Exploring New Therapeutic Targets for Myasthenia Gravis: A Plasma and Cerebrospinal Fluid Proteomics Mendelian Randomization Study

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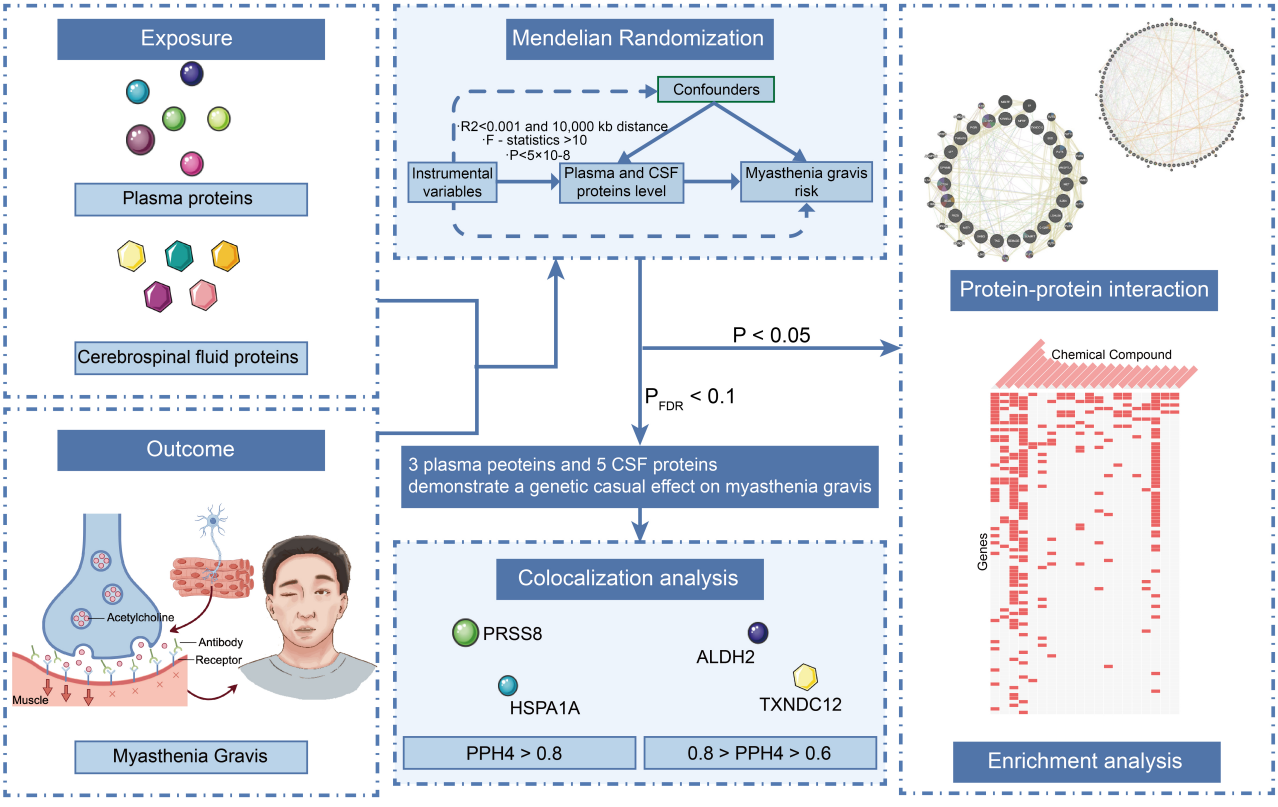
Graphical Abstract



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Research Article

Life Conflux

Exploring New Therapeutic Targets for Myasthenia Gravis: A Plasma and Cerebrospinal Fluid Proteomics Mendelian Randomization Study

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Abstract

Background: Myasthenia Gravis (MG) is a chronic autoimmune neuromuscular disorder that severely impacts patients' quality of life. Identifying plasma and cerebrospinal fluid (CSF) proteins with a genetic causal relationship to MG may provide novel therapeutic targets.
Methods: This study employed the Mendelian randomization (MR) approach, in combination with Bayesian colocalization analysis, to assess the causal relationship between 4,185 plasma proteins and 832 CSF proteins and the risk of MG. Sensitivity analyses were also performed to validate the robustness of the MR results. Additionally, protein–protein interaction networks and candidate drug predictions were utilized to elucidate the complex interactions between proteins and identify potential drug targets.
Results: Three plasma proteins and five CSF proteins were significantly associated with MG risk. ALDH2, HSPA1A, PRSS8, MFRP, CTSH, SHBG, and TXNDC12 were found to increase MG risk, while IL36A was negatively correlated. Further colocalization analysis revealed strong evidence for the associations between PRSS8 and HSPA1A with MG (pph4 > 0.8), and substantial evidence for TXNDC12 and ALDH2 (0.8 > pph4 > 0.6).
Conclusion: This study employed proteomics-based MR to identify several plasma and CSF proteins significantly associated with the risk of MG. Notably, PRSS8, HSPA1A, TXNDC12, and ALDH2 emerge as potential therapeutic targets for MG. While these findings offer valuable insights into the pathological mechanisms of MG and the development of novel therapeutic strategies, further research is required to evaluate the feasibility and clinical efficacy of these candidate proteins.
Keywords: Myasthenia Gravis; Mendelian Randomization; Proteomics; Cerebrospinal Fluid Proteins; Colocalization; Therapeutic Target.

Introduction

Myasthenia Gravis (MG) is a chronic autoimmune neuromuscular junction disorder [1]. Ocular weakness is the most common initial symptom, but the condition often progresses to involve the medullary muscles, limbs, axial muscles, and respiratory muscles, ultimately leading to generalized MG [2]. Studies indicate that the global prevalence of MG is approximately 20 to 50 cases per 100,000 people, with an annual incidence ranging from 0.3 to 2.8 cases per 100,000 people. MG can occur at any age, though it is most common in young women and elderly men [3]. Standard treatment options include acetylcholinesterase inhibitors, corticosteroids, and immunosuppressants [4]. For patients who show inadequate responses to drug therapy, thymectomy, intravenous immunoglobulin, and plasmapheresis are commonly utilized [5]. Although these treatments help control symptoms, they still have significant limitations. Drug therapy often requires lifelong treatment, which potentially lead to side effects such as gastrointestinal discomfort, muscle spasms, and an increased risk of infections [5, 6]. Plasmapheresis and immunoglobulin therapy provide rapid symptom relief but have a short duration of effect and are costly [7]. Thymectomy may benefit some patients, but it carries surgical risks, and its effectiveness in cases of late-onset or antibody-negative MG remains uncertain [5, 8]. Additionally, approximately 10-20% of patients show limited or no response to standard treatments [5], highlighting the urgent need for further research into the pathological mechanisms of MG and the identification of novel therapeutic targets.
Proteins play a crucial role in the pathophysiology of MG. In MG patients, autoantibodies bind to target antigens at the neuromuscular junction, activating the complement system,

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inflammation, and the damage or functional alteration of acetylcholine receptors, thus impairing neuronal signal transmission [9]. The circulating proteome comprises proteins from various cells and tissues throughout the body, which may be actively secreted into the bloodstream or passively released during cell damage or turnover, serving as biomarkers for disease [10]. A cohort study of MG patients found elevated serum amyloid A (SAA) levels in MG patients and promoted the expansion of CD4+ T cells and CD19+ B cell subpopulations [11]. Furthermore, IL-17 expression levels were correlated with the severity of the disease in MG patients, suggesting its potential promotive role in the pathogenesis of MG [12]. While no direct studies have yet utilized cerebrospinal fluid (CSF) proteins to identify therapeutic targets for MG a review article pointed out the importance of CSF analysis in MG patients to better understand the immune pathological processes associated with the disease, particularly for developing personalized treatment strategies for different MG subtypes [13]. Moreover, proteins are the targets of most pharmacological agents [14]. A randomized, double-blind, placebo-controlled, multicenter phase 3 clinical trial (ADAPT study, NCT03669588) demonstrated that efgartigimod improved clinical symptoms in MG patients by inhibiting the neonatal Fc receptor, significantly enhancing their quality of life [15]. However, despite the identification of associations between certain circulating proteins and MG, clarifying their causal relationship is hindered by factors such as small sample sizes and observational study designs. Conducting large-scale randomized controlled trials to explore potential causal links between numerous proteins and MG remains impractical.

Mendelian Randomization (MR) is a method that uses genetic variation as an instrumental variable to assess causal relationships between exposures and outcomes. Genetic variations are determined before birth and are generally not influenced by postnatal environmental or behavioral factors, effectively minimizing the impact of confounding variables [16]. Proteomics-based MR, which integrates pQTL of plasma and CSF proteins and genome-wide association study (GWAS) data on MG, analyzes proteins that may influence MG, thereby completely avoiding reverse causality. As such, proteomics-based MR offers a novel approach to elucidating the molecular mechanisms underlying MG and can also aid in identifying genetically supported drug therapy targets. This approach has the potential to enhance the success rate of clinical drug development.

Methods

Study Design

In this study, we utilized pQTL data from large-scale plasma and CSF proteomics studies and applied MR to investigate the genetic causal relationship between these proteins and MG. Additionally, we conducted protein-protein interaction (PPI) network construction and colocalization analysis on the statistically significant proteins identified in the MR analysis, with the goal of pinpointing the most promising therapeutic targets and predicting potential drugs. This approach aims to bridge basic research and clinical applications (Figure 1).

Exposure Data Source

We obtained single nucleotide polymorphisms (SNPs) data associated with plasma protein levels from the Fenland study. This genome-proteome association study included 10,708 participants of European descent and collected data on 4,775 plasma proteins using SomaScan v4 (http://www.omicscience.org/apps/pgwas) [17]. Since the selected circulating proteins were based on cis-acting protein quantitative trait loci (cis-pQTL), a total of 4,185 plasma proteins were included in the subsequent analysis.

The SNP data associated with CSF protein levels were derived from a genome-proteome association study conducted by the Washington University School of Medicine, which included 971 participants. Using an aptamer-based high-throughput proteomics platform, 1,305 proteins were detected. Following stringent quality control, 835 CSF protein data points were obtained [18]. After excluding duplicate-sequenced proteins, 832 CSF proteins were included in the subsequent analysis.

Outcome Data Source

In this study, our outcome data were sourced from the largest MG meta-GWAS study conducted in the United States and Italy. The study included 1,873 acetylcholine receptor antibody-positive (AChR+) MG patients and 36,370 healthy controls, excluding all muscle-specific kinase antibody-positive (MuSK+) patients. Data were collected through collaboration among institutions including Johns Hopkins University, the National Institute on Aging, the University of Pisa, and the Catholic University of Rome, with ethical approval from all participating institutional review boards [19].

