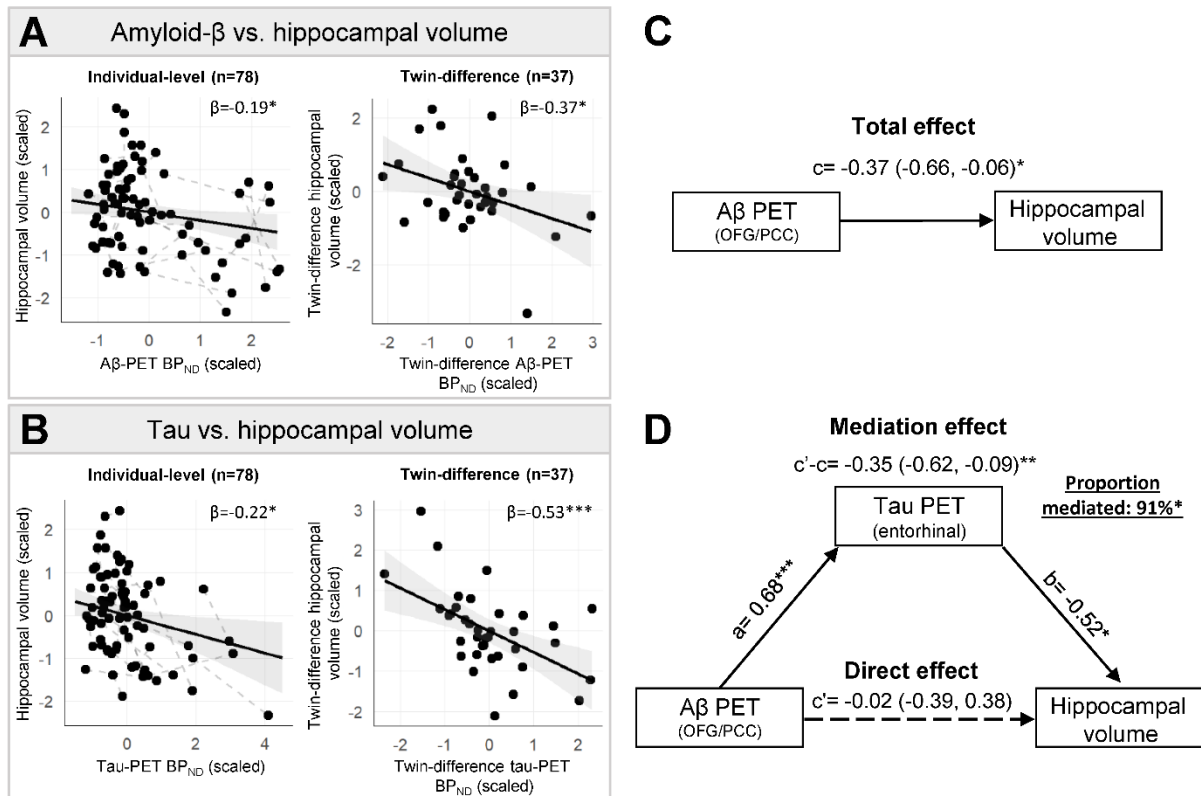
E.M.C., J.T., L.L., M.K., R.R., S.M.L., M.H., S.S.V.G., M.Y., E.J.C.G. and A.B. report no competing interests. R.O. has given a lecture in a symposium sponsored by GE Healthcare (fee paid to the institution), and is an editorial board member of the European Journal of Nuclear Medicine and Molecular Imaging & of Alzheimer's Research & Therapy. L.E.C. has received research support from GE Healthcare (paid to institution). A.D.W. is Editor-in-Chief of Nuclear Medicine and Biology. F.B. is in a steering committee or iDMC member for Biogen, Merck, Roche, Eisai and Prothena. Consultant for Roche, Biogen, Merck, IXICO, Jansen, Combinostics. Research agreements with Merck, Biogen, GE Healthcare, Roche. Co-founder and shareholder of Queen Square Analytics LTD. FB is supported by the NIHR biomedical research centre at UCLH. P.S. has received consultancy fees (paid to the university) from Alzheon, Brainstorm Cell and Green Valley. Within his university affiliation he is global PI of the phase 1b study of AC Immune, Phase 2b study with FUJI-film/Toyama and phase 2 study of UCB. He is chair of the EU steering committee of the phase 2b program of Vivoryon, the phase 2b study of Novartis Cardiology and co-chair of the phase 3 study with NOVO-Nordisk. He is also an employee of EQT Life Sciences (formerly LSP). BMT and PJV are inventors on a patent (#P122938EP10, #P1222938PC00, owner: Stichting VUmc). BvB has received research support from EU-FP7, CTMM, ZonMw, NWO and Alzheimer Nederland, has

performed contract research for Rodin, IONIS, AVID, Eli Lilly, UCB, DIAN-TU and Janssen, was a speaker at a symposium organized by Springer Healthcare, has a consultancy agreement with IXICO for the reading of PET scans, and is a trainer for GE. BvB only receives financial compensation from Amsterdam UMC.



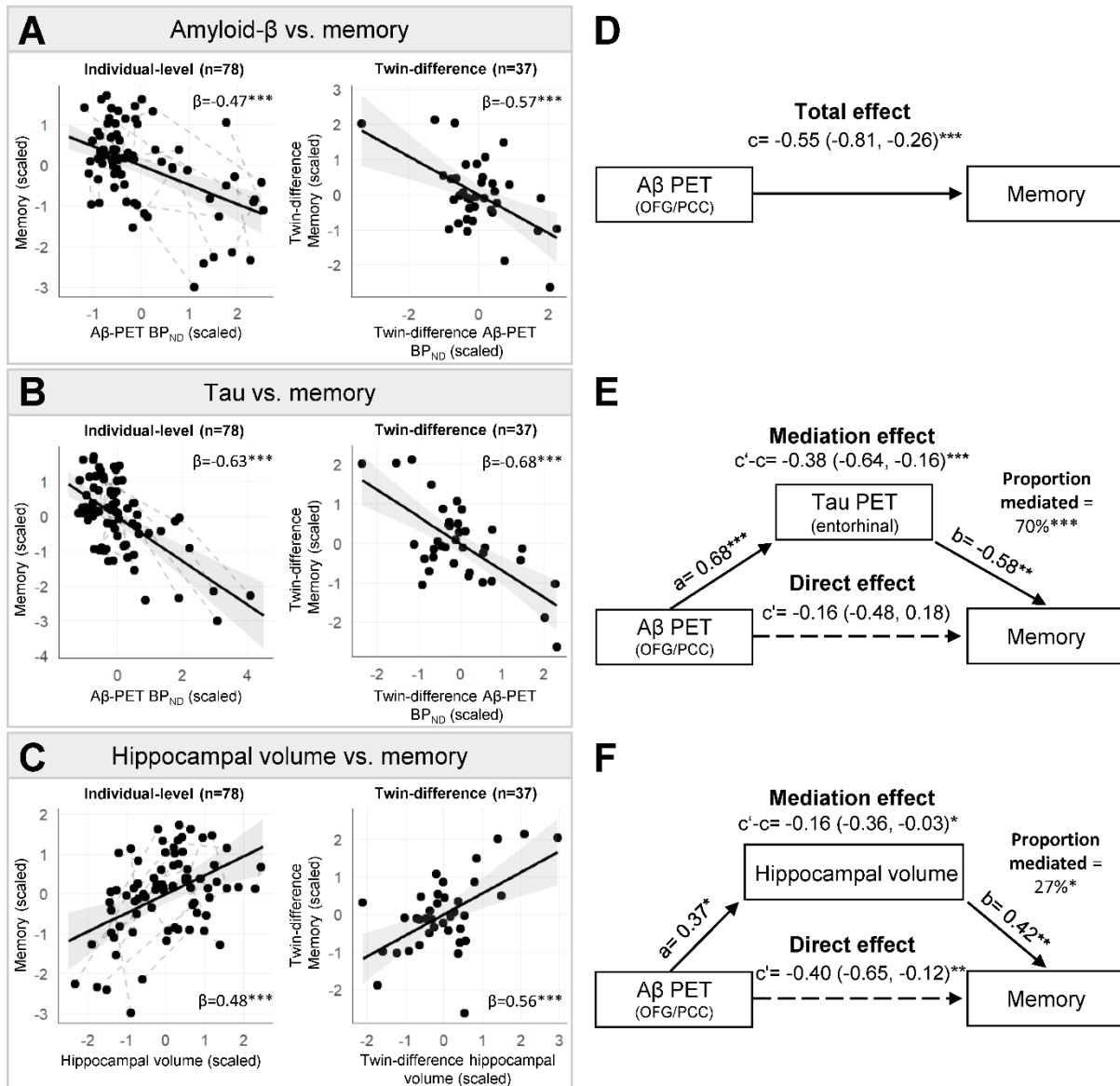
**Figure 1 Individual-level and twin-difference associations between amyloid-β and tau.**

