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Sex-Dependent Glial Cell Density in the Prefrontal Cortex and Its Relevance to Glioblastoma

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Abstract

Glioblastoma is the most lethal primary brain tumor in adults, and median survival has not meaningfully improved since temozolomide, an oral alkylating chemotherapy agent, was adopted as standard of care in 2005. Female patients consistently outlive males by approximately 3 to 5 months, even after controlling for known prognostic variables. The biological basis for this survival gap remains unresolved. Identifying the cellular and molecular mechanisms underlying this sex-specific survival difference represents a critical unmet need in neuro-oncology. Nearly all existing research examines glial cell activity after a tumor has developed, leaving baseline sex differences in the glial landscape before tumor formation largely uncharacterized. Two complementary analyses addressed this gap. In Study 1, glial cell density was quantified across three subregions of the rat medial prefrontal cortex. Males showed a significant dorsal-to-ventral glial density difference; females showed a parallel pattern that did not reach significance at this sample size. In Study 2, GFAP and AIF1 gene expression were analyzed in 37 human glioblastoma blocks from the Ivy Glioblastoma Atlas Project. Neither marker differed by sex in isolation. However, when patients were stratified by EGFR amplification status, amplification was significantly associated with an elevated astrocyte-to-microglial expression ratio in male patients ($p = 0.010$) but not females. Together, these exploratory findings indicate that sex-dependent glial responsiveness to spatial and oncogenic gradients warrants further investigation in relation to the female survival advantage in GBM. Sex-stratified analysis of EGFR amplification status may also help identify subgroups for which combined molecular and microenvironment-directed therapies could be evaluated.

Keywords: glioblastoma (GBM), sex differences, tumor microenvironment (TME), glial cells, EGFR amplification, astrocyte-microglia interactions, gene expression analysis

1. Introduction

1.1. Glioblastoma and the Sex Survival Gap

Glioblastoma (GBM) is the most common malignant primary brain tumor in adults, accounting for approximately 14.6% of all primary brain tumors and nearly half of all malignant diagnoses (Ostrom et al., 2020). Even with the current standard of care, including surgical resection followed by concurrent radiotherapy and temozolomide chemotherapy, median overall survival remains 12 to 15 months, and the five-year relative survival rate is approximately 5% (Ostrom et al., 2020; Stupp et al., 2005). The tumor almost always recurs regardless of treatment response, acquiring resistance through rapid genetic diversification within the tumor mass and actively suppressing immune surveillance (Brennan et al., 2013). The five-year survival rate has not meaningfully changed since temozolomide was adopted as standard chemotherapy in 2005 (Ostrom et al., 2020; Stupp et al., 2005). Identifying the biological factors contributing to this persistently poor prognosis is a pressing priority in neuro-oncology, and examining the consistent survival disparity between male and female patients offers one of the most promising avenues toward that goal.

One of the most consistently replicated epidemiological observations in glioblastoma is a survival advantage for female patients. Across analyses encompassing tens of thousands of cases, females outlive males by approximately 3 to 5 months at the median, even after controlling for age at diagnosis, extent of surgical resection, and MGMT promoter methylation status (Ostrom et al., 2018). The consistency of this pattern across independent datasets strongly suggests a real biological difference. Yet the mechanism responsible for it has not been established. Proposed explanations include sex-hormone effects on tumor cell proliferation, differences in immune activation, and sex-specific molecular subtype distributions (Rubin et al., 2020). Each of these mechanisms has supporting evidence, but none fully accounts for the consistent 3- to 5-month gap observed across datasets.

1.2. Glial Cells in the Tumor Microenvironment

The tumor microenvironment (TME) has become a central focus of glioblastoma sex difference research. Within the TME, astrocytes and microglia are the two most abundant non-neuronal cell populations. Astrocytes, identified by the protein glial fibrillary acidic protein (GFAP), form a reactive structural scaffold around tumors and contribute to immune modulation, growth factor signaling, and angiogenesis (Sofroniew & Vinters, 2010). Microglia, identified by allograft inflammatory factor 1 (AIF1, also known as IBA1), are the brain's resident immune cells and can constitute up to one-third of the total tumor mass in glioblastoma (Ransohoff, 2016; Hambardzumyan et al., 2016; Graeber et al., 2002). Together, the balance of astrocytic and microglial activity determines the immunosuppressive state of the tumor microenvironment and the immune system's ability to respond to the tumor.

One nuance specific to glioblastoma is that GFAP is expressed not only by reactive astrocytes but also by the tumor cells themselves, because glioblastoma arises from glial lineage and the tumor cells retain astrocytic transcriptional programs (Brennan et al., 2013; Louis et al., 2016). This distinction matters for interpreting the ratio of GFAP to AIF1 expression: in the context of GBM, this ratio is not a simple count of astrocytes versus microglia. A high ratio indicates that the astrocyte-and-tumor-cell compartment dominates; a low ratio indicates that the microglial immune compartment dominates. This ratio is not a direct count of cell types but a measure of the transcriptional balance between two major biological identities within the tumor.

1.3. Sex Differences in Glial Biology

Sex-dependent differences in glial cell activity have been documented in both healthy brain and tumor contexts. Adult male and female mice differ in microglial morphology, gene expression profiles, and inflammatory responsiveness even under baseline conditions (Villa et al., 2018; Guneykaya et al., 2018). In the GBM TME, microglial activation states and macrophage infiltration levels have been shown to differ by sex (Hambardzumyan et al., 2016; Villa et al., 2018; Guneykaya et al., 2018). Together, these observations point toward sex-dependent differences in glial biology as a likely contributor to the female survival advantage in glioblastoma.

Although baseline sex differences in glial biology are well established, few studies have examined how these pre-existing differences may influence glioblastoma development or the tumor microenvironment. If males and females already differ in how their glial cells are organized and distributed before cancer develops, those baseline differences could shape the cellular composition of the TME from the moment a tumor begins to form. This pre-tumor baseline has not been systematically characterized.

1.4. EGFR Amplification and the GFAP/AIF1 Ratio

The epidermal growth factor receptor (EGFR) gene is amplified in approximately 40 to 60% of primary GBMs, making it the most common recurrent amplification in this cancer (Brennan et al., 2013). EGFR amplification is the defining molecular feature of the Classical GBM subtype, characterized by high EGFR copy number, high-level GFAP expression by the tumor cells themselves, and a transcriptional profile dominated by astrocytic gene programs (Verhaak et al., 2010). This means EGFR amplification directly drives GFAP expression upward in tumor cells, not because of astrocyte activity but because the tumor itself more strongly expresses astrocytic identity genes.

This leads to a testable prediction. EGFR amplification drives GFAP upward through the tumor's own astrocytic gene programs. If female microglia simultaneously maintain a more homeostatic transcriptional state under oncogenic pressure, EGFR amplification should push the GFAP/AIF1 ratio substantially higher in males while having a much smaller effect in females, because the GFAP increase would not be counterbalanced in males to the same degree it would be in females. Examining either marker individually would not detect this, because the asymmetry lives in the ratio rather than in any single variable. Together, these considerations from baseline glial architecture and oncogenic perturbation point toward a shared analytical principle: sex-dependent glial biology may be most clearly revealed not by direct male-versus-female comparisons of single measurements, but by examining how glial populations are arranged across, and respond to, biological gradients. This principle motivates the two-study design that follows.

1.5. Study Rationale and Hypotheses

To characterize pre-tumor glial architecture (the spatial organization and density of glial cells in healthy brain tissue before any tumor has formed), the medial prefrontal cortex (mPFC) was selected as the study region for Study 1. GBM arises in the frontal lobe in approximately 25 to 43% of cases (Ostrom et al., 2020), making the prefrontal cortex among the most clinically relevant regions for this disease. Importantly, astrocytes and microglia are not uniformly distributed throughout the central nervous system; both populations show regional heterogeneity in morphology, density, and gene expression, with subregional differences detectable even within individual cortical areas (Sofroniew & Vinters, 2010; Hambardzumyan et al.,

2016). This regional heterogeneity is relevant to glioma biology because tumor cells emerge from and interact with the local glial substrate, and the molecular character of that substrate is likely to shape the eventual composition of the tumor microenvironment. The rat mPFC is one of the best-characterized brain regions for sex-dependent glial organization; it contains sex hormone receptors and shows sex-dependent differences in glutamatergic signaling and microglial distribution (Heidbreder & Groenewegen, 2003; Knouse et al., 2022). Structurally, the mPFC contains two functionally distinct zones: the dorsal anterior cingulate area (ACA), which connects to executive control circuitry, and the ventral infralimbic and prelimbic areas (ILA and PL), which connect densely to the amygdala, hypothalamus, and brainstem stress-response systems (Heidbreder & Groenewegen, 2003). These ventral subregions are established sites of elevated glial reactivity and host dense projections to immune-modulatory subcortical nuclei, properties that make the dorsal-to-ventral axis a biologically meaningful gradient rather than an arbitrary anatomical contrast. Knouse et al. (2022) specifically reported sex-dependent differences in microglial and glutamatergic signaling within this axis, supporting its use as a framework for detecting sex-organized glial architecture. Direct evidence linking mPFC dorsal-versus-ventral glial heterogeneity to GBM outcomes does not yet exist; the dorsal-to-ventral gradient is therefore proposed as a candidate organizing axis to be tested rather than an established mechanistic link.

Two complementary analyses were conducted. Study 1 used rat mPFC histology to establish whether baseline glial density differs by sex across dorsal versus ventral subregions, providing a model of pre-tumor glial architecture. Study 2 used human glioblastoma gene expression data from the publicly available Ivy Glioblastoma Atlas Project (Puchalski et al., 2018) to test whether EGFR amplification shifts the GFAP/AIF1 expression ratio differently by sex. Both studies were motivated by the same question: does sex shape how glial populations are organized and how they respond to biological perturbation, rather than simply how many of them are present? In both cases, the key was applying a reference gradient (spatial organization in rats, EGFR amplification status in humans) to ask whether that gradient reveals sex-dependent differences that a direct male-versus-female comparison of individual markers would miss.

We hypothesized that female rats would show higher baseline glial density in the ventral mPFC relative to males, and that EGFR amplification would be associated with a higher GFAP/AIF1 ratio in male glioblastoma patients than in female patients, reflecting sex-dependent differences in how the tumor microenvironment responds to a major oncogenic driver.

2. Methods

Study 1: Rat mPFC Glial Density

Animals and tissue preparation. Adult male and female *Rattus norvegicus* (n = 3 per sex; age 3 months) were obtained from AMSBIO. Brains were obtained following euthanasia and immersion-fixed in 4% paraformaldehyde for 24 hours at 4°C, then cryoprotected in 30% sucrose in phosphate-buffered saline. Coronal sections (40 μm) were cut through the full anterior-posterior extent of the mPFC. Three subregions were identified per hemisphere using the Paxinos and Watson rat brain atlas (7th edition): the ACA (AP +2.7 to +3.2 mm from bregma; dorsal-most subregion, approximately 0.5 to 1.2 mm from dorsal surface), the prelimbic area (PL; AP +2.0 to +2.7 mm; intermediate, approximately 1.5 to 3.0 mm depth), and the infralimbic area (ILA; AP +2.0 to +2.7 mm; most ventral, approximately 3.0 to 4.5 mm depth). Estrous cycle stage of female rats was not confirmed at tissue collection, noted as a limitation.

Cell identification and imaging. Sections were stained with a proprietary nuclear counterstain supplied with the IHCeasy kit (AMSBIO) and imaged under brightfield microscopy. Glial nuclei were distinguished from neuronal nuclei using established nuclear morphology criteria: glial nuclei are notably smaller and more uniformly rounded than neuronal nuclei, which are larger and more irregularly shaped (Peters et al., 1991). This approach counts astrocytes, microglia, and oligodendrocytes collectively and does not resolve individual glial subtypes. Images were captured at 20× magnification; three non-overlapping fields of view were acquired per animal per region. All acquisition and counting were performed by a single analyst; counting was not performed blind to the animal's sex.

Automated cell counting. Glial nuclei were counted in ImageJ using a color thresholding approach to isolate counterstained nuclei, followed by the Analyze Particles function. Particles were filtered by area (minimum 6 px^2) and circularity (0.5 to 1.0). Density was expressed as objects per 100,000 px^2. The three field values per region per animal were averaged to yield one density value per animal per region.

