

Increased Tau, Amyloid- β , and IBA1 in the Middle Temporal Gyrus Is Associated With Lower Cognitive Function in SEA-AD Donors

[school redacted]

Abstract

Alzheimer's Disease (AD) is a neurodegenerative disorder characterized by mechanisms including neuroinflammation, neurofibrillary tangles of phosphorylated tau, accumulation of amyloid plaques, and microglial aggregation. In AD, disease pathology is closely related to cognitive function, measured using the Mini-Mental-State-Examination (MMSE), a clinical screening tool. This study examined relationships among tau burden, amyloid deposition, and microglial activity in the middle temporal gyrus across three MMSE-defined stages of cognitive decline. Data, including quantifications of AT8, 6e10, and IBA1, were obtained from the Seattle Alzheimer's Disease Brain Cell Atlas. One-way ANOVAs were used to study the change in AT8 positive area (phosphorylated tau), IBA1-positive cell counts and activation state (microglia), 6e10 cell counts (amyloid-beta), and IBA1+/6e10 colocalization, alongside MMSE-defined cognitive decline. Our analysis revealed that neuropathological measures of AT8 positive area, IBA1/6e10 colocalized objects, 6e10 cell count, and IBA1 inactive:active ratio all reflected a graded increase as MMSE scores decreased. Although general IBA1+ cell counts were comparable across MMSE groups, the inactive:active ratio greatly differed, with lower MMSE scores exhibiting higher ratios. These findings lead to the hypothesis that decreasing MMSE scores may be associated with increased tau burden and amyloid deposition, as well as alterations in microglial activation. Specifically, the microglia may exhibit increased association with sites of pathology whilst displaying reduced functional activation in more advanced stages of cognitive decline. Together, these findings inform a broader understanding of the link between pathological burden and cognitive function in a multidimensional model of AD.

Keywords: Alzheimer's Disease (AD), MMSE, dementia, tau pathology, amyloid beta, microglial activation

1. Introduction

Alzheimer's disease (AD) is the most common cause of dementia and is characterized by progressive cognitive decline. It is accompanied by the accumulation of extracellular amyloid- β (A β) plaques, intracellular neurofibrillary tangles composed of hyperphosphorylated tau, and widespread neuroinflammation. Although these pathological hallmarks have been studied extensively, the mechanisms linking such protein aggregation to cognitive impairment are still unclear.

The accumulation of extracellular amyloid plaques, formed from self-aggregating peptides, has long been regarded as the first hallmark of Alzheimer's disease, inhibiting neuronal synaptic function (Reitz, 2012). Amyloid deposition is known to begin years before the onset of clinical symptoms. On the other hand, tau accumulation, presenting as neurofibrillary tangles toxic to neurons, more closely tracks the timeline of dementia-related neuronal dysfunction, synapse loss, and worsening cognition (Cody et al., 2024). Consequently, increasing tau burden is commonly considered one of the strongest neuropathological indicators of disease severity (Murray et al., 2022). Previous studies have also affirmed that tau pathology is more strongly associated with cognitive impairment than amyloid burden alone (Guo et al., 2026). Amyloid pathology nevertheless remains an important marker, as it is believed to initiate a cascade of downstream events that contribute to tau aggregation, neurodegeneration, and ultimately dementia (Reitz, 2012).

In addition to protein deposition, neuroinflammation is another major hallmark of dementia, with emerging studies suggesting that inflammation may be both a consequence and an initiator of AD pathology like tau accumulation and amyloid plaques (Zhang et al., 2023). Under healthy conditions, microglia contribute to tissue maintenance through debris clearance and immune surveillance (McKinney et al., 2026). They are the brain's resident immune cells and macrophages, continuously monitoring the brain environment and responding to accumulating amyloid deposits and neuroinflammation (Muzio et al., 2021). In AD, however, microglia aggregate around amyloid plaques and undergo substantial changes in morphology, gene expression, and function (Bolmont et al., 2008).

Experimental and genetic evidence suggests that microglia initially exert protective effects by containing plaques and phagocytosis of pathological proteins, but prolonged pathological stress may yet lead to impaired clearance mechanisms and contribute to neurodegeneration by accelerating the spreading of proteins like A β and tau. (Gao et al., 2023). Furthermore, evidence argues that certain activation states of microglia may mediate synapse loss, worsen tau pathology, and secrete inflammatory factors that directly harm neurons (Hansen et al., 2018). Microglia are able to transform from one phenotype to the other—in particular, the M1 microglia, which release inflammatory mediators, and the M2 microglia, which release anti-inflammatory mediators, may provide further insight on the effective activation states of microglia (Guo et al., 2022). Despite growing recognition of the importance of neuroinflammation, it still remains unclear whether microglia continue to respond effectively as disease progresses or whether they transition into dysfunctional states that contribute to further pathological accumulation. This study therefore investigates how the accumulation of microglia around sites of pathology may change across different stages of neurodegeneration.

In AD, neuroinflammation spreads heavily across the brain to areas like the cerebral cortex, the outer layer of the brain responsible for thinking, memory, and language (Ni & Wu, 2021). The cerebral cortex is organized into distinct layers that all

73 differ in cellular composition, connectivity, and functional roles, meaning pathology may behave differently across layers.
74 Emerging evidence suggests that neurodegenerative pathology may not be distributed uniformly throughout cortical tissue,
75 and analyses restricted to just gray matter measurements may obscure important layer-specific patterns (Willumsen et al.,
76 2022). The middle temporal gyrus (MTG) is particularly relevant in AD because of its involvement in language processing,
77 semantic memory, and other higher order cognitive functions that become impaired during disease progression (Chen et al.,
78 2022). Investigating pathology across individual cortical layers may therefore provide additional insight into selective regional
79 differences and vulnerabilities.

80 Accordingly, the present study investigated relationships among tau burden, amyloid deposition, and microglial activity
81 within the MTG across MMSE-defined stages of cognitive decline. Particular emphasis was placed on examining the
82 pathology within individual cortical layers 1 to 6, following initial bulk gray matter analyses.

83 A previous study of these markers in the SEA-AD dataset investigated the APOE4 genotype and an individual's genetic
84 predisposition to dementia (Wang, 2025). However, this present study uses MMSE grouping to better understand the
85 relationships between disease pathology and an individual's ongoing cognitive capacities.

86 I hypothesized that lower MMSE scores would be associated with higher levels of tau burden and amyloid deposition, as well
87 as alterations in microglial activation. More specifically, the microglia were expected to increasingly associate with sites of
88 pathology whilst displaying reduced functional activation in more advanced stages of cognitive decline. Furthermore,
89 layer-specific analyses were expected to reveal patterns that would not be apparent from the gray matter measurements
90 alone.

91 2. Materials and Methods

92 De-identified postmortem human brain data and quantitative neuropathological measurements of the middle temporal gyrus
93 were accessed from the Allen Brain Institute's publicly available SEA-AD dataset (Hawrylycz et al., 2024). All experimentation
94 and data were conducted by the Kaiser Permanente Washington Health Research Institute ACT Study researchers and the
95 University of Washington ADRC at the UW BioRepository and Integrated Neuropathology (BRaIN) laboratory. The dataset
96 focused on different patients' histological pathology, obtained via immunolabeling and microscopy across cortical layers and
97 protein markers.

98 Data from the Quantitative Neuropathology Summary Data (MTG) and Donor Metadata from the Allen Brain Institute's
99 SEA-AD data set were aligned and compiled in an Excel spreadsheet in order to match neuropathology data to donor MMSE
100 status. Donors were stratified into three groups based on their respective Mini-Mental State Examination (MMSE) scores:
101 27–30, 23–26, and 0–22.

102 2.1. Diagnosing Dementia

103 Cognitive status was classified into donor groups according to Mini-Mental State Examination (MMSE) scores obtained from
104 SEA-AD donor metadata. The MMSE is a widely used clinical screening tool for cognitive impairment, with scores ranging
105 from 0 to 30, where lower scores indicate greater cognitive dysfunction (Creavin et al., 2016; Yang et al., 2024).



Donors were stratified into three groups based on established thresholds used in dementia research: MMSE 27–30 (cognitively normal or minimally impaired), MMSE 23–26 (mild cognitive impairment), and MMSE 0–22 (moderate to severe cognitive impairment consistent with dementia). These thresholds were selected based on prior studies demonstrating that scores below 24 are strongly associated with clinical dementia—additionally, scores in the 24–26 range have been shown to represent a transitional state, with individuals at increased risk of progression to dementia (Braekhus et al., 1995; Kukull et al., 1994).

The MMSE cutoff of 24 is commonly used in both clinical and research settings to distinguish cognitively impaired from non-impaired individuals and has been validated against structural brain changes (Sakurai et al., 2021). Based on this framework, the present study used MMSE stratification to approximate stages of cognitive decline and to enable comparison of neuropathological burden across disease severity.

Due to the nature of dementia disease progression, age at death is an essential consideration when analyzing the severity of pathological burden. Thus, when deciding the MMSE ranges for the three experimental groups, a one-way ANOVA was used to run a replicate data column analysis with multiple comparisons of donor ages at death. When grouping MMSE scores into three groups: 0–22, 23–26, and 27–30, the multiple comparison mean values yielded no significant difference between groups (mean of 0–22 vs. 23–26 being 89.29, mean of 0–22 vs. 27–30 being 89.87, and mean of 23–26 vs. 27–30 being 89.87). Thus, these cutoffs provided a more balanced partition of donor data rather than relying on arbitrary thresholds.

In our dataset, before outlier removal, the group with MMSE scores 27–30 consisted of 31 donors, the group with MMSE scores 23–26 consisted of 31 donors, and the group with MMSE scores 0–22 consisted of 17 donors.

All downstream analyses were performed using these MMSE-defined groups to evaluate relationships between cognitive status and pathological markers in the middle temporal gyrus.

2.2. Neuropathological Quantification

Detailed tissue processing and staining protocols have been previously described in a published paper as well as an online procedural overview (Gabbitto et al., 2024b; Seattle Alzheimer's Disease Data Set, n.d.). Briefly, postmortem human brain tissue was obtained through the Adult Changes in Thought (ACT) study conducted by the Kaiser Permanente Washington Health Research Institute in collaboration with the University of Washington.

Briefly, researchers collected middle temporal gyrus (MTG) samples, fixing and embedding each in paraffin prior to sectioning. Sections underwent duplex immunohistochemical staining to detect various hallmark Alzheimer's disease proteins (Hawrylycz et al., 2024). Phosphorylated tau was labeled using the AT8 antibody, while amyloid- β (A β) deposits were identified using the 6E10 monoclonal antibody. Quantification of AT8-positive area, 6E10-positive objects, and IBA1-associated measures was performed on digitized images of the MTG cortical layers. IBA1-positive cells were classified as active or inactive according to dataset-provided morphological criteria.

The Allen Brain Institute collected and compiled the data for access to the general public. Data were accessed June 6th, 2026, via:

139 <https://portal.brain-map.org/explore/seattle-alzheimers-disease/seattle-alzheimers-disease-brain-cell-atlas-download?edit&language=en>.
140

141 2.3. Outlier Removal

142 Initial outlier detection was performed using the ROUT method ($Q = 1\%$) in GraphPad Prism. Then, donors who were
143 consistently identified as outliers across datasets were excluded. A total of six donors were excluded, including four males,
144 and two females. Of the excluded donors, four were classified as having dementia, and two were classified as having no
145 dementia. Excluded donors were: H20.33.028, H20.33.043, H21.33.020, H21.33.029, H20.33.036, and H21.33.031.

146 2.4. Statistical Analysis

147 Statistical analyses were performed using ordinary one-way ANOVA with multiple comparisons in GraphPad Prism.
148 Layer-specific analyses were conducted within MMSE groups.

149 Data are presented as bars with mean $\pm$ SEM. Statistical significance thresholds were defined as: * $p < .05$, ** $p < .01$, *** $p < .001$.
150

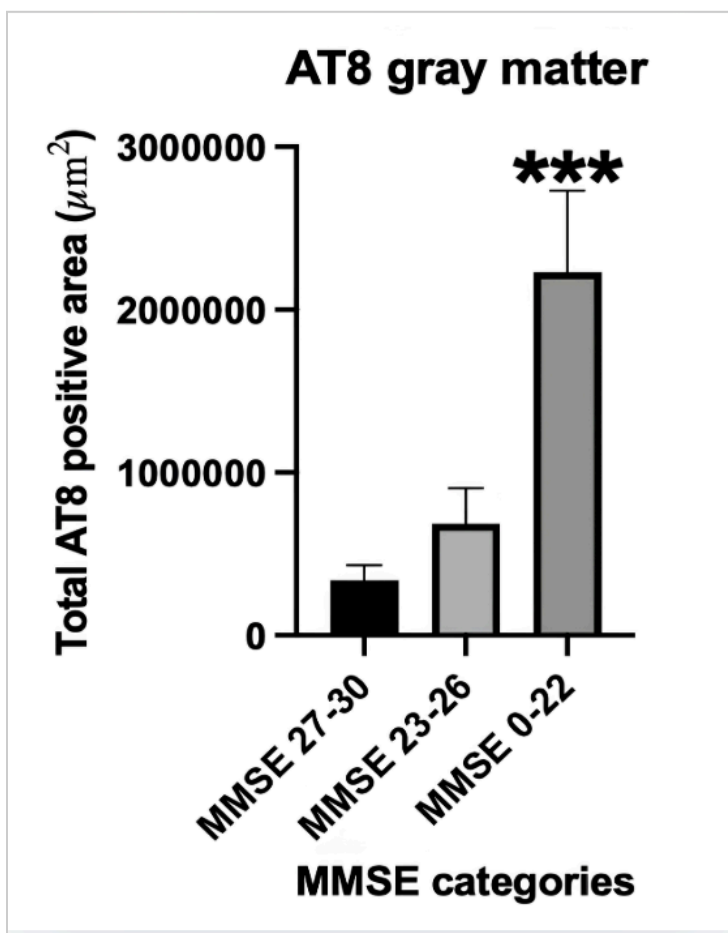
151 3. Results

152 3.1. AT8 Pathology in the Middle Temporal Gyrus Higher with Cognitive Decline

153 To assess the relationship between tau pathology and cognitive decline, total AT8-positive area was quantified across the
154 gray matter of the middle temporal gyrus (MTG) and stratified by MMSE category (Figure 1). AT8 burden was higher in
155 donors with decreased MMSE scores, with the lowest MMSE group (0–22) exhibiting a greatly elevated and significant total
156 AT8-positive area in micrometres squared. The intermediate group (23–26) showed values between the high and low MMSE
157 groups, indicating a graded relationship between tau accumulation and cognitive impairment.

158 This trend was consistent across donors within each group, with statistical comparisons demonstrating a significant
159 increase in AT8-positive area in the 0–22 group relative to both higher MMSE groups (*** $p < .001$).





160

161 **Figure 1. Total AT8-positive area in MTG gray matter across MMSE categories.** Significance (***) was found between

162 3.2. Increased Microglial Association with Amyloid Pathology Across Cortical Layers

163 To evaluate microglial interaction with amyloid pathology, the number of IBA1/6E10 co-localized objects was quantified
164 across cortical layers of the MTG (Figure 2). Co-localized object counts were higher with decreased MMSE scores, with the
165 greatest values observed in the 0-22 group.

166 Layer-specific analysis revealed a pronounced enrichment in layer 3 across all MMSE groups. This increase was statistically
167 significant in the 23-26 group (**p < .01) and further elevated in the 0-22 group (***) (***p < .001). Other layers demonstrated
168 comparatively lower counts and less consistent differences between MMSE categories.

169 The progressive increase in co-localized objects across MMSE groups indicates an enhanced microglial presence in
170 proximity to amyloid deposits as cognitive impairment worsens.



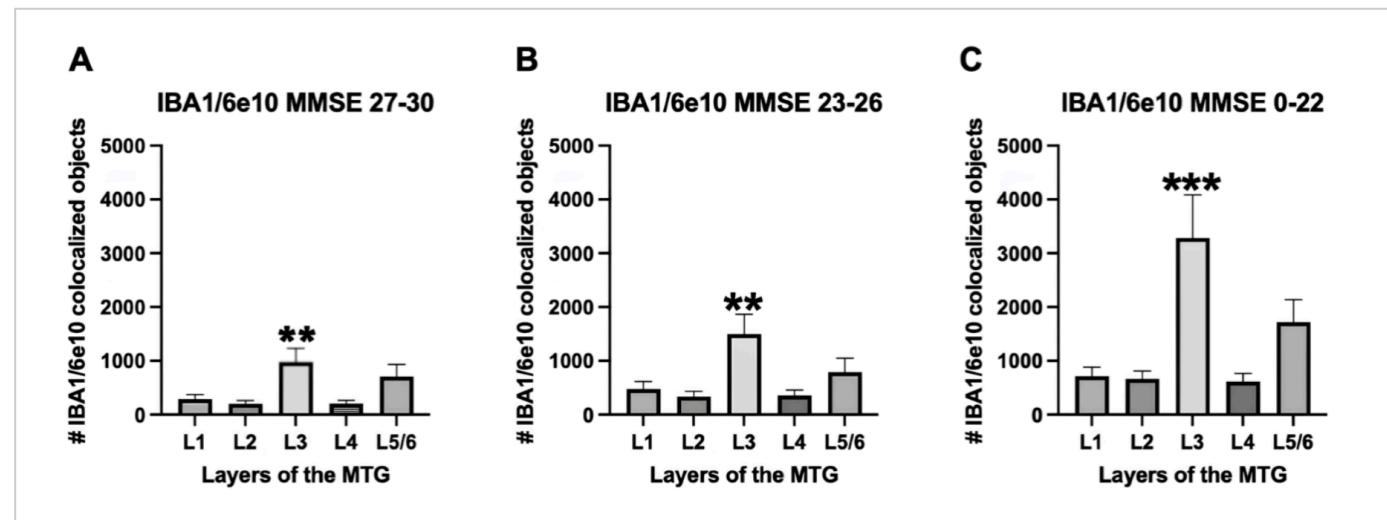


Figure 2. Number of IBA1/6E10 co-localized objects across MTG cortical layers by MMSE category. In graph A (MMSE group 27-30), significance was found between L3 and all other layers (** $p < .01$). In graph B (MMSE group 23-26), significance was found between L3 and all other layers (** $p < .01$). In graph C (MMSE group 0-22), significance was found between L3 and all other layers (*** $p < .001$).

3.3. Layer-Specific Distribution of Amyloid- β Deposition

To determine whether the observed layer-specific patterns were driven by amyloid distribution, 6E10-positive objects were quantified independently across MTG layers (Figure 3). A consistent significance of 6E10-positive objects was observed in layer 3 across all MMSE groups (*** $p < 0.001$).

While total amyloid burden was higher in lower MMSE scores, the relative distribution across cortical layers remained stable. Layer 3 consistently exhibited the highest number of 6E10-positive objects, while other layers showed lower and more uniform distributions.

Notably, the number of 6E10-positive objects exceeded the number of IBA1/6E10 co-localized objects across all groups, indicating that only a subset of amyloid deposits were associated with microglial localization.



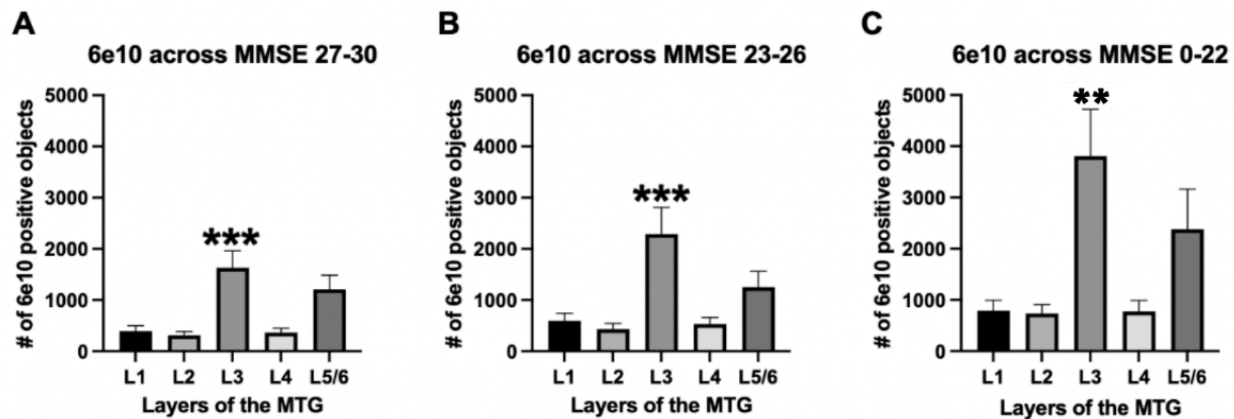


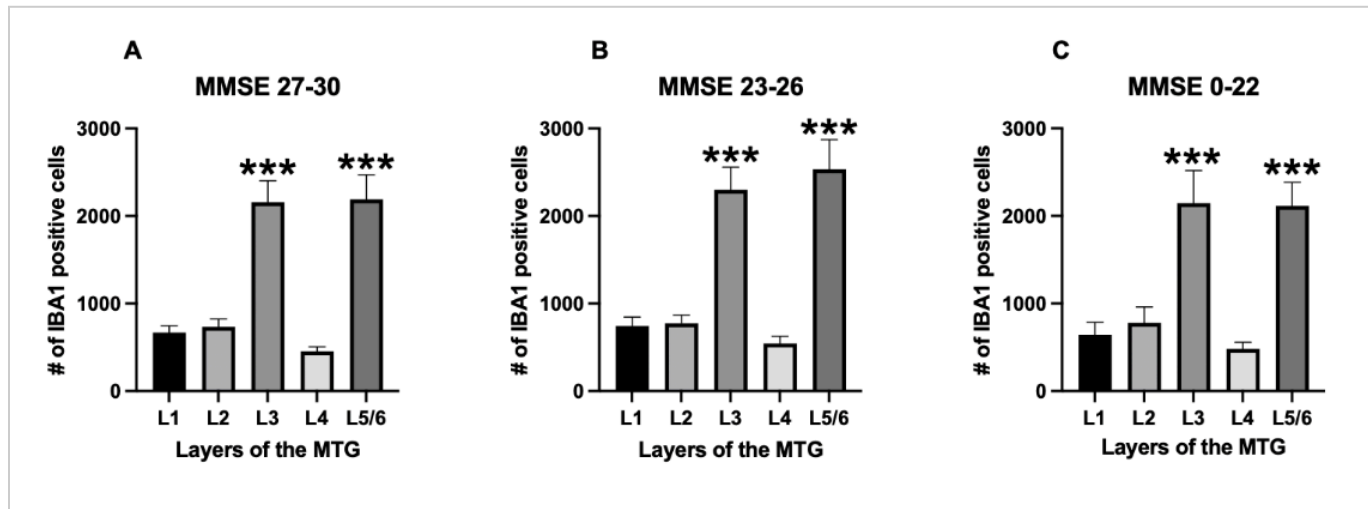
Figure 3. Number of 6E10-positive objects across MTG cortical layers by MMSE category. In graph A (MMSE 27-30), significance was found between L3 and all other layers ($***p < .001$). In graph B (MMSE 23-26), significance was found between L3 and all other layers ($***p < .001$). In graph C (MMSE 0-22), significance was found between L3 and all other layers ($***p < .001$).

3.4. Total Microglial Activation Appears Comparable Across Cognitive Status

To assess whether the survival of microglial cells affected amyloid colocalization, the total amount of IBA1-positive cells was first compared across cortical layers and MMSE groups (Figure 4). Notably, levels of IBA1 positive cell counts were similar across all three MMSE groups, indicating that there must be another explanation for the amyloid colocalization differences from Figure 2.

Layer-specific analysis revealed a pronounced enrichment in layer 3 across all MMSE groups. This increase was statistically significant ($***p < 0.001$) in all groups. Other layers demonstrated comparatively lower counts and less consistent differences between MMSE categories.





196

197 **Figure 4. Number of total IBA1 positive cells across MTG cortical layers by MMSE category.** In graph A (MMSE group 27-30), graph B
198 (MMSE group 23-26), and graph C (MMSE group 0-22), significance was found between L3 and all other layers (***p < .001), and
199 between L5/6 and all other layers (***p < .001).

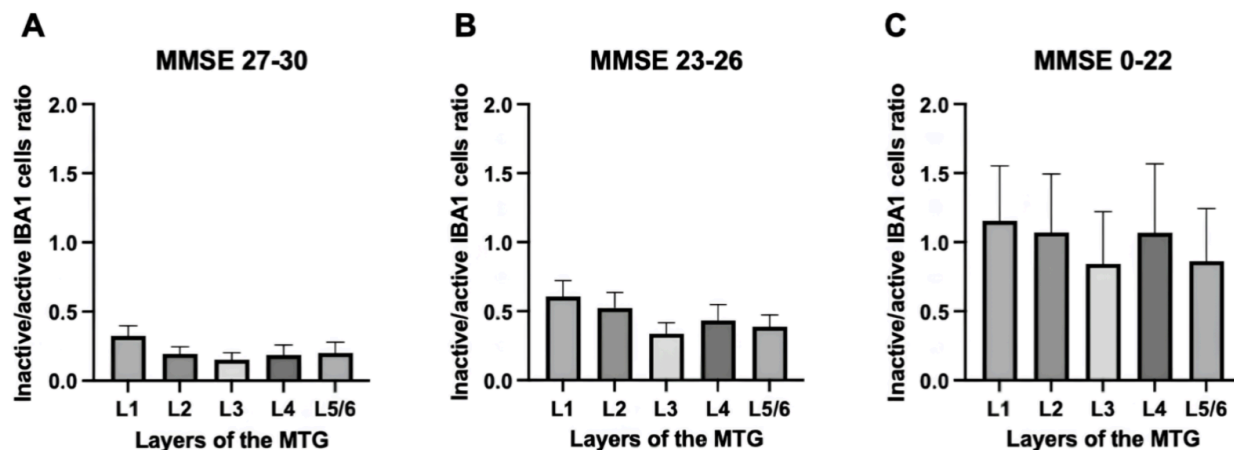
200 3.5. Microglial Activation Decreases with Worsening Cognitive Status

201 To assess microglial functional state itself, the ratio of inactive to active IBA1-positive cells was calculated across cortical
202 layers and MMSE groups (Figure 5). A progressive increase in the activation ratio was observed across declined MMSE
203 scores, with the highest ratios in the 27-30 group and the lowest in the 0-22 group.

204 Although differences between cortical layers were not statistically significant, the upward trend in activation ratio was
205 consistent across all layers. In the lowest MMSE group, the ratio reflected a relative increase in activated microglial states.

206 These findings indicate that while microglia remain comparably present in regions of pathology, their activation state
207 changes with disease progression, with more cells becoming inactive relative to active as disease severity worsens.





208

209 **Figure 5. Ratio of inactive to active IBA1-positive microglia across MTG layers by MMSE category.** No significance was found.

210 3.6. Summary of Pathological Trends Across MMSE Groups

211 Across all measures, lower MMSE scores were associated with higher pathological burden and altered microglial dynamics.
212 AT8-positive area and 6E10-positive object counts were higher in donors with worse cognitive status, while IBA1/6E10
213 co-localization also showed higher levels across layers, particularly in layer 3. Total IBA1 positive cell counts were relatively
214 constant across MMSE scores, indicative of almost no difference related to disease severity. In contrast, the ratio of
215 inactive to active microglia was higher in donors with lower MMSE scores.

216 4. Discussion

217 4.1. Progressive Pathological Burden Accompanies Cognitive Decline

218 The present study investigated relationships between tau pathology, amyloid deposition, and microglial activity within the
219 middle temporal gyrus (MTG) across MMSE-defined stages of cognitive decline. It was hypothesized that lower MMSE scores
220 would be associated with higher pathological burden and altered microglial activation states. Overall, the findings supported
221 this hypothesis and affirmed expected changes in disease pathology and neuroimmune dynamics in individuals with varying
222 degrees of cognitive deficits.

223 Tau burden, measured by the AT8-positive area in the study, was substantially higher in donors with lower MMSE scores and
224 exhibited a graded pattern across the three cognitive groups. Donors with the lowest MMSE scores (0-22) demonstrated the
225 greatest accumulation of phosphorylated tau within MTG gray matter, supporting the close relationship between tau
226 pathology and cognitive deterioration. Whereas amyloid accumulation often precedes the onset of clinical symptoms and
227 may eventually plateau, tau pathology has been shown to track more closely with neuronal dysfunction and disease severity



(Mormino & Papp, 2018). The present findings therefore support that tau accumulation is a key pathological feature associated with cognitive decline in Alzheimer's disease.

Collectively, the simultaneous increase in both tau and amyloid pathology across MMSE groups is consistent with current models of Alzheimer's disease, which propose close interactions between protein aggregation, neuronal dysfunction, and cognitive decline.

