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Pathway-Centric Transcriptomic Profiling of Middle Ear Cholesteatoma Identifies Selective Dysregulation of the IL-6/JAK-STAT/MAPK and MMP9/ZEB1 Pathways

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Abstract

Background: Cholesteatoma is a destructive lesion of the middle ear characterized by hyperproliferative keratinocytes and chronic bone erosion. Although histologically benign, its aggressive behaviour causes severe otologic complications. The precise molecular mechanisms dividing active drivers from unaltered regulators within key pathogenic pathways remain poorly defined.
Methods: Public transcriptomic data from the GSE42256 dataset were analyzed to compare human cholesteatoma tissue against normal middle ear mucosa. Differential gene expression analysis was performed using linear modeling (R limma) in R/Bioconductor. P-values were adjusted using the Benjamini-Hochberg procedure, with significance set at adjusted $p < 0.05$ and $|\log_2 FC| > 0.5$. Targeted analysis was mapped onto TNF-α, IL-6, S100, and invasive signaling cascades.
Results: Specific transcriptomic profiles revealed highly selective, non-generalized pathway dysregulations. Within the S100 family, S100A7, S100A8, S100A9, and S100A12 were significantly upregulated, driving leukocyte recruitment. The TNF-α cascade exhibited a highly constrained pattern in CASP8, FADD, and TNFRSF1B genes, triggering localised remodelling. The IL-6 axis demonstrated a comprehensive and coordinated upregulation of its intracellular downstream machinery, specifically concentrated in the JAK1, STAT3, MAPK3, GRB2, SHC1, ELK1, and MAP2K1 genes, fueling continuous proliferation. Finally, tissue invasion was characterized by the marked upregulation of matrix metalloproteinases (MMP2/MMP9) and the transcription factor ZEB1, orchestrating extracellular matrix degradation.
Conclusions: Cholesteatoma pathogenesis is driven by targeted intracellular relays rather than a generalised transcriptional surge. Pinpointing the extensive dysregulation of the IL-6/JAK-STAT/MAPK and MMP9/ZEB1 pathways isolates precise molecular checkpoints, providing novel therapeutic opportunities to halt keratinocyte hyperproliferation and osteolytic bone destruction.

Keywords: Cholesteatoma, Keratinocyte hyperproliferation, IL-6 axis, MMP9/ZEB1 axis, S100 alarmins, R limma package.

Introduction

Cholesteatoma is a destructive, non-neoplastic lesion of the middle ear characterised by the destructive accumulation of hyperproliferative keratinizing squamous epithelium. Clinically, despite its histologically benign nature, cholesteatoma exhibits an aggressive behaviour characterised by persistent local expansion, chronic inflammation, and the progressive destruction of surrounding bony structures, including the ossicular chain and the otic capsule [1-3]. This osteolytic process leads to severe complications such as conductive or sensorineural hearing loss, vestibular dysfunction, and life-threatening intracranial infections. To date, surgical excision remains the gold standard treatment; however, high recurrence rates driven by incomplete resection and persistent hyperproliferative triggers highlight an urgent, unmet clinical need for targeted pharmacological interventions.

At the cellular level, the pathogenesis of cholesteatoma is sustained by a profound disruption of keratinocyte homeostasis [4,5], shifting from controlled differentiation to unconstrained proliferation. This phenotype is orchestrated by a complex network of signalling cascades within the middle ear microenvironment. Pro-inflammatory cytokines, particularly tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), act as primary drivers of this chronic inflammatory state, activating intracellular propagation pathways that alter cell cycle checkpoints and suppress terminal differentiation [6,7]. Concurrently, the chronic recruitment of leucocytes is fuelled by the sustained release of endogenous danger signals, such as the S100 alarmin family [8,9], which perpetuate keratinocyte activation and foster a self-sustaining loop of tissue hyperproliferation.

The destructive and invasive capability of cholesteatoma relies heavily on the remodelling of the extracellular matrix (ECM) and cellular transdifferentiation. Enhanced expression of matrix metalloproteinases, specifically MMP2 and MMP9, allows the expanding keratinous cyst to degrade the basement membrane and erode bone [10,11]. This process is further amplified by transcription factors such as ZEB1, a master regulator of the epithelial-mesenchymal transition (EMT) that confers migratory and invasive properties to epithelial cells [12,13]. While the individual involvement of these mediators has been documented, a comprehensive, pathway-specific transcriptomic evaluation is required to delineate the precise boundaries between activated core effectors and stable, unaltered regulatory components within these cascades.

To address this gap, the present study utilizes public transcriptomic data from the GSE42256 dataset to map out specific molecular shifts in human cholesteatoma tissue against normal middle ear mucosa. By leveraging high-throughput differential expression analysis via linear modeling, we systematically evaluate the targeted dysregulation of the TNF-α, *IL-6*, S100, and *EGFL8* MMP/ZEB1 pathways. Our findings isolate the exact intracellular relays that drive the disease phenotype while identifying a significant pool of unaltered candidate genes, thereby revealing highly precise, non-generalized molecular checkpoints. Ultimately, this pathway-centric dissection pinpoints novel diagnostic markers and specific therapeutic targets capable of disrupting the proliferative and bone-eroding capacity of cholesteatomas.

Methodology

Data Retrieval and Preprocessing

Publicly available transcriptomic expression data were retrieved from the NCBI Gene Expression Omnibus (GEO) database under the accession number GSE42256 [14]. This dataset comprises gene expression profiles from human cholesteatoma tissue and normal middle ear mucosa controls. Raw

data were subjected to quality control assessment and background correction. Log_2 transformation and quantile normalization were performed using the oligo package in R/Bioconductor to ensure variance stabilization and cross-array comparability before downstream statistical testing.

Differential Gene Expression Analysis

To identify differentially expressed genes between cholesteatoma samples and normal controls, a linear modeling approach was applied using the R limma (Linear Models for Microarray Data) package [15,16]. Empirical Bayes (eBayes) smoothing was utilized to borrow strength across genes and moderate the standard errors, thereby enhancing statistical power for low-replicate conditions. To control the false discovery rate resulting from multiple hypothesis testing, p-values were adjusted using the Benjamini-Hochberg procedure. Genes meeting a threshold of adjusted p-value < 0.05 and an absolute log_2 fold change ($|log_2FC|$) > 0.5 were considered statistically significant.

Pathway and Candidate Gene Enrichment

A targeted pathway analysis was conducted to evaluate specific molecular cascades driving cholesteatoma pathogenesis. Predefined gene sets representing the tumor necrosis factor-alpha (TNF-α) signaling cascade, interleukin-6 (*IL-6*) axis, S100 alarmin family, and cellular invasion *EGFL8* modulators (including *MMP2, MMP9*, and *ZEB1*) were systematically mapped onto the differential expression results. Volcano plots were generated by plotting the negative log_{10} of the adjusted p-values against the log_2 fold changes for each candidate gene to visualise specific transcriptomic shifts and quantify the proportion of unaltered components within each pathway.

Results and Discussion

Transcriptomic profiling of cholesteatoma tissue against normal middle ear mucosa using the GSE42256 dataset revealed distinct, pathway-specific alterations. Regarding the S100 protein family (Table 1, Figure 1), differential expression analysis identified a selective cluster of dysregulated targets (Figure 2). While key regulatory genes remained transcriptomically stable and fell below the statistical threshold, a specific subset of alarmins demonstrated prominent activation. Notably, *S100A7, S100A8, S100A9*, and *S100A12* (to a lesser degree, *S100A3*) exhibited significant upregulation (Log Ratio > 0.5, $p < 0.05$), which correlates with chronic leucocyte recruitment and hyperproliferation [17,18]. Conversely, *S100A1* was significantly downregulated, demonstrating that cholesteatoma triggers a highly specific reshaping of the calcium-binding protein profile rather than a generalized activation of the locus (Figure 2).

The tumour necrosis factor-alpha (TNF-α) signalling cascade exhibited a highly constrained yet statistically robust activation pattern. Out of the key candidate genes analysed within this pathway (Table 2, Figure 1), only 5 demonstrated statistically significant dysregulation in the volcano plot, whereas the remaining components showed no significant expression changes (Figure 2). The significant upregulation of downstream effectors *CASP8* and *FADD*, alongside the membrane receptor *TNFRSF1B*, indicates a molecular shift toward programmed cell death and tissue remodelling [19,20]. Concurrently, the upregulation of *IKBKG* coupled with the downregulation of *IKBKB* (Figure 2) implies a fine-tuned, non-canonical regulation of the NF-κB complex. This highly localised gene expression profile provides a molecular explanation for the concurrent tissue destruction and survival signalling characteristic of keratinocytes in the middle ear.

Analysis of the interleukin-6 (*IL-6*) pathway (Table 3, Figure 1) revealed a coordinated upregulation of downstream effectors, while upstream receptors remained at baseline expression levels. This significant transcriptomic acceleration was highly concentrated across the entire intracellular

propagation machinery (Figure 2C). Specifically, key components of both the *JAK/STAT* and Ras-MAPK arms demonstrated robust upregulation, including *JAK1*, *STAT3*, *MAPK3* (*ERK1*), MAP2K1 (*MEK1*), and the nuclear transcription factor *ELK1*. Furthermore, the significant positive log ratios of the molecular adaptors *GRB2* and *SHC1* firmly connect this cascade to accelerated growth factor signaling and upstream receptor tyrosine kinase coupling. Because key pathway components were comprehensively triggered while intermediate transmembrane receptors remained unaltered, these data suggest that the *IL-6* axis in cholesteatoma operates via hyper-sensitized, interconnected intracellular relays that drive continuous keratinocyte proliferation and prevent normal terminal differentiation [21-25].

Regarding the upstream modulator *EGFL8* and proximal regulators and terminal targets driving cellular proliferation, invasive phenotypes, and hyperpolarisation (Table 4, Figure 1) demonstrated pronounced, statistically significant shifts. The matrix metalloproteinases *MMP2* and *MMP9* were heavily upregulated alongside the zinc finger transcription factor *ZEB1*, confirming a transcriptomic program geared toward extracellular matrix degradation and epithelial-mesenchymal transition (EMT). This invasive profile is further reinforced by the upregulation of *CXCL16* and *VEGFA*, driving angiogenesis and immune regulation. While several cell cycle checkpoint genes and baseline Notch signaling receptors did not cross the significance threshold, the targeted activation of the *MMP2/MMP9/ZEB1* axis (Figure 2) provides the definitive molecular rationale for the aggressive, osteolytic, and invasive phenotype of this benign keratinous cyst [26-28].

Conclusions

pathogenesis is not driven by a random transcriptional surge, but by the precise, selective dysregulation of key molecular checkpoints. Hyperproliferation is mediated by the comprehensive intracellular hyperactivation of the interconnected IL-6/JAK-STAT and Ras-MAPK/ERK signaling cascades, expanding beyond isolated nodes to encompass essential adaptors and downstream effectors such as JAK1, STAT3, MAPK3, GRB2, SHC1, MAP2K1, and ELK1. Concurrently, tissue destruction and local invasion are orchestrated by the targeted upregulation of specific S100 alarmins, TNF-α death-domain effectors, and matrix-degrading metalloproteinases (MMP2/9). The observation that a substantial fraction of candidate genes within each pathway remained unaffected highlights the highly specific, non-generalized nature of this disease process. These findings pinpoint precise therapeutic checkpoints, suggesting that targeted disruption of these hyper-sensitized intracellular networks—particularly the expanded IL-6 signaling machinery or the MMP9/ZEB1 axis—could effectively halt the unconstrained expansion and bone-eroding capacity of middle ear cholesteatomas.

Competing interests

The authors declare no competing interest

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Table 1. Key human S100 calcium-binding protein family members

Gene Symbol	Gene ID	Gene Title	System
S100A1	6271	S100 calcium binding protein A1	Proteínas S100
S100A2	6273	S100 calcium binding protein A2	Proteínas S100
S100A3	6274	S100 calcium binding protein A3	Proteínas S100
S100A4	6275	S100 calcium binding protein A4	Proteínas S100
S100A5	6276	S100 calcium binding protein A5	Proteínas S100
S100A6	6277	S100 calcium binding protein A6	Proteínas S100
S100A7	6278	S100 calcium binding protein A7	Proteínas S100
S100A8	6279	S100 calcium binding protein A8	Proteínas S100
S100A9	6280	S100 calcium binding protein A9	Proteínas S100
S100A10	6281	S100 calcium binding protein A10	Proteínas S100
S100A11	6282	S100 calcium binding protein A11	Proteínas S100
S100A12	6283	S100 calcium binding protein A12	Proteínas S100
S100A13	6284	S100 calcium binding protein A13	Proteínas S100
S100A14	57402	S100 calcium binding protein A14	Proteínas S100
S100A16	142246	S100 calcium binding protein A16	Proteínas S100
S100B	6285	S100 calcium binding protein B	Proteínas S100
S100G	795	S100 calcium binding protein G	Proteínas S100
S100P	6286	S100 calcium binding protein P	Proteínas S100
S100Z	170505	S100 calcium binding protein Z	Proteínas S100

The selection of the S100 multigene family members was compiled and verified using the official nomenclature database of the **HUGO Gene Nomenclature Committee (HGNC)** and the **NCBI Gene** repository. Official gene symbols, unique gene identifiers (Gene ID), and functional gene titles correspond to the current genomic consensus version, ensuring specific biological concordance for the human organism.

Table 2: Core essential genes and molecular components of the tumor necrosis factor-alpha (TNF-α) signaling pathway

Gene Symbol	Gene ID	Gene Title	System
TNF	7124	tumor necrosis factor	TNF-alpha
TNFRSF1A	7132	TNF receptor superfamily member 1A	TNF-alpha
TNFRSF1B	7133	TNF receptor superfamily member 1B	TNF-alpha
RELA	5970	RELA proto-oncogene, NF-kB subunit	TNF-alpha
NFKB1	4790	nuclear factor kappa B subunit 1	TNF-alpha
IKBKB	3551	inhibitor of nuclear factor kappa B kinase subunit beta	TNF-alpha
CHUK	1147	component of inhibitor of nuclear factor kappa B kinase complex	TNF-alpha
IKBKG	8517	inhibitor of nuclear factor kappa B kinase subunit gamma	TNF-alpha
TRAF2	7186	TNF receptor associated factor 2	TNF-alpha
TRAF1	7185	TNF receptor associated factor 1	TNF-alpha
TRADD	8717	TNFRSF1A associated via death domain	TNF-alpha
FADD	8772	Fas associated via death domain	TNF-alpha
RIPK1	8737	receptor interacting serine/threonine kinase 1	TNF-alpha
CASP8	841	caspase 8	TNF-alpha
MAP3K7	6885	mitogen-activated protein kinase kinase kinase 7	TNF-alpha
TAB1	10454	TGF-beta activated kinase 1 binding protein 1	TNF-alpha
TAB2	23118	TGF-beta activated kinase 1 binding protein 2	TNF-alpha
TNFAIP3	7128	TNF alpha induced protein 3	TNF-alpha

The repertoire of downstream molecular components and central effectors of the TNF-α signaling cascade was structured based on reference transduction networks from **KEGG Pathway (Kyoto Encyclopedia of Genes and Genomes)** and the functional annotations of the **Gene Ontology (GO)** consortium. All standardized symbols and numerical identifiers for ligands, receptors (TNFRSF), and downstream transcription factors (NF-κB/Rel complex) were cross-validated through the **NCBI Entrez Gene** platform.

Table 3: Critical genes and molecular intermediates involved in the Interleukin 6 (IL-6) cascade and the JAK/STAT signaling axis

Gene Symbol	Gene ID	Gene Title	System
IL6	3569	interleukin 6	IL-6
IL6R	3570	interleukin 6 receptor	IL-6
IL6ST	3572	interleukin 6 cytokine family signal transducer	IL-6
JAK1	3716	Janus kinase 1	IL-6
JAK2	3717	Janus kinase 2	IL-6
STAT3	6774	signal transducer and activator of transcription 3	IL-6
CEBPB	1051	CCAAT enhancer binding protein beta	IL-6
GRB2	2885	growth factor receptor bound protein 2	IL-6
SHC1	6464	SHC adaptor protein 1	IL-6
SOS1	6654	SOS Ras/Rac guanine nucleotide exchange factor 1	IL-6
HRAS	3265	HRas proto-oncogene, GTPase	IL-6
RAF1	5894	Raf-1 proto-oncogene, serine/threonine kinase	IL-6
MAP2K1	5604	mitogen-activated protein kinase kinase 1	IL-6
MAPK3	5595	mitogen-activated protein kinase 3	IL-6
ELK1	2002	ETS transcription factor ELK1	IL-6
FOS	2353	Fos proto-oncogene, AP-1 transcription factor subunit	IL-6
JUN	3725	Jun proto-oncogene, AP-1 transcription factor subunit	IL-6
SOCS3	9006	suppressor of cytokine signaling 3	IL-6

The cataloged genes encompass the core ligand (IL-6), its receptor complex components (IL6R/IL6ST), the JAK/STAT family kinase transducers, associated transcriptional effectors (e.g., CEBPB, AP-1), downstream MAPK/ERK pathway regulators, and negative feedback loops (SOCS3). This set of essential elements was systematically retrieved from the **Gene Ontology (GO)** biological process hierarchical classifications and the **NCBI Gene** molecular reference database.

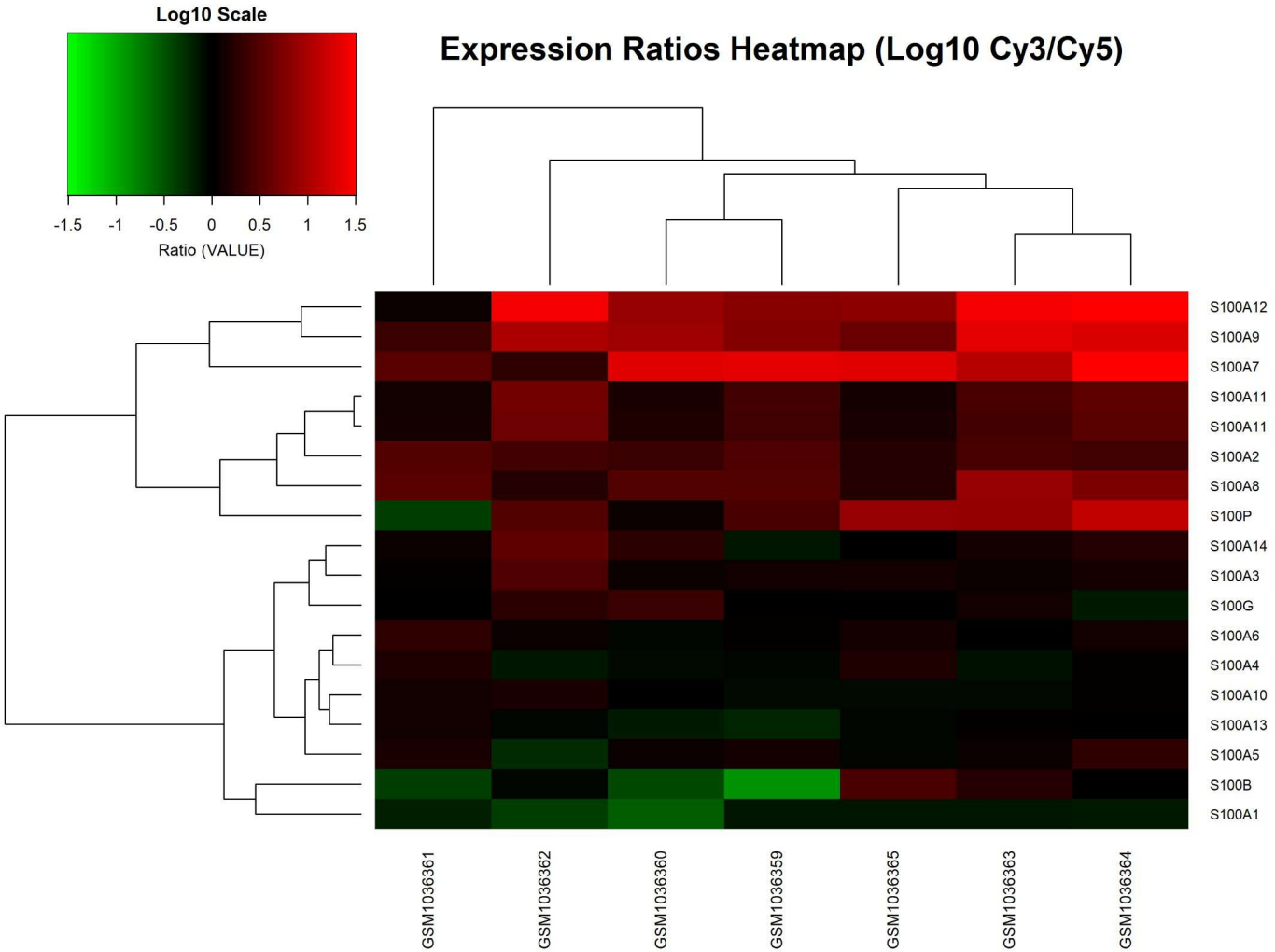
Table 4. Key genes involved in biological modulation by the upstream factor EGFL8 and its integration with Notch, ERK/MAPK, cell cycle, apoptosis, and angiogenesis molecular networks

Gene Symbol	Gene ID	Gene Title	System
EGFL8	115797	epidermal growth factor like domain multiple 8	General upstream modulator
NOTCH1	4851	notch receptor 1	Notch Signaling
NOTCH2	4853	notch receptor 2	Notch Signaling
HES1	3280	hes family bHLH transcription factor 1	Notch Signaling
HEY1	23462	hes related family bHLH transcription factor with YRPW motif 1	Notch Signaling
MMP2	4313	matrix metallopeptidase 2	Notch Signaling / Invasion
MMP9	4318	matrix metallopeptidase 9	Notch Signaling / Invasion
ZEB1	6935	zinc finger E-box binding homeobox 1	Notch Signaling / EMT
MAPK1	5594	mitogen-activated protein kinase 1 (ERK2)	ERK/MAPK pathway
MAPK3	5595	mitogen-activated protein kinase 3 (ERK1)	ERK/MAPK pathway
RAF1	5894	Raf-1 proto-oncogene, serine/threonine kinase	ERK/MAPK pathway
MAP2K1	5604	mitogen-activated protein kinase kinase 1 (MEK1)	ERK/MAPK pathway
FOS	2353	FOS proto-oncogene, AP-1 transcription factor subunit	ERK/MAPK pathway
JUN	3725	JUN proto-oncogene, AP-1 transcription factor subunit	ERK/MAPK pathway
CDK1	983	cyclin dependent kinase 1	Cell Cycle / Apoptosis
CDK4	1019	cyclin dependent kinase 4	Cell Cycle / Apoptosis
CDK6	1021	cyclin dependent kinase 6	Cell Cycle / Apoptosis
CCND1	595	cyclin D1	Cell Cycle / Apoptosis
BCL2	596	BCL2 apoptosis regulator	Cell Cycle / Apoptosis
BCL2L1	598	BCL2 like 1 (Bcl-xL)	Cell Cycle / Apoptosis
FASLG	356	Fas ligand	Cell Cycle / Apoptosis
VEGFA	7422	vascular endothelial growth factor A	Angiogenesis / Immune Regulation
THBS1	7057	thrombospondin 1	Angiogenesis / Immune Regulation
NRP1	8829	neuropilin 1	Angiogenesis / Immune Regulation
ANGPTL1	9068	angiopoietin like 1	Angiogenesis / Immune Regulation
CXCL5	6374	C-X-C motif chemokine ligand 5	Angiogenesis / Immune Regulation
CXCL10	3627	C-X-C motif chemokine ligand 10	Angiogenesis / Immune Regulation
CXCL16	58191	C-X-C motif chemokine ligand 16	Angiogenesis / Immune Regulation
CCL20	6364	C-C motif chemokine ligand 20	Angiogenesis / Immune Regulation
CD74	972	CD74 molecule	Angiogenesis / Immune Regulation
NFKB2	4791	nuclear factor kappa B subunit 2	Angiogenesis / Immune Regulation
IRF7	3665	interferon regulatory factor 7	Angiogenesis / Immune Regulation

This dataset consolidates the master upstream modulating gene *EGFL8* alongside representative gene sets from its primary downstream effector pathways and coupled biological processes (including epithelial-mesenchymal transition [EMT], tissue invasion, and immune regulation). The thematic delimitation of the molecular blocks and the standardization of the official nomenclature (*HGNC symbols*) were derived and cross-referenced using the **AmiGO / Gene Ontology** database and the **NCBI Reference Sequence (RefSeq)** indexed records for the human species.

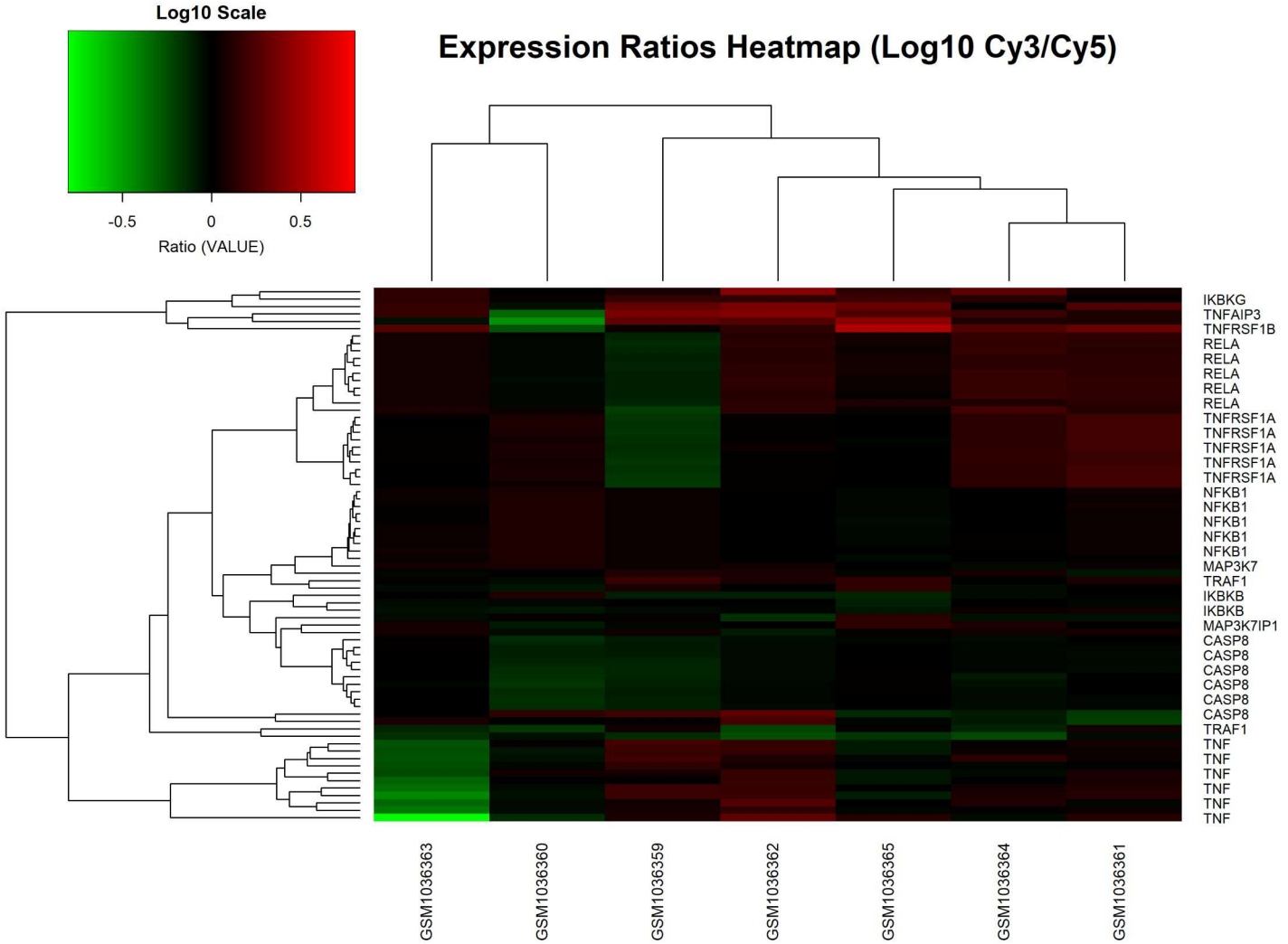
Figure 1. Pathway-centric expression heatmaps displaying distinct transcriptomic signatures in middle ear cholesteatoma (GSE42256 dataset)