Statistical analysis. A two-way repeated-measures ANOVA assessed main effects of sex and region (ACA, PL, ILA) and their interaction, with animal as the repeated unit, using Šídák's post-hoc test. To specifically examine dorsal-to-ventral organization, a second two-way ordinary ANOVA compared ACA density to a pooled composite ventral mPFC measure (summed ILA + PL density per animal), using Fisher's LSD post-hoc. This composite value represents the sum of ILA and PL density values for each animal, consolidating both ventral subregions into a single measure of total ventral glial burden; it is therefore not directly equivalent to a single-region density and should be interpreted as a relative composite measure. Given the small sample size (n = 3 per sex), the appropriateness of non-parametric testing was considered. However, at n = 3 per group, the Mann–Whitney U test has a minimum achievable two-tailed p-value of 0.100, because $C(6,3) = 20$ possible rank arrangements and the most extreme arrangement yields $p = 0.10$ two-tailed. The test, therefore, cannot reach $\alpha = 0.05$ regardless of the observed difference, confirming that Study 1 is underpowered for null-hypothesis significance testing by any standard method at this sample size. The ANOVA framework is retained for structural consistency with the two-way repeated-measures design; all Study 1 comparisons are treated as explicitly exploratory. Effect sizes were calculated as Hedges' *g*. All statistics were computed in GraphPad Prism 10.

Study 2: Human GBM Gene Expression (Ivy GAP)

Dataset. Gene expression and clinical data were obtained from the Ivy Glioblastoma Atlas Project (Ivy GAP), an anatomically stratified transcriptional atlas of human GBM described by Puchalski et al. (2018). The Ivy GAP cohort comprised 41 patients (42 tumors). For each tumor block, five anatomically defined zones were isolated by laser microdissection and profiled by RNA-seq; expression values are reported as $\log^2$-normalized FPKM. The present analysis used per-block expression values averaged across all sequenced zones per block to obtain a whole-tumor expression profile rather than a single-zone measurement. Our analyzed subset comprised 37 tumor blocks from 36 unique patients (patient W22 contributed two tumor blocks; 19 male blocks, 18 female blocks). For the GFAP/AIF1 ratio analysis, nine blocks with unavailable EGFR status were excluded, yielding n = 28 for the stratified analysis (Table 2).

Gene expression variables. GFAP and AIF1 are the canonical markers for the astrocytic/tumor-cell and microglial compartments of the GBM TME, respectively (Sofroniew & Vinters, 2010; Ransohoff, 2016). As described in the Introduction, GFAP expression in this dataset reflects contributions from both reactive astrocytes and the tumor cells themselves (Brennan

et al., 2013; Louis et al., 2016). The GFAP/AIF1 expression ratio was calculated as $\log^2$(GFAP/AIF1) using mean per-block FPKM values. EGFR amplification status was obtained from the clinical metadata provided by Ivy GAP.

Statistical analysis. Sex differences in individual marker expression were assessed by the Mann-Whitney U test. A two-way ANOVA examined the main effects of sex (male/female) and EGFR amplification status (amplified/non-amplified) on the $\log^2$(GFAP/AIF1) ratio, followed by Fisher's LSD post-hoc comparisons. Survival differences between sexes were assessed by the Mann-Whitney U test and reported as Cliff's delta. All analyses were performed in GraphPad Prism 10. P-values were not corrected for multiple comparisons.

3. Results

Study 1: Glial Density Across mPFC Subregions

Study 1 examined whether baseline glial density differs between sexes across dorsal and ventral subregions of the rat mPFC. Figure 1 and Table 1 present mean glial cell density across the three individual subregions (ACA, PL, and ILA, ordered dorsal to ventral) by sex. Females showed consistently higher mean glial density (non-significant in all individual pairwise comparisons) than males across all three subregions, with the largest numerical differences in the ACA (Hedges' g = 1.91; female mean 132.9, male mean 78.3 objects per 100,000 px²) and the ILA (g = 1.18; female 124.6, male 85.9), and a negligible difference in the PL (g = 0.29; female 96.0, male 89.9). The three-region repeated-measures ANOVA found no significant main effects of sex, region, or their interaction. The large effect sizes in the ACA and ILA indicate that, despite non-significance, these differences are not trivial; a power analysis for g = 1.91 at 80% power and α = 0.05 indicates approximately n = 6 per sex would likely be sufficient to detect such a difference. When examining the three subregions individually, the data show a directional trend consistent with lower glial density in the dorsal ACA relative to the ventral ILA and PL, but this pattern does not constitute a statistically demonstrable monotonic gradient across all three regions at this sample size (see Limitations).

Table 1: Mean glial cell density by mPFC subregion and sex (ordered dorsal to ventral).

Region	Female mean (SEM)	Male mean (SEM)	Δ F vs M	Hedges' g	***p*-value**
ACA (dorsal mPFC)	132.9 (17.1)	78.3 (1.2)	+70%	1.91	0.226 (ns)
PL (ventral mPFC)	96.0 (18.5)	89.9 (33.4)	+7%	0.29	0.995 (ns)
ILA (ventral mPFC)	124.6 (9.2)	85.9 (18.1)	+45%	1.18	0.438 (ns)

Note: Values in objects per 100,000 px². Two-way RM ANOVA with Šídák's multiple comparisons. n = 3 per sex. ns = not significant at α = 0.05. Subregions ordered dorsal to ventral: ACA, PL, ILA. Values in parentheses are SEM.

