

# Costimulatory molecule-related gene-based prognostic signature and immune characteristics in osteosarcoma

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## Abstract

**Introduction:** Osteosarcoma (OSA) is a primary malignant bone tumor with a poor prognosis. Costimulatory molecule-related genes (CMRGs) are crucial for T cell-mediated antitumor immunity, yet their prognostic and therapeutic value in OSA remains unclear.

**Material and methods:** Non-negative matrix factorization (NMF) clustering was used to define CMRG-based subtypes. Prognostic signature genes were selected using univariate Cox, LASSO, and multivariate regression analyses. The predictive performance of the model was assessed using ROC curves, calibration plots, and decision curve analysis (DCA). Immune infiltration and immune checkpoint differences were evaluated via ssGSEA. Drug sensitivity was predicted using CellMiner, and gene expression was validated by qRT-PCR in OSA cells.

**Results:** This study identified five characteristic genes related to the prognosis of OSA and constructed a prognostic model. The evaluation results indicated that the model has favorable predictive performance, with calibration curves and DCA indicating good agreement between predicted and observed survival probabilities. Furthermore, immunotherapy response analysis revealed that patients with lower risk scores had more favorable immune responses and lower rates of disease metastasis. Notably, DMAPT, Obatoclax, 8-Chloro-adenosine, and Palbociclib showed a significant positive correlation with MYC. Additionally, qRT-PCR results indicated that EPYC, SCD1, and AOC3 were significantly overexpressed in OSA cells.

**Conclusions:** This study integrates CMRG-based subtyping, risk modeling, immune profiling, and experimental validation in OSA. The identified biomarkers may help inform personalized treatment and survival risk stratification in OSA patients.

**Key words:** osteosarcoma, costimulatory molecule, prognostic gene signature, immunological characteristics.

## Introduction

Osteosarcoma (OSA) is a highly aggressive primary malignant bone tumor originating from mesenchymal cells, predominantly affecting children and adolescents [1]. It is characterized by a high propensity for early metastasis, particularly to the lungs, contributing to poor prognosis [2, 3]. While localized OSA has a 5-year survival rate of ~60%, this rate drops to ~20% in metastatic cases [4, 5]. Despite surgical and chemotherapeutic advances, overall survival has not significantly improved over the past two decades [6], largely due to tumor heterogeneity, genetic alterations, and chemoresistance [7–9].

Recent studies have highlighted that non-coding RNAs and epigenetic modifications are important contributors to OSA chemoresistance. For instance, METTL3-mediated m6A methylation stabilizes LINC00520, which enhances glycolysis and cisplatin resistance by stabilizing ENO1 protein [10]. In addition, ferroptosis has emerged as a key mechanism in OSA progression and therapy response. Exosome-mediated delivery of miR-144-3p was shown to inhibit OSA cell proliferation and invasion by promoting ferroptosis through ZEB1 suppression [11].

Moreover, dysregulated cell death pathways such as autophagy and ferroptosis may be therapeutically exploitable. Overexpression of apolipoprotein E (APOE) has been reported to suppress OSA growth by inducing both autophagy and ferroptosis via inhibition of the mTOR/Stat3 signaling pathway [12]. These findings underscore the need for better prognostic markers and novel therapeutic strategies in OSA.

The tumor microenvironment (TME) – comprising tumor cells, immune cells, stromal components, and cytokines – plays a critical role in cancer progression, metastasis, and immunotherapy efficacy [13]. In this context, costimulatory molecules are essential in mediating T cell activation and immune modulation [8, 14, 15]. These molecules, primarily members of the B7-CD28 and TNF superfamilies, are involved in delivering the second signal required for effective T cell responses [16–21]. While studies have implicated B7 family members such as PD-L1 and B7-H1 in OSA immune regulation [18], a comprehensive understanding of costimulatory molecule-related genes (CMRGs) in OSA remains lacking.

To address this, we conducted a systematic analysis of CMRG expression patterns, identified molecular subtypes, and developed a prognostic model using characteristic CMRGs. We further evaluated immune infiltration, predicted drug sensitivity, and validated key genes via qRT-PCR. This integrative approach aims to elucidate the immunological and prognostic significance of CMRGs in OSA and to support the development of individualized treatment strategies.

## Material and methods

### Data origin

mRNA expression data related to TARGET-OS were retrieved from the TCGA database (<https://portal.gdc.cancer.gov/>). Patient clinical information was sourced from the TARGET database (<https://ocg.cancer.gov/programs/target>). Additionally, microarray datasets were obtained from the Gene Expression Omnibus (GEO) database (GSE21257 and GSE99671), while single-cell data

came from GSE152048. To identify 60 CMRGs, we reviewed existing literature (Supplementary Table SI) [22]. The IMvigor210CoreBiologies package was employed to gather data on immunotherapy cohorts and relevant clinical details for bladder cancer (BLCA) patients undergoing immune checkpoint therapy.

### Differential analysis and molecular subtype construction based on CMRGs

Using the “edgeR” package, differential analysis was performed on the GSE99671 dataset, comparing the normal group ( $N = 18$ ) and the tumor group (OSA,  $N = 18$ ) with criteria of  $|\log \text{fold change (FC)}| > 0.585$  and  $p < 0.05$ . The differentially expressed genes (OSA-DEGs) were intersected with CMRGs, resulting in CMRG-related differentially expressed genes (DECMRGs, Supplementary Table SII). The expression levels of the DECMRGs in both the normal and tumor groups were quantified, and a heatmap was generated. Based on the DECMRGs, the non-negative matrix factorization (NMF) clustering method was employed to classify the TARGET-OS dataset. Survival analysis of the three subtypes was conducted using Kaplan-Meier (KM) curves.

### Immune status analysis of molecular subtypes

Using the “GSVA” package, single-sample gene set enrichment analysis (ssGSEA) was conducted to investigate the differences in immune-related functions and immune cell infiltration among the three subtypes. The ESTIMATE algorithm was employed to evaluate the stromal score, immune score, ESTIMATE score and tumor purity for each sample across the three subtypes. Clusters 2 and 3 were merged to form a new subtype. Subsequently, the “clusterProfiler” package was used to perform Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis on the differentially expressed genes (group-DEGs) between Cluster 1 and the merged subtype (Supplementary Table SIII).

### Selection of prognostic feature genes for OSA and construction of a prognostic model

Clinical data were merged with the expression levels of differentially expressed genes. The intersection of group-DEGs ( $|\log \text{FC}| > 1$ ,  $p < 0.01$ ) and OSA-DEGs ( $|\log \text{FC}| > 0.585$ ,  $p < 0.05$ ) was used as candidate genes. To reduce the risk of model overfitting, least absolute shrinkage and selection operator (LASSO) regression analysis was performed on the candidate genes using the “glmnet” package. Cross-validation was employed to select the

penalty parameter  $\lambda$ , which helped select relevant genes and reduce model complexity. A multivariate regression analysis of the candidate genes was conducted using the “survival” package to construct the prognostic model.

Risk score =  $\sum_{i=1}^n \beta_i \times \text{Exp}_i$ .  $\beta_i$  is the risk coefficient, and  $\text{Exp}_i$  is the gene expression level.

Based on this model, a risk score for each patient was calculated using the expression levels and risk coefficients of the genes, and patients were categorized into high- and low-risk groups according to the median score. Survival curves were generated based on the risk scores. The “timeROC” package was used to plot receiver operating characteristic (ROC) curves and calculate the area under the curve (AUC) values for 1-year, 3-year, and 5-year survival rates. Additionally, score distribution plots, survival status distribution plots, and heatmaps of expression levels were created for the high- and low-risk groups.

#### Construction of a nomogram for independent prognostic analysis

We conducted univariate and multivariate regression analyses on the training set samples, integrating clinical information and prognostic risk scores, and generated corresponding forest plots. Utilizing the “rms” package, we constructed a nomogram to predict patient survival probabilities at 1, 3, and 5 years. Calibration curves and decision curve analysis (DCA) were subsequently performed to assess the predictive performance of the nomogram and to establish its role as an independent prognostic factor. Furthermore, we validated the predictive accuracy of the nomogram using calibration curves derived from the independent dataset GSE21257.

#### Immunological evaluation

We employed the “GSVA” and “estimate” packages to conduct ssGSEA analysis, quantifying the extent of immune cell infiltration. Violin plots were generated to illustrate immune scores, stromal scores, ESTIMATE scores, and tumor purity for both high- and low-risk groups. Box plots were created to statistically compare the expression levels of immune checkpoints and the degree of immune cell infiltration between these groups. The Tumor Immune Phenotype (TIP) (<http://biocc.hrbmu.edu.cn/TIP/>) is a web-based analysis platform designed to assess the activation levels of the seven steps in the anti-cancer immune cycle [23]. We evaluated the associations between the activation levels of this immune cycle and risk scores, along with characteristic genes. Furthermore, we used the ssGSEA algorithm, TIMER algorithm, and MCP-Counter to analyze the relation-

ships between characteristic genes and immune cells.

#### Investigation of biological functions and active pathways

Differential analysis was performed between the high-risk and low-risk groups of OSA using the edgeR package ( $|\log\text{FC}| > 1$ ,  $p < 0.05$ ). GO and KEGG enrichment analyses were conducted on the differentially expressed genes using the “clusterProfiler” package and “GOplot.”

#### Analyzing the efficacy of risk scores in predicting immune therapy response

Anti-PD-1 and anti-PD-L1 checkpoint inhibitor therapies have emerged as significant components of immunotherapy, garnering considerable attention [24]. To assess the ability of risk scores to predict responses to immune checkpoint blockade, we gathered transcriptomic and clinical data from patients receiving anti-PD-L1 treatment within the IMvigor210 cohort (Supplementary Table SIV). Utilizing the risk scores, we categorized the samples into high-risk and low-risk groups.

#### Single-cell sequencing analysis based on OSA

This study employed the Read10X function from the “Seurat” package to analyze gene expression data from 11 samples in GSE152048. We identified the top 2,000 highly variable genes using the FindVariableFeatures function, and removed batch effects through anchor-based data integration with the FindIntegrationAnchors function. Low-quality cells, defined as those with fewer than 300 detected genes or exhibiting more than 10% mitochondrial gene expression, were excluded, and the remaining cells were normalized. Using the “SCISSOR” package, we categorized cells into high-risk and low-risk groups based on patient expression data and risk stratification information from TARGET-OS. Finally, we analyzed and visualized the intercellular communication network within the TME using the “CellChatR” package.

#### Potential drug targets and prediction of drug inhibitory concentrations

The present study aimed to identify potential therapeutic targets and agents. We utilized the CellMiner database (<https://discover.nci.nih.gov/cellminer/>) to screen for antitumor drugs significantly associated with drug sensitivity and the expression of prognostic genes. Additionally, we explored the DGIdb database (<https://www.dgidb.org>) to identify approved drugs that interact with the feature genes, discussing the potential drug

targets and mechanisms linked to disease susceptibility genes. The “pRRophetic” package was employed to predict the half-maximal inhibitory concentration ( $IC_{50}$ ) of various drugs in high-risk and low-risk groups. A lower  $IC_{50}$  indicates greater effectiveness of the drug in tumor treatment.

### Cell acquisition and cell culture

hFOB1.19, SAOS2 and U2OS cell lines were obtained from the American Type Culture Collection (ATCC) and cultured in DMEM. All cell cultures were maintained in a 37°C incubator with 5%  $CO_2$ .

### RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from cells using RNAiso Trizol reagent (Sangon Biotech, China). cDNA was synthesized through reverse transcription with the GeniScrip III Select RT Kit for qPCR (Youji, China). For qRT-PCR, the uGreener Fex qPCR 2X MiX (Youji, China) was utilized on the LightCycler 480 qPCR system (Roche, Switzerland), with the primer sequences listed below. The results were normalized to GAPDH, and relative expression levels were calculated using the  $2^{-\Delta\Delta CT}$  method (Table I).

### Cytotoxicity assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method was used to determine the toxic effects of drugs on cells. U2OS and SAOS2 cells were seeded into a 96-well plate. After cell attachment, the cells were treated with various concentrations of temozolomide or cisplatin for 48 h, including a blank control group. Subsequently, 10  $\mu$ l of MTT solution was added to each well and the plate was incubated at 37°C for 2 h. The supernatant was then aspirated, and DMSO was added to dissolve the formazan. The absorbance of each well was measured at a wavelength of 490 nm.

### Cell apoptosis assay

Terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL) assay was used to assess the effect of drugs on cell apoptosis. U2OS

and SAOS2 cells were seeded onto culture plates containing coverslips. After cell attachment, cells were treated with temozolomide or cisplatin for 48 h, including a solvent control group. Following treatment, TUNEL staining was performed: cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, incubated with TUNEL reaction mixture to label DNA fragmentation, and counterstained with DAPI. After washing, coverslips were mounted with antifade mounting medium and observed under a fluorescence microscope. Multiple fields of view were randomly selected, and the numbers of TUNEL-positive and DAPI-positive cells were counted to calculate the apoptosis rate.

## Results

### Identification of CMRGs linked to OSA and development of molecular subtypes from DECMRGs

This study initially conducted a differential analysis of the normal and OSA groups in the GSE99671 dataset (Normal: 18, OSA: 18) with criteria set at  $|\log FC| > 0.585$  and  $p < 0.05$ , resulting in 1,046 upregulated and 1,365 downregulated genes (OSA-DEGs, Figure 1 A). By intersecting OSA-DEGs with CMRGs, we identified 13 differentially expressed CMRG-related genes (DECMRGs, Figure 1 B). A PPI network was constructed for the 13 DECMRGs using the STRING database, as illustrated in Figure 1 C, revealing strong interactions among CTLA4, TNFRSF4, CD86, CD276, and CD28. The expression levels of DECMRGs in both the normal and OSA groups were analyzed, and a heatmap was generated, indicating that CTLA4, TNFRSF4, CD86, CD276, and CD28 were significantly overexpressed in the OSA group (Figure 1 D).

The objective of this study was to conduct a comprehensive analysis of the key characteristics of OSA tissues to inform the design of personalized treatment strategies for OSA patients. To accomplish this, we used NMF clustering techniques to classify the TARGET-OS samples into three subtypes based on DECMRGs (Sample: Cluster 1: 26, Cluster 2: 13, Cluster 3: 46, Figure 1 E). K-M curve analysis demonstrated significant

**Table I.** Primer sequences used for quantitative real-time PCR (qRT-PCR)

| Gene name    | Forward primer sequence (5'-3') | Reverse primer sequence (5'-3') |
|--------------|---------------------------------|---------------------------------|
| <i>EPYC</i>  | AGGTCAGAGCCAAAGGAAAGC           | TAGAGTTGGGGCAGTCACAG            |
| <i>SCD1</i>  | TTCCCGACGTGGCTTTTCT             | AGCCAGGTTTGTAGTACCTCC           |
| <i>AOC3</i>  | CAAGTGTCAGAGCACACCCT            | TCCAGTCCTGCTACATCCAGA           |
| <i>MYC</i>   | TGGAAAACAGCCTCCCG               | TTCTCCTCCTCGTCGCAGTA            |
| <i>KLK2</i>  | TGGAGGGGAAAGGGTGATTC            | CCACTTCCGGTAATGCACCA            |
| <i>GAPDH</i> | ACATCGCTCAGACACCATG             | TGTAGTTGAGGTCAATGAAGGG          |

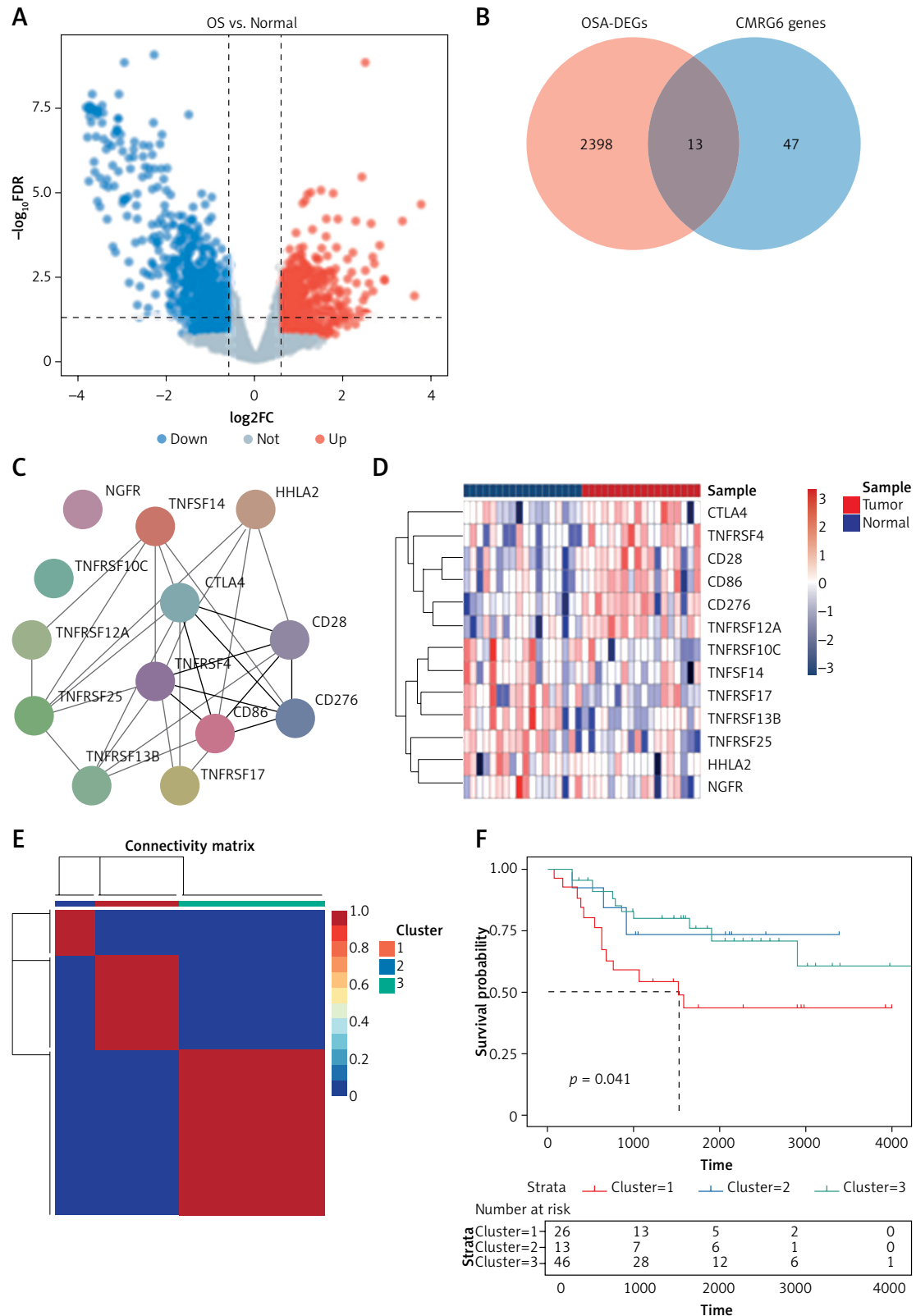


**Figure 1.** Identification of prognostic candidate genes and subtype construction. **A** – Volcano plot depicting the expression of differentially expressed genes between normal tissue and OSA tissue, (upregulated: 1046, down-regulated: 1365). **B** – Venn diagram showing the intersecting genes between OSA-DEGs and CMRGs gene sets. **C** – Results of the PPI network diagram for the 13 DECMRGs. **D** – Heatmap showing the differences in expression of the 13 DECMRGs in normal and OSA tissues. **E** – NMF clustering results show that the optimal number of clustering results is 3. **F** – KM curve analysis describes the survival outcomes for the three subgroups of patients, with those in subtype 1 having the poorest survival

differences in survival outcomes among the three subtypes, with Cluster 1 showing the poorest survival rates (Figure 1 F).

### Analysis of immune function and immune cell infiltration among molecular subtypes based on DECMRGs

To explore the differences in immune-related functions and immune cell infiltration among the three subtypes, we conducted ssGSEA analysis to evaluate immune cell infiltration across these subtypes (Figure 2 A). Our findings indicate that subtype 3 exhibited significantly higher levels of immune cell infiltration compared to subtypes 1 and 2. The results illustrated in Figures 2 B and C further confirm that immune cell infiltration is notably more pronounced in subtype 3. Furthermore, stromal score comparisons revealed that subtype 3 had a significantly higher stromal score than subtype 1 ( $p = 0.0085$ ).

In contrast, subtype 1 exhibited significantly higher tumor purity than subtype 3 ( $p = 0.049$ ), while its ESTIMATE score was significantly lower than that of subtype 3 ( $p = 0.049$ ) (Figure 2 D).

### Differential analysis and biological function exploration among subtypes

We analyzed DECMRG expression across the three subtypes and found that TNFRSF12A showed the lowest expression in subtype 1, while NGFR was significantly higher in subtype 2 compared to the other subtypes. Additionally, CD276 was most highly expressed in subtype 1, and TNFRSF10C was most highly expressed in subtype 3 (Figure 3 A). To further investigate the clinical characteristics of the three patient subtypes, we conducted a statistical analysis of disease metastasis. As shown in Figure 3 B, the metastasis rates were 23% in subtype 1, 15% in subtype 2, and

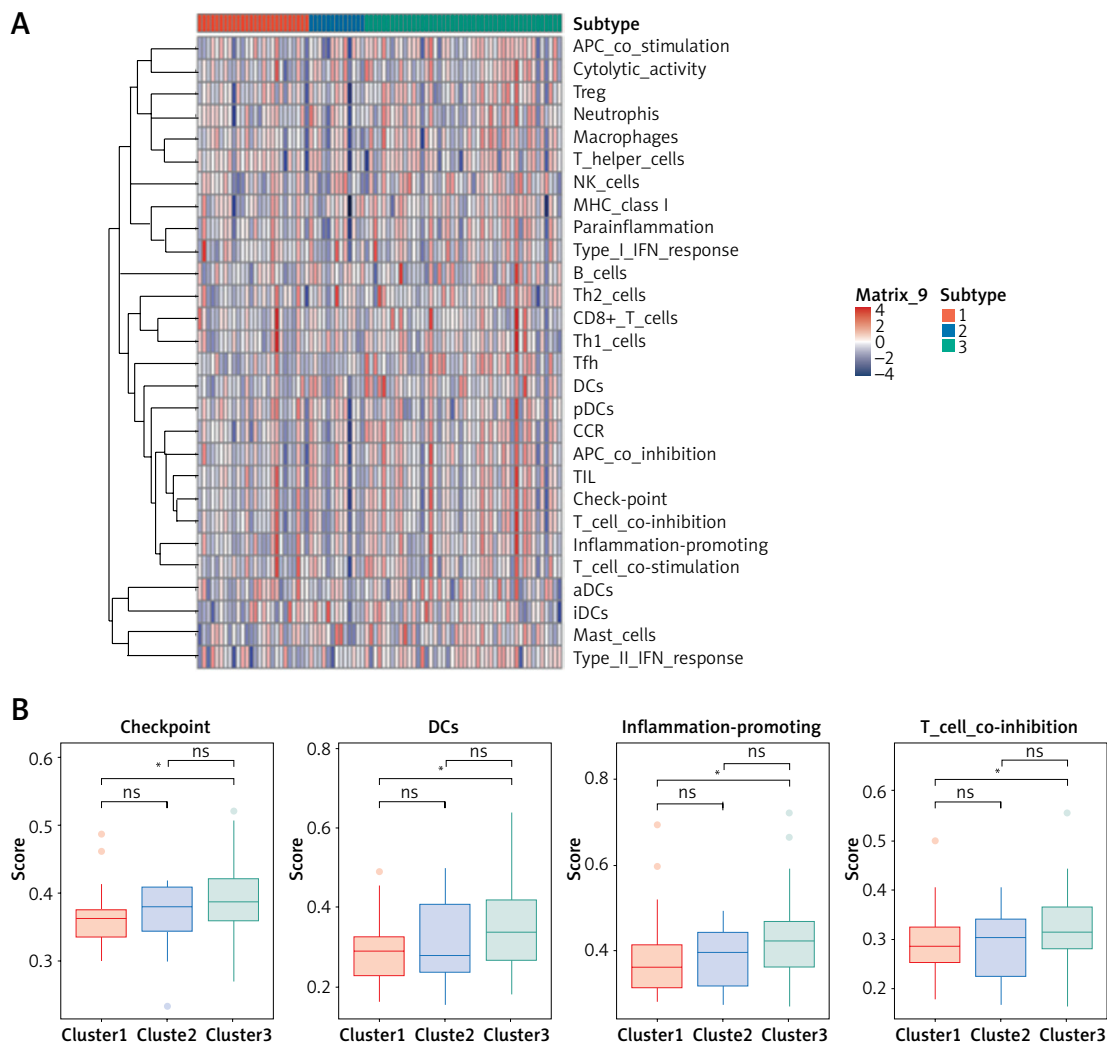


**Figure 2.** Analysis of immune function and immune infiltration. **A** – Heatmap depicting the differences in immune cell infiltration in the three subtypes groups. **B** – Box plots showing analysis of differences in the degree of infiltration of each immune cell in the three subtyping groups. ns denotes no significant difference, \*signifies  $p < 0.05$ , \*\*represents  $p < 0.01$ , and \*\*\*corresponds to  $p < 0.001$

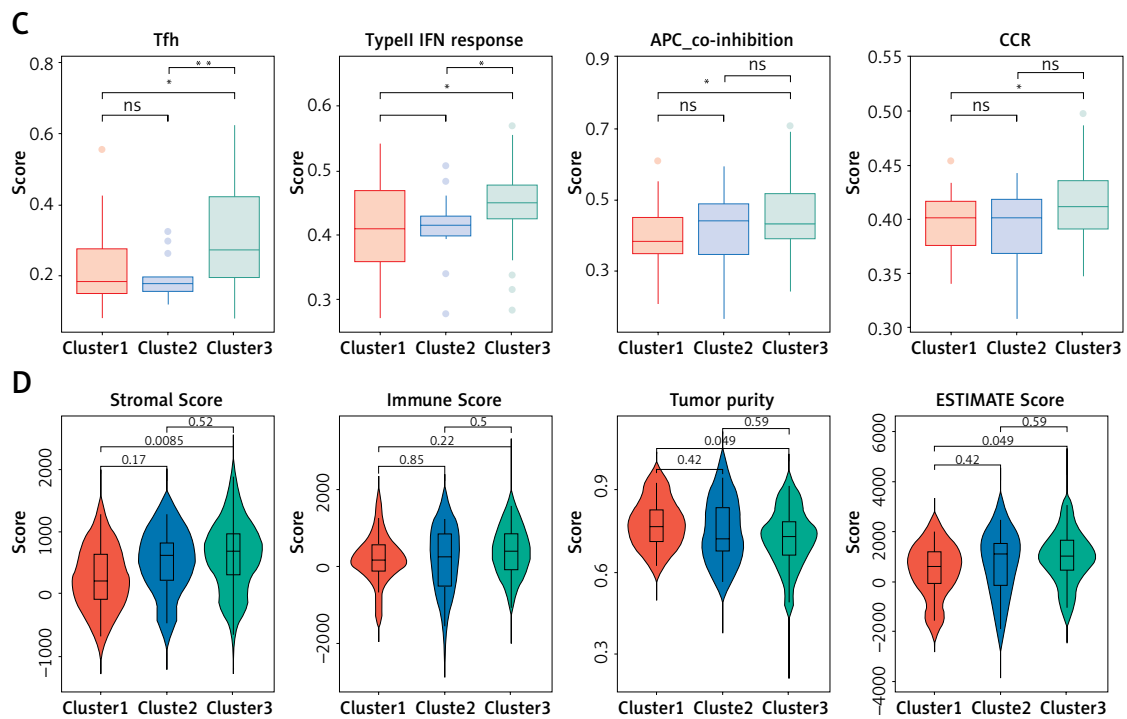


**Figure 2.** Cont. **C** – Variations in immune cell infiltration among the three subtypes. **D** – Violin plot illustrating the findings related to the stromal score, immune score, tumor purity, and ESTIMATE score across the three subtypes. ns denotes no significant difference, \*signifies  $p < 0.05$ , \*\*represents  $p < 0.01$ , and \*\*\*corresponds to  $p < 0.001$

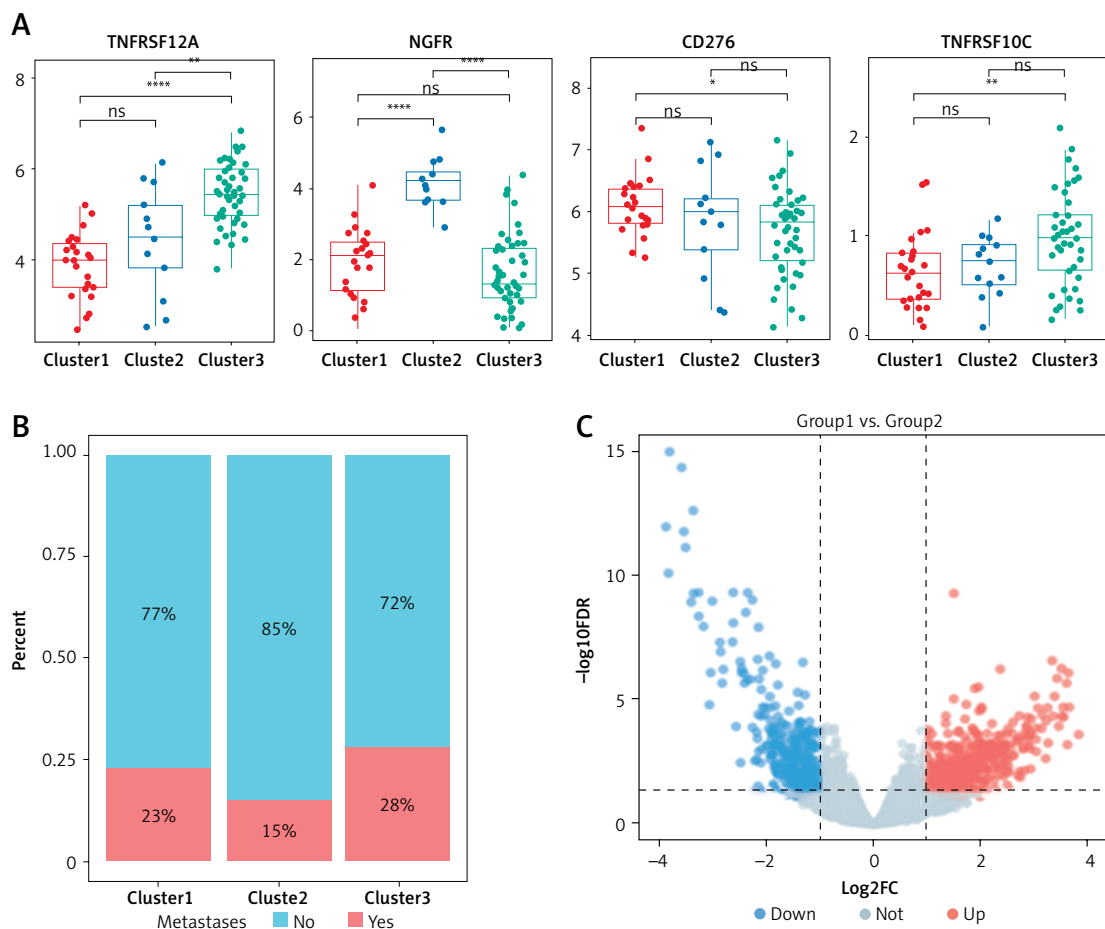


**Figure 3.** Analysis of differences between subtypes and functional exploration. **A** – Analysis of DECMRG expression among subtypes. Expression levels are plotted on the vertical axis, while the horizontal axis represents the different subtypes. **B** – Metastasis of illness in patients with various subtypes. **C** – Volcano plot showing the number of differentially expressed genes between the merged subtypes (subtype 2 and 3) and subtype 1, upregulated genes: 802, downregulated genes: 385. ns indicates no significant difference, \*indicates significant difference, \*\* and \*\*\* indicate increasing levels of statistical significance

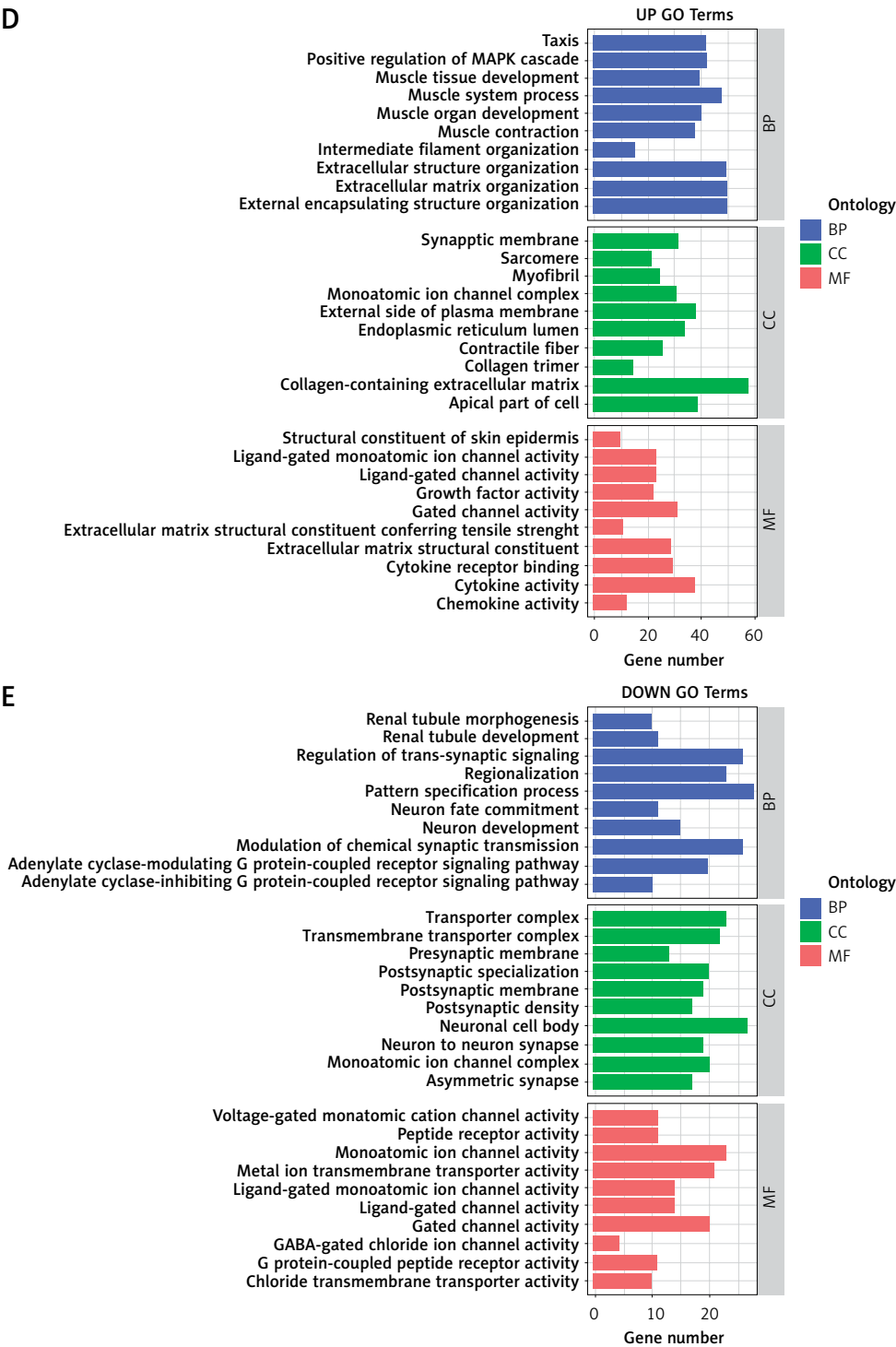


**Figure 3.** Cont. **D** – Results of GO enrichment analysis of upregulated genes. **E** – Results of GO enrichment analysis of downregulated genes. ns indicates no significant difference, \* indicates significant difference, \*\* and \*\*\* indicate increasing levels of statistical significance

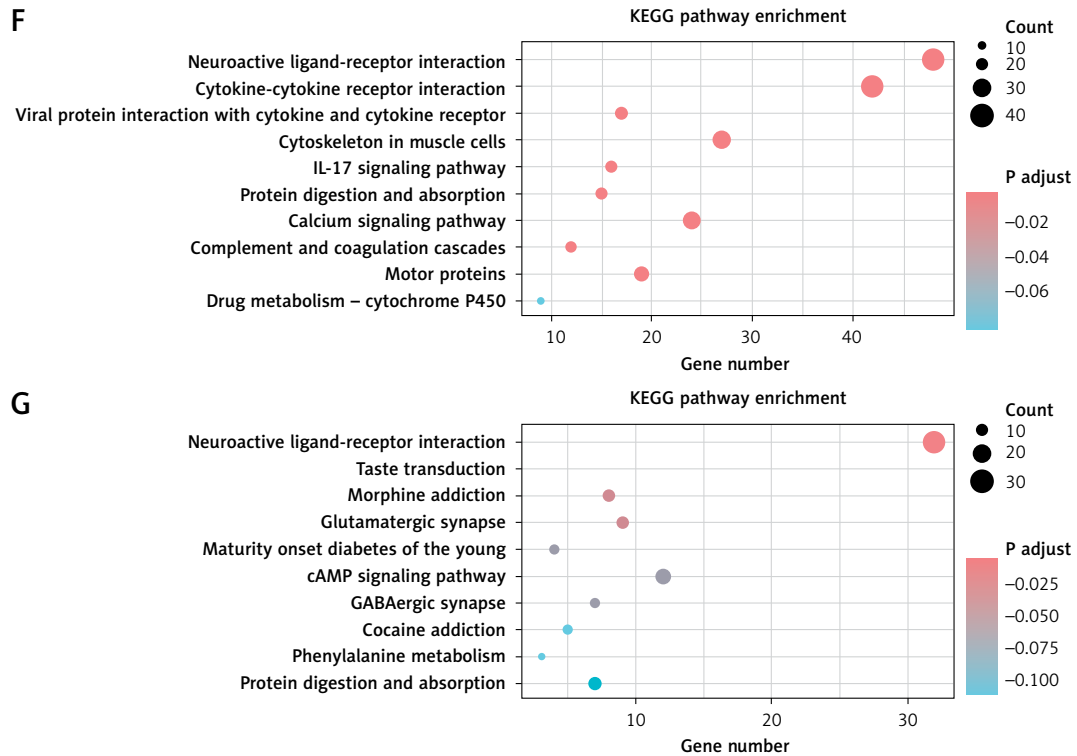


**Figure 3.** Cont. **F** – KEGG enrichment analysis of upregulated genes. **G** – Outcomes of KEGG enrichment analysis of downregulated genes. ns indicates no significant difference, \* indicates significant difference, \*\* and \*\*\* indicate increasing levels of statistical significance

28% in subtype 3. To explore differences between the subtypes, we merged the data from subtypes 2 and 3 based on survival outcomes. We then performed a differential analysis of the combined gene set compared with subtype 1, using thresholds of  $\log_2|FC| > 1$  and  $p < 0.01$ . This yielded 1,187 differentially expressed genes (group-DEGs), including 802 upregulated and 385 downregulated genes (Figure 3 C). Subsequently, we conducted GO and KEGG analyses to further explore the biological functions and pathway activities associated with the groups-DEGs. As illustrated in Figure 3 D, upregulated genes were enriched in extracellular structure and muscle tissue-related processes. In contrast, downregulated genes were mainly associated with neuroregulation, transmembrane transport, and protein function (Figure 3 E). KEGG analysis showed that upregulated genes were significantly involved in neural activity regulation, inflammatory signaling, and protein metabolism-related pathways (Figure 3 F), while downregulated genes were primarily enriched in protein metabolism-related pathways (Figure 3 G).

#### Identification of prognosis-related feature genes for developing OSA prognosis models

To further analyze the prognostic characteristics of OSA, this study identified the intersection of the OSA-DEGs gene set and the group-DEGs gene

set, resulting in 274 overlapping genes (Figure 4 A). Subsequently, univariate Cox analysis was performed, which yielded 54 genes significantly associated with survival ( $p < 0.05$ ) (Figure 4 B). LASSO regression analysis was then employed to refine the list of candidate genes (Figure 4 C). Based on cross-validation results, we selected an optimal model with 13 genes (Figure 4 D). Further multivariate regression analysis resulted in the selection of 5 characteristic genes to construct the prognostic model: risk score =  $-0.3182 \times EPYC + 0.2907 \times SCD1 + 0.5353 \times AOC3 + 0.6530 \times MYC + 2.0331 \times KCLK2$  (Figure 4 E). To evaluate model performance, ROC curves were plotted. As shown in Figure 4 F, the AUC values for the training set were 0.895 (1 year), 0.834 (3 years), and 0.862 (5 years). For the validation set, the AUCs were 0.719, 0.714, and 0.698 at the respective time points (Figure 4 G). These results indicate good predictive performance in the training set and moderate predictive performance in the validation set. Based on the median risk score, OSA patients were classified into low-risk and high-risk groups. As illustrated in the KM curve results in Figure 4 H, the survival probability of patients in the low-risk group was significantly higher than that of the high-risk group ( $p < 0.0001$ ). Additionally, survival status and risk scores were more favorable in the low-risk group (Figure 4 I). Among the feature genes,

SCD1, MYC, AOC3, and KLK2 were overexpressed in the high-risk group, while EPYC was expressed at lower levels (Figure 4 J).

### Constructing a nomogram for independent prognostic analysis

Survival analysis of the feature genes revealed that while EPYC showed a survival trend,

the difference was not statistically significant ( $p = 0.05384$ , Figure 5 A). In contrast, MYC exhibited a significant difference between risk groups (Figure 5 B). We then performed univariate and multivariate regression analyses integrating clinical features and risk scores, identifying M stage and risk score as independent prognostic factors ( $p < 0.05$ , Figures 5 C, D). To facilitate clinical application, we developed a nomogram integrating

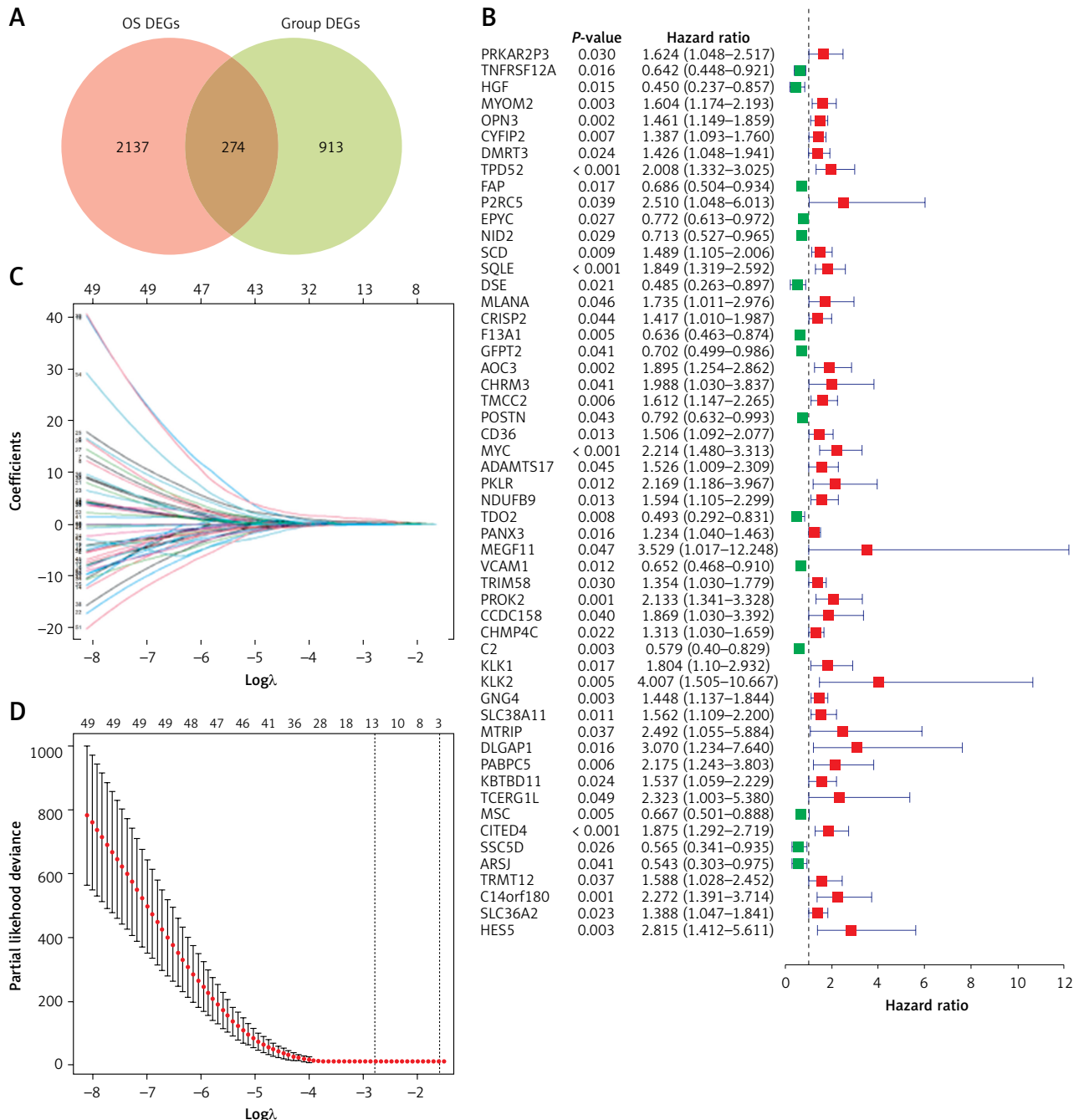


**Figure 4.** Identification of characteristic genes and construction of prognostic models. **A** – Venn diagram representing the intersection of the OSA-DEGs gene set and the group-DEGs gene set, with 274 intersecting genes. **B** – Results of the univariate regression analysis showing genes with a significance level less than 0.05. **C** – LASSO regression coefficient path plot showing each line representing a variable, with the vertical axis indicating coefficient values and the horizontal axis representing  $\log(\lambda)$  values. **D** – Cross-validation curve of the LASSO regression showing the partial likelihood deviance across different values of  $\log(\lambda)$

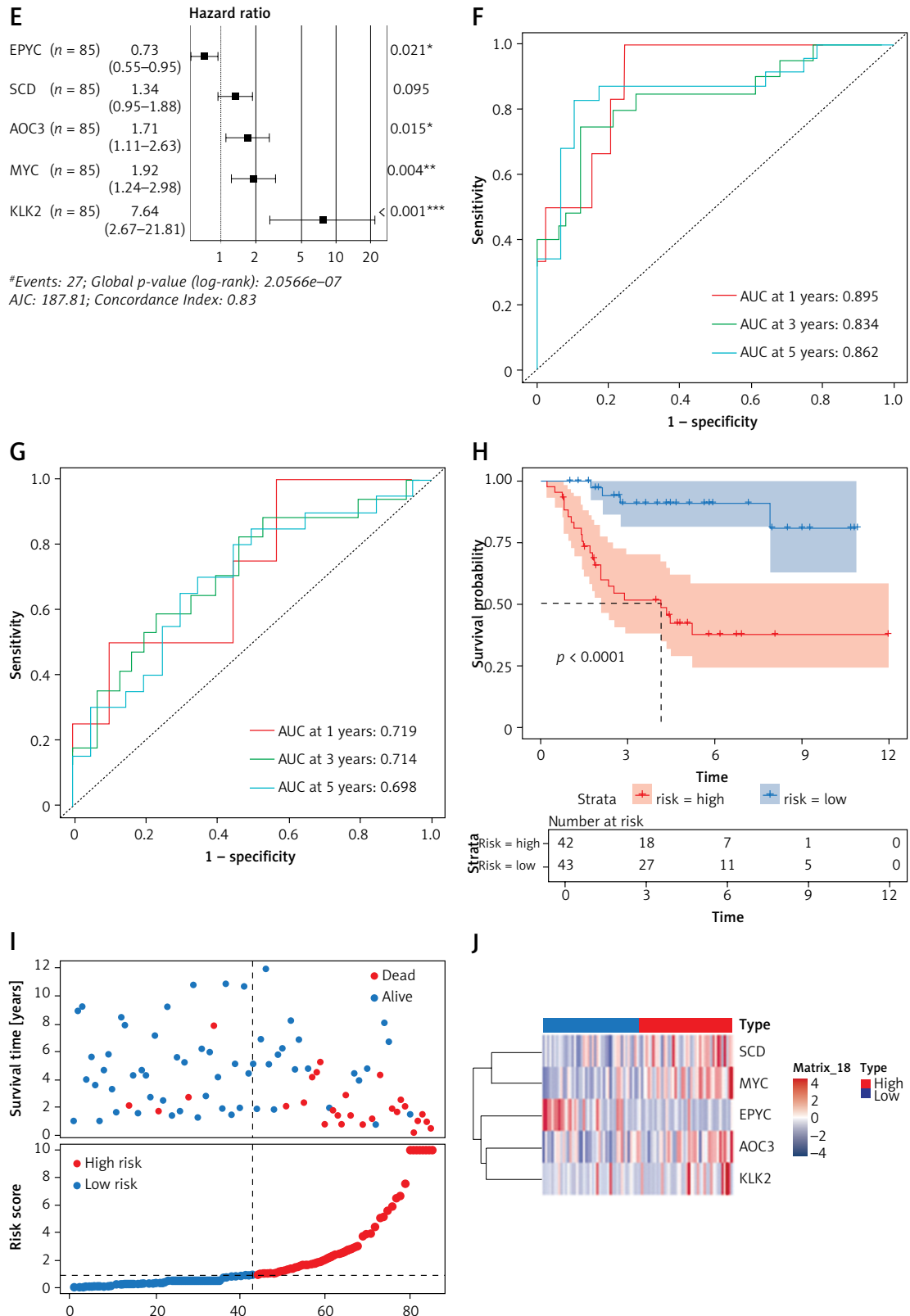


**Figure 4.** Cont. **E** – Results of the multivariate regression analysis identifying 5 characteristic genes. **F** – ROC analysis results for the training set indicating AUC values of 0.895, 0.834, and 0.862 at 1, 3, and 5 years, respectively. **G** – AUC values of the ROC curve for the validation set are 0.719, 0.714, and 0.698 at 1, 3, and 5 years, respectively. **H** – KM curve showing that the survival outcomes of the low-risk group are more favorable than those of the high-risk group. **I** – Distribution of survival status and risk scores for the high-risk and low-risk groups. **J** – Heatmap showing the expression levels of characteristic genes in the two risk groups

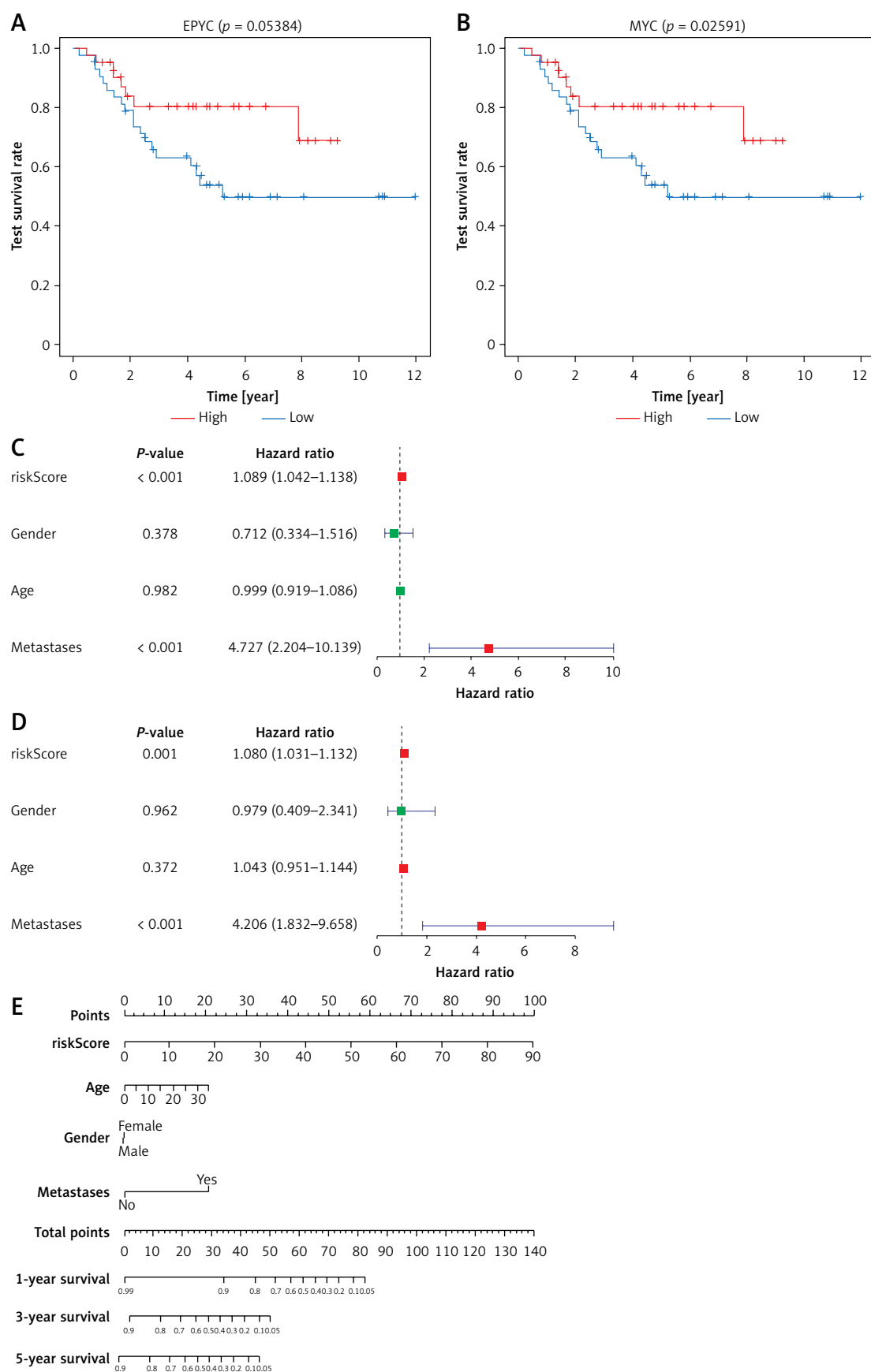
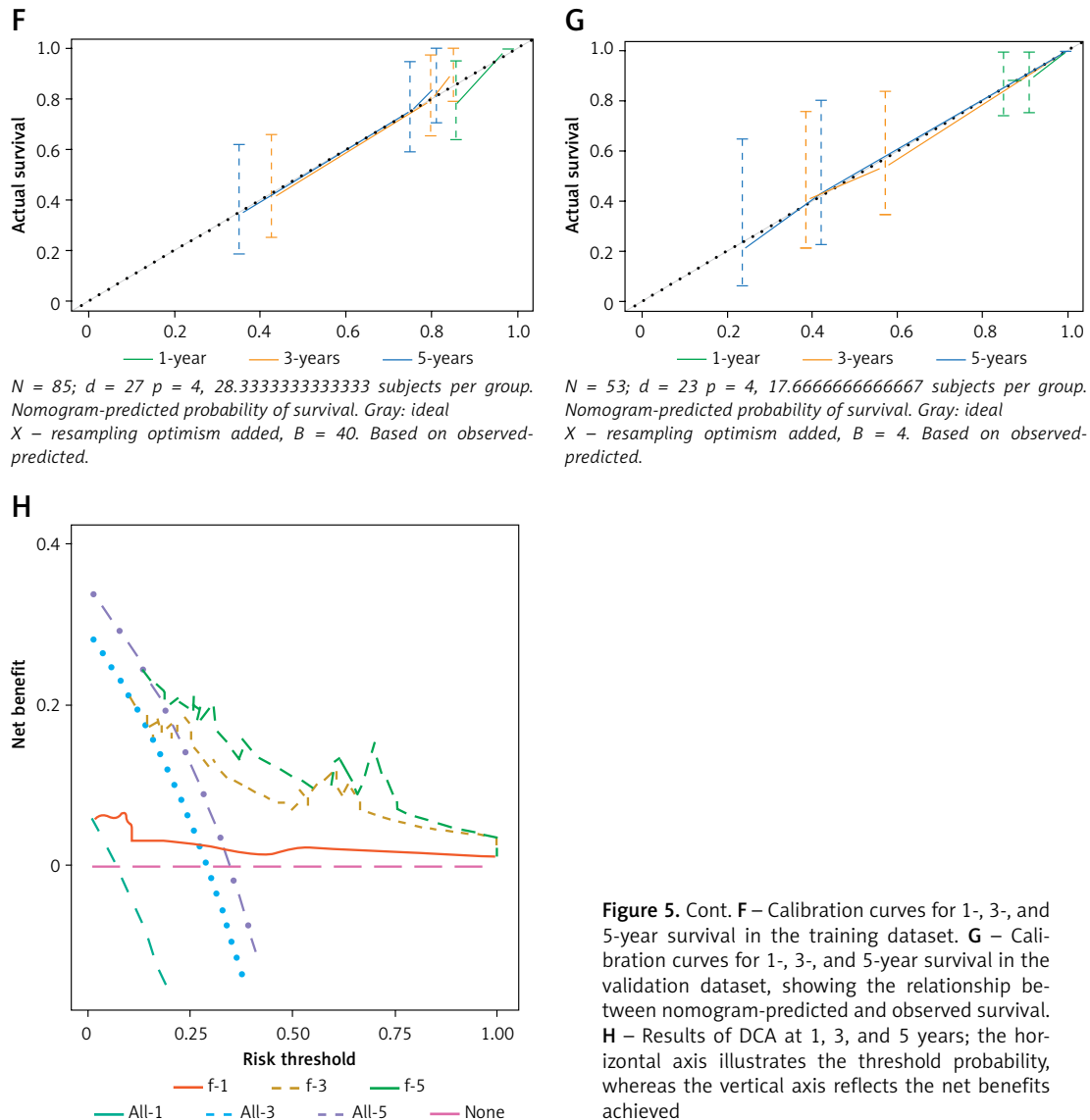


**Figure 5.** Development and assessment of nomograms. **A** – KM curve for EPYC. **B** – The KM curve illustrates a higher survival rate for MYC in the low-risk category compared to the high-risk category,  $p = 0.02591$ . **C** – Results of univariate regression analyses utilizing risk scores and clinical data. **D** – Results of multivariate regression analyses based on risk score and clinical information. **E** – Nomogram for comprehensively assessing the prognostic performance of OSA



risk score and clinical variables to predict 1-, 3-, and 5-year survival (Figure 5 E). Calibration curves (Figure 5 F) showed that the nomogram's predicted outcomes closely matched observed survival in the training set. Similar results were observed in the validation set (Figure 5 G). Decision curve analysis (DCA) further demonstrated that the nomogram provided potential net clinical benefit for predicting survival at 1, 3, and 5 years (Figure 5 H). In summary, our findings suggest that the nomogram has good predictive performance, and the 5-gene-based model may serve as an independent prognostic indicator for OSA.

#### Examination of the immune environment between high-risk and low-risk groups

To comprehensively assess immune infiltration and prognosis across risk groups, ssGSEA was used to compare immune-related functions

and cell infiltration between high- and low-risk groups. As shown in Figure 6 A, immune infiltration was markedly higher in the low-risk group. Figure 6 B further supports this finding, with box plots demonstrating elevated infiltration levels for most immune cell types. Consistently, the low-risk group showed significantly higher ESTIMATE, immune, and stromal scores, while tumor purity was increased in the high-risk group (Figure 6 C). Metastasis rates were also lower in the low-risk group (Figure 6 D).

Immune checkpoint gene expression was broadly upregulated in the low-risk group (Figure 6 E). Correlation analysis revealed that AOC3 and EPYC were positively associated with B cells, while MYC showed a negative correlation. Similarly, AOC3 and KLK2 positively correlated with pDCs, whereas MYC was negatively correlated. AOC3 (Figure 6 F), KLK2 (Figure 6 G), and EPYC (Figure 6 H) were positively correlated with most immune

cells, while MYC (Figure 6 I) and SCD1 (Figure 6 J) showed negative correlations.

To investigate interactions with oncogenic pathways, we analyzed the expression correlation between canonical oncogenes and signa-

ture genes in the TARGET-OS cohort. As shown in Supplementary Figure S1, KLK2 was negatively correlated with FAS ( $r = -0.41$ ,  $p < 0.01$ ), and SCD1 positively correlated with NRAS ( $r = 0.32$ ,  $p < 0.01$ ), indicating potential associations be-

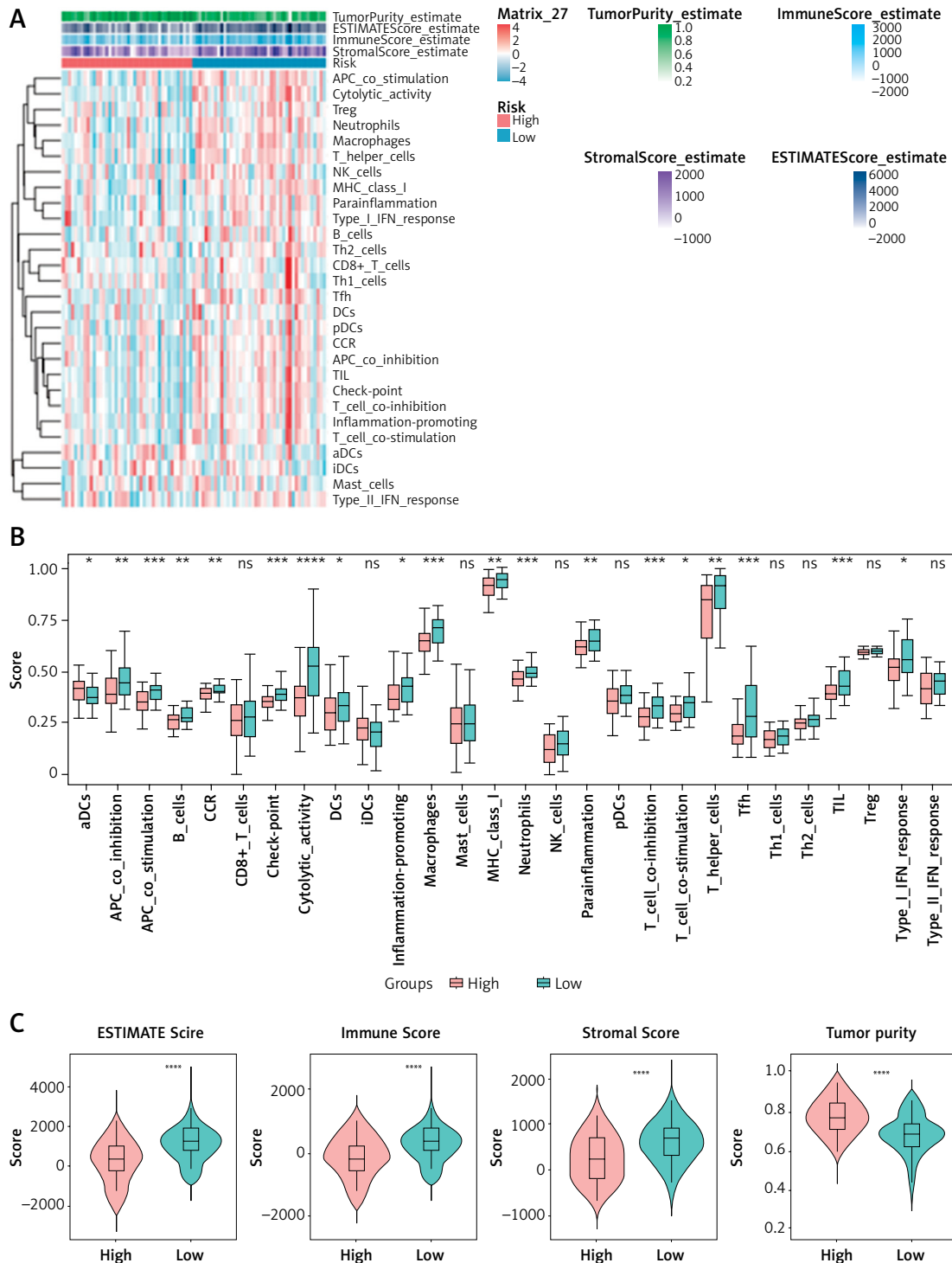


**Figure 6.** Evaluation of immune activity and infiltration of immune cells across the two risk categories. **A** – Heat-map displaying variations in immune cell infiltration between the two risk groups. **B** – Box plots illustrating variations in immune cell infiltration expression between the two risk groups. **C** – Violin plot illustrating the variation in multiple scores across the two risk groups. ns denotes no significant difference, \*signifies  $p < 0.05$ , \*\*represents  $p < 0.01$ , and \*\*\*indicates  $p < 0.001$

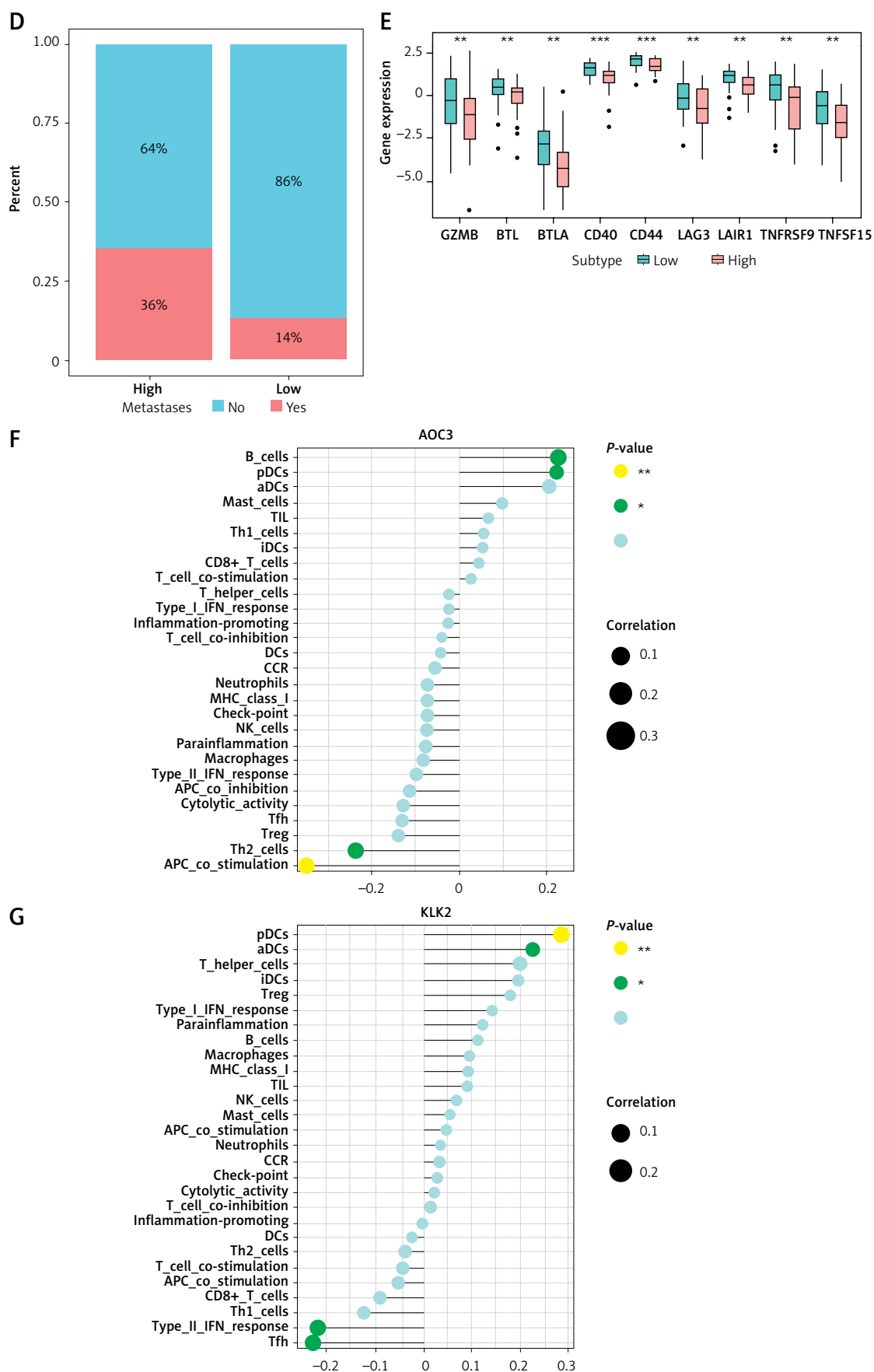
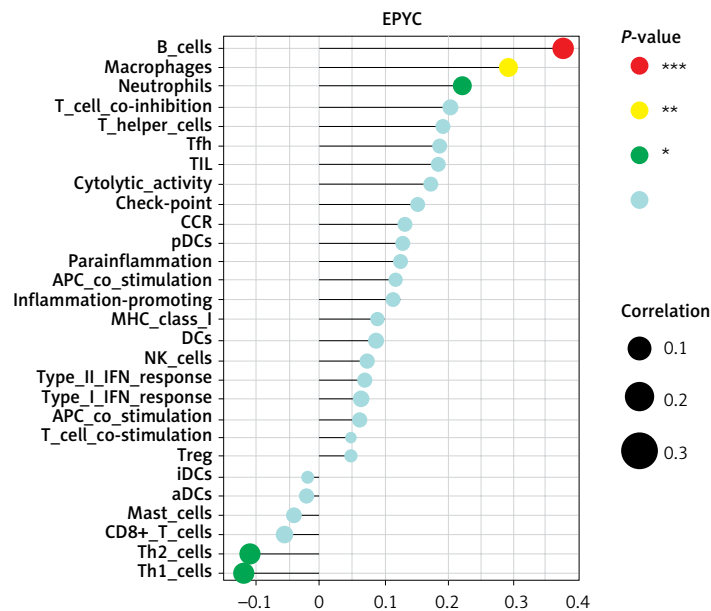
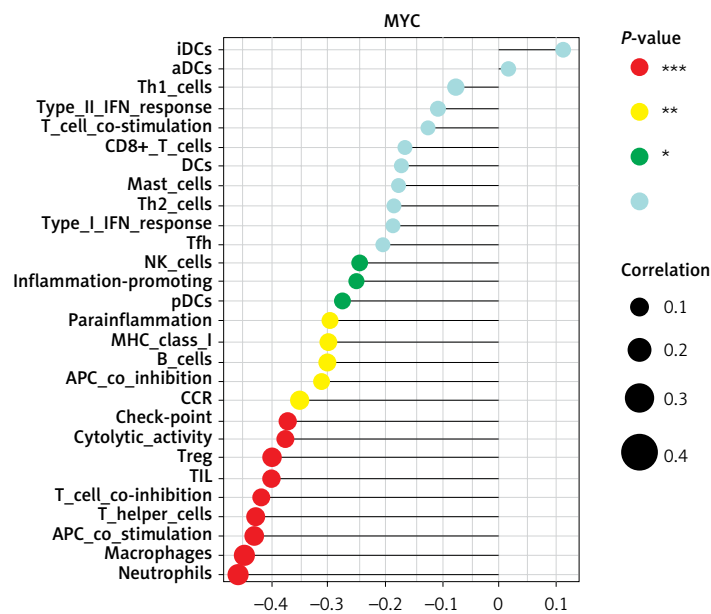


**Figure 6.** Cont. **D** – Comparison of disease metastasis between high- and low-risk groups. **E** – Comparison of immune checkpoint gene expression in high-risk versus low-risk groups. Correlation analysis diagram of characteristic genes AOC3 (**F**), KLK2 (**G**). ns denotes no significant difference, \*signifies  $p < 0.05$ , \*\*represents  $p < 0.01$ , and \*\*\*indicates  $p < 0.001$

H



I



J

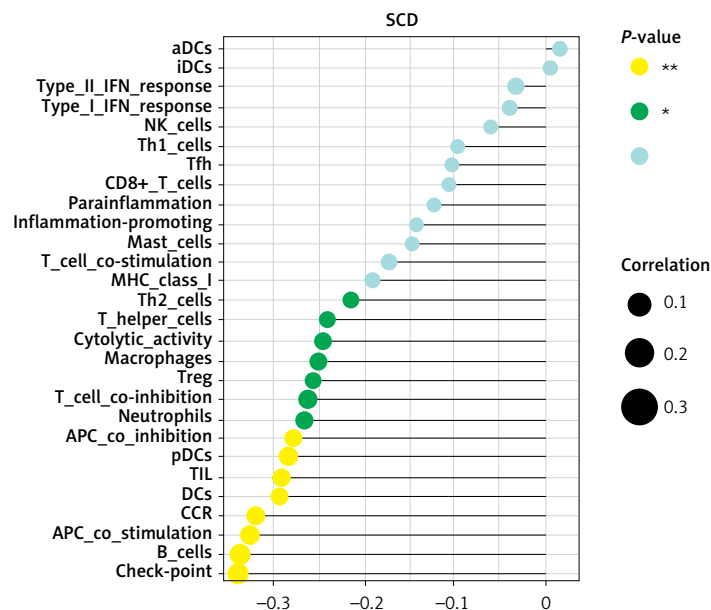


**Figure 6.** Cont. Correlation analysis diagram of characteristic genes EPYC (H), MYC (I), and SCD1 (J) with immune cell infiltration. ns denotes no significant difference, \*signifies  $p < 0.05$ , \*\*represents  $p < 0.01$ , and \*\*\*indicates  $p < 0.001$

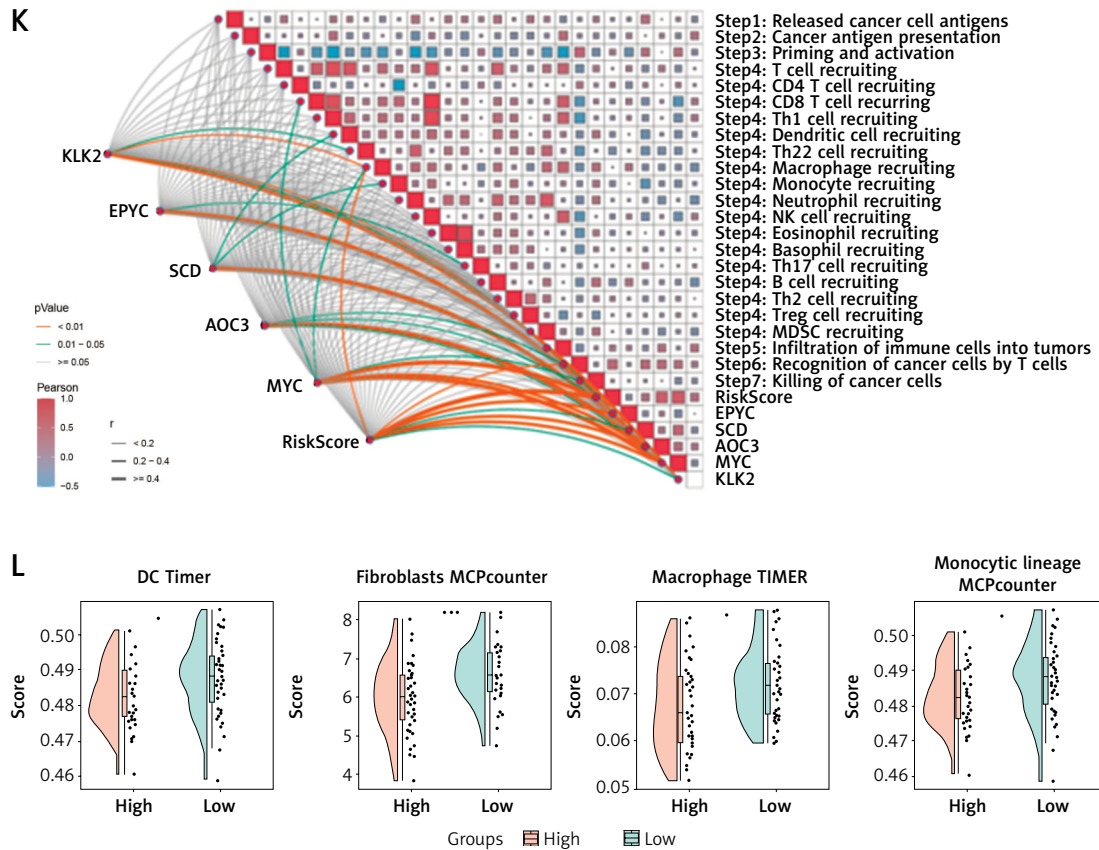


**Figure 6.** Cont. **K** – Heatmap of the association between signature genes, risk scores, and the immunization cycle. **L** – Infiltration levels for various cell types, such as dendritic cells (DC), fibroblasts, macrophages, and cells from the monocytic lineage, were compared between high-risk and low-risk groups. ns denotes no significant difference, \*signifies  $p < 0.05$ , \*\*represents  $p < 0.01$ , and \*\*\*indicates  $p < 0.001$

tween CMRG-derived markers and oncogenic signaling in osteosarcoma.

We further evaluated the association between the risk score and the tumor immune cycle. As illustrated in Figure 6 K, the risk score and most characteristic genes were negatively correlated with step 4 (immune cell recruitment). Additionally, Figure 6 L shows that infiltration scores for immune and tissue repair cells were significantly higher in the low-risk group.

Overall, these findings indicate a more active immune microenvironment in low-risk patients, suggesting that these patients may be more responsive to immunotherapy.

#### Response to PD-L1 inhibition immunotherapy according to risk scores

Following the assessment of immune infiltration, we extracted data from the IMvigor210 cohort regarding responses to PD-L1 inhibitor immunotherapy from public databases. In this cohort of 348 patients, responses to PD-L1 blockade varied and included stable disease (SD), partial response (PR), complete response (CR), and disease progression (PD). Our analysis using KM curves demon-

strated that the survival outcomes for the low-risk group were significantly better than those of the high-risk group (Figure 7 A). Importantly, we classified SD and PD as NR (not responding) and CR and PR as R (responding). The analysis indicated that patients in the R group exhibited significantly lower risk scores compared to those in the NR group (Figure 7 B). Additionally, the proportion of NR patients in the low-risk group was significantly lower than that in the high-risk group (Figure 7 C).

#### Functional enhancement of differentially expressed genes in high- and low-risk cohorts

To further investigate the biological functions and underlying molecular mechanisms across risk groups, we conducted differential expression analysis between the high- and low-risk groups ( $|\log FC| > 1$ ,  $p < 0.05$ , Figure 8 A). This analysis identified 908 upregulated and 413 downregulated genes, which were subsequently subjected to GO and KEGG enrichment analyses. GO analysis revealed that differentially expressed genes were significantly enriched in cell membrane structure-related pathways within the Biological Pro-

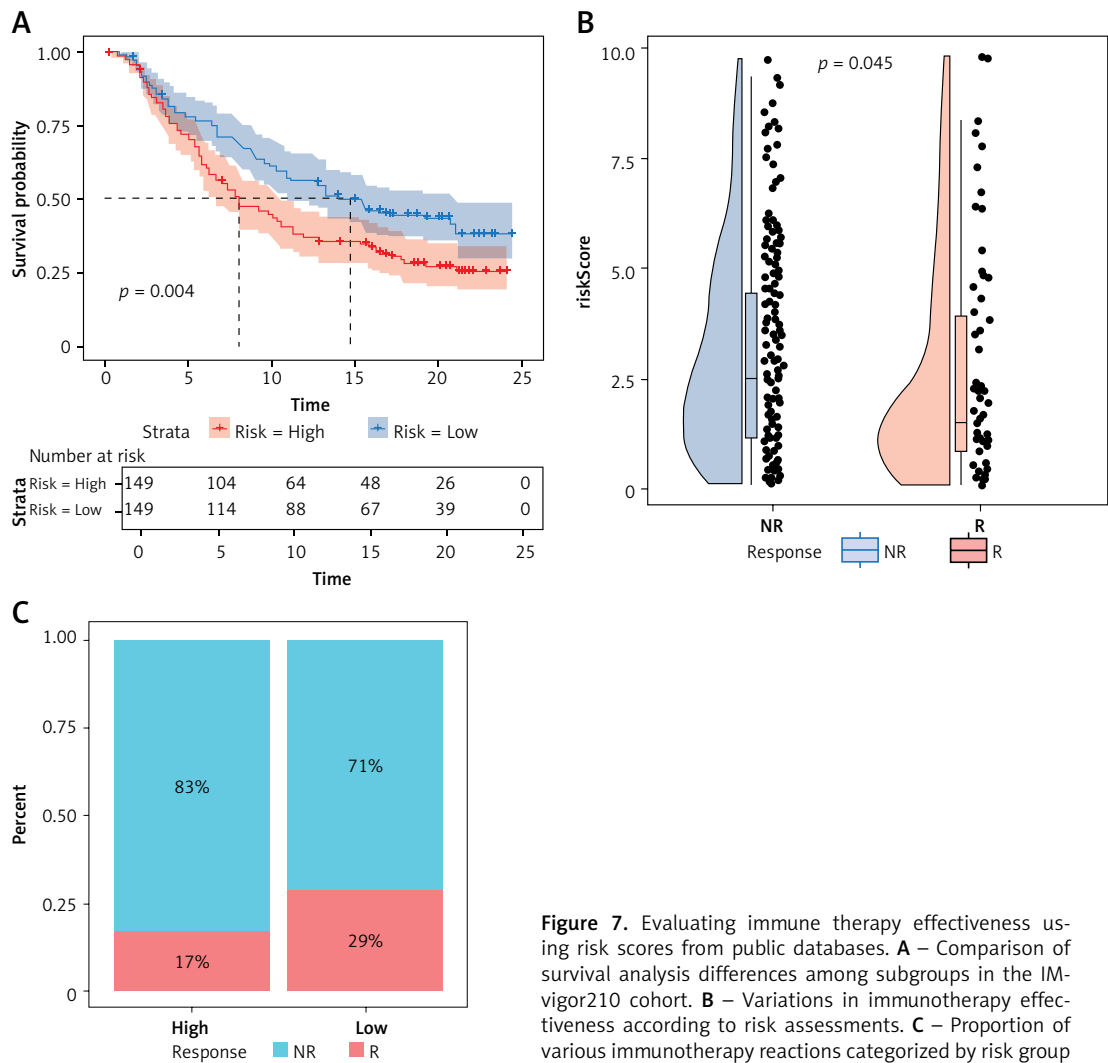


**Figure 7.** Evaluating immune therapy effectiveness using risk scores from public databases. **A** – Comparison of survival analysis differences among subgroups in the IM-vigor210 cohort. **B** – Variations in immunotherapy effectiveness according to risk assessments. **C** – Proportion of various immunotherapy reactions categorized by risk group

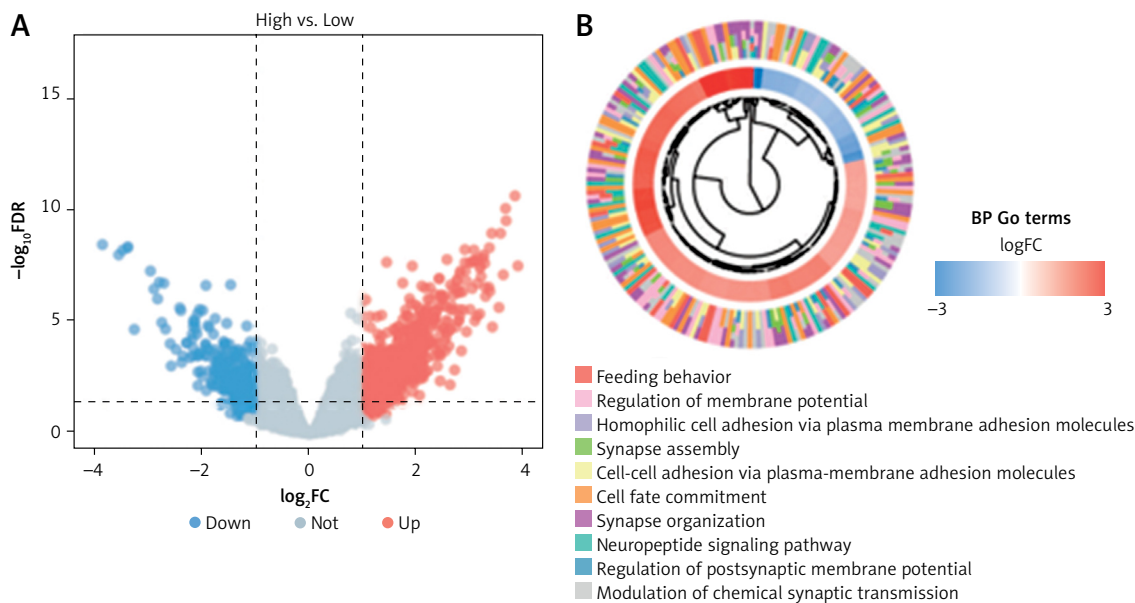


**Figure 8.** Investigation of biological roles and pathway activities in high- and low-risk groups. **A** – Volcano map illustrating genes expressed differently between high- and low-risk groups. Functional analysis in **(B)** Biological Activities (BP)

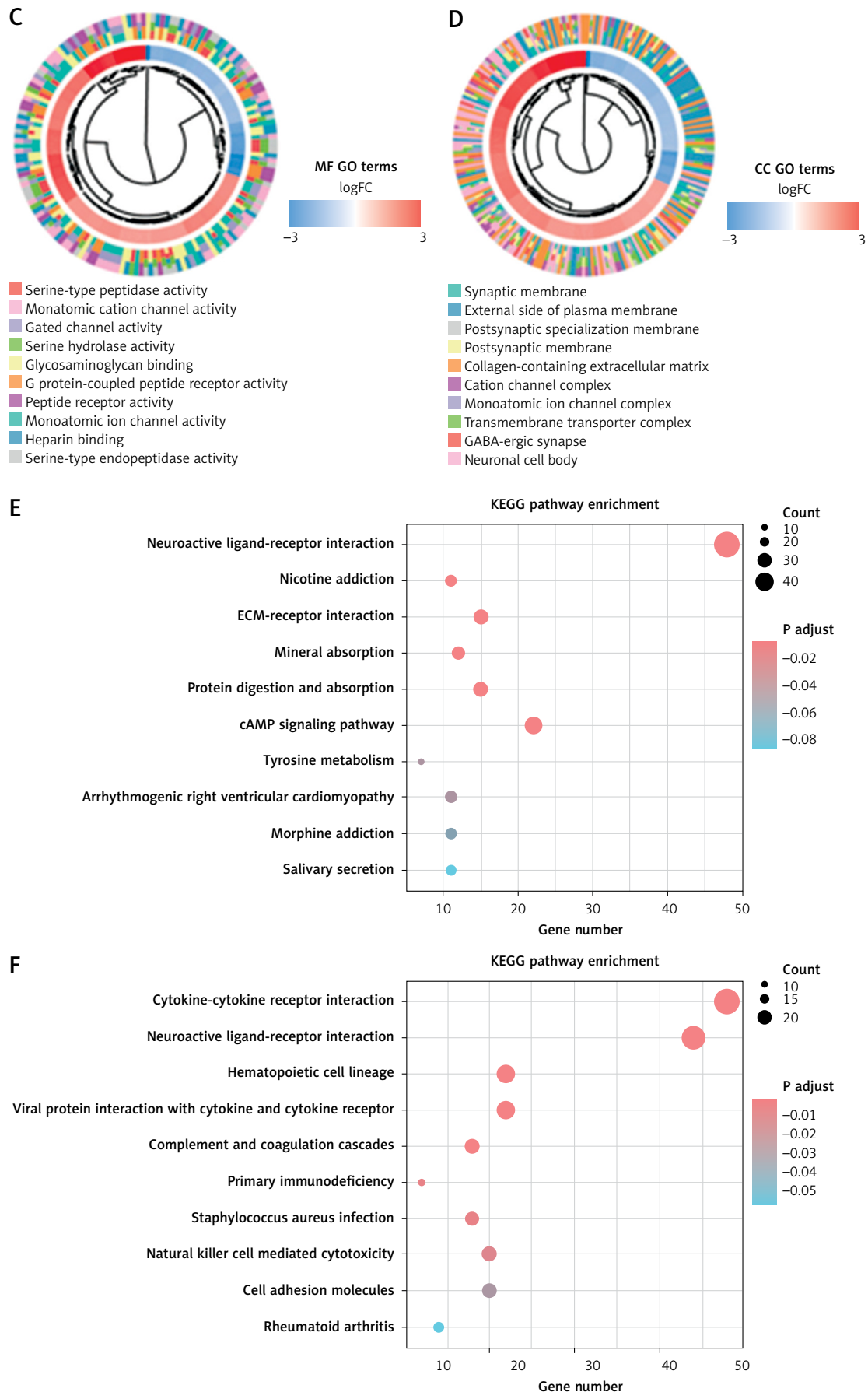


**Figure 8.** Cont. Functional analysis in (C) Molecular Roles (MF), and (D) Cellular Structures (CC). E – KEGG enrichment assessment of increased gene expression. F – KEGG enrichment assessment of downregulated genes

cess (BP) category (Figure 8 B), protein metabolism pathways within the Molecular Function (MF) category (Figure 8 C), and neuroregulation-related pathways within the Cellular Component (CC) category (Figure 8 D). Additionally, KEGG analysis indicated that upregulated genes were enriched in neuroregulation and protein metabolism pathways (Figure 8 E), whereas downregulated genes were associated with immune regulation-related pathways (Figure 8 F).

### Analysis of the relationship between TME and risk score-related cell types based on single-cell sequencing

To investigate the relationship between the TME and cell types associated with risk scores, we analyzed single-cell sequencing data from 11 OSA

patients in the GSE152048 dataset. After quality control and batch effect removal, we clustered and annotated the filtered cells into nine major cell types: macrophages, chondroblastic cells, osteoclasts, T cells, cycling cells, monocytes, osteoblastic cells, pericytes, endothelial cells, NK cells, and mast cells (Figure 9 A). Using the SCISSOR algorithm alongside sequencing data from TARGET-OS patients and risk group information, we identified cell types correlated with different risk levels (Figure 9 B) [25]. Consistent with expectations, we observed more high-risk-associated cell types in patients with recurrence and metastasis (Figure 9 C).

We then employed CellChat to infer overall intercellular communication based on ligand-receptor signaling. As illustrated in Figure 9 D, the results depict the number of cells involved in in-

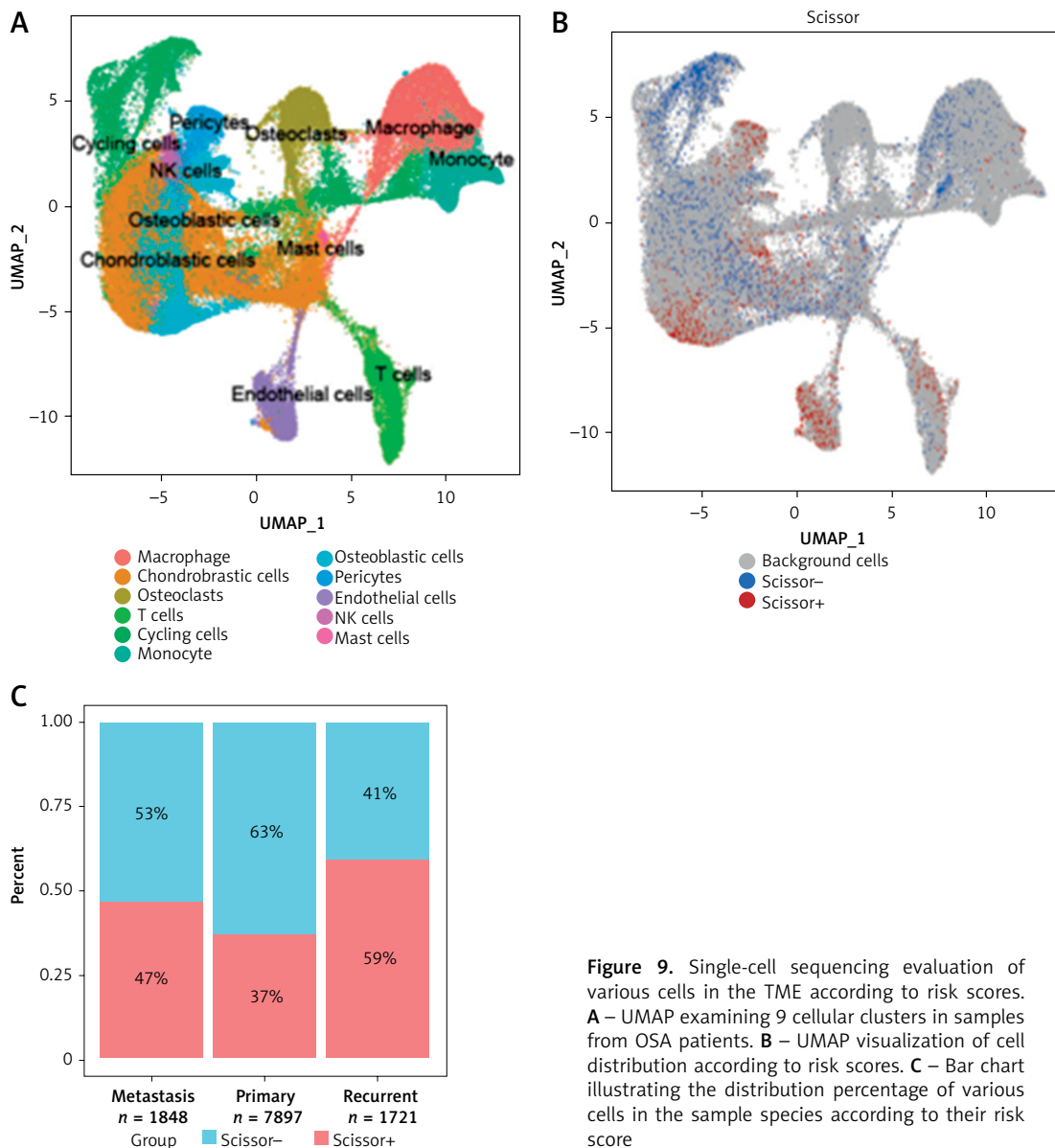


**Figure 9.** Single-cell sequencing evaluation of various cells in the TME according to risk scores. **A** – UMAP examining 9 cellular clusters in samples from OSA patients. **B** – UMAP visualization of cell distribution according to risk scores. **C** – Bar chart illustrating the distribution percentage of various cells in the sample species according to their risk score

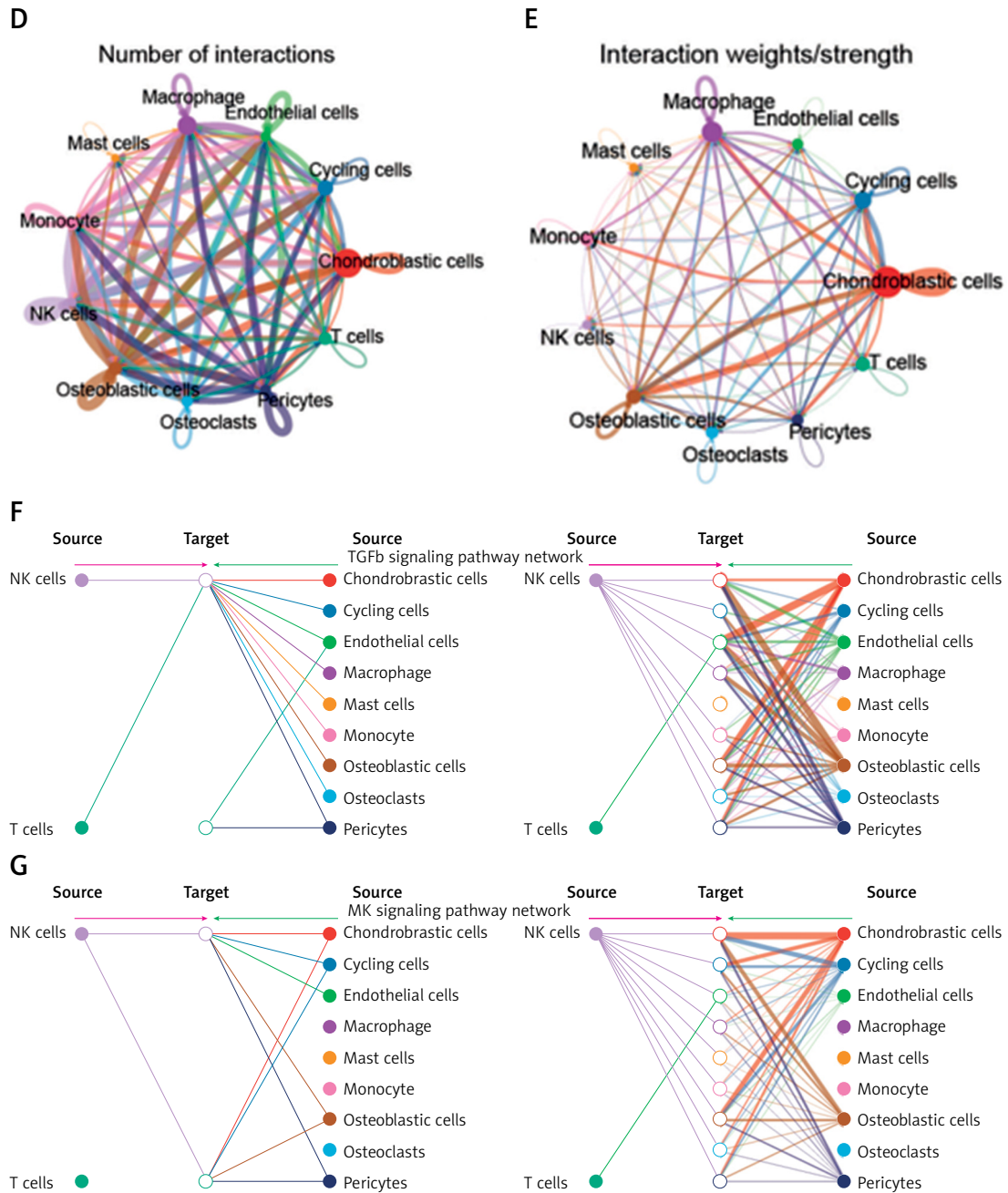


**Figure 9.** Cont. **D** – The number of cells engaged in communication and interaction with one another is depicted. Different colored circles represent the cell count, with larger circles signifying a higher number of cells involved. **E** – Intensity of intercellular communication exchanges: the thicker the line, the greater the intensity. Examination of cell interactions in the TGFβ (**F**) and MKK signaling pathway (**G**)

teractions, while Figure 9 E shows the frequency of interactions among different cell types. Collectively, these findings indicate significant cell-cell communication, which is essential for understanding immune cell functions within the TME. Furthermore, transforming growth factor  $\beta$  (TGF $\beta$ , Figure 9 F) and mitogen-activated protein kinase kinase kinase (MKK, Figure 9 G) signaling pathways were identified as potentially involved in cell proliferation, differentiation, and immune

regulation, possibly playing multifaceted roles in tumor progression. We further analyzed the relationships between the identified cell types and these pathways, and found distinct intercellular communication patterns targeting these pathways. Additionally, we assessed the distribution of characteristic genes across immune cell types and observed that MYC and SCD1 had superior cell-type-specific expression compared to other genes (Supplementary Figure S2).

## Comprehensive analysis of potential anti-OSA drugs

This study employed the CellMiner database to predict the correlation between feature genes and drug sensitivity, identifying a significant positive relationship between MYC and DMAPT (Figure 10 A), obatoclax (Figure 10 B), 8-chloro-adenosine (Figure 10 C), and palbociclib (Figure 10 D)

D). We also conducted a statistical analysis of approved drugs targeting feature genes, providing theoretical support for selecting suitable therapeutic agents for OSA patients (Figure 10 E). Notably, our analysis of drug sensitivity between risk groups showed that patients in the low-risk group had significantly lower IC<sub>50</sub> values for cisplatin (Figure 10 F) and temozolomide (Figure 10 G).

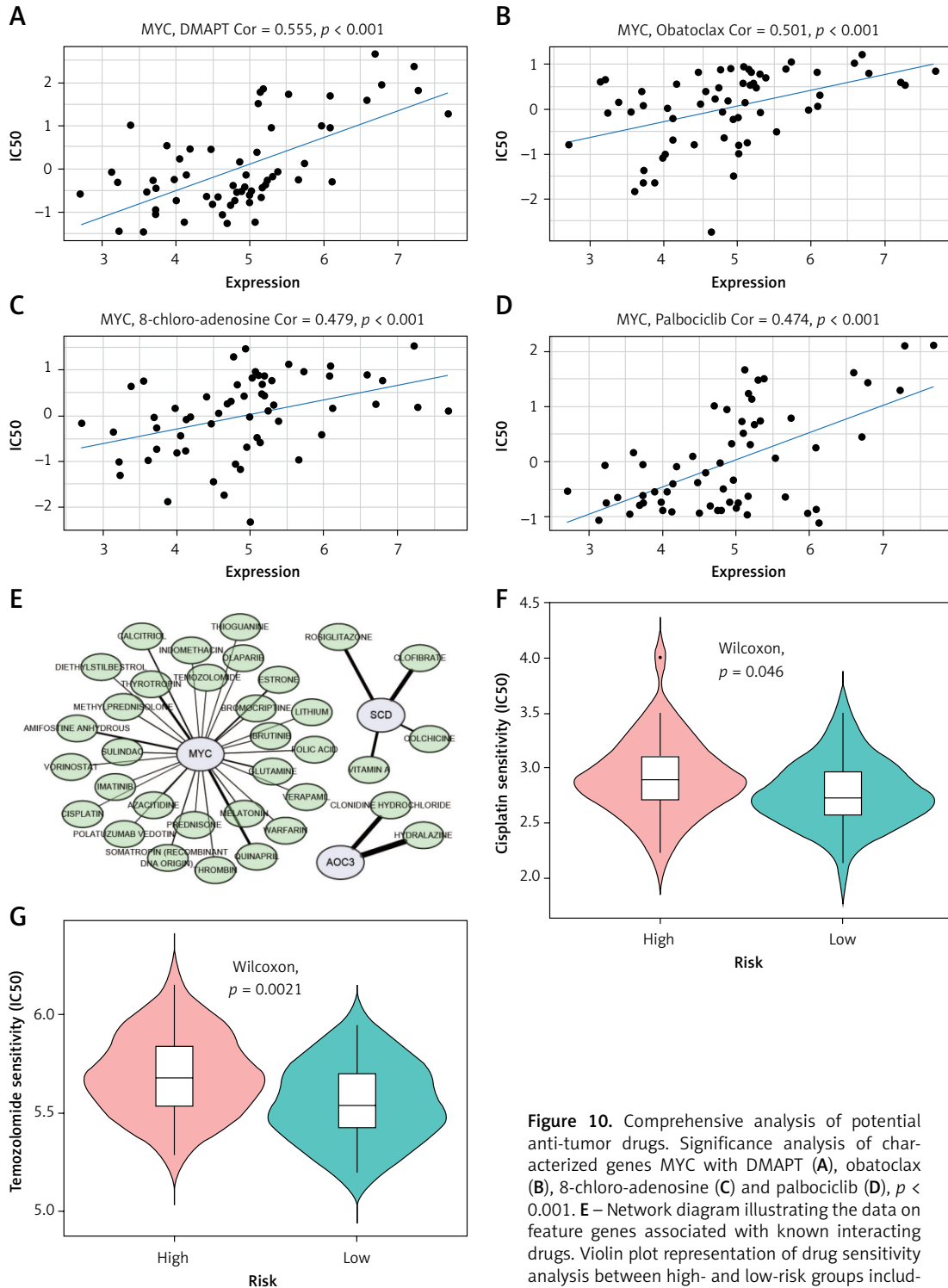


**Figure 10.** Comprehensive analysis of potential anti-tumor drugs. Significance analysis of characterized genes MYC with DMAPT (A), obatoclax (B), 8-chloro-adenosine (C) and palbociclib (D),  $p < 0.001$ . E – Network diagram illustrating the data on feature genes associated with known interacting drugs. Violin plot representation of drug sensitivity analysis between high- and low-risk groups including cisplatin (F) and temozolomide (G)

(Figure 10 G), indicating that these drugs may be more effective in low-risk patients.

### Comprehensive experimental analysis of characteristic gene expression, cell apoptosis, and cytotoxicity

Next, we validated the expression levels of the characteristic genes in the osteoblastic cell line hFOB1.19 and the OSA cell line U2OS. The qRT-PCR results (Figure 11 A) showed that EPYC was significantly overexpressed in U2OS cells. Similarly, SCD1 and AOC3 were also significantly upregulated in OSA cells. In contrast, no significant differences were observed in the expression of MYC and KLK2. These findings further confirm the differential expression of characteristic genes between normal and OSA cells. The MTT cytotoxicity assay results (Figure 11 B) demonstrated that both temozolomide and cisplatin exhibited concentration-dependent enhanced inhibitory effects on the proliferation of OSA cell lines U2OS and SAOS2. SAOS2 cells showed higher sensitivity to both drugs, with a significantly lower  $IC_{50}$  (81.52  $\mu$ M) for temozolomide compared to U2OS cells

(112.4  $\mu$ M), and a markedly lower  $IC_{50}$  (4.087  $\mu$ M) for cisplatin (9.314  $\mu$ M). TUNEL apoptosis assays further corroborated the heightened sensitivity of SAOS2 cells, revealing a significantly higher proportion of TUNEL positive cells in SAOS2 cells following treatment with either temozolomide or cisplatin, compared to U2OS cells (Figures 11 C, D). Collectively, these data consistently demonstrate that SAOS2 cells possess higher sensitivity than U2OS cells to temozolomide- and cisplatin-induced cytotoxicity and apoptosis.

### Discussion

Immunotherapy plays a crucial role in tumor treatment. Although it has not yet been approved for OSA, several approaches – including immune checkpoint inhibitors, bispecific antibodies, and chimeric antigen receptor (CAR) T cells – are actively undergoing clinical development [26]. Consequently, research on immunotherapy for OSA is expected to become a key focus in the field. CMRGs are essential for regulating T cell activity, maintaining immune balance, and modulating immune responses [27, 28]. This study evaluated the

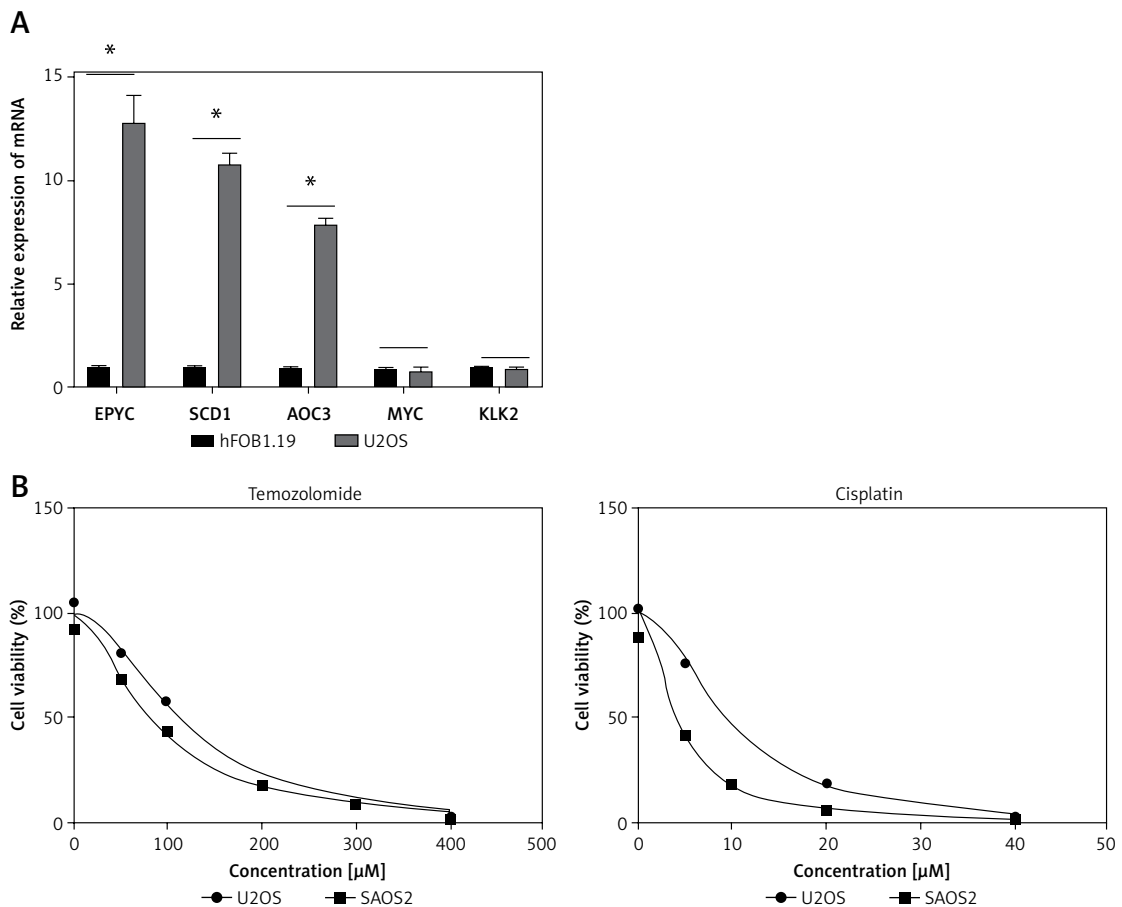


**Figure 11.** Comprehensive experimental analysis of characteristic gene expression, cell apoptosis, and cytotoxicity. **A** – qRT-PCR Results of EPYC, SCD1, and AOC3 in normal cells and OSA cells. **B** – MTT assay to detect the viability of U2OS and SAOS2 cells treated with different concentrations of temozolomide or cisplatin for 48 h. ns denotes no significant difference, \* signifies  $p < 0.05$

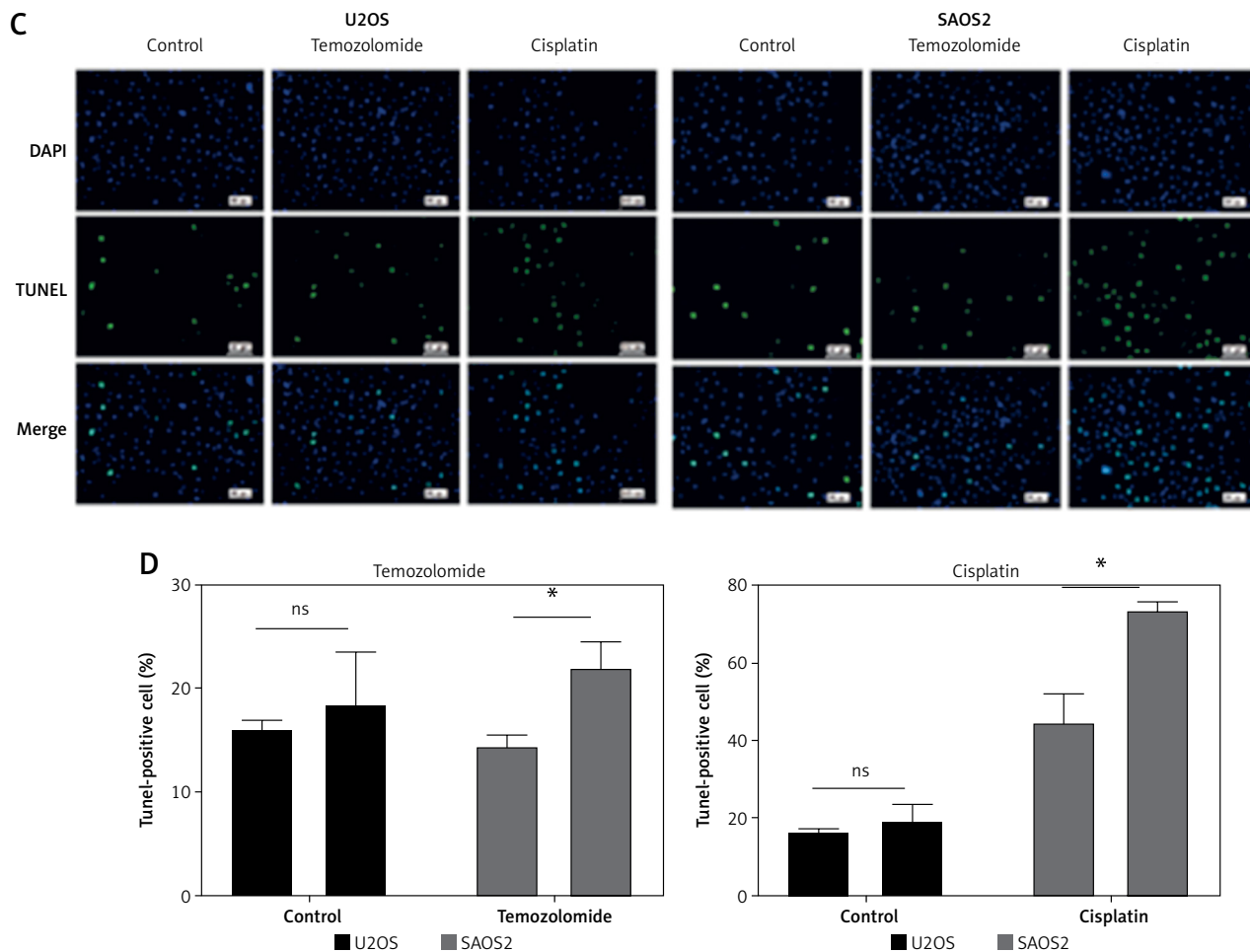


**Figure 11.** Cont. **C** – Representative images of TUNEL assay for cell apoptosis. **D** – Quantitative analysis results of TUNEL assay for cell apoptosis. ns denotes no significant difference, \* signifies  $p < 0.05$

prognostic outcomes of OSA patients based on CMRGs, and identified five characteristic genes: EPYC, SCD1, AOC3, MYC, and KLK2.

Epiphygan (EPYC), located on human chromosome 12, is a protein-coding gene composed of seven exons [29]. Its primary function is to regulate fibril formation through interactions with collagen fibrils and other extracellular matrix proteins [30]. EPYC has been implicated in various diseases. Liang *et al.* demonstrated that EPYC serves as a diagnostic marker for osteoarthritis, supported by both machine learning and in vitro experiments [31]. Similarly, Zhang *et al.* confirmed EPYC as a characteristic gene for osteoarthritis and reported a significant correlation between EPYC expression and immune infiltration [32]. In addition, EPYC has been identified as a prognostic biomarker for pancreatic cancer [30]. Our results revealed that EPYC is highly expressed in OSA tissues, and that patients in the low-risk group exhibited significantly better survival outcomes. Furthermore, EPYC expression was positively correlated with immune cell infil-

tration, suggesting its potential as a prognostic biomarker for OSA.

Stearoyl-CoA desaturase 1 (SCD1) is an enzyme located in the endoplasmic reticulum, which catalyzes the desaturation of saturated fatty acids (SFAs) [33]. A review study reported that SCD1 acts as an autophagy inducer, affecting cancer cell proliferation and metastasis [34]. In colorectal cancer, SCD1 plays a key role in drug-induced growth inhibition, and SCD1 knockout enhances sensitivity to ferroptosis [35]. Notably, Cai *et al.* found that SCD1 regulates the Akt/mTOR pathway, promoting lipid metabolism and enhancing OSA cell proliferation [36].

Amine oxidase copper containing 3 (AOC3) encodes a cell adhesion protein that is involved in lymphocyte extravasation and recirculation [37]. Dysregulation of AOC3 expression has been shown to influence cancer progression. Studies report that AOC3 expression is decreased in colorectal cancer tissues and serum, with low levels correlating with poor prognosis [38, 39]. Conversely, high AOC3 expression in thymic carcinoma has

been significantly associated with unfavorable prognosis [40].

MYC is a transcription factor and a key driver of human tumorigenesis. The MYC protein mainly regulates transcriptional processes, including elongation, DNA replication, and cell cycle progression [41, 42]. Targeting MYC has emerged as a potential strategy for cancer therapy [43, 44].

Human kallikrein 2 (KLK2) belongs to the kallikrein gene family and functions as a serine protease [45, 46]. Recent studies suggest that KLK2 may be a therapeutic target in prostate cancer, where elevated KLK2 expression correlates with increased proliferation and reduced apoptosis [47]. KLK2 has also been established as a prognostic biomarker for prostate cancer [48]. Our study explored KLK2 as a component of a prognostic model for OSA, demonstrating its potential value in predicting survival.

In conclusion, this study identifies five characteristic genes based on CMRGs to construct a prognostic model, which may support personalized treatment strategies for OSA patients and inform clinical decision-making.

Most immunotherapy strategies rely on the patient's intrinsic immune response, typically progressing through three stages: immune surveillance, self-regulation, and immune evasion [14]. Immune checkpoint inhibitors are central to these mechanisms and have shown promise in treating OSA [14, 49]. CTLA-4 binds to B7 on Tregs and antigen-presenting cells, thereby suppressing T cell activity. Blocking this interaction can restore T cell functionality [49]. Notably, anti-PD-1 therapy in an OSA mouse model led to increased expression of GZMB in T cells [50, 51]. Yoshida *et al.* reported a positive correlation between GZMB and IFN- $\gamma$  expression, with elevated GZMB levels linked to improved survival in OSA patients [52]. These findings highlight the potential relevance of GZMB in immunotherapy efficacy. Our analysis revealed elevated GZMB expression in the low-risk group, along with other immune checkpoint-related genes such as BTK, BTLA, CD40, CD44, LAG3, LAIR1, TNFRSF9, and TNFSF15, suggesting that low-risk patients may have greater potential to respond to immunotherapy.

Dimethylaminoparthenolide (DMAPT) is an anti-inflammatory compound that has been reported to inhibit prostate cancer [53] and bladder cancer progression [54]. Obatoclax, a BCL2 family inhibitor, induces thyroid cancer cell death by disrupting lysosomal function [55]. 8-chloro-adenosine is a nucleoside analog that has been investigated in leukemia research [56]. Palbociclib is an orally available CDK4/6 inhibitor, which suppresses proliferation and induces cell cycle arrest, with broad application in breast cancer therapy [57, 58]. Our

study identified a significant positive correlation between MYC expression and the IC<sub>50</sub> values of these drugs. Moreover, our analysis revealed that low-risk patients showed greater predicted sensitivity to cisplatin and temozolomide, supporting their potential clinical utility in this subgroup.

Our correlation analysis (Supplementary Figure S1) revealed associations between the CMRG-based signature and classic oncogenes. The negative correlation between KLK2 and FAS may indicate an association between KLK2 expression and FAS-mediated apoptosis, a mechanism known to contribute to chemotherapy resistance [59]. Conversely, the positive correlation between SCD1 and NRAS is consistent with NRAS's role in activating SREBP-1c-driven lipogenesis – a pathway that increases the production of monounsaturated fatty acids (MUFA) through SCD1-mediated desaturation, thereby potentially enhancing membrane fluidity, oncogenic signaling, and tumor cell proliferation [60, 61]. Notably, the absence of correlation with TP53 – a gene frequently mutated in OSA – may indicate that our CMRG signature reflects biological pathways distinct from canonical tumor suppressor mechanisms.

To our knowledge, this is the first comprehensive study to construct a CMRG-based prognostic model for OSA that integrates subtype classification, immune profiling, drug sensitivity prediction, and experimental validation, thereby expanding the understanding of immune regulation in OSA and proposing novel clinical biomarkers. However, several limitations warrant consideration: the analyses primarily rely on public databases, which may introduce population bias; although qRT-PCR validated gene expression, further *in vivo* and clinical studies are needed to confirm the functional roles of these genes; and the risk model's generalizability requires external validation due to the lack of prospective cohorts.

In conclusion, this study subdivided OSA samples into three subtypes based on CMRGs and analyzed the differences in immune cell infiltration levels among the subtypes, revealing that subtype 3 exhibited significantly higher immune infiltration levels than subtypes 1 and 2. By merging subtypes 2 and 3, we identified differentially expressed genes, ultimately selecting five characteristic genes through LASSO and multivariable regression analysis to construct a prognostic model for OSA. Our results showed that the low-risk group had better prognostic outcomes, suggesting that patients in this group may have greater potential to respond to immunotherapy. Additionally, single-cell sequencing results characterized the distribution of immune cells and the expression levels of characteristic genes within these cells. In summary, the prognostic prediction model devel-

oped in this study may help inform strategies to enhance survival rates in OSA patients, providing a theoretical basis for personalized treatment approaches.

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## Ethical approval

Not applicable.

## Conflict of interest

The authors declare no conflict of interest.

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