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. 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. 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 trend that did not reach significance. 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 findings suggest that sex-dependent glial microenvironment differences may contribute to the female survival advantage in GBM, and that sex-stratified EGFR analysis may inform treatment approaches for this disease. Median survival in glioblastoma has not improved since 2005.

Keywords: glioblastoma, sex differences, glial cells, tumor microenvironment, EGFR amplification

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).

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 how immunosuppressive the tumor microenvironment is and how effectively the immune system can 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.

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. 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). This dorsal-to-ventral distinction makes the mPFC well suited for detecting spatial differences in glial organization by sex.

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.

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 animal 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 px²) and circularity (0.5 to 1.0). Density was expressed as objects per 100,000 px². 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 Šíčák's post-hoc test. To specifically examine dorsal-to-ventral organization, a second two-way ordinary ANOVA compared ACA density to pooled ventral mPFC density (ILA + PL), using Fisher's LSD post-hoc. Effect sizes were calculated as Hedges' *g*. All statistics were computed in GraphPad Prism 10. P-values were not corrected for multiple comparisons.

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 1).

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 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 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.

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 2 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 n = 5 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 2. *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 Šíčák'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.

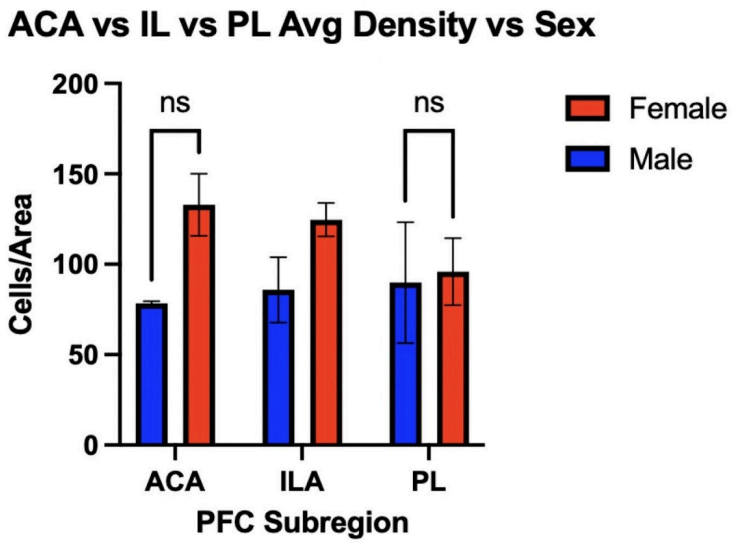

Figure 1. *Mean glial cell density across three mPFC subregions by sex (n = 3 per sex). Red bars = female; blue bars = male. Subregions are ordered dorsal to ventral* (ACA, PL, ILA). *Females showed consistently higher mean glial density (non-significant) than males in all subregions, with the ACA and ILA comparisons showing large effect sizes (Hedges' g = 1.91 and 1.18, respectively). No significance brackets are shown for non-significant comparisons. Error bars = ± SEM. Two-way RM ANOVA with Šíčák's post-hoc.*

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 pooled male ventral density (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: $p = 0.312$).

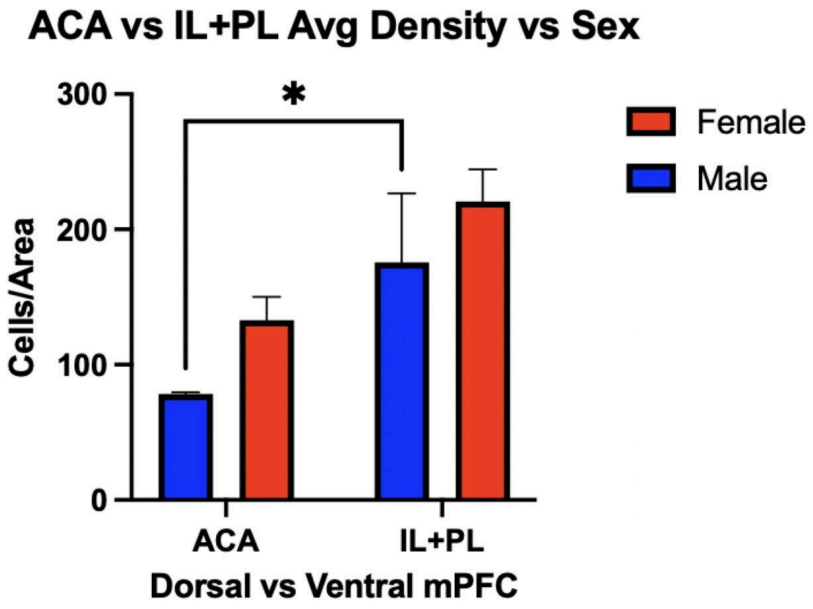

Figure 2. *Glial density in dorsal ACA versus pooled ventral mPFC (ILA + PL) by sex. Red bars = female; blue bars = male. Male rats showed a statistically significant dorsal-to-ventral difference* (p = 0.047, *Fisher's LSD), with substantially lower glial density in the ACA than in ventral subregions. Female rats showed a comparable directional pattern that narrowly missed significance* (p = 0.068). *Two-way ordinary* ANOVA. *Error bars* = ± SEM. *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—though insufficient sample size alone cannot be confirmed as the sole explanation. 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 1. *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) 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 shapes not 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.

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), trending toward 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.

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	Trending, ns
Non-amplified (8M, 6F)	0.319	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.

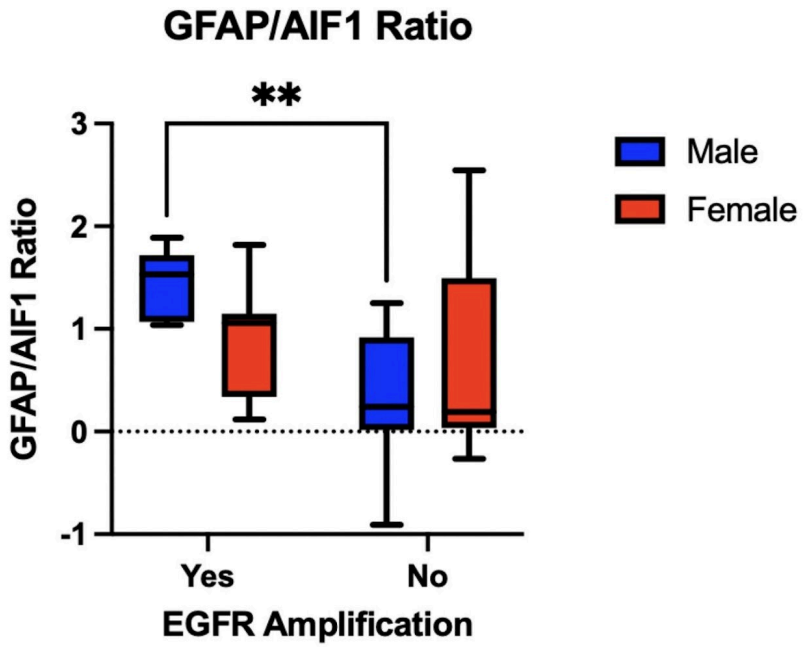

Figure 3. *log²(GFAP/AIF1) ratio by EGFR amplification status and sex. Red bars = female; blue bars = male. EGFR amplification was a significant main effect overall* (p = 0.021). *Within males, EGFR-amplified tumors showed a significantly higher ratio than non-amplified tumors* (p = 0.010). *Within females,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

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—differences that 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—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—whether spatial (the dorsal-to-ventral density difference) or 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—with female glial populations 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 show no equivalent ratio shift, suggesting their microglial compartment is not equivalently displaced by the EGFR-driven GFAP increase—a resistance potentially reflecting 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—one for whom combined EGFR-targeted and microenvironment-directed therapies may be warranted. Current glioblastoma trials do not stratify patients by both EGFR status and sex, leaving this potential subgroup distinction unexamined.