A



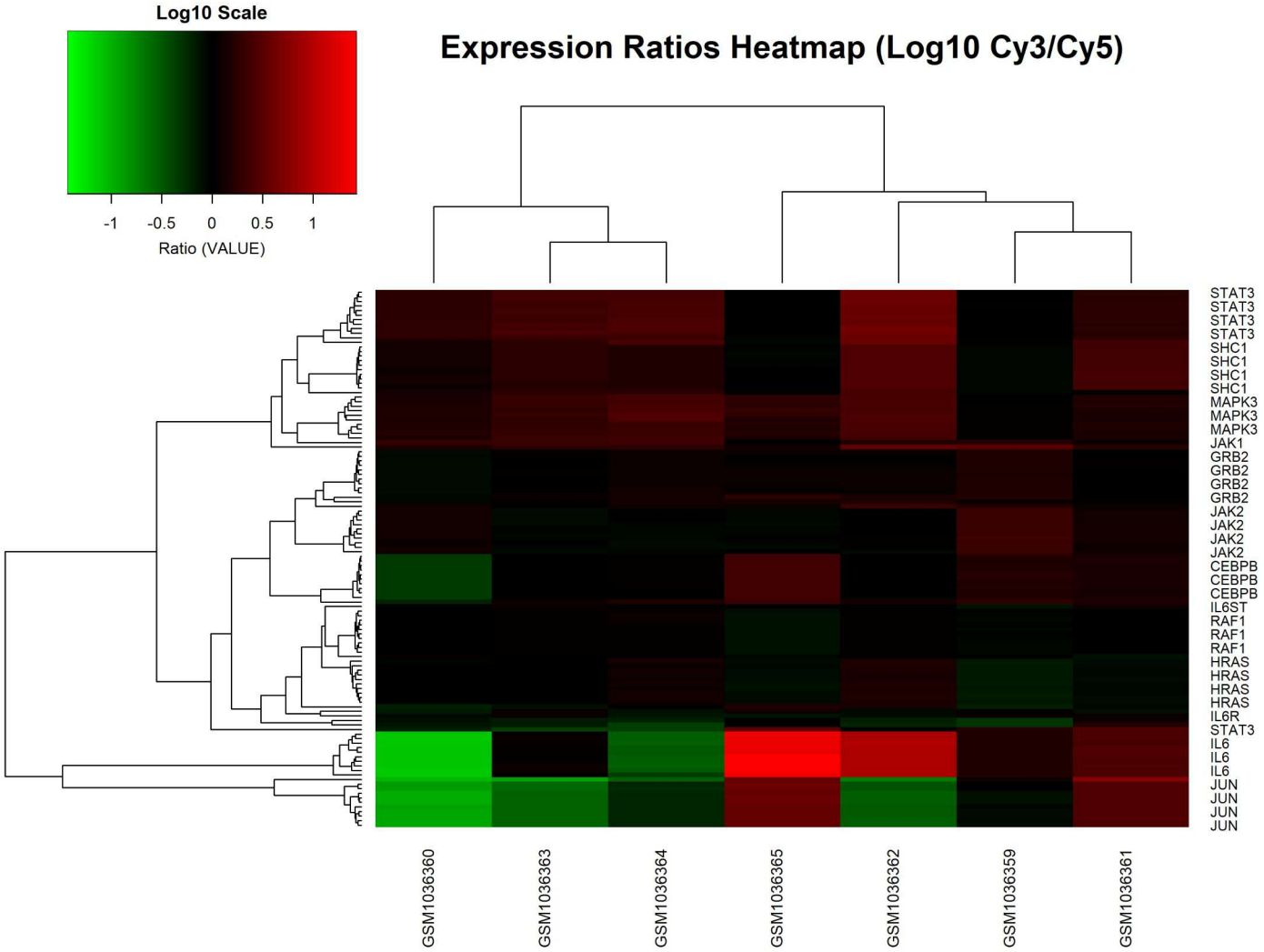
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B



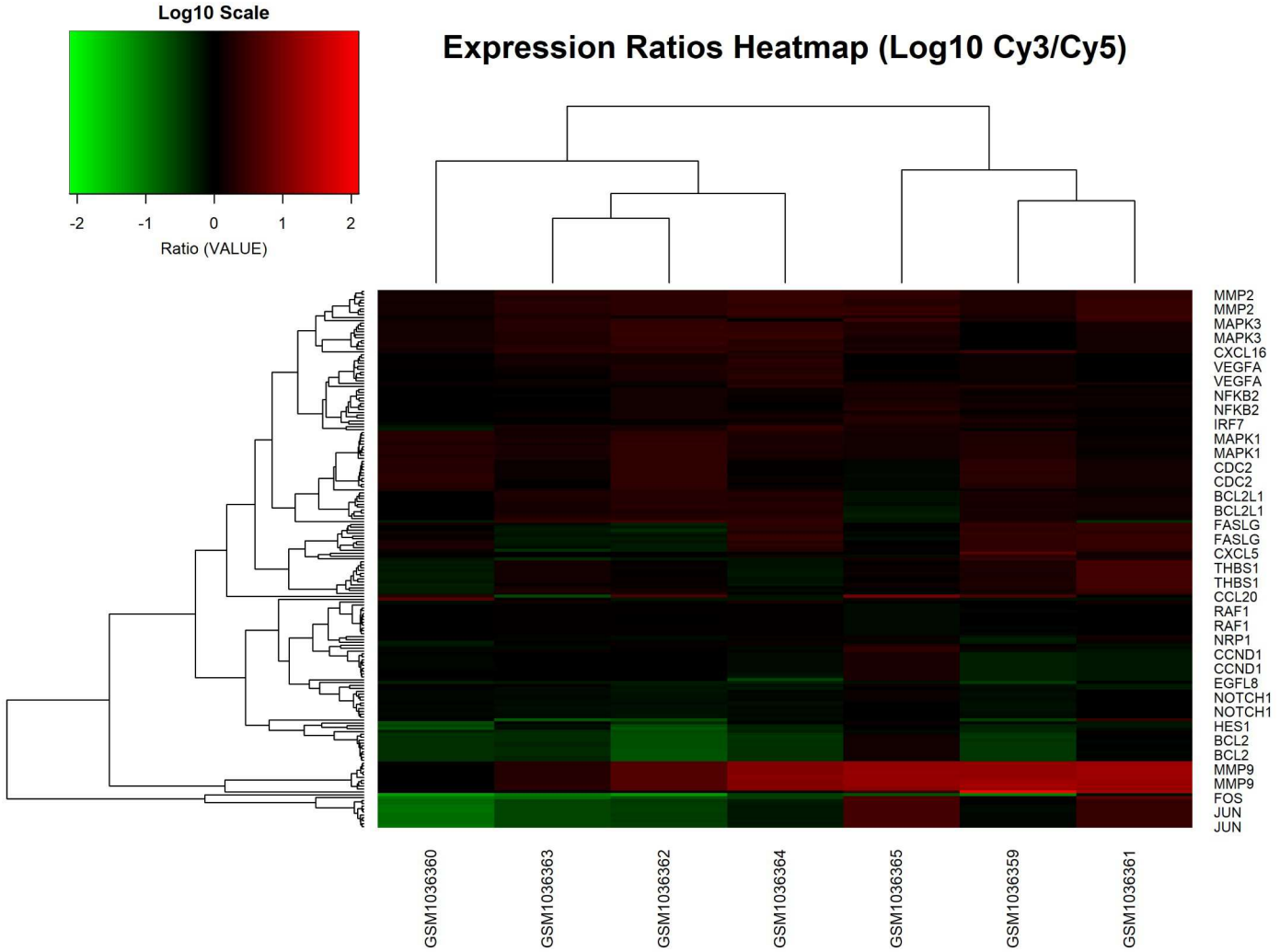
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C



(Figure 1 continued)

D

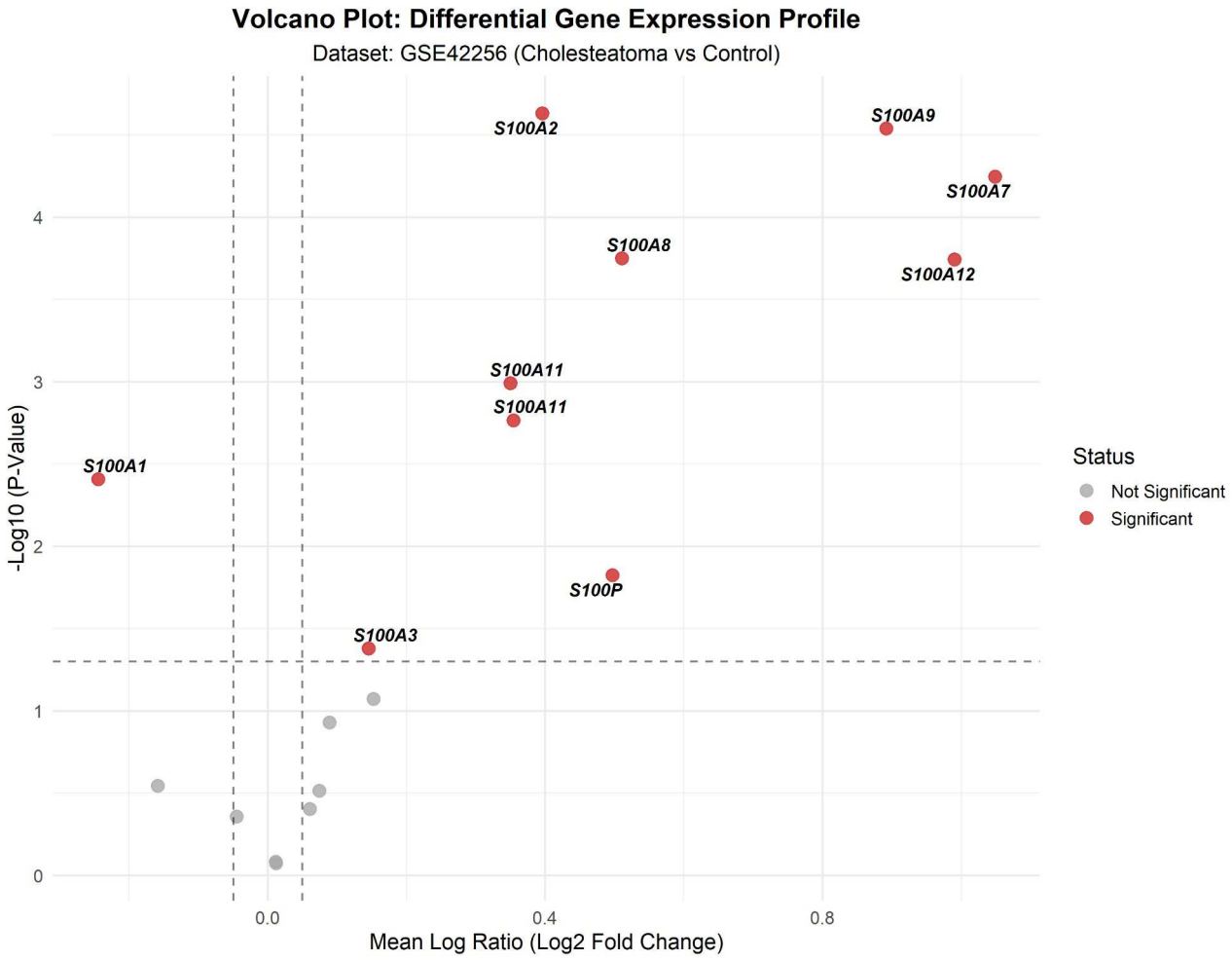


Rows represent individual candidate genes within each specific molecular cascade, and **columns** represent individual microarray samples from human cholesteatoma tissue and normal middle ear mucosa controls (GSE42256 dataset). Dendrograms represent unsupervised hierarchical clustering based on Euclidean distance and complete linkage, illustrating the transcriptomic separation between pathological and control tissues. The color gradient signifies the stabilized **$\log_{10}$** expression ratios (**Cy3/Cy5 fluorescence value**), where **green** indicates downregulation, **red** indicates upregulation, and the intermediate spectrum reflects stable, unaltered gene expression levels.

- **(A)** S100 Alarmin Family Locus: Hierarchical clustering reveals a highly coordinated expression block, isolating the robust transcriptomic upregulation of *S100A7, S100A8, S100A9*, and S100A12 across the pathological cohort against baseline control values.
- **(B)** Tumor Necrosis Factor-Alpha (TNF-α) Signaling Cascade: Visual profiling highlights a highly restricted internal cluster activation, visually grouping the selective deregulation of *CASP8, FADD*, and *TNFRSF1B* effectors, while neighboring regulatory genes maintain an unaltered, homogeneous pattern.
- **(C)** Interleukin-6 (IL-6) Axis Relays: The dense and clustered red spectrum represents the accelerated and uniform transcriptomic activation of downstream intracellular propagation machinery, specifically *JAK1, STAT3*, and MAPK3 variants.
- **(D)** Upstream Factor EGFL8 and Modulators and Invasive Phenotype: Targeted profiling demonstrates a robust transcriptomic shift toward an invasive phenotype, defined by a distinct cluster containing heavily upregulated matrix metalloproteinases (*MMP2*, *MMP9*) and the master epithelial-mesenchymal transition (EMT) transcription factor ZEB1.

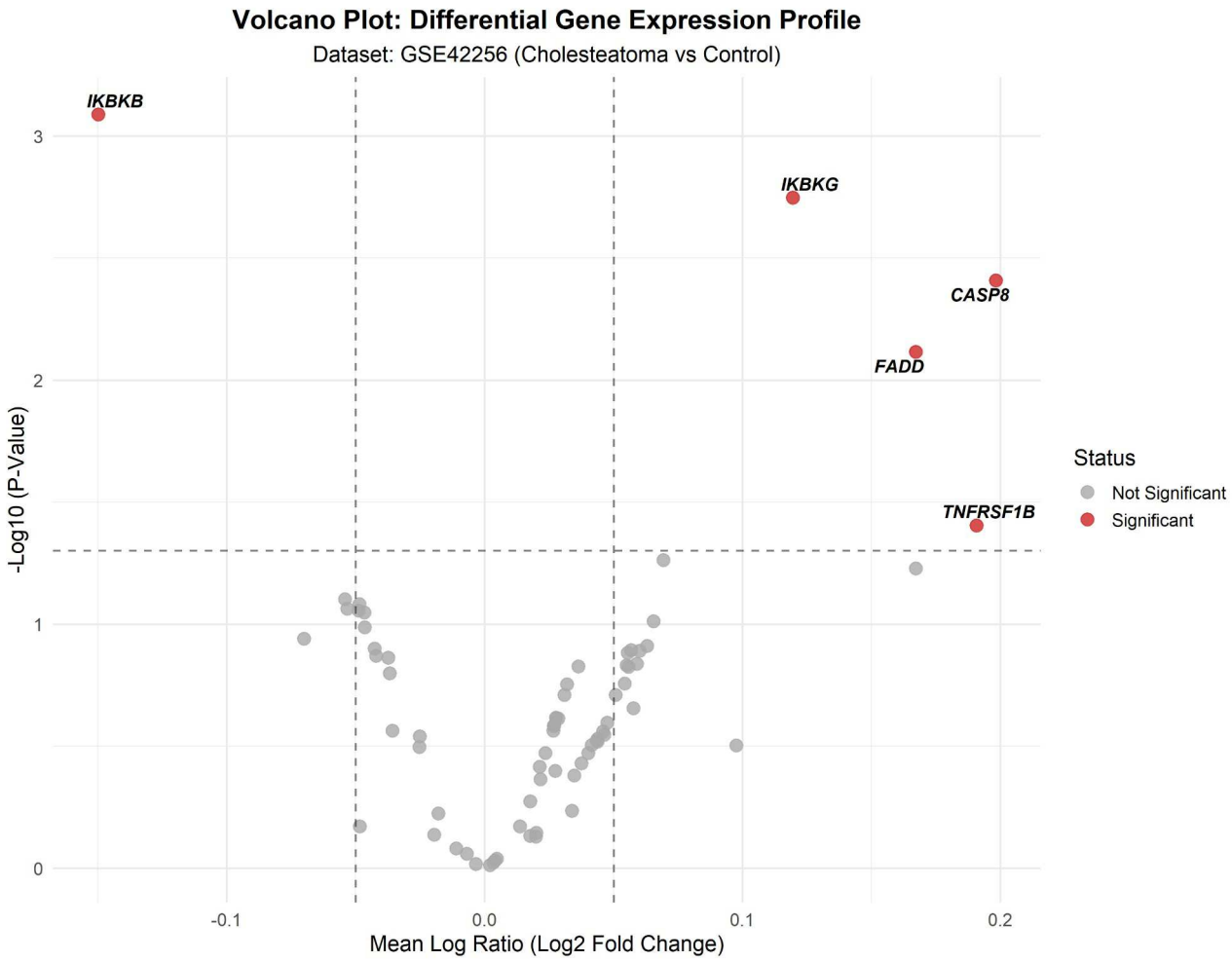
Figure 2. Pathway-centric volcano plots illustrating targeted transcriptomic alterations in middle ear cholesteatoma (GSE42256 dataset)

A



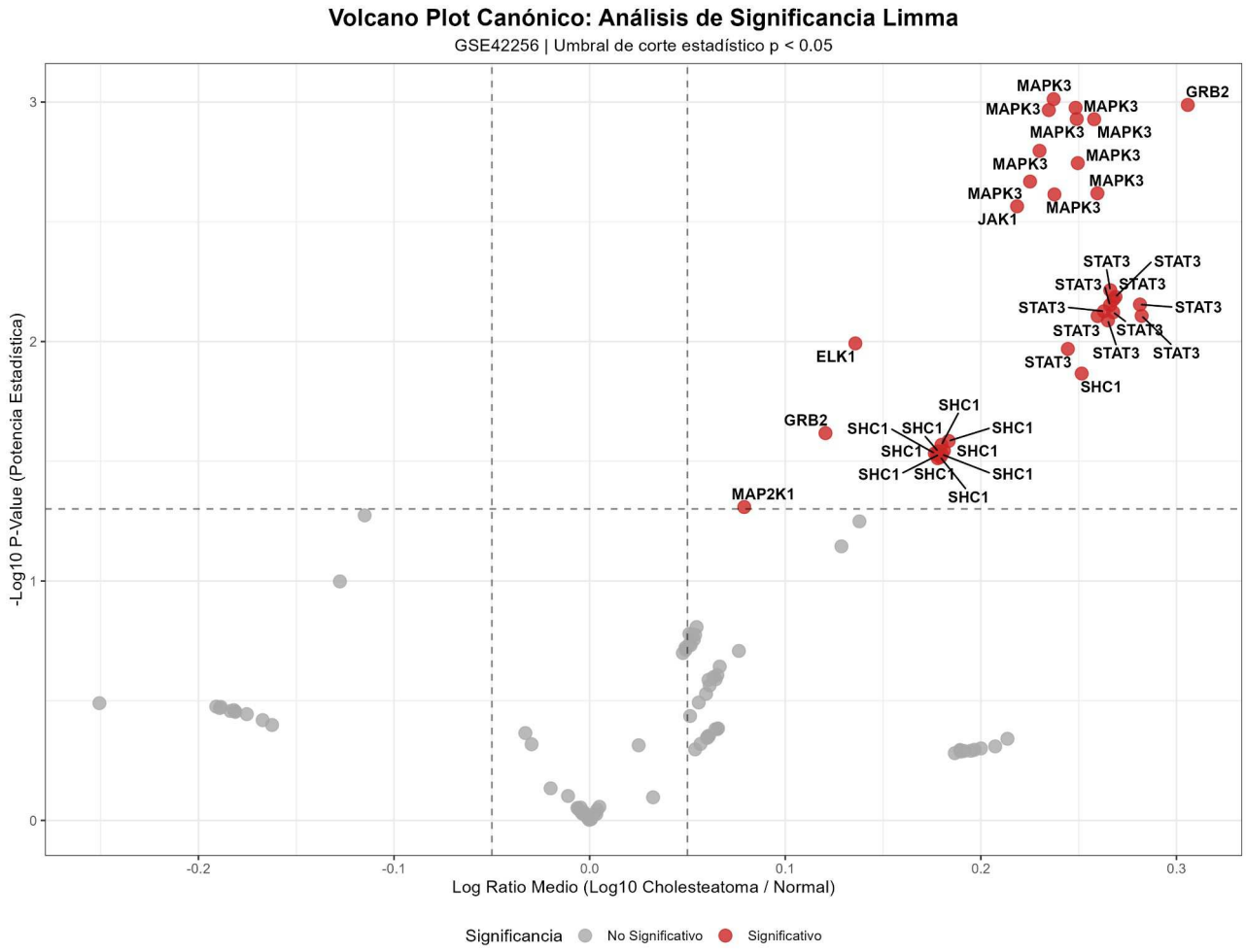
(Figure 2 continued)

B



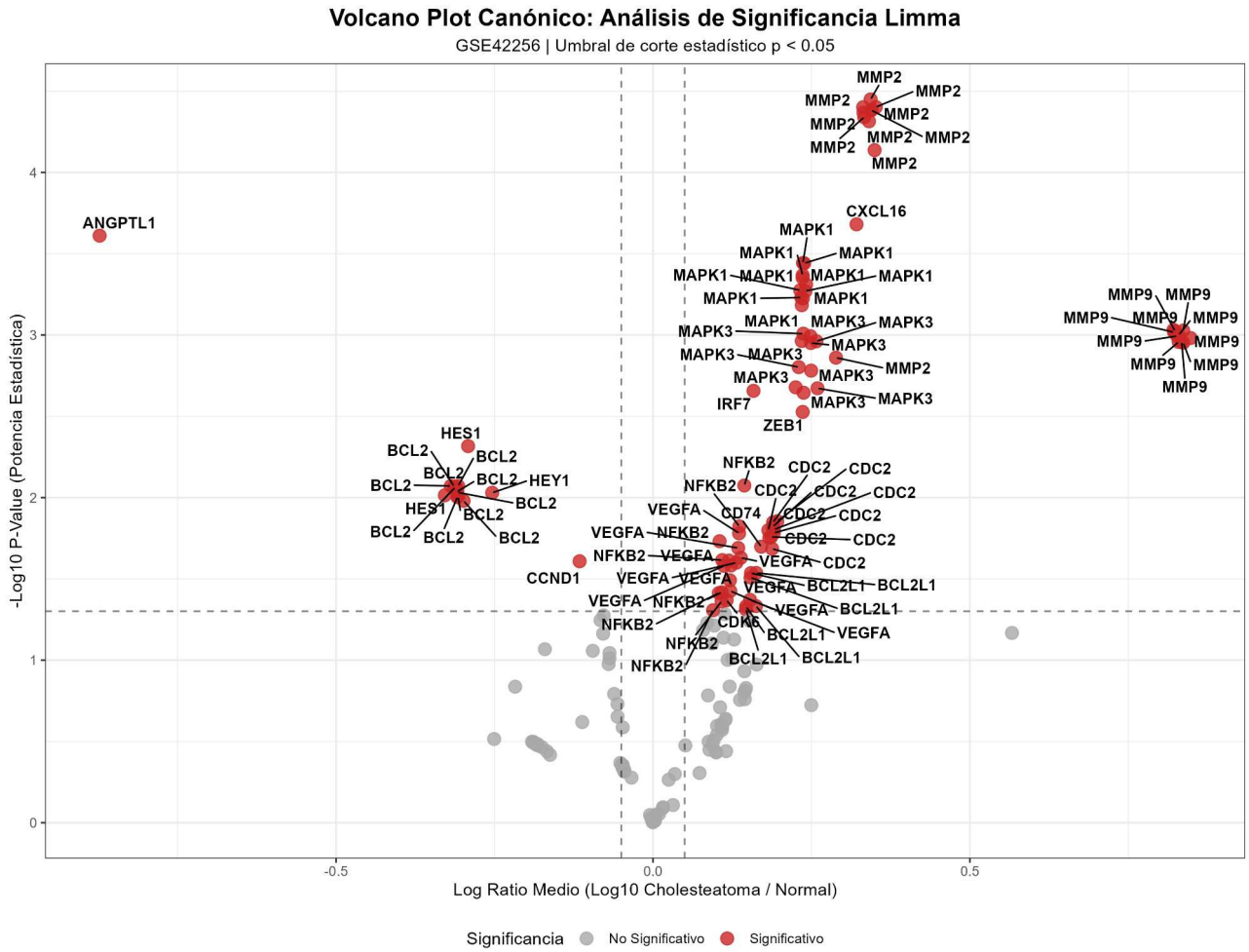
(Figure 2 continued)

C



(Figure 1 continued)

D



The negative **$\log_{10}$** of the adjusted p-values is plotted against the **$\log_2$ fold change ($\log_2$FC)** for each analyzed gene node. The horizontal dashed line denotes the statistical significance threshold **(adjusted p-value < 0.05, Benjamini-Hochberg corrected)**, and the vertical dashed lines represent the biological threshold **($\log_2$FC > 0.5)**. Red dots represent significantly upregulated genes, blue dots indicate significantly downregulated genes, and grey dots represent stable, transcriptomically unaltered pathway components.

- **(A)** S100 Protein Family: Selective dysregulation of the alarmin locus, showing massive upregulation of *S100A7, S100A8, S100A9*, and *S100A12*, contrasting with the downregulation of *S100A1*.
- **(B)** Tumor Necrosis Factor-Alpha (TNF-α) Pathway: Constrained activation pattern where only 5 core candidate genes (*CASP8, FADD, TNFRSF1B, IKBKG*, and *IKBKB*) cross the statistical threshold, signaling localized programmed cell death.
- **(C)** Interleukin-6 *(IL-6)* Signaling Axis: Coordinated transcriptomic acceleration concentrated within the intracellular core machinery, specifically involving *JAK1, STAT3, MAPK3*, and *GRB2* variants.
- **(D)** Upstream Factor EGFL8 and Modulators and Invasive Phenotype: Targeted shifts driving local invasiveness, characterized by the pronounced upregulation of matrix metalloproteinases (*MMP2, MMP9*) and the epithelial-mesenchymal transition (EMT) master regulator *ZEB1*.