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Familial Glioma in a Father and Son: Integrated Genomic and Multi-compartment Proteomic Profiling

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Abstract

Background/Aim: Genetic predisposition to glioblastoma multiforme (GBM) is rare and is often associated with syndromes such as Li–Fraumeni syndrome and neurofibromatosis. However, a subset of cases involves familial aggregation of glioma, and its molecular pathogenesis remains poorly understood. This study aimed to elucidate the proteomic and genomic profiles of familial glioma cases.

Case Report: A 60-year-old male (father) and his 35-year-old son presented with gliomas. Clinical histories, imaging, surgical procedures, and histological analyses were documented. Proteomic analysis of tissue, plasma-derived extracellular vesicles (EVs), and urine was performed, and a systems biology approach was used to compare molecular profiles. Computational evaluation and pathway analysis were performed. Gliomas were diagnosed according to World Health Organization (WHO) guidelines. The father was diagnosed with glioblastoma, isocitrate dehydrogenase (IDH)-wildtype, WHO grade 4, with an unmethylated O6-methylguanine-DNA methyltransferase (MGMT) promoter, whereas the son presented with astrocytoma, IDH-mutant, WHO grade 3, with a methylated MGMT promoter. Proteomic

continued

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analysis identified 1,268 proteins, with approximately 200 differentially abundant proteins (DAPs) in each biological matrix. Pathway analysis highlighted patient-specific differences, including inflammatory markers and GBM-associated proteins, with particularly notable variation in urine samples, including glycoproteins.
Conclusion: This study provides insight into proteomic differences between familial gliomas, showing that they can develop through distinct molecular profiles and pathogenic pathways despite a shared genetic background. Proteomic analysis across tissue, plasma-derived EVs, and urine revealed tumor-associated proteins that could serve as non-invasive biomarkers, capturing patient-specific molecular features that were less evident in plasma.

Keywords: Glioma, familial aggregation, proteomic analysis, molecular pathogenesis, personalized treatment.

Introduction

Gliomas represent a heterogeneous group of primary brain tumors defined by extensive molecular, cellular, and clinical diversity (1-4). While large-scale genomic studies have refined the classification of sporadic cases by resolving major molecular subtypes and evolutionary trajectories (5-9), they offer limited insight into dynamic functional states, metabolic adaptations, and the tumor microenvironment, which are more directly captured by microenvironmental and proteomic approaches (10-12). This complexity is further amplified in familial gliomas, which account for 5-10% of cases and show reproducible aggregation among first-degree relatives (13-17). Although a minority are linked to hereditary cancer syndromes (18), experimental models of NF1-associated glioma biology and broader reviews of adult glioma genetics support the contribution of inherited susceptibility pathways (19, 20). Germline mutations in genes such as *POT1* (a member of the shelterin complex involved in telomere maintenance) have been identified in certain families (21-24). Nevertheless, the vast majority of familial gliomas occur without identifiable monogenic alterations (25-27).

High-grade gliomas (HGGs) exhibit profound intra-tumoral heterogeneity, with intrinsic programs driving distinct biological behaviors even among genetically related cases (5-9). Complementing this genomic knowledge, proteomic profiling allows for the direct analysis of stress-response pathways, bridging the gap between genomic alterations and clinical phenotypes (12). Integrating tissue-derived data with analyses of biological fluids, such as plasma-derived extracellular vesicles (EVs) and urine, allows us to capture this heterogeneity across different biological compartments (28-32). Because EV isolation, characterization, and functional interpretation are highly method-dependent, internationally recognized standards and classification frameworks are essential for reproducible analysis (33-37). Although the detection of brain-derived proteins in urine is hindered by the blood–brain barrier and renal filtration, urinary exosomes and proteins can provide a readout of systemic responses to tumor progression (38).

Isolated cases of familial glioma not associated with known hereditary syndromes are extremely rare, making their proteomic landscape largely unexplored (25, 27). By integrating tissue-derived data with compartment-specific protein signatures from biological fluids, we can gain a deeper understanding of the pathogenesis of familial glioblastomas (GBMs) and support molecularly informed therapeutic strategies based on functional precision medicine, targeted sequencing, and genomic identification of actionable pathways (39-41).

In this article, we present an integrative proteomic and genomic analysis of two familial gliomas that arose almost simultaneously in a father and son. By analyzing profiles from tumor tissues, plasma extracellular vesicles (EVs), and urine, we examined how a shared genetic background intersects with divergent somatic evolution. This approach provides a functional perspective on the biology of familial gliomas, supporting the potential of systemic biological fluids for the translational identification of biomarkers.

Methods

We present a familial case of two patients, father and son, both affected by high-grade gliomas. Following surgical resection, both patients underwent the standard Stupp protocol. To further investigate the molecular basis of this rare familial occurrence, we performed an integrative analysis including genomics and high-resolution proteomics on tumor tissue, urine and plasma-derived EVs.

Both participants provided written informed consent, and the study protocol was approved by the Local Ethical Committee in accordance with the principles of the Helsinki Declaration.

Environmental assessment. To evaluate potential shared environmental risk factors, an environmental impact assessment was conducted at the patients' residence by the Regional Agency for Environmental Protection (ARPA). Electromagnetic field measurements, performed according to CEI 211/7 standards, showed exposure levels well below regulatory safety limits (effective field: 0.58 V/m; maximum field: 0.81 V/m), excluding relevant environmental electromagnetic exposure.

DNA extraction and dosage. DNA from paraffin-embedded tumor sections was extracted using QIAamp DNA FFPE tissue kit (Qiagen, Hilden, Germany), quantified using a Qiaxpert (Qiagen, Hilden, Germany), and assessed for quality using automated electrophoresis on Tape Station 2000 (Agilent Technologies, Santa Clara, CA, USA). The DNA yield was quantified using a TaqMan RNase P Detection Reagent Kit (Thermo Fisher Scientific, Waltham, MA, USA) on Real-Time PCR AriaDx (Agilent Technologies).

LC-MS/MS proteomics and systems biology analysis. Proteomic profiles were generated from whole urine, plasma-derived EVs, and FFPE tissue samples. Urine (1.5 ml) was thawed on ice and centrifuged (1,000 × g, 10 min, 4°C). Proteins were purified *via* ProteoSpin™ Kit (Norgen Biotek Corp., Thorold, ON, Canada). Plasma EVs were isolated using ExoQuick® ULTRA Kit (System Biosciences, Palo Alto, CA, USA) from samples previously centrifuged at 2,200 × *g*. FFPE sections (5 per subject) were deparaffinized in hexane, scraped, and de-crosslinked in 0.5% RapiGest SF (Waters Corp., Milford, MA, USA) at 100°C for 20 min and 80°C for 2 h. Protein concentration was determined using the Qubit™ Assay (Invitrogen™, Thermo Fisher Scientific, Rockford, IL, USA). Digestion was performed using a Trypsin/Lys-C Mix at a 1:25 enzyme-to-protein ratio overnight at 37°C. Reactions were quenched with trifluoroacetic acid (0.5% final concentration) (TFA; Sigma-Aldrich Inc., St. Louis, MO, USA). Peptides were purified by C18 spin columns (Pierce™ PepClean™, Thermo Fisher Scientific for Urine and EVs; G-Biosciences, St. Louis, MO, USA for FFPE), dried, and reconstituted in 0.1% formic acid for nano-liquid chromatography-tandem mass spectrometry (nLC-MS/MS) analysis.

Proteomic analyses were performed using nano-flow liquid chromatography systems coupled to high-resolution mass spectrometers. Urine and plasma-EV peptide mixtures were analyzed on an Eksigent nanoLC-Ultra 2D/cHiPLC-nanoflex system (Eksigent Technologies, Dublin, CA, USA) coupled to an Orbitrap Exploris 120 mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) in trap-elute mode. FFPE tissue samples were analyzed using an Eksigent nanoLC 415 system (SCIEX, Framingham, MA, USA) interfaced with an OptiFlow Turbo V-equipped TripleTOF 6600+ mass spectrometer (AB SCIEX, Concord, ON, Canada). For all matrices, peptides were first loaded onto a C18 trap column at a flow rate of 3 µl/min and subsequently separated on C18 analytical columns at a flow rate of 300 nl/min. Data acquisition was performed in data-dependent mode (DDA).

Raw MS data were processed using Proteome Discoverer (v2.5, Thermo Fisher Scientific) and MaxQuant (v2.5.2.0, Max Planck Institute of Biochemistry, Martinsried, Germany). Database searches were performed on UniProt using the *Homo sapiens* reference proteome (November 2023) and a trypsin digestion model. Precursor mass tolerance was 10 ppm and fragment tolerance was 0.02 Da. A stringent False Discovery Rate (FDR) threshold of <0.01 was applied.

Multivariate statistical analysis was conducted using JMP® Pro (v17, JMP Statistical Discovery LLC, Cary, NC). Linear Discriminant Analysis (LDA) was applied as a dimensionality reduction and pattern recognition method to identify proteins contributing to variability across biological matrices and subjects. To ensure analytical robustness given the limited sample size, the analysis was performed considering technical replicates for each condition. A significance threshold of $p<0.05$ was used for the selection of Differentially Abundant Proteins (DAPs). These DAPs were subsequently utilized as the input for Protein-Protein Interaction (PPI) network reconstruction in Cytoscape (v3.10, Cytoscape Consortium, San Diego, CA/Seattle, WA) using the STRING database. Detailed protocols, instrumental configurations, and bioinformatic parameters are available in the Supplementary Materials.

Case Report

Tumor tissue, plasma-derived EVs, and urine from the two familial glioma cases (father and son) were profiled using proteomics, identifying matrix-specific DAPs and pathway alterations consistent with their distinct genomic backgrounds.

Case 1 (Father). A 60-year-old male, a plumber by trade, was referred to our hospital and presented with headache. His previous clinical history was negative. Computed tomography (CT) and head magnetic resonance imaging (MRI) with gadolinium scan showed a left-sided temporal intraxial tumor 3×3 cm with peripheral contrast enhancement and central necrosis (Figure 1A). The patient underwent left pterional craniotomy for en-bloc resection of the lesion (Figure 1B). The surgical procedure was aided by neuronavigation, intraoperative neurophysiology monitoring and intraoperative 5-aminolevulinic acid (5-ALA) fluorescence (42). No neurological deficits were detected in the postoperative period and histology characterized the tumor as glioblastoma, IDH-wildtype, WHO CNS grade 4, MGMT promoter unmethylated. Genetic tests conducted postoperatively, in accordance with the 2021 World Health Organization (WHO) guidelines, showed the absence of pathogenic mutations and 1p/19q codeletion. The patient started standard Stupp protocol and developed pulmonary embolisms after two months that were successfully treated with medical therapy. The patient was followed up and despite no recurrence or residual tumor at 10 months, a new 2 mm enhancement spot posterior to the previous known lesion was noted on imaging (Figure 1C). This new lesion remained stable till two years after surgery when a new MRI showed tumor progression further confirmed using a methionine positron emission tomography (MET-PET) head scan, showing infiltration of the basal ganglia.

A second craniotomy was performed [aided by navigated transcranial magnetic stimulation (nTMS) mapping, Figure 1D], achieving left temporal lobectomy, with no new neurological deficits; postoperative imaging showed gross total removal of the lesion extending to the basal ganglia (Figure 1E). The last head MRI with gadolinium 10 months after the second surgery and 34 months after the first craniotomy showed tumor progression, not eligible for surgery (Figure 1F). The patient developed another pulmonary embolism successfully treated with oral medication and started Lomustine with significant regression of the lesion at last follow-up after three years from the first surgery (Figure 1G). The patient is still able to stand and walk (mRS score 4) but requires support for daily activity.

Case 2 (Son). A 35-year-old male, left-handed veterinarian, son of the previous patient, presented to our clinic three months after his father due to generalized seizures that required sedation and intubation. His clinical history was positive for eye surgery during his childhood for strabismus. CT scan and MRI with gadolinium showed a right premotor lesion, 5×4 cm with a smaller hyperintense region in T2 TSE and T2 FLAIR sequences (Figure 1H-I). Since the lesion affected the dominant hemisphere, the patient underwent advanced preoperative planning with tractography (Figure 1J), spectroscopy (Figure 1K), and

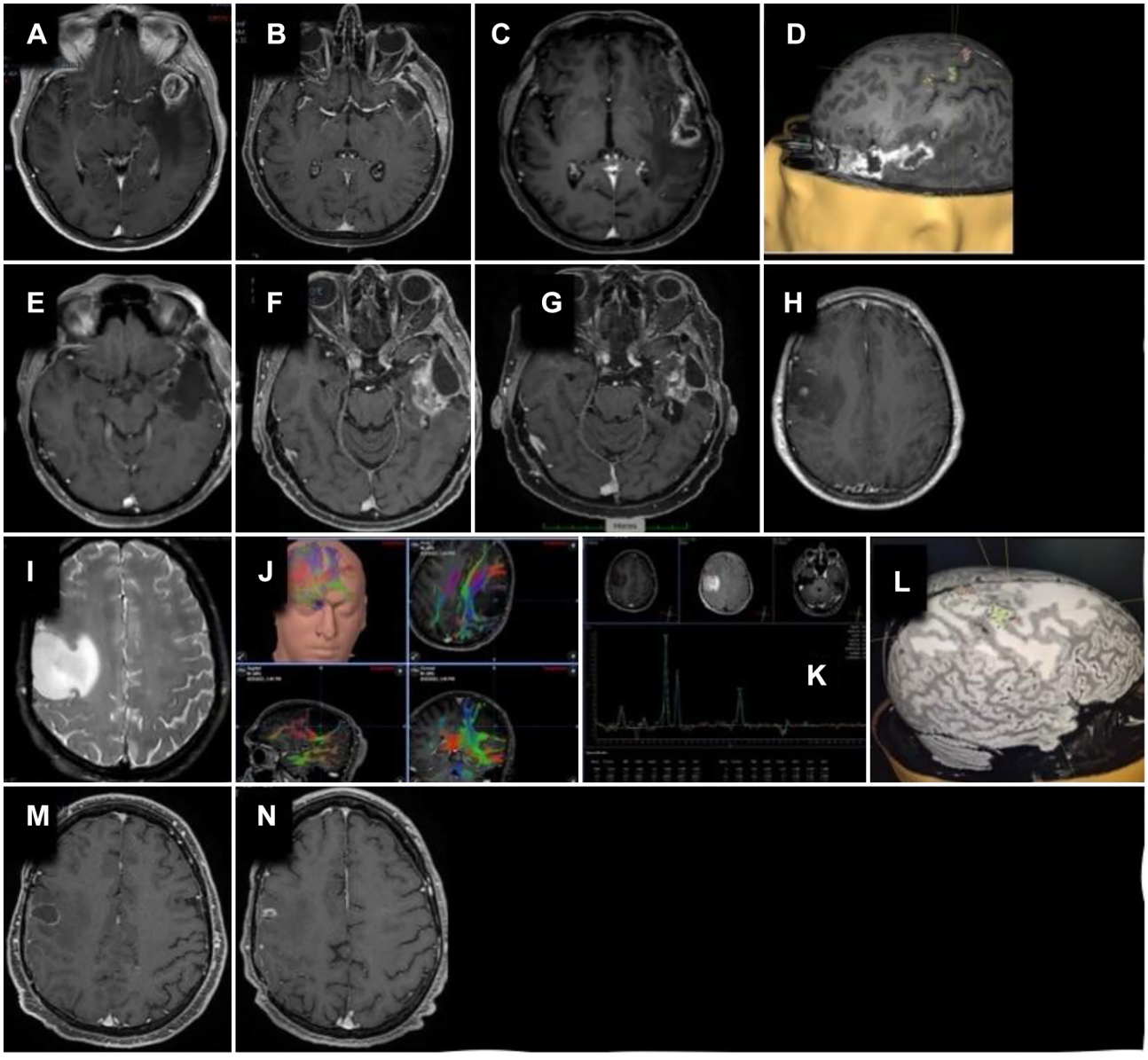


Figure 1. *Case illustrations. (A) Preoperative axial T1-weighted magnetic resonance imaging (MRI) with gadolinium of Case 1 (father) showing a left temporal lesion. (B) Postoperative axial T1-weighted MRI with gadolinium of Case 1 showing gross total removal of the lesion and sparing of the middle cerebral artery M1-M2 segments. (C) Documentation of the first recurrence of Case 1. (D) Preoperative navigated transcranial magnetic stimulation (nTMS) cortical mapping before the second surgery of Case 1 (dots locate motor area). (E) Postoperative axial T1-weighted MRI with gadolinium of Case 1 after the second surgery, showing gross total removal of the lesion extending to the basal ganglia and sparing of the middle cerebral artery M1-M2 segments. (F) Second recurrence of Case 1. (G) Reduction of the second recurrence after treatment with lomustine. (H) Preoperative axial T1-weighted MRI with gadolinium of Case 2 (son) showing a right dominant fronto-parietal lesion with a spot of enhancement. (I) T2 TSE axial MRI of Case 2 showing the extension of the glioma. (J) Preoperative MRI tractography reconstruction for intraoperative neuronavigation of Case 2. (K) Preoperative MRI spectroscopy of Case 2. (L) Preoperative nTMS cortical mapping before surgery of Case 2 (dots locate motor area). (M) Early postoperative axial T1-weighted MRI with gadolinium of Case 2 showing the surgical extent of resection, limited to the eloquent area infiltrated by the lesion. (N) Last follow-up at 3 years showing postoperative axial T1-weighted MRI with gadolinium of Case 2, documenting the surgical scar and the regression of the surrounding lower-grade lesion.*

cortical nTMS mapping (Figure 1L), that documented functional parenchyma crossing the lesion.

The patient was provided with psychological support to help him in this phase, particularly difficult owing to the concomitant condition of the father. Due to the high risk of seizures and the psychological status of the patient, we planned a fully asleep, low-risk surgery, aiming to remove the contrast enhanced spot while avoiding any neurological deficit. A right frontal craniotomy was centered on the lesion with assistance of neuronavigation (43). After opening of the dura, the lesion appeared pallid and swollen, clearly different from the healthy tissue. Direct cortical stimulation confirmed the nTMS mapping. Aided by 5-ALA and cortical-subcortical stimulation with suction probe, the contrast enhanced portion was located and removed (Figure 1M).

Histological examination documented astrocytoma, IDH-mutant, WHO CNS grade 3, MGMT promoter methylated. Postoperative genetic testing, performed according to the 2021 WHO guidelines, revealed IDH1 R132H and TP53 R175H mutations and no 1p/19q codeletion. The patient was addressed to standard STUPP protocol. Post treatment, he returned to work. The patient is stable at the last follow-up after 34 months with no recurrence or tumor progression, showing regression of the surrounding lower grade lesion (Figure 1N).

EV characterization. Extracellular vesicle concentration and size distribution were characterized *via* Nanoparticle Tracking Analysis (NTA) and validated using Tunable Resistive Pulse Sensing (TRPS). Plasma samples generally showed higher vesicle concentrations compared to urine. In plasma, average EV sizes were 161 nm and 135 nm for father and son, with concentrations of 1.51×10^{10} and 1.06×10^{11} particles/ml, respectively. While the subsequent proteomic analysis was conducted on whole urine to maximize biomarker recovery, NTA was performed on isolated urinary EVs to confirm their presence and morphology. Urinary EVs exhibited larger average sizes (218 nm and 160 nm) and lower concentrations (3.91×10^{9} to 6.65×10^{9} particles/ml) compared to plasma.

Proteomic data. Proteomic profiling allowed a complete characterization of the molecular profiles of both subjects across tissue, plasma-derived EVs, and urine. A total of 916 proteins were identified in tissue samples, 718 in urine, and 263 in plasma-derived EVs.

To evaluate protein distribution across biological matrices, Venn diagrams were generated for each subject (Figure 2A and 2B). In both cases, most of the proteome was matrix-specific. The consistency of the proteomic profiles between the two subjects was further assessed through inter-subject comparisons for each matrix, showing a high degree of overlap within the same sample type (Figure 3).

The datasets from the three matrices were merged and filtered (to include only IDs present in at least two replicates), resulting in a core list of 1,268 non-redundant proteins. To assess the overall proteomic profile, hierarchical clustering was performed on the 716 DAPs identified *via* LDA. This analysis revealed a primary segregation of samples based on their biological matrix (Figure 4). Notably, this matrix-based clustering remained predominant even when, in a secondary analysis, the samples were divided into two groups (father *vs*. son); the primary segregation continued to reflect biological origin rather than individual identity (Figure 5).

This confirms that matrix-specific proteomic signatures outweigh interindividual variability, reinforcing the consistency of our multi-compartmental analysis. To identify the proteins driving the variability within each biological compartment, a matrix-specific LDA was performed, yielding 183 DAPs for tissues, 230 for urine, and 72 for plasma-derived EVs. The hierarchical clustering for each matrix and the corresponding matrix-specific DAP sets are shown in Figure 6.

The overlap among these protein sets, illustrated in Figure 7, was remarkably limited; this underscores the highly distinct and complementary molecular insights provided by each biological matrix. To visualize the functional relationships and specific pathways of each matrix, protein-protein interaction networks were generated for tissue (Figure 8), urine (Figure 9), and plasma-derived EVs (Figure 10).

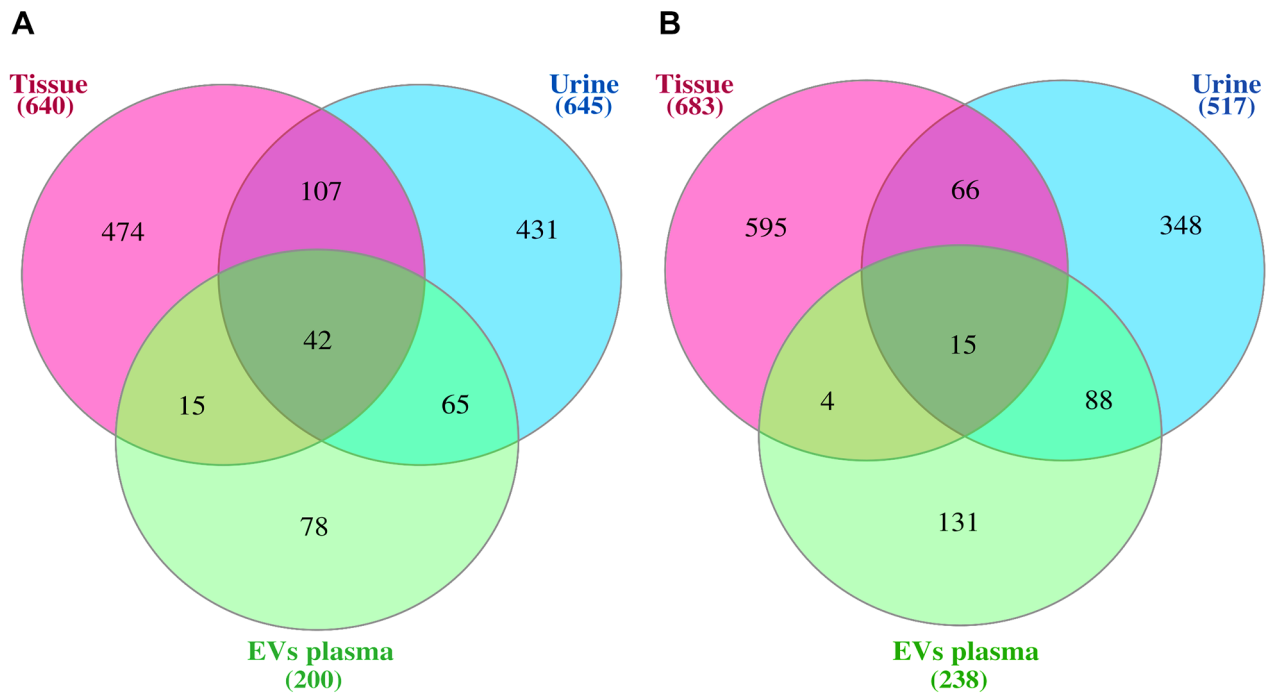


Figure 2. *Matrix-specific protein distribution. Venn diagrams showing the overlap of proteins identified in tissue (red), urine (blue), and plasma-derived extracellular vesicles (EVs) (green) for the father (anonymized as CT_01; A) and the son (anonymized as CT_02; B).*

PPI networks. These networks were constructed using DAPs, and their biological significance was summarized using bubble plots (Figure 11). The protein networks revealed distinct clusters for each matrix: while the tissue proteome directly reflects the tumor mass, biofluids (especially urine) highlight systemic responses and compartment-specific oncogenic modules.

The tissue PPI network revealed distinct expression profiles between the two subjects: clusters associated with the innate immune response, glycolysis, and lipid metabolism were composed predominantly of proteins that were upregulated in the father. The son's profile, however, was characterized by pathways related to neurological development, cytoskeletal organization (tubulins and intermediate filaments), and gene expression signaling, suggesting a more metabolic/inflammatory profile in the IDH-wild-type patient compared to a structural/developmental profile in the IDH-mutant patient.

The urinary PPI network showed significant functional overlap with tissues, particularly regarding the immune system, which was predominantly upregulated in the father. Furthermore, the urinary proteome revealed clusters such as the Extracellular Matrix and Oncogenic Signaling (including Apoptosis and Gene Expression Regulation), associated with proteins overexpressed in the father. In this matrix, the proteostasis cluster (characterized by serine proteases and heat shock proteins) was prominent in the father, likely reflecting the systemic filtration of tumor-derived stress markers. In contrast, the son's urine samples were characterized by the prevalence of proteins involved in antioxidant pathways and cellular signaling, consistent with the more adaptive and structural profile observed at the tissue level.

Finally, the plasma EV network exhibited a more streamlined functional profile. In particular, the immune response was more pronounced in the father, thus reflecting the inflammatory signals observed in both tissues and urine. Furthermore, the presence of upregulated cellular signaling clusters in the son, along with a metabolic profile shared by all three matrices,

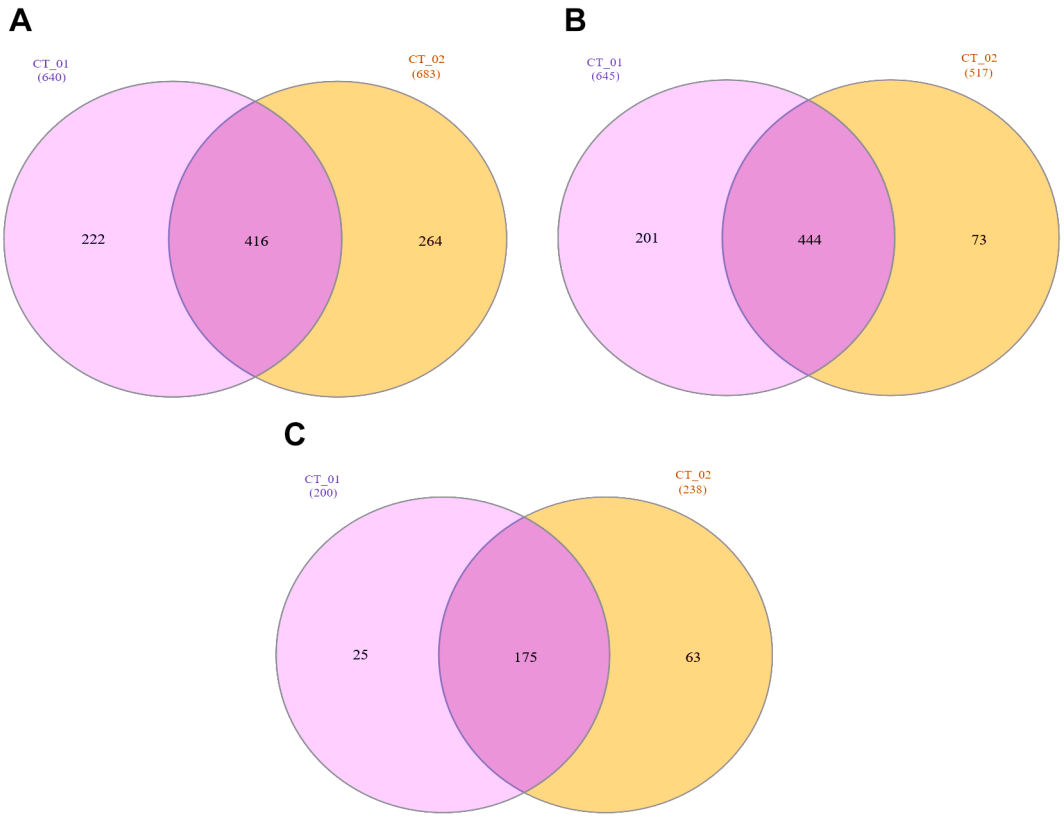


Figure 3. *Inter-subject protein overlap. Venn diagrams showing the overlap of proteins identified between the father (anonymized as CT_01) and the son (anonymized as CT_02) in: (A) tissue, (B) urine, and (C) plasma-derived extracellular vesicles (EVs).*

suggests that plasma EVs may act as selective carriers of systemic signaling mediators rather than as cellular debris.

Discussion

The near-simultaneous development of high-grade gliomas in a father and son represents an exceptionally rare familial presentation of an otherwise sporadic disease, offering a unique framework to interrogate how inherited susceptibility, somatic evolution, and tumor-derived signaling pathway activation intersect in gliomagenesis. Although familial gliomas account for only a small fraction of cases, epidemiological studies consistently report an approximately two-fold increased risk among first-degree relatives of affected individuals, supporting the existence of shared biological determinants beyond chance alone (13-17). Consistent with this, environmental factors were ruled out *via* standardized electromagnetic field assessments (ARPA protocols), which showed no abnormal exposure, further pointing toward a genetic or molecular predisposition.

While well-defined cancer predisposition syndromes such as Li–Fraumeni syndrome, neurofibromatosis type 1, and others explain a subset of familial gliomas (18-20), many familial cases arise in the absence of identifiable syndromic alterations. This observation aligns with emerging evidence implicating subtler mechanisms, including telomere dysfunction and impaired genome maintenance, with rare variants in genes such as *POT1* contributing to glioma susceptibility beyond classical hereditary cancer pathways (21-24).

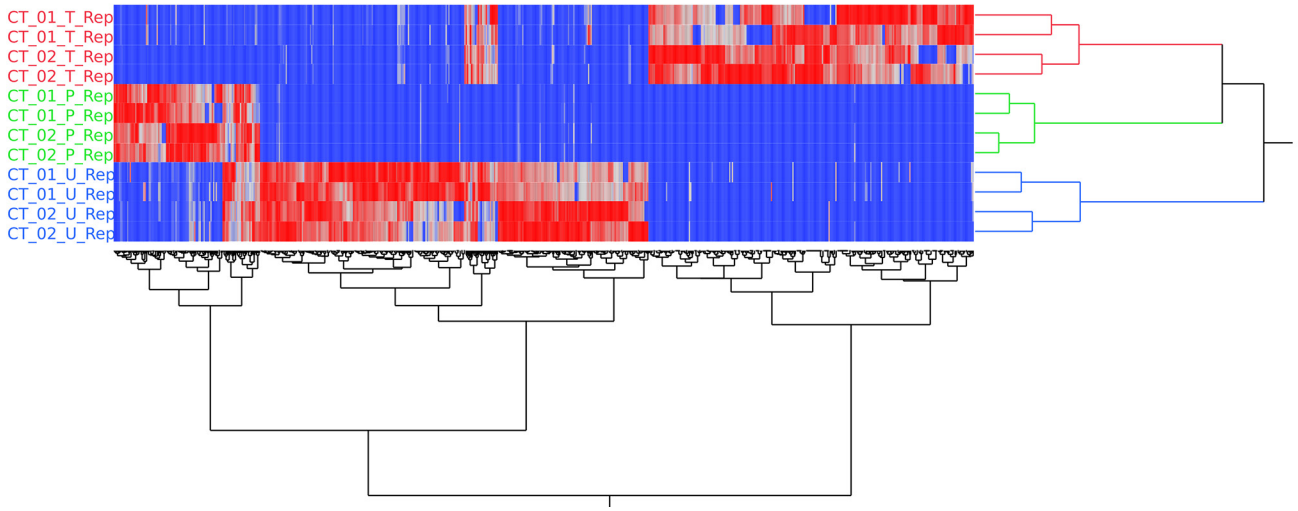


Figure 4. *Hierarchical clustering of the proteome. The heatmap illustrates the segregation of samples according to their biological matrix, based on the differentially abundant proteins. Samples are grouped by origin: tissue (red), urine (blue), and plasma-derived extracellular vesicles (green). Father anonymized as CT_01; son anonymized as CT_02.*

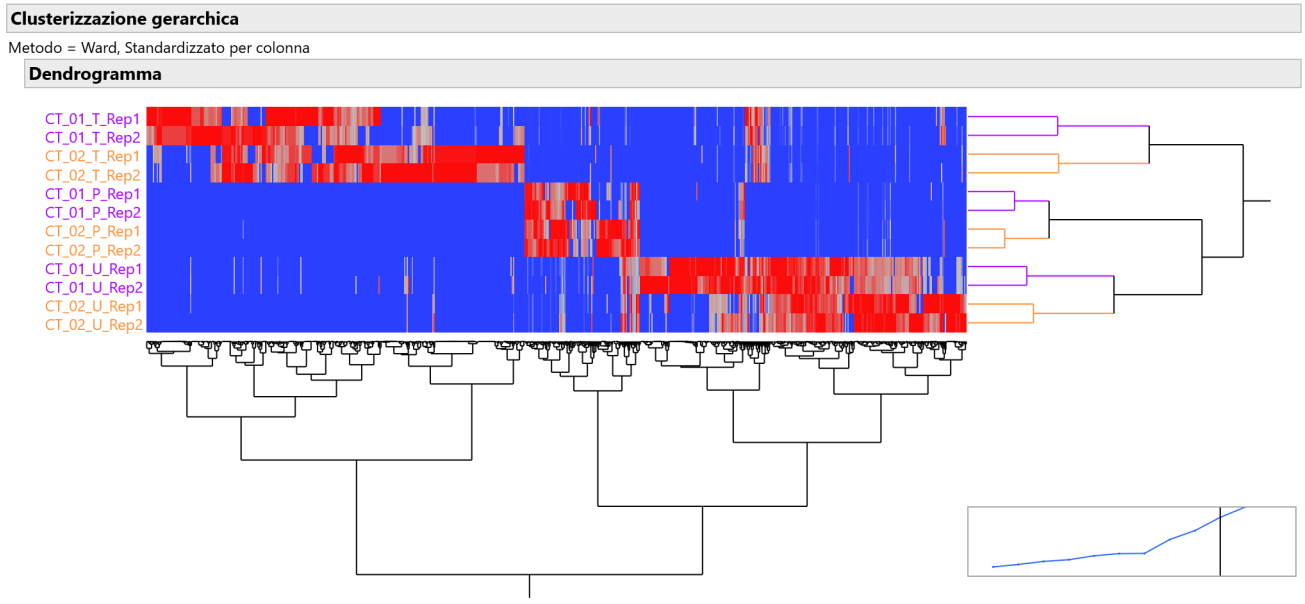


Figure 5. *Hierarchical clustering by subject. Samples are grouped as father (anonymized as CT_01) and son (anonymized as CT_02). This analysis assesses inter-individual variability, confirming that samples predominantly segregate by biological matrix despite subject-based grouping.*

The father's glioblastoma, characterized by an IDH-wildtype and MGMT-unmethylated profile, conforms to the molecular archetype of primary glioblastoma, which is typically associated with aggressive clinical behavior, extensive metabolic reprogramming, and poor outcome (5, 6, 44). In contrast, the son's tumor, harboring an IDH1 R132H mutation and a TP53 R175H mutation together with MGMT promoter methylation, is consistent with an

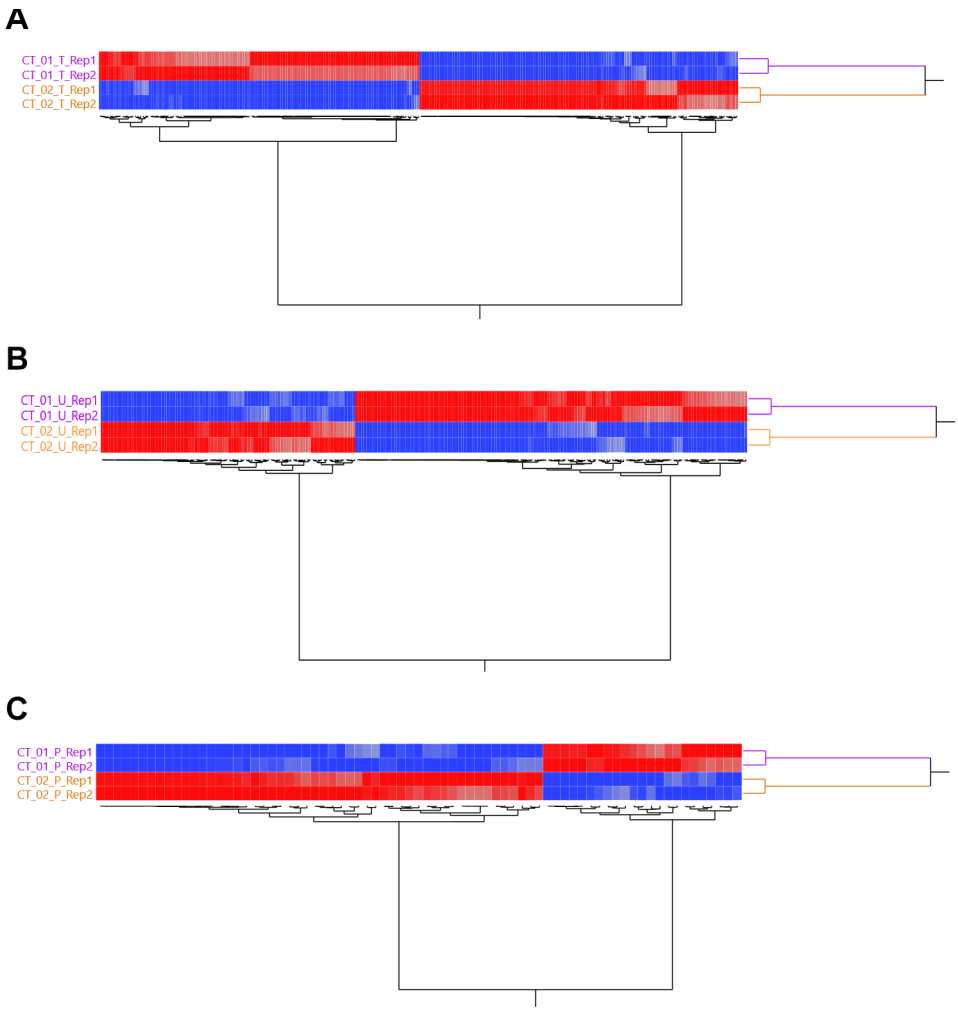


Figure 6. *Hierarchical clustering of biological matrices. The heatmaps show the separation of samples based on differentially abundant proteins identified via linear discriminant analysis (LDA) in: (A) tissue, (B) urine, and (C) plasma-derived extracellular vesicles (EVs).*

IDH-mutant high-grade astrocytoma arising from a lower-grade precursor lesion (1, 7-9, 45, 46). Despite their shared familial background, the two tumors followed clearly divergent molecular trajectories, reflecting distinct somatic evolutionary pathways. These observations suggest that familial predisposition to glioma does not necessarily result in molecular convergence but may instead provide a permissive background upon which independent tumor-specific evolutionary programs unfold.

By integrating proteomic profiling across tumor tissue, urine, and plasma-derived extracellular vesicles, our study provides a multi-compartmental view of tumor biology that extends beyond genomic classification (12). EVs reflect tumor metabolic, immune, and stress-related states, providing a functional readout complementary to genomic classification (28-32). The pronounced divergence in EV-associated proteomes between the father and the son across all matrices mirrors their genomic and clinical differences, suggesting that EV profiles are primarily shaped by tumor-intrinsic biology.

At the tissue level, the father's tumor exhibited enrichment of pathways related to glycolysis, lipid and

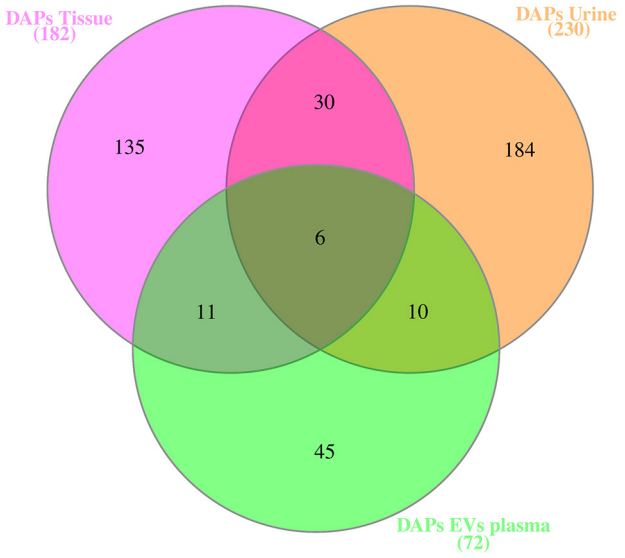


Figure 7. *Distribution of matrix-specific differentially abundant proteins (DAPs). Venn diagram illustrating the overlap of DAPs in tissue, urine, and plasma-derived extracellular vesicles (EVs). The limited core of only 6 shared proteins underscores the highly distinct molecular signatures characteristic of each biological compartment.*

glycogen metabolism, integrin signaling, focal adhesion, innate immune response, ferroptosis, and cellular senescence. This constellation of pathways is characteristic of IDH-wildtype glioblastoma, which relies on metabolic plasticity and increased bioenergetic flux to sustain rapid proliferation and invasion (5, 6, 10-12). Increased glycolytic activity likely exacerbates oxidative stress, potentially explaining the concurrent activation of ferroptosis- and senescence-related pathways. Ferroptosis has emerged as a context-dependent process in glioblastoma, capable of both limiting tumor growth and contributing to therapy resistance depending on cellular and microenvironmental cues. The concomitant activation of inflammatory and adhesion-related pathways further supports a highly invasive, microenvironment-interacting phenotype typical of primary glioblastoma (10, 11).

In contrast, the son's tumor showed enrichment of oxidative phosphorylation–related pathways, amino acid metabolism, neuronal system pathways, gliogenesis, and neurogenesis, together with proteins of the keratin and serpin families. IDH mutation-associated metabolic reprogramming likely underlies the observed enrichment in amino acid metabolism and oxidative pathways (1, 7-9, 46). This profile suggests a tumor state that retains elements of neural lineage identity and differentiation, consistent with the biology of IDH-mutant gliomas (1, 7, 8). Rather than a purely homeostatic state, this signature likely reflects the cellular plasticity and slower evolutionary trajectory typical of IDH-mutant lineages under therapeutic pressure.

Urinary proteomics further amplified these biological distinctions, revealing extensive differences between the father and the son. In the father, enrichment of glycolytic enzymes, lysosomal proteins, heat shock proteins, serine proteases, and mediators of the acute inflammatory response mirrors the metabolic and proteostatic stress observed in tumor tissue, validating urine as a sensitive "systemic mirror" of the primary lesion (12, 38). Lysosomal activation and enhanced proteolysis are increasingly recognized as characteristic features of aggressive cancers, including glioblastoma, supporting nutrient scavenging and resistance to metabolic stress (10-12). Conversely, urine samples from the son were enriched in antioxidant pathways, amino acid metabolism, immune regulatory processes, and proteoglycan-related signaling, consistent with a more homeostatic and adaptive tumor phenotype.

At the systemic level, plasma EVs displayed fewer differentially abundant proteins, likely reflecting systemic dilution, while retaining key biological signals despite overall signal attenuation. EVs from the father were enriched in coagulation, complement activation, extracellular matrix remodeling, inflammatory pathways, and MAPK signaling. The inflammatory and immune-modulatory components are consistent with the immunobiology of glioblastoma and with broader frameworks distinguishing immune-active and immune-suppressed tumors (10, 11, 47, 48). In contrast, plasma EVs from the son showed enrichment in proteoglycan- and glycosaminoglycan-related pathways, lipid and cholesterol metabolism, and immune regulatory processes, consistent with a more adaptive and