The Study 2 finding has a straightforward mechanistic interpretation grounded in what EGFR amplification actually does. 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 is not driving 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 shows up as spatial differences in glial density that are detectable by dorsal-to-ventral contrast but not by region-by-region comparison. In tumor tissue, it shows up 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 n = 5 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 animal 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. Finally, bar graphs displaying group means were used for all figures; these do not fully convey the distribution of individual data points, which is particularly relevant given the argument that female biological responses show greater individual variability. Future work should present violin or box plots alongside means to better characterize distributional differences between sexes.

Conclusion

Both studies show 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 trended in the same direction 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 maintain a more stable glial balance even when a major oncogenic driver is 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.

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Mentor Contribution Statement

[Mentor] provided mentorship, laboratory training, and oversight throughout this project. [He/she] 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/she] helped frame the post-hoc EGFR findings as exploratory and provided extensive feedback on the

interpretation and presentation of results. [Collaborator] assisted with the human gene expression dataset analysis.

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[Author] is a high school junior at [High School] in [City], California, conducting cancer biology research at [Laboratory], with a focus on sex-dependent glial biology and glioblastoma. [Author's] broader research experience includes single-cell RNA sequencing of bladder cancer models at a UC campus laboratory, multi-omics analysis of renal cell carcinoma at a UC Comprehensive Cancer Center, and cancer biology research at Cambridge University. [Author] is interested in translational oncology and sex-stratified approaches to precision medicine. [Identifying details redacted for blind review; full biography submitted via portal.]

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I have reviewed the manuscript, and while I was in favor of moving on to peer review based on the content, my manual review of the references is very concerning. Even from the first five in the list, three of them have incorrect information, the DOIs don't match, etc (all of which are AI hallmarks).

For example, the fourth reference listed below has a doi that actually links to this article:

Guneykaya, D., Ivanov, A., Hernandez, D. P., Haage, V., Wojtas, B., Meyer, N., Maricos, M., Jordan, P., Lehnardt, S., Kaminska, B., Bhatt, D., & Wolf, S. A. (2018). Transcriptional and translational differences of microglia from male and female brains. Cell Reports, 24(10), 2773–2783. https://doi.org/10.1016/j.celrep.2018.08.070

The DOI from the intended article is CLOSE to the DOI listed in the bibliography (Intended article here), but the repeated errors make me think that this was an AI-induced inappropriate assignment of citation information.

I would recommend not moving to peer review until the student (and/or mentor) can explain the discrepancies.

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

[Author details redacted for blind review]

Abstract

Glioblastoma is the most lethal primary brain tumor in adults. 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. 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 trend that did not reach significance. 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 findings suggest that sex-dependent glial microenvironment differences may contribute to the female survival advantage in GBM, and that sex-stratified EGFR analysis may inform treatment approaches for this disease. Median survival in glioblastoma has not improved since 2005.

Keywords: glioblastoma, sex differences, glial cells, tumor microenvironment, EGFR amplification

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).

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 how immunosuppressive the tumor microenvironment is and how effectively the immune system can 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.

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. 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). This dorsal-to-ventral distinction makes the mPFC well suited for detecting spatial differences in glial organization by sex.

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.

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 animal 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 px²) and circularity (0.5 to 1.0). Density was expressed as objects per 100,000 px². 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 Šíčák's post-hoc test. To specifically examine dorsal-to-ventral organization, a second two-way ordinary ANOVA compared ACA density to pooled ventral mPFC density (ILA + PL), using Fisher's LSD post-hoc. Effect sizes were calculated as Hedges' *g*. All statistics were computed in GraphPad Prism 10. P-values were not corrected for multiple comparisons.

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 1).

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 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 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.

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 2 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 n = 5 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 2. *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 Šíčák'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.

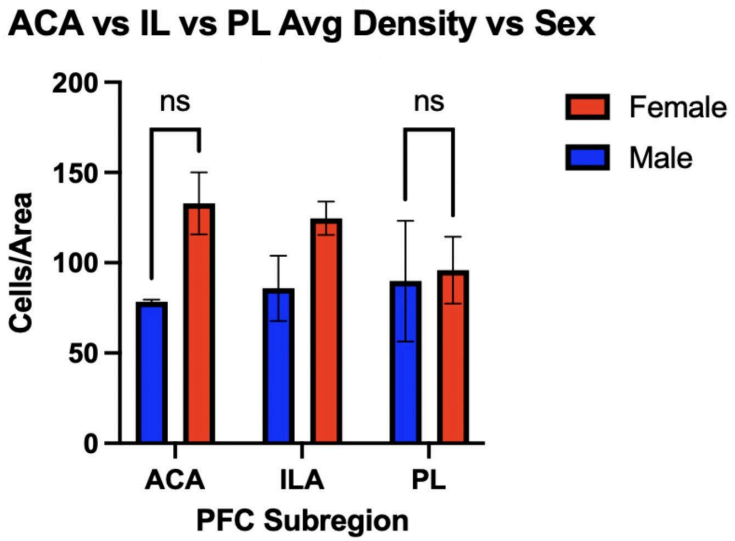

Figure 1. *Mean glial cell density across three mPFC subregions by sex (n = 3 per sex). Red bars = female; blue bars = male. Subregions are ordered dorsal to ventral* (ACA, PL, ILA). *Females showed consistently higher mean glial density (non-significant) than males in all subregions, with the ACA and ILA comparisons showing large effect sizes (Hedges' g = 1.91 and 1.18, respectively). No significance brackets are shown for non-significant comparisons. Error bars = ± SEM. Two-way RM ANOVA with Šíčák's post-hoc.*

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 pooled male ventral density (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: $p = 0.312$).

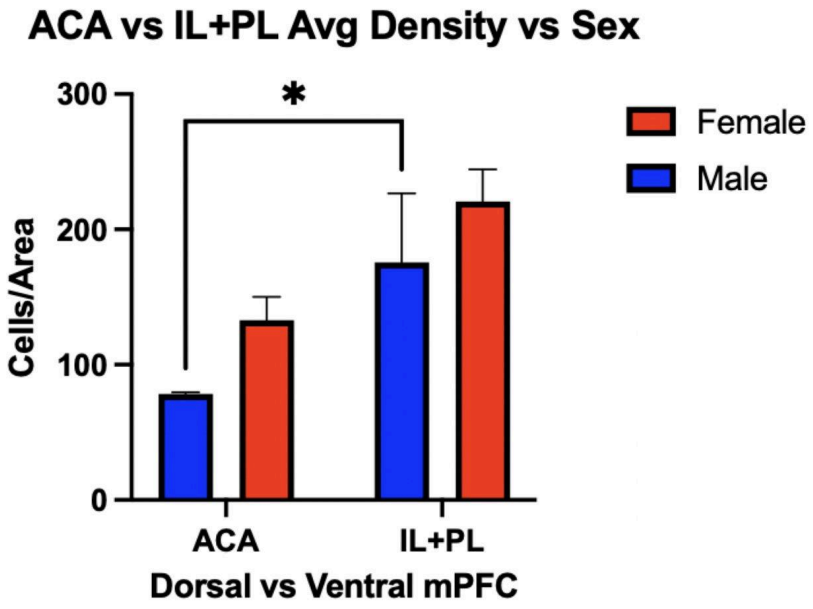

Figure 2. *Glial density in dorsal ACA versus pooled ventral mPFC (ILA + PL) by sex. Red bars = female; blue bars = male. Male rats showed a statistically significant dorsal-to-ventral difference* (p = 0.047, *Fisher's LSD), with substantially lower glial density in the ACA than in ventral subregions. Female rats showed a comparable directional pattern that narrowly missed significance* (p = 0.068). *Two-way ordinary* ANOVA. *Error bars* = ± SEM. *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—though insufficient sample size alone cannot be confirmed as the sole explanation. 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 1. *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) 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 shapes not 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.

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), trending toward 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.

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	Trending, ns
Non-amplified (8M, 6F)	0.319	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.

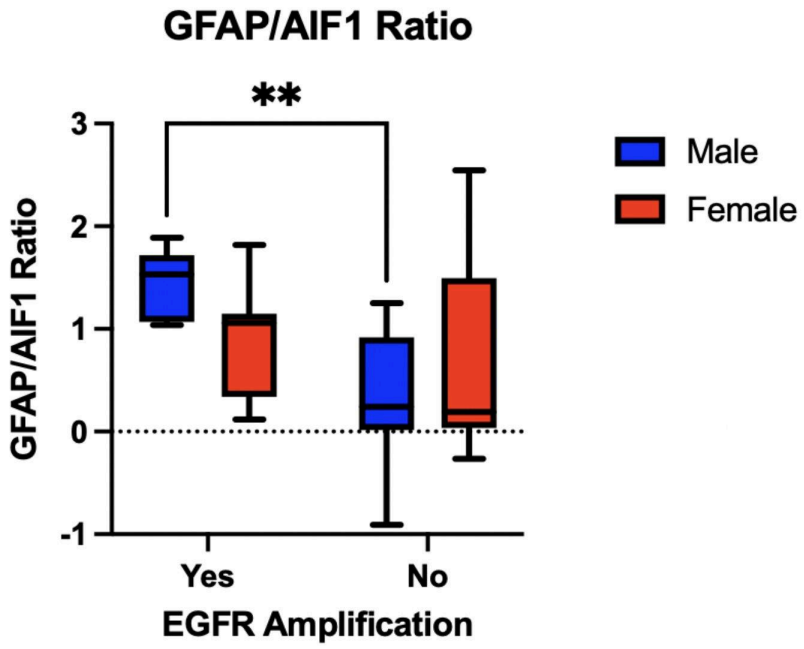

Figure 3. *log²(GFAP/AIF1) ratio by EGFR amplification status and sex. Red bars = female; blue bars = male. EGFR amplification was a significant main effect overall* (p = 0.021). *Within males, EGFR-amplified tumors showed a significantly higher ratio than non-amplified tumors* (p = 0.010). *Within females,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

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—differences that 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—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—whether spatial (the dorsal-to-ventral density difference) or 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—with female glial populations 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 show no equivalent ratio shift, suggesting their microglial compartment is not equivalently displaced by the EGFR-driven GFAP increase—a resistance potentially reflecting 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—one for whom combined EGFR-targeted and microenvironment-directed therapies may be warranted. Current glioblastoma trials do not stratify patients by both EGFR status and sex, leaving this potential subgroup distinction unexamined.

The Study 2 finding has a straightforward mechanistic interpretation grounded in what EGFR amplification actually does. 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 is not driving 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 shows up as spatial differences in glial density that are detectable by dorsal-to-ventral contrast but not by region-by-region comparison. In tumor tissue, it shows up 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 n = 5 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 animal 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. Finally, bar graphs displaying group means were used for all figures; these do not fully convey the distribution of individual data points, which is particularly relevant given the argument that female biological responses show greater individual variability. Future work should present violin or box plots alongside means to better characterize distributional differences between sexes.

Conclusion

Both studies show 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 trended in the same direction 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 maintain a more stable glial balance even when a major oncogenic driver is 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.

Acknowledgements

The author thanks [Mentor] for mentorship, laboratory training, and extensive guidance throughout this project, including the suggestion to pool the ventral mPFC subregions for the dorsal-to-ventral gradient analysis, which produced the study's primary significant finding. [Collaborator] assisted with the human gene expression dataset analysis. This research was conducted through Pasadena Bio Collaborative Labs. Human gene expression and clinical data were obtained from the Ivy Glioblastoma Atlas Project (Ivy GAP), developed by the Allen Institute for Brain Science and the Ben and Catherine Ivy Foundation.

Mentor Contribution Statement

[Mentor] provided mentorship, laboratory training, and oversight throughout this project. [He/she] 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/she] helped frame the post-hoc EGFR findings as exploratory and provided extensive feedback on the

interpretation and presentation of results. [Collaborator] assisted with the human gene expression dataset analysis.

Author Biography

[Author] is a high school junior at [High School] in [City], California, conducting cancer biology research at [Laboratory], with a focus on sex-dependent glial biology and glioblastoma. [Author's] broader research experience includes single-cell RNA sequencing of bladder cancer models at a UC campus laboratory, multi-omics analysis of renal cell carcinoma at a UC Comprehensive Cancer Center, and cancer biology research at Cambridge University. [Author] is interested in translational oncology and sex-stratified approaches to precision medicine. [Identifying details redacted for blind review; full biography submitted via portal.]

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Editor Recommendation:

Both reviewers commend the student for their thoughtful and sophisticated manuscript, and both recommend minor text revisions prior to acceptance.

Reviewer 1:

Overall, the paper is well written with a clear goal of using two study designs to assess survival differences between sexes in glioblastoma (GBM) to provide mechanistic insight into how these survival differences are promoted in GBM. The integration of a rodent model of GBM paired with assessment of GBM patient samples provides translational insight into potential mechanisms promoting differences in survival observed in GBM patients. The two reported studies were well designed and thought out. Outcomes from these two studies provide good initial insight into how glial profiles and EGFR amplification status may promote changes in the TME molecular architecture resulting in survival differences observed in GBM thus addressing a significant gap in the current literature. Minor revisions are included below to bolster the manuscript for acceptance:

In the Introduction section, line 98, I would recommend adding in a smoother transition sentence into your hypothesis to make the previous paragraph logically flow into this last paragraph for section 1.4 (prior to getting into section 1.5 with the study rationale and hypotheses).

The study rationale and hypotheses are well thought out and detailed with clear, concise sentences to describe the experimental design to the reader. Well done!

For all sentences that have "–" in them please consider restructuring the sentences to make the sentences clearer and concise when reporting interpretations or suggesting mechanistic pathways. Some of these sentences feel too long at times and can be broken up into smaller clearer sentences.

In the discussion section lines 388-398 I would recommend rewording this to include something along the lines of "…grounded in the canonical function of EGFR amplification.".

In the Discussion section line 403 please make "show" past tense as the experiment was completed and you are reporting these concluded results.

In the Discussion section lines 416-418 consider using different verbiage for "shows up" when describing the healthy brain versus tumor tissue.

In the Discussion section line 424 animal should be "animal's" sex.

In the last paragraph of your conclusions section, I would recommend expanding on how your findings can inform better therapeutic interventions/treatments to address the survival differences between male vs female patients. How can the insights from your studies enhance treatment to decrease the survival differences between male and female GBM patients? Strengthening this will end the manuscript on a stronger note.

Reviewer Final Recommendation: Accept with minor revisions

Reviewer 2:

Comments:

This manuscript investigates sex-dependent differences in glial organization using two distinct datasets: (1) glial density measurements in rat medial prefrontal cortex and (2) GFAP/AIF1

expression ratios in human glioblastoma stratified by EGFR amplification status. The authors propose that sex acts primarily as an “organizing variable,” influencing glial responsiveness to perturbation rather than baseline abundance. The manuscript is conceptually ambitious and clearly written. The student demonstrates a good understanding of the limitations of the work and makes a reasonable effort to address them in the Discussion section, while also attempting to interpret the broader implications of the findings. Several key claims would benefit from more cautious framing. Overall, this is a well-written and thoughtfully developed manuscript that addresses an interesting and potentially important question. However, several issues need to be addressed before the manuscript can be accepted.

Major comments:

1) The rationale for examining dorsal versus ventral glial density in the mPFC requires further strengthening and a clearer connection to glioblastoma biology. While the manuscript notes that the frontal lobe is a common site of GBM occurrence and that the mPFC shows sex-dependent glial organization, the justification for using the dorsal–ventral axis as the primary framework remains underdeveloped. At present, the link between regional glial architecture in healthy rat mPFC and sex-dependent differences in the human GBM microenvironment appears largely conceptual rather than mechanistically grounded.

In particular, the manuscript should more clearly explain why dorsal -versus-ventral glial distribution is relevant to GBM progression, immune regulation, or sex-dependent outcomes. It would also strengthen the work to discuss whether glial density is known to vary along this axis and whether prior studies report sex-dependent spatial differences in mPFC glial organization, ideally supported by relevant literature on regional glial heterogeneity.

Additionally, because the central framework is that sex differences emerge through “organizational gradients” rather than absolute abundance, the authors should better justify why the dorsal–ventral axis represents a biologically meaningful gradient for glioma-related processes. Overall, the framework is interesting but remains somewhat speculative in its current form, and would benefit from clearer mechanistic grounding and integration with prior work.

2) Several statistical concerns should be addressed to improve the rigor of the analysis and interpretation. First, given the extremely small sample size in Study 1 (n = 3 per sex), the use of repeated-measures and two-way ANOVA may not be well justified. Because the primary comparisons appear to involve male-versus-female differences within individual regions, simpler

nonparametric approaches such as rank-sum (Mann–Whitney) tests may be more appropriate and more robust to violations of normality assumptions. The current ANOVA framework may overstate the apparent structure of the data relative to what can realistically be supported at this sample size.

In addition, the interpretation of borderline p-values should be made more cautiously. In Table 3, the comparison with $p = 0.169$ should not be described as “trending,” as this terminology may imply biological support that is not statistically warranted. Similarly, the dorsal-versus-ventral glial density comparison yielded $p = 0.047$ in males and $p = 0.068$ in females, indicating that the difference between sexes is itself relatively modest. Given the very small sample sizes and overlapping significance thresholds, the manuscript should avoid strong claims regarding sex-specific organizational effects based on these comparisons alone. Overall, the statistical interpretation would benefit from more conservative language and clearer acknowledgment of the exploratory nature of the findings.

3) Abstract - Together, these findings suggest that sex-dependent glial microenvironment differences may contribute to the female survival advantage in GBM, and that sex-stratified EGFR analysis may inform treatment approaches for this disease.

“Median survival in glioblastoma has not improved since 2005.” The final sentence is somewhat ambiguous and reads as if part of the abstract or a concluding claim may be missing, particularly regarding how the stated mechanism is meant to connect to a survival advantage. In addition, the assertion that sex differences contribute to a survival advantage is not clearly supported or explicitly developed in the main text, and as currently phrased it represents a relatively strong interpretive leap beyond the presented data.

<u>Minor Comments:</u>

1) Future work should present violin or box plots alongside means to better characterize distributional differences between sexes. -> The author may want to include violin or box plots

2) Figure 1: A statistical comparison for the ILA region was not reported.

Final Recommendation

- Accept with minor revisions (acceptance conditional on satisfactory minor revisions)

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

[Author details redacted for blind review]

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, sex differences, glial cells, tumor microenvironment, EGFR amplification

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 how immunosuppressive the tumor microenvironment is and how effectively the immune system can 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.

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 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 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.

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 px^2) 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.