Selection of Instrumental Variables

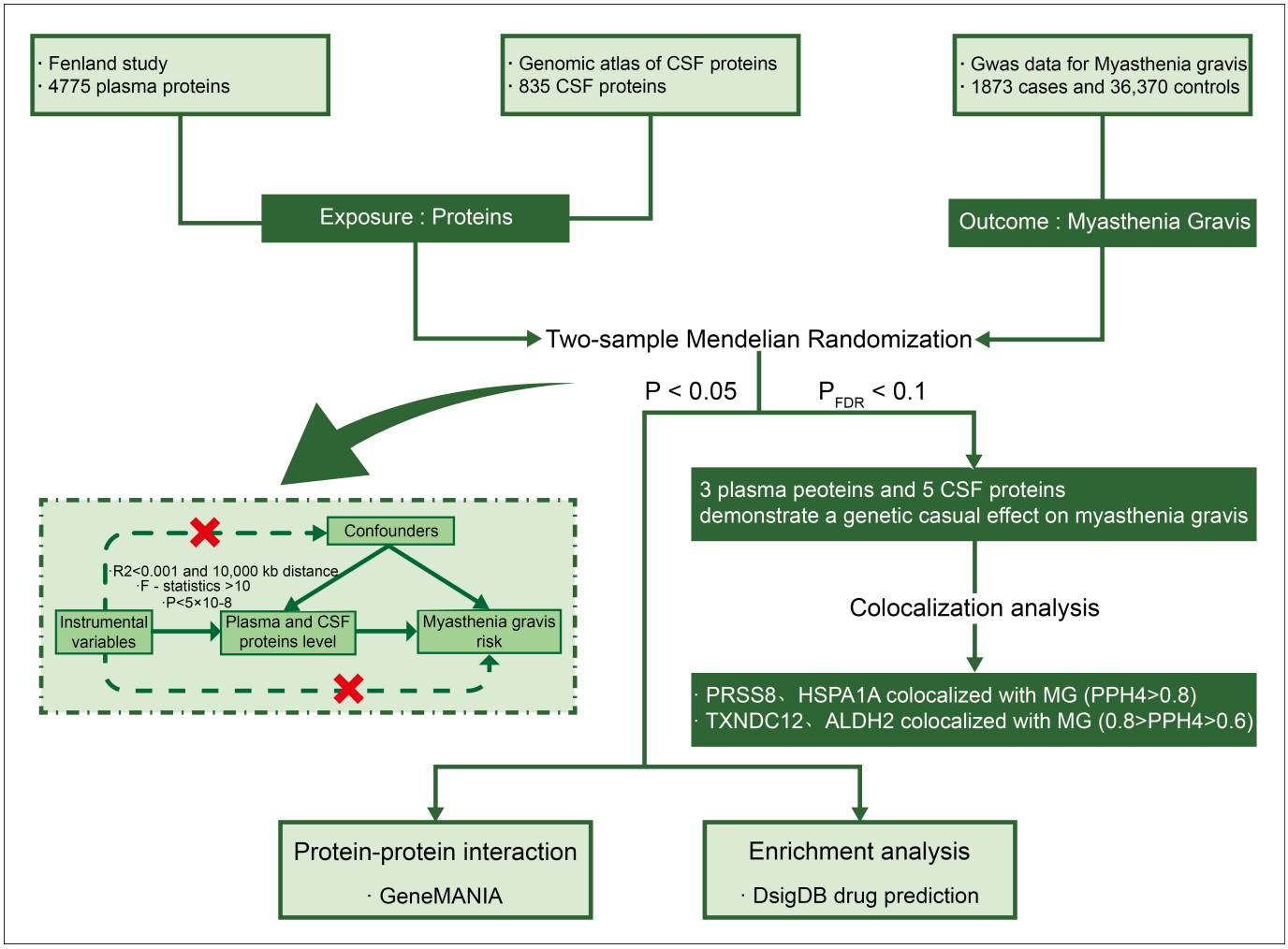
In conducting the MR analysis, we selected cis-pQTLs that directly regulate protein expression levels as instrumental variables (IVs), with cis-pQTLs spanning a gene range of ±1Mb. The selection of SNPs was performed based on three main assumptions: 1) SNPs highly correlated with plasma proteins were selected, and these SNPs met the significance threshold (P-value < 5×10^{-8}); 2) The linkage disequilibrium (LD) parameter threshold (r2) was set to 0.001, and SNPs were required to be at least 10,000 kb apart to ensure independence between SNPs and minimize the impact of LD; 3) Weak IVs, defined as those with an F-statistic < 10 were excluded to ensure a stable and reliable association with the exposure; 4) SNPs strongly associated with the outcome (P-value > 5×10^{-8}) were excluded.

Statistical Analysis

In this MR study, we employed several statistical methods to assess the potential causal relationship between circulating proteins and MG. When two or more IVs were available, the inverse variance weighted (IVW) method was chosen as the primary analytical tool [20]. For proteins with only a single instrumental variable, the Wald ratio method was applied to estimate the change in the log-odds ratio of MG risk for each standard deviation (SD) increase in protein levels. To control for the expected false-discovery rate, we applied the Benjamini-Hochberg (B-H) correction to adjust for p-values resulting from multiple tests and set the statistical significance threshold at $P_{FDR} < 0.1$ to enhance the reliability of the results [21].

Additionally, we conducted sensitivity analyses using Cochran's Q test, MR-Egger, and MR-PRESSO methods. Cochran's Q statistic was used to assess the heterogeneity among the

Figure 1. Flow Chart of the Overall Study Design. It begins with the exposure of proteins derived from the Fenland study (4775 plasma proteins) and the Genomic Atlas of CSF proteins (835 proteins). The outcome is MG, based on GWAS data for 1873 cases and 36, 370 controls. The study employs two-sample Mendelian randomization to assess causal relationships, with significant findings (P < 0.05 and PFDR < 0.1) showing that three plasma proteins and five CSF proteins have a genetic causal effect on MG. Further analysis includes colocalization and enrichment analyses.



selected IVs, with a P-value < 0.05 indicating significant heterogeneity. The MR-Egger method evaluates causality under the InSIDE assumption and can be performed even in the absence of valid IVs. A significant intercept in the MR-Egger method suggests the possibility of pleiotropy, indicated by a P-value < 0.05. The MR-PRESSO method, implemented through the "MR-PRESSO" package, identifies and removes SNP outliers with horizontal pleiotropy. However, in cases where the number of SNPs is small, it may be insufficient for effective analysis of heterogeneity and pleiotropy [22]. Finally, the MR-Steiger test assesses the directionality of causal effects by comparing the variance ratio of IVs between the exposure and outcome variables, thus evaluating the applicability of the IVs [23].

pQTL-GWAS Colocalization Analysis

To assess whether two traits share causal variants in a single region, we performed colocalization analysis using the default prior probabilities of the coloc R package [24]. For each cis-protein gene locus, the Bayesian method tested five mutually exclusive hypotheses: 1) no association with either trait (H_0); 2) only associated with protein levels (H_1); 3) only associated with MG risk (H_2); 4) associated with both protein levels and MG risk, but with independent genetic variants for each trait (H_3); 5) protein levels and MG risk share the same genetic variants (H_4). In this study, the degree of colocalization support was measured using the posterior probability (pph4).

PPI Network and Potential Drug Prediction

Due to the presence of the blood-brain barrier (BBB), the connection between plasma pQTLs and CSF pQTLs may be relatively weak. To further identify protein targets associated with MG risk and to understand protein interactions in different tissue environments, we constructed PPInetworks for plasma and CSF proteins using the GeneMANIA tool [25].

Proteins are the fundamental units of bodily functions and represent one of the key categories of druggable targets [26], this study subsequently utilized the Drug Signatures Database (DSigDB) to predict potential drugs and assess the druggability of genes corresponding to these target proteins [27]. The database contains 22,527 gene sets, encompassing 17,389

compounds and 19,531 genes, offering researchers a powerful tool for identifying and validating potential drug-target genes.

Results

Plasma and CSF Proteins and MG Risk: MR Analysis

Our team employed the IVW method and the Wald ratio method to comprehensively assess the effects of 4,185 plasma pQTLs and 832 CSF pQTLs on the risk of MG. Ultimately, we identified 95 plasma proteins and 23 CSF proteins associated with MG risk (P < 0.05). We then applied B-H correction to adjust the p-values, and the results indicated that three plasma proteins—Aldehyde Dehydrogenase 2 (ALDH2), Heat Shock Protein Family A Member 1A (HSPA1A), and Serine Protease 8 (PRSS8)—and five CSF proteins—Interleukin 36 Alpha (IL36A), Membrane Frizzled-Related Protein (MFRP), Cathepsin H (CTSH), Sex Hormone Binding Globulin (SHBG), Thioredoxin Domain Containing 12 (TXNDC12)—have a genetic causal relationship with MG risk (P_{FDR} < 0.1). Specifically, higher expression levels of plasma ALDH2 (OR = 2.32, 95% CI: 1.48-3.62, P = 2.22e-04, P_{FDR} = 0.079), HSPA1A (OR = 2.68, 95% CI: 1.59-4.51, P = 2.16e-04, P_{FDR} = 0.079), and PRSS8 (OR = 6.89, 95% CI: 3.00-15.86, P = 5.62e-06, P_{FDR} = 0.008) increase the risk of MG (Figure 2). Regarding CSF proteins, higher expression levels of MFRP (OR = 2.81, 95% CI: 1.74-4.54, P = 2.38e-05, P_{FDR} = 0.005), CTSH (OR = 2.75, 95% CI: 1.68-4.49, P = 5.49e-05, P_{FDR} = 0.006), SHBG (OR = 6.20, 95% CI: 2.02-19.09, P = 1.47e-03, P_{FDR} = 0.062), and TXNDC12 (OR = 21.17, 95% CI: 3.66-122.49, P = 6.52e-04, P_{FDR} = 0.046) were found to increase the risk of MG, whereas higher expression of IL36A (OR = 0.35, 95% CI: 0.19-0.66, P = 1.25e-03, P_{FDR} = 0.062) was negatively correlated with MG risk (Figure 3). Detailed data are shown in the Supplementary Materials.

Sensitivity analysis further confirmed the reliability of the study findings. MR-Steiger testing validated the positive causal effect of the eight identified proteins on MG risk. Additionally, Cochran's Q test, MR-Egger, and MR-PRESSO analysis for proteins with more than three SNPs all yielded p-values greater than 0.05, indicating no significant heterogeneity or horizontal pleiotropy among the selected IVs.

Bayesian Colocalization Analysis

The Bayesian colocalization results (Table 1) revealed that PRSS8 and HSPA1A exhibited strong colocalization with MG (pph4 > 0.8), suggesting these proteins share the same genetic variants as MG and can be considered as primary drug candidate targets [28]. Meanwhile, TXNDC12 and ALDH2 showed pph4 values between 0.6 and 0.8, indicating moderate colocalization strength, and may be considered as secondary candidate therapeutic targets. Detailed data are shown in the Supplementary Materials.

Table 1. The pph4 values from the colocalization analysis of proteins with P_{FDR} < 0.1.

Protein	pph4
PRSS8	9.85E-01
HSPA1A	8.82E-01
TXNDC12	7.31E-01
ALDH2	6.51E-01
SHBG	3.96E-01
CTSH	1.26E-02
IL36A	1.62E-05
MFRP	2.53E-06

*pph4: The posterior probability that proteins and MG share the same causal variant

Figure 2. Forest Plot Displaying Plasma Proteins Genetically Associated with MG. The data shown represent the odds ratios with 95% confidence intervals, where P_{FDR} < 0.1 was considered statistically significant. Proteins identified include PRSS8, HSPA1A, and ALDH2, with the corresponding P-values and P_{FDR} values indicated for each protein.