4.2. Microglia Remain Present but May Be Functionally Compromised

The microglial findings revealed a more complex pattern than just higher or lower levels of cellular activity. Initially, quantification of IBA1/6E10 co-localized objects demonstrated the expected increase in microglial association with amyloid deposits as MMSE scores declined. At first glance, this finding suggests a natural heightened immune response accompanying increasing pathological burden.

Despite increased co-localization with amyloid pathology, total IBA1-positive cell counts actually remained relatively constant across the three cognitive groups. Therefore, more microglial presence near plaques in terms of cell abundance alone is not a reliable reflection of the immune response to amyloid plaques. Instead, changes in functional state may better explain the observed pattern.

While microglia continued to localize around amyloid deposits in predictable numbers, the change in inactive:active IBA1 cell ratios suggested a reduction in effective microglial responsiveness in donors with lower MMSE scores. As MMSE scores decreased, the ratio of the number of inactive:active IBA1 cells progressively increased. Together, these findings raise an important possibility—even though microglia continue to recognize and cluster around sites of pathology, they may lose the ability to efficiently clear amyloid plaques.

Rather than representing an absent immune response, these results may reflect a state of chronic dysfunction or cellular exhaustion. Under conditions of sustained pathological stress, microglia may remain physically associated with amyloid deposits but become progressively less capable of executing protective functions such as plaque clearance, debris removal, or maintenance of tissue homeostasis. Such a loss-of-function could be a major contributor to continued pathological accumulation despite persistent microglial presence.

This interpretation is consistent with emerging literature describing differing disease-associated microglial states in Alzheimer's disease (Cheng & Ho, 2025; Kuhn & Proctor, 2025). Several studies have reported that microglia surrounding plaques exhibit altered transcriptional and functional profiles, including impaired phagocytic activity and reduced homeostatic signaling (Hassan et al., 2025). Experimental models have similarly demonstrated that prolonged exposure to amyloid pathology can shift microglia from protective to maladaptive states (McKinney et al., 2026). The present findings contribute to this ongoing discussion by suggesting that physical proximity to pathology does not necessarily indicate effective functional engagement.

4.3. Selective Layer Enrichment



A notable feature across multiple analyses was the consistent enrichment of pathological and microglial measures within layer 3 of the MTG. Increased amyloid deposition, greater IBA1/6E10 co-localization, and elevated total IBA1-positive cell counts were repeatedly observed within this cortical layer regardless of MMSE category.

Importantly, the observed microglial pattern may not necessarily indicate preference of microglia toward layer 3 itself. Because amyloid burden was similarly enriched within this layer, higher levels of microglial localization likely reflects the distribution of pathology that microglia are responding to, rather than an independent microglial preference. In other words, microglial cells may just be responding proportionally to where pathology is concentrated—indicating that layer 3 itself may be uniquely vulnerable to neurodegeneration.

Nevertheless, the repeated prominence of layer 3 is notable. Cortical layers differ significantly in neuronal composition, connectivity, and functional organization, and these differences may influence selective vulnerability during neurodegeneration. Analyses limited to bulk gray matter measurements risk masking such spatial patterns. The layer-specific approach used here therefore provides additional resolution and suggests that important disease-related changes may occur within discrete anatomical compartments rather than uniformly throughout cortical tissue.

The enrichment of pathology within layer 3 may be particularly significant given the role of this layer in corticocortical communication. Layer 3 pyramidal neurons form extensive long-range connections with other cortical association areas and are heavily integrated into large-scale neural networks involved in cognition (Planert et al., 2026). Previous studies have suggested that highly connected neuronal populations may be especially vulnerable to pathological protein spread and metabolic stress (Bloomingdale et al., 2022; Brettschneider et al., 2015). Research has also suggested that neurons in layers 2 and 3 of the MTG may be more vulnerable to degenerative mechanisms in early stages of AD (Chen et al., 2021). Consequently, the concentration of amyloid pathology and microglial responses within layer 3 may reflect underlying network properties that increase a region's susceptibility to neurodegeneration (Escarião et al., 2025).

Interestingly, elevated microglial abundance was also observed within deeper cortical layers, particularly layers 5 and 6. Past research has shown that layers 3 and 4 of the neocortex, including the MTG, are the first to accumulate neurofibrillary tangles of tau (Leng et al., 2021). Evidence also suggests that neurons in layers 2, 3, and 5 of the temporal lobe, including the MTG, may be selectively vulnerable to tau pathology (Chen et al., 2022). Together, these findings raise the possibility that distinct cortical layers may contribute differently to disease progression. Further investigation is needed to confirm whether these layer-specific patterns are unique to the MTG or represent broader cortical inhomogeneity in Alzheimer's disease.

4.4. Findings Extend Existing Models of Disease Progression

The overall findings broadly align with current models of Alzheimer's disease progression while also highlighting potential limitations of simplified frameworks. Increasing amyloid burden alongside progressive tau accumulation is consistent with the amyloid cascade hypothesis, which proposes that amyloid deposition initiates downstream pathological events leading ultimately to neurodegeneration and cognitive decline (Wu et al., 2022).

However, the present findings suggest that immune responses may play a more active and complex role than originally proposed. While classical models often frame neuroinflammation as a secondary consequence of pathology, growing evidence suggests that microglial dysfunction itself may contribute meaningfully to disease progression (Cheng & Ho, 2025;

McKinney et al., 2026). The present data support this possibility – microglial cells appeared increasingly associated with pathology but simultaneously exhibited changes suggestive of diminished functional activity.

Presence alone does not necessarily imply effective response, and thus cells may occupy regions of pathology while simultaneously undergoing shifts towards a loss-of-function state (McKinney et al., 2026). The transition from protective surveillance to dysfunctional activity may be a critical feature of disease progression and could help explain why pathological burden continues to increase despite persistent immune response (Guo et al., 2022; Lier et al., 2021).

These findings are consistent in conceptualizing Alzheimer's disease as a multifactorial disease involving more complex interactions among protein aggregation, neuroinflammation, synaptic dysfunction, and neuronal loss. Rather than acting solely downstream of amyloid deposition, microglia may well influence other stages of disease progression by affecting neuroinflammation and becoming unable to clear pathological proteins. Consequently, therapeutic strategies targeting microglia may need to focus not only on enhancing microglial activity but also on preserving beneficial, active states and preventing transitions toward chronic inactivation.

4.5. Limitations and Future Directions

Several limitations should be considered when interpreting these findings. Firstly, because the present study relied on observational postmortem data, clear causal relationships cannot be established. Although significant associations were identified between MMSE status and neuropathological measures, it remains unclear whether altered microglial states drive disease progression or emerge as a consequence of accumulating pathology.

Additionally, MMSE-based grouping provides only an approximate measure of disease severity. Cognitive decline is a multidimensional process influenced by numerous biological and clinical factors, and thus individuals with similar MMSE scores may actually differ substantially in their true pathological profiles. Future studies incorporating both additional cognitive assessments and clinical data may provide a more comprehensive understanding of the relationship between cognition and neuropathology.

The two microglial activation (active and inactive) states were determined according to morphology-based classifications provided by the dataset, but morphology alone may be insufficient in showing the functional diversity of microglial states. Future studies incorporating transcriptomic, proteomic, and molecular approaches may better help determine whether the observed changes represent dysfunction, adaptive responses, or distinct disease-associated phenotypes.

The layer-specific findings observed in this study also need further investigation. The consistent enrichment of amyloid pathology and microglial measures within layer 3 raises important concerns regarding what controls selective cortical vulnerability. Future studies must examine whether differences in neuronal connectivity, synaptic density, or structural communication patterns contribute to the observed distribution of pathology. Further research on layer 3 may provide insight into how pathological processes spread through large-scale brain networks.

Finally, the present analysis focused only on the middle temporal gyrus. Expanding similar layer-resolved analyses to other brain regions commonly affected in Alzheimer's disease, like the hippocampus, entorhinal cortex, frontal cortex, etc., could



determine whether the observed relationships between pathology and microglial dynamics represent a generalized feature of disease progression or are a region-specific phenomenon (Leng et al., 2021).

4.6. Conclusions

In summary, lower cognitive function was associated with progressively higher tau and amyloid pathology within the MTG, accompanied by changes in microglial dynamics. Tau burden exhibited a particularly strong relationship with declining MMSE scores, supporting previous evidence that tau pathology closely tracks clinical impairment. On the other hand, microglia increasingly localized near amyloid deposits despite evidence suggesting altered activation states as disease severity progressed.

Together, these findings support a model where pathological burden and neuroimmune changes evolve in parallel with Alzheimer's disease progression. The results further suggest that microglial presence alone may be insufficient to assess neuroimmune function, as cells can remain associated with pathology while potentially undergoing shifts toward less effective or maladaptive states. Additionally, the layer-specific patterns identified within the MTG highlight the importance of considering spatial organization when investigating such neurodegenerative mechanisms. Understanding why certain cortical layers and regions exhibit heightened vulnerability and the changes in pathology associated with these regions may ultimately lead to the development of more targeted therapeutic strategies.

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457 *Transduction and Targeted Therapy*, 8(1), 267. <https://doi.org/10.1038/s41392-023-01486-5>

458 Data Availability Statement

459 All datasets analyzed and methodology are available through the Allen Institute's BrainMap in the Seattle Alzheimer's Disease
460 Brain Cell Atlas:
461 [https://portal.brain-map.org/explore/seattle-alzheimers-disease/seattle-alzheimers-disease-brain-cell-atlas-download?ed](https://portal.brain-map.org/explore/seattle-alzheimers-disease/seattle-alzheimers-disease-brain-cell-atlas-download?edit&language=en)
462 [it&language=en](https://portal.brain-map.org/explore/seattle-alzheimers-disease/seattle-alzheimers-disease-brain-cell-atlas-download?edit&language=en).

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473 Author Biography

474 [redacted]

475 Mentor Contribution Statement

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EDITOR RECOMMENDATION:

The reviewers commend the author for an ambitious paper, and have several suggestions for ways to improve the strength of the manuscript. As the reviews are quite detailed, I would focus efforts on ways to improve the text to appropriately temper conclusions where noted, and to add clarity to the methods and their strengths/limitations.

REVIEWER 1:

Review of: Increased Tau, Amyloid- β , and IBA1 in the Middle Temporal Gyrus Is Associated With Lower Cognitive Function in SEA-AD Donors

Recommendation: Accept with revisions

Overall assessment

This manuscript investigates an important question in Alzheimer's disease research: how tau, amyloid- β , and microglial changes within the middle temporal gyrus relate to cognitive impairment. The manuscript's strengths include the use of a human postmortem data, the focus on layer-specific pathology rather than bulk measurements, and the attempt to interpret microglial changes beyond simple differences in cell abundance. The discussion around distinguishing microglial presence from potential changes in microglial state is interesting and is a strong contribution. However, some revisions are needed before publication including more careful interpretation of microglial activation states, softening of claims of conclusions, and clearly distinguishing observed associations from proposed mechanisms

Major Comments:**Introduction**

- The introduction provides a strong overview of AD pathology and relevance of tau, amyloid, and microglia. The specific gap motivating this study could be more clearly developed. E.g., what remains unknown about the MTG, layer-specific patterns, relationship between microglial localization and cognitive decline.
- The layer-specific analysis is one of the manuscript's strongest contributions, which could be emphasized more earlier on. You mention that bulk gray matter measurements may obscure layer-specific patterns, but it could help to explain what prior studies have shown and what remains unknown about how AD pathology and microglial responses vary across cortical layers.
- In the introduction, some statements could be softened to better reflect current evidence. While amyloid, tau, and neuroinflammation are strongly associated with AD, the causal relationships among these processes remain debated. Consider more language such as "has been implicated in", "is associated with", "may contribute to"
- In the introduction, M1/M2 terminology may oversimplify current understanding that microglial states are heterogeneous and context-dependent rather than just pro-inflammatory versus anti-inflammatory. Check out this paper: [https://www.cell.com/neuron/fulltext/S0896-6273\(22\)00859-5](https://www.cell.com/neuron/fulltext/S0896-6273(22)00859-5) and consider briefly acknowledging this nuance.
- The importance of the MTG and rationale for focusing on this region could be expanded and more developed. Consider mentioning why this region is particularly vulnerable or informative in AD compared to other regions
- Some claims lack citations such as : "Alzheimer's disease is the most common cause of dementia", "Amyloid deposition is known to begin years before the onset of clinical symptoms", "The cerebral cortex is organized into distinct layers that all differ in cellular composition..."

Methods:

- Some language makes it difficult to distinguish between analyses performed on this public data versus the experiments conducted by the SEA-AD researchers. E.g., manuscript states “all experimentation and data were conducted by...” Consider revising this language to clearly state in the beginning of methods that previously collected data were accessed and reanalyzed in the present study
- Please clarify if corrections for multiple comparisons were performed given the large number of statistical tests across markers and cortical layers. If no correction was applied, please acknowledge this as a limitation.
- Outlier removal explanation is unclear. The author says subjects “consistently identified as outliers across datasets” – but this is difficult to interpret.

Results:

- I may be misunderstanding but there is inconsistency in the y-axis metric and reported interpretation of Figure 5. Since the y-axis of Fig 5 represents the inactive/active IBA1 cell ratio, an increased ratio indicates relatively more inactive cells compared to active cells, whereas a decreased ratio would indicate relatively greater active microglial representation. The text currently states that lower MMSE groups show increased activation, but the reported ratio appears to suggest the opposite. Please clarify if the ratio was calculated as inactive/active or active/inactive and revise the interpretation accordingly.
- Be cautious when interpreting IBA1 morphology as a direct measure of microglial activation/function. Morphological differences may reflect altered microglial states, but don't necessarily indicate functional changes. In the results section, try to report the direct findings and expand on what this might mean in the discussion

Discussion/Limitations:

- The interpretation that microglia become functionally compromised is interesting and potentially important, but the current data don't directly measure microglial function. Increased inactive/active ratios based on morphology may indicate altered microglial states, but they don't necessarily demonstrate reduced phagocytosis, impaired plaque clearance, or loss of homeostatic function. I suggest revising this to: “consistent with altered microglial states” or “suggestive of reduced responsiveness”. Link this to future directions of functional assays needed to establish this mechanism.
- The clinical implications discussed are thought-provoking but should acknowledge that translating morphology-based observations into therapeutics is challenging. Consider emphasizing that these findings may help generate hypotheses for future therapeutic investigation instead of directly informing treatment
- When talking about DAM, clarify your study doesn't directly measure DAM signatures and that it is a conceptual connection.
- Limitations/future directions section is strong and acknowledges several important limitations.

Minor Comments:

- Text size changes
- Unclear, reword for clarity: “a one-way ANOVA was used to run a replicate data column analysis”
- In figure legends, make sure to explain everything present in figure. E.g., what error bars represent, statistical tests, if comparisons are between groups or layers
- Soften language e.g., “indicating that there must be another explanation” □ could be “...unlikely to be explained solely”
- “microglia increasingly localized” - not totally accurate to what data show, should be something like “greater microglial localization was observed”

REVIEWER 2:

This is a clear study that proposes statistical analysis of the SEA-AD dataset and provides a few strong findings, which however, have not been reported consistently. The layer-resolved analysis of the MTG and the tau/MMSE gradient are clear and convincing analyses, and the distinction between microglial number and functional state is a useful contribution. However, one central result is apparently stated in two contradicting directions and the title slightly overstates the finding.

Recommendation: Major revisions

Main points (in order of importance)

- Section 3.5 says the inactive:active ratio is highest in the 27–30 group and lowest in the 0–22 group whereas the heading and sections 3.6, and 4.2 state the opposite. The paper's main novelty and conclusion depends on this direction. Section 3.5 needs to be updated to reconcile the claims in all four locations.
- Section 3.4 reports total IBA1 counts were comparable across groups; what changed was co-localization and activation state. The title suggests a significant difference and needs to be revised to state the actual finding.
- Exclusions need to be justified. Six donors were removed via ROUT (four with dementia); excluding high-pathology cases could attenuate the reported effects. Please state what drove the exclusions and confirm results hold on the full sample.
- Layer counts will need to be normalised for comparability. Layer 3 is enriched and is anatomically thick. It would be helpful to explain if the reported counts are per-area; if they are raw values, perhaps the layer-3 claim may need to be reconsidered.
- The paper by Escarió et al. (2025) is very close in concept and dataset but cited only in passing. Could the author explain briefly what is replicated from that paper and what is new in this study?
- Figure 1 caption appears incomplete, please write this in full.
- A two-way ANOVA would be the most suitable choice for the statistics of this study. The design is MMSE group $\times$ layer with layers repeated within donor; separate one-way ANOVAs risk pseudoreplication.
- A few comments of editorial nature: "I/my" vs "we/our" should be consistently used and p-value formatting is inconsistent. There are a few broken Zotero citation links and a few uncited references

Increased Tau, A β , and IBA1 Inactivation in the Middle Temporal Gyrus Is Associated With Lower Cognitive Function in SEA-AD Donors

Abstract

Alzheimer's Disease (AD) is a neurodegenerative disorder characterized by mechanisms including neuroinflammation, neurofibrillary tangles of phosphorylated tau, accumulation of amyloid plaques, and microglial aggregation. In AD, disease pathology is closely related to cognitive function, measured using the Mini-Mental-State-Examination (MMSE), a clinical screening tool. This study examined relationships among tau burden, amyloid deposition, and microglial activity in the middle temporal gyrus across three MMSE-defined stages of cognitive decline. Data, including quantifications of AT8, 6e10, and IBA1, were obtained from the Seattle Alzheimer's Disease Brain Cell Atlas. Two-way ANOVAs were used to study the change in percent AT8+ area (phosphorylated tau), IBA1+ cells per area and activation (microglia), 6e10+ cells per area (amyloid-beta), and IBA1+/6e10+ colocalized objects per area, alongside MMSE-defined cognitive decline. Our analysis revealed that neuropathological measures of percent AT8+ area, IBA1/6e10 colocalized objects per area, and IBA1 inactive:active ratio reflected a significant overall MMSE effect, while measures of IBA1+/6e10+ colocalized objects per area, IBA1+ cells per area, and 6e10+ cells per area reflected a significant overall layer effect. Although general IBA1+ cell per area counts were comparable across MMSE groups, the inactive:active ratio differed, with the lowest MMSE group having the highest ratios. These findings lead to the hypothesis that lower MMSE scores may be associated with more tau burden, amyloid deposition, and amyloid/microglia colocalization, accompanied by alterations in microglial state. Together, these findings inform a broader understanding of the link between pathological burden and cognitive function in a multidimensional model of AD.

Keywords: Alzheimer's Disease (AD), MMSE, dementia, tau pathology, amyloid beta, microglial activation, cortical layers

1. Introduction



Alzheimer's disease (AD) is the most common cause of dementia and is characterized by progressive cognitive decline (Atri, 2019; US Department of Health and Human Services, 2017). It is accompanied by the accumulation of extracellular amyloid- β (A β) plaques, intracellular neurofibrillary tangles composed of hyperphosphorylated tau, and widespread neuroinflammation (Khachaturian, 1985; Reitz, 2012). Although these pathological hallmarks have been studied extensively, the mechanisms linking such protein aggregation to cognitive impairment are still unclear.

The accumulation of extracellular amyloid plaques, formed from self-aggregating peptides, has long been regarded as the first hallmark of Alzheimer's disease, inhibiting neuronal synaptic function (Reitz, 2012). Amyloid deposition is known to begin years before the onset of clinical symptoms (Wu et al., 2022). On the other hand, tau accumulation, presenting as neurofibrillary tangles toxic to neurons, is implicated to more closely track the timeline of dementia-related neuronal dysfunction, synapse loss, and worsening cognition (Cody et al., 2024). Consequently, increasing tau burden is commonly considered one of the strongest neuropathological indicators of disease severity (Murray et al., 2022). Previous studies have also suggested that tau pathology is more strongly associated with cognitive impairment than amyloid burden alone (Guo et al., 2026). Amyloid pathology nevertheless remains an important marker, as it is commonly believed to initiate a cascade of downstream events that contribute to tau aggregation, neurodegeneration, and ultimately dementia – a process often referred to as the “amyloid cascade hypothesis” (Reitz, 2012).

In addition to protein deposition, neuroinflammation is another major hallmark of dementia, with emerging studies suggesting that inflammation may be both a consequence and an initiator of AD pathology like tau accumulation and amyloid plaques (Zhang et al., 2023). Under healthy conditions, microglia contribute to tissue maintenance through debris clearance and immune surveillance (McKinney et al., 2026). They are the brain's resident immune cells and macrophages, continuously monitoring the brain environment and responding to accumulating amyloid deposits and neuroinflammation (Muzio et al., 2021). In AD, however, microglia aggregate around amyloid plaques and undergo substantial changes in morphology, gene expression, and function (Bolmont et al., 2008).

Experimental evidence suggests that microglia initially exert protective effects by containing plaques and phagocytosis of pathological proteins, but being exposed to prolonged pathological stress may yet lead to impaired clearance mechanisms and ultimately contribute to neurodegeneration by accelerating the spreading of proteins like A β and tau. (Gao et al., 2023). Furthermore, evidence argues that microglia have specific activation states which may mediate synapse loss, worsen tau pathology, and secrete inflammatory factors that directly harm neurons (Hansen et al., 2018). Microglia are implicated to be able to transform from one phenotype to others—many researchers believe that the M1 microglia, which release inflammatory mediators, and the M2 microglia, which release anti-inflammatory mediators, may provide further insight on the effective activation states of microglia (Guo et al., 2022). However, current understanding has not reached a consensus on whether microglial states can be explicitly divided into simply anti-inflammatory and pro-inflammatory subtypes, due to the complex heterogeneity and heavily context-dependent nature of microglial states (Paolicelli et al., 2022). Despite growing recognition of the importance of neuroinflammation, it still remains unclear whether microglia continue to respond effectively as disease progresses or whether they transition into altered functional states that contribute to further pathological accumulation. This study therefore investigates how the accumulation of microglia around sites of pathology may change across different stages of neurodegeneration.

In AD, neuroinflammation spreads heavily across the brain to areas like the cerebral cortex, the outer layer of the brain responsible for thinking, memory, and language (Ni & Wu, 2021). The cerebral cortex is organized into distinct layers that all differ in cellular composition, connectivity, and functional roles, meaning pathology may behave differently across layers (Chen et al., 2021). Emerging evidence suggests that neurodegenerative pathology may not be distributed uniformly throughout cortical tissue, and analyses restricted to just gray matter measurements may obscure important layer-specific patterns (Willumsen et al., 2022). Across past studies, AD neuropathology has been known to show strong regional specificity when developing across the brain (Yang et al., 2026). Neurofibrillary tangles typically spread from the entorhinal cortex to the hippocampus, limbic system, and neocortex, amyloid plaques show graded accumulation across brain regions, and microglia in the prefrontal cortex, cerebellum, and hippocampus are known to have significant differences in their highly expressed genes (Asher & Priefer, 2022; Jacobsen et al., 2005; Yang et al., 2026).

The middle temporal gyrus (MTG) is particularly relevant in AD because of its involvement in language processing, semantic memory, and other higher order cognitive functions that become impaired during disease progression (Chen et al., 2022). Therapeutic strategies developed over the years rely heavily on gray matter analysis and often encounter obstacles once in clinical trials (Asher & Priefer, 2022). Investigating pathology across individual cortical layers may therefore provide additional insight into selective regional differences and vulnerabilities.

Despite extensive research conducted on AD biomarkers, critical gaps in the scientific understanding of disease mechanisms still exist. It remains unclear whether layer-specific microglial clustering directly damages local circuitry or merely reacts to underlying synaptic failure and plaque growth, and when microglia transition activation states (Escarião et al., 2025). In the MTG itself, the precise functional organization and the exact division of labor for semantic processing remain unknown, meaning a layer-specific approach to examining the MTG may provide more insight (Escarião et al., 2025).

Accordingly, the present study investigated relationships among tau burden, amyloid deposition, and microglial activity within the MTG across MMSE-defined stages of cognitive decline. Particular emphasis was placed on examining the pathology within individual cortical layers 1 to 6, following initial bulk gray matter analyses.

A previous study of these markers in the SEA-AD dataset investigated the APOE4 genotype and an individual's genetic predisposition to dementia (Wang, 2025). However, this present study uses MMSE grouping to better understand the relationships between disease pathology and an individual's ongoing cognitive capacities.

I hypothesized that lower MMSE scores would be associated with higher levels of tau burden and amyloid deposition, as well as accompanied alterations in microglial activation. More specifically, the microglia were expected to increasingly associate with sites of pathology whilst displaying signs of change in activation. Furthermore, layer-specific analyses were expected to reveal patterns that would not be apparent from the gray matter measurements alone.

2. Materials and Methods

Previously collected data were accessed and reanalyzed in the present study. De-identified postmortem human brain data and quantitative neuropathological measurements of the middle temporal gyrus were accessed from the Allen Brain Institute's publicly available SEA-AD dataset (Hawrylycz et al., 2024). All initial experimentation and data collection were conducted by the Kaiser Permanente Washington Health Research Institute ACT Study researchers and the University of

Washington ADRC at the UW BioRepository and Integrated Neuropathology (BRaIN) laboratory. The dataset focused on different patients' histological pathology, obtained via immunolabeling and microscopy across cortical layers and protein markers.

For my study, the data from the Quantitative Neuropathology Summary Data (MTG) and Donor Metadata from the Allen Brain Institute's SEA-AD data set were aligned and compiled in an Excel spreadsheet in order to match neuropathology data to donor MMSE status. Donors were stratified into three groups based on their respective Mini-Mental State Examination (MMSE) scores: 27–30, 23–26, and 0–22.

2.1. MMSE-Based Cognitive Stratification

Cognitive status was classified into donor groups according to Mini-Mental State Examination (MMSE) scores obtained from SEA-AD donor metadata. The MMSE is a widely used clinical screening tool for cognitive impairment, with scores ranging from 0 to 30, where lower scores indicate greater cognitive dysfunction (Creavin et al., 2016; Yang et al., 2024).

Donors were stratified into three groups based on established thresholds used in dementia research: MMSE 27–30 (cognitively normal or minimally impaired), MMSE 23–26 (mild cognitive impairment), and MMSE 0–22 (moderate to severe cognitive impairment consistent with dementia).

Across studies, there exist many different categorizations of MMSE scores, with no consensus yet on what threshold may be truly significant in terms of cognitive status. The Mini-Mental State Examination (MMSE) faces significant diagnostic inconsistency because fixed cutoff scores fail to account for demographic variations — while a score of 24 serves as the traditional clinical threshold, its diagnostic accuracy is heavily confounded by a patient's age and education level, often yielding false negatives in highly educated individuals and false positives in those with limited schooling (Creavin et al., 2016). Consequently, researchers and clinicians increasingly employ stratified thresholds ranging from 21 to 27 to enhance sensitivity and specificity across diverse populations (Salis et al., 2023). Therefore, MMSE grouping is heavily dependent on the dataset being analyzed. Based on this framework, the present study used MMSE stratification to approximate stages of cognitive decline and to enable comparison of neuropathological burden across disease severity.

Due to the nature of dementia disease progression, age at death is an essential consideration when analyzing the severity of pathological burden. Thus, when deciding the MMSE ranges for the three experimental groups, instead of solely relying on numbers used in past studies, I used a one-way ANOVA to run a replicate data column analysis with multiple comparisons of donor ages at death. When grouping MMSE scores into the three groups: 0–22, 23–26, and 27–30, the multiple comparison mean values yielded no significant difference between groups (mean of 0–22 vs. 23–26 being 89.29, mean of 0–22 vs. 27–30 being 89.87, and mean of 23–26 vs. 27–30 being 89.87). Thus, these cutoffs provided a more balanced partition of donor data rather than relying on arbitrary and inconsistent thresholds.

In my dataset, the group with MMSE scores 27–30 consisted of 31 donors, the group with MMSE scores 23–26 consisted of 31 donors, and the group with MMSE scores 0–22 consisted of 17 donors.

All downstream analyses were performed using these MMSE-defined groups to evaluate relationships between cognitive status and pathological markers in the middle temporal gyrus.

145 2.2. Neuropathological Quantification

146 Detailed tissue processing and staining protocols have been previously described in a published paper as well as an online
147 procedural overview (Gabbitto et al., 2024; Seattle Alzheimer's Disease Data Set, n.d.).

148 Briefly, postmortem human brain tissue was obtained through the Adult Changes in Thought (ACT) study conducted by the
149 Kaiser Permanente Washington Health Research Institute in collaboration with the University of Washington. Researchers
150 collected middle temporal gyrus (MTG) samples, fixing and embedding each in paraffin prior to sectioning. Sections
151 underwent duplex immunohistochemical staining to detect various hallmark Alzheimer's disease proteins (Hawrylycz et al.,
152 2024). Phosphorylated tau was labeled using the AT8 antibody, while amyloid- β (A β) deposits were identified using the 6E10 monoclonal
153 antibody. Quantification of percent AT8-positive area, 6E10-positive objects per area, and IBA1-associated measures was performed on
154 digitized images of the MTG cortical layers. IBA1-positive cells were classified as active or inactive according to dataset-provided
155 morphological criteria.

156 Cortical layers are established by the SEA-AD dataset annotations, in which Layer 5 and Layer 6 are combined. Thus, the
157 layers are analyzed accordingly as L1, L2, L3, L4, and L5/6.

158 The Allen Brain Institute collected and compiled the data for access to the general public. Data were accessed June 6th, 2026,
159 via:

160 [https://portal.brain-map.org/explore/seattle-alzheimers-disease/seattle-alzheimers-disease-brain-cell-atlas-download?ed](https://portal.brain-map.org/explore/seattle-alzheimers-disease/seattle-alzheimers-disease-brain-cell-atlas-download?edit&language=en)
161 [it&language=en](https://portal.brain-map.org/explore/seattle-alzheimers-disease/seattle-alzheimers-disease-brain-cell-atlas-download?edit&language=en).

162 2.3. Outlier Removal

163 To preserve the statistical validity and reproducibility of my pathological assessments, outlier detection was performed for
164 all pathological measurements using the ROUT method (Q = 1%) in GraphPad Prism. No outliers were identified consistently
165 across all measures, therefore no donors were excluded from my analyses.

166 2.4. Statistical Analysis

167 Statistical analyses were largely performed using two-way mixed effect model ANOVAs with repeated measures, with
168 MMSE group as the between-subjects factor and cortical layer as the repeated within-donor factor. This design is required
169 because multiple cortical layers are sampled from the same individual, violating the assumption of independent
170 observations necessary for standard one-way ANOVAs. By modeling donor identity as a random effect, this approach
171 accounts for the inherent statistical dependency within each subject, properly partitions the variance, prevents the
172 artificial inflation of the degrees of freedom, and allows for the critical evaluation of the interaction effect between MMSE
173 group and layer (Wilcox & Rousselet, 2023). A one-way ANOVA was performed for the AT8 measure due to the fact that it is
174 a plain gray matter analysis with no cortical layer analyses.

175 Multiple comparisons were performed for all analyses by comparing cell means with others in its row and column. Šidák's
176 multiple-comparisons correction was used to control the family-wise Type I error rate arising from multiple pairwise
177 comparisons while providing greater statistical power than the more conservative Bonferroni correction. (Abdi, 2007)



To ensure valid cross-layer comparisons within the MTG, normalized counts were used for analysis of all measures. This normalization is statistically required to control for the prominent anatomical variations in laminar thickness inherent to the MTG (Zachlod et al., 2022). Failing to account for these structural volume differences would conflate a higher absolute cell count with a true increase in cellular density, thereby introducing systematic bias into our regional comparative analyses. Normalized measures are presented as AT8 percent area (%), IBA1+/6e10+ colocalized objects per area ($\#/\mu\text{m}^2$), IBA1+ objects per area ($\#/\mu\text{m}^2$), 6E10+ objects per area ($\#/\mu\text{m}^2$), and IBA1+ inactive microglial cells per area : IBA1+ active microglial cells per area (#).

Data are presented as bars with mean $\pm$ SEM. Statistical significance thresholds were defined per APA style 7th edition as: * $p < .05$, ** $p < .01$, *** $p < .001$.

I calculated the overall main effect of MMSE, layers, and the MMSE x layer interaction for all two-way ANOVAs. A significant overall main effect of MMSE group (M) demonstrates that MMSE grouping has a statistically significant effect on the dependent variable. Similarly, a significant overall main effect of cortical layers (L), then, means that the layers of the MTG have a statistically significant effect on the dependent variable. A significant MMSE x cortical layer interaction indicates that there is a statistically significant interaction between both the effects of MMSE grouping and layer. Thus, while the main effects describe overall differences associated with either cognitive status or cortical layers, the interaction significance provides evidence that the two factors jointly influence the distribution of the biomarker across the middle temporal gyrus.

3. Results

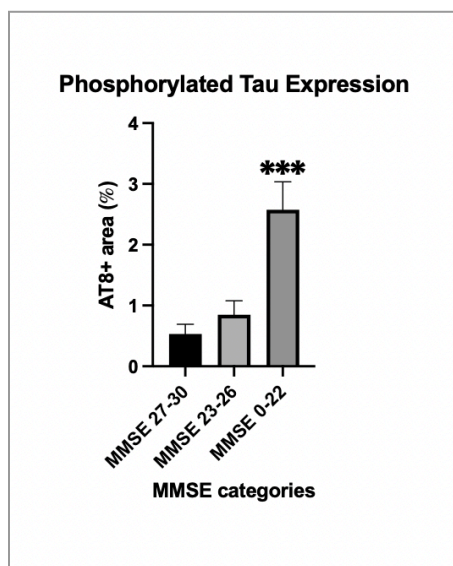
3.1. AT8 Pathology in the Middle Temporal Gyrus Higher with Cognitive Decline

To assess the relationship between tau pathology and cognitive decline, percent AT8-positive area was quantified across the gray matter of the middle temporal gyrus (MTG) and stratified by MMSE category (Figure 1). My analyses compared donors with low, medium, and high MMSE scores, where a standard one-way ANOVA revealed a significant difference between groups ($F_{2,76} = 14.31$, *** $p < .001$). Šídák-adjusted post-hoc tests showed significance of *** $p < .001$ for both MMSE 27-30 vs. MMSE 0-22 and MMSE 23-26 vs. MMSE 0-22.

AT8 burden was higher in donors with decreased MMSE scores, with the lowest MMSE group (0-22) exhibiting a greatly elevated and significant percent AT8-positive area. The intermediate group (23-26) showed values between the high and low MMSE groups, indicative of a graded relationship between tau accumulation and cognitive impairment.

This trend was consistent across donors within each group, with statistical comparisons demonstrating a significant increase in AT8-positive area in the 0-22 group relative to both higher MMSE groups (*** $p < .001$).





207

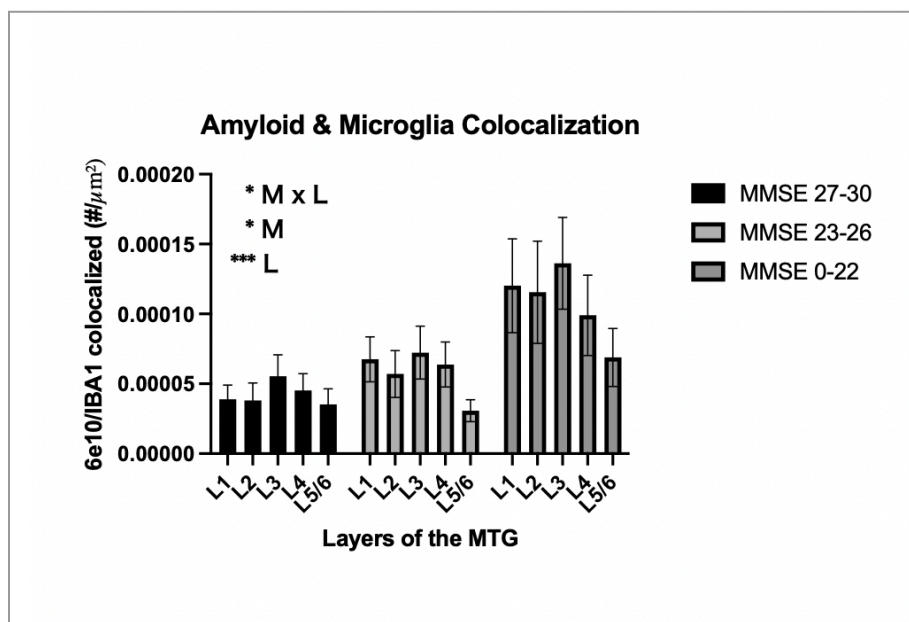
208 **Figure 1. Percent AT8-positive area in MTG gray matter across MMSE categories.** Significance (***) $p < .001$ was found between
209 MMSE group 0-22 and all other MMSE groups.

210 3.2. Increased Microglial Association with Amyloid Pathology Across Cortical Layers

211 To evaluate microglial interaction with amyloid pathology, the number of IBA1/6E10 co-localized objects per area was
212 quantified across cortical layers of the MTG (Figure 2). Co-localized object per area counts were higher with decreased
213 MMSE scores, with the greatest values observed in the 0-22 group. The progressive increase in co-localized objects per
214 area across MMSE groups may indicate an enhanced microglial presence in proximity to amyloid deposits as cognitive
215 impairment worsens.

216 Two-way ANOVA analyses with MMSE group as the between-subjects factor and cortical layer as the repeated
217 within-donor factor revealed a significant overall layer effect ($F_{2,528,192.1} = 14.58$, $***p < .001$), a significant overall MMSE
218 effect ($F_{2,76} = 3.290$, $*p = .043$) and a significant overall interaction effect of layer and MMSE group ($F_{5,056,192.1} = 2.367$, $*p =$
219 $.041$). However, no statistical significance was found between individual layers.





220

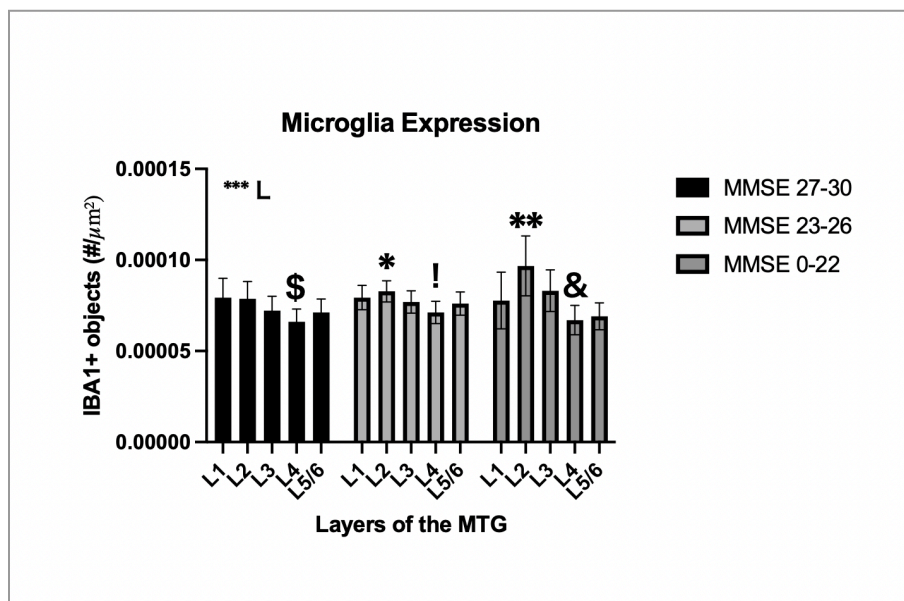
221 **Figure 2. Number of IBA1/6E10 co-localized objects per area.** Significance was observed for the overall interaction effect of layer and
222 MMSE group (* M x L), the overall MMSE group effect (* M), and the overall layer effect (*** L).

223 3.3. Total Microglial Activation Appears Comparable Across Cognitive Status

224 To assess whether the survival of microglial cells affected amyloid colocalization, the amount of IBA1-positive cells per area
225 was first compared across cortical layers and MMSE groups (Figure 3). Notably, layer-specific multiple comparison analysis
226 revealed significance between layers within each MMSE group, but not across MMSE groups themselves.

227 Two-way ANOVA analyses with MMSE group as the between-subjects factor and cortical layer as the repeated
228 within-donor factor revealed a significant overall layer effect ($F_{1.618,122.9} = 14.62$, $***p < .001$), but no significant overall MMSE
229 effect or significant overall interaction effect of layer and MMSE group.





230

231 **Figure 3. Number of total IBA1 positive cells per area.** Significance was observed for the overall layer effect (*** L). In MMSE 27-30,
232 significance marked by "\$" was found between L4 and L2 (**p < .01), L4 and L5/6 (**p < .01), and L4 and L3 (***p < .001). In MMSE
233 23-26, significance was found between L3 and L2 (*p < .05). Significance (***p < .001) marked by "!" was found between L2 and L4,
234 and L3 and L4. In MMSE 0-22, significance was found between L1 and L2 (**p < .01). Significance (*p < .05) marked by "&" was found
235 between L3 and L4.

236 3.4. Layer-Specific Distribution of Amyloid- β Deposition

237 To determine whether the observed layer-patterns from Figure 2 were driven by amyloid distribution, 6E10-positive
238 objects per area were quantified independently across MTG layers (Figure 4). A consistent significance of 6E10-positive
239 objects per area was observed in layer 3 across all MMSE groups.

240 Two-way ANOVA analyses with MMSE group as the between-subjects factor and cortical layer as the repeated
241 within-donor factor revealed a significant overall layer effect as well ($F_{2,529,192.2} = 14.45$, ***p < .001), but no significant overall
242 MMSE effect or significant overall interaction effect of layer and MMSE group.

243 Notably, the number of 6E10-positive objects per area exceeded the number of IBA1/6E10 co-localized objects per area
244 across all groups and layers, perhaps indicating that only a subset of detected 6E10+ objects met the criteria for
245 colocalization.



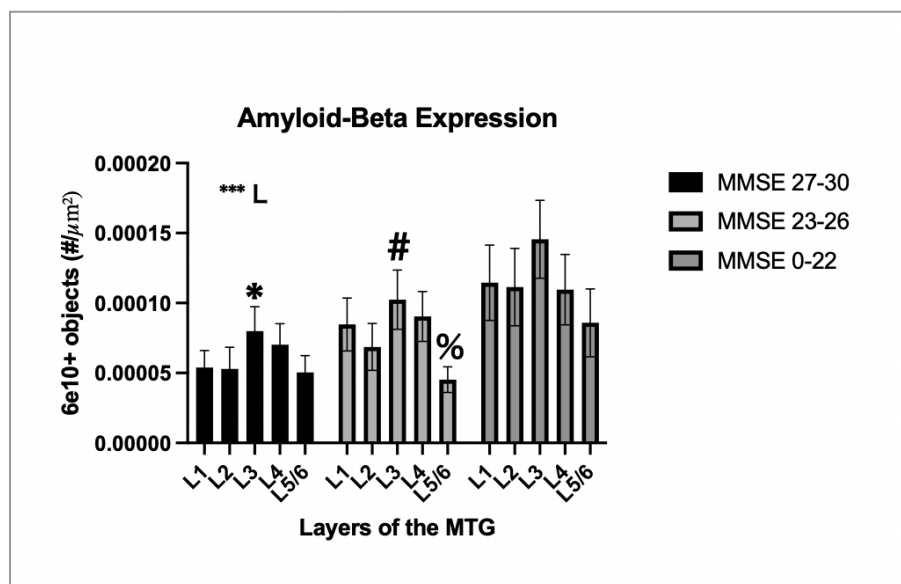


Figure 4. Number of 6E10-positive objects per area. Significance was observed for the overall layer effect (*** L). In MMSE 27-30, significance was found between L3 and L2 (* $p < .05$). In MMSE 23-26, significance (*** $p < .001$) marked by “#” was found between L3 and L2 (*** $p < .001$). Significance (* $p < .05$) marked by “%” was found between L5/6 and L3, and L5/6 and L4. No significance was found between individual layers of MMSE 0-22.

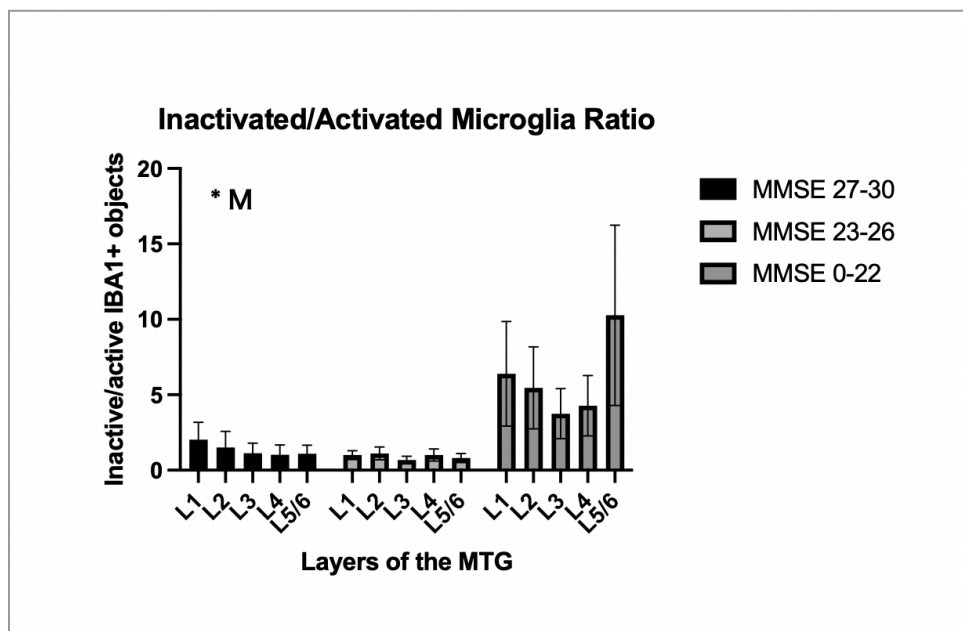
3.5. Microglial Inactivation Is Higher with Worse Cognitive Status

To assess microglial morphology and states, the ratio of morphologically-classified inactive to active IBA1-positive microglial cells per area was calculated (inactive cells per area divided by active cells per area) across cortical layers and MMSE groups (Figure 5). A prominent difference in the inactivation ratio was observed between the 0-22 MMSE group and all other MMSE groups.

Two-way ANOVA analyses with MMSE group as the between-subjects factor and cortical layer as the repeated within-donor factor revealed a significant overall MMSE group effect ($F_{2,76} = 3.959$, * $p = .023$), but no significant overall layer effect or significant overall interaction effect of layer and MMSE group.

Although differences between specific cortical layers and MMSE groups were not statistically significant, the increase in inactivation ratio was clear across MMSE groups. In the lowest MMSE group, the ratio indicated a relative increase in inactivated IBA1 cells, with the highest inactive:active cell per area ratio.

These findings indicate that while microglia remain comparably present in regions of pathology as seen in Figure 3, their activation state may change with disease progression, with more IBA1 cells per area becoming inactive relative to active as disease severity worsens.



265

266 **Figure 5. Ratio of inactive to active IBA1-positive cells per area.** Significance was observed for the overall MMSE group effect (*M) only.

267 3.6. Summary of Pathological Trends Across MMSE Groups

268 Across measures, lower MMSE scores were associated with higher pathological burden and altered microglial dynamics.
 269 AT8-positive percent area, IBA1/6E10 co-localized objects per area, 6E10-positive objects per area, and the IBA1
 270 inactive:active cell per area ratio were highest in donors with an MMSE score between 0-22. IBA1 positive cell per area
 271 counts were relatively constant across MMSE scores, indicative of almost no difference related to disease severity. In
 272 contrast, the ratio of inactive to active microglia was higher in donors with lower MMSE scores. Overall layer significance
 273 was observed in all analyses except for the IBA1 ratio.

274 4. Discussion

275 4.1. Progressive Pathological Burden Accompanies Cognitive Decline

276 The present study investigated relationships between tau pathology, amyloid deposition, and microglial activity within the
 277 middle temporal gyrus (MTG) across MMSE-defined stages of cognitive decline. I hypothesized that lower MMSE scores
 278 would be associated with higher pathological burden across measures, accompanied by altered microglial activation states.
 279 Overall, my findings supported this hypothesis and affirmed expected changes in disease pathology and neuroimmune
 280 dynamics in individuals with varying degrees of cognitive deficits.



281 Tau burden, measured by the percent AT8-positive area in the study, was substantially higher in donors with lower MMSE
282 scores and exhibited a graded pattern across the three cognitive groups. Donors with the lowest MMSE scores (0-22)
283 demonstrated the greatest accumulation of phosphorylated tau within MTG gray matter, supporting the close relationship
284 between tau pathology and cognitive deterioration.

285 Post-hoc comparisons confirmed significantly greater AT8 levels in the low-MMSE group compared with both the medium-
286 and high-MMSE groups. This relationship between more AT8 pathology and lower MMSE provided an initial indication that
287 cognitive status was associated with meaningful differences in neuropathology within the MTG and motivated further
288 investigation of whether other pathological measures might exhibit similar relationships with MMSE and whether these
289 relationships vary across cortical layers.

290 Whereas amyloid accumulation is generally believed to precede the onset of clinical symptoms and eventually plateau, tau
291 pathology has been shown to track more closely with disease severity (Mormino & Papp, 2018). The present findings therefore
292 support that tau accumulation is a key pathological feature closely associated with cognitive decline in Alzheimer's disease.
293 Collectively, the simultaneous increase in both tau and amyloid pathology across MMSE groups is consistent with current
294 models of Alzheimer's disease.

295 4.2. Microglia Associate With Amyloid Plaques

296 Initially, quantification of IBA1/6E10 co-localized objects per area demonstrated the expected increase in microglial
297 association with amyloid deposits as MMSE scores declined. At first glance, this finding suggests a natural heightened
298 immune response accompanying increasing pathological burden.

299 My analysis of IBA1+/6e10+ colocalized objects per area demonstrated significant main effects of MMSE group and cortical
300 layer, as well as a significant MMSE group x cortical layer interaction. The significant main effect of MMSE group indicates
301 that the density of IBA1+/6e10+ colocalized objects is dependent on the MMSE group. Consistent with this effect, a
302 progressive increase in colocalized objects was observed across the MMSE groups, suggesting greater microglial association
303 with amyloid pathology in donors with greater cognitive impairment. The significant main effect of cortical layer indicates
304 that the density is also dependent on the layer, which suggests that different regions of the MTG may vary in properties or
305 vulnerability to accumulating amyloid plaques (Leng et al., 2021; Willumsen et al., 2022; Yang et al., 2026).

306 The significant MMSE x layer interaction indicates that the density of IBA1+/6e10+ colocalized objects is dependent on the
307 interaction between MMSE group and layer. In interpreting this interaction effect, I ran two more secondary multiple
308 comparisons tests to specifically identify where the significant MMSE effect (comparing MMSE means) and the layer effect
309 (comparing layer means) occurred. The secondary MMSE effect analysis highlighted a significance (***p <.001) between the
310 MMSE 0-22 group and both other groups, whereas the MMSE 23-26 group and the MMSE 27-30 group did not significantly
311 differ from each other. The secondary main layer effect analysis then yielded a significance (*p <.05) between L3 and L5/6.
312 Thus, the interaction effect can be interpreted as meaning that the difference between L3 and L5/6 of the MMSE 0-22 group
313 is significantly greater than both the difference between L3 and L5/6 of the MMSE 23-26 group, as well as the difference
314 between L3 and L5/6 of the MMSE 27-30 group. This suggests that the regional variation of amyloid/microglia colocalization
315 may be more pronounced with lower cognition, and therefore differences in regional properties may also be more



pronounced in donors with low cognition (Chen et al., 2022; Escarião et al., 2025). These findings therefore prompted further investigation into whether a significant layer effect, MMSE effect, or MMSE x layer interaction effect was also observed for IBA1+ alone or 6e10+ alone.

4.3. Microglia Remain Present but May Transition Towards An Altered State

Despite increased co-localization with amyloid pathology, IBA1-positive cell per area counts actually remained relatively constant across the three cognitive groups. My analysis of IBA1+ objects per area demonstrated a significant main layer effect, indicating that the density of IBA1+ objects is dependent on the layer. In interpreting this significant layer effect, I ran a secondary multiple comparisons test to specifically identify where the layer effect (comparing layer means) occurred. The analysis highlighted a significance ($*p < .05$) between L2 and L4, whereas the other layers did not significantly differ from each other. This finding suggests that different regions of the MTG may vary in microglial presence, which could make certain areas more or less vulnerable to injury and accumulation of proteins (Chen et al., 2021). However, no significant main effect of MMSE group or MMSE x layer interaction was observed, indicating that overall IBA1+ object density was not dependent on MMSE or the interaction between MMSE and layer.

My analysis of 6e10+ colocalized objects per area demonstrated a significant main layer effect, indicating that the amyloid density is dependent on the cortical layer. In interpreting the significant overall layer effect, I ran a secondary multiple comparisons test to specifically identify where the layer effect (comparing layer means) occurred. The analysis highlighted a significance ($*p < .05$) between L3 and L5/6, whereas the other layers did not significantly differ from each other. This finding further supports that the MTG may exhibit region-specific vulnerability to accumulating amyloid plaques (Leng et al., 2021; Willumsen et al., 2022). No significant main effect of MMSE group or MMSE x layer interaction was observed, indicating that overall 6e10+ object density did not depend on the donor's MMSE grouping or the interaction between MMSE and layer. Interestingly, 6e10+ object-per-area counts were consistently higher than the corresponding counts of colocalized objects, suggesting that not all amyloid deposits were associated with detectable IBA1-positive microglial colocalization and that microglial association with amyloid deposits may therefore be incomplete.

Nevertheless, while microglia continued to localize around amyloid deposits, the change in inactive divided by active IBA1 cell per area ratios suggested a relative increase in microglial inactivation in donors with lower MMSE scores. My analysis of the IBA1+ inactive:active ratio demonstrated a significant main MMSE effect, indicating that the relative proportion of inactive to active microglia differed according to cognitive status. In interpreting the MMSE effect, I ran a secondary multiple comparisons test to specifically identify where the MMSE effect (comparing MMSE means) occurred. The secondary MMSE effect analysis highlighted a significance ($***p < .001$) between the MMSE 0-22 group and both other groups, whereas the MMSE 23-26 group and the MMSE 27-30 group did not significantly differ from each other. Essentially, lower ratios indicate relatively less inactive cells as compared to active, while higher ratios indicate relatively more inactive cells as compared to active. Therefore, the donors exhibiting higher ratios for this measure, which are the low MMSE group, could be experiencing changes in microglial state that cause such a shift in activation (Guo et al., 2022).

However, no significant main effect of MMSE group or MMSE x layer interaction was observed, indicating that the IBA1+ inactive:active ratio was not dependent on MMSE or the interaction between MMSE and layer. Thus, unlike the layer-dependent effect observed for IBA1+/6e10+ colocalization, IBA1+ alone, and 6e10+ alone, the pronounced shift in the

inactive:active ratio was primarily dependent on MMSE grouping. This suggests an intriguing relationship between declining cognitive status and changes in microglial activation—moreover, this relationship may be observed across regions, independent of layer dynamics.

Rather than representing an entirely absent immune response, however, these results may be indicative of a state of chronic dysfunction or cellular exhaustion. Past research has shown that under conditions of sustained pathological stress, microglia may remain physically associated with amyloid deposits but transition to an altered state (Cheng & Ho, 2025; Gao et al., 2023). This state may include reduced ability to execute protective functions such as plaque clearance, debris removal, or maintenance of tissue homeostasis (Lier et al., 2021). Such a reduced-activation state could be a major contributor to continued pathological accumulation despite persistent microglial presence.

This interpretation is consistent with emerging literature describing differing disease-associated microglial (DAM) states in Alzheimer's disease (Cheng & Ho, 2025; Kuhn & Proctor, 2025). Although the present study does not directly measure DAM signatures, they provide an interesting conceptual connection that may inform future research. Several studies have reported that microglia surrounding plaques exhibit altered transcriptional profiles, including impaired phagocytic activity and reduced homeostatic signaling (Hassan et al., 2025). Experimental models have similarly demonstrated that prolonged exposure to amyloid pathology can shift microglia from protective to maladaptive states (McKinney et al., 2026).

The present findings contribute to this ongoing discussion by suggesting that physical proximity to pathology does not necessarily indicate effective functional engagement. However, although changes in inactive to active ratios based on morphology may provide insight into altered microglial states, the current data cannot conclusively measure or affirm microglial function.

4.4. Selective Layer Enrichment

A notable feature across multiple analyses was the significant overall layer effect. Significance was observed in L3 of the 6e10+ measure and L2 of the IBA1+ measure. Additionally, in the secondary multiple comparisons tests (comparing layer means) for both IBA1+/6e10+ colocalized objects per area and the 6e10+ objects per area, significance (* $p < .05$) was found between L3 and L5/6. Moreover, in the secondary multiple comparisons tests (comparing layer means) for the IBA1+ objects per area, significance (* $p < .05$) was found between L2 and L4.

Cortical layers differ significantly in neuronal composition, connectivity, and functional organization, and these differences may influence selective vulnerability during neurodegeneration (Chen et al., 2021). This is particularly true for the MTG, where there are structural and microarchitectural asymmetries between the left and right hemispheres, regional variations in surface area, and differences in cortical thickness (Zachlod et al., 2022). Analyses limited to bulk gray matter measurements risk masking such spatial patterns. The layer-specific approach used here therefore provides additional resolution and suggests that important disease-related changes may occur within discrete anatomical compartments rather than uniformly throughout cortical tissue.

The enrichment of amyloid pathology within Layer 3 may be particularly significant given the role of this layer in corticocortical communication. Interestingly, a significantly lower 6e10+ object-per-area count was observed in layer 5/6 as well, further suggesting that layer dynamics should be a strong consideration for future studies.

Layer 3 pyramidal neurons form extensive long-range connections with other cortical association areas and are heavily integrated into large-scale neural networks involved in cognition (Planert et al., 2026). Previous studies have suggested that highly connected neuronal populations may be especially vulnerable to pathological protein spread and metabolic stress (Bloomingdale et al., 2022; Brettschneider et al., 2015). Research has also suggested that neurons in layers 2 and 3 of the MTG may be more vulnerable to degenerative mechanisms in early stages of AD (Chen et al., 2021). Consequently, the concentration of amyloid pathology within layer 3 may reflect underlying network properties that increase a region's susceptibility to neurodegeneration (Escarião et al., 2025).

The IBA1+ analyses revealed significant enrichment of microglial presence within Layer 2, and a significantly lower count within Layer 4. The high microglial localization found in Layer 2 and the low microglial localization found in Layer 4 do not necessarily indicate that the microglia themselves exhibit a natural preference towards layers — rather, they may be responding to accumulating pathology in more vulnerable areas of the MTG. This layer-specific angle of researching AD must consider the possibility that certain layers are more susceptible to some biomarkers — and these vulnerable areas may be different for different biomarkers.

Past research has shown that layers 3 and 4 of the neocortex, including the MTG, are the first to accumulate neurofibrillary tangles of tau (Leng et al., 2021). Evidence also suggests that neurons in layers 2, 3, and 5 of the temporal lobe, including the MTG, may be selectively vulnerable to tau pathology (Chen et al., 2022). Together, these findings raise the possibility that distinct cortical layers may contribute differently to disease progression. Further investigation is needed to confirm whether these layer-specific patterns are unique to the MTG or represent broader cortical inhomogeneity in Alzheimer's disease.

4.5. Findings Extend Existing Models of Disease Progression

The overall findings broadly align with current models of Alzheimer's disease progression while also highlighting potential limitations of simplified frameworks. Increasing amyloid burden alongside progressive tau accumulation is consistent with the amyloid cascade hypothesis, which proposes that amyloid deposition initiates downstream pathological events leading ultimately to neurodegeneration and cognitive decline (Wu et al., 2022).

However, the present findings suggest that immune responses may play a more active and complex role than originally proposed. While classical models often frame neuroinflammation as a secondary consequence of pathology, growing evidence suggests that microglial dysfunction itself may contribute meaningfully to disease progression (Cheng & Ho, 2025; McKinney et al., 2026). The present data support this possibility — microglial cells appeared increasingly associated with pathology but simultaneously exhibited changes suggestive of diminished activity.

In their 2025 manuscript analyzing the SEA-AD dataset, Escarião et al. noted that higher PAM (plaque-associated microglia) expression correlated with lower MMSE scores—my finding of greater IBA1+/6e10 colocalized density in the low MMSE group complements this, while further demonstrating that this occurs alongside an increased inactive:active microglial ratio (Escarião et al., 2025). My study integrates multiple pathological dimensions and specifically examines the relationship between microglial-amyloid colocalization, total IBA1+ density, amyloid density, and morphology-based inactive:active microglial state across MMSE groups.



421 Presence alone does not necessarily imply effective response, and thus cells may occupy regions of pathology while
422 simultaneously undergoing shifts towards a reduced activation state (McKinney et al., 2026). The observed shift in
423 morphology raises the possibility that altered microglial states may be a critical feature of disease progression and could help
424 inform future explanations of why pathological burden continues to increase despite persistent immune response, although
425 functional and causal relationships are yet to be confirmed (Guo et al., 2022; Lier et al., 2021).

426 These findings are consistent in conceptualizing Alzheimer's disease as a multifactorial disease involving more complex
427 interactions among protein aggregation, neuroinflammation, synaptic dysfunction, and neuronal loss. Rather than acting
428 solely downstream of amyloid deposition, microglia may well influence other stages of disease progression by affecting
429 neuroinflammation and becoming unable to clear pathological proteins. Consequently, therapeutic strategies targeting
430 microglia may need to focus not only on enhancing microglial activity but also on preserving beneficial, active states and
431 preventing transitions toward chronic inactivation.

432 4.6. Limitations and Future Directions

433 Several limitations should be considered when interpreting these findings. Firstly, because the present study relied on
434 observational postmortem data, clear causal relationships cannot be established. Although significant associations were
435 identified between MMSE status and neuropathological measures, it remains unclear whether altered microglial states drive
436 disease progression or emerge as a consequence of accumulating pathology. These findings should help generate hypotheses
437 for future therapeutic investigation, rather than directly informing treatment.

438 Additionally, MMSE-based grouping provides only an approximate measure of disease severity. Cognitive decline is a
439 multidimensional process influenced by numerous biological and clinical factors, and thus individuals with similar MMSE
440 scores may actually differ substantially in their true pathological profiles. Future studies incorporating both additional
441 cognitive assessments and clinical data may provide a more comprehensive understanding of the relationship between
442 cognition and neuropathology.

443 The two microglial activation (active and inactive) states were determined according to morphology-based classifications
444 provided by the dataset, but morphology alone may be insufficient in showing the functional diversity of microglial states.
445 Future studies incorporating transcriptomic, proteomic, and molecular approaches may better help determine whether the
446 observed changes represent dysfunction, adaptive responses, or distinct disease-associated phenotypes.

447 The layer-specific findings observed in this study also need further investigation. The observed enrichment of amyloid
448 pathology within Layer 3 and microglia within Layer 2, along with the consistent significant overall layer effect in several of
449 the two-way ANOVAs, raises important concerns regarding what truly controls selective cortical vulnerability. Future studies
450 must examine whether differences in neuronal connectivity, synaptic density, or structural communication patterns
451 contribute to the observed distribution of pathology. Further research on layer-specific attributes may provide insight into
452 how pathological processes spread through large-scale brain networks.

453 Finally, the present analysis focused only on the middle temporal gyrus. Expanding similar layer-resolved analyses to other
454 brain regions commonly affected in Alzheimer's disease, like the hippocampus, entorhinal cortex, frontal cortex, etc., could
455 determine whether the observed relationships between pathology and microglial dynamics represent a generalized feature of

disease progression or are a region-specific phenomenon (Leng et al., 2021). Future large-scale studies utilizing automated spatial transcriptomics or volumetric modeling would benefit from nested mixed-effects linear models to further isolate intra-donor laminar variance.

4.7. Conclusions

In summary, lower cognitive function was associated with higher tau and amyloid pathology within the MTG, accompanied by changes in microglial dynamics. Tau burden exhibited a particularly strong relationship with declining MMSE scores, supporting previous evidence that tau pathology closely tracks clinical impairment. On the other hand, greater microglial localization was observed near amyloid deposits in lower MMSE scores despite evidence suggesting altered activation states as disease severity progressed.

Together, these findings support a model where pathological burden and neuroimmune changes evolve in parallel with Alzheimer's disease progression. The results further suggest that microglial presence alone may be insufficient to assess neuroimmune function, as cells can remain associated with pathology while potentially undergoing shifts toward less effective or maladaptive states. Additionally, the layer-specific patterns identified within the MTG highlight the importance of considering spatial organization when investigating such neurodegenerative mechanisms. Understanding why certain cortical layers and regions exhibit heightened vulnerability and the changes in pathology associated with these regions may ultimately lead to the development of more targeted therapeutic strategies.

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Data Availability Statement

All datasets analyzed and methodology are available through the Allen Institute's BrainMap in the Seattle Alzheimer's Disease Brain Cell Atlas: <https://portal.brain-map.org/explore/seattle-alzheimers-disease/seattle-alzheimers-disease-brain-cell-atlas-download?edit&language=en>.

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Author Biography

[REDACTED] is a high school junior at the [REDACTED] in [REDACTED] with a strong interest in various fields of biomedicine. Her experience includes both wet-lab research and data analysis in neuroscience, genetics, cell biology, toxicology, and computational biology. Outside of research, [REDACTED] volunteers at a pediatric hospital and teaches taekwondo to younger students. At school, she seeks leadership positions as co-head of Science Club and The Catalyst, the school STEM magazine. She hopes to pursue a career in biomedical research and medicine after college.



Mentor Contribution Statement

615

616 Dr. [REDACTED] provided comprehensive mentorship and scientific guidance throughout the development of this research
617 project.

618 Dr. [REDACTED] introduced the author to Alzheimer's disease research and provided access to the publicly available datasets,
619 analytical framework, computational workflows, and software protocols used throughout the study. Through his
620 instruction, the author learned to organize, analyze, visualize, and interpret high-dimensional transcriptomic and genomic
621 data independently.

622 Through the research process, Dr. [REDACTED] offered continuous feedback on the study design, statistical analyses,
623 interpretation of the results, and scientific writing. His thoughtful critiques strengthened the rigor, clarity, and overall
624 quality of the manuscript while encouraging the author's independent scientific reasoning and problem-solving skills.

625 Finally, Dr. [REDACTED] carefully reviewed multiple manuscript revisions prior to submission, providing constructive and extensive
626 suggestions to improve the presentation and communication of the findings. His mentorship was essential in the successful
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2 **Increased Tau, Amyloid β , and IBA1 in the Middle Temporal Gyrus Is**
3 **Associated With Lower Cognitive Function in SEA-AD Donors**
4 **Increased**
5 **Tau, A β , and IBA1 Inactivation in the Middle Temporal Gyrus Is**
6 **Associated With Lower Cognitive Function in SEA-AD Donors**

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Abstract

Alzheimer's Disease (AD) is a neurodegenerative disorder characterized by mechanisms including neuroinflammation, neurofibrillary tangles of phosphorylated tau, accumulation of amyloid plaques, and microglial aggregation. In AD, disease pathology is closely related to cognitive function, measured using the Mini-Mental-State-Examination (MMSE), a clinical screening tool. This study examined relationships among tau burden, amyloid deposition, and microglial activity in the middle temporal gyrus across three MMSE-defined stages of cognitive decline. Data, including quantifications of AT8, 6e10, and IBA1, were obtained from the Seattle Alzheimer's Disease Brain Cell Atlas. Two-way ANOVAs were used to study the change in percent AT8+ area (phosphorylated tau), IBA1+ cells per area and activation (microglia), 6e10+ cells per area (amyloid-beta), and IBA1+/6e10+ colocalized objects per area, alongside MMSE-defined cognitive decline. Our analysis revealed that neuropathological measures of percent AT8+ area, IBA1/6e10 colocalized objects per area, and IBA1 inactive:active ratio reflected a significant overall MMSE effect, while measures of IBA1+/6e10+ colocalized objects per area, IBA1+ cells per area, and 6e10+ cells per area reflected a significant overall layer effect. Although general IBA1+ cell per area counts were comparable across MMSE groups, the inactive:active ratio differed, with the lowest MMSE group having the highest ratios. These findings lead to the hypothesis that lower MMSE scores may be associated with more tau burden, amyloid deposition, and amyloid/microglia colocalization, accompanied by alterations in microglial state. Together, these findings inform a broader understanding of the link between pathological burden and cognitive function in a multidimensional model of AD.



Keywords: Alzheimer's Disease (AD), MMSE, dementia, tau pathology, amyloid beta, microglial activation, cortical layers

1. Introduction

Alzheimer's disease (AD) is the most common cause of dementia and is characterized by progressive cognitive decline (Atri, 2019; US Department of Health and Human Services, 2017). It is accompanied by the accumulation of extracellular amyloid- β (A β) plaques, intracellular neurofibrillary tangles composed of hyperphosphorylated tau, and widespread neuroinflammation (Khachaturian, 1985; Reitz, 2012). Although these pathological hallmarks have been studied extensively, the mechanisms linking such protein aggregation to cognitive impairment are still unclear.

The accumulation of extracellular amyloid plaques, formed from self-aggregating peptides, has long been regarded as the first hallmark of Alzheimer's disease, inhibiting neuronal synaptic function (Reitz, 2012). Amyloid deposition is known to begin years before the onset of clinical symptoms (Wu et al., 2022). On the other hand, tau accumulation, presenting as neurofibrillary tangles toxic to neurons, is implicated to more closely track the timeline of dementia-related neuronal dysfunction, synapse loss, and worsening cognition (Cody et al., 2024). Consequently, increasing tau burden is commonly considered one of the strongest neuropathological indicators of disease severity (Murray et al., 2022). Previous studies have also affirmed suggested that tau pathology is more strongly associated with cognitive impairment than amyloid burden alone (Guo et al., 2026). Amyloid pathology nevertheless remains an important marker, as it is commonly believed to initiate a cascade of downstream events that contribute to tau aggregation, neurodegeneration, and ultimately dementia — a process often referred to as the “amyloid cascade hypothesis” (Reitz, 2012).

In addition to protein deposition, neuroinflammation is another major hallmark of dementia, with emerging studies suggesting that inflammation may be both a consequence and an initiator of AD pathology like tau accumulation and amyloid plaques (Zhang et al., 2023). Under healthy conditions, microglia contribute to tissue maintenance through debris clearance and immune surveillance (McKinney et al., 2026). They are the brain's resident immune cells and macrophages, continuously monitoring the brain environment and responding to accumulating amyloid deposits and neuroinflammation (Muzio et al., 2021). In AD, however, microglia aggregate around amyloid plaques and undergo substantial changes in morphology, gene expression, and function (Bolmont et al., 2008).

Experimental and genetic evidence suggests that microglia initially exert protective effects by containing plaques and phagocytosis of pathological proteins, but being exposed to prolonged pathological stress may yet lead to impaired clearance mechanisms and ultimately contribute to neurodegeneration by accelerating the spreading of proteins like A β and tau. (Gao et al., 2023). Furthermore, evidence argues that microglia have certain specific activation states of microglia may which may mediate synapse loss, worsen tau pathology, and secrete inflammatory factors that directly harm neurons (Hansen et al., 2018). Microglia are implicated to be able to transform from one phenotype to the others—in particular, many researchers believe that the M1 microglia, which release inflammatory mediators, and the M2 microglia, which release anti-inflammatory mediators, may provide further insight on the effective activation states of microglia (Guo et al., 2022). However, current understanding has not reached a consensus on whether microglial states can be explicitly divided into simply anti-inflammatory and pro-inflammatory subtypes, due to the complex heterogeneity and heavily context-dependent nature



74 of microglial states (Paolicelli et al., 2022). Despite growing recognition of the importance of neuroinflammation, it still
75 remains unclear whether microglia continue to respond effectively as disease progresses or whether they transition into
76 dysaltered functional states that contribute to further pathological accumulation. This study therefore investigates how the
77 accumulation of microglia around sites of pathology may change across different stages of neurodegeneration.

78 In AD, neuroinflammation spreads heavily across the brain to areas like the cerebral cortex, the outer layer of the brain
79 responsible for thinking, memory, and language (Ni & Wu, 2021). The cerebral cortex is organized into distinct layers that all
80 differ in cellular composition, connectivity, and functional roles, meaning pathology may behave differently across layers
81 (Chen et al., 2021). Emerging evidence suggests that neurodegenerative pathology may not be distributed uniformly
82 throughout cortical tissue, and analyses restricted to just gray matter measurements may obscure important layer-specific
83 patterns (Willumsen et al., 2022). Across past studies, AD neuropathology has been known to show strong regional specificity
84 when developing across the brain (Yang et al., 2026). Neurofibrillary tangles typically spread from the entorhinal cortex to the
85 hippocampus, limbic system, and neocortex, amyloid plaques show graded accumulation across brain regions, and microglia
86 in the prefrontal cortex, cerebellum, and hippocampus are known to have significant differences in their highly expressed
87 genes (Asher & Priefer, 2022; Jacobsen et al., 2005; Yang et al., 2026).

88 The middle temporal gyrus (MTG) is particularly relevant in AD because of its involvement in language processing, semantic
89 memory, and other higher order cognitive functions that become impaired during disease progression (Chen et al., 2022).
90 Therapeutic strategies developed over the years rely heavily on gray matter analysis and often encounter obstacles once in
91 clinical trials (Asher & Priefer, 2022). Investigating pathology across individual cortical layers may therefore provide additional
92 insight into selective regional differences and vulnerabilities.

93 Despite extensive research conducted on AD biomarkers, critical gaps in the scientific understanding of disease mechanisms
94 still exist. It remains unclear whether layer-specific microglial clustering directly damages local circuitry or merely reacts to
95 underlying synaptic failure and plaque growth, and when microglia transition activation states (Escarião et al., 2025). In the
96 MTG itself, the precise functional organization and the exact division of labor for semantic processing remain unknown,
97 meaning a layer-specific approach to examining the MTG may provide more insight (Escarião et al., 2025).

98 Accordingly, the present study investigated relationships among tau burden, amyloid deposition, and microglial activity
99 within the MTG across MMSE-defined stages of cognitive decline. Particular emphasis was placed on examining the
100 pathology within individual cortical layers 1 to 6, following initial bulk gray matter analyses.

101 A previous study of these markers in the SEA-AD dataset investigated the APOE4 genotype and an individual's genetic
102 predisposition to dementia (Wang, 2025). However, this present study uses MMSE grouping to better understand the
103 relationships between disease pathology and an individual's ongoing cognitive capacities.

104 I hypothesized that lower MMSE scores would be associated with higher levels of tau burden and amyloid deposition, as well
105 s well as accompanied alterations in microglial activation. More specifically, the microglia were expected to increasingly
106 associate with sites of pathology whilst displaying ~~reduced functional activation in more advanced stages of cognitive~~



declines in change in activation. Furthermore, layer-specific analyses were expected to reveal patterns that would not be apparent from the gray matter measurements alone.

2. Materials and Methods

Previously collected data were accessed and reanalyzed in the present study. De-identified postmortem human brain data and quantitative neuropathological measurements of the middle temporal gyrus were accessed from the Allen Brain Institute's publicly available SEA-AD dataset (Hawrylycz et al., 2024). All ~~experimentation~~ initial experimentation and data and data collection were conducted by the Kaiser Permanente Washington Health Research Institute ACT Study researchers and the University of Washington ADRC at the UW BioRepository and Integrated Neuropathology (BRaIN) laboratory. The dataset focused on different patients' histological pathology, obtained via immunolabeling and microscopy across cortical layers and protein markers.

For my study, the data from the Quantitative Neuropathology Summary Data (MTG) and Donor Metadata from the Allen Brain Institute's SEA-AD data set were aligned and compiled in an Excel spreadsheet in order to match neuropathology data to donor MMSE status. Donors were stratified into three groups based on their respective Mini-Mental State Examination (MMSE) scores: 27–30, 23–26, and 0–22.

2.1. ~~Diagnosing Dementia~~ MMSE-Based Cognitive Stratification

Cognitive status was classified into donor groups according to Mini-Mental State Examination (MMSE) scores obtained from SEA-AD donor metadata. The MMSE is a widely used clinical screening tool for cognitive impairment, with scores ranging from 0 to 30, where lower scores indicate greater cognitive dysfunction (Creavin et al., 2016; Yang et al., 2024).

Donors were stratified into three groups based on established thresholds used in dementia research: MMSE 27–30 (cognitively normal or minimally impaired), MMSE 23–26 (mild cognitive impairment), and MMSE 0–22 (moderate to severe cognitive impairment consistent with dementia). ~~These thresholds were selected based on p~~

Across studies, there exist many different categorizations of MMSE scores, with no consensus yet on what threshold may be truly significant in terms of cognitive status. The Mini-Mental State Examination (MMSE) faces significant diagnostic inconsistency because fixed cutoff scores fail to account for demographic variations — while a score of 24 serves as the traditional clinical threshold, its diagnostic accuracy is heavily confounded by a patient's age and education level, often yielding false negatives in highly educated individuals and false positives in those with limited schooling (Creavin et al., 2016). Consequently, researchers and clinicians increasingly employ stratified thresholds ranging from 21 to 27 to enhance sensitivity and specificity across diverse populations (Salis et al., 2023). ~~rior studies demonstrating that scores below 24 are strongly associated with clinical dementia—additionally, scores in the 24–26 range have been shown to represent a transitional state, with individuals at increased risk of progression to dementia (Brackhus et al., 1995; Kukull et al., 1994).~~



The MMSE cutoff of 24 is commonly used in both clinical and research settings to distinguish cognitively impaired from non-impaired individuals and has been validated against structural brain changes (Sakurai et al., 2021). Therefore, MMSE grouping is heavily dependent on the dataset being analyzed. Based on this framework, the present study used MMSE stratification to approximate stages of cognitive decline and to enable comparison of neuropathological burden across disease severity.

Due to the nature of dementia disease progression, age at death is an essential consideration when analyzing the severity of pathological burden. Thus, when deciding the MMSE ranges for the three experimental groups, instead of solely relying on numbers used in past studies, I used a one-way ANOVA was used to run a replicate data column analysis with multiple comparisons of donor ages at death. When grouping MMSE scores into the three groups: 0-22, 23-26, and 27-30, the multiple comparison mean values yielded no significant difference between groups (mean of 0-22 vs. 23-26 being 89.29, mean of 0-22 vs. 27-30 being 89.87, and mean of 23-26 vs. 27-30 being 89.87). Thus, these cutoffs provided a more balanced partition of donor data rather than relying on arbitrary and inconsistent thresholds.

In our my dataset, before outlier removal, the group with MMSE scores 27-30 consisted of 31 donors, the group with MMSE scores 23-26 consisted of 31 donors, and the group with MMSE scores 0-22 consisted of 17 donors.

All downstream analyses were performed using these MMSE-defined groups to evaluate relationships between cognitive status and pathological markers in the middle temporal gyrus.

2.2. Neuropathological Quantification

Detailed tissue processing and staining protocols have been previously described in a published paper as well as an online procedural overview (Gabbito et al., 2024b; Seattle Alzheimer's Disease Data Set, n.d.).

Briefly, postmortem human brain tissue was obtained through the Adult Changes in Thought (ACT) study conducted by the Kaiser Permanente Washington Health Research Institute in collaboration with the University of Washington.

Briefly, Researchers collected middle temporal gyrus (MTG) samples, fixing and embedding each in paraffin prior to sectioning. Sections underwent duplex immunohistochemical staining to detect various hallmark Alzheimer's disease proteins (Hawrylycz et al., 2024). Phosphorylated tau was labeled using the AT8 antibody, while amyloid- β (A β) deposits were identified using the 6E10 monoclonal antibody. Quantification of percent AT8-positive area, 6E10-positive objects per area, and IBA1-associated measures was performed on digitized images of the MTG cortical layers. IBA1-positive cells were classified as active or inactive according to dataset-provided morphological criteria.

Cortical layers are established by the SEA-AD dataset annotations, in which Layer 5 and Layer 6 are combined. Thus, the layers are analyzed accordingly as L1, L2, L3, L4, and L5/6.

The Allen Brain Institute collected and compiled the data for access to the general public. Data were accessed June 6th, 2026, via:



168 <https://portal.brain-map.org/explore/seattle-alzheimers-disease/seattle-alzheimers-disease-brain-cell-atlas-download?edit&language=en>.

170 2.3. Outlier Removal

171 Initial outlier detection was performed using the ROUT method ($Q = 1\%$) in GraphPad Prism. Then, donors who were
 172 consistently identified as outliers across datasets were excluded. A total of six donors were excluded, including four males,
 173 and two females. Of the excluded donors, four were classified as having dementia, and two were classified as having no
 174 dementia. Excluded donors were: H20.33.028, H20.33.043, H21.33.020, H21.33.029, H20.33.036, and H21.33.031. To preserve
 175 the statistical validity and reproducibility of my pathological assessments, outlier detection was performed for all
 176 pathological measurements using the ROUT method ($Q = 1\%$) in GraphPad Prism. No outliers were identified consistently
 177 across all measures, therefore no donors were excluded from my analyses.

178 2.4. Statistical Analysis

179 Statistical analyses were largely performed using ~~ordinary one-way~~ two-way mixed effect model ANOVAs with multiple
 180 comparisons in GraphPad Prism. Layer-specific analyses were conducted within MMSE groups.

181 with repeated measures, with MMSE group as the between-subjects factor and cortical layer as the repeated within-donor
 182 factor. This design is required because multiple cortical layers are sampled from the same individual, violating the
 183 assumption of independent observations necessary for standard one-way ANOVAs. By modeling donor identity as a random
 184 effect, this approach accounts for the inherent statistical dependency within each subject, properly partitions the variance,
 185 prevents the artificial inflation of the degrees of freedom, and allows for the critical evaluation of the interaction effect
 186 between MMSE group and layer (Wilcox & Rousselet, 2023). A one-way ANOVA was performed for the AT8 measure due to
 187 the fact that it is a plain gray matter analysis with no cortical layer analyses.

188 Multiple comparisons were performed for all analyses by comparing cell means with others in its row and column. Šidák's
 189 multiple-comparisons correction was used to control the family-wise Type I error rate arising from multiple pairwise
 190 comparisons while providing greater statistical power than the more conservative Bonferroni correction. (Abdi, 2007)

191 To ensure valid cross-layer comparisons within the MTG, normalized counts were used for analysis of all measures. This
 192 normalization is statistically required to control for the prominent anatomical variations in laminar thickness inherent to
 193 the MTG (Zachlod et al., 2022). Failing to account for these structural volume differences would conflate a higher absolute
 194 cell count with a true increase in cellular density, thereby introducing systematic bias into our regional comparative
 195 analyses. Normalized measures are presented as AT8 percent area (%), IBA1+/6E10+ colocalized objects per area ($\#/\mu\text{m}^2$),
 196 IBA1+ objects per area ($\#/\mu\text{m}^2$), 6E10+ objects per area ($\#/\mu\text{m}^2$), and IBA1+ inactive microglial cells per area : IBA1+ active
 197 microglial cells per area (#).

198 Data are presented as bars with mean $\pm$ SEM. Statistical significance thresholds were defined per APA style 7th edition -as:
 199 * $p < .05$, ** $p < .01$, *** $p < .001$.



I calculated the overall main effect of MMSE, layers, and the MMSE x layer interaction for all two-way ANOVAs. A significant overall main effect of MMSE group (M) demonstrates that MMSE grouping has a statistically significant effect on the dependent variable. Similarly, a significant overall main effect of cortical layers (L), then, means that the layers of the MTG have a statistically significant effect on the dependent variable. A significant MMSE x cortical layer interaction indicates that there is a statistically significant interaction between both the effects of MMSE grouping and layer. Thus, while the main effects describe overall differences associated with either cognitive status or cortical layers, the interaction significance provides evidence that the two factors jointly influence the distribution of the biomarker across the middle temporal gyrus.

3. Results

3.1. AT8 Pathology in the Middle Temporal Gyrus Higher with Cognitive Decline

To assess the relationship between tau pathology and cognitive decline, ~~total~~percent AT8-positive area was quantified across the gray matter of the middle temporal gyrus (MTG) and stratified by MMSE category (Figure 1). My analyses compared donors with low, medium, and high MMSE scores, where a standard one-way ANOVA revealed a significant difference between groups ($F_{2,76} = 14.31$, $***p < .001$). Šídák-adjusted post-hoc tests showed significance of $***p < .001$ for both MMSE 27-30 vs. MMSE 0-22 and MMSE 23-26 vs. MMSE 0-22.

AT8 burden was higher in donors with decreased MMSE scores, with the lowest MMSE group (0-22) exhibiting a greatly elevated and significant ~~total~~percent AT8-positive area ~~a in micrometres squared~~. The intermediate group (23-26) showed values between the high and low MMSE groups, indicating ~~ingive~~ of a graded relationship between tau accumulation and cognitive impairment.

This trend was consistent across donors within each group, with statistical comparisons demonstrating a significant increase in AT8-positive area in the 0-22 group relative to both higher MMSE groups ($***p < .001$).



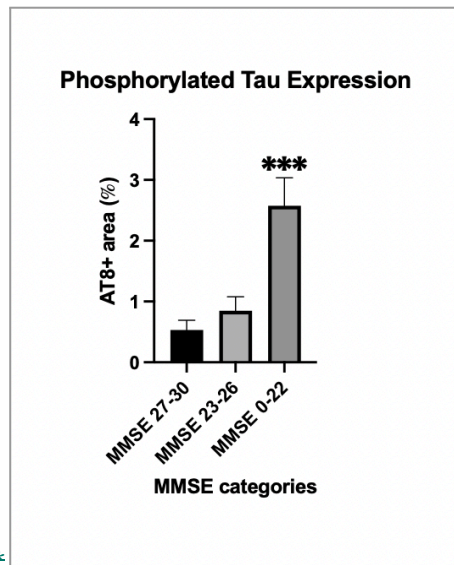


Figure 1. TotalPercent AT8-positive area in MTG gray matter across MMSE categories. Significance (** $p < .001$) was found between MMSE group 0-22 and all other MMSE groups.

3.2. Increased Microglial Association with Amyloid Pathology Across Cortical Layers

To evaluate microglial interaction with amyloid pathology, the number of IBA1/6E10 co-localized objects per area was quantified across cortical layers of the MTG (Figure 2). Co-localized object per area counts- were higher with decreased MMSE scores, with the greatest values observed in the 0-22 group. The progressive increase in co-localized objects per area across MMSE groups may indicate an enhanced microglial presence in proximity to amyloid deposits as cognitive impairment worsens.

Two-way ANOVA analyses with MMSE group as the between-subjects factor and cortical layer as the repeated within-donor factor revealed a significant overall layer effect ($F_{2,528,192.1} = 14.58$, ** $p < .001$), a significant overall MMSE effect ($F_{2,76} = 3.290$, * $p = .043$) and a significant overall interaction effect of layer and MMSE group ($F_{5,056,192.1} = 2.367$, * $p = .041$).

Layer-specific analysis revealed a pronounced enrichment in layer 3 across all MMSE groups. This increase was statistically significant in the 23-26 group (** $p < .01$) and further elevated in the 0-22 group (** $p < .001$). Other layers demonstrated comparatively lower counts and less consistent differences between MMSE categories. However, no statistical significance was found between individual layers.

The progressive increase in co-localized objects across MMSE groups indicates an enhanced microglial presence in

proximity to amyloid deposits as cognitive impairment worsens.

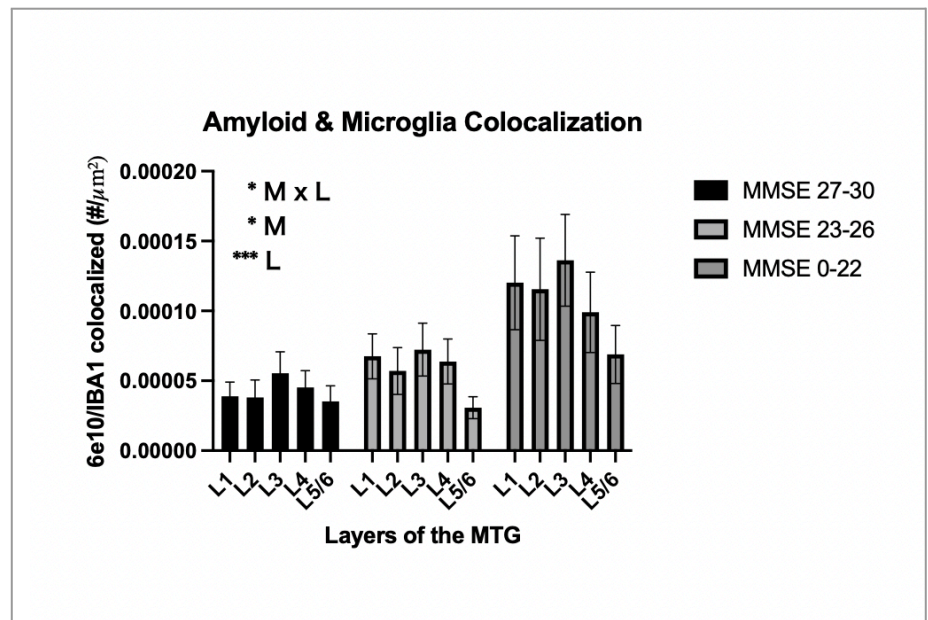
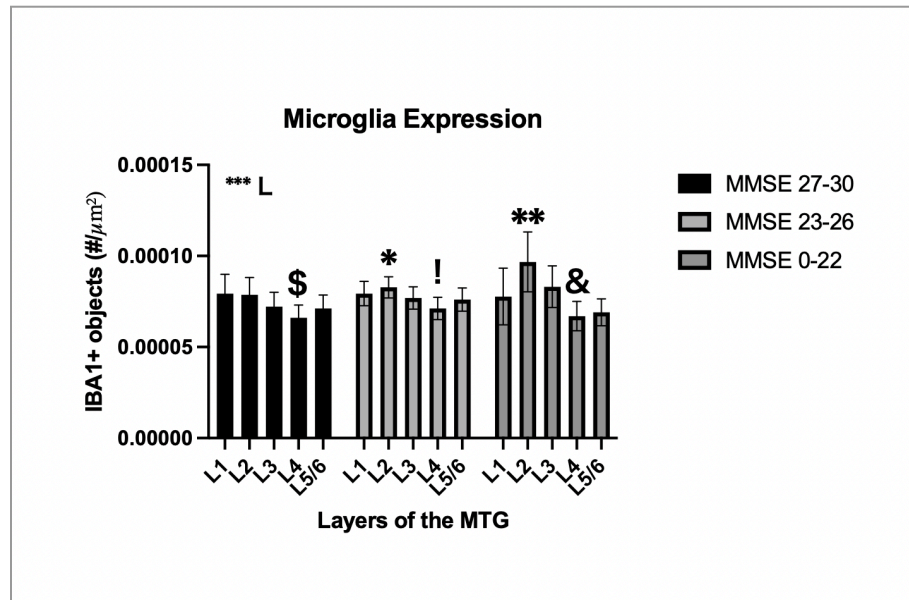


Figure 2. Number of IBA1/6E10 co-localized objects per area across MTG cortical layers by MMSE category. In graph A (MMSE group 27–30), significance was found between L3 and all other layers (** $p < .01$). In graph B (MMSE group 23–26), significance was found between L3 and all other layers (** $p < .01$). In graph C (MMSE group 0–22), significance was found between L3 and all other layers (** $p < .001$). Significance was observed for the overall interaction effect of layer and MMSE group (* $M \times L$), the overall MMSE group effect (* M), and the overall layer effect (** L).

3.3. Total Microglial Activation Appears Comparable Across Cognitive Status

To assess whether the survival of microglial cells affected amyloid colocalization, the amount of IBA1-positive cells per area was first compared across cortical layers and MMSE groups (Figure 3). Notably, layer-specific multiple comparison analysis revealed significance between layers within each MMSE group, but not across MMSE groups themselves.

Two-way ANOVA analyses with MMSE group as the between-subjects factor and cortical layer as the repeated within-donor factor revealed a significant overall layer effect ($F_{1,618,122.9} = 14.62$, *** $p < .001$), but no significant overall MMSE effect or significant overall interaction effect of layer and MMSE group.



255

256 **Figure 3. Number of total IBA1 positive cells per area.** Significance was observed for the overall layer effect (***) L. In MMSE 27-30,
257 significance marked by "\$" was found between L4 and L2 (**p < .01), L4 and L5/6 (**p < .01), and L4 and L3 (***) p < .001). In MMSE
258 23-26, significance was found between L3 and L2 (*p < .05). Significance (***) p < .001) marked by "!" was found between L2 and L4,
259 and L3 and L4. In MMSE 0-22, significance was found between L1 and L2 (**p < .01). Significance (*p < .05) marked by "&" was found
260 between L3 and L4.

261 3.34. Layer-Specific Distribution of Amyloid- β Deposition

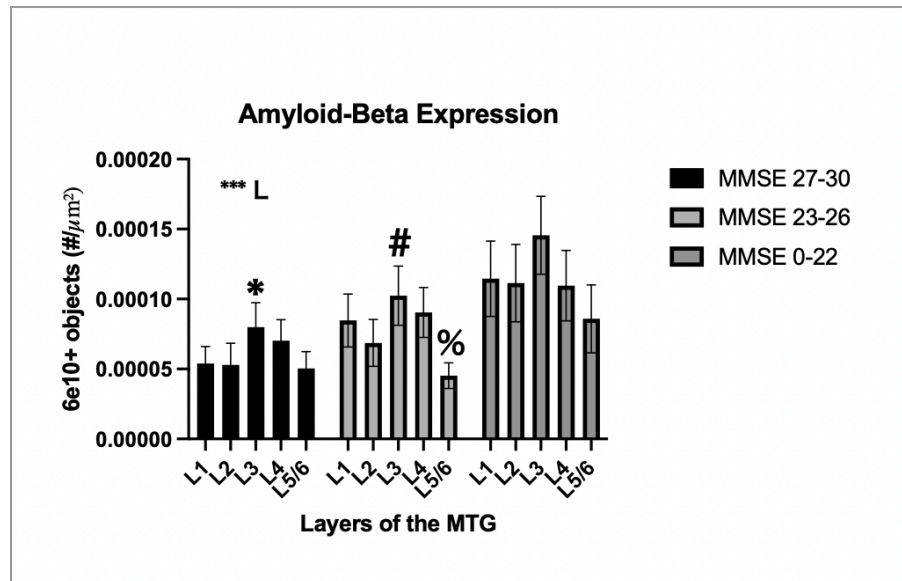
262 To determine whether the observed layer-specific patterns from Figure 2 were driven by amyloid distribution,
263 6E10-positive objects per area were quantified independently across MTG layers (Figure 34). A consistent-consistent
264 significance of 6E10-positive objects per area was observed in layer 3 across all MMSE groups (***) p < 0.001).

265 Two-way ANOVA analyses with MMSE group as the between-subjects factor and cortical layer as the repeated
266 within-donor factor revealed a significant overall layer effect as well ($F_{2,529,192.2} = 14.45$, ***p < .001), but no significant overall
267 MMSE effect or significant overall interaction effect of layer and MMSE group.

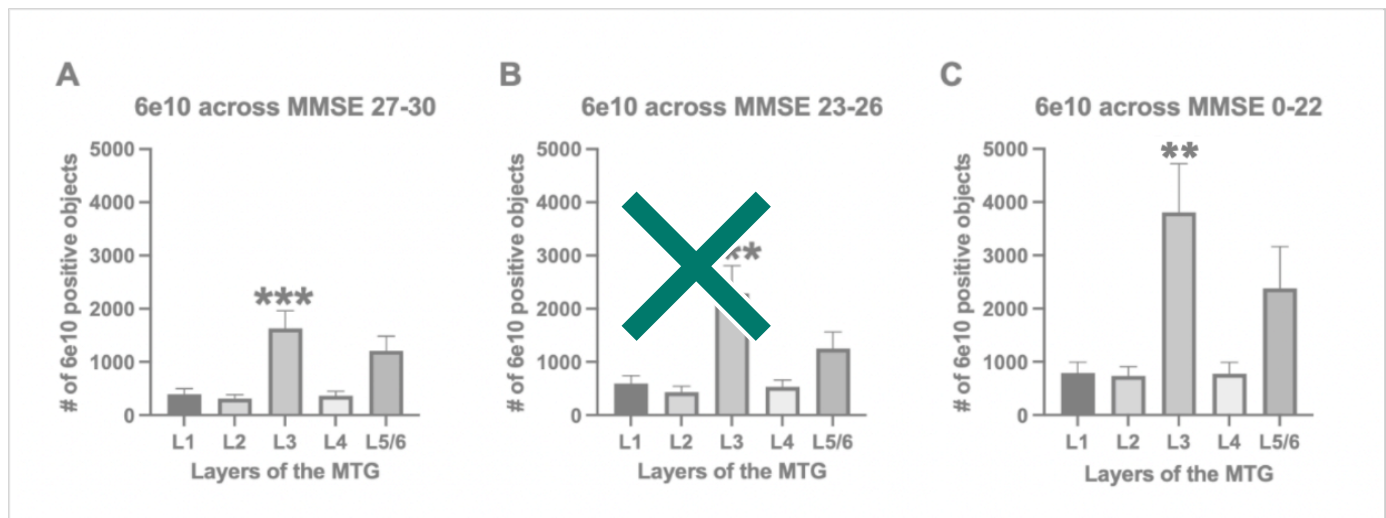
268 While total amyloid burden was higher in lower MMSE scores, the relative distribution across cortical layers remained
269 stable. Layer 3 consistently exhibited the highest number of 6E10-positive objects, while other layers showed lower and
270 more uniform distributions.

271 Notably, the number of 6E10-positive objects per area exceeded the number of IBA1/6E10 co-localized objects per area

across all groups and layers, perhaps indicating that only a subset of amyloid deposits were associated with microglial localization. detected 6E10+ objects met the criteria for colocalization.



274



275

Figure 34. Number of 6E10-positive objects per area across MTG cortical layers by MMSE category. Significance was observed for the overall layer effect (*** L). In graph A (MMSE 27-30), significance was found between L3 and all other layers L2 (***p < .0015). In graph B (MMSE 23-26), significance (***p < .001) marked by “#” was found between L3 and all other layers L2 (***p < .001). Significance

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277

278

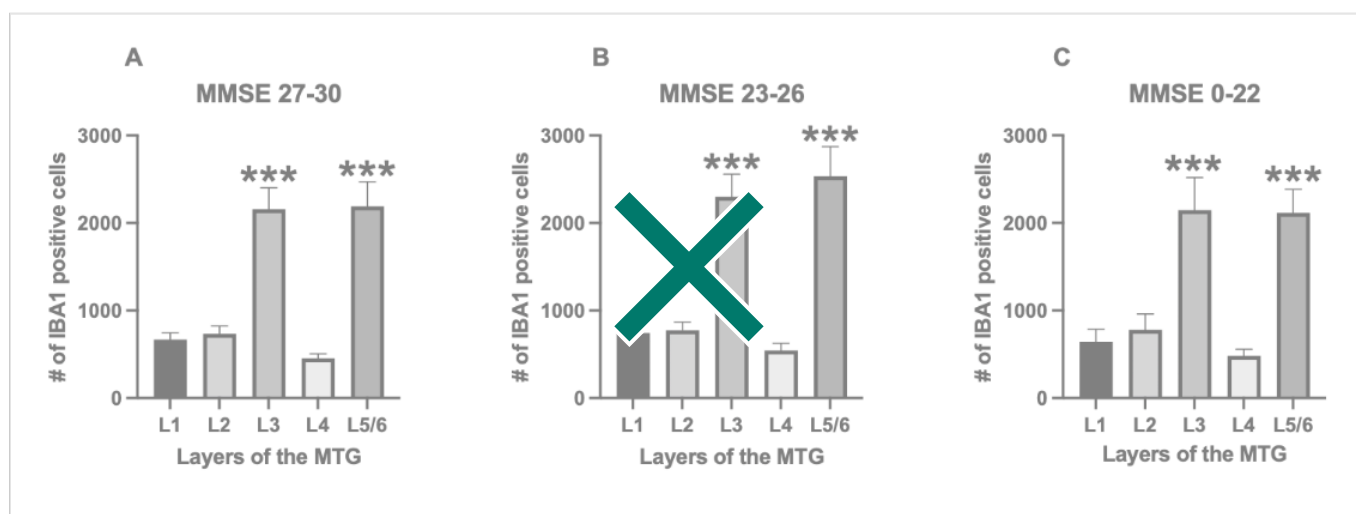


279 (* $p < .05$) marked by “%” was found between L5/6 and L3, and L5/6 and L4. No significance was found in graph C (between individual
 280 layers of MMSE 0-22); significance was found between L3 and all other layers ($***p < .001$).

281 3.4. Total Microglial Activation Appears Comparable Across Cognitive Status

282 To assess whether the survival of microglial cells affected amyloid colocalization, the total amount of IBA1-positive cells was
 283 first compared across cortical layers and MMSE groups (Figure 4). Notably, levels of IBA1-positive cell counts were similar
 284 across all three MMSE groups, indicating that there must be another explanation for the amyloid colocalization differences
 285 from Figure 2.

286 Layer-specific analysis revealed a pronounced enrichment in layer 3 across all MMSE groups. This increase was statistically
 287 significant ($***p < 0.001$) in all groups. Other layers demonstrated comparatively lower counts and less consistent
 288 differences between MMSE categories.



290 **Figure 4. Number of total IBA1 positive cells across MTG cortical layers by MMSE category.** In graph A (MMSE group 27-30), graph B
 291 (MMSE group 23-26), and graph C (MMSE group 0-22), significance was found between L3 and all other layers ($***p < .001$), and
 292 between L5/6 and all other layers ($***p < .001$).

293 3.5. Microglial AInactivation DecreasesIs Higher with Worsening Cognitive Statusive Status

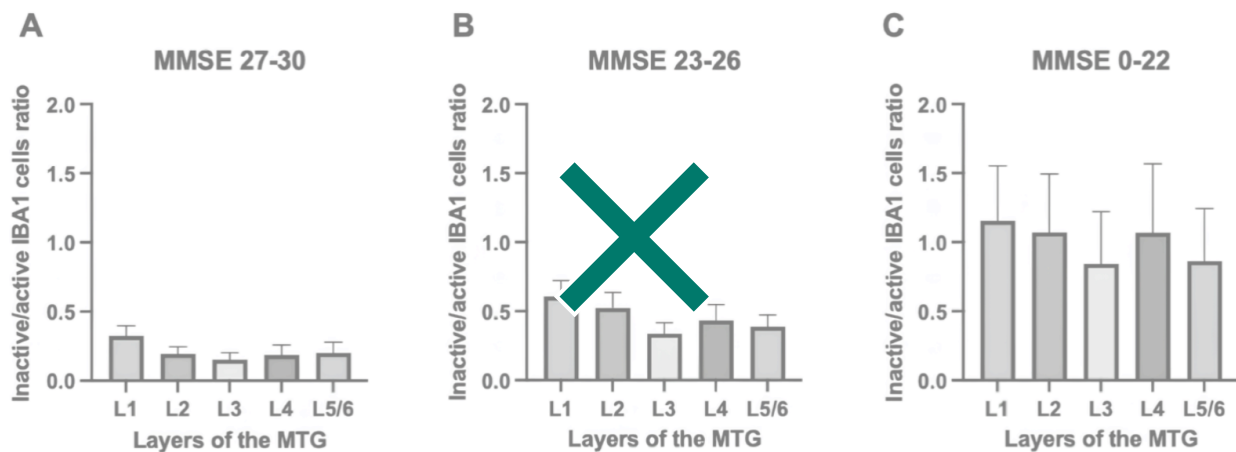
294 To assess microglial functional morphology and state itself, the ratio of morphologically-classified inactive to active
 295 IBA1-positive microglial cells per area was calculated (inactive cells per area divided by active cells per area) across cortical
 296 layers and MMSE groups (Figure 5). A progressive increase prominent difference in the inactivation ratio was observed
 297 across declined MMSE scores, with the highest ratios in the 27-30 between the 0-22 MMSE group and the lowest in the

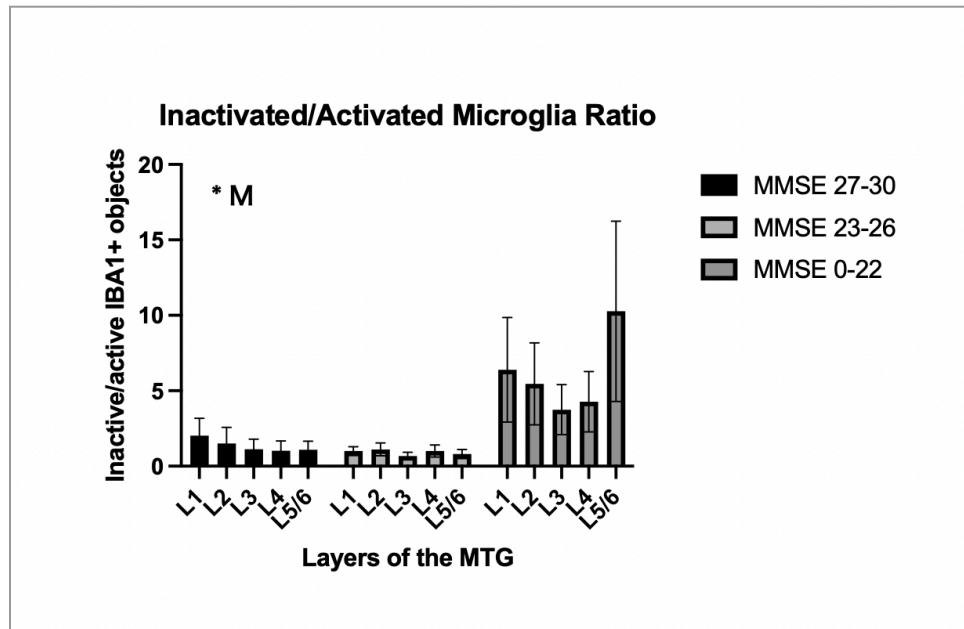
298 ~~0-22 group~~ all other MMSE groups.

299 Two-way ANOVA analyses with MMSE group as the between-subjects factor and cortical layer as the repeated
 300 within-donor factor revealed a significant overall MMSE group effect ($F_{2,76} = 3.959$, $*p = .023$), but no significant overall layer
 301 effect or significant overall interaction effect of layer and MMSE group.

302 Although differences between specific cortical layers and MMSE groups were not statistically significant, the upward
 303 trend/increase in inactivation ratio was consistent/clear across all layers/MMSE groups. In the lowest MMSE group, the ratio
 304 reflected/indicated a relative increase in inactivated microglial states/IBA1 cells, with the highest inactive:active cell per area
 305 ratio.

306 These findings indicate that while microglia remain comparably present in regions of pathology as seen in Figure 3, their
 307 activation state may changes with disease progression, with more IBA1 cells per area becoming inactive relative to active as
 308 disease severity worsens.





310

311 **Figure 5. Ratio of inactive to active IBA1-positive microglia across MTG layers by MMSE category cells per area. No significance was**
312 **found. Significance was observed for the overall MMSE group effect (*M) only.**

313 3.6. Summary of Pathological Trends Across MMSE Groups

314 Across all measures, lower MMSE scores were associated with higher pathological burden and altered microglial dynamics.
315 AT8-positive percent area, IBA1/6E10 co-localized objects per area, and 6E10-positive object counts per area, and the IBA1
316 inactive:active cell per area ratio were higherst in donors with worse cognitive status, while IBA1/6E10 co-localization also
317 showed higher levels across layers, particularly in layer 3an MMSE score between 0-22. Total IBA1 positive cell per area
318 counts were relatively constant across MMSE scores, indicative of almost no difference related to disease severity. In
319 contrast, the ratio of inactive to active microglia was higher in donors with lower MMSE scores. Overall layer significance
320 was observed in all analyses except for the IBA1 ratio.

321 4. Discussion

322 4.1. Progressive Pathological Burden Accompanies Cognitive Decline

323 The present study investigated relationships between tau pathology, amyloid deposition, and microglial activity within the
324 middle temporal gyrus (MTG) across MMSE-defined stages of cognitive decline. It was hypothesized that lower MMSE scores
325 would be associated with higher pathological burden and across measures, accompanied by altered microglial activation



states. Overall, ~~the my~~ findings supported this hypothesis and affirmed expected changes in disease pathology and neuroimmune dynamics in individuals with varying degrees of cognitive deficits.

Tau burden, measured by the ~~percent~~ AT8-positive area in the study, was substantially higher in donors with lower MMSE scores and exhibited a ~~graded~~ pattern across the three cognitive groups. Donors with the lowest MMSE scores (0-22) demonstrated the greatest accumulation of phosphorylated tau within MTG gray matter, supporting the close relationship between tau pathology and cognitive deterioration.

Post-hoc comparisons confirmed significantly greater AT8 levels in the low-MMSE group compared with both the medium- and high-MMSE groups. This relationship between more AT8 pathology and lower MMSE provided an initial indication that cognitive status was associated with meaningful differences in neuropathology within the MTG and motivated further investigation of whether other pathological measures might exhibit similar relationships with MMSE and whether these relationships vary across cortical layers.

Whereas amyloid accumulation ~~is generally believed to often precedes~~ precede the onset of clinical symptoms and ~~may~~ eventually plateau, tau pathology has been shown to track more closely with ~~neuronal dysfunction and~~ disease severity (Mormino & Papp, 2018). The present findings therefore support that tau accumulation is a key pathological feature closely associated with cognitive decline in Alzheimer's disease:

. Collectively, the simultaneous increase in both tau and amyloid pathology across MMSE groups is consistent with current models of Alzheimer's disease, ~~which propose close interactions between protein aggregation, neuronal dysfunction, and cognitive decline.~~

4.2. Microglia Remain Present but May Be Functionally Compromised

~~The microglial findings revealed a more complex pattern than just higher or lower levels of cellular activity.~~ Microglia Associate With Amyloid Plaques

Initially, quantification of IBA1/6E10 co-localized objects ~~per area~~ demonstrated the expected increase in microglial association with amyloid deposits as MMSE scores declined. At first glance, this finding suggests a natural heightened immune response accompanying increasing pathological burden.

My analysis of IBA1+/6e10+ colocalized objects per area demonstrated significant main effects of MMSE group and cortical layer, as well as a significant MMSE group x cortical layer interaction. The significant main effect of MMSE group indicates that the density of IBA1+/6e10+ colocalized objects is dependent on the MMSE group. Consistent with this effect, a progressive increase in colocalized objects was observed across the MMSE groups, suggesting greater microglial association with amyloid pathology in donors with greater cognitive impairment. The significant main effect of cortical layer indicates



that the density is also dependent on the layer, which suggests that different regions of the MTG may vary in properties or vulnerability to accumulating amyloid plaques (Leng et al., 2021; Willumsen et al., 2022; Yang et al., 2026).

The significant MMSE x layer interaction indicates that the density of IBA1+/6e10+ colocalized objects is dependent on the interaction between MMSE group and layer. In interpreting this interaction effect, I ran two more secondary multiple comparisons tests to specifically identify where the significant MMSE effect (comparing MMSE means) and the layer effect (comparing layer means) occurred. The secondary MMSE effect analysis highlighted a significance ($***p < .001$) between the MMSE 0-22 group and both other groups, whereas the MMSE 23-26 group and the MMSE 27-30 group did not significantly differ from each other. The secondary main layer effect analysis then yielded a significance ($*p < .05$) between L3 and L5/6. Thus, the interaction effect can be interpreted as meaning that the difference between L3 and L5/6 of the MMSE 0-22 group is significantly greater than both the difference between L3 and L5/6 of the MMSE 23-26 group, as well as the difference between L3 and L5/6 of the MMSE 27-30 group. This suggests that the regional variation of amyloid/microglia colocalization may be more pronounced with lower cognition, and therefore differences in regional properties may also be more pronounced in donors with low cognition (Chen et al., 2022; Escarião et al., 2025). These findings therefore prompted further investigation into whether a significant layer effect, MMSE effect, or MMSE x layer interaction effect was also observed for IBA1+ alone or 6e10+ alone.

4.3. Microglia Remain Present but May Transition Towards An Altered State

Despite increased co-localization with amyloid pathology, ~~total~~ IBA1-positive cell per area counts actually remained relatively constant across the three cognitive groups. My analysis of IBA1+ objects per area demonstrated a significant main layer effect, indicating that the density of IBA1+ objects is dependent on the layer. In interpreting this significant layer effect, I ran a secondary multiple comparisons test to specifically identify where the layer effect (comparing layer means) occurred. The analysis highlighted a significance ($*p < .05$) between L2 and L4, whereas the other layers did not significantly differ from each other. This finding suggests that different regions of the MTG may vary in microglial presence, which could make certain areas more or less vulnerable to injury and accumulation of proteins (Chen et al., 2021). However, no significant main effect of MMSE group or MMSE x layer interaction was observed, indicating that overall IBA1+ object density was not dependent on MMSE or the interaction between MMSE and layer.

My analysis of 6e10+ colocalized objects per area demonstrated a significant main layer effect, indicating that the amyloid density is dependent on the cortical layer. ~~Therefore, more microglial presence near plaques in terms of cell abundance alone is not a reliable reflection of the immune response to amyloid plaques. Instead, changes in functional state may better explain the observed pattern.~~

In interpreting the significant overall layer effect, I ran a secondary multiple comparisons test to specifically identify where the layer effect (comparing layer means) occurred. The analysis highlighted a significance ($*p < .05$) between L3 and L5/6, whereas the other layers did not significantly differ from each other. This finding further supports that the MTG may exhibit region-specific vulnerability to accumulating amyloid plaques (Leng et al., 2021; Willumsen et al., 2022). No significant main effect of MMSE group or MMSE x layer interaction was observed, indicating that overall 6e10+ object density did not depend



on the donor's MMSE grouping or the interaction between MMSE and layer. Interestingly, 6e10+ object-per-area counts were consistently higher than the corresponding counts of colocalized objects, suggesting that not all amyloid deposits were associated with detectable IBA1-positive microglial colocalization and that microglial association with amyloid deposits may therefore be incomplete.