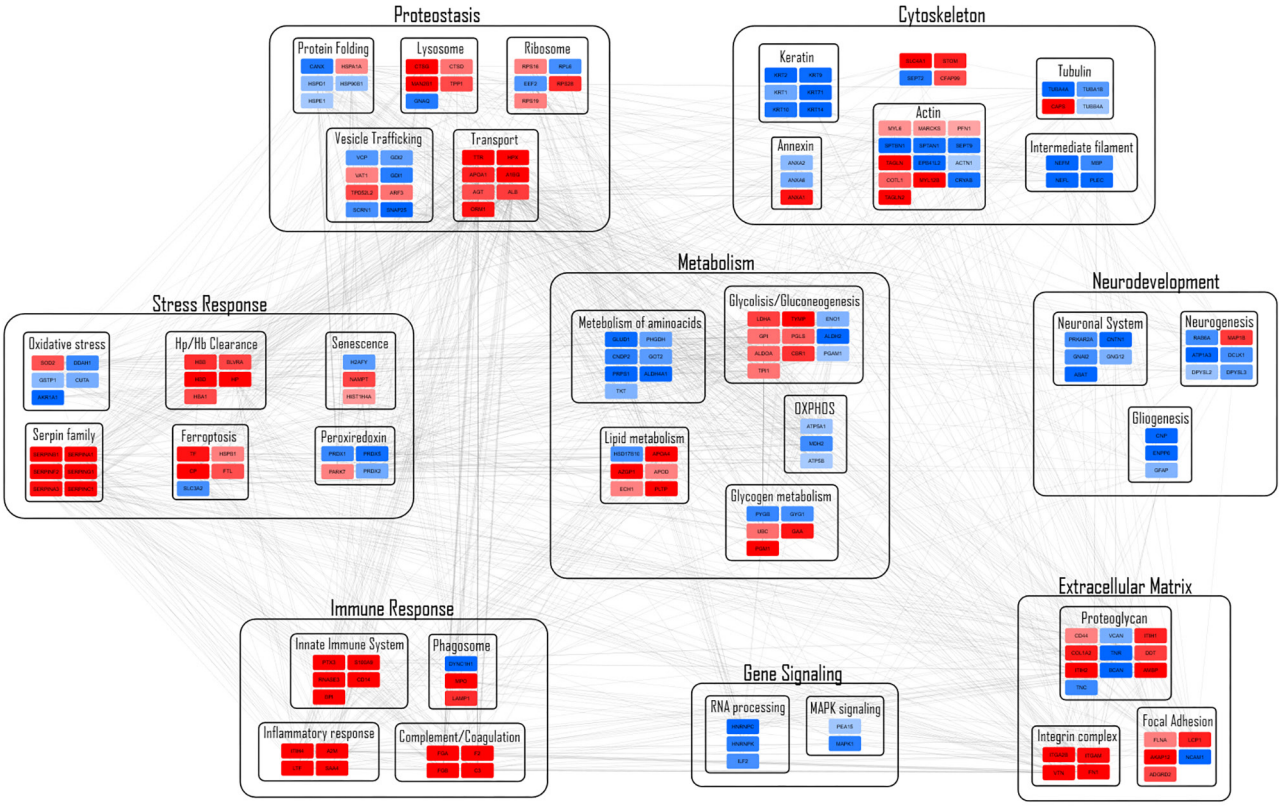


Figure 8. *Protein-protein interaction (PPI) network in tissue. Interaction network of differentially abundant proteins in formalin-fixed, paraffin-embedded (FFPE) samples. Nodes represent proteins, and edges represent evidence of functional or physical interactions retrieved from the STRING database. Node color reflects a gradient of relative abundance: 0 (blue, enriched in the son) to 100 (red, enriched in the father).*

homeostatic biological profile (49). Taken together, plasma-derived EVs and urine proteomic profiles provide complementary views of tumor-associated biology, shaped by the distinct properties of each matrix.

By focusing on glioblastoma-relevant proteins, including LGALS3BP, CD44, SPP1, IDH1, FGL2, and PKM, we further highlighted functionally distinct tumor states across the different biological matrices. In the father, up-regulation of PKM, CD44, LGALS3BP, and SPP1 in both tissue and urinary EVs is indicative of a metabolism-driven, pro-inflammatory, and invasive phenotype characteristic of IDH-wildtype glioblastoma (6, 12). SPP1, in particular, has been described as a hypoxia-responsive mediator of HIF1α/VEGF-dependent angiogenesis in solid tumors (50), a mechanism consistent with the pro-angiogenic phenotype of aggressive glioblastoma. In contrast, in the son, enrichment of FGL2 and MAPK-related proteins aligns with immune modulation and adaptive signaling pathways previously associated with IDH-mutant gliomas (1, 9).

Notably, several of these proteins were more detectable in urine than in plasma EVs, supporting the concept that urine may provide a less diluted and more sensitive readout of tumor-derived proteomic signals (12, 38). This observation is consistent with the broader feasibility of urinary exosome proteomics, likely reflecting systemic clearance mechanisms and EV trafficking routes (38).

The histopathological and molecular diagnoses of both tumors were interpreted according to the 2021 WHO framework (51). The divergent clinical outcomes observed in the two patients, despite standard-of-care high-grade glioma treatment with the Stupp protocol

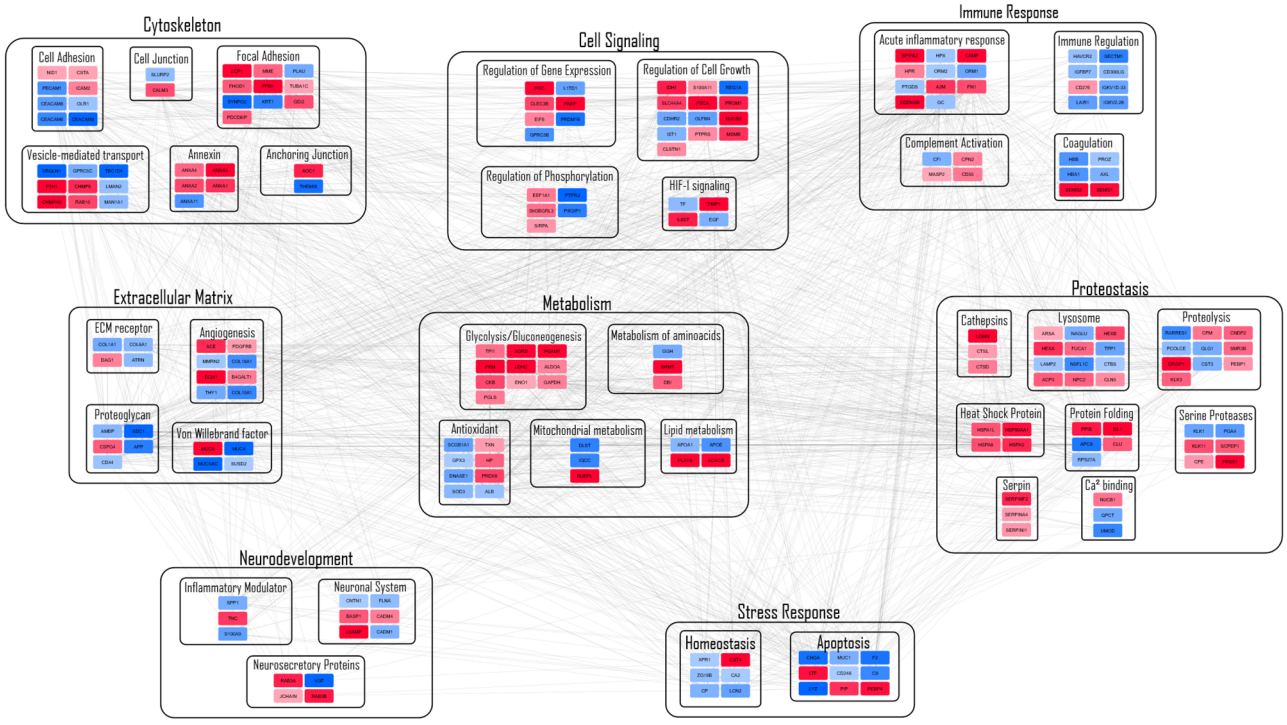


Figure 9. *Protein-protein interaction (PPI) network in urine. Interaction network of differentially abundant proteins in urine samples. Nodes represent proteins, and edges represent evidence of functional or physical interactions retrieved from the STRING database. Node color reflects a gradient of relative abundance: 0 (blue, enriched in the son) to 100 (red, enriched in the father).*

(52), further underscore the biological relevance of these proteomic differences. In particular, MGMT promoter methylation in the son but not in the father likely contributed to differential therapeutic sensitivity (45), highlighting the limitations of uniform treatment strategies given molecular heterogeneity. These findings reinforce the rationale for personalized therapeutic approaches, including molecularly informed stratification, patient-derived models, and immune or metabolic targeting tailored to glioblastoma subtype (39-41, 53), reflecting distinct molecular endotypes defined by different underlying biological mechanisms.

Study limitations. First, this study is based on only two biologically related patients from a single family and should therefore be considered exploratory. Consequently, the findings cannot be generalized to familial glioma. Second, no independent cohort or sporadic glioma controls were available for validation of the identified proteomic signatures. Third, although extensive proteomic analyses were performed, the biological significance of the identified proteins was not experimentally validated by complementary molecular techniques such as immunohistochemistry or western blotting. Fourth, comprehensive germline genetic analyses were not available, preventing definitive conclusions regarding inherited susceptibility. Finally, while environmental electromagnetic exposure was assessed, other shared environmental or occupational factors cannot be completely excluded.