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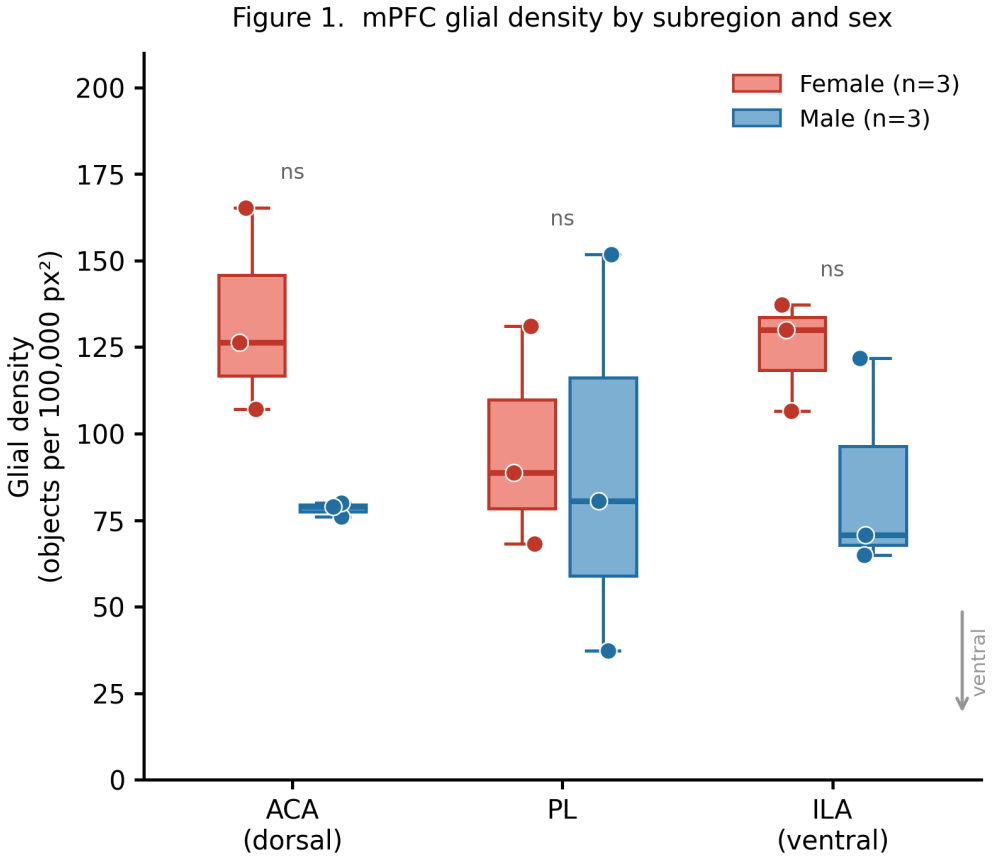

Figure 1: Mean glial cell density across three mPFC subregions by sex (n = 3 per sex). Red = female; blue = male. Subregions are ordered dorsal to ventral (ACA, PL, ILA). Females showed consistently higher mean glial density (non-significant) than males in all subregions, with the ACA and ILA comparisons showing large effect sizes (Hedges' g = 1.91 and 1.18, respectively). Pairwise sex comparisons by subregion (Šídák post-hoc): ACA p = 0.226 ns, PL p = 0.995 ns, ILA p = 0.438 ns. Box: median and IQR; whiskers: min–max; dots: individual animals. Two-way RM ANOVA.

To more directly test dorsal-to-ventral organization, ILA and PL were pooled into a single ventral mPFC composite measure and compared to the dorsal ACA (Figure 2). This pooling approach was used to consolidate the two anatomically connected ventral subregions and increase statistical resolution. A two-way ordinary ANOVA revealed a significant main effect of region ($F(1,8) = 9.883$, $p = 0.014$), with sex not significant as a main effect ($F(1,8) = 2.856$, $p = 0.129$) and no interaction ($F(1,8) = 0.027$, $p = 0.874$). Post-hoc comparisons showed that male ACA density (78.3 objects per 100,000 px²) was significantly lower than the pooled male ventral composite measure (summed ILA + PL = 175.7; $p = 0.047$), establishing a statistically detectable dorsal-to-ventral difference in glial density within males. In female rats, a directionally comparable pattern was present (ACA

= 132.9 vs. ILA + PL = 220.6) but narrowly missed significance (p = 0.068). Sex comparisons within each zone were non-significant (ACA: p = 0.226; ILA + PL: p = 0.312).

Figure 2. Dorsal vs. pooled ventral mPFC glial density by sex

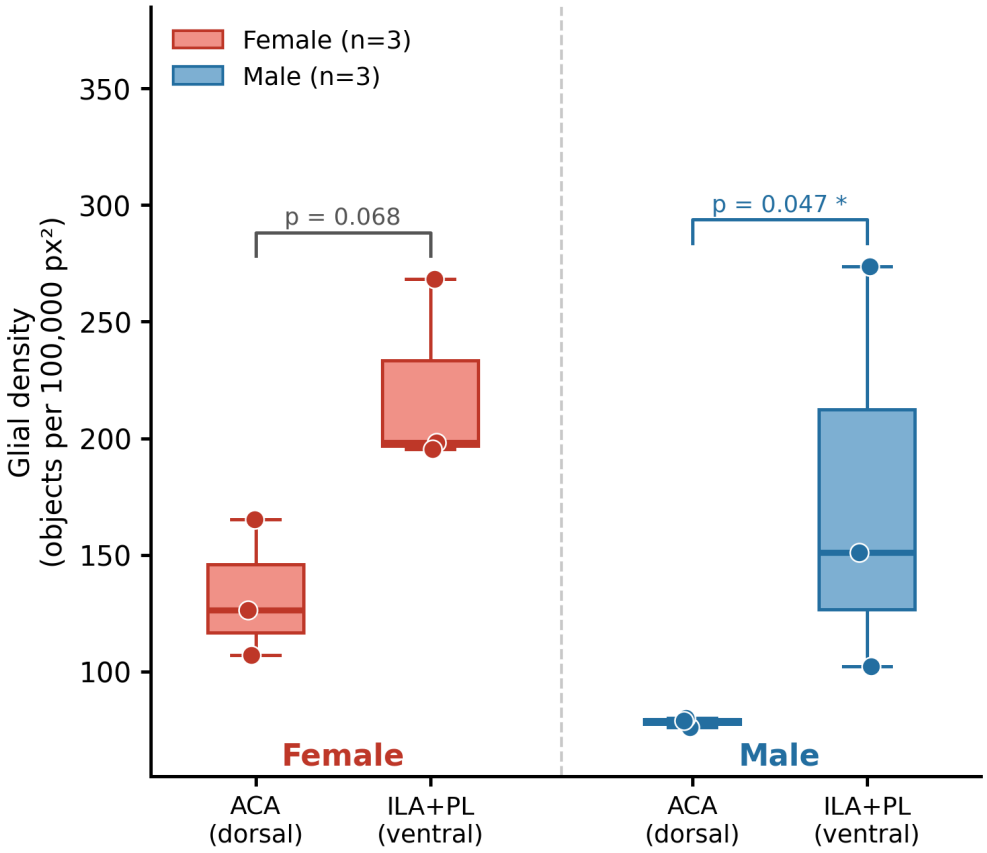

Figure 2: Dorsal ACA glial density versus pooled ventral mPFC composite measure (summed ILA + PL) by sex. Red = female; blue = male. Male rats showed a statistically significant dorsal-to-ventral difference (p = 0.047, Fisher's LSD), with substantially lower glial density in the ACA than in ventral subregions. Female rats showed a comparable directional pattern that narrowly missed significance (p = 0.068). Box: median and IQR; whiskers: min–max; dots: individual animals. Note: ventral values are summed ILA + PL densities (composite burden measure) and are not directly equivalent to single-region ACA density. Two-way ordinary ANOVA. n = 3 per sex.

The female dorsal-to-ventral difference is numerically comparable in magnitude (Δ = 87.7 units vs. 97.4 in males), but individual values are more variable across animals, which may have contributed to the non-significant result. However, insufficient sample size alone cannot be confirmed as the sole explanation. Because the male dorsal-to-ventral p-value (0.047) and the female value (0.068) straddle the conventional α = 0.05 threshold at a very small sample size, the apparent

sex-specific organizational difference should be interpreted cautiously, and the difference between sexes is itself modest rather than categorical. The pattern appears to be present directionally in both sexes; what may differ is the consistency with which it is expressed across individuals, an issue that could reflect hormonal variability, given that estrous cycle stage was not confirmed at tissue collection.

Study 2: GFAP and AIF1 Expression in Human Glioblastoma

Table 2: Ivy GAP cohort composition by EGFR amplification status and sex (n = 28).

Variable	EGFR+ Male (n = 5)	EGFR− Male (n = 8)	EGFR+ Female (n = 9)	EGFR− Female (n = 6)
$\log^2$(GFAP/AIF1) mean	1.420	0.319	0.876	0.658
± SEM	0.158	0.238	0.179	0.423

Note: Blocks with unavailable EGFR status (n = 9) were excluded from ratio analysis. EGFR amplification rate in this cohort (50% female vs. 26% male) likely reflects sampling variation in this small dataset.

Study 2 uses gene expression data from resected human glioblastoma tissue. Unlike Study 1, it does not measure baseline architecture in the healthy brain; it examines whether sex shapes the astrocytic-to-microglial transcriptional balance within established tumors, and whether EGFR amplification shifts that balance differently depending on sex. Female patients in this Ivy GAP subset survived longer than males (median 493 vs. 351 days; Cliff's $\delta = -0.367$; Mann-Whitney $p = 0.097$), consistent with published registry data (Ostrom et al., 2018), confirming that this cohort reflects the broader sex survival pattern in GBM.

When GFAP and AIF1 were examined individually, neither differed significantly by sex. Mean GFAP expression was 6.08 $\log^2$ FPKM in males versus 5.85 in females (Mann-Whitney $p = 0.347$), and mean AIF1 was 4.98 in males versus 5.01 in females ($p = 0.909$). This is consistent with prior work showing that bulk-level expression of individual glial markers does not reliably capture sex differences without a stratifying variable (Villa et al., 2018; Guneykaya et al., 2018). These null results are informative: if sex does not shape the quantity of each glial population but how those populations respond to an oncogenic perturbation, no single-marker comparison will detect it. The ratio analysis was designed specifically to test that possibility.

EGFR Amplification Modifies the GFAP/AIF1 Ratio Differently by Sex

A two-way ANOVA of the $\log^2$(GFAP/AIF1) ratio (n = 28 blocks: five EGFR-amplified males, eight non-amplified males, nine EGFR-amplified females, six non-amplified females) revealed a significant main effect of EGFR amplification ($F(1,24) = 6.117$, $p =$

0.021) but not of sex ($F(1,24) = 0.148$, $p = 0.704$). The sex-by-EGFR interaction was not significant ($F(1,24) = 2.738$, $p = 0.111$). Post-hoc comparisons are summarized in Table 3 and Figure 3.

Table 3: $\log^2$(GFAP/AIF1) ratio by EGFR amplification status and sex.

Group comparison	Male mean (SEM)	Female mean (SEM)	**p-value**	Interpretation
EGFR-amplified (5M, 9F)	1.420 ± 0.158	0.876 ± 0.179	0.169	ns
Non-amplified (8M, 6F)	0.319 ± 0.238	0.658 ± 0.423	0.370	ns
Males: EGFR-amp vs. non-amp	—	—	**0.010**	**Significant ***
Females: EGFR-amp vs. non-amp	—	—	0.552	ns

Note: Two-way ANOVA with Fisher's LSD post-hoc. n = 28 blocks (nine excluded for unavailable EGFR status). p* < 0.05. EGFR amplification rate in this cohort (50% female vs. 26% male) is atypical of larger GBM registries and likely reflects sampling variation in the small Ivy GAP dataset.

In male patients, EGFR amplification was significantly associated with a higher $\log^2$(GFAP/AIF1) ratio (amplified: 1.420 vs. non-amplified: 0.319; $p = 0.010$). In female patients, the same comparison produced no significant difference (amplified: 0.876 vs. non-amplified: 0.658; $p = 0.552$). The same genetic alteration produced a large, significant shift in one sex and essentially no shift in the other. Within EGFR-amplified tumors, males showed a higher mean ratio than females (1.420 vs. 0.876), although this comparison did not reach statistical significance ($p = 0.169$). Within non-amplified tumors, the direction reversed: non-amplified males showed a lower ratio (0.319) than non-amplified females (0.658; $p = 0.370$), though this comparison is notably underpowered given that the majority of non-amplified patients are male in this cohort.

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Figure 3. GFAP/AIF1 ratio by EGFR amplification status and sex

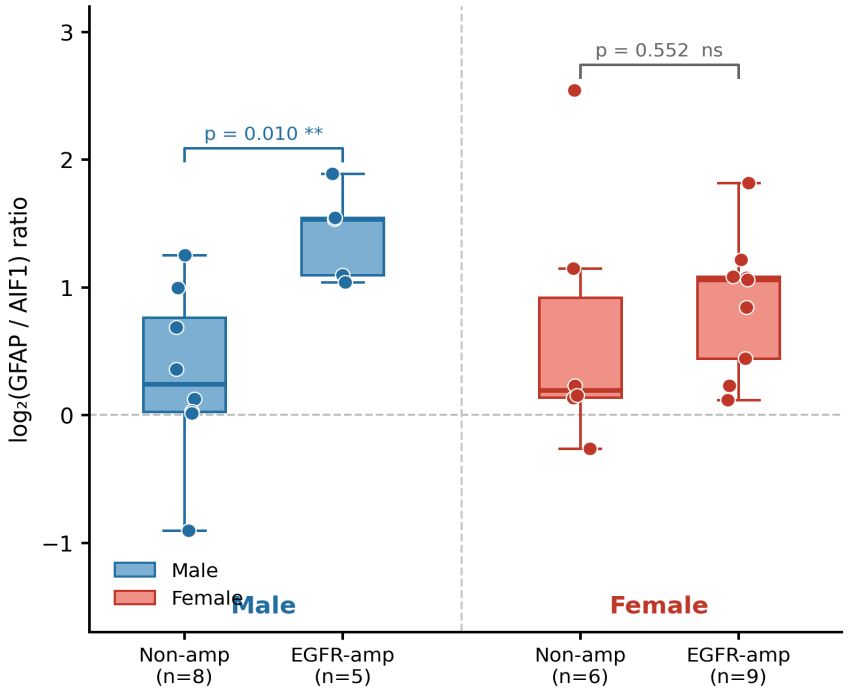

Figure 3: log²(GFAP/AIF1) ratio by EGFR amplification status and sex. Red = female; blue = male. EGFR amplification was a significant main effect overall (p = 0.021). Within males, EGFR-amplified tumors showed a significantly higher ratio than non-amplified tumors (p = 0.010). Within females, no significant shift was observed (p = 0.552). Two-way ANOVA, Fisher's LSD post-hoc.

4. Discussion

Taken together, these findings suggest that sex shapes glial biology primarily as an organizing variable, not as a scaling one. In both datasets, the sex difference was invisible in direct male-versus-female comparisons of any single measurement. It only became detectable when a reference gradient was introduced: a spatial dorsal-to-ventral contrast in the rat brain, and EGFR amplification status in human tumor tissue. That the same structural pattern emerged across two independent datasets, two species, and two entirely different types of biological measurement points toward a consistent organizing principle: sex-dependent glial biology appears to be expressed through how populations are arranged and how they respond to perturbation, rather than through their absolute quantity. This resonates with the transcriptomic work of Villa et al. (2018) and Guneykaya et al. (2018), in which male and female microglia differed not in total cell number but in gene expression profiles and inflammatory signaling pathways. These differences were measurable only when comparing activation states, not bulk marker levels.

The Study 1 finding of a significant dorsal-to-ventral glial density difference in males and a parallel but non-significant pattern in females is consistent with the known functional architecture of the rodent mPFC. The ventral ILA and PL are densely connected to subcortical stress-response systems, including the amygdala, hypothalamus, and brainstem (Heidbreder & Groenewegen, 2003), and are established sites of elevated glial reactivity. This is consistent with the higher glial density observed in these ventral regions in both sexes. The dorsal ACA, by contrast, connects primarily to cortical executive circuits and may have lower baseline glial activity. Sex differences in glutamatergic and microglial signaling within these circuits have been further described by Knouse et al. (2022). In males, the density difference between dorsal and ventral regions is sharp enough to reach statistical detection at n = 3. In females, the numerical magnitude of the difference is comparable, but individual values are more variable across animals, compressing the signal below significance at this sample size. One candidate explanation is that estrogen receptor signaling exerts anti-inflammatory effects on microglia through ERα and ERβ-mediated mechanisms (Villa et al., 2016; Acaz-Fonseca et al., 2015), which could dampen and desynchronize ventral glial density across individual female animals depending on hormonal state. Because estrous cycle stage was not confirmed at tissue collection, this cannot be tested directly from the current data.

An observation that extends beyond group mean differences is the pattern of individual variability across both studies. Male animals and patients showed more consistent, rigid responses to biological organization cues, both spatial (the dorsal-to-ventral density difference) and oncogenic (EGFR amplification shifting the GFAP/AIF1 ratio). Female subjects showed greater intragroup variability in both cases: the female dorsal-to-ventral pattern missed significance partly due to greater individual spread, and female tumor GFAP/AIF1 ratios did not shift significantly in either EGFR-amplified or non-amplified groups. This raises the possibility that sex differences in glial biology are not primarily differences in means but differences in the structure of variance. Female glial populations may be subject to more dynamic, hormonally modulated regulation that varies across individuals and contexts. If this interpretation is correct, the female survival advantage in GBM (Ostrom et al., 2018) may partly reflect this regulatory flexibility: female tumor microenvironments may be more capable of dynamic adjustment under oncogenic pressure, maintaining a more balanced astrocyte-to-microglial ratio even under EGFR-driven perturbation. This is broadly consistent with the higher baseline inflammatory responsiveness and maintained homeostatic profiles documented in female microglia under perturbation by Villa et al. (2018) and Guneykaya et al. (2018).

The sex-specific EGFR amplification finding has specific implications for understanding male vulnerability in glioblastoma. Non-amplified male tumors in this cohort showed the lowest mean GFAP/AIF1 ratio of any group (0.319), consistent with prior evidence that male GBM tumors exhibit higher baseline microglial and macrophage infiltration when major tumor-intrinsic immunosuppressive mechanisms are not fully active (Hambardzumyan et al., 2016; Villa et al., 2018). The addition of EGFR amplification in males then dramatically shifts this balance toward an astrocyte-dominant state (ratio 1.420, $p = 0.010$ vs. non-amplified males), creating a particularly unfavorable combination: EGFR-driven GFAP expression from the tumor cells themselves (Brennan et al., 2013; Verhaak et al., 2010) dominates over the microglial compartment. Astrocyte-dominant tumor microenvironments are specifically associated with suppression of cytotoxic T cell infiltration through TGF-β secretion and physical barrier formation (Sofroniew & Vinters, 2010), which are primary immunosuppressive mechanisms in GBM and key barriers to immunotherapy efficacy. Female patients with EGFR-amplified tumors showed no equivalent ratio shift, suggesting their microglial compartment is not equivalently displaced by the EGFR-driven GFAP increase. This resistance may reflect estrogen receptor-mediated maintenance of microglial homeostasis (Villa et al., 2016; Acaz-Fonseca et al., 2015). If confirmed in larger datasets, EGFR-amplified male GBM patients may represent a biologically distinct subgroup with an astrocyte-dominant TME that is specifically resistant to checkpoint inhibitor-based immunotherapy. This subgroup may

benefit from combined EGFR-targeted and microenvironment-directed therapies. Many current glioblastoma trial designs do not routinely stratify patients by both EGFR status and sex, leaving this potential subgroup distinction largely unexamined.