While Nevertheless, while microglia continued to localize around amyloid deposits in predictable numbers, the change in inactive:divided by active IBA1 cell per area ratios suggested a reduction relative increase in effective-microglial responsiveness inactivation in donors with lower MMSE scores. As MMSE scores decreased, the ratio of the number of inactive:active IBA1 cells progressively increased. Together, these findings raise an important possibility—even though microglia continue to recognize and cluster around sites of pathology, they may lose the ability to efficiently clear amyloid plaques.

My analysis of the IBA1+ inactive:active ratio demonstrated a significant main MMSE effect, indicating that the relative proportion of inactive to active microglia differed according to cognitive status. In interpreting the MMSE effect, I ran a secondary multiple comparisons test to specifically identify where the MMSE effect (comparing MMSE means) occurred. The secondary MMSE effect analysis highlighted a significance (**p <.001) between the MMSE 0-22 group and both other groups, whereas the MMSE 23-26 group and the MMSE 27-30 group did not significantly differ from each other. Essentially, lower ratios indicate relatively less inactive cells as compared to active, while higher ratios indicate relatively more inactive cells as compared to active. Therefore, the donors exhibiting higher ratios for this measure, which are the low MMSE group, could be experiencing changes in microglial state that cause such a shift in activation (Guo et al., 2022).

However, no significant main effect of MMSE group or MMSE x layer interaction was observed, indicating that the IBA1+ inactive:active ratio was not dependent on MMSE or the interaction between MMSE and layer. Thus, unlike the layer-dependent effect observed for IBA1+/6e10+ colocalization, IBA1+ alone, and 6e10+ alone, the pronounced shift in the inactive:active ratio was primarily dependent on MMSE grouping. This suggests an intriguing relationship between declining cognitive status and changes in microglial activation—moreover, this relationship may be observed across regions, independent of layer dynamics.

Rather than representing an entirely absent immune response, however, these results may reflect the indicative of a state of chronic dysfunction or cellular exhaustion. Past research has shown that under conditions of sustained pathological stress, microglia may remain physically associated with amyloid deposits but transition to an altered state become (Cheng & Ho, 2025; Gao et al., 2023). This state may include progressively less capable of reduced ability to executing the protective functions such as plaque clearance, debris removal, or maintenance of tissue homeostasis (Lier et al., 2021). Such a loss of function reduced-activation state could be a major contributor to continued pathological accumulation despite persistent microglial presence.

421



This interpretation is consistent with emerging literature describing differing disease-associated microglial (DAM) states in Alzheimer's disease (Cheng & Ho, 2025; Kuhn & Proctor, 2025). Although the present study does not directly measure DAM signatures, they provide an interesting conceptual connection that may inform future research. Several studies have reported that microglia surrounding plaques exhibit altered transcriptional and functional profiles, including impaired phagocytic activity and reduced homeostatic signaling (Hassan et al., 2025). Experimental models have similarly demonstrated that prolonged exposure to amyloid pathology can shift microglia from protective to maladaptive states (McKinney et al., 2026).

The present findings contribute to this ongoing discussion by suggesting that physical proximity to pathology does not necessarily indicate effective functional engagement. However, although changes in inactive to active ratios based on morphology may provide insight into altered microglial states, the current data cannot conclusively measure or affirm microglial function.

432

4.34. Selective Layer Enrichment

A notable feature across multiple analyses was the consistent enrichment of pathological and microglial measures within layer 3 of the MTG significant overall layer effect. Increased amyloid deposition, greater IBA1/6E10 co-localization, and elevated total IBA1 positive cell counts were repeatedly observed within this cortical layer regardless of MMSE category.

Importantly, the observed microglial pattern may not necessarily indicate preference of microglia toward layer 3 itself. Because amyloid burden was similarly enriched within this layer, higher levels of microglial localization likely reflects the distribution of pathology that microglia are responding to, rather than an independent microglial preference. In other words, microglial cells may just be responding proportionally to where pathology is concentrated indicating that layer 3 itself may be uniquely vulnerable to neurodegeneration. Significance was observed in L3 of the 6e10+ measure and L2 of the IBA1+ measure. Additionally, in the secondary multiple comparisons tests (comparing layer means) for both IBA1+/6e10+ colocalized objects per area and the 6e10+ objects per area, significance (* $p < .05$) was found between L3 and L5/6. Moreover, in the secondary multiple comparisons tests (comparing layer means) for the IBA1+ objects per area, significance (* $p < .05$) was found between L2 and L4.

Nevertheless, the repeated prominence of layer 3 is notable. Cortical layers differ significantly in neuronal composition, connectivity, and functional organization, and these differences may influence selective vulnerability during neurodegeneration (Chen et al., 2021). This is particularly true for the MTG, where there are structural and microarchitectural asymmetries between the left and right hemispheres, regional variations in surface area, and differences in cortical thickness (Zachlod et al., 2022). Analyses limited to bulk gray matter measurements risk masking such spatial patterns. The layer-specific approach used here therefore provides additional resolution and suggests that important disease-related changes may occur within discrete anatomical compartments rather than uniformly throughout cortical tissue.



453 The enrichment of amyloid pathology within Layer 3 may be particularly significant given the role of this layer in
 454 corticocortical communication. Interestingly, a significantly lower 6e10+ object-per-area count was observed in layer 5/6 as
 455 well, further suggesting that layer dynamics should be a strong consideration for future studies.

456 Layer 3 pyramidal neurons form extensive long-range connections with other cortical association areas and are heavily
 457 integrated into large-scale neural networks involved in cognition (Planert et al., 2026). Previous studies have suggested that
 458 highly connected neuronal populations may be especially vulnerable to pathological protein spread and metabolic stress
 459 (Bloomingdale et al., 2022; Brettschneider et al., 2015). Research has also suggested that neurons in layers 2 and 3 of the MTG
 460 may be more vulnerable to degenerative mechanisms in early stages of AD (Chen et al., 2021). Consequently, the
 461 concentration of amyloid pathology and microglial responses within layer 3 may reflect underlying network properties that
 462 increase a region's susceptibility to neurodegeneration (Escarião et al., 2025).

463 The IBA1+ analyses revealed significant enrichment of microglial presence within Layer 2, and a significantly lower count
 464 within Layer 4. The high microglial localization found in Layer 2 and the low microglial localization found in Layer 4 do not
 465 necessarily indicate that the microglia themselves exhibit a natural preference towards layers — rather, they may be
 466 responding to accumulating pathology in more vulnerable areas of the MTG.

467 Interestingly, elevated microglial abundance was also observed within deeper cortical layers, particularly layers 5 and 6. This
 468 layer-specific angle of researching AD must consider the possibility that certain layers are more susceptible to some
 469 biomarkers — and these vulnerable areas may be different for different biomarkers.

470 Past research has shown that layers 3 and 4 of the neocortex, including the MTG, are the first to accumulate neurofibrillary
 471 tangles of tau (Leng et al., 2021). Evidence also suggests that neurons in layers 2, 3, and 5 of the temporal lobe, including the
 472 MTG, may be selectively vulnerable to tau pathology (Chen et al., 2022). Together, these findings raise the possibility that
 473 distinct cortical layers may contribute differently to disease progression. Further investigation is needed to confirm whether
 474 these layer-specific patterns are unique to the MTG or represent broader cortical inhomogeneity in Alzheimer's disease.

475 4.45. Findings Extend Existing Models of Disease Progression

476 The overall findings broadly align with current models of Alzheimer's disease progression while also highlighting potential
 477 limitations of simplified frameworks. Increasing amyloid burden alongside progressive tau accumulation is consistent with
 478 the amyloid cascade hypothesis, which proposes that amyloid deposition initiates downstream pathological events leading
 479 ultimately to neurodegeneration and cognitive decline (Wu et al., 2022).

480 However, the present findings suggest that immune responses may play a more active and complex role than originally
 481 proposed. While classical models often frame neuroinflammation as a secondary consequence of pathology, growing
 482 evidence suggests that microglial dysfunction itself may contribute meaningfully to disease progression (Cheng & Ho, 2025;
 483 McKinney et al., 2026). The present data support this possibility — microglial cells appeared increasingly associated with
 484 pathology but simultaneously exhibited changes suggestive of diminished functional activity.



485 In their 2025 manuscript analyzing the SEA-AD dataset, Escarião et al. noted that higher PAM (plaque-associated microglia)
 486 expression correlated with lower MMSE scores—my finding of greater IBA1+ /6e10 colocalized density in the low MMSE group
 487 complements this, while further demonstrating that this occurs alongside an increased inactive:active microglial ratio
 488 (Escarião et al., 2025). My study integrates multiple pathological dimensions and specifically examines the relationship
 489 between microglial-amyloid colocalization, total IBA1+ density, amyloid density, and morphology-based inactive:active
 490 microglial state across MMSE groups.

491 Presence alone does not necessarily imply effective response, and thus cells may occupy regions of pathology while
 492 simultaneously undergoing shifts towards a ~~reduced loss-of-function~~ activation state (McKinney et al., 2026). The ~~transition~~
 493 observed shift in morphology ~~from protective surveillance to dysfunctional activity~~ raises the possibility that altered
 494 microglial states may be a critical feature of disease progression and could help inform future explanations of why
 495 pathological burden continues to increase despite persistent immune response, although functional and causal relationships
 496 are yet to be confirmed (Guo et al., 2022; Lier et al., 2021).

497

498 These findings are consistent in conceptualizing Alzheimer's disease as a multifactorial disease involving more complex
 499 interactions among protein aggregation, neuroinflammation, synaptic dysfunction, and neuronal loss. Rather than acting
 500 solely downstream of amyloid deposition, microglia may well influence other stages of disease progression by affecting
 501 neuroinflammation and becoming unable to clear pathological proteins. Consequently, therapeutic strategies targeting
 502 microglia may need to focus not only on enhancing microglial activity but also on preserving beneficial, active states and
 503 preventing transitions toward chronic inactivation.

504 4.56. Limitations and Future Directions

505 Several limitations should be considered when interpreting these findings. Firstly, because the present study relied on
 506 observational postmortem data, clear causal relationships cannot be established. Although significant associations were
 507 identified between MMSE status and neuropathological measures, it remains unclear whether altered microglial states drive
 508 disease progression or emerge as a consequence of accumulating pathology. *These findings should be used to help inform*
 509 *and generate hypotheses for future therapeutic studies, rather than to directly inform treatment.*

510

511 Additionally, MMSE-based grouping provides only an approximate measure of disease severity. Cognitive decline is a
 512 multidimensional process influenced by numerous biological and clinical factors, and thus individuals with similar MMSE
 513 scores may actually differ substantially in their true pathological profiles. Future studies incorporating both additional
 514 cognitive assessments and clinical data may provide a more comprehensive understanding of the relationship between
 515 cognition and neuropathology.



The two microglial activation (active and inactive) states were determined according to morphology-based classifications provided by the dataset, but morphology alone may be insufficient in showing the functional diversity of microglial states. Future studies incorporating transcriptomic, proteomic, and molecular approaches may better help determine whether the observed changes represent dysfunction, adaptive responses, or distinct disease-associated phenotypes.

The layer-specific findings observed in this study also need further investigation. The ~~consistent-observed~~ enrichment of amyloid pathology ~~and microglial measures~~ within Layer 3- and microglia within Layer 2, along with the consistent significant overall layer effect in several of the two-way ANOVAs, raises important concerns regarding what truly controls selective cortical vulnerability. Future studies must examine whether differences in neuronal connectivity, synaptic density, or structural communication patterns contribute to the observed distribution of pathology. Further research on layer 3-specific attributes may provide insight into how pathological processes spread through large-scale brain networks.

Finally, the present analysis focused only on the middle temporal gyrus. Expanding similar layer-resolved analyses to other brain regions commonly affected in Alzheimer's disease, like the hippocampus, entorhinal cortex, frontal cortex, etc., could determine whether the observed relationships between pathology and microglial dynamics represent a generalized feature of disease progression or are a region-specific phenomenon (Leng et al., 2021).

Future large-scale studies utilizing automated spatial transcriptomics or volumetric modeling would benefit from nested mixed-effects linear models to further isolate intra-donor laminar variance.

4.67. Conclusions

In summary, lower cognitive function was associated with ~~progressively~~ higher tau and amyloid pathology within the MTG, accompanied by changes in microglial dynamics. Tau burden exhibited a particularly strong relationship with declining MMSE scores, supporting previous evidence that tau pathology closely tracks clinical impairment. On the other hand, ~~microglia increasingly localized~~ greater microglial localization was observed near amyloid deposits in lower MMSE -scores despite evidence suggesting altered activation states as disease severity progressed.

Together, these findings support a model where pathological burden and neuroimmune changes evolve in parallel with Alzheimer's disease progression. The results further suggest that microglial presence alone may be insufficient to assess neuroimmune function, as cells can remain associated with pathology while potentially undergoing shifts toward less effective or maladaptive states. Additionally, the layer-specific patterns identified within the MTG highlight the importance of considering spatial organization when investigating such neurodegenerative mechanisms. Understanding why certain cortical layers and regions exhibit heightened vulnerability and the changes in pathology associated with these regions may ultimately lead to the development of more targeted therapeutic strategies.

5. References



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Data Availability Statement

- 681 All datasets analyzed and methodology are available through the Allen Institute's BrainMap in the Seattle- Alzheimer's Disease
682 Brain Cell Atlas:
683 [https://portal.brain-map.org/explore/seattle-alzheimers-disease/seattle-alzheimers-disease-brain-cell-atlas-download?ed](https://portal.brain-map.org/explore/seattle-alzheimers-disease/seattle-alzheimers-disease-brain-cell-atlas-download?edit&language=en)
684 [it&language=en](https://portal.brain-map.org/explore/seattle-alzheimers-disease/seattle-alzheimers-disease-brain-cell-atlas-download?edit&language=en).

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689 encouraged me to think critically, ask meaningful questions, and approach research with a passion and rigor that it is
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695

Author Biography

696 [REDACTED] is a high school junior at the [REDACTED] in [REDACTED] with a strong interest in various fields of
697 biomedicine. Her experience includes both wet-lab research and data analysis in neuroscience, genetics, cell biology,
698 toxicology, and computational biology. Outside of research, [REDACTED] volunteers at a pediatric hospital and teaches
699 taekwondo to younger students. At school, she seeks leadership positions as co-head of Science Club and The Catalyst, the
700 school STEM magazine. She hopes to pursue a career in biomedical research and medicine after college.

701

Mentor Contribution Statement

702 Dr. [REDACTED] provided comprehensive mentorship and scientific guidance throughout the development of this research
703 project.

704 Dr. [REDACTED] introduced the author to Alzheimer's disease research and provided access to the publicly available datasets,
705 analytical framework, computational workflows, and software protocols used throughout the study. Through his
706 instruction, the author learned to organize, analyze, visualize, and interpret high-dimensional transcriptomic and genomic
707 data independently.

708 Through the research process, Dr. [REDACTED] offered continuous feedback on the study design, statistical analyses,
709 interpretation of the results, and scientific writing. His thoughtful critiques strengthened the rigor, clarity, and overall
710 quality of the manuscript while encouraging the author's independent scientific reasoning and problem-solving skills.

711 Finally, Dr. [REDACTED] carefully reviewed multiple manuscript revisions prior to submission, providing constructive and extensive
712 suggestions to improve the presentation and communication of the findings. His mentorship was essential in the successful
713 completion of this project and in the author's personal growth as an emerging researcher.



REVIEWER 1:

Review of: Increased Tau, Amyloid- β , and IBA1 in the Middle Temporal Gyrus Is Associated With Lower Cognitive Function in SEA-AD Donors

Recommendation: Accept with minor revisions

Overall assessment

This manuscript investigates an important question in Alzheimer's disease research: how tau, amyloid- β , and microglial changes within the middle temporal gyrus relate to cognitive impairment. The manuscript's strengths include the use of a human postmortem data, the focus on layer-specific pathology rather than bulk measurements, and the attempt to interpret microglial changes beyond simple differences in cell abundance. The discussion around distinguishing microglial presence from potential changes in microglial state is interesting and is a strong contribution. However, some revisions are needed before publication including more careful interpretation of microglial activation states, softening of claims of conclusions, and clearly distinguishing observed associations from proposed mechanisms

Major Comments:

Introduction

- The introduction provides a strong overview of AD pathology and relevance of tau, amyloid, and microglia. The specific gap motivating this study could be more clearly developed. E.g., what remains unknown about the MTG, layer-specific patterns, relationship between microglial localization and cognitive decline.

I thank the reviewer for suggesting this revision. I have done further research and elaborated upon the gap in current AD knowledge and the aim of the study in section 1.0.

- The layer-specific analysis is one of the manuscript's strongest contributions, which could be emphasized more earlier on. You mention that bulk gray matter measurements may obscure layer-specific patterns, but it could help to explain what prior studies have shown and what remains unknown about how AD pathology and microglial responses vary across cortical layers.

I thank the reviewer for this important point. I have included more relevant studies and elaborated upon the cortical layers of the MTG in section 1.0.

- In the introduction, some statements could be softened to better reflect current evidence. While amyloid, tau, and neuroinflammation are strongly associated with AD, the causal relationships among these processes remain debated. Consider more language such as "has been implicated in", "is associated with", "may contribute to"

I thank the reviewer for this point. I have adjusted my language throughout the paper to avoid overly strong language that may suggest a consensus in understanding.

- In the introduction, M1/M2 terminology may oversimplify current understanding that microglial states are heterogeneous and context-dependent rather than just pro-inflammatory versus anti-inflammatory. Check out this paper: [https://www.cell.com/neuron/fulltext/S0896-6273\(22\)00859-5](https://www.cell.com/neuron/fulltext/S0896-6273(22)00859-5) and consider briefly acknowledging this nuance.

I thank the reviewer for providing this paper and bringing up this nuance. I have added more context in section 1.0 for the microglial states I am addressing in my paper and addressed that microglia are heavily context-dependent.

- The importance of the MTG and rationale for focusing on this region could be expanded and more developed. Consider mentioning why this region is particularly vulnerable or informative in AD compared to other regions

I thank the reviewer for this point. I have added further points and explanations on how AD develops through the brain and why the MTG is uniquely informative for my purposes at the end of section 1.0.

- Some claims lack citations such as : “Alzheimer’s disease is the most common cause of dementia”, “Amyloid deposition is known to begin years before the onset of clinical symptoms”, “The cerebral cortex is organized into distinct layers that all differ in cellular composition...”

I thank the reviewer for this important point. I have added citations to all my claims across the paper and confirmed that they are reputable sources.

Methods:

- Some language makes it difficult to distinguish between analyses performed on this public data versus the experiments conducted by the SEA-AD researchers. E.g., manuscript states “all experimentation and data were conducted by...” Consider revising this language to clearly state in the beginning of methods that previously collected data were accessed and reanalyzed in the present study

I thank the reviewer for this important point. I have adjusted my language in the methods, section 2.0 to be clearer, and explicitly stated that the SEA-AD researchers conducted initial experimentation and data collection, which I then accessed and reanalyzed in the present study.

- Please clarify if corrections for multiple comparisons were performed given the large number of statistical tests across markers and cortical layers. If no correction was applied, please acknowledge this as a limitation.

I thank the reviewer for this point. Šidák’s multiple-comparison corrections were performed, in which I compared each cell means with others in its row and column. I conducted further research into Type I and Type II errors and the justification behind using Šidák corrections for multiple comparisons. I have explained my reasoning for choosing Šidák and provide further details regarding the multiple-comparisons in my paper, which can be found in section 2.4.

*I also calculated whether there was a significant overall main effect of MMSE, layer, or the MMSE x layer interaction for all two-way ANOVAs. Each effect is thoroughly explained in context in the **Discussion**, and brief definitions of the effects have been added to section 2.4.*

*Finally, when interpreting any overall main effects, in order to ensure that I have thoroughly analyzed and appropriately interpreted the data, I ran secondary multiple comparisons tests to specifically identify where the MMSE effect (comparing MMSE means) or the layer effect (comparing layer means) occurred. I then reported the significance found in the secondary analyses for each measure in the **Discussion**.*

- Outlier removal explanation is unclear. The author says subjects “consistently identified as outliers across datasets” – but this is difficult to interpret.

I thank the reviewer for their comment concerning outlier exclusion. After reviewer 2 raised a question regarding the normalisation of counts, I re-ran each analyses with normalised

counts using two-way ANOVAs. As such, with these new analyses, I performed a new outlier removal test. No outliers were identified, therefore no donors are excluded from my analyses. I have explained this process in section 2.3.

Results:

- I may be misunderstanding but there is inconsistency in the y-axis metric and reported interpretation of Figure 5. Since the y-axis of Fig 5 represents the inactive/active IBA1 cell ratio, an increased ratio indicates relatively more inactive cells compared to active cells, whereas a decreased ratio would indicate relatively greater active microglial representation. The text currently states that lower MMSE groups show increased activation, but the reported ratio appears to suggest the opposite. Please clarify if the ratio was calculated as inactive/active or active/inactive and revise the interpretation accordingly.

I thank the reviewer for bringing up this very critical point. The ratio was indeed calculated as inactive/active, and Section 3.5 has been updated to reflect the correct claim. I have clarified there that an increased ratio with lower MMSE scores should be interpreted as relatively more inactivated IBA1+ cells as compared to activated.

- Be cautious when interpreting IBA1 morphology as a direct measure of microglial activation/function. Morphological differences may reflect altered microglial states, but don't necessarily indicate functional changes. In the results section, try to report the direct findings and expand on what this might mean in the discussion

I thank the reviewer for this point. I have researched this further and added more context for the microglial states I am addressing in my paper, and addressed that microglia are heavily context-dependent. I have also revised the paper to clarify that the IBA1 cells may have experienced alterations in states, but that my data does not confirm functional changes. In the discussion, I further interpret the IBA1 ratio changes and possible explanations for change in state in section 4.1.

Discussion/Limitations:

- The interpretation that microglia become functionally compromised is interesting and potentially important, but the current data don't directly measure microglial function. Increased inactive/active ratios based on morphology may indicate altered microglial states, but they don't necessarily demonstrate reduced phagocytosis, impaired plaque clearance, or loss of homeostatic function. I suggest revising this to: "consistent with altered microglial states" or "suggestive of reduced responsiveness". Link this to future directions of functional assays needed to establish this mechanism.

I thank the reviewer for this point. In section 4.3, I have added more context for the microglial states I am addressing in my paper and addressed that microglia are heavily context-dependent. I have also revised the paper to clarify that the IBA1 cells may have experience alterations in states, but do not confirm functional changes. I have also added this into the future directions in section 4.6.

- The clinical implications discussed are thought-provoking but should acknowledge that translating morphology-based observations into therapeutics is challenging. Consider emphasizing that these findings may help generate hypotheses for future therapeutic investigation instead of directly informing treatment

I thank the reviewer for suggesting this revision. I have explicitly emphasized that my study's findings cannot directly inform treatment and should not be used as such for clinical therapy in section 4.6.

- When talking about DAM, clarify your study doesn't directly measure DAM signatures and that it is a conceptual connection.

I thank the reviewer for bringing up this point. I have added in a clarification in section 4.3 that my study does not directly measure any DAM signatures, and instead it is a connection that I made and used to inform my discussion.

- Limitations/future directions section is strong and acknowledges several important limitations.

I thank the reviewer for their feedback.

Minor Comments:

- Text size changes
- Unclear, reword for clarity: "a one-way ANOVA was used to run a replicate data column analysis"
- In figure legends, make sure to explain everything present in figure. E.g., what error bars represent, statistical tests, if comparisons are between groups or layers
- Soften language e.g., "indicating that there must be another explanation" □ could be "...unlikely to be explained solely".
- "microglia increasingly localized" - not totally accurate to what data show, should be something like "greater microglial localization was observed"

I thank the reviewer for their careful reading of the manuscript and for highlighting these important editorial details. I have thoroughly audited the text to standardize text size and write with more clarity. My figures have been edited accordingly and I have also changed my language to be more specific. These corrections have greatly improved the overall polish and presentation of my manuscript. I greatly appreciate the time and clear attention to detail that they have committed to my paper and will utilize their feedback.

REVIEWER 2:

This is a clear study that proposes statistical analysis of the SEA-AD dataset and provides a few strong findings, which however, have not been reported consistently. The layer-resolved analysis of the MTG and the tau/MMSE gradient are clear and convincing analyses, and the distinction between microglial number and functional state is a useful contribution. However, one central result is apparently stated in two contradicting directions and the title slightly overstates the finding.

Recommendation: Resubmit with major revisions

Main points (in order of importance)

- Section 3.5 says the inactive:active ratio is highest in the 27–30 group and lowest in the 0–22 group whereas the heading and sections 3.6, and 4.3 state the opposite. The paper's main novelty and conclusion depends on this direction. Section 3.5 needs to be updated to reconcile the claims in all four locations.

I thank the reviewer for bringing up this very critical point. Section 3.5 has been updated to reflect the correct claim: that the ratio is highest in the 0-22 group, and lowest in the 27-30 group.

- Section 3.4 reports total IBA1 counts were comparable across groups; what changed was co-localization and activation state. The title suggests a significant difference and needs to be revised to state the actual finding.

I thank the reviewer for their attention to the inconsistent reporting. The title has been adjusted to: "Increased Tau, A β , and IBA1 Inactivation in the Middle Temporal Gyrus Is Associated With Lower Cognitive Function in SEA-AD Donors" to reflect the finding that the IBA1 count is not increased, but the relative IBA1 inactivation is increased.

- Exclusions need to be justified. Six donors were removed via ROUT (four with dementia); excluding high-pathology cases could attenuate the reported effects. Please state what drove the exclusions and confirm results hold on the full sample.

I thank the reviewer for their comment concerning outlier exclusion. After running the new, two-way ANOVA analyses using normalised counts for all measures, no consistent outliers were identified. Therefore, no donors will be excluded. I sincerely thank the reviewer. This update can be found in section 2.3.

- Layer counts will need to be normalised for comparability. Layer 3 is enriched and is anatomically thick. It would be helpful to explain if the reported counts are per-area; if they are raw values, perhaps the layer-3 claim may need to be reconsidered.

I thank the reviewer for this consideration. The original reported counts are raw values. I have now produced new graphs using two-way ANOVAs and normalised counts for all measures. The paper has been updated to accommodate these revised graphs.

After running the new analyses and producing updated graphs, the layer-3 enrichment is still observed in the 6E10/IBA1 colocalized objects per area graph and similarly the 6E10 (alone) objects per area graph. This layer-3 enrichment is not observed in the IBA1 (alone) objects per area graph.

Additionally, I conducted further research into the anatomy of the MTG and its layer-specific attributes. I added them into section 2.4 of the paper to justify my reasoning for normalizing layer counts.

The paper by Escari  et al. (2025) is very close in concept and dataset but cited only in passing. Could the author explain briefly what is replicated from that paper and what is new in this study?

I thank the reviewer for pointing out the need for a clearer distinction. Both studies do utilize postmortem tissue from the Middle Temporal Gyrus (MTG) within the Seattle Alzheimer's Disease Brain Cell Atlas (SEA-AD) and evaluate how pathology relates to Mini-Mental State Examination (MMSE) scores. However, my study diverges from and expands upon Escarião et al. in several key ways:

- I quantified phosphorylated tau (AT8 positive area), allowing for a multi-protein analysis (Tau, Amyloid, and Microglia) rather than focusing strictly on amyloid-microglia interactions.*
- While Escarião et al. focused heavily on Plaque-Associated Microglia (PAM) and total densities, my study introduces a distinct analysis of the inactive-to-active IBA1+ microglial ratio, discovering a paradoxical increase in inactive states in advanced cognitive decline.*
- Escarião et al. analyzed correlations across continuous scores and layer-specific anatomy. In contrast, my study categorizes cognitive decline into three distinct MMSE-defined clinical stages using ANOVAs to model stage-by-stage progression.*

I have updated the Introduction - section 1.0 and Discussion - section 4.5 of the revised manuscript to explicitly contextualize my findings alongside Escarião et al., ensuring their work is properly credited rather than cited in passing.

- Figure 1 caption appears incomplete, please write this in full.*

I thank the reviewer for raising this important point. Upon review, the Figure 1 caption was cut off when converting to DOCX and has now been restored. It has been confirmed to retain once converted to DOCX again.

- A two-way ANOVA would be the most suitable choice for the statistics of this study. The design is MMSE group × layer with layers repeated within the donor; separate one-way ANOVAs risk pseudoreplication.*

I thank the reviewer for their attention to this experimental design flaw. New statistical analyses have been performed using two-way mixed ANOVAs, with MMSE group as the between-subjects factor and cortical layer as the repeated within-donor factor. This design is indeed required because multiple cortical layers are sampled from the same individual, violating the assumption of independent observations necessary for standard one-way ANOVAs. By modeling donor identity as a random effect, this approach accounts for the inherent statistical dependency within each subject, properly partitions the variance, prevents the artificial inflation of the degrees of freedom, and allows for the critical evaluation of the interaction effect between MMSE group and layer. The paper has been updated to accommodate these new graphs.