Conclusion

The presented cases of a father and son both affected by high-grade gliomas highlight the value of integrated proteomic analyses in the study of familial glioma. Despite

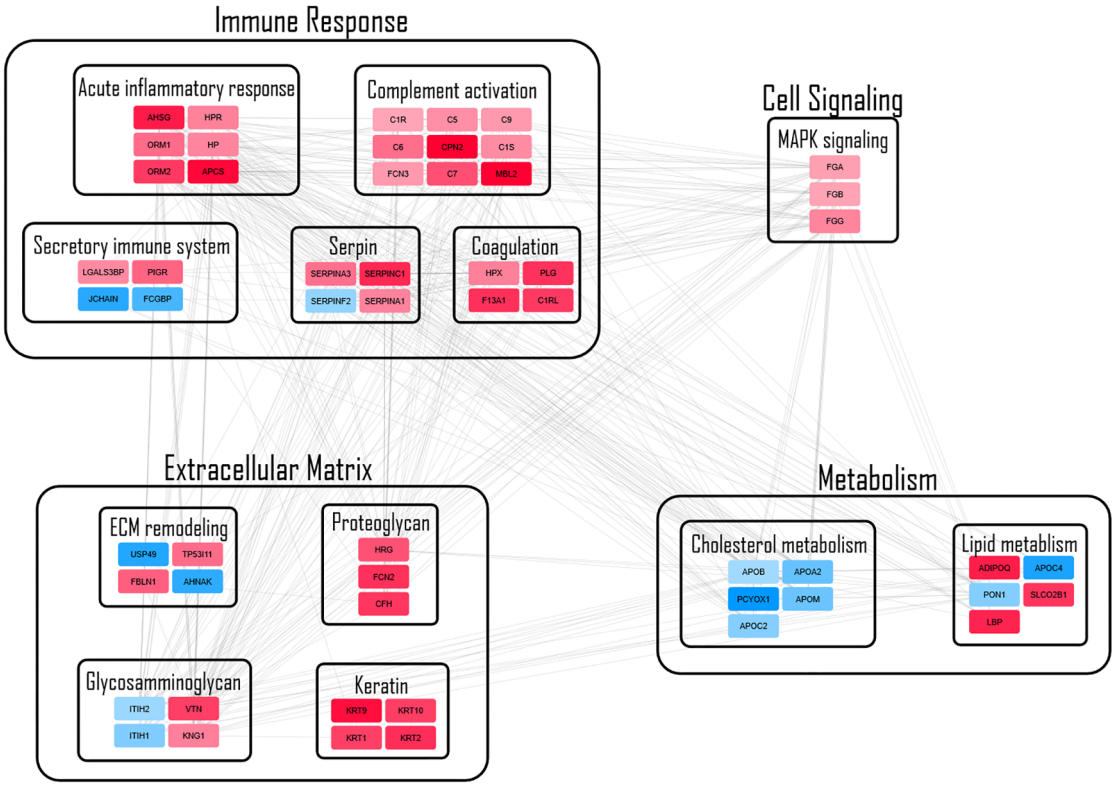


Figure 10. *Protein-protein interaction (PPI) network in plasma-derived extracellular vesicles. Interaction network of differentially abundant proteins in plasma-derived extracellular vesicle samples. Nodes represent proteins, and edges represent evidence of functional or physical interactions retrieved from the STRING database. Node color reflects a gradient of relative abundance: 0 (blue, enriched in the son) to 100 (red, enriched in the father).*

their familial relationship, the father's tumor was IDH-wildtype with an unmethylated *MGMT* promoter, whereas the son's tumor harbored *IDH1* R132H and *TP53* R175H mutations with a methylated MGMT promoter, reflecting significant biological variability and independent molecular evolution even within the same family. Proteomic analysis, particularly from urine, identified candidate non-invasive biomarkers, capturing patient-specific molecular features that may be less evident in plasma. These findings suggest that urine-based proteomic profiling provides sensitive insight into tumor biology and could help guide personalized treatment strategies. Further research in additional familial or genetically related cases will be required to validate these findings and to deepen our understanding of glioma development and progression.

Supplementary Material

Available at: https://figshare.com/s/09aa8a460f89e84daa9a10.6084/m9.figshare.33165167.

Conflicts of Interest

The Authors declare no conflicts of interest in relation to this study.

Authors' Contributions

Giuseppe E. Umana: Conceptualization, writing – original draft, writing – review & editing, investigation (surgical procedures); Giusy Ciuffreda: Formal analysis, data curation, writing – original draft, writing – review &

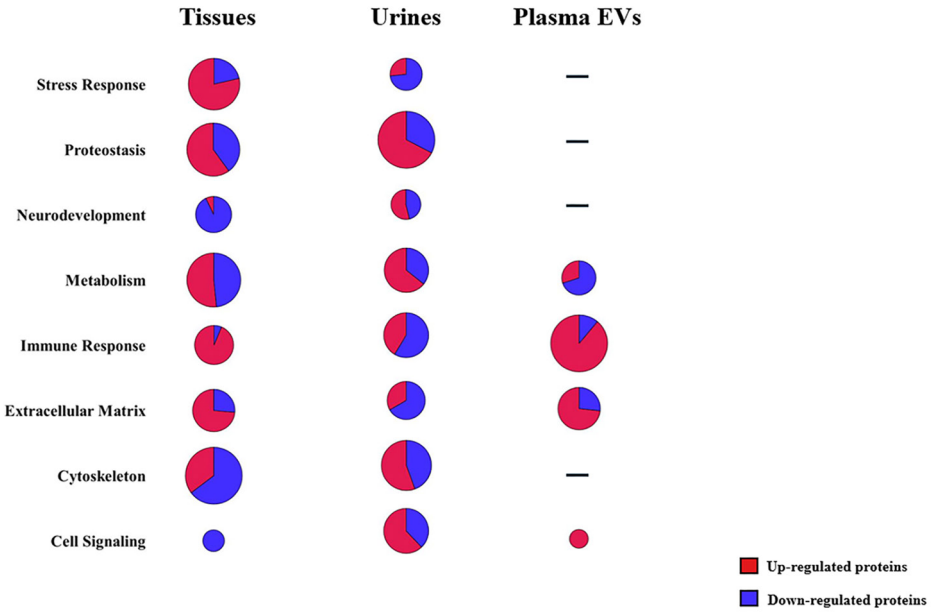


Figure 11. *Comparative functional mapping. Bubble plot of protein-protein interaction (PPI)-derived pathways across matrices. Bubble size indicates protein count; bubble charts show relative abundance for the father (red) and the son (blue). Dashes (–) indicate no proteins identified for that pathway. EVs: Extracellular vesicles.*

editing, investigation (proteomic analysis); Elena Cortese: Data curation, writing – review & editing; Chiara Corso: Data curation, writing – review & editing; Giovanni Raffa: Investigation (nTMS cortical mapping), data curation; Claudia R. Vecchio: Review, validation, methodology; Sruthi Ranganathan: Writing – review & editing; Angela Pettinato: Investigation (histological examination, molecular analysis), data curation; Mara Curduman: Investigation (histological examination, molecular analysis), data curation; Saverio Spadola: Investigation (histological examination, molecular analysis), data curation; Gianluca Ferini: Investigation (radiotherapy), validation; Raffaello Viganò: Methodology, investigation (sample preparation, proteomic analysis); Francesca Brambilla: Methodology, investigation (sample preparation); Dario Di Silvestre: Data curation (proteomic data); Yvan Torrente: Methodology, investigation (vesicle preparation and characterization); Pierluigi Mauri: Conceptualization, supervision, writing – review & editing. All Authors have read and agreed to the published version of the manuscript.

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Artificial Intelligence (AI) Disclosure

During the preparation of this manuscript, a large language model (ChatGPT, OpenAI; GPT-5.6 Pro) was used solely for language editing and stylistic improvements in select paragraphs. No sections involving the generation, analysis, or interpretation of research data were produced by generative AI. All scientific content was created and verified by the authors. Furthermore, no figures or visual

data were generated or modified using generative AI or machine learning–based image enhancement tools.

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