The Study 2 finding has a straightforward mechanistic interpretation grounded in the canonical function of EGFR amplification. In the Classical GBM subtype, EGFR amplification drives GFAP upward through the tumor cells' own astrocytic gene programs, not through reactive astrocyte activity (Brennan et al., 2013; Verhaak et al., 2010). In male patients, this EGFR-driven GFAP increase is sufficient to significantly elevate the GFAP/AIF1 ratio relative to non-amplified male tumors. In female patients, the same EGFR-driven GFAP increase does not produce a detectable ratio shift. The most likely explanation is that female microglia maintain a more homeostatic transcriptional state under oncogenic stress (Villa et al., 2018; Guneykaya et al., 2018), potentially through estrogen receptor-mediated pathways in microglial cells (Villa et al., 2016). If female microglia maintain higher AIF1 expression in the presence of EGFR-driven tumor expansion, the ratio stays more stable even as GFAP rises. The sex-by-EGFR interaction term did not reach significance (p = 0.111), so this sex-dependent pattern should be treated as a hypothesis to test in larger datasets rather than a confirmed finding.

The directional reversal in non-amplified tumors, where males show a lower ratio (0.319) than females (0.658), is noteworthy and not contradictory. In non-amplified tumors, EGFR does not drive GFAP upward in tumor cells. The ratio, therefore, reflects the native balance between host astrocytic activity and microglial activity without the EGFR-driven GFAP boost. That non-amplified male tumors appear more AIF1-dominant than any other group is consistent with prior reports of higher microglial infiltration in male GBM tumors in the absence of major immunosuppressive oncogenic drivers (Hambardzumyan et al., 2016; Villa et al., 2018). However, with n = 8 non-amplified males in this cohort, including one outlier patient (W34) with a ratio of −0.907, the mechanistic interpretation of this group is premature.

Across both studies, sex-dependent glial biology appears to be expressed primarily as a difference in organizational consistency and perturbation responsiveness, not as a global difference in cell quantity or baseline marker levels. In the healthy brain, this is reflected in spatial differences in glial density that are detectable by dorsal-to-ventral contrast but not by region-by-region comparison. In tumor tissue, it emerges as a difference in how the GFAP/AIF1 balance responds to an oncogenic driver that directly amplifies the GFAP compartment.

Several limitations affect the interpretation of both studies. In Study 1, n = 3 per sex is underpowered; a power analysis for the ACA effect size (g = 1.91) at 80% power and α = 0.05 indicates approximately n = 6 per sex would likely be sufficient, while n = 8 to 10 provides more stable estimates. Estrous cycle stage was not confirmed, introducing potential hormonal variability in female animals. Counting was not performed blind to the animal's sex, which introduces potential observer bias. Nuclear morphology counting using the kit-supplied proprietary counterstain identifies all glial subtypes collectively and cannot resolve astrocytes, microglia, and oligodendrocytes separately, which limits the direct comparability to Study 2's astrocyte/microglial gene expression ratio. Additionally, the term "dorsal-to-ventral gradient" refers specifically to the pooled ACA versus ILA + PL comparison; when examining all three subregions individually, the pattern is directional but not a monotonically increasing gradient. In Study 2, within-group sample sizes are as small as n = 5 (EGFR-amplified males), and the EGFR amplification rate in this cohort (50% of females vs. 26% of males) is atypical of larger GBM registries, most likely reflecting sampling variation. This imbalance means the non-amplified group is predominantly male, which limits the power of within-non-amplified sex comparisons. Across both studies, p-values were not corrected for multiple comparisons, which increases the risk of type I error across the multiple post-hoc tests performed. Specifically, Study 1's three-region post-hoc comparisons used Šídák's correction, which controls the family-wise error rate; however, the dorsal-to-ventral Fisher's LSD

comparisons in Study 1 and all Fisher's LSD post-hoc comparisons in Study 2 rely on the protection afforded by a significant omnibus F-test rather than a per-comparison alpha adjustment, leaving some residual multiple comparisons risk for those specific tests. In this revision, all three figures have been re-rendered as box plots with individual data points overlaid, replacing the original bar graphs; this addresses the distributional representation concern and allows the individual variability in female responses to be directly visible, particularly the high-variance non-amplified female group in Figure 3.

5. Conclusion

Both studies showed that sex shapes glial biology through differential responsiveness to spatial and oncogenic gradients, not through differences in absolute cell quantity or individual marker expression. In the rat medial prefrontal cortex, a significant dorsal-to-ventral glial density difference was detectable in males and showed a directionally similar but non-significant pattern in females, suggesting sex-dependent differences in how glial cells are spatially organized within this region. In human glioblastoma, EGFR amplification significantly elevated the GFAP/AIF1 expression ratio in male patients but produced no detectable shift in female patients, indicating that female tumors appeared to maintain a more stable glial balance in this cohort even when a major oncogenic driver was pushing the GFAP compartment upward.

Several specific next steps would build directly on these findings. Replication of the sex-by-EGFR interaction in the GFAP/AIF1 ratio in TCGA-GBM, which contains over 150 patients with EGFR amplification data, would determine whether the sex-dependent ratio response is robust. If confirmed, examination of cytotoxic T cell marker expression (e.g., CD8A) stratified by both sex and EGFR status would directly test whether EGFR-amplified male patients carry a more immunosuppressed TME. Integration with single-cell RNA sequencing data would allow the relative contributions of tumor-cell GFAP and reactive astrocyte GFAP to be disentangled. For Study 1, addressing whether the glial density differences detected here are driven primarily by astrocytes, microglia, or both would require replication with n = 8 to 10 per sex using combined IBA1 and GFAP immunostaining alongside the nuclear counterstain, with estrous cycle stage confirmed at tissue collection. Including an ovariectomy group with defined hormone replacement would allow direct testing of whether estrogen suppresses ventral mPFC glial density and increases individual variability in females. If sex shapes the spatial organization of astrocytes and microglia differently in the healthy brain, and if that organizational difference persists into the tumor microenvironment, it may prove to be one of the biological foundations of the female survival advantage in glioblastoma (Ostrom et al., 2018).

Sex is not just a demographic variable in glioblastoma. It appears to change the molecular architecture of the tumor microenvironment in ways that interact with specific oncogenic drivers. Treating sex as a variable to adjust for rather than a biological question to investigate directly leaves part of the disease's biology unexamined, and may help explain why some treatment approaches have not produced durable outcomes across patient populations. Several specific therapeutic directions follow from these observations. First, the apparent astrocyte-dominant tumor microenvironment in EGFR-amplified male patients suggests that this subgroup may benefit from combined treatment strategies that pair EGFR-targeted agents with microenvironment-directed therapies, in particular those that disrupt astrocyte-derived immunosuppressive signaling (for example, TGF-β pathway inhibitors) or restore microglial immune function. Second, the differential response of male and female tumors to EGFR amplification implies that checkpoint inhibitor-based immunotherapy may require sex-stratified evaluation: if female tumors maintain a more balanced glial compartment under oncogenic pressure, female EGFR-amplified patients may show a different baseline immune-suppression profile, and therefore a different response to immune-checkpoint blockade, than their male counterparts. Third, prospective glioblastoma trials should consider stratifying enrollment by both EGFR amplification status and sex, an analytical step that

many current trial designs omit, and that could clarify whether sex-specific dosing strategies, response biomarkers, or combination regimens may help narrow the male–female survival gap. Even modest improvements in identifying patients most likely to benefit from specific therapeutic combinations could meaningfully contribute to closing the 3- to 5-month survival difference that has persisted despite two decades of stable median survival in glioblastoma.

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AI Use Statement

The AI tools used in the preparation of this manuscript were Claude (claude.ai), developed by Anthropic, and Grammarly. Both tools were used exclusively for grammar, punctuation, and sentence-level clarity editing on completed draft sections. At no point were either of these tools used to generate ideas, formulate arguments, develop hypotheses, produce or paraphrase literature summaries, or contribute to the scientific content, analysis, or interpretation of the manuscript in any form.

All aspects of the scientific work underlying this paper were conducted and interpreted independently. The research questions and hypotheses were developed through the author's own scientific reasoning under the mentorship of Dr. Jorge Avila at Pasadena Bio Collaborative Labs. Study 1 involved direct laboratory work, including tissue preparation, DAPI nuclear staining, brightfield imaging at 20x magnification, and automated cell counting using ImageJ's Analyze Particles function. All imaging acquisition, threshold calibration, and particle filtering were performed by the author personally. Statistical analyses for both studies, including two-way repeated-measures ANOVA, ordinary two-way ANOVA, Mann-Whitney U tests, and effect size calculations, were performed by the author in GraphPad Prism 10. Study 2 involved the author's independent access to the Ivy Glioblastoma Atlas Project public database, construction of per-block mean expression variables, calculation of the log2(GFAP/AIF1) ratio, stratification by EGFR amplification status and sex, and all downstream statistical modeling.

The literature review was conducted by the author through direct engagement with primary sources accessed via PubMed and individual journal websites. Each cited article was read and evaluated independently for methodological relevance and interpretive significance. All writing in this manuscript, including the introduction, methods, results, discussion, and conclusion sections, was composed by the author in full before any AI-assisted grammar review. Claude and Grammarly were applied only after complete drafts existed, and their use was confined strictly to the sentence level for grammar and fluency. Neither tool was used to generate, restructure, or rewrite any passage, argument, or claim.

Regarding the citation errors identified during the initial desk review: these errors reflect inaccuracies in citation metadata that were not caught before the initial submission. All nineteen references have since been individually verified against primary sources, with each DOI resolved directly and each author list confirmed against the published article. The corrected reference list is included in this revised submission.

Author Biography

Daniel Zhang is a rising senior at Canyon Crest Academy in San Diego, California, pursuing an MD-PhD pathway in cancer biology and bioinformatics. He received a full $13,600 scholarship as 1 of 2 students worldwide selected for the Indigo Research Global Innovators Lab at Pasadena Bio Collaborative, where he performed immunohistochemistry on rat prefrontal cortex tissue and analyzed 37 human glioblastoma RNA-seq datasets from the Ivy Glioblastoma Atlas Project, producing this research paper. As 1 12 high school Affiliate Scholars at the UC Davis Comprehensive Cancer Center, he analyzed 1,450+ renal cell carcinoma samples across 45 TCGA and GEO cohorts, identified immune checkpoint targets associated with therapy resistance, and presented first-author findings at ICBES 2026 in London. He was also selected as 1 of 36 students from more than 4,800 applicants for the SSP Bacterial Genomics program at Purdue University and serves as 1 of 5 global student organizers for the ISCB Youth Bioinformatics Symposium at ISMB 2026.

Daniel is the co-founder and International President of STEAMLabs International, a 501(c)(3) nonprofit that has reached more than 1,500 K-8 students across 11 cities, 7 states, and Nigeria through 50+ STEM events supported by 110+ volunteer mentors and $27,000+ in grants and technology credits. He is also CEO and co-founder of Tumor Tactics, a 72-card cancer education game that earned finalist recognition in the ECGBL 14th International Educational Games Competition and will be featured at the 2026 UN Science Summit.

An All-State pianist with MTAC Level 10 State Honors, Daniel has performed at Carnegie Hall, the Berlin Philharmonie, Vienna Glass Hall, and Tokyo Opera City Hall. A 2026 Carson Scholar, his work is inspired by the loss of his grandmother to pancreatic cancer when he was nine, an experience that shaped his commitment to cancer education and research.

Mentor Contribution Statement

Dr. Jorge Avila provided mentorship, laboratory training, and oversight throughout this project. He guided the experimental design, advised on statistical methodology, and suggested the pooled ILA + PL dorsal-to-ventral gradient analysis that produced the study's primary significant finding. He helped frame the post-hoc EGFR findings as exploratory and provided extensive feedback on the interpretation and presentation of results. Louisa Schilling assisted with the human gene expression dataset analysis.