Figure 1. mPFC glial density by subregion and sex

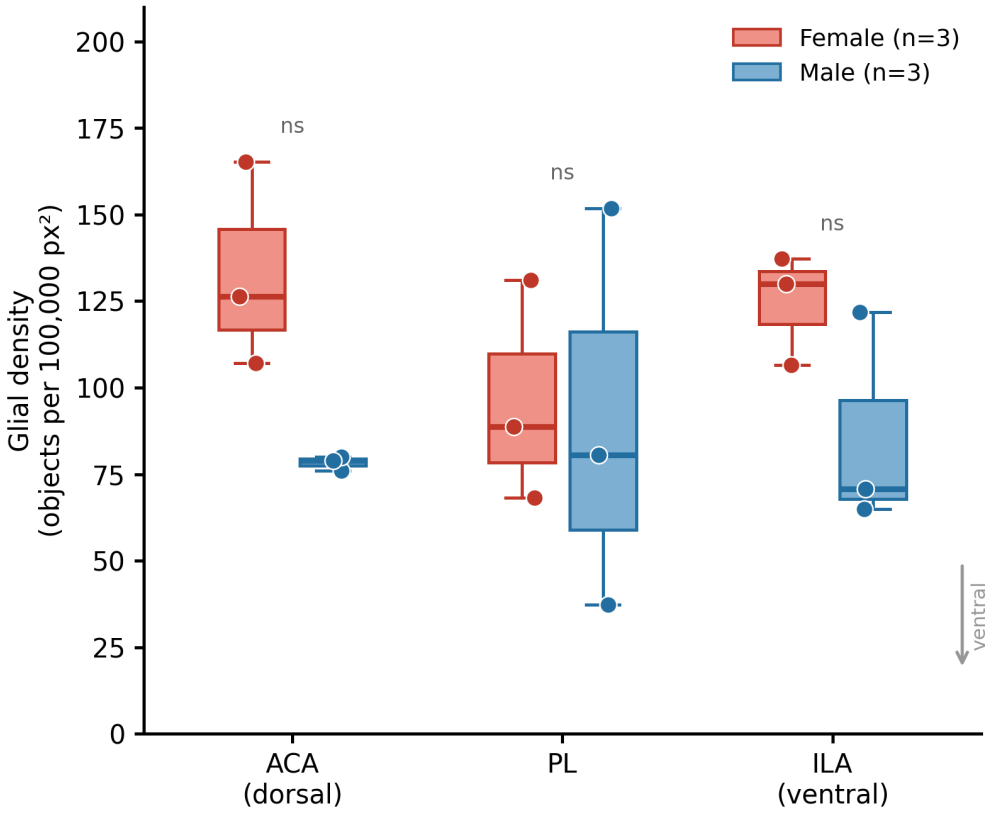

Figure 1. Mean glial cell density across three mPFC subregions by sex (n = 3 per sex). Red = female; blue = male. Subregions are ordered dorsal to ventral (ACA, PL, ILA). Females showed consistently higher mean glial density (non-significant) than males in all subregions, with the ACA and ILA comparisons 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 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

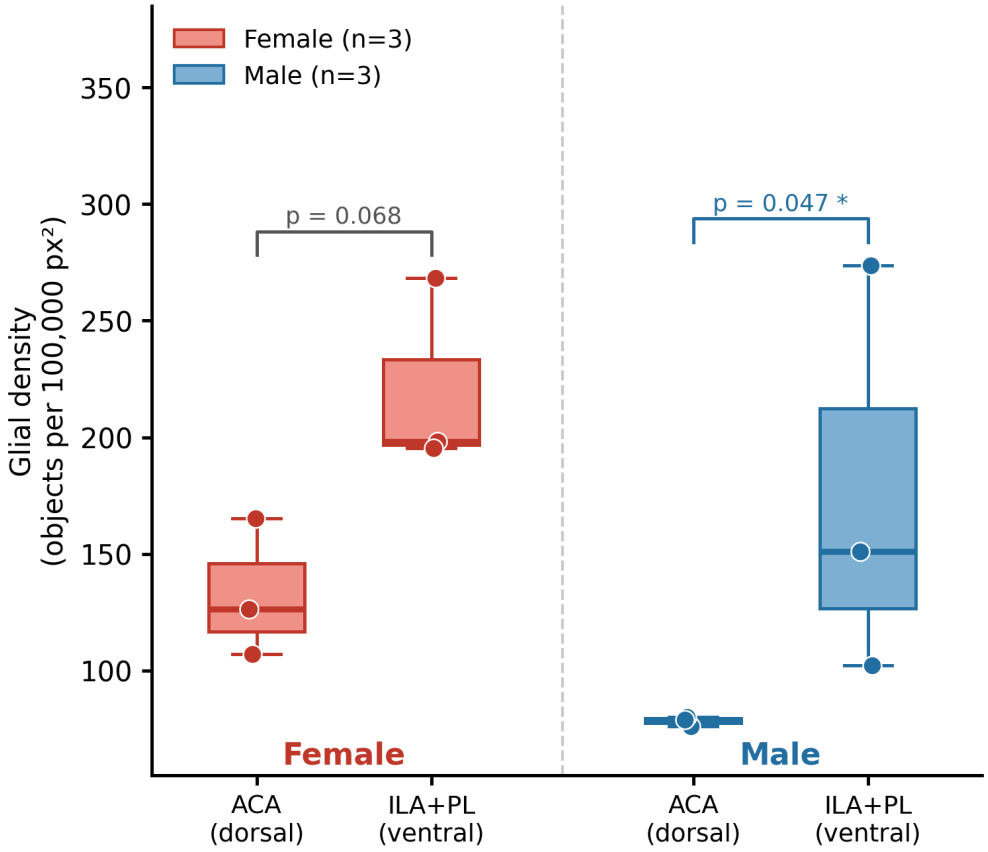

Figure 2. Dorsal ACA glial density versus pooled ventral mPFC composite measure (summed ILA + PL) by sex. Red = female; blue = male. Male rats showed a statistically significant dorsal-to-ventral difference (p = 0.047, Fisher's LSD), with substantially lower glial density in the ACA than in ventral subregions. 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 $\alpha = 0.05$ 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) 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 shapes not 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.

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.

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.

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

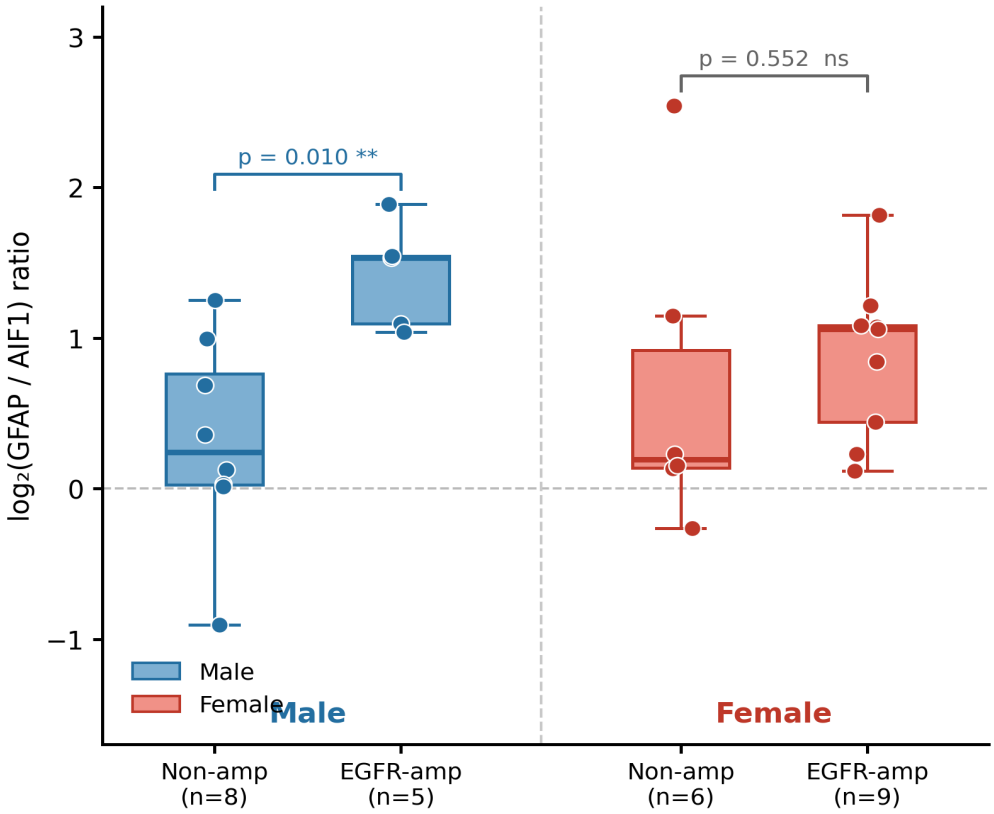

Figure 3. log²(GFAP/AIF1) ratio by EGFR amplification status and sex. Red = female; blue = male. EGFR amplification was a significant main effect overall (p = 0.021). Within males, EGFR-amplified tumors showed a significantly higher ratio than non-amplified tumors (p = 0.010). Within females, no significant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

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 is not driving 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.