Shown are individual-level and twin-difference associations between (A) cross-sectional amyloid-β versus tau, and (B) annual change in amyloid-β versus tau. For both plots, amyloid-β PET and tau PET  $BP_{ND}$  in the early-AD ROI is shown (OFG/PCC for amyloid-β PET and entorhinal cortex for tau PET). In the individual-level scatterplots, each dot reflects a twin, and twins that belong to the same pair are connected with the dashed line. In the twin-difference scatterplots, each dot reflects a twin-pair. **Voxel-wise results reflect the association between voxel-wise tau PET  $BP_{ND}$  as the outcome variable, and amyloid-β PET  $BP_{ND}$  in the early-AD ROI (OFG/PCC) as the predictor variable at (A) the individual-level and (B) within identical twin-pairs. Voxel-wise results at the individual-level are corrected for age and sex. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .**



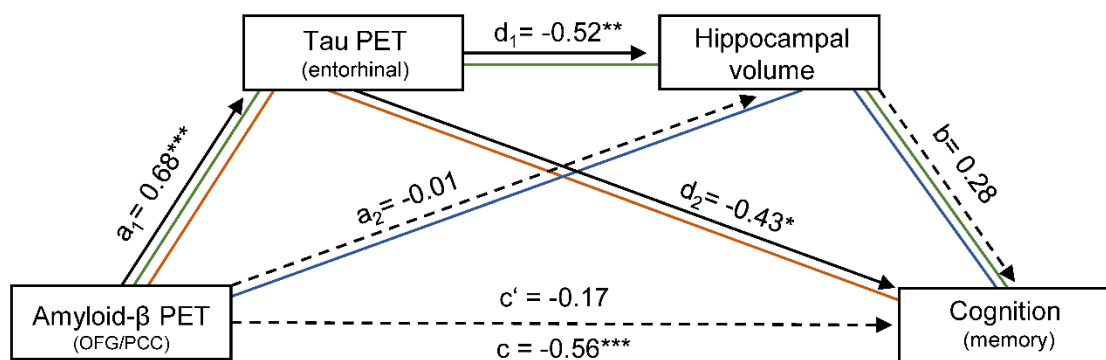
**Figure 2 Individual-level and twin-difference associations between amyloid- $\beta$ , tau and hippocampal volume**

Shown are individual-level and twin-difference associations between (A) amyloid- $\beta$  and hippocampal volume, and (B) tau and hippocampal volume. For both plots, amyloid- $\beta$  PET and tau PET  $BP_{ND}$  in the early-AD ROI is shown (OFG/PCC for amyloid- $\beta$  PET and entorhinal cortex for tau PET). In the individual-level scatterplots, each dot reflects a twin, and twins that belong to the same pair are connected with the dashed line. In the twin-difference scatterplots, each dot reflects a twin-pair. In (C) the direct effect of within-pair differences in amyloid- $\beta$  PET on within-pair differences in hippocampal volume is shown. In (D) the mediating effect of within-pair differences in tau PET in the association between within-pair differences in amyloid- $\beta$  PET and within-pair differences in hippocampal volume is shown. Dashed lines indicate pathways that were not significant. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .



**Figure 3 Individual-level and twin-difference associations between amyloid-β, tau, hippocampal volume and memory**

Shown are individual-level and twin-difference associations between (A) amyloid-β and composite memory, (B) tau and composite memory, and (C) hippocampal volume and composite memory. For both plots, amyloid-β PET and tau PET BP<sub>ND</sub> in the early-AD ROI is shown (OFG/PCC for amyloid-β PET and entorhinal cortex for tau PET). In the individual-level, each dot reflects a twin, and twins that belong to the same pair are connected with the dashed line. In the twin-difference scatterplots, each dot reflects a twin-pair. In (D) the direct effect of within-pair differences in amyloid-β PET on within-pair differences in composite memory is shown. In (E) the mediating effect of within-pair differences in tau PET is shown, and in (F) the mediating effect of within-pair differences in hippocampal volume is shown. Dashed lines indicate pathways that were not significant. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .



Total proportion mediated ( $a_1*d_2 + a_2*b + a_1*d_1*b$ ) = 69.9% ( $p < 0.001$ )

Proportion mediated A>T>C ( $a_1*d_2$ ) = 51.6% ( $p = 0.03$ )

Proportion mediated A>N>C ( $a_2*b$ ) = 0.7% ( $p = 0.94$ )

Proportion mediated A>T>N>C ( $a_1*d_1*b$ ) = 17.8% ( $p = 0.26$ )

#### Figure 4 Serial mediation model

Serial mediation model including pathways between within-pair differences in amyloid-β, tau, hippocampal volume and memory functioning (n=37). For the direct effect of amyloid-β on memory functioning, the total proportion that was mediated via pathways including tau and neurodegeneration was 69.9%. The pathway leading from amyloid-β to tau to memory functioning (highlighted in orange) revealed the largest proportion mediated (51.6%). Dashed lines indicate pathways that were not significant. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

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