*In addition to the new analyses, I conducted further research into Type I and Type II errors and the justification behind using Šidák corrections for multiple comparisons. I also calculated whether there was a significant overall main effect of MMSE, layer, or the MMSE x layer interaction for all two-way ANOVAs. Each effect is thoroughly explained in the context of the biomarker being analyzed in the **Discussion**, and brief definitions of the effects have been added to section 2.4.*

*Finally, when interpreting any overall main effects, in order to ensure that I have thoroughly analyzed and appropriately interpreted the data, I ran secondary multiple comparisons tests to specifically identify where the MMSE effect (comparing MMSE means) or the layer effect (comparing layer means) occurred. I then reported the significance found in the secondary analyses for each measure in the **Discussion**.*

- A few comments of editorial nature: "I/my" vs "we/our" should be consistently used and p-value formatting is inconsistent. There are a few broken Zotero citation links and a few uncited references*

I thank the reviewer for their careful reading of the manuscript and for highlighting these important editorial details. I have thoroughly audited the text to standardize my pronouns for

a consistent voice, and I have verified all statistical reporting to ensure that p-value notation is perfectly uniform across all sections, tables, and figures. Additionally, I repaired the broken Zotero citation links, cross-referenced my library, and confirmed that every listed reference is properly cited within the text. These corrections have greatly improved the overall polish and presentation of my manuscript. I greatly appreciate the time and clear attention to detail that they have committed to my paper and will utilize their feedback.

REVIEWER 1:

The student did a great job on a thorough revision of the manuscript. They addressed the major concerns raised by myself and the other reviewer, which has made the manuscript much clearer and gives a more accurate reporting of the results. I do recommend one more final round of revisions addressing the following points:

- There is a contradiction in the reporting of the IBA1 inactive ratio. Section 3.5 reports a significant main effect of MMSE, but Section 4.3 mentions there was no significant main effect of MMSE. Please check the reported results are correct and fix this discrepancy between the Results and Discussion.
- Additionally, since only the lowest-MMSE group differed significantly from the other groups, the student should be careful to avoid language implying a continuous increase across all groups. The manuscript sometimes describes the ratio as increasing across MMSE groups. Try to make the wording more precise to reflect the post-hoc results (i.e., the lowest-MMSE group had a higher ratio than the two higher-MMSE groups) rather than implying a graded relationship across all three groups.
- I also recommend a more cautious description of the amyloid results. In Section 3.4, the amyloid density looks highest in the lowest MMSE group, but the authors report no significant MMSE effect for this measure. Thus, make it more clear that this is just a descriptive pattern and there is no direct evidence that amyloid density increases with cognitive decline in this analysis.
- I would also soften causal language in the paper (particularly the Discussion and Conclusion). Be sure that statements like "microglia change with disease progression" or contribute to progression of the disease are clearly framed as hypotheses. Specifically, Section 3.5 of the Results and Section 4.5 of the Discussion.

Otherwise, the paper is an impressive and interesting contribution! With these revisions, I believe the manuscript will definitely be suitable for publication and I recommend acceptance.

REVIEWER 2:

The manuscript has improved considerably after this revision and contains a clear well-reported main finding with relevant support. The research is strong and novel overall. There are a few minor issues that need to be addressed before publication.

1. Line ~619 states the analysis "demonstrated a significant main MMSE effect," consistent with Results 3.5 ($F_{2,76} = 3.959$, $p = .023$). But line ~634 then says "no significant main effect of MMSE group or MMSE $\times$ layer interaction was observed.", which contradicts the sentence it follows. It should read "no significant main effect of layer or MMSE $\times$ layer interaction."
2. The age-matching ANOVA (Section 2.1) is not reported fully. The passage reports "the multiple comparison mean values yielded no significant difference (mean of 0-22 vs. 23-26 being 89.29, mean of 0-22 vs. 27-30 being 89.87, and mean of 23-26 vs. 27-30 being 89.87)." Two of the three values are identical (89.87), and these are described as means of comparisons. Please report group mean ages $\pm$ SD for each of the three groups, then F and p, then adjusted p-values for the pairwise contrasts.
3. Several F-statistics carry fractional degrees of freedom (e.g., $F_{2.528,192.1}$), which means a sphericity correction was applied. The Methods should state that sphericity was tested (Mauchly) and G-G (or H-F) correction applied when violated.
4. Section 3.3 heading conflates presence with activation. "Total Microglial Activation Appears Comparable Across Cognitive Status" — but the measure (IBA1+ cells per area) is microglial density/presence, not activation. Activation is the inactive:active ratio in 3.5.

Suggest "Total Microglial Presence Appears Comparable." The distinction is actually central to your argument (present but altered), so keeping it crisp helps.

5. Title terminology — "IBA1 Inactivation." IBA1 is a marker so it cannot be activated or inactivated. Consider "...and Microglial Inactivation..." to be precise, since it's the microglia (indexed by IBA1) whose state changes.

Increased Tau, A β , and Microglial Inactivation in the Middle Temporal Gyrus Is Associated With Lower Cognitive Function in SEA-AD Donors

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Abstract

Alzheimer's Disease (AD) is a neurodegenerative disorder characterized by mechanisms including neuroinflammation, neurofibrillary tangles of phosphorylated tau, accumulation of amyloid plaques, and microglial aggregation. In AD, disease pathology is closely related to cognitive function, measured using the Mini-Mental-State-Examination (MMSE), a clinical screening tool. This study examined relationships among tau burden, amyloid deposition, and microglial activity in the middle temporal gyrus across three MMSE-defined stages of cognitive decline. Data, including quantifications of AT8, 6e10, and IBA1, were obtained from the Seattle Alzheimer's Disease Brain Cell Atlas. Two-way ANOVAs were used to study the change in percent AT8+ area (phosphorylated tau), IBA1+ cells per area and activation (microglia), 6e10+ cells per area (amyloid-beta), and IBA1+/6e10+ colocalized objects per area, alongside MMSE-defined cognitive decline. Our analysis revealed that neuropathological measures of percent AT8+ area, IBA1/6e10 colocalized objects per area, and IBA1 inactive:active ratio reflected a significant overall MMSE effect, while measures of IBA1+/6e10+ colocalized objects per area, IBA1+ cells per area, and 6e10+ cells per area reflected a significant overall layer effect. Although general IBA1+ cell per area counts were comparable across MMSE groups, the inactive:active ratio differed, with the lowest MMSE group having the highest ratios. These findings lead to the hypothesis that lower MMSE scores may be associated with more tau burden, amyloid deposition, and amyloid/microglia colocalization, accompanied by alterations in microglial state. Together, these findings inform a broader understanding of the link between pathological burden and cognitive function in a multidimensional model of AD.

Keywords: Alzheimer's Disease (AD), MMSE, dementia, tau pathology, amyloid beta, microglial activation, cortical layers

1. Introduction



Alzheimer's disease (AD) is the most common cause of dementia and is characterized by progressive cognitive decline (Atri, 2019; US Department of Health and Human Services, 2017). It is accompanied by the accumulation of extracellular amyloid- β (A β) plaques, intracellular neurofibrillary tangles composed of hyperphosphorylated tau, and widespread neuroinflammation (Khachaturian, 1985; Reitz, 2012). Although these pathological hallmarks have been studied extensively, the mechanisms linking such protein aggregation to cognitive impairment are still unclear.

The accumulation of extracellular amyloid plaques, formed from self-aggregating peptides, has long been regarded as the first hallmark of Alzheimer's disease, inhibiting neuronal synaptic function (Reitz, 2012). Amyloid deposition is known to begin years before the onset of clinical symptoms (Wu et al., 2022). On the other hand, tau accumulation, presenting as neurofibrillary tangles toxic to neurons, is implicated to more closely track the timeline of dementia-related neuronal dysfunction, synapse loss, and worsening cognition (Cody et al., 2024). Consequently, increasing tau burden is commonly considered one of the strongest neuropathological indicators of disease severity (Murray et al., 2022). Previous studies have also suggested that tau pathology is more strongly associated with cognitive impairment than amyloid burden alone (Guo et al., 2026). Amyloid pathology nevertheless remains an important marker, as it is commonly believed to initiate a cascade of downstream events that contribute to tau aggregation, neurodegeneration, and ultimately dementia – a process often referred to as the “amyloid cascade hypothesis” (Reitz, 2012).

In addition to protein deposition, neuroinflammation is another major hallmark of dementia, with emerging studies suggesting that inflammation may be both a consequence and an initiator of AD pathology like tau accumulation and amyloid plaques (Zhang et al., 2023). Under healthy conditions, microglia contribute to tissue maintenance through debris clearance and immune surveillance (McKinney et al., 2026). They are the brain's resident immune cells and macrophages, continuously monitoring the brain environment and responding to accumulating amyloid deposits and neuroinflammation (Muzio et al., 2021). In AD, however, microglia aggregate around amyloid plaques and undergo substantial changes in morphology, gene expression, and function (Bolmont et al., 2008).

Experimental evidence suggests that microglia initially exert protective effects by containing plaques and phagocytosis of pathological proteins, but being exposed to prolonged pathological stress may yet lead to impaired clearance mechanisms and ultimately contribute to neurodegeneration by accelerating the spreading of proteins like A β and tau. (Gao et al., 2023). Furthermore, evidence argues that microglia have specific activation states which may mediate synapse loss, worsen tau pathology, and secrete inflammatory factors that directly harm neurons (Hansen et al., 2018). Microglia are implicated to be able to transform from one phenotype to others—many researchers believe that the M1 microglia, which release inflammatory mediators, and the M2 microglia, which release anti-inflammatory mediators, may provide further insight on the effective activation states of microglia (Guo et al., 2022). However, current understanding has not reached a consensus on whether microglial states can be explicitly divided into simply anti-inflammatory and pro-inflammatory subtypes, due to the complex heterogeneity and heavily context-dependent nature of microglial states (Paolicelli et al., 2022). Despite growing recognition of the importance of neuroinflammation, it still remains unclear whether microglia continue to respond effectively as disease progresses or whether they transition into altered functional states that contribute to further pathological accumulation. This study therefore investigates how the accumulation of microglia around sites of pathology may change across different stages of neurodegeneration.

In AD, neuroinflammation spreads heavily across the brain to areas like the cerebral cortex, the outer layer of the brain responsible for thinking, memory, and language (Ni & Wu, 2021). The cerebral cortex is organized into distinct layers that all differ in cellular composition, connectivity, and functional roles, meaning pathology may behave differently across layers (Chen et al., 2021). Emerging evidence suggests that neurodegenerative pathology may not be distributed uniformly throughout cortical tissue, and analyses restricted to just gray matter measurements may obscure important layer-specific patterns (Willumsen et al., 2022). Across past studies, AD neuropathology has been known to show strong regional specificity when developing across the brain (Yang et al., 2026). Neurofibrillary tangles typically spread from the entorhinal cortex to the hippocampus, limbic system, and neocortex, amyloid plaques show graded accumulation across brain regions, and microglia in the prefrontal cortex, cerebellum, and hippocampus are known to have significant differences in their highly expressed genes (Asher & Priefer, 2022; Jacobsen et al., 2005; Yang et al., 2026).

The middle temporal gyrus (MTG) is particularly relevant in AD because of its involvement in language processing, semantic memory, and other higher order cognitive functions that become impaired during disease progression (Chen et al., 2022). Therapeutic strategies developed over the years rely heavily on gray matter analysis and often encounter obstacles once in clinical trials (Asher & Priefer, 2022). Investigating pathology across individual cortical layers may therefore provide additional insight into selective regional differences and vulnerabilities.

Despite extensive research conducted on AD biomarkers, critical gaps in the scientific understanding of disease mechanisms still exist. It remains unclear whether layer-specific microglial clustering directly damages local circuitry or merely reacts to underlying synaptic failure and plaque growth, and when microglia transition activation states (Escarião et al., 2025). In the MTG itself, the precise functional organization and the exact division of labor for semantic processing remain unknown, meaning a layer-specific approach to examining the MTG may provide more insight (Escarião et al., 2025).

Accordingly, the present study investigated relationships among tau burden, amyloid deposition, and microglial activity within the MTG across MMSE-defined stages of cognitive decline. Particular emphasis was placed on examining the pathology within individual cortical layers 1 to 6, following initial bulk gray matter analyses.

A previous study of these markers in the SEA-AD dataset investigated the APOE4 genotype and an individual's genetic predisposition to dementia (Wang, 2025). However, this present study uses MMSE grouping to better understand the relationships between disease pathology and an individual's ongoing cognitive capacities.

I hypothesized that lower MMSE scores would be associated with higher levels of tau burden and amyloid deposition, as well as accompanied alterations in microglial activation. More specifically, the microglia were expected to increasingly associate with sites of pathology whilst displaying signs of change in activation. Furthermore, layer-specific analyses were expected to reveal patterns that would not be apparent from the gray matter measurements alone.

2. Materials and Methods

Previously collected data were accessed and reanalyzed in the present study. De-identified postmortem human brain data and quantitative neuropathological measurements of the middle temporal gyrus were accessed from the Allen Brain Institute's publicly available SEA-AD dataset (Hawrylycz et al., 2024). All initial experimentation and data collection were conducted by the Kaiser Permanente Washington Health Research Institute ACT Study researchers and the University of

Washington ADRC at the UW BioRepository and Integrated Neuropathology (BRaIN) laboratory. The dataset focused on different patients' histological pathology, obtained via immunolabeling and microscopy across cortical layers and protein markers.

For my study, the data from the Quantitative Neuropathology Summary Data (MTG) and Donor Metadata from the Allen Brain Institute's SEA-AD data set were aligned and compiled in an Excel spreadsheet in order to match neuropathology data to donor MMSE status. Donors were stratified into three groups based on their respective Mini-Mental State Examination (MMSE) scores: 27–30, 23–26, and 0–22.

2.1. MMSE-Based Cognitive Stratification

Cognitive status was classified into donor groups according to Mini-Mental State Examination (MMSE) scores obtained from SEA-AD donor metadata. The MMSE is a widely used clinical screening tool for cognitive impairment, with scores ranging from 0 to 30, where lower scores indicate greater cognitive dysfunction (Creavin et al., 2016; Yang et al., 2024).

Donors were stratified into three groups based on established thresholds used in dementia research: MMSE 27–30 (cognitively normal or minimally impaired), MMSE 23–26 (mild cognitive impairment), and MMSE 0–22 (moderate to severe cognitive impairment consistent with dementia).

Across studies, there exist many different categorizations of MMSE scores, with no consensus yet on what threshold may be truly significant in terms of cognitive status. The Mini-Mental State Examination (MMSE) faces significant diagnostic inconsistency because fixed cutoff scores fail to account for demographic variations — while a score of 24 serves as the traditional clinical threshold, its diagnostic accuracy is heavily confounded by a patient's age and education level, often yielding false negatives in highly educated individuals and false positives in those with limited schooling (Creavin et al., 2016). Consequently, researchers and clinicians increasingly employ stratified thresholds ranging from 21 to 27 to enhance sensitivity and specificity across diverse populations (Salis et al., 2023). Therefore, MMSE grouping is heavily dependent on the dataset being analyzed. Based on this framework, the present study used MMSE stratification to approximate stages of cognitive decline and to enable comparison of neuropathological burden across disease severity.

Due to the nature of dementia disease progression, age at death is an essential consideration when analyzing the severity of pathological burden. Thus, when deciding the MMSE ranges for the three experimental groups, instead of solely relying on numbers used in past studies, I used a one-way ANOVA to run a replicate data column analysis with multiple comparisons of donor ages at death. The group mean of 0–22 was 88.76 with a SD of 7.289, the group mean of 23–26 was 89.29 with a SD of 7.349, and the group mean of 27–30 was 89.87 with a SD of 7.315. Pairwise contrasts between groups were: 0.971 for 0–22 vs 23–26, 0.968 for 0–22 vs 27–30, and 0.960 for 23–26 vs 27–30. The multiple comparison mean values yielded no significant difference between groups ($F_{2,76} = 0.1247$, $p = .883$). Thus, these cutoffs provided a more balanced partition of donor data rather than relying on arbitrary and inconsistent thresholds.

In my dataset, the group with MMSE scores 27–30 consisted of 31 donors, the group with MMSE scores 23–26 consisted of 31 donors, and the group with MMSE scores 0–22 consisted of 17 donors.



All downstream analyses were performed using these MMSE-defined groups to evaluate relationships between cognitive status and pathological markers in the middle temporal gyrus.

2.2. Neuropathological Quantification

Detailed tissue processing and staining protocols have been previously described in a published paper as well as an online procedural overview (Gabbitto et al., 2024; Seattle Alzheimer's Disease Data Set, n.d.).

Briefly, postmortem human brain tissue was obtained through the Adult Changes in Thought (ACT) study conducted by the Kaiser Permanente Washington Health Research Institute in collaboration with the University of Washington. Researchers collected middle temporal gyrus (MTG) samples, fixing and embedding each in paraffin prior to sectioning. Sections underwent duplex immunohistochemical staining to detect various hallmark Alzheimer's disease proteins (Hawrylycz et al., 2024). Phosphorylated tau was labeled using the AT8 antibody, while amyloid- β (A β) deposits were identified using the 6E10 monoclonal antibody. Quantification of percent AT8-positive area, 6E10-positive objects per area, and IBA1-associated measures was performed on digitized images of the MTG cortical layers. IBA1-positive cells were classified as active or inactive according to dataset-provided morphological criteria.

Cortical layers are established by the SEA-AD dataset annotations, in which Layer 5 and Layer 6 are combined. Thus, the layers are analyzed accordingly as L1, L2, L3, L4, and L5/6.

The Allen Brain Institute collected and compiled the data for access to the general public. Data were accessed June 6th, 2026, via:

<https://portal.brain-map.org/explore/seattle-alzheimers-disease/seattle-alzheimers-disease-brain-cell-atlas-download?edit&language=en>.

2.3. Outlier Removal

To preserve the statistical validity and reproducibility of my pathological assessments, outlier detection was performed for all pathological measurements using the ROUT method (Q = 1%) in GraphPad Prism. No outliers were identified consistently across all measures, therefore no donors were excluded from my analyses.

2.4. Statistical Analysis

Statistical analyses were largely performed using two-way mixed effect model ANOVAs with repeated measures, with MMSE group as the between-subjects factor and cortical layer as the repeated within-donor factor. This design is required because multiple cortical layers are sampled from the same individual, violating the assumption of independent observations necessary for standard one-way ANOVAs. By modeling donor identity as a random effect, this approach accounts for the inherent statistical dependency within each subject, properly partitions the variance, prevents the artificial inflation of the degrees of freedom, and allows for the critical evaluation of the interaction effect between MMSE group and layer (Wilcox & Rousselet, 2023). A one-way ANOVA was performed for the AT8 measure due to the fact that it is a plain gray matter analysis with no cortical layer analyses.



Multiple comparisons were performed for all analyses by comparing cell means with others in its row and column. Šidák's multiple-comparisons correction was used to control the family-wise Type I error rate arising from multiple pairwise comparisons while providing greater statistical power than the more conservative Bonferroni correction. (Abdi, 2007) Sphericity was tested (Mauchly) when applicable and G-G correction applied when violated.

To ensure valid cross-layer comparisons within the MTG, normalized counts were used for analysis of all measures. This normalization is statistically required to control for the prominent anatomical variations in laminar thickness inherent to the MTG (Zachlod et al., 2022). Failing to account for these structural volume differences would conflate a higher absolute cell count with a true increase in cellular density, thereby introducing systematic bias into our regional comparative analyses. Normalized measures are presented as AT8 percent area (%), IBA1+/6E10+ colocalized objects per area ($\#/\mu\text{m}^2$), IBA1+ objects per area ($\#/\mu\text{m}^2$), 6E10+ objects per area ($\#/\mu\text{m}^2$), and IBA1+ inactive microglial cells per area : IBA1+ active microglial cells per area ($\#$).

Data are presented as bars with mean $\pm$ SEM. Statistical significance thresholds were defined per APA style 7th edition as: * $p < .05$, ** $p < .01$, *** $p < .001$.

I calculated the overall main effect of MMSE, layers, and the MMSE $\times$ layer interaction for all two-way ANOVAs. A significant overall main effect of MMSE group (M) demonstrates that MMSE grouping has a statistically significant effect on the dependent variable. Similarly, a significant overall main effect of cortical layers (L), then, means that the layers of the MTG have a statistically significant effect on the dependent variable. A significant MMSE $\times$ cortical layer interaction indicates that there is a statistically significant interaction between both the effects of MMSE grouping and layer. Thus, while the main effects describe overall differences associated with either cognitive status or cortical layers, the interaction significance provides evidence that the two factors jointly influence the distribution of the biomarker across the middle temporal gyrus.

3. Results

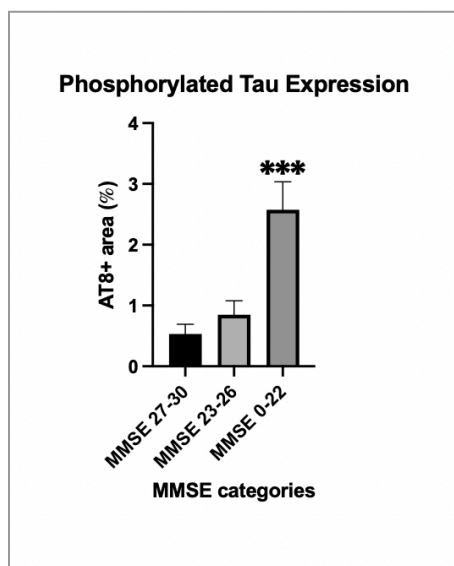
3.1. AT8 Pathology in the Middle Temporal Gyrus Higher with Cognitive Decline

To assess the relationship between tau pathology and cognitive decline, percent AT8-positive area was quantified across the gray matter of the middle temporal gyrus (MTG) and stratified by MMSE category (Figure 1). My analyses compared donors with low, medium, and high MMSE scores, where a standard one-way ANOVA revealed a significant difference between groups ($F_{2,76} = 14.31$, *** $p < .001$). Šidák-adjusted post-hoc tests showed significance of *** $p < .001$ for both MMSE 27-30 vs. MMSE 0-22 and MMSE 23-26 vs. MMSE 0-22.

AT8 burden was higher in donors with decreased MMSE scores, with the lowest MMSE group (0-22) exhibiting a greatly elevated and significant percent AT8-positive area. The intermediate group (23-26) showed values between the high and low MMSE groups, indicative of a graded relationship between tau accumulation and cognitive impairment.

This trend was consistent across donors within each group, with statistical comparisons demonstrating a significant increase in AT8-positive area in the 0-22 group relative to both higher MMSE groups (*** $p < .001$).





209

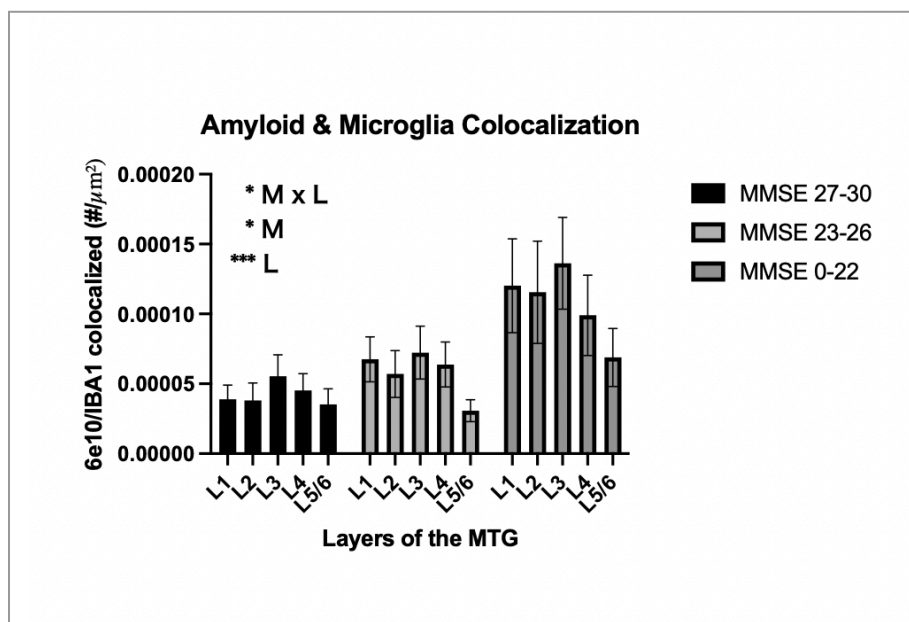
210 **Figure 1. Percent AT8-positive area in MTG gray matter across MMSE categories.** Significance (***) was found between
211 MMSE group 0-22 and all other MMSE groups.

212 3.2. Increased Microglial Association with Amyloid Pathology Across Cortical Layers

213 To evaluate microglial interaction with amyloid pathology, the number of IBA1/6E10 co-localized objects per area was
214 quantified across cortical layers of the MTG (Figure 2). Co-localized object per area counts were higher with decreased
215 MMSE scores, with the greatest values observed in the 0-22 group. The progressive increase in co-localized objects per
216 area across MMSE groups may indicate an enhanced microglial presence in proximity to amyloid deposits as cognitive
217 impairment worsens.

218 Two-way ANOVA analyses with MMSE group as the between-subjects factor and cortical layer as the repeated
219 within-donor factor revealed a significant overall layer effect ($F_{2,528,192.1} = 14.58$, $***p < .001$), a significant overall MMSE
220 effect ($F_{2,76} = 3.290$, $*p = .043$) and a significant overall interaction effect of layer and MMSE group ($F_{5,056,192.1} = 2.367$, $*p =$
221 $.041$). However, no statistical significance was found between individual layers.





222

223 **Figure 2. Number of IBA1/6E10 co-localized objects per area.** Significance was observed for the overall interaction effect of layer and
224 MMSE group (* M x L), the overall MMSE group effect (* M), and the overall layer effect (*** L).

225 3.3. Total Microglial Presence Appears Comparable Across Cognitive Status

226 To assess whether the survival of microglial cells affected amyloid colocalization, the amount of IBA1-positive cells per area
227 was first compared across cortical layers and MMSE groups (Figure 3). Notably, layer-specific multiple comparison analysis
228 revealed significance between layers within each MMSE group, but not across MMSE groups themselves.

229 Two-way ANOVA analyses with MMSE group as the between-subjects factor and cortical layer as the repeated
230 within-donor factor revealed a significant overall layer effect ($F_{1.618,122.9} = 14.62$, $***p < .001$), but no significant overall MMSE
231 effect or significant overall interaction effect of layer and MMSE group.



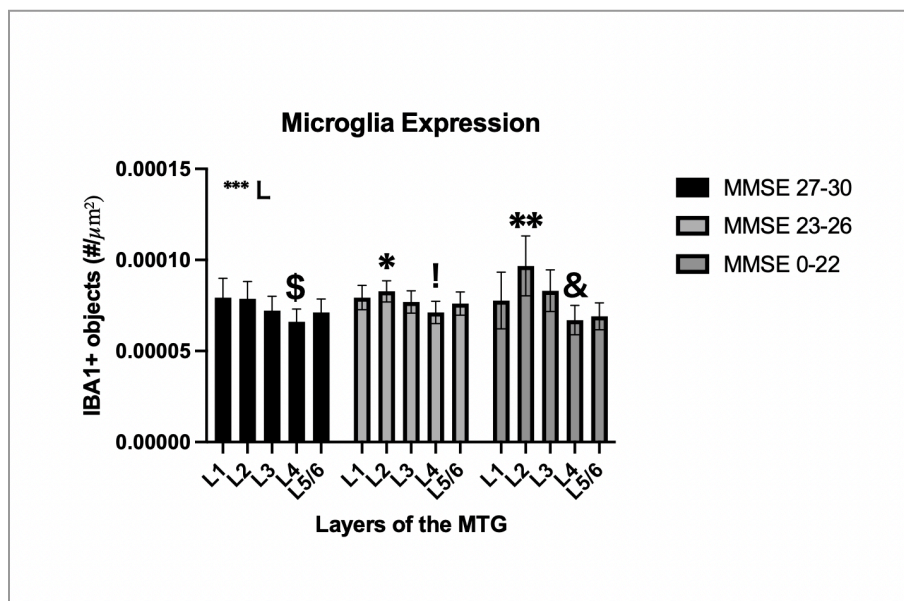


Figure 3. Number of total IBA1 positive cells per area. Significance was observed for the overall layer effect (*** L). In MMSE 27-30, significance marked by “\$” was found between L4 and L2 (**p < .01), L4 and L5/6 (**p < .01), and L4 and L3 (***p < .001). In MMSE 23-26, significance was found between L3 and L2 (*p < .05). Significance (***p < .001) marked by “!” was found between L2 and L4, and L3 and L4. In MMSE 0-22, significance was found between L1 and L2 (**p < .01). Significance (*p < .05) marked by “&” was found between L3 and L4.

3.4. Layer-Specific Distribution of Amyloid- β Deposition

To determine whether the observed layer-patterns from Figure 2 were driven by amyloid distribution, 6E10-positive objects per area were quantified independently across MTG layers (Figure 4). A consistent significance of 6E10-positive objects per area was observed in layer 3 across all MMSE groups.

Two-way ANOVA analyses with MMSE group as the between-subjects factor and cortical layer as the repeated within-donor factor revealed a significant overall layer effect as well ($F_{2,529,192.2} = 14.45$, ***p < .001), but no significant overall MMSE effect or significant overall interaction effect of layer and MMSE group.

It is appreciably observed that the low MMSE group exhibits higher densities of 6E10+ objects per area than both other MMSE groups. However, because this is just a descriptive pattern, there is no direct evidence that amyloid density increases with cognitive decline in this analysis.

Notably, the number of 6E10-positive objects per area exceeded the number of IBA1/6E10 co-localized objects per area across all groups and layers, perhaps indicating that only a subset of detected 6E10+ objects per area met the criteria for colocalization.



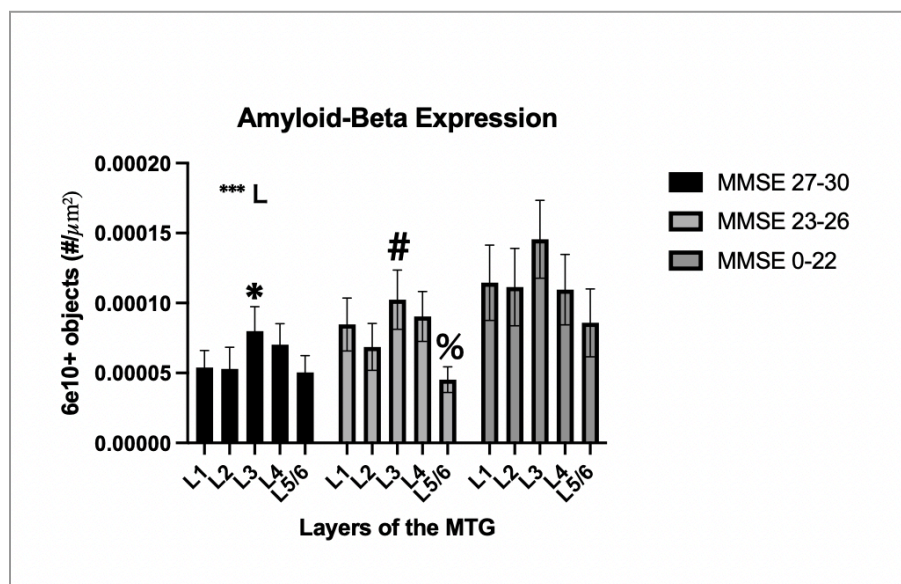


Figure 4. Number of 6E10-positive objects per area. Significance was observed for the overall layer effect (*** L). In MMSE 27-30, significance was found between L3 and L2 (* $p < .05$). In MMSE 23-26, significance (*** $p < .001$) marked by “#” was found between L3 and L2 (*** $p < .001$). Significance (* $p < .05$) marked by “%” was found between L5/6 and L3, and L5/6 and L4. No significance was found between individual layers of MMSE 0-22.

3.5. Microglial Inactivation Is Higher with Worse Cognitive Status

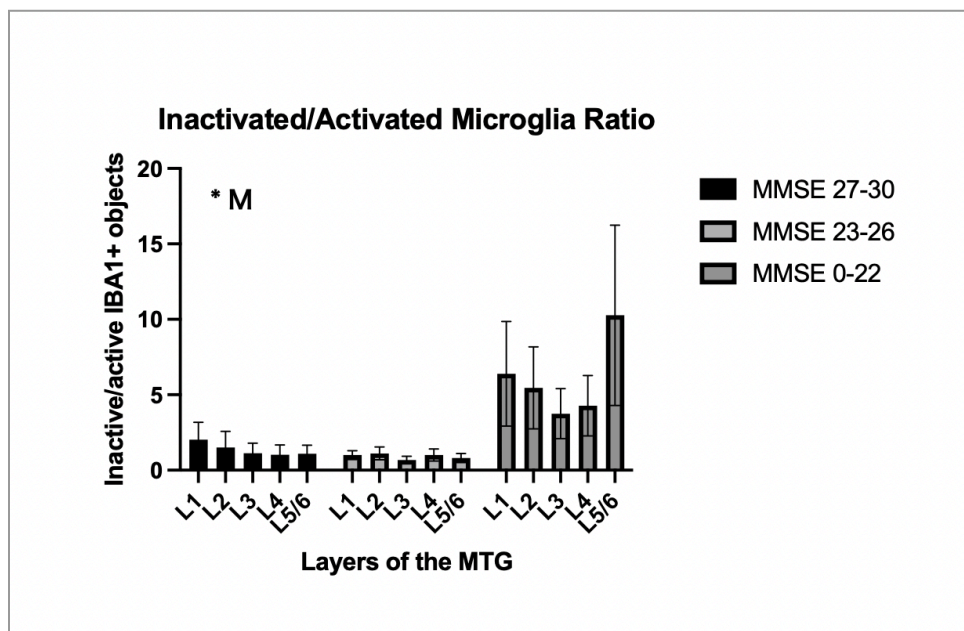
To assess microglial morphology and states, the ratio of morphologically-classified inactive to active IBA1-positive microglial cells per area was calculated (inactive cells per area divided by active cells per area) across cortical layers and MMSE groups (Figure 5). A prominent difference in the inactivation ratio was observed between the 0-22 MMSE group and all other MMSE groups.

Two-way ANOVA analyses with MMSE group as the between-subjects factor and cortical layer as the repeated within-donor factor revealed a significant overall MMSE group effect ($F_{2,76} = 3.959$, * $p = .023$), but no significant overall layer effect or significant overall interaction effect of layer and MMSE group.

Although differences between specific cortical layers and MMSE groups were not statistically significant, the increase in inactivation ratio was clear across MMSE groups. In the lowest MMSE group, the ratio indicated a relative increase in inactivated IBA1 cells, with the highest inactive:active cell per area ratio.

These findings indicate that while microglia remain comparably present in regions of pathology as seen in Figure 3, their activation state may change with disease progression, with more IBA1 cells per area becoming inactive relative to active as disease severity worsens.





270

271 **Figure 5. Ratio of inactive to active IBA1-positive cells per area.** Significance was observed for the overall MMSE group effect (*M) only.

272 3.6. Summary of Pathological Trends Across MMSE Groups

273 Across measures, lower MMSE scores were associated with higher pathological burden and altered microglial dynamics.
274 AT8-positive percent area, IBA1/6E10 co-localized objects per area, 6E10-positive objects per area, and the IBA1
275 inactive:active cell per area ratio were highest in donors with an MMSE score between 0-22. IBA1 positive cell per area
276 counts were relatively constant across MMSE scores, indicative of almost no difference related to disease severity. In
277 contrast, the ratio of inactive to active microglia was higher in donors with lower MMSE scores. Overall layer significance
278 was observed in all analyses except for the IBA1 ratio.

279 4. Discussion

280 4.1. Progressive Pathological Burden Accompanies Cognitive Decline

281 The present study investigated relationships between tau pathology, amyloid deposition, and microglial activity within the
282 middle temporal gyrus (MTG) across MMSE-defined stages of cognitive decline. I hypothesized that lower MMSE scores
283 would be associated with higher pathological burden across measures, accompanied by altered microglial activation states.
284 Overall, my findings supported this hypothesis and affirmed expected changes in disease pathology and neuroimmune
285 dynamics in individuals with varying degrees of cognitive deficits.



Tau burden, measured by the percent AT8-positive area in the study, was substantially higher in donors with lower MMSE scores and exhibited a graded pattern across the three cognitive groups. Donors with the lowest MMSE scores (0-22) demonstrated the greatest accumulation of phosphorylated tau within MTG gray matter, supporting the close relationship between tau pathology and cognitive deterioration.

Post-hoc comparisons confirmed significantly greater AT8 levels in the low-MMSE group compared with both the medium- and high-MMSE groups. This relationship between more AT8 pathology and lower MMSE provided an initial indication that cognitive status was associated with meaningful differences in neuropathology within the MTG and motivated further investigation of whether other pathological measures might exhibit similar relationships with MMSE and whether these relationships vary across cortical layers.

Whereas amyloid accumulation is generally believed to precede the onset of clinical symptoms and eventually plateau, tau pathology has been shown to track more closely with disease severity (Mormino & Papp, 2018). The present findings therefore support that tau accumulation is a key pathological feature closely associated with cognitive decline in Alzheimer's disease. Collectively, the simultaneous increase in both tau and amyloid pathology across MMSE groups is consistent with current models of Alzheimer's disease.

4.2. Microglia Associate With Amyloid Plaques

Initially, quantification of IBA1/6E10 co-localized objects per area demonstrated the expected increase in microglial association with amyloid deposits as MMSE scores declined. At first glance, this finding suggests a natural heightened immune response accompanying increasing pathological burden.

My analysis of IBA1+/6E10+ colocalized objects per area demonstrated significant main effects of MMSE group and cortical layer, as well as a significant MMSE group x cortical layer interaction. The significant main effect of MMSE group indicates that the density of IBA1+/6E10+ colocalized objects is dependent on the MMSE group. Consistent with this effect, a progressive increase in colocalized objects was observed across the MMSE groups, suggesting greater microglial association with amyloid pathology in donors with greater cognitive impairment. The significant main effect of cortical layer indicates that the density is also dependent on the layer, which suggests that different regions of the MTG may vary in properties or vulnerability to accumulating amyloid plaques (Leng et al., 2021; Willumsen et al., 2022; Yang et al., 2026).

The significant MMSE x layer interaction indicates that the density of IBA1+/6E10+ colocalized objects is dependent on the interaction between MMSE group and layer. In interpreting this interaction effect, I ran two more secondary multiple comparisons tests to specifically identify where the significant MMSE effect (comparing MMSE means) and the layer effect (comparing layer means) occurred. The secondary MMSE effect analysis highlighted a significance ($***p < .001$) between the MMSE 0-22 group and both other groups, whereas the MMSE 23-26 group and the MMSE 27-30 group did not significantly differ from each other. The secondary main layer effect analysis then yielded a significance ($*p < .05$) between L3 and L5/6. Thus, the interaction effect can be interpreted as meaning that the difference between L3 and L5/6 of the MMSE 0-22 group is significantly greater than both the difference between L3 and L5/6 of the MMSE 23-26 group, as well as the difference between L3 and L5/6 of the MMSE 27-30 group. This suggests that the regional variation of amyloid/microglia colocalization may be more pronounced with lower cognition, and therefore differences in regional properties may also be more



pronounced in donors with low cognition (Chen et al., 2022; Escarião et al., 2025). These findings therefore prompted further investigation into whether a significant layer effect, MMSE effect, or MMSE x layer interaction effect was also observed for IBA1+ alone or 6e10+ alone.

4.3. Microglia Remain Present but May Transition Towards An Altered State

Despite increased co-localization with amyloid pathology, IBA1-positive cell per area counts actually remained relatively constant across the three cognitive groups. My analysis of IBA1+ objects per area demonstrated a significant main layer effect, indicating that the density of IBA1+ objects is dependent on the layer. In interpreting this significant layer effect, I ran a secondary multiple comparisons test to specifically identify where the layer effect (comparing layer means) occurred. The analysis highlighted a significance ($*p < .05$) between L2 and L4, whereas the other layers did not significantly differ from each other. This finding suggests that different regions of the MTG may vary in microglial presence, which could make certain areas more or less vulnerable to injury and accumulation of proteins (Chen et al., 2021). However, no significant main effect of MMSE group or MMSE x layer interaction was observed, indicating that overall IBA1+ object density was not dependent on MMSE or the interaction between MMSE and layer.

My analysis of 6e10+ colocalized objects per area demonstrated a significant main layer effect, indicating that the amyloid density is dependent on the cortical layer. In interpreting the significant overall layer effect, I ran a secondary multiple comparisons test to specifically identify where the layer effect (comparing layer means) occurred. The analysis highlighted a significance ($*p < .05$) between L3 and L5/6, whereas the other layers did not significantly differ from each other. This finding further supports that the MTG may exhibit region-specific vulnerability to accumulating amyloid plaques (Leng et al., 2021; Willumsen et al., 2022). No significant main effect of MMSE group or MMSE x layer interaction was observed, indicating that overall 6e10+ object density did not depend on the donor's MMSE grouping or the interaction between MMSE and layer. Interestingly, 6e10+ object-per-area counts were consistently higher than the corresponding counts of colocalized objects, suggesting that not all amyloid deposits were associated with detectable IBA1-positive microglial colocalization and that microglial association with amyloid deposits may therefore be incomplete.

Nevertheless, while microglia continued to localize around amyloid deposits, the change in inactive divided by active IBA1 cell per area ratios suggested a relative increase in microglial inactivation in donors with lower MMSE scores. My analysis of the IBA1+ inactive:active ratio demonstrated a significant main MMSE effect, indicating that the relative proportion of inactive to active microglia differed according to cognitive status. In interpreting the MMSE effect, I ran a secondary multiple comparisons test to specifically identify where the MMSE effect (comparing MMSE means) occurred. The secondary MMSE effect analysis highlighted a significance ($***p < .001$) between the MMSE 0-22 group and both other groups, whereas the MMSE 23-26 group and the MMSE 27-30 group did not significantly differ from each other. Essentially, lower ratios indicate relatively less inactive cells as compared to active, while higher ratios indicate relatively more inactive cells as compared to active. Therefore, the donors exhibiting higher ratios for this measure, which are the low MMSE group, could be experiencing changes in microglial state that cause such a shift in activation (Guo et al., 2022).

However, no significant main effect of cortical layer or MMSE x layer interaction was observed, indicating that the IBA1+ inactive:active ratio was not dependent on layer or the interaction between MMSE and layer. Thus, unlike the layer-dependent effect observed for IBA1+/6e10+ colocalization, IBA1+ alone, and 6e10+ alone, the pronounced shift in the

inactive:active ratio was primarily dependent on MMSE grouping. This suggests an intriguing relationship between declining cognitive status and changes in microglial activation—moreover, this relationship may be observed across regions, independent of layer dynamics.

Rather than representing an entirely absent immune response, however, these results may be indicative of a state of chronic dysfunction or cellular exhaustion. Past research has shown that under conditions of sustained pathological stress, microglia may remain physically associated with amyloid deposits but transition to an altered state (Cheng & Ho, 2025; Gao et al., 2023). This state may include reduced ability to execute protective functions such as plaque clearance, debris removal, or maintenance of tissue homeostasis (Lier et al., 2021). Such a reduced-activation state could be a major contributor to continued pathological accumulation despite persistent microglial presence.

This interpretation is consistent with emerging literature describing differing disease-associated microglial (DAM) states in Alzheimer's disease (Cheng & Ho, 2025; Kuhn & Proctor, 2025). Although the present study does not directly measure DAM signatures, they provide an interesting conceptual connection that may inform future research. Several studies have reported that microglia surrounding plaques exhibit altered transcriptional profiles, including impaired phagocytic activity and reduced homeostatic signaling (Hassan et al., 2025). Experimental models have similarly demonstrated that prolonged exposure to amyloid pathology can shift microglia from protective to maladaptive states (McKinney et al., 2026).

The present findings contribute to this ongoing discussion by suggesting that physical proximity to pathology does not necessarily indicate effective functional engagement. However, although changes in inactive to active ratios based on morphology may provide insight into altered microglial states, the current data cannot conclusively measure or affirm microglial function.

4.4. Selective Layer Enrichment

A notable feature across multiple analyses was the significant overall layer effect. Significance was observed in L3 of the 6e10+ measure and L2 of the IBA1+ measure. Additionally, in the secondary multiple comparisons tests (comparing layer means) for both IBA1+/6e10+ colocalized objects per area and the 6e10+ objects per area, significance (* $p < .05$) was found between L3 and L5/6. Moreover, in the secondary multiple comparisons tests (comparing layer means) for the IBA1+ objects per area, significance (* $p < .05$) was found between L2 and L4.

Cortical layers differ significantly in neuronal composition, connectivity, and functional organization, and these differences may influence selective vulnerability during neurodegeneration (Chen et al., 2021). This is particularly true for the MTG, where there are structural and microarchitectural asymmetries between the left and right hemispheres, regional variations in surface area, and differences in cortical thickness (Zachlod et al., 2022). Analyses limited to bulk gray matter measurements risk masking such spatial patterns. The layer-specific approach used here therefore provides additional resolution and suggests that important disease-related changes may occur within discrete anatomical compartments rather than uniformly throughout cortical tissue.

The enrichment of amyloid pathology within Layer 3 may be particularly significant given the role of this layer in corticocortical communication. Interestingly, a significantly lower 6e10+ object-per-area count was observed in layer 5/6 as well, further suggesting that layer dynamics should be a strong consideration for future studies.



Layer 3 pyramidal neurons form extensive long-range connections with other cortical association areas and are heavily integrated into large-scale neural networks involved in cognition (Planert et al., 2026). Previous studies have suggested that highly connected neuronal populations may be especially vulnerable to pathological protein spread and metabolic stress (Bloomingdale et al., 2022; Brettschneider et al., 2015). Research has also suggested that neurons in layers 2 and 3 of the MTG may be more vulnerable to degenerative mechanisms in early stages of AD (Chen et al., 2021). Consequently, the concentration of amyloid pathology within layer 3 may reflect underlying network properties that increase a region's susceptibility to neurodegeneration (Escarião et al., 2025).

The IBA1+ analyses revealed significant enrichment of microglial presence within Layer 2, and a significantly lower count within Layer 4. The high microglial localization found in Layer 2 and the low microglial localization found in Layer 4 do not necessarily indicate that the microglia themselves exhibit a natural preference towards layers — rather, they may be responding to accumulating pathology in more vulnerable areas of the MTG. This layer-specific angle of researching AD must consider the possibility that certain layers are more susceptible to some biomarkers — and these vulnerable areas may be different for different biomarkers.

Past research has shown that layers 3 and 4 of the neocortex, including the MTG, are the first to accumulate neurofibrillary tangles of tau (Leng et al., 2021). Evidence also suggests that neurons in layers 2, 3, and 5 of the temporal lobe, including the MTG, may be selectively vulnerable to tau pathology (Chen et al., 2022). Together, these findings raise the possibility that distinct cortical layers may contribute differently to disease progression. Further investigation is needed to confirm whether these layer-specific patterns are unique to the MTG or represent broader cortical inhomogeneity in Alzheimer's disease.

4.5. Findings Extend Existing Models of Disease Progression

The overall findings broadly align with current models of Alzheimer's disease progression while also highlighting potential limitations of simplified frameworks. Increasing amyloid burden alongside progressive tau accumulation is consistent with the amyloid cascade hypothesis, which proposes that amyloid deposition initiates downstream pathological events leading ultimately to neurodegeneration and cognitive decline (Wu et al., 2022).

However, the present findings suggest that immune responses may play a more active and complex role than originally proposed. While classical models often frame neuroinflammation as a secondary consequence of pathology, growing evidence suggests that microglial dysfunction itself may contribute meaningfully to disease progression (Cheng & Ho, 2025; McKinney et al., 2026). The present data support this possibility — microglial cells appeared increasingly associated with pathology but simultaneously exhibited changes suggestive of diminished activity.

In their 2025 manuscript analyzing the SEA-AD dataset, Escarião et al. noted that higher PAM (plaque-associated microglia) expression correlated with lower MMSE scores—my finding of greater IBA1+/6e10 colocalized density in the low MMSE group complements this, while further demonstrating that this may occur alongside an increased inactive:active microglial ratio (Escarião et al., 2025). My study integrates multiple pathological dimensions and specifically examines the relationship between microglial-amyloid colocalization, total IBA1+ density, amyloid density, and morphology-based inactive:active microglial state across MMSE groups.



426 Presence alone does not necessarily imply effective response, and thus cells may occupy regions of pathology while
427 simultaneously undergoing shifts towards a reduced activation state (McKinney et al., 2026). The observed shift in
428 morphology raises the possibility that altered microglial states may be a critical feature of disease progression and could help
429 inform future explanations of why pathological burden continues to increase despite persistent immune response, although
430 functional and causal relationships are yet to be confirmed (Guo et al., 2022; Lier et al., 2021).

431 These findings are consistent in conceptualizing Alzheimer's disease as a multifactorial disease involving more complex
432 interactions among protein aggregation, neuroinflammation, synaptic dysfunction, and neuronal loss. Rather than acting
433 solely downstream of amyloid deposition, microglia may well influence other stages of disease progression by affecting
434 neuroinflammation and becoming unable to clear pathological proteins. Consequently, therapeutic strategies targeting
435 microglia may need to focus not only on enhancing microglial activity but also on preserving beneficial, active states and
436 preventing transitions toward chronic inactivation.

437 4.6. Limitations and Future Directions

438 Several limitations should be considered when interpreting these findings. Firstly, because the present study relied on
439 observational postmortem data, clear causal relationships cannot be established. Although significant associations were
440 identified between MMSE status and neuropathological measures, it remains unclear whether altered microglial states drive
441 disease progression or emerge as a consequence of accumulating pathology. These findings should be used to help inform
442 and generate hypotheses for future therapeutic studies, rather than to directly inform treatment.

443 Additionally, MMSE-based grouping provides only an approximate measure of disease severity. Cognitive decline is a
444 multidimensional process influenced by numerous biological and clinical factors, and thus individuals with similar MMSE
445 scores may actually differ substantially in their true pathological profiles. Future studies incorporating both additional
446 cognitive assessments and clinical data may provide a more comprehensive understanding of the relationship between
447 cognition and neuropathology.

448 The two microglial activation (active and inactive) states were determined according to morphology-based classifications
449 provided by the dataset, but morphology alone may be insufficient in showing the functional diversity of microglial states.
450 Future studies incorporating transcriptomic, proteomic, and molecular approaches may better help determine whether the
451 observed changes represent dysfunction, adaptive responses, or distinct disease-associated phenotypes.

452 The layer-specific findings observed in this study also need further investigation. The observed enrichment of amyloid
453 pathology within Layer 3 and microglia within Layer 2, along with the consistent significant overall layer effect in several of
454 the two-way ANOVAs, raises important concerns regarding what truly controls selective cortical vulnerability. Future studies
455 must examine whether differences in neuronal connectivity, synaptic density, or structural communication patterns
456 contribute to the observed distribution of pathology. Further research on layer-specific attributes may provide insight into
457 how pathological processes spread through large-scale brain networks.

458 Finally, the present analysis focused only on the middle temporal gyrus. Expanding similar layer-resolved analyses to other
459 brain regions commonly affected in Alzheimer's disease, like the hippocampus, entorhinal cortex, frontal cortex, etc., could
460 determine whether the observed relationships between pathology and microglial dynamics represent a generalized feature of



disease progression or are a region-specific phenomenon (Leng et al., 2021). Future large-scale studies utilizing automated spatial transcriptomics or volumetric modeling would benefit from nested mixed-effects linear models to further isolate intra-donor laminar variance.

4.7. Conclusions

In summary, lower cognitive function was associated with higher tau and amyloid pathology within the MTG, accompanied by changes in microglial dynamics. Tau burden exhibited a particularly strong relationship with declining MMSE scores, supporting previous evidence that tau pathology closely tracks clinical impairment. On the other hand, greater microglial localization was observed near amyloid deposits in lower MMSE scores despite evidence suggesting altered activation states as disease severity progressed.

Together, these findings support a model where pathological burden and neuroimmune changes evolve in parallel with Alzheimer's disease progression. The results further suggest that microglial presence alone may be insufficient to assess neuroimmune function, as cells can remain associated with pathology while potentially undergoing shifts toward less effective or maladaptive states. Additionally, the layer-specific patterns identified within the MTG highlight the importance of considering spatial organization when investigating such neurodegenerative mechanisms. Understanding why certain cortical layers and regions exhibit heightened vulnerability and the changes in pathology associated with these regions may ultimately lead to the development of more targeted therapeutic strategies.

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Data Availability Statement

All datasets analyzed and methodology are available through the Allen Institute's BrainMap in the Seattle Alzheimer's Disease Brain Cell Atlas: <https://portal.brain-map.org/explore/seattle-alzheimers-disease/seattle-alzheimers-disease-brain-cell-atlas-download?edit&language=en>.

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I am also sincerely thankful for the opportunity to explore computational neuroscience and Alzheimer's disease research under his expert guidance. This experience has only fortified my devotion to biomedical research and has given me a deeper appreciation for the collaborative nature of scientific discovery. I am beyond grateful for the time, encouragement, and mentorship Dr. Avila has invested in my academic development, all of which made this manuscript possible.

Author Biography

Amber Zhou is a high school junior at the Brearley School in New York City with a strong interest in various fields of biomedicine, specifically multiple myeloma genetics and clinical care, because conducting wet-lab research and seeing its real patient impact genuinely intrigues her.

Beyond the classroom, Amber participates in a wide variety of extracurricular activities such as volunteering at a pediatric hospital, working at a podiatric surgery clinic, and teaching taekwondo to younger students. To accompany this, she has a



strong commitment to school leadership, seeking roles as co-head of Science Club and The Catalyst, the school's STEM magazine, as she believes that fostering scientific curiosity is integral to a balanced community. Moreover, she regularly engages in service and mentorship to support both younger peers and patients in need, culminating in presenting her research on blood cancer at the prestigious ASH Annual Conference this December.

Amber sees herself as forward-thinking, ambitious, and grounded, helped by her dedication to both advanced scientific inquiry and community impact. Attending a top-tier university and pursuing a career in biomedical research and medicine is one of Amber's long-term goals because, after gaining deep exposure to both clinical environments and advanced laboratory settings, she is ready to harness the resources these institutions have to offer to grow as a scientist.

Mentor Contribution Statement

Dr. Jorge A. Avila provided comprehensive mentorship and scientific guidance throughout the development of this research project.

Dr. Avila introduced the author to Alzheimer's disease research and provided access to the publicly available datasets, analytical framework, computational workflows, and software protocols used throughout the study. Through his instruction, the author learned to organize, analyze, visualize, and interpret high-dimensional transcriptomic and genomic data independently.

Through the research process, Dr. Avila offered continuous feedback on the study design, statistical analyses, interpretation of the results, and scientific writing. His thoughtful critiques strengthened the rigor, clarity, and overall quality of the manuscript while encouraging the author's independent scientific reasoning and problem-solving skills.

Finally, Dr. Avila carefully reviewed multiple manuscript revisions prior to submission, providing constructive and extensive suggestions to improve the presentation and communication of the findings. His mentorship was essential in the successful completion of this project and in the author's personal growth as an emerging researcher.



Decision: Minor edits before publication.

Review: Thank you for your careful revisions in response to the last round of post-revision feedback; you have addressed most of the requested corrections, including the IBA1/MMSE contradiction in the Discussion, the reported ANOVA values, and the use of “microglial inactivation” instead of “IBA1 inactivation.” However, in comparing the revisions vs. the post-revision feedback, I felt the following issues remain:

1. Section 3.5 still states that the increase in inactivation ratio was clear across MMSE groups and worsens with disease severity. However, the results seem to support a difference between the MMSE 0-22 group and each of the two higher MMSE groups. Moreover, there are no significant differences seemingly between MMSE 23-26 and 27-30. Please state these observations directly and further clarify the MMSE-group pattern for the inactive vs. active microglial ratio. In my view, **you should avoid all wording that implies a continuous or monotonic increase across all three groups, as the data do not really demonstrate that.**
2. Section 3.4 now appropriately states that the higher 6E10 density in the lowest-MMSE group is a descriptive pattern and that there was no significant overall MMSE effect. However, later sections still refer to a “simultaneous increase” in tau and amyloid across MMSE groups, and the Conclusion states that lower cognitive function was associated with higher tau and amyloid pathology. **Please revise all statements and make the results consistent throughout the manuscript (incl. perhaps even in the title and abstract) so that the statistically supported tau findings are distinguished from the descriptive amyloid-density pattern.**
3. The revision adds some good cautionary language in the discussion and limitations, but Section 3.5 and the conclusion still use phrases about how microglial activation states change “with disease progression” or “as disease severity progressed.” Since the study uses observational postmortem data, **you should reframe these as cross-sectional associations or hypotheses rather than clearer evidence of longitudinal progression or causation.**
4. The age-matched pairwise statistics should be stated explicitly as **adjusted pairwise p-values** rather than simply “pairwise contrasts.” These are not really comparison means either, so avoid using the phrase “multiple comparison mean values.” “Pairwise contrasts” refers to the tests themselves, as far as I know, and the *results* of these pairwise contrasts are what you reported.