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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Mentor Contribution Statement

[Mentor] provided mentorship, laboratory training, and oversight throughout this project. [He/she] 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/she] helped frame the post-hoc EGFR findings as exploratory and provided extensive feedback on the interpretation and presentation of results. [Collaborator] assisted with the human gene expression dataset analysis.

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

[Author details redacted for blind review]

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 ~~chemotherapy~~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, sex differences, glial cells, tumor microenvironment, EGFR amplification

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 how immunosuppressive the tumor microenvironment is and how effectively the immune system can 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.

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. ~~P-values were not corrected for multiple comparisons.~~

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 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 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.

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.

Figure 1. mPFC glial density by subregion and sex

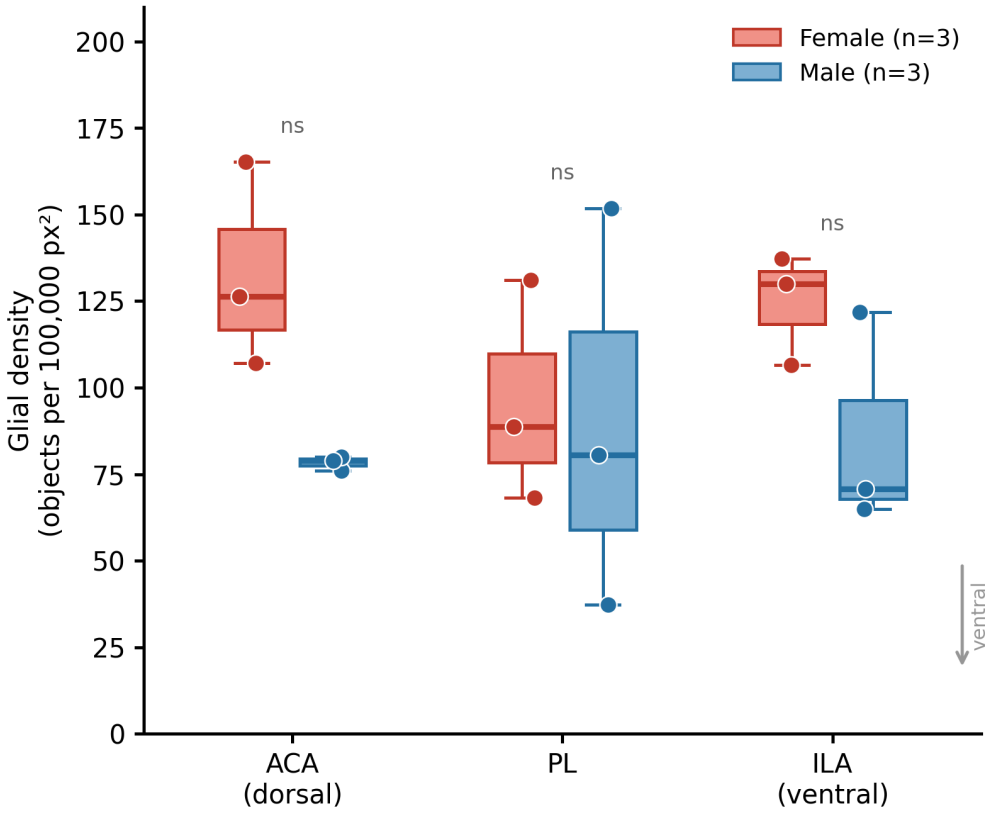

Figure 1. Mean glial cell density across three mPFC subregions by sex (n = 3 per sex). Red = female; blue = male. Subregions are ordered dorsal to ventral (ACA, PL, ILA). Females showed consistently higher mean glial density (non-significant) than males in all subregions, with the ACA and ILA comparisons 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 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

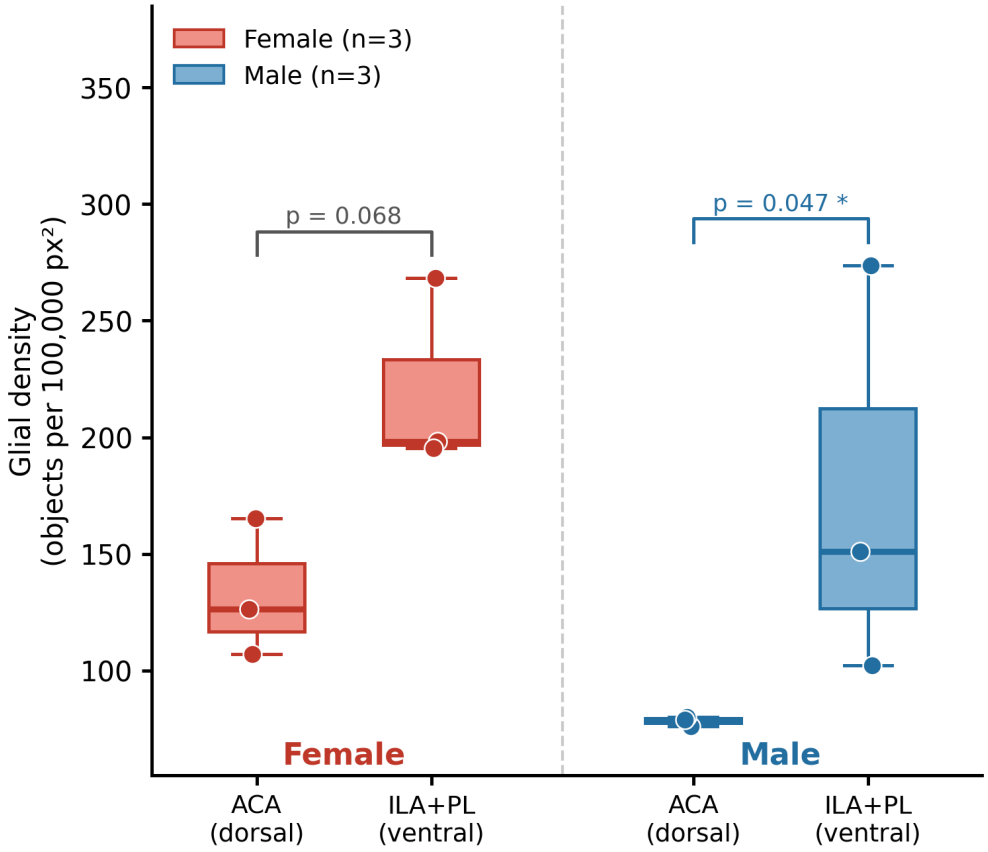

Figure 2. Dorsal ACA glial density versus pooled ventral mPFC composite measure (summed ILA + PL) by sex. Red = female; blue = male. Male rats showed a statistically significant dorsal-to-ventral difference (p = 0.047, Fisher's LSD), with substantially lower glial density in the ACA than in ventral subregions. 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 $\alpha = 0.05$ 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) 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 shapes not 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.

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.

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.

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

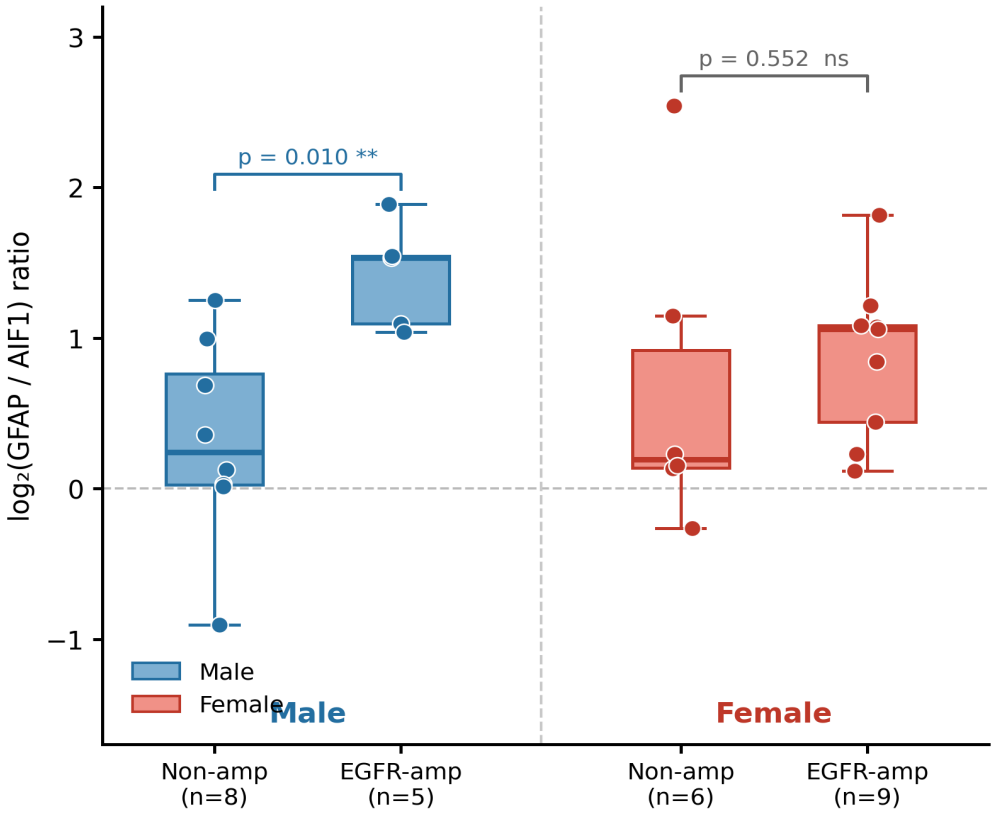

Figure 3. log²(GFAP/AIF1) ratio by EGFR amplification status and sex. Red = female; blue = male. EGFR amplification was a significant main effect overall (p = 0.021). Within males, EGFR-amplified tumors showed a significantly higher ratio than non-amplified tumors (p = 0.010). Within females, no significant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

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 is not driving 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.

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.

Acknowledgements

The author thanks [Mentor] for mentorship, laboratory training, and extensive guidance throughout this project, including the suggestion to pool the ventral mPFC subregions for the dorsal-to-ventral gradient analysis, which produced the study's primary significant finding. [Collaborator] assisted with the human gene expression dataset analysis. This research was conducted through Pasadena Bio Collaborative Labs. Human gene expression and clinical data were obtained from the Ivy Glioblastoma Atlas Project (Ivy GAP), developed by the Allen Institute for Brain Science and the Ben and Catherine Ivy Foundation.

Mentor Contribution Statement

[Mentor] provided mentorship, laboratory training, and oversight throughout this project. [He/she] 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/she] helped frame the post-hoc EGFR findings as exploratory and provided extensive feedback on the interpretation and presentation of results. [Collaborator] assisted with the human gene expression dataset analysis.

Author Biography

[Author] is a high school junior at [High School] in [City], California, conducting cancer biology research at [Laboratory], with a focus on sex-dependent glial biology and glioblastoma. [Author's] broader research experience includes single-cell RNA sequencing of bladder cancer models at a UC campus laboratory, multi-omics analysis of renal cell carcinoma at a UC Comprehensive Cancer Center, and cancer biology research at Cambridge University. [Author] is interested in translational oncology and sex-stratified approaches to precision medicine. [Identifying details redacted for blind review; full biography submitted via portal.]

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Response to Reviewer 1

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

Submission ID: 100178 (Revision 2)

Journal: Convergence Journal

Date: June 2026

Dear Reviewer 1,

Thank you for your thorough and constructive feedback on my revised manuscript. Your comments have allowed me to strengthen the paper further. Below I address each of your three comments in turn. All changes are indicated in the accompanying tracked-changes document.

Comment 1 for my Abstract: Define temozolomide

Reviewer comment:

> *"Please briefly explain what temozolomide is when mentioned."*

Response:

I agree that readers unfamiliar with standard GBM treatment would benefit from a brief definition at first mention. The abstract has been revised to include a parenthetical definition directly after the word:

Revised text (Abstract): *"…and median survival has not meaningfully improved since temozolomide, an oral alkylating chemotherapy agent, was adopted as standard of care in 2005."*

No other changes were made to this sentence.

Comment 2 for Figure 3: Overlapping legend labels

Reviewer comment:

> *"The male and female figure legends appear to overlap and should be adjusted for clarity and readability."*

Response:

This overlap was introduced by the automatic legend placement in the original matplotlib code. The Figure 3 visualization script has been revised: the legend has been repositioned explicitly to the upper-right area of the plot with a fixed bounding box anchor and increased padding from the axis, eliminating the overlap between the Female (red) label and the Male axis label. A revised Figure 3 is included in the manuscript.

Note: Reviewer 2 raised the same concern about Figure 3, and the same fix addresses both comments.

Comment 3 for Statistical contradiction in Methods

Reviewer comment:

"There is a direct contradiction in the statistical reporting within the Methods section. The authors state that Šídák's post-hoc test was used for the two-way repeated measures ANOVA (Study 1). However, the final sentence of the same section explicitly states, "P-values were not corrected for multiple comparisons." By definition, Šídák's method is a family-wise error rate control procedure designed specifically to correct for multiplicity. These two statements cannot coexist."

Response:

You are correct. This is an internal contradiction. Šídák's post-hoc test is a family-wise error rate correction procedure; the sentence stating that p-values were not corrected for multiple comparisons directly conflicts with its stated use. This sentence has been deleted from the Study 1 statistical analysis section.

Change to Study 1 Statistical Analysis (Methods): *Deleted: "P-values were not corrected for multiple comparisons." The paragraph now ends: "…Effect sizes were calculated as Hedges' g. All statistics were computed in GraphPad Prism 10."*

In addition, the Limitations section previously carried a similar statement, "Across both studies, p-values were not corrected for multiple comparisons". which was also inaccurate with respect to Study 1's RM ANOVA. This sentence has been revised to accurately characterize the correction procedures used:

Revised text (Limitations): *"…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."*

I am very grateful to Reviewer 1 for these precise and actionable comments, which have improved the statistical transparency of the manuscript. Wishing you the best summer!

Sincerely,

Daniel Zhang

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Response to Reviewer 2

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

Submission ID: 100178 (Revision 2)

Journal: Convergence Journal

Date: June 2026

Dear Reviewer 2,

Thank you for your kind assessment of the revision and for the thoughtful additional comments. I appreciate the recognition of improvements to the manuscript's logical flow and conceptual grounding. Below I've addressed each of your comments in turn, and all changes are indicated in the accompanying tracked-changes document.

Comment 1 about the Abstract: Unmet medical need statement

Reviewer comment:

> *"In the Abstract, following lines 5–7 or in addition to these lines, I suggest adding in a statement about a significant gap in the literature that must be addressed to increase survivability in patients diagnosed with glioblastoma or a statement highlighting this unmet medical need in the population."*

Response:

I definitely agree that explicitly framing the sex survival gap as an unmet need strengthens the abstract's clinical relevance. A sentence has been added directly after the statement that the biological basis of the gap remains unresolved:

Added sentence (Abstract): *"Identifying the cellular and molecular mechanisms underlying this sex-specific survival difference represents a critical unmet need in neuro-oncology."*

This addition appears in the third sentence of the abstract, immediately before the description of the existing research gap.

Comment 2 for the Introduction §1.1: Transitional sentence linking paragraphs 1 and 2

Reviewer comment:

> *"In the introduction section, section 1.1 lines 48–50, I would recommend adding in a closing sentence/transitional sentence that links the first paragraph and the second paragraph more strongly. You can highlight that there is a significant gap in the literature that must be addressed to understand the lack of improved mortality rates and one way to do this is to understand the disparity in mortality amongst sexes."*

Response:

I 100% agree that paragraph 1 of section 1.1, which describes the grim treatment landscape and stagnant prognosis, would benefit from a transition that motivates the sex

survival discussion that follows in paragraph 2. A closing sentence has been added at the end of paragraph 1:

Added sentence (Introduction §1.1, end of paragraph 1): *"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."*

This sentence now serves as a bridge between the treatment-failure context of paragraph 1 and the introduction of the sex survival advantage in paragraph 2, as suggested.

Comment 3 regarding the Statistical analysis section: Delete contradictory final sentence

Reviewer comment:

"In the statistical analysis section, Sidak's post-hoc tests correct p-values to control for family-wise error rate so consider deleting the last sentence of the paragraph (line 225–226)."

Response:

The reviewer is correct. The sentence "P-values were not corrected for multiple comparisons" at the end of the Study 1 statistical analysis section is directly contradicted by the use of Šídák's post-hoc test, which by definition corrects for family-wise error rate. This sentence has been deleted.

Change to Study 1 Statistical Analysis (Methods): *Deleted: "P-values were not corrected for multiple comparisons." The paragraph now ends: "…Effect sizes were calculated as Hedges' g. All statistics were computed in GraphPad Prism 10."*

Additionally, I noticed that the Limitations section previously contained the statement "Across both studies, p-values were not corrected for multiple comparisons," which was similarly inaccurate for Study 1's RM ANOVA. This sentence has been revised to distinguish accurately between the Šídák-corrected comparisons (Study 1's three-region RM ANOVA) and the Fisher's LSD comparisons in both studies, which rely on omnibus F-test protection rather than a per-comparison alpha adjustment.

Note: Reviewer 1 raised the same concern about this statistical contradiction, and the same fix addresses both comments.

Comment 4 about Figure 3: Overlapping legend labels

Reviewer comment:

"Please edit the figure legend in Figure 3 as the red=female indicator is slightly overlapping the blue Male axis/subset label."

Response:

I have revised the Figure 3 Python visualization script to explicitly reposition the legend to the upper-right of the plot with a fixed bounding box anchor and increased clearance from the axis labels, eliminating the overlap between the red Female legend entry and the blue Male axis label. A revised Figure 3 is included in the manuscript.

I also note that Reviewer 1 raised the same concern, and the same revision addresses both comments.

I am sincerely grateful for Reviewer 2's continued engagement with this work. The suggested additions have measurably improved both the abstract's clinical grounding and the introduction's logical flow. Wishing a fantastic summer to Reviewer 2!

Sincerely,

Daniel Zhang

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Editor Recommendation: Accept with Minor Revisions

REVIEWER 1:

I appreciate the author's detailed and thoughtful response to the statistical concerns, particularly the transparent discussion of the limitations imposed by the extremely small sample size in Study 1. Overall, the manuscript appears to have been substantially improved in response to the reviewer comments, and the revisions have strengthened both the framing and interpretation of the findings.

Recommendation: Accept with minor revisions.

Minor comments:
1. Abstract: Please briefly explain what temozolomide is when mentioned.
2. Figure 3: The male and female figure legends appear to overlap and should be adjusted for clarity and readability.
3. There is a direct contradiction in the statistical reporting within the Methods section. The authors state that **Šídák's post-hoc test** was used for the two-way repeated measures ANOVA (Study 1). However, the final sentence of the same section explicitly states, **"P-values were not corrected for multiple comparisons."** By definition, Šídák's method is a family-wise error rate control procedure designed specifically to correct for multiplicity. These two statements cannot coexist.

REVIEWER 2:

Post-Revision Critical Review: The authors have done a wonderful job addressing the reviewer comments and with the revisions the updated manuscript has a stronger logical flow and concrete throughline. Outlined in this paragraph are minor revisions to strengthen the manuscript further.

In the Abstract, following lines 5-7 or in addition to these lines, I suggest adding in a statement about a significant gap in the literature that must be addressed to increase survivability in patients diagnosed with glioblastoma or a statement highlighting this unmet medical need in the population.

In the introduction section, section 1.1 lines 48-50, I would recommend adding in a closing sentence/transitional sentence that links the first paragraph and the second paragraph more strongly. You can highlight that there is a significant gap in the literature that must be addressed to understand the lack of improved mortality rates and one way to do this is to understand the disparity in mortality amongst sexes. The additions to the rest of the introduction subsections flow well and elevate the manuscript's conceptual knowledge base to set the stage for the Methods and Results sections.

The updates to the statistical analysis section provide additional clarity in how the data were analyzed and incorporate enough detail for replication of the study's analyses. In

the statistical analysis section, Sidak's post-hoc tests correct p-values to control for family-wise error rate so consider deleting the last sentence of the paragraph (line 225-226).

Please edit the figure legend in Figure 3 as the red=female indicator is slightly overlapping the blue Male axis/subset label.

The revisions to the Discussion and Conclusion sections further strengthen and contextualize the study's findings in relation to the current literature and how the findings from these studies advance the field of glioblastoma. Lines 612-631 (new additions since the previous manuscript version) thoroughly explain how results from the current studies can be used to advance glioblastoma therapeutic intervention/treatments to increase the survivability for glioblastoma patients and ends the manuscript on a very strong note. Well done!

Final Recommendation: Accept with minor revisions