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Sex-specific effects of microglial activation on Alzheimer's disease proteinopathy in older adults

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Females show a disproportionate burden of Alzheimer's disease pathology and higher Alzheimer's disease dementia prevalences compared to males, yet the mechanisms driving these vulnerabilities are unknown. There is sexual dimorphism in immunological functioning, and neuroimmune processes are implicated in Alzheimer's disease genesis. Using neuropathology indicators from human brain tissue, we examined the mediational role of microglial activation on the relationship between amyloid and tau and how it differs by sex.

187 decedents (64% female; 89 mean age at death; 62% non-demented) from the Rush Memory and Aging Project completed neuropathological evaluations with brain tissue quantified for microglial activation, amyloid-β and tau. Proportion of morphologically activated microglia was determined via immunohistochemistry (HLA-DP-DQ-DR) and morphological staging (stage I, II or III). Amyloid-β and tau burden were quantified via immunohistochemistry (M00872 or AT8, respectively). Using causal counterfactual modelling, we estimated the mediational effect of microglial activation on the amyloid-β to tau relationship in the whole sample and stratified by sex (amyloid-β → microglial activation → tau). Alternative models tested the role of microglia activation as the precipitating event (microglial activation → amyloid-β → tau).

Microglial activation significantly mediated 33% [95% confidence interval (CI) 10–67] of the relationship between amyloid-β and tau in the whole sample; stratified analyses suggested this effect was stronger and only statistically significant in females. 57% (95% CI 22–100) of the effect of amyloid-β on tau was mediated through microglial activation in females, compared to 19% (95% CI 0–64) in males. Regional analyses suggested that mediational effects were driven by greater cortical versus subcortical microglial activation. Relationships were independent of cerebrovascular disease indices. Alternative models suggested that in females, microglial activation was a significant exposure both preceding the amyloid-β to tau relationship (mediational effect: 50%, 95% CI 23–90) and directly related to tau burden (microglia direct effect: 50%, 95% CI 10–77). By contrast, in males, only the direct effect of microglial activation to tau reached significance (74%, 95% CI 32–100) (mediational effect: 26%, 95% CI 0–68).

Our models suggest a reciprocal, bidirectional relationship between amyloid-β and microglial activation that significantly accounts for tau burden in females. By contrast, in males, direct independent (non-mediational) relationships between microglial activation or amyloid-β with tau were observed. Microglial activation may be disproportionately important for Alzheimer's disease pathogenesis in females. Determining sex-specific vulnerabilities to Alzheimer's disease development both inform fundamental pathophysiology and support precision health approaches for this heterogeneous disease.

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Introduction

Alzheimer's disease is the most common neurodegenerative disease, yet our understanding of how the hallmark amyloid-β plaques and tau tangles develop is still being unravelled. Biological sex and gender (herein referred to as 'sex') is one factor that is associated with Alzheimer's disease pathophysiology and its clinical manifestation. Females account for two-thirds of Alzheimer's disease dementia cases, and show later manifestation of verbal memory impairment and steeper cognitive decline with incipient Alzheimer's disease risk (e.g. *APOEe4* carriage, cerebral amyloid accumulation).[1,2] Despite having clinical statuses comparable to males, several converging studies indicate that females carry higher levels of Alzheimer's disease pathology at autopsy and greater *in vivo* tau density for their amyloid-β burden.[3–9] A leading hypothesis of Alzheimer's disease pathogenesis posits a temporal sequence starting with formation of amyloid-β plaques that promotes the formation of tau tangles and finally neurodegeneration and cognitive decline.[10] These data indicate there may be differential pathways connecting amyloid-β to tau and contributing to the clinical expression of Alzheimer's pathology in females compared to males.

Neuroimmune processes, including microglial functioning, are increasingly implicated in Alzheimer's disease pathogenesis and may provide insights into sex-specific development of disease.

Microglia are resident innate immune cells primarily responsible for surveillance and repair of the CNS.[11,12] In the context of Alzheimer's disease, microglia colocalize with amyloid-β and with prolonged amyloid-β exposure, become activated and differentiate into heterogeneous, proinflammatory response states.[13,14] Continuously activated microglia are then closely tied to the initiation and progression of tau pathology.[15] For instance, microglia release proinflammatory cytokines (e.g. IL1α/β) that can induce and exacerbate tau phosphorylation.[16–18] Microglia also internalize tau, but appear to be inefficient in the breakdown of tau seeds[19] and subsequently become hypofunctional and potentiate tau propagation in both *in vivo* and *in vitro* models of Alzheimer's disease.[20–22] Further, unbiased genomic studies of Alzheimer's disease consistently uncover genes expressed by glial cells (e.g. TREM2, APOE, CD33, PICALM, BIN1) as important contributory and/or modifying pathways in the development of amyloid-β and tau.[23,24] Human PET data additionally indicate that tangle pathology is more strongly correlated with microglial activation than amyloid-β burden, and microglial activation may better predict future spatiotemporal spread of tau compared to amyloid-β.[25,26] Microglial activation may therefore represent an integral intermediary process linking canonical amyloid-β and tau processes.

There is well-described sexual dimorphism of the systemic immune system and accumulating data to suggest these differences

translate into the CNS.[27,28] Peripherally, females tend to show a more robust immunological response to pathogens, greater vaccine-induced antibody production, faster wound healing and are at increased risk of autoimmune diseases.[27] Sex-specific investigation of microglia additionally suggest females show faster 'microglial maturation' in typical development[29] and heightened activation response to prolonged amyloid-β exposure.[13] In amyloid rodent models, there is upregulation of disease associated microglial genes (i.e. proinflammatory), correspondingly less amyloid coverage and compaction, and poorer phagocytic activity in female microglia compared to males.[30,31]

Taken together, microglial activation is initiated by amyloid-β and has the capacity to propagate abnormal tau development and spread. Furthermore, sex differences are observed across microglial, amyloid-β and tau processes. We therefore evaluated the mediational role of microglial activation in the relationship between amyloid-β and tau and tested how this association may differ by sex. We leveraged the Rush Memory and Aging Project (MAP) neuropathology cohort with quantitative measurement of total amyloid-β and tau burden via immunohistochemistry, and activated microglia markers via immunohistochemistry (HLA-DP-DQ-DR) and morphological staging.[32] We used causal counterfactual modelling to estimate the mediational effect of microglial activation on the relationship between amyloid-β and tau relationship stratified by sex, including consideration of regionality and temporality (microglia → amyloid → tau versus amyloid → microglia → tau) of associations.

Materials and methods

Participants

Decedents from the Rush MAP cohort[33] with neuropathological evaluations quantifying brain tissue microglial activation, amyloid-β and tau markers were included in analyses ($n = 187$). Microglial activation markers are not part of the standard neuropathological evaluation in Religious Orders Study/MAP/Minority Aging Research Study (ROS/MAP/MARS), but instead were in support of a substudy.[32] Participants were selected for microglial quantification in chronical (consecutive) order based on the substudy goals within the larger MAP cohort. Participants needed to be dementia free at study entry but were followed to autopsy regardless of clinical status and represent older adults in retirement facilities in the Chicago, Illinois metropolitan area. The only other exclusion criteria used in MAP was an inability to sign an informed consent and the Anatomical Gift Act. Given the relatively smaller sample size and to determine the generalizability of analyses to the larger Rush Alzheimer's Research Center sample, we used inverse probability weights (see the 'Statistical analyses' section) to weight the analytic sample to the demographics (age, sex, education) of all deceased Rush Alzheimer's Research Center participants (ROS/MAP/MARS, $n = 2310$) in a sensitivity analysis. All participants signed a repository consent to allow their data to be repurposed. ROS/MAP/MARS were approved by a Rush University Medical Center Institutional Review Board and are conducted in accordance with the latest Declaration of Helsinki, including written informed consent from all participants.

Neuropathological evaluation

Tissue preparation

Brains from decedents were removed using a standardized protocol. After being weighed, brains were cut coronally into 1-cm thick slabs, immersed in 4% paraformaldehyde for 48–72 h and placed in 2% dimethylsulphoxide/2% glycerol in PBS for storage. See previous studies for in-depth neuropathological evaluation information.[34–36]

Amyloid-β and tau burden

Tissue from eight regions of interest were dissected into 0.5-cm-thick blocks and embedded in paraffin: hippocampus, entorhinal cortex, midfrontal cortex, inferior temporal cortex, angular gyrus, calcarine cortex, anterior cingulate cortex and superior frontal cortex. Amyloid-β was labelled with M00872 (1:100), which binds to both 1–40 and 1–42 lengths of amyloid-β fragments. Paired helical filament tau was labelled with AT8 (1:800), an antibody specific for phosphorylated tau, in 4% horse serum. Immunohistochemical analysis used diaminobenzidine as the reporter, with 2.5% nickel sulphate to enhance immunoreaction product contrast, as previously described.[37,38] Quantification of tau densities was performed using stereological image analyses via Leica DMRBE microscope and computer (Millennia Mme; Micron Electronic) and StereoInvestigator software, version 8.0 (50% region sample with 150×150 μm counting frame at ×400 magnification).[37,38] A standardized, custom algorithm carried was applied to video images of amyloid-β stained sections for quantitative analysis of plaque deposition.[37,38] 24-bit colour images at each sampling site were converted into 8-bit greyscale images and percentage area occupied by amyloid-β immunoreactive pixels was estimated using Object-Image 1.62p15. For both amyloid and tau, the percentage area occupied was estimated per region and then averaged across the eight regions.

Microglial activation quantification

Brain tissue was analysed for the presence of major histocompatibility complex II (MHCII) related microglia activation at three stages of morphological severity (stage I, II, III) across four brain regions: midfrontal gyrus, inferior temporal gyrus, posterior putamen and ventromedial caudate. Activated microglia were tagged via immunohistochemistry using an Automated Leica Bond immunostainer (Leica Microsystems Inc) and anti-human HLA-DP-DQ-DR antibodies (clone CR3/43; DaktoCytomation, 1:100 dilution; catalogue #MA1-25914). Separately, cells were staged by a blinded investigator on the basis of morphology: stage I (thin ramified processes), stage II (rounded cell body >14 μm with thickened processes) or stage III (appearance of macrophages, cell body >14 μm) using StereoInvestigator software, v.8.0 (4.0% region sample with 200×150 μm counting frame at ×400 magnification). Two adjacent blocks of tissue were quantified (0.5 to 1 cm apart) and activated microglia counts were upweighted by stereology software to estimate total number by stage in defined regions. For each stage, several activated microglia were summed, divided by area and multiplied by 10^6 to obtain a composite average density by region. In the brain, MHCII is primarily expressed on microglia/monocytes and considered to be a marker of reactive cells[39,40]; therefore, the HLA-DP-DQ-DR isoforms quantified here reflect densities of 'activated microglia' (versus total microglial count).

A summary index of the proportion of morphologically activated microglia (PAM) was calculated following a recently validated approach[32]:

$$PAM_r = \sqrt{\frac{S3_r}{S1_r + S2_r + S3_r}} \qquad (1)$$

In which r represents each of the four regions and $S1$-3 represent microglial densities across activation stages. PAM was developed to be a sensitive index of the most activated microglia states consistent with disease.[32] Iterative model fitting and sensitivity analyses demonstrated that PAM was a better discriminator of Alzheimer's disease pathology compared to examination of microglia at individual stages. As such, we selected PAM as our primary outcome to reflect a global index of microglia activation.

Global PAM was used for primary analyses. However, given regional vulnerabilities of pathologies, cortical microglial activation may be more indicative of cortical pathologies such as amyloid-β and tau, whereas subcortical microglial activation may be a stronger indicator of vascular disease. Therefore, in secondary analyses, we examined effects of cortical (middle frontal and inferior temporal gyri) versus subcortical (posterior putamen and ventromedial caudate) PAM.

Covariates

APOE genotype was determined from peripheral blood mononuclear cells or brain tissue using Agencourt Bioscience Corporation via high-throughput sequence of codon 112 (position 3937) and codon 158 (position 407) of exon 4 on the APOE gene on chromosome 19. Participants were categorized as carriers versus noncarriers based on presence of APOEe4 allele (homozygote or heterozygote). All models adjusted for APOEe4 status (yes/no), as well as age at death and education. Additionally, given reported associations between both microglial activation and vascular pathology, we further adjusted final models for total vascular disease burden to estimate effects independent of vascular injury. A vascular pathology composite was created by taking the sum of microinfarcts (yes/no) × 3 + macroinfarcts (yes/no) × 3 + arteriolosclerosis (0–3) + atherosclerosis (0–3). Each vascular pathology was weighted to be equally represented in the composite, an approach that has been previously used in the creation of summary scores of vascular dysfunction.[41,42]

Statistical analyses

We used descriptive statistics to describe the sample overall and stratified by sex. We used Wilcoxon signed-rank and chi-squared tests to assess the statistical significance of differences between males and females. Due to the skewed nature of neuropathological variables, we applied a square root transformation for amyloid-β, tau and microglial activation based on visual inspection of QQ-plots in exploratory analyses. We additionally applied a z-score transformation to all neuropathological variables to aid in interpretation of results. We used linear regression and added variable plots to assess the relationships between amyloid-β burden, microglial activation and tau burden among males and females, while adjusting for age at death, APOEe4 status and years of educational attainment.

To assess the mediating effect of microglial activation on the relationship between amyloid and tau burden, we used a causal mediation framework to quantify the natural direct effect and the natural indirect effect. The natural direct effect captures the effect of the exposure (amyloid-β) on the outcome (tau) not through the mediator (microglia activation). The natural indirect effect quantifies the effect of the exposure on the outcome that is due to the effect of the exposure on the mediator.[43,44] To describe these estimands, we defined M0 as the level of microglial activation (mediator) that an individual would have if amyloid-β burden (exposure) was 0, and M1 as the level of microglial activation (mediator) that an individual would have if amyloid-β burden was 1. Given these definitions, the natural direct effect is the difference in the counterfactual outcomes comparing two duplicate hypothetical populations: all individuals with amyloid-β burden = 1 and microglial activation = M0, and all individuals with amyloid-β burden = 0 and microglial activation = M0. Likewise, the natural indirect effect is the contrast in the counterfactual between two different hypothetical populations: all individuals with amyloid-β burden = 1 and microglial activation = M1 and all individuals with amyloid burden = 1 and microglial activation = M0. Additional details on causal mediation analysis and natural direct and indirect effects can be found elsewhere.[45,46] We used 1000 bootstrap replications to quantify the variability of estimated effect sizes and defined the 95% uncertainty intervals as the 2.5th and 97.5th values of the ordered replications. We used bootstrap uncertainty intervals rather than traditional confidence intervals (CIs) to summarize the variability of estimates from mediation analyses. However, to streamline terminology, throughout the paper we use the term CI to refer to uncertainty estimates from either regression models or bootstrap procedures. Details on model assumptions and the quantification of these estimands using regression analysis can be found in the Appendix.

Based on a priori hypotheses, our primary analysis considered amyloid-β burden as the exposure, microglial activation as the mediator, and tau burden as the outcome. Due to the relatively small sample size, which limited our power, we used a threshold of 0.3 as a marker of an important effect size rather than solely rely on hypothesis testing (P-values) in the interpretation of our findings.[47] We also used four-way decomposition methods to compare the overall impact of mediation versus interaction in considering the effect of amyloid-β and microglial activation on tau.[48,49] These methods algebraically decompose the total effect of an exposure on an outcome into four distinct components, and compare the components associated with interaction versus those associated with mediation. In secondary analyses we further explored whether there were differential effects by regionality (subcortical versus cortical) of microglial activation. Additionally, some models hypothesize that neuroinflammation may be a very early event even preceding development of amyloid-β or tau.[50] We therefore considered an alternative model under which microglial activation preceded the amyloid-tau relationship and was considered the exposure, with amyloid-β burden as the mediator.

Sensitivity analyses

We further examined the robustness of our final models to: (i) limiting the sample to only those without clinical dementia at their last visit; (ii) adding the vascular pathology summary score as a covariate; (iii) replicating analyses limiting the amyloid-β, tau and microglial activation measurements to only include the two overlapping brain regions in which all three indicators were assessed (middle frontal and inferior temporal); and (iv) applying inverse probability weighting to account for selection of individuals in the current study and generalize findings to the broader ROS/MAP/MARS combined sample who were eligible for autopsy (n = 2310).[51] For inverse probability weighting, we used logistic regression with basis splines (three degrees) on both age at death and education separately by sex to estimate stabilized weights and account for differences in these variables between those included in our sample and those excluded. We winsorized weights at the 98th percentile to prevent large increases in the variance of estimates due to large weights.

Data availability

MAP data can be requested at https://www.radc.rush.edu.

Table 1 Clinical and demographic characteristics of study sample (*n* = 187) overall and by sex

	Total (*n* = 187)	Females (*n* = 119)	Males (*n* = 68)	P-value	Effect size
Age at death, years	89.68 (6.74)	89.44 (6.54)	90.36 (6.83)	0.93	0.008
Education, years	14 (4)	14 (4)	16 (3.25)	<0.001	−0.34
Race (%, *n*)	–	–		0.42	0.1
White	98.4%, 184	97.5%, 116	100%, 68	–	–
Black	1.1%, 2	1.7%, 2	0	–	–
Other	0.5%, 1	0.8%, 1	0	–	–
MMSE	28 (3)	28 (2)	27 (4)	0.006	0.24
Neurocognitive diagnosis (%, *n*)	–	–	–	0.17	0.17
Clinically normal	33.7%, 63	37.8%, 45	26.5%, 18	–	–
Mild cognitive impairment	27.8%, 52	26.9%, 32	29.4%, 20	–	–
Dementia	38.5%, 72	35.3%, 42	44.1%, 30	–	–
APOEe4 (%, *n*)	23%, 43	23.5%, 28	22.1%, 15	0.96	0.02
Pathological Alzheimer's disease (Reagan criteria, %, *n*)	60.4%, 113	61.3%, 73	58.8%, 40	0.97	0.04
Amyloid-β	3.64 (6.74)	3.83 (7.31)	3.47 (6.52)	0.44	0.07
Tau	3.84 (6.21)	3.84 (5.93)	3.86 (6.69)	0.54	0.05
% Activated microglia					
Global	0.09 (0.06)	0.09 (0.05)	0.09 (0.07)	0.94	0.007
Cortical	0.08 (0.09)	0.09 (0.09)	0.08 (0.08)	0.38	0.08
Subcortical	0.07 (0.07)	0.07 (0.06)	0.08 (0.07)	0.34	−0.08

Effect sizes refer to Cliff's delta for nonparametric continuous variables, Cohen's *D* for parametric continuous variables and Cramer's *V* for categorical variables.

Results

Our sample included 187 participants; there were more females (*n* = 119) than males (*n* = 68). The mean age at death was 89.18 years [standard deviation (SD) = 5.76] and did not differ between females and males (Table 1). Males had slightly higher mean years of education (15.49, SD = 2.61), compared to females (14.00, SD = 2.33). Most of the sample was White, with only two Black participants and one participant identifying as 'other', all of whom were females. Males were more likely to have a clinical diagnosis of mild cognitive impairment and dementia at the last clinical visit, and females had slightly higher rates of pathological diagnoses of Alzheimer's disease; however, neither of these differences were statistically significant. Both amyloid-β and tau levels were slightly, although non-significantly, higher in females compared to males. The PAM [stage III/(stages I + II + III)], which was the indicator used in all analyses, was similar across males and females (Table 1). Regarding absolute stage III microglia density, while the average values did not differ, the distribution of stage III microglia density was shifted higher in females compared to males, and the difference in mean stage III density between males and females was of borderline significance (*P* = 0.06) (Supplementary Fig. 1). Among females, there were also more individuals at the tail of the distribution of activated microglial density with the highest levels of activated microglia.

Added variable plots showed strong and positive associations between amyloid-β and tau, amyloid-β and microglial activation, as well as microglial activation and tau in both males and females after controlling for age at death, education and *APOEe4* status (Fig. 1). In females, a difference of 1 SD in amyloid-β burden was associated with 0.45 SD (95% CI 0.30–0.61) higher microglial activation. This association was slightly smaller in males [standardized coefficient: 0.36 (95% CI 0.10–0.62)], but differences in the estimates for females as compared to males did not reach statistical significance. The association between microglial activation and tau was similar between males [standardized coefficient: 0.41 (95% CI 0.21–0.60)] and females [standardized coefficient: 0.35 (95% CI 0.18–0.51)]. There was also no statistically significant difference in the association between amyloid-β and tau for males compared to

females; however, the magnitude of the point estimate was larger in males [standardized coefficient: 0.51 (95% CI 0.30–0.72)] compared to females [standardized coefficient: 0.35 (95% CI 0.20–0.51)].

In the whole sample, mediation models showed that microglial activation mediated the effect of amyloid-β on tau (significant indirect effect). However, in stratified analysis, this effect was stronger in females. Females with high amyloid burden (+1 SD) demonstrated 0.43 SD (95% CI 0.15–0.77) greater tau burden due to the effect of amyloid on microglial activation compared to females with low (−1 SD) amyloid burden (Figs 2A and 3A). The direct effect of amyloid on tau through pathways other than microglial activation was still large (estimate >0.3) but did not reach statistical significance. The proportion of the total estimated effect of amyloid on tau in females mediated through microglial activation was 57% (95% CI 22–100). By contrast, for males, the indirect effect of amyloid on tau mediated through microglial activation was smaller and not statistically significant. In males, those with high amyloid burden had only 0.19 SD (95% CI −0.04–0.70) greater tau burden than those with low amyloid burden due to the effect of amyloid on microglial activation. Only 19% (95% CI 0–64) of the relationship between amyloid and tau was explained by microglial activation in males, compared to 57% (95% CI 22–100) in females.

Examining the direct effect between amyloid-β and tau (independent of microglial activation) in these models also showed sex differences. The magnitude of the direct effect of amyloid-β on tau was large and statistically significant in males. Comparing males with high versus low amyloid-β burden (±1 SD), there was an estimated 0.81 SD (95% CI 0.32–1.28) difference in tau burden attributable to pathways other than microglial activation. However, in females, while the direct effect of amyloid-β on tau through pathways other than microglial activation was still large (estimate >0.3), it did not reach statistical significance. Taken together, the effect of amyloid-β on tau not explained by microglial activation was ~4.3 times the effect that was mediated through microglial activation in males. In contrast, in females, the effect of amyloid-β on tau not explained by microglial activation was only 1.3 times the effect that was mediated through microglial activation. We examined the same models using regional microglial
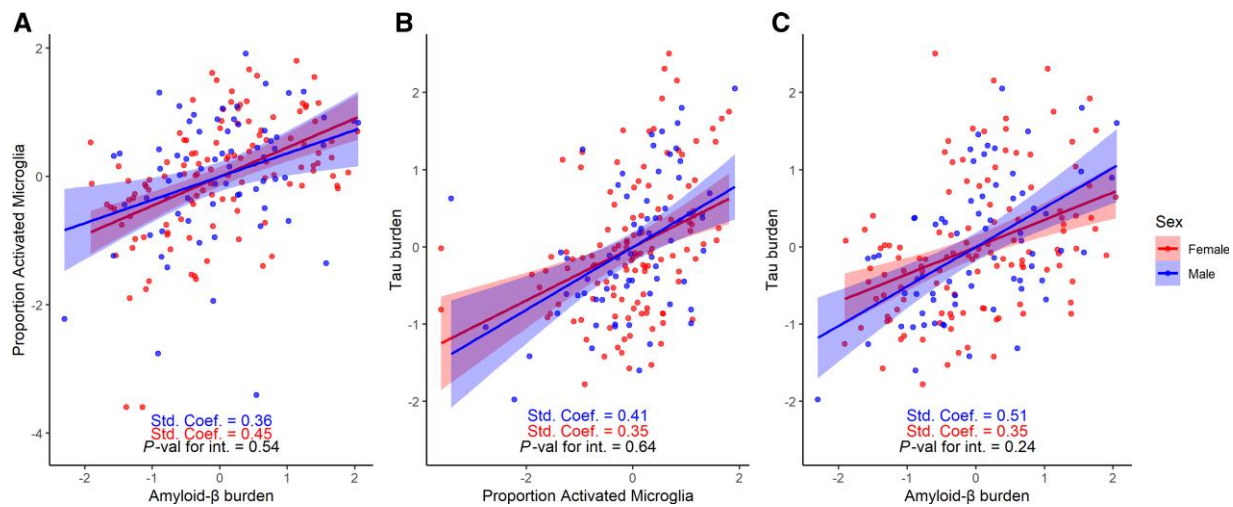
Figure 1 Added variable plots illustrating the relationship between (A) amyloid-β and microglial activation, (B) microglial activation and tau and (C) amyloid-β and tau, in males versus females. The plots show the sex-stratified relationship in the residuals of amyloid-β burden, microglial activation and tau burden, after regressing out common covariation with age at death, education and APOE4 status. Shaded areas represent 95% CIs. Results indicate positive associations between amyloid-β, microglial activation and tau burden. There was some evidence of a difference by sex in the magnitude of the association between amyloid-β and tau, although differences were not statistically significant. Standardized coefficients (std. coef.) and P-values for the interaction by sex are reported in each panel.
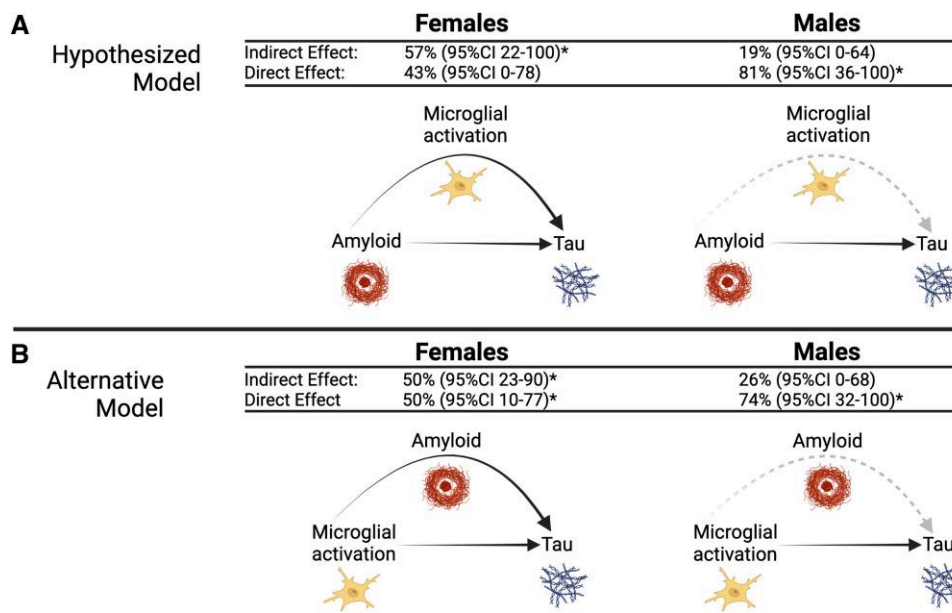

Figure 2 Summary of mediation results for the hypothesized model (amyloid-β leads to tau, partially mediated by microglial activation) and the alternative model (microglial activation leads to tau, partially mediated by amyloid-β). Asterisk denotes P-values < 0.05. Numbers indicate the proportion of the total effect explained by either the direct or indirect effect. Black arrows correspond to standardized beta estimates >0.30 and grey arrows correspond to standardized beta estimates <0.03. Both (A) indirect (hypothesized model) and (B) direct and indirect (alternative model) effects of microglial activation are observed on the amyloid-β-tau relationship in females, but not males.

activation indicators (cortical or subcortical, separately). We found that the observed sex differences were driven by greater cortical rather than subcortical microglial activation (Fig. 3).

A four-way decomposition to compare the magnitude of the mediating versus moderating (interaction) effect of microglial activation on the relationship between amyloid-β and tau confirmed the importance of the mediating relationship. A larger proportion of the overall effect was due to mediation [32% (95% CI 9–77)], compared to interaction [10% (95% CI 0–28)].

We tested alternative causal mediation models in which microglial activation preceded the amyloid-β to tau relationship (microglial activation → amyloid-β → tau). Both direct [standardized coefficient: 0.41 (95% CI 0.20–0.66)] and indirect [standardized coefficient: 0.42 (95% CI 0.07–0.80)] effects were strong and statistically significant in females (Fig. 2B). In comparison, for males, the direct effect was much larger [standardized coefficient: 0.62 (95% CI 0.15–1.12)] than the indirect effect [standardized coefficient: 0.21 (95% CI 0–0.54)].

Although sample size and power were reduced in sensitivity analyses restricting the sample to those without clinical dementia, we found that in this subset of the sample, the point estimate for the effect of amyloid on tau mediated by microglial activation was slightly larger than the point estimate for the effect in the full sample, and retained statistical significance in females [standardized coefficient: 0.54 (95% CI 0.05–1.19)]. In males, the effect of amyloid on tau mediated by microglial activation among those without a clinical diagnosis of dementia was similar to that observed in the full sample [standardized coefficient: 0.14 (95% CI
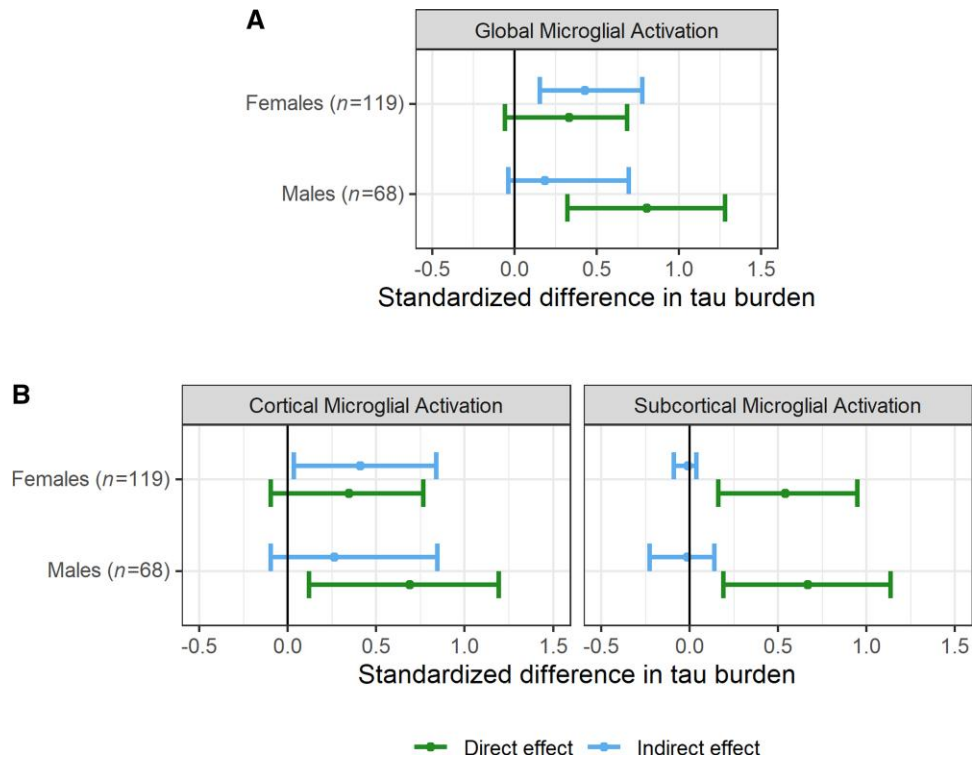

Figure 3 The effect of amyloid-β on tau burden via microglial activation (indirect effect) and via other pathways (direct effect) overall (A) and stratified by brain regions (B) from causal mediation analyses. Error bars represent 95% CIs. Results indicate a double dissociation in strength of direct (amyloid-β → tau) versus indirect (amyloid-β → microglia → tau) effects of mediational models in males versus females, which is driven by cortical greater than subcortical microglial activation.
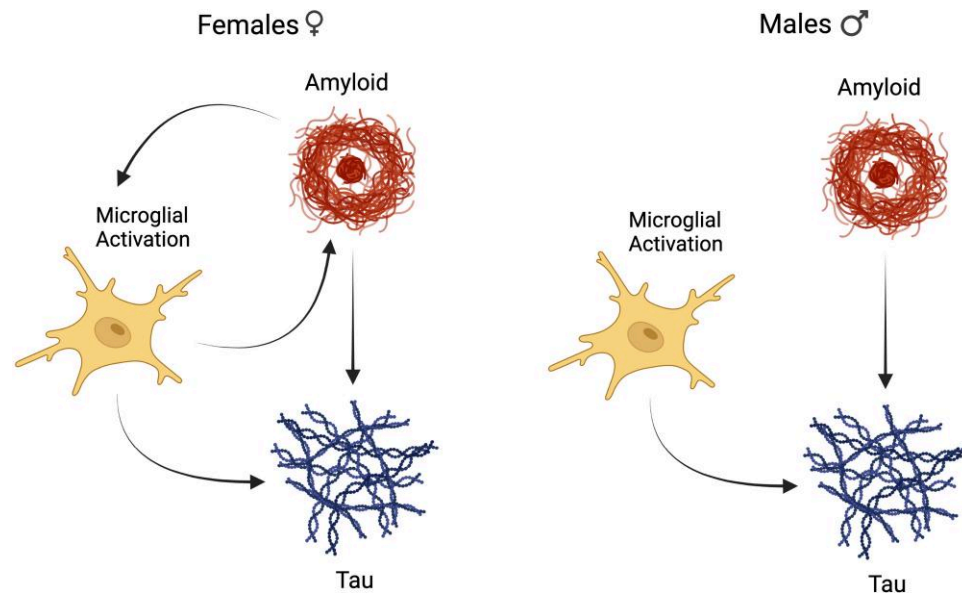

Figure 4 Conceptual summary reflecting study findings from causal counterfactual analytic models. In females, results of the hypothesized and alternative models suggest that there are complex inter-relationships between microglial activation, amyloid-β burden and tau, wherein a reciprocal bidirectional relationship exists between microglial activation and amyloid-β burden, which both then exacerbate tau burden. In contrast, findings in males suggest that the effects of microglial activation and amyloid-β on tau are more independent.

–0.19–1.27)]. Among both females and males, the direct effect of amyloid on tau, via paths other than through microglial activation was smaller in those without clinical dementia as compared to the full sample. Further sensitivity analyses including restricting the sample to those without clinical dementia, adjusting for vascular pathology, using inverse probability weights to generalize findings to the larger ROS/MAP/MARS cohort and using neuropathology summaries of brain regions in common among measures led to some small changes, including shifts in the statistical significance of some findings. However, the effect sizes and patterns of results remained consistent (Supplementary Fig. 2).

Taken together, these models suggest that in females, there may be a bidirectional feedback loop between amyloid-β burden and microglial activation that account for both the effect of amyloid-β on tau and the effect of microglial activation on tau. For males, while both amyloid-β and microglial activation were associated with tau, the mediating relationships between these two variables were weaker and not statistically significant, perhaps indicating that the effects of amyloid-β and microglial activation are more independent in males (Fig. 4).

Discussion

Our data indicate that microglial activation mediates the relationship between amyloid-β and tau burden in females but not males, using counterfactual causal inference models in human neuropathology data. There was a double dissociation such that microglial activation (indirect effect) but not other pathways (direct effect) explained the relationship between amyloid-β and tau relation in females, whereas other pathways (direct effect) but not microglial activation (indirect effect) significantly explained the relationship between amyloid-β and tau in males. We further showed that mediation but not moderation best fit the neuropathology data; this highlights the role of microglia activation as a pathway connecting amyloid-β and tau versus an effect modifier in the amyloid-tau relationship. Results did not change after applying demographic weighting to the larger ROS/MAP/MARS cohort or adjusting for vascular pathology, and appeared driven by relationships in cortical (more susceptible to Alzheimer's disease) versus subcortical regions. Given the molecular sequence of events cannot be fully determined from our observational data and reported bidirectionality between inflammation and amyloid-β development,[50] we tested an alternative model in which microglial activation preceded amyloid-β and tau development. This model further indicated that amyloid-β mediated the relationship between microglial activation and tau in females, whereas the direct effect between microglial activation and tau was strongest in males. Together, our models suggest a reciprocal, bidirectional relationship between amyloid-β and microglial activation in females that accounted for tau burden, with more direct relationships between either amyloid-β or microglial activation with tau in males. These findings suggest that microglial activation may be a more relevant pathway specific to Alzheimer's disease pathology development in females compared to males. Our findings have critical implications and raise the importance of evaluating sex effects in ongoing clinical trials that target (e.g. CSF1R; NSAIDS) and/or risk stratification based on microglial/immunologic dysfunction.

Several converging human studies using autopsy, molecular imaging and CSF indicate overall greater Alzheimer's disease pathology burden, susceptibility to APOEε4, and greater tau given amyloid-β levels in females compared to males.[3–8] Although probably multifactorial, our data indicate that one differentially contributing pathway may be microglia activation. The microglial marker our data used an antibody indicative of reactive microglial states (HLA-DR-DQ-DL) and morphology most related to disease responses (stage 3 morphological activation).[39] Our findings therefore suggest that dysfunctional glial processes are disproportionately relevant in the development of the amyloid-tau relationship in females, both as precipitating and mediating events. However, microglia are highly dynamic cells and additional research is needed to disentangle sex-specific impact of potentially protective states (e.g. 'M2' states) in Alzheimer's disease development. Microglial activation and 'brain inflammation' are also strongly linked with cerebrovascular injury, and vascular disease has a stronger impact on brain aging in postmenopausal females.[52,53] We found that microglial activation in cortical (Alzheimer's disease vulnerable), but not subcortical (vascular vulnerable) regions drove the mediational models in females, and that models remained unchanged adjusting for vascular pathologies. Together, our models may reflect a more Alzheimer's disease-specific, amyloid-related immune response in females. Importantly, there were no sex differences in absolute levels of microglial activation, suggesting that activation states may be equally present in both sexes but function differently. In males, while microglial activation and amyloid-β strongly related to tau burden, mediation and moderation analyses suggest that these biological pathways were relatively independent. Our data highlight a potential role for amyloid-related microglial activation as a female specific explanatory pathway for the disproportionate representation of Alzheimer's disease and especially tau burden in females.

Although the underlying mechanisms of female microglial vulnerability are unknown, our study converges with recent animal work demonstrating sex-specific microglial signalling in Alzheimer's disease. Recent studies have demonstrated faster but more dysfunctional microglial signalling in female Alzheimer's disease transgenic mice, including less amyloid-β phagocytosis and plaque coverage.[13,30] Although the overall number of microglia do not appear to differ by sex, microglial signalling factors and morphology did, suggesting functional differences in microglial responses in female versus male Alzheimer's disease models,[13] consistent with our findings. For instance, microglial TREM2 levels, which may be a key promoter of amyloid-β phagocytosis, were lower in EFAD female mice.[31] Additionally, male APP/PS1 mice evidenced more ameboid microglia, considered to be more mobile and phagocytic.[31] These animal models align with our findings indicating a bidirectional relationship between dysfunctional microglial activation and amyloidosis that may promote tau development in females. Together, these observations suggest that initially dysfunctional microglial responses to amyloid-β in females may perpetuate the presence of amyloid-β and further promote proinflammatory microglial signalling that can contribute to tau hyperphosphorylation and spread.

There are several important limitations to our study. First, the observational nature of the neuropathology data precludes causal temporal sequencing, and our indicator of microglial activation was limited to proinflammatory, disease-related states at autopsy. Although our hypothesized models were guided by temporal ordering from previous in vitro and in vivo experiments, in vivo clinical studies are needed to carefully parse out the timing and signalling states most relevant by sex and in disease development. It is increasingly recognized that microglia are a highly heterogeneous cells and play influential roles in brain development throughout the lifespan that may cumulatively affect Alzheimer's disease risk and development; lifespan estimation of microglial signalling (e.g. pre- and post-menopause) are needed to better understand differential sex risks. We additionally had a relatively small and

homogeneous sample, which probably affected the precision of our estimates. Most notably, we had fewer males than females, which may have limited power in stratified analyses. However, it is notable that pattern of findings was also qualitatively different in males versus females (e.g. double dissociation) and effect sizes were large. There were notable differences between the full ROS/MAP/MARS cohort and our analytic sample; while there was a statistically significant sex difference in amyloid-β and tau burden in the full sample there was no sex difference in our primary analytic sample (Supplementary Fig. 3). However, our mediation findings were also consistent in sensitivity analyses using inverse probability weights, suggesting that findings are generalizable to the larger, more diverse ROS/MAP/MARS cohort; however, inverse probability weights were a function of only age, sex and education. Importantly, we were unable to use inverse weighting as a function of race due to the fact our sample had very few non-White participants. It is imperative for future brain autopsy studies to recruit more diverse racial/ethnic samples to expand research in this area into the broader population. Given consistently reported sex by APOE effects in Alzheimer's disease risk, larger samples in future studies are needed to explore how the observed sex differences were impacted by APOE4 genotype.[4,54] These are the first human analyses showing a sex-specific mediational role of microglial activation in females compared to males. Replication of these findings is needed to test veracity, particularly against influences due to cohort-specific selection and survival bias.

Microglia are increasingly implicated in Alzheimer's disease genesis and pathology spread, and we found a sex-specific role for proinflammatory microglial activation in canonical Alzheimer's disease proteinopathy pathways. We identified a positive feedback loop between microglial activation and amyloid-β that mediated tau levels in females, whereas in males, direct (non-mediational), independent relationships between microglial activation or amyloid-β with tau were observed. These data indicate that microglial activation plays a disproportionate role in Alzheimer's disease development in females, whereas other pathways are more relevant to Alzheimer's disease development in males. Our data highlight the importance of evaluating sex-specific vulnerabilities in pathways to Alzheimer's disease development. Clinical trials and risk biomarkers targeting microglial dysfunction may benefit from careful sex-specific applications.

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Competing interests

The authors report no competing interests.

Supplementary material

Supplementary material is available at *Brain* online.

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