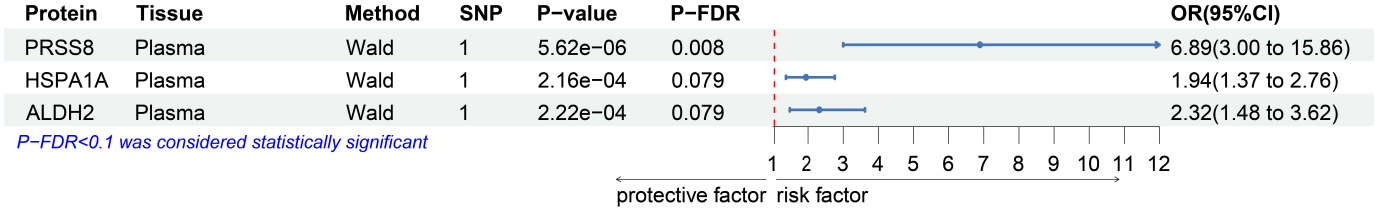
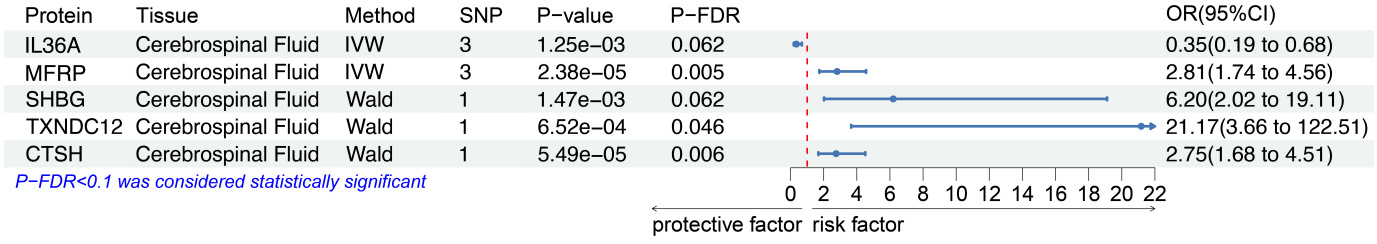


Figure 3. Forest Plot Displaying CSF Proteins Genetically Associated with MG. The data shown represent the odds ratios with 95% confidence intervals, where P_{FDR} < 0.1 was considered statistically significant. Proteins identified include IL36A, MFRP, SHBG, TXNDC12, and CTSH, with the corresponding P-values and P_{FDR} values indicated for each protein.



PPI Network and Potential Drug Prediction

Plasma and CSF proteins significantly associated with MG risk ($P < 0.05$) were input into GeneMANIA for protein network construction. In addition to the input proteins, each network was expanded to include 20 potential interacting genes. The plasma protein network was primarily driven by co-expression mechanisms (55.75%), predictive models (17.10%), physical interactions (11.86%), and colocalization (9.29%). The functions of this network mainly included axonogenesis, glycosaminoglycan binding, neuronal projection guidance, regulation of chemotaxis, positive regulation of cell adhesion, ERK1 and ERK2 signaling cascades, and positive regulation of α-β T cell activation ($P_{FDR} < 0.05$). Detailed data are shown in the Supplementary Materials.

The CSF protein network was primarily connected through co-expression mechanisms (63.96%), with additional connections involving colocalization (16.67%), shared protein domains (14.07%), and genetic interactions (5.30%). The network's functions mainly include fucosyltransferase activity, positive regulation of cell adhesion, CD4-positive α-β T cell cytokine production, and T cell- and lymphocyte-mediated immunity, which form the core mechanisms of the immune response. Detailed data are shown in the Supplementary Materials. Notably, IL18 was identified as a potential interacting gene in both protein networks.

Subsequently, we compiled the same-named genes of IL18 and the significant proteins from the two proteomics datasets and used the DSigDB database via the open-source Enrichr platform (https://maayanlab.cloud/Enrichr/) to predict candidate drugs associated with these genes [29]. The results were ranked by adjusted p-values, from smallest to largest. Figure 4 displays the clustering of the top 20 chemical compounds with adjusted p-values less than 0.05, along with all target genes. The results show that arsenous acid (Arsenous acid CTD 00000922) had the smallest adjusted p-value, indicating the highest significance. Most of the genes interacted with retinoic acid (Retinoic acid CTD 00006918), benzo(a)pyrene (benzo[a]pyrene CTD 00005488), and estradiol (estradiol CTD 00005920). Detailed data are shown in the Supplementary Materials.

Discussion

To our knowledge, this study is the first to combine plasma and CSF proteomics to explore new therapeutic targets for MG. Through rigorous multi-condition restrictions, we identified three plasma proteins and five CSF proteins that have a significant genetic causal relationship with MG risk. The results demonstrate that high expression of PRSS8, HSPA1A, TXNDC12, ALDH2, SHBG, CTSH, MFRP, and low expression of IL36A are positively associated with MG susceptibility. Among these, PRSS8 and HSPA1A showed strong colocalization support (pph4 > 0.8), while TXNDC12 and ALDH2 exhibited moderate colocalization support (0.8 > pph4 > 0.6). Additionally, the complex interactions between proteins were visualized through the PPInetwork. Based on the expanded results from both plasma and CSF protein interaction networks, we inferred that IL18 is also closely related to MG risk. Finally, drug predictions may facilitate the translation of basic research findings into clinical applications, optimize the research and development process, and reduce drug development costs.

PRSS8, also known as Prostasin, is a member of the serine protease family. Previous studies have shown that PRSS8 (Prostasin) is co-expressed with Matriptase during embryonic development, and that Prostasin can indirectly participate in extracellular matrix remodeling by activating Matriptase [30]. Additionally, Prostasin influences the migration and adhesion of immune cells, a mechanism confirmed in the tumor microenvironment [31]. We hypothesize that, during the pathogenesis of MG, PRSS8 may contribute to abnormal attacks on the neuromuscular junction by affecting the migration and localization of lymphocytes, thereby exacerbating disease progression. Moreover, one study suggests that PRSS8 can regulate the Toll-like receptor 4 (TLR4)-mediated signaling pathway, affecting the expression of inflammatory factors such as TNF-α, IL-6, and IL-1β [32]. Another study indicates that PRSS8 can modulate the expression of inflammatory factors (including IL-6 and IL-8) in prostate epithelial cells through the regulation of PAR-2 and associated signaling pathways [33]. These inflammatory factors, including TNF-α, IL-6, and IL-1β, have been established as key regulators in the autoimmune response of MG [34]. Based on existing research and our MR analysis, we propose that changes in the expression levels and activity of PRSS8 could be a crucial molecular event leading to the onset of MG. Future studies should focus on investigating the expression of PRSS8 and its related signaling pathways in MG patients to further validate this hypothesis.

HSPA1A, a member of the heat shock protein 70 (HSP70) family, primarily functions as a molecular chaperone that prevents the aggregation of misfolded proteins [35]. It plays a central role in various biological processes, including stress response, signal transduction, and cell cycle regulation [36]. HSPA1A can bind to peptide fragments generated within the cell due to stress or injury and subsequently release them extracellularly. These HSPA1A-peptide complexes can be recognized and internalized by antigen-presenting cells (APCs), where they are presented to T cells via MHC molecules, subsequently triggering a specific T cell response against self-antigens [37]. In the context of MG, if these peptides originate from the acetylcholine receptor, they may lead to misrecognition of these self-antigens by T cells, activating B cells to produce corresponding autoantibodies, thus exacerbating the disease. Furthermore, the interaction between HSPs and APCs can promote the secretion of inflammatory cytokines by macrophages, further amplifying the autoimmune response [37, 38]. Interestingly, studies have shown that compared to healthy controls, the mRNA expression level of HSPA1A in the peripheral blood mononuclear cells (PBMCs) of MG patients is significantly reduced [35]. This suggests a more complex potential relationship than initially expected, warranting further research to resolve this paradox and better understand the role of HSPA1A in the pathogenesis of MG [35].

TXNDC12, also known as AGR1, TLP19, or ERP18/19, is a member of the protein disulfide isomerase (PDI) family and plays a crucial role in the proper folding of proteins [39]. While research on the relationship between TXNDC12 and the risk of MG remains limited, an increasing body of evidence suggests that sustained protein misfolding can initiate apoptotic cascades, contributing to the development of various neurological diseases [40]. ALDH2 is primarily responsible for the metabolism of acetaldehyde in the body and is a key enzyme in

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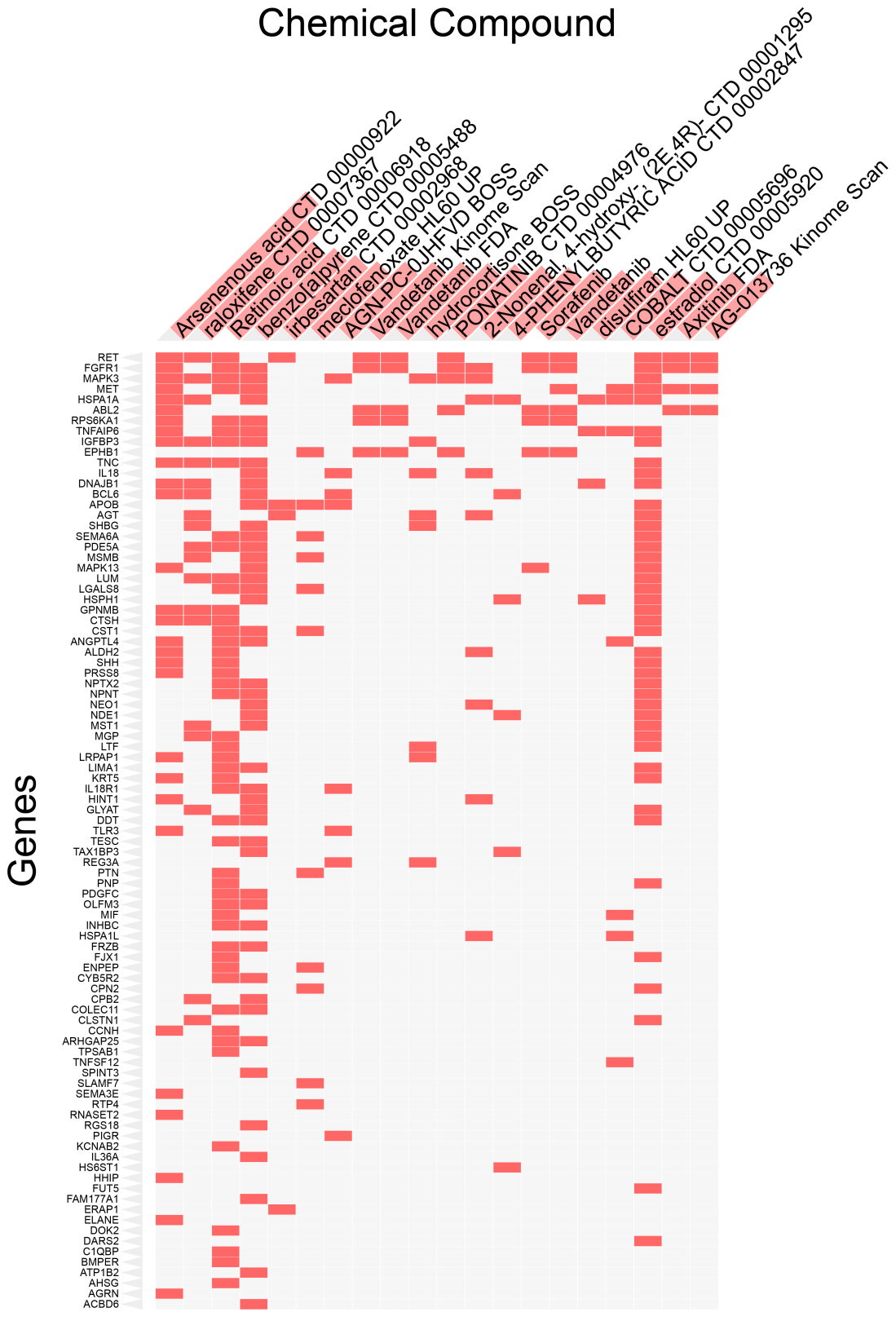
Figure 4. Top 20 Chemical Compounds Targeting Genes Corresponding to Proteins Associated with MG Risk. This figure visualizes the results from the DSigDB database via the Enrichr platform, which predicts candidate drugs associated with genes whose corresponding proteins are linked to MG risk (P < 0.05). The chemical compounds are ranked by adjusted p-values, with the top 20 compounds having p-values less than 0.05 displayed. The red bars along the horizontal axis represent the strength of the association between each chemical compound and the corresponding genes, with longer bars indicating stronger associations.



maintaining cellular metabolic balance and reducing oxidative stress [41]. Like TXNDC12, the association between ALDH2 and MG risk has been infrequently reported. However, in this multi-omics MR study, the levels of TXNDC12 and ALDH2 were found to exhibit strong positive correlations with MG risk. This genetic finding implies a complex pathophysiological relationship between TXNDC12, ALDH2, and MG. Therefore, future research should focus on elucidating the biological functions of these proteins and the associated molecular pathways involved in the pathogenesis of MG, with the aim of identifying potential new therapeutic targets for the disease.

This study leveraged a large-scale Finnish plasma protein dataset, along with data from over 900 CSF proteins, and the largest available GWAS on MG to conduct a MR analysis, thereby enhancing the robustness of the findings. Additionally, we employed multiple methodological strategies to minimize potential confounding factors. Specifically, we used the Steiger test to mitigate the influence of reverse causality. For plasma proteins, we prioritized cis-pQTLs, located at or near the gene encoding the target proteins, over trans-pQTLs and eQTLs due to their substantial contribution to explaining protein expression [42]. The B-H correction was applied to control for the false-positive rate, and gene colocalization analysis was conducted to further enhance the robustness of the statistical results. Finally, through PPInetworks and potential drug predictions, we offer novel insights for the development of MG therapies.

However, our study has several limitations. First, this research is based on data from populations of European ancestry, which limits the generalizability of the results to different ancestral groups. Second, although the study encompassed a broad range of proteins, it may have overlooked other potential therapeutic targets due to the selection of variables based on stringent significance thresholds. Furthermore, drug predictions and the construction of interaction networks were based on the gene names corresponding to the proteins. The function and regulation of proteins are influenced by multiple factors, including environmental interactions and epigenetic modifications, which were not fully considered in this study, potentially oversimplifying the pathways through which proteins affect MG. Finally, MG exists in two subtypes, AChR+ and MuSK+, but we did not distinguish between these subtypes when selecting the outcome data sources, potentially overlooking target specificity.

Conclusion

In conclusion, our study suggests that the levels of three plasma proteins and five CSF proteins are causally associated with the risk of MG. Among these, PRSS8, HSPA1A, TXNDC12, and ALDH2 are promising candidates for new therapeutic targets. Our research provides new perspectives for understanding the pathogenesis of MG. However, further studies are needed to confirm the association between these candidate plasma proteins and MG risk to establish their clinical relevance.

Abbreviations

Acetylcholine receptor antibody-positive: AChR+; Aldehyde Dehydrogenase 2: ALDH2; Benjamini-Hochberg: B-H; Blood-brain barrier: BBB; Cathepsin H: CTSH; Cerebrospinal fluid: CSF; Cis-acting protein quantitative trait loci: cis-pQTL; Drug Signatures Database: DSigDB; Heat Shock Protein Family A Member 1A: HSPA1A; Heat shock protein 70: HSP70; Instrumental variables: IVs; Interleukin 36 Alpha: IL36A; Inverse variance weighted: IVW; Membrane Frizzled-Related Protein: MFRP; Mendelian Randomization: MR; Myasthenia Gravis: MG; Muscle-specific kinase antibody-positive: MuSK+; Peripheral blood mononuclear cells: PBMCs; Protein disulfide isomerase: PDI; Protein-protein interaction: PPI; Serine Protease 8: PRSS8; Serum amyloid A: SAA; Single nucleotide polymorphisms: SNPs; Standard deviation: SD; Toll-like receptor 4: TLR4.

Author Contributions

Hongmei Miao: Writing-review & editing, Writing-original draft, Conceptualization, Visualization, Formal analysis. Yinying Chai: Writing-review & editing, Validation, Writing-original draft, Supervision, Data curation, Formal analysis. Qianran Hong: Writing-review & editing, Validation, Formal analysis. Yuxuan Song: Writing-review & editing, Validation, Funding acquisition, Data curation. Yibo He: Writing-review & editing, Conceptualization, Supervision. All authors read and approved the final manuscript.

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Ethics Approval and Consent to Participate

Not Applicable.

Competing Interests

The authors declare that they have no existing or potential commercial or financial relationships that could create a conflict of interest at the time of conducting this study.

Data Availability

The data that supports the findings of this study are available in the supplementary materials of this article.

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Construction of prognostic model and tumor microenvironment landscape based on cuproptosis-related subtypes in melanoma

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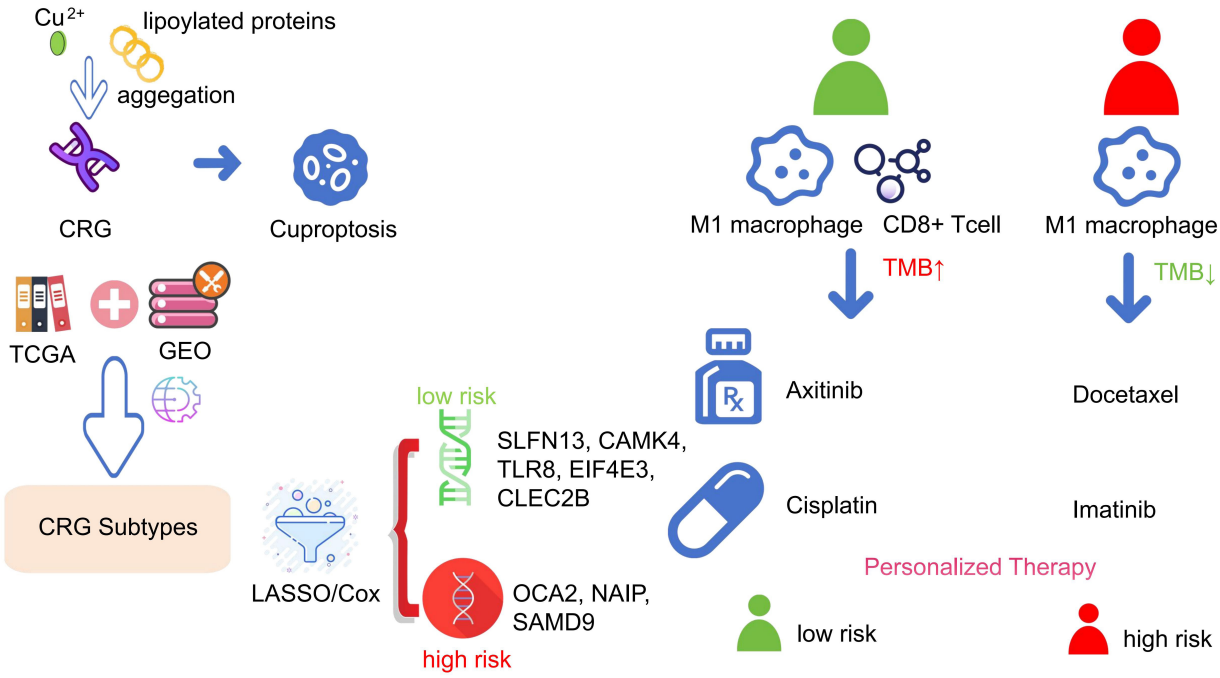
Graphical Abstract



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Research Article Life Conflux

Construction of prognostic model and tumor microenvironment landscape based on cuproptosis-related subtypes in melanoma

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Abstract

Background: Melanoma, known for its aggressive nature and poor prognosis, may be impacted by cuproptosis, a recently discovered form of programmed cell death. Despite its unclear mechanisms, preliminary studies suggested a link between cuproptosis and cancer progression and metastasis. We aimed to investigate the association between cuproptosis-related genes (CRGs) and melanoma to enhance prognostic and therapeutic strategies.

Method: In this study, we downloaded transcriptome RNA-seqs and clinical information of all melanoma patients from The Cancer Genome Atlas (TCGA) database, selected a dataset from Gene Expression Omnibus (GEO) databases, and merged the two datasets. After univariate regression analysis, all the samples were categorized into three groups based on expression levels of CRGs. Differential expression analysis was carried out for three CRG clusters to obtain the significant differentially expressed genes (DEGs). After univariate Cox regression analysis, multivariate Cox regression analysis and the least absolute shrinkage and selection operator (LASSO) algorithm were performed on DEGs, the prognosis related genes were screened to establish a prognosis prediction model. The model's accuracy was validated through Kaplan-Meier analysis, receiver operating characteristic (ROC) curve, nomogram, and independent prognostic analysis. Additionally, we compared the immune scores of the tumor microenvironment, tumor mutation burden, tumor immune dysfunction and exclusion, and drug sensitivity between high-risk and low-risk groups.

Results: Through algorithm analysis, eight genes significantly related to prognosis were identified, among which SLFN13, CAMK4, TLR8, EIF4E3, and CLEC2B were low-risk genes, OCA2, NAIP, and SAMD9 were high-risk genes. Using these genes, we established a prognostic model that effectively distinguishes between different survival outcomes, with the low-risk group showing a markedly higher long-term survival rate.

Conclusion: In conclusion, based on the research of cuproptosis subtypes, we identify the DEG with predictive potential and establish a prognosis prediction model. This study may provide a reference for the prognosis and clinical treatment of melanoma patients from the perspective of cuproptosis.

Keywords: melanoma; cuproptosis; tumor microenvironment; differentially expressed genes; risk score; bioinformatics analysis.

Introduction

Melanoma, a malignant tumor that originates from melanocytes, typically manifests in the skin [1,2]. The development of melanoma is influenced by both environmental and genetic factors [3]. Often resembling melanocytic nevi, its early symptoms can be subtle, complicating early detection and diagnosis [4]. By the time symptoms appear, melanoma frequently advances to a late stage characterized by rapid progression, widespread metastasis, and poor prognosis [5, 6].

The primary treatment for metastatic melanoma has long been surgical resection combined with chemotherapy [7]. For decades, immunotherapy and targeted drugs, such as PD1 – PDL1 inhibitors, small molecule BRAF and MEK inhibitors, cytotoxic T lymphocyte antigen 4 (CTLA4) inhibitors, and the combination of multiple drugs have been explored to revolutionize the treatment of malignant melanoma [8, 9]. However, not all melanoma patients respond effectively [10], and resistance to these therapies is emerging [11]. This underscores the critical need for new biomarkers that can predict prognosis and effective therapeutic targets.

Copper, an essential trace element, plays a pivotal role in various cellular functions due to its inherent redox properties [12], serving as a cofactor for enzymes involved in mitochondrial respiration, antioxidant defense, and the biosynthesis of hormones, neurotransmitters, and pigments [13]. Recent studies have highlighted that disruptions in copper homeostasis can lead to cytotoxic effects [14-16]. Tsvetkov et al. showed a unique cell programmed death mode caused by excessive copper accumulation called cuproptosis [17]. This process involves the binding of excess copper to lipoylated proteins

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in the tricarboxylic acid (TCA) cycle, triggering protein aggregation, loss of Fe-S cluster proteins, and resultant proteotoxic stress. Interestingly, previous studies have shown that cancer cells exhibit higher copper levels than normal tissues, suggesting that they exploit copper for energy needs while avoiding cuproptosis [13, 18, 19]. This seems to offer a potential therapeutic avenue targeting copper metabolism in cancer cells.

In this study, we aimed to define the role of cuproptosis in melanoma by analyzing cuproptosis-related gene (CRG) expression in patient samples. We categorized melanoma patients based on CRG expression profiles into distinct subtypes, assessed their immune characteristics, and developed a new prognostic model using differentially expressed genes (DEGs) linked to these CRG clusters. This approach may provide valuable insights for enhancing melanoma diagnosis and treatment strategies.

Materials and Methods

Data Collection and Preparation

Transcriptomic RNA-seq and clinical data were acquired from The Cancer Genome Atlas (TCGA) database and the GSE65904 dataset from Gene Expression Omnibus (GEO). After screening, samples lacking complete survival information or from normal tissues were excluded. The remaining transcriptomic and clinical data were merged from both sources. Additionally, somatic mutation and copy number variation (CNV) were downloaded from GDC and UCSC Xena, respectively. We utilized 18 CRGs (NFE2L2, NLRP3, ATP7B, ATP7A, SLC31A1, FDX1, LIAS, LIPT1, DLD, DLAT, PDHA1, PDHB, MTF1, GLS, CDKN2A, DBT, GCSH, DLST) identified from previous studies [13, 17, 18, 20, 21].

CNV analysis and prognosis analysis of CRGs

CNV of CRGs was extracted from the CNV file downloaded from TCGA. We analyzed the difference and used the R package "RCircos" (version 1.2.2) for visualization. To validate the prognostic value of CRGs, survival analysis and univariate Cox regression analysis were conducted on the merged data using the R package "limma" (version 3.64.3) and "survival" (version 3.8.3). According to the relationship between high and low gene expression and survival information, CRGs were divided into "Favorable factors" and "Risk factors".

Consensus clustering analysis with CRGs

R package "ConsensusClusterPlus" (version 1.58.0) was run to cluster the expression differences of these 18 CRGs in the merged sample dataset. The samples were divided into different clusters based on the result of cuproptosis clustering. Kaplan-Meier analysis was conducted to compare survival probability differences among different CRG clusters. In addition, the principal component analysis (PCA) diagram showed the geometric distance between subclusters. The heatmap showed the difference of CRGs expression. Gene set variation analysis (GSVA) was conducted to present the differences in immune pathway enrichment between the three clusters. Single sample gene-set enrichment analysis (ssGSEA) algorithm was performed to compare the immune cell infiltration of different CRG clusters, and we visualized the results with R package "ggpubr" (version 0.6.1).

Identification of CRG clusters related DEGs and function enrichment analysis

Differential expression analysis was carried out for three CRG clusters to obtain the DEGs. The intersection of DEGs across the three clusters was further analyzed. GO and KEGG function enrichment analyses were conducted for these DEGs.

Obtaining DEG clusters

We performed univariate Cox regression analysis on the DEGs to get the significant DEGs and conducted the consensus unsupervised clustering analysis for these DEGs. The merged sample data was divided into different DEG clusters. Kaplan-Meier (K-M) survival analysis was performed to show the survival differences among DEG clusters. The heat map was drawn to describe the differential expression of DEG clusters, and the boxplot described the differential expression of CRGs among DEG clusters.

Prognostic Model Construction and Validation

Prognostic genes were determined using multivariate Cox regression, and LASSO algorithm. To prevent overfitting, the optimal penalty coefficient was obtained through cross validation of 1000 iterations. The prognostic CRG clusters related DEGs optimal group was determined, and a prognostic risk model was established using multivariate Cox regression from DEG signature, with patients' risk scores calculated as follows: Risk score = $\sum_{i=1}^{n}$ exp (Xi) * coef (Xi), "exp" means gene's expression, "coef" means corresponding coefficient. The patients were randomly divided into training and test sets (1:1 ratio), and the training set, the test set, and all patients were further divided into high-risk and low-risk groups based on median risk scores, respectively. Kaplan-Meier analysis was carried out by "survival" R package to compare the long-term survival probability between the training set, test set, low-risk group, and all patients. In addition, based on the "survival" (version 3.8.3), "survminer" (version 0.4.2), "timeROC" (version 0.4) R package, we created the receiver operating characteristic (ROC) curves of 1-, 3-, and 5- years and calculated the area under the curve (AUC) to compare the testing effectiveness.

Establishment of Predictive Nomogram

We combined various key clinical factors with risk scores and used the "rms", "regplot" R package to construct 1-year, 3-year, and 5-year nomographs to predict the long-term survival rate of melanoma patients. And to verify the reliability of the model, we drew a calibration curve according to the Hosmer – Lemeshow test. The independence of the prognostic model from clinical factors such as sex, age, and pathological stage was confirmed through univariate regression and multivariate regression analysis.

Analysis of immune microenvironment (TME), tumor mutation burden (TMB), and tumor immune dysfunction and exclusion (TIDE)

The CIBERSORT method was used to analyze the difference in immune infiltration of total melanoma samples. We used the R package "ESTIMATE" to evaluate immune scores, stromal scores, and estimate scores of TME. This algorithm can use gene expression characteristics to estimate the level of stromal cells and immune cells in malignant tumor tissues. We also run the "maftools" R package (version 2.24.0) to analyze

the TMB and compare the gene mutation differences between high-risk group and low-risk group. And TIDE was downloaded from TIDE website (http://tide.dfci.harvard.edu) to predict patients' response to immunotherapy [22, 23].

Drug sensitivity analysis

According to the Genomics of Drug Sensitivity in Cancer (GDSC, https://www.cancerrxgene.org/) database, the "pRRophetic" package (version 0.5) in R was applied to compare the difference between high-risk groups and low-risk groups in sensitivity to chemotherapy drugs.

Statistical analysis

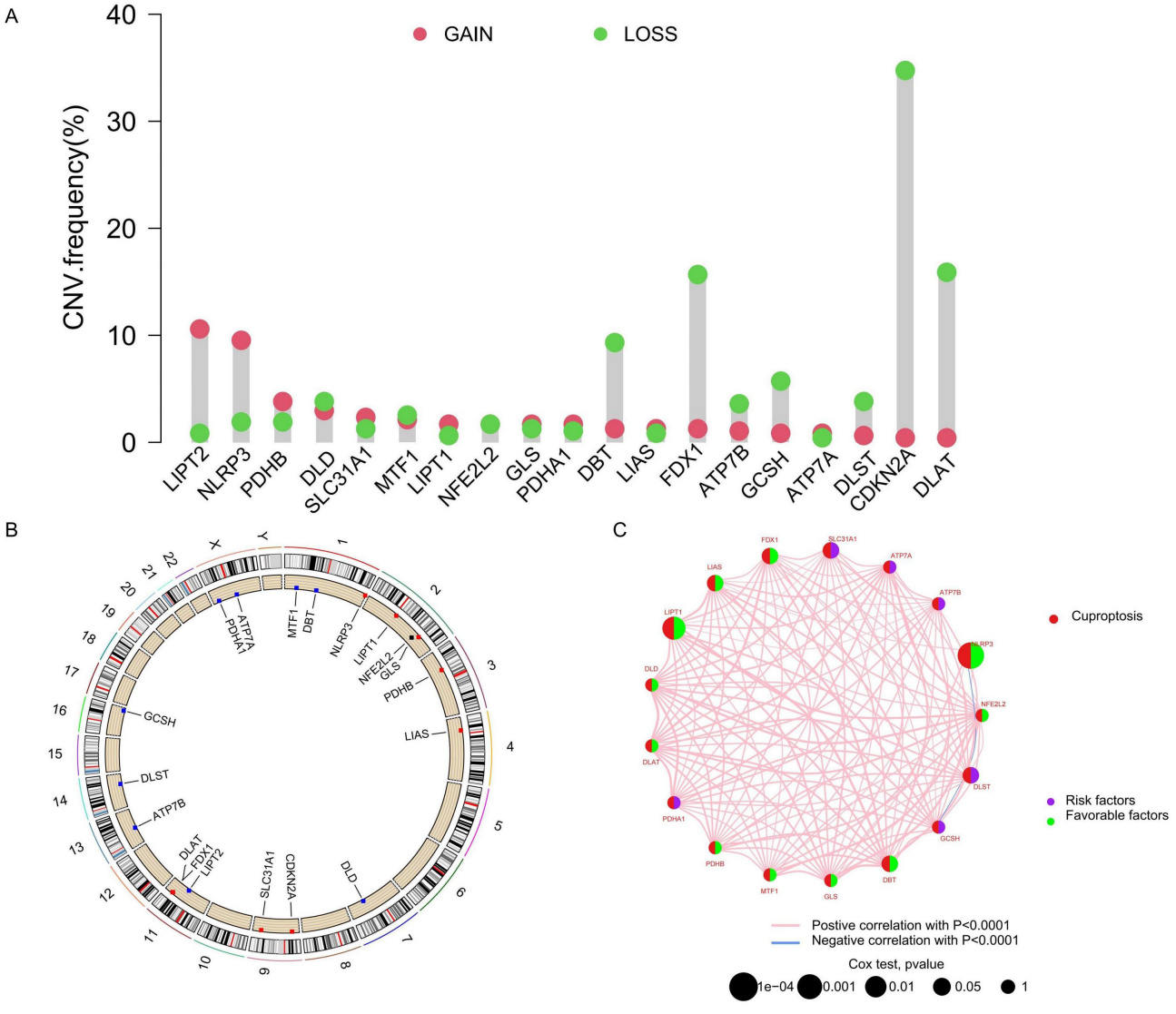
All statistical analyses in this study were performed using R software (version 3.6.1) and PERL. A p-value of less than 0.05 (two-sided) was considered to indicate statistically significant differences. Univariate Cox regression analysis was utilized to identify DEGs with prognostic value. We constructed the prognostic prediction model using the LASSO regression algorithm, univariate Cox regression analysis, and multivariate Cox regression analysis.

Result

CNV and prognosis value of CRGs

Analysis of the CNV in 18 CRGs highlighted significant reductions in CDKN2A, DLAT, GCSH, FDX1, and DBT, with increases observed in NLRP3. These variations suggested distinct patterns of transcription and expression of CRGs in tumor samples (Figure 1A), potentially reflecting their involvement in tumor development, progression, or other molecular mechanisms. Chromosomal locations of CRGs, with increases marked in red and decreases in blue, are displayed in Figure1B. To further assess the prognostic significance of these CRGs, we integrated transcriptome RNA sequencing data with clinical information from the TCGA and GEO databases and conducted Kaplan-Meier survival analysis. The analysis revealed

Figure 1. Genomic variation of CRG. (**A**) The change of CNV frequency of CRGs. (**B**) CRG position of CNV on the chromosome. (**C**) The interaction between CRGs in melanoma, where the width of the line represents the strength of the correlation between CRGs.



significant differences in overall survival between high and low expression groups for 15 CRGs, including ATP7A, ATP7B, CDKN2A, DBT, DLD, DLST, FDX1, GCSH, LIAS, LIPT1, MTF1, NFE2L2, NLRP3, PDHA1, and SLC31A1 (see Supplementary Figure S1A-O online). Based on the survival curves from this analysis, we categorized the CRGs into "Risk factors" and "Favorable factors," which are illustrated in a network diagram (Figure 1C).

Consensus clustering analysis with CRGs

To clearly delineate the characteristic distribution of CRGs across varying expression levels in all samples, we performed consensus clustering analysis on the transcriptome data, simulating group numbers from k=2 to k=9. The classification was most distinct at k=3, effectively reflecting the differences in expression and potential biological diversity among the samples. Consequently, we divided the samples into three CRG clusters: A (n=276), B (n=280), and C (n=126), based on their expression characteristics related to risk and Favorable factors (Figure 2A). PCA results revealed significant differences in gene expression profiles among the three CRG clusters (Figure 2B), suggesting that different clusters may represent distinct biological states. The heat map showed the differential expression of CRG among the three clusters and different clinical features (Figure 2C). Further, K-M survival analysis of the three CRG clusters indicated significant differences in survival outcomes, with CRG cluster A exhibiting a notably higher long-term survival probability than clusters B and C (Figure 2D). In addition, GSVA results highlighted the top 20 most significant pathways differing among clusters A, B, and C (see Supplementary Figure S2A-C online). Analysis of immune cell proportions in the three clusters was conducted using ssGSEA (Figure 2E). The results demonstrated varying types of immune cell infiltration across the melanoma samples, identifying potential therapeutic targets within these immunological variations

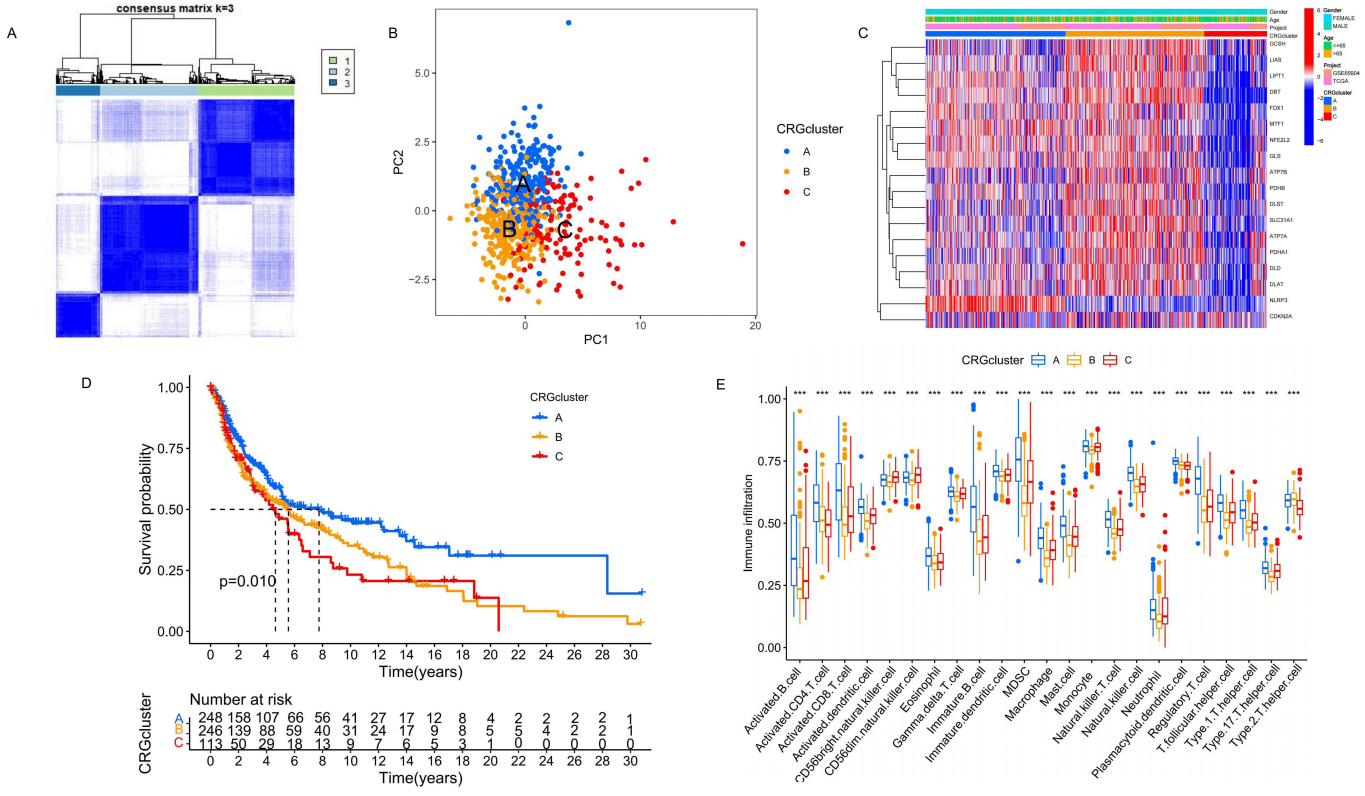
Identification of CRG clusters related DEGs and immune function enrichment analysis

Differential expression analysis across the three CRG clusters identified intersecting DEGs, presented in a Venn diagram (Figure 3A). Subsequent immune function enrichment analyses using GO and KEGG were conducted on these intersecting DEGs. The GO analysis identified significant enrichment in Molecular Function (MF) and Biological Process (BP) categories (Figure 3B, Supplementary Figure S3A). KEGG enrichment analysis further demonstrated significant differences in the expression of DEGs within cytokine-cytokine receptor interaction, Toll-like receptor signaling pathway, and PI3K-Akt signaling pathway, etc (Figure 3C, Supplementary Figure S3B). These findings highlight the significant impact of DEGs associated with CRG clusters on immune regulation within melanoma.

Obtaining DEGs clusters

Significant DEGs were obtained through univariate Cox regression analysis. Based on the expression differences, we conducted a grouping simulation, finding that categorizing the samples into two clusters (A and B) provided the most distinct grouping performance (Figure 4A). Subsequently, K-M survival analysis revealed that the long-term survival probability of

Figure 2. Identification and analysis of the CRG clusters. (**A**) Unsupervised consensus clustering identified three molecular subtypes of cuproptosis. (**B**) The PCA results show the distribution of the three CRG clusters. (**C**) It shows the differential expression of CRG among the three CRG clusters and different clinical features. (**D**) The K-M survival analysis of the 3 CRG clusters. (**E**) The immune infiltration difference of TME in the three clusters.



samples in cluster A was significantly higher than in cluster B (Figure 4B). Additionally, we combined the characteristic of CRG clusters and population drew a heatmap of gene expression differences (Figure 4C). The differences in CRG expression between the two DEG clusters were further detailed in a boxplot (Figure 4D), where risk factors such as ATP7B, DLST, GCSH, and PDHA1 showed notably higher expression in cluster B. These findings highlight the potential of these DEGs in predicting prognosis in melanoma patients, and also suggested the possible role of CRGs in melanoma progression.

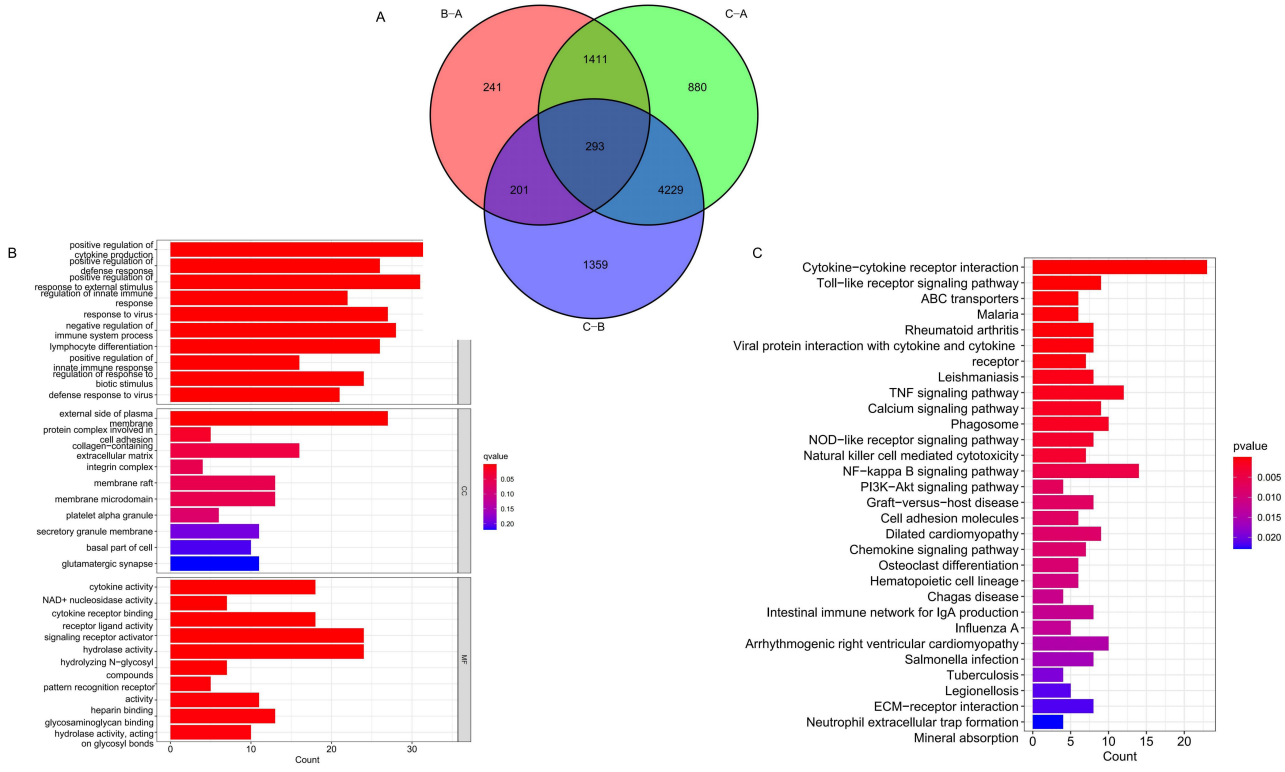
Construction of prognostic model

The LASSO algorithm analysis and multivariate Cox regression analysis were applied to 293 DEGs intersecting across three CRG clusters, as shown in Figure 5A and 5B. After 1,000 iterations, this analysis identified a prognostic model composed of eight genes—SLFN13, CAMK4, TLR8, EIF4E3, CLEC2B, OCA2, NAIP, and SAMD9—which exhibited substantial prognostic relevance. The risk score for this model was calculated as follows: Risk score = exp (TLR8) × (-0.266) + exp (SAMD9) × 0.252 + exp (NAIP) × 0.465 + exp (EIF4E3) × (-0.152) + exp (CLEC2B) × (-0.271) + exp (SLFN13) × (-0.121) + exp (CAMK4) × (-0.103) + exp (OCA2) × 0.091. Using this signature, we calculated risk scores for all samples, classifying them into high and low-risk groups based on the median score. A Sankey diagram (Figure 5C) illustrated the relationships between CRG clusters, DEG clusters, risk groups, and survival outcomes, highlighting the efficacy of CRG and DEG classifications in predicting melanoma patient risk and survival. The boxplot showed the risk score variations in CRG clusters (Figure 5D) and DEG clusters (Figure 5E), revealing that groups with higher long-term survival probabilities, specifically CRG Cluster A and DEG Cluster A, had lower risk scores. Additionally, boxplots comparing high and low-risk groups (Figure 5F) showed significant differences in the expression of CRGs, where risk factors such as SLC31A1, ATP7A, ATP7B, DLST, GCSH, and PDHA1 are significantly elevated in the high-risk group. These findings underscore the reliability of our prognostic model.

Verification of the Prognostic Model

A total of 607 melanoma patients were randomly divided into a test set (303 samples) and a training set (304 samples), nearly a 1:1 ratio, to assess the effectiveness of the risk prediction model. K-M survival analysis was conducted on all samples, training set and test set categorized by high and low-risk groups, consistently showed that the long-term survival probability of the low-risk group was significantly higher than that of the high-risk group (Figure 6A-C). This finding confirmed that the risk prediction model effectively differentiates patients with varying prognostic levels. Expression differences of prognostic signature genes between the high-risk and low-risk groups were visualized using heatmaps across all samples, training, and test sets (Figure 6D-F). Scatter plots depicting the survival time against increasing risk scores indicated that higher scores were associated with a significant increase in mortality and a notable decrease in survival time (Figure 6G-L). To evaluate the sensitivity and specificity of the prognostic model, we drew the ROC curve, the AUC of 1-, 3- and 5-year were 0.680, 0.758, and 0.785 in training set, and the minimum AUC of all the samples and test set was 0.647 (Figure 6M-O). These results emphasize the model's strong predictive capability for long-term prognosis, even at the lowest AUC value.

Figure 3. GO and KEGG analysis of the CRG clusters related DEGs. (**A**) Intersection DEGs of three CRG clusters. (**B-C**) Visualization of GO and KEGG analysis results.



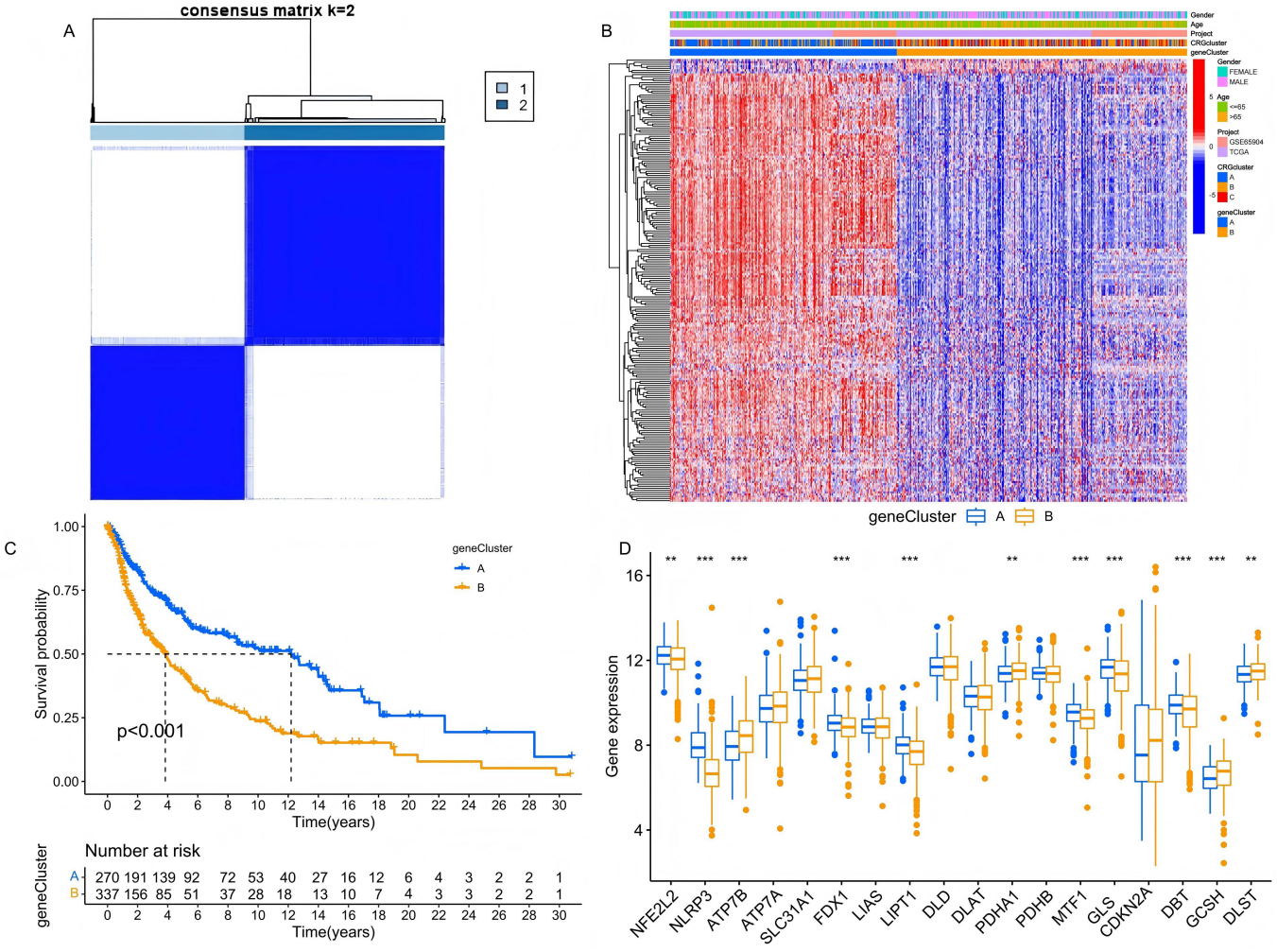
Further verification of the model's reliability as an independent predictor was conducted using univariate and multivariate Cox regression analyses. The hazard ratio (HR) values of risk score showed that it could be regarded as an independent prognostic indicator alongside clinical characteristics (Figure 7A-B). To enhance clinical applicability, a nomogram integrating clinicopathological features and risk scores was developed to quantitatively predict 1-year, 3-year, and 5-year survival probabilities for melanoma patients (Figure 7C). As shown in Figure 7C, if the total risk score of a patient is 258 points, then in the prediction of this model, the survival probability of this patient in the next 1 year, 3 years and 5 years is 90.5%, 46.8% and 28.3% respectively. The accuracy of the nomogram was affirmed by calibration curves, which showed high consistency between actual observations and predictions (Figure 7D). These findings suggest that the constructed risk prediction model not only effectively forecasts the survival prognosis of melanoma patients but also holds substantial potential for clinical application due to its high predictive accuracy and consistency.

Analysis of immune microenvironment, TMB and TIDE

To understand the relationship between prognostic genes, risk scores, and immune cell infiltration, we utilized the CIBERSORT algorithm (Figure 8A). The analysis revealed that higher risk scores were negatively correlated with the infiltration of M1 macrophages, plasma cells, activated CD4 memory T cells, and CD8 T cells, but positively correlated with M0 macrophages. This suggests that a higher risk score reflects a more immunosuppressive TME. Further evaluation of immune, stromal, and estimate scores within the TME showed significantly higher scores in the low-risk group compared to the high-risk group (Figure 8B). This indicated a more robust immune presence in the low-risk group, underscoring the importance of the TME in patient prognosis.

Immune checkpoint blockade (ICB) therapy has shown substantial clinical benefits in treating melanoma; however, its effectiveness varies, and some patients experience considerable side effects [24]. Recent studies have identified TMB as a valuable predictor of tumor immune response, potentially indicating the efficacy of ICB therapy [22, 25, 26]. Quantitative TMB analysis revealed that the high-risk group had a higher concentration of mutations across more genes than the low-risk group, which may correspond to a higher TMB (Figure 8C-D). K-M analysis further demonstrated that patients with high TMB had better survival probabilities than those with low TMB. Moreover, integrating risk model predictions, we found that the

Figure 4. Identification and analysis of the DEG clusters. (**A**) Unsupervised consensus clustering identified two DEG clusters. (**B**) The K-M survival analysis of the DEG clusters. (**C**) The clinical characteristics and cuproptosis subtypes differences between the two DEG subtypes. (**D**) The differences in CRG expression between the two DEG clusters.



highest long-term survival probability was observed in patients with high TMB and low-risk scores, whereas the lowest was in those with low TMB and high-risk scores (Figure 8E-F).
Additionally, we obtained immunotherapy scores for patient samples from the TIDE website and conducted a matching analysis with our prognostic model, calculating TIDE scores for the two groups. The results showed significant differences in TIDE scores, with higher scores observed in the low-risk group compared to the high-risk group (Figure 8G). When combined with the TMB analysis, these results suggested that patients in the high-risk group may have a more active response to immunotherapy.

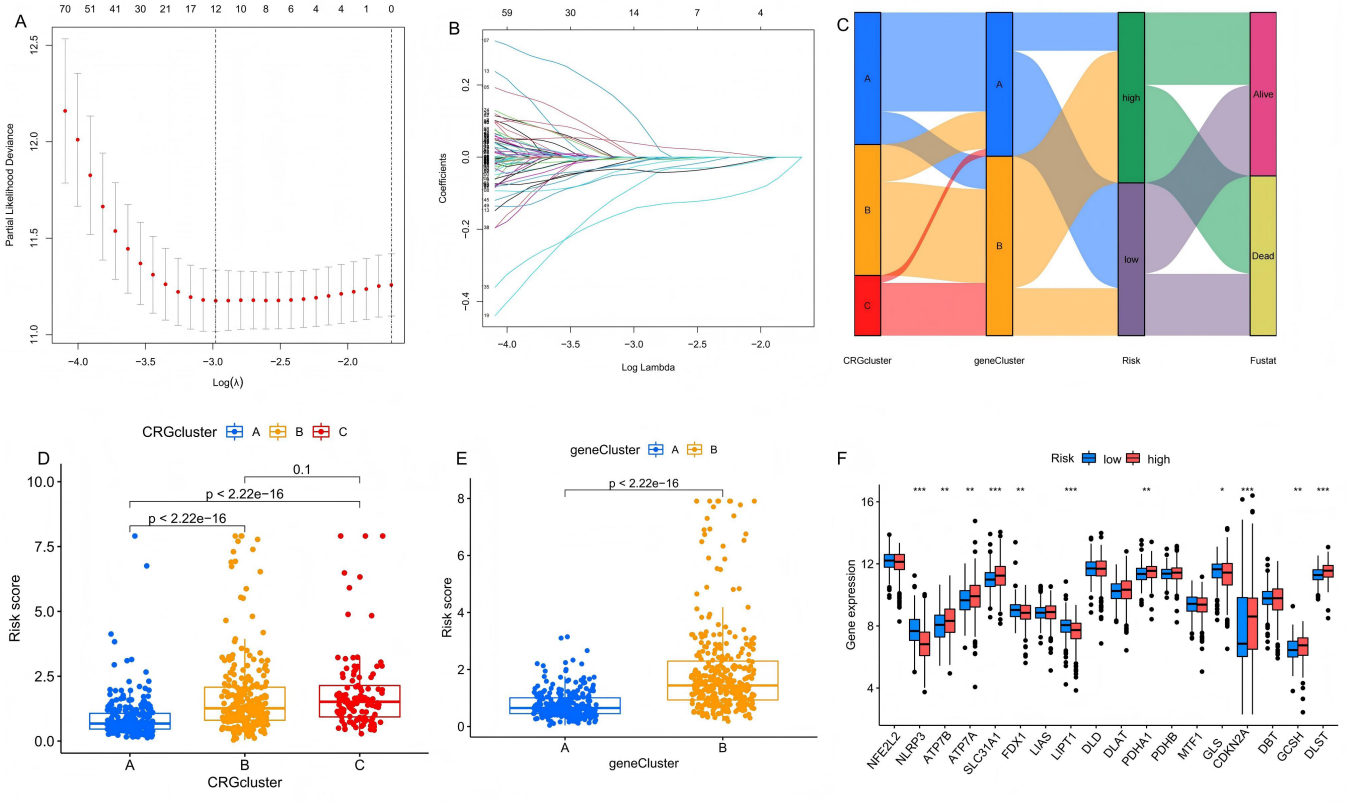
Drug sensitivity analysis

To enhance the clinical utility of our prognostic model and improve treatment efficacy, we compared the drug sensitivity between the high-risk and low-risk groups to identify potential drugs for more effective immune or targeted therapies. The analysis of half-maximal inhibitory concentration (IC50) for various drugs revealed significant differences between the two groups. The low-risk group demonstrated greater sensitivity to several immunotherapeutic and targeted drugs, including Axitinib, Cisplatin, Gemcitabine, Methotrexate, Nilotinib, Rapamycin, Sunitinib, and Temsirolimus (Figure 9A-H). Conversely, the high-risk group exhibited higher sensitivity to drugs such as Docetaxel, Elesclomol, Imatinib, and Thapsigargin (Figure 9I-L). These findings provided valuable insights into tailoring treatment strategies based on the risk profile, potentially leading to more effective therapeutic interventions for patients.

Discussion

Melanoma, the most prevalent and deadly form of skin cancer, often goes undetected in its early stages due to non-obvious symptoms, leading to diagnoses at more advanced stages with metastatic lesions and consequently poor prognoses [27, 28]. While the development and application of immunotherapy and targeted therapies, such as BRAF inhibitors, BRAF/MEK combination targeted therapy, and PD-1/PD-L1/CTLA-4 blockers, have significantly improved outcomes for many patients, resistance to these therapies frequently develops through mutations that promote irreversible drug resistance [10, 29, 30].
The progression of tumor cells is primarily driven by accumulations of gene mutations, which lead to uncontrolled cell proliferation [31]. A critical aspect of many cancers, including melanoma, is the activation of the MAPK pathway, which stimulates growth-promoting genes, leading to anchoring loss and inhibition of intercellular contact, resulting in uncontrolled cell proliferation and transformation [1, 32].Normally, cells can initiate various regulated cell death (RCD) mechanisms to maintain cellular homeostasis, including necroptosis, pyroptosis, ferroptosis, autophagic cell death, programmed cell death and apoptosis [33]. In addition, a novel form of cell death termed cuproptosis, characterized by copper-induced cell death, has been identified [17]. The research showed that excessive cop-

Figure 5. Construction of the prognostic model. (**A-B**) LASSO regression analysis screened prognostic signatures from the DEGs to build the model. (**C**) The relationship among CRG clusters, DEG clusters, risk groups and survival status. (**D**) Distribution of risk scores across the three CRG clusters. (**E**) Distribution of risk scores across the two DEG clusters. (**F**) Comparison of CRG expression between the high-risk group and low-risk group.

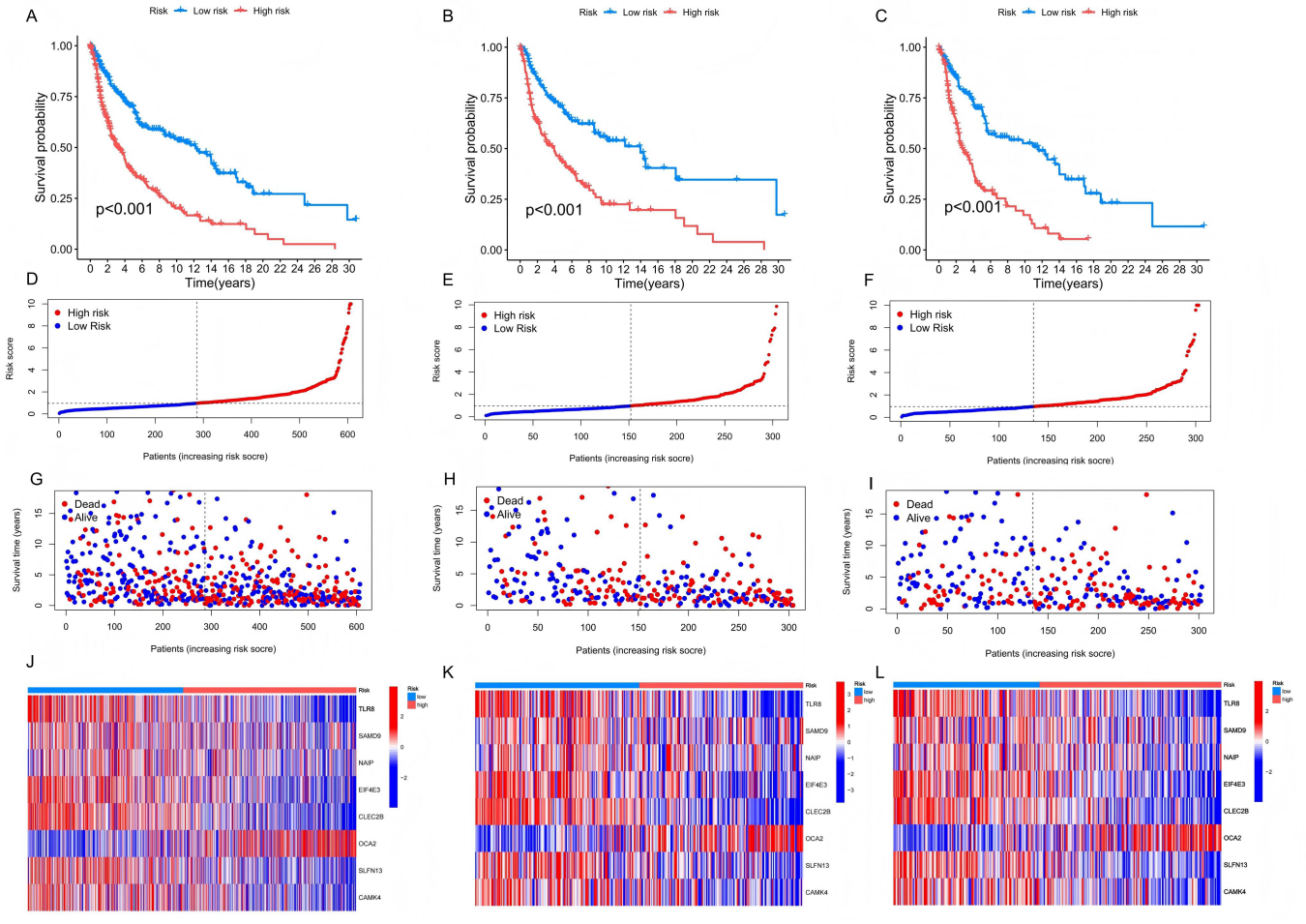


per accumulates in cells and directly combines with the lipoylated components of the TCA, leading to the aggregation of lipoylated proteins and the loss of Fe-S cluster proteins, which in turn leads to protein toxicity stress and eventually leads to cell death. And they proved that FDX1 (a cuproptosis related gene) was involved in regulating the lipoylation of proteins. In addition, the analysis of cancer dependency graph showed that the expression of FDX1 was positively correlated with the level of lipoic acid in tumor tissue, and the deletion of FDX1 could inhibit the lipoylation of dihydrolipoamide S-acetyltransferase (DLAT) (an enzyme in TCA). This showed that the new field of cuproptosis may provide a new perspective to develop therapeutic targets for cancer treatment.

In this study, we categorized melanoma samples into three distinct cuproptosis-related subtypes based on the expression profiles of 18 CRGs. Survival analysis revealed significant prognostic differences among these subtypes. Further analysis identified DEGs associated with these subtypes that were involved in cytotoxic production, immune response regulation, and various signaling pathways such as PI3K-Akt, potentially impacting tumor cell metabolism and evasion of immune surveillance. potentially impacting tumor cell metabolism and evasion of immune surveillance. Our research focused on the CRG clusters related DEGs, and through algorithm simulation, we obtained eight significant prognostic signatures and established a prognostic model. Previous studies have established prognostic models for bladder cancer, prostate cancer, hepatocellular carcinoma, and other diseases and shown good predictive ability [34-36]. And we also verified the performance of our prognostic models through survival analysis, ROC curve and independent prognostic analysis, etc. The results indicated that our prognostic model has the ability to group patients according to the risk score and predict the prognosis of patients.

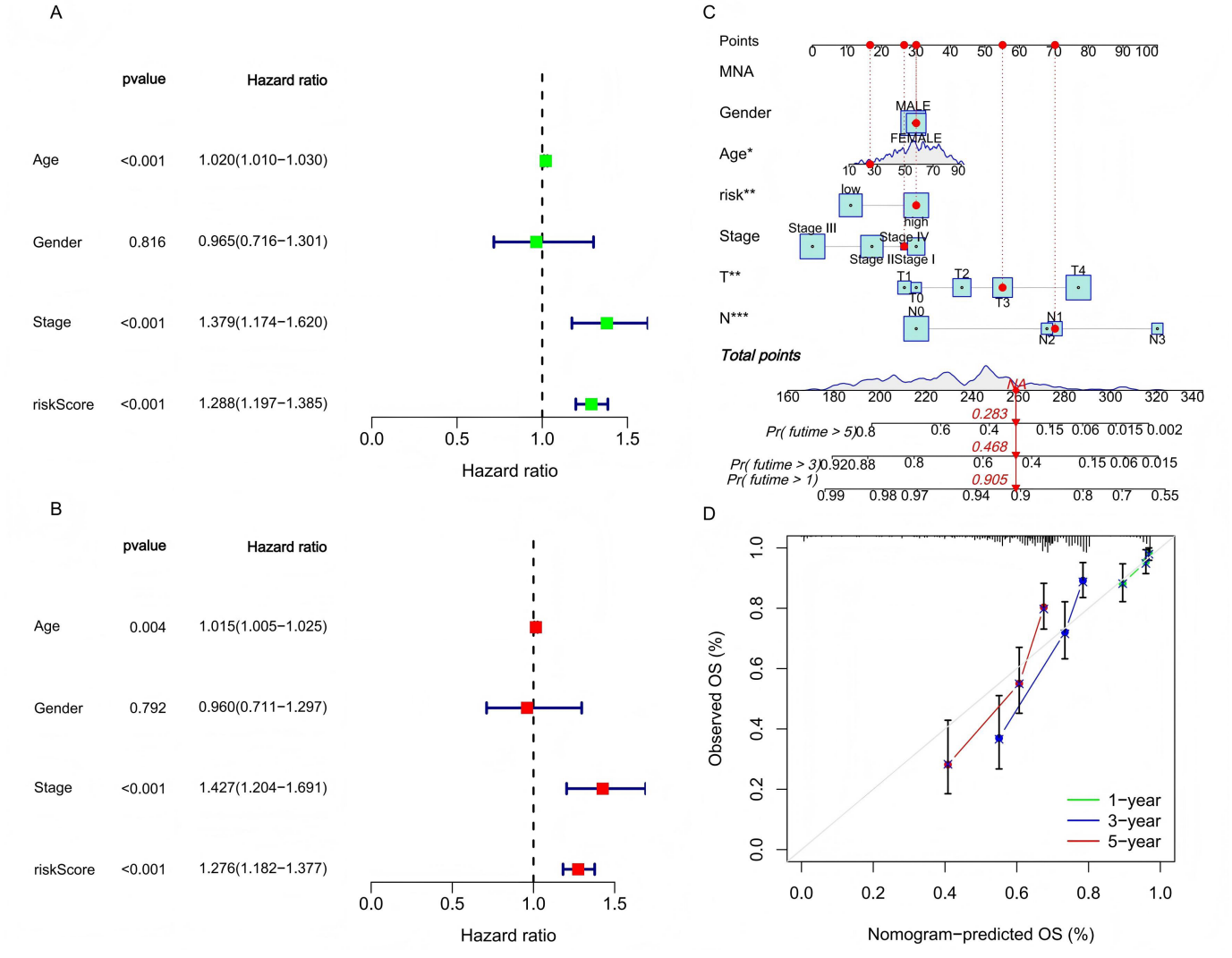
The eight screened-out DEGs related to CRG clusters are CAMK4, TLR8, EIF4E3, CLEC2B, OCA2, SLFN13, SAMD9 and NAIP. Notably, research by Li et al. demonstrated that microRNA-129-5p targeted calmodulin-dependent protein kinase IV (CAMK4) to inhibit the proliferation, migration, and invasion of hepatocytes, suggesting that CAMK4 could mitigate cancer progression by inhibiting the MAPK pathway [37]—a key promoter of tumor growth and angiogenesis. This finding indicated that CAMK4 may be a promising target for melanoma, especially since current treatments like Vemurafenib and Trametinib target the MAPK pathway to control disease progression [10]. Toll-like receptors (TLRs), critical to innate immunity, are garnering attention in immunotherapy. With the development of immunotherapy, the TLRs family has also been paid more and more attention. Motolimod, a TLR8 agonist, has shown potential in preclinical models, underscoring

Figure 6. The validation of the prognostic model. The survival analysis results, risk score distribution, survival status, the expression of genes related to prognosis in the high and low risk groups, and AUC of all samples (**A, D, G, J, M**), training set (**B, E, H, K, N**), and test set (**C, F, I, L, O**).



the relevance of the TLRs in cancer treatment, particularly as resistance to existing therapies increases [38]. With the targeting and immune therapy of melanoma, drug resistance is gradually increasing. The development or combination of new drugs may improve the therapeutic effect. Another noteworthy gene, EIF4E3, part of the EIF4E family, acts as a tissue-specific tumor suppressor by binding to the methyl-7-guanosine cap, thus preventing carcinogenic transformation [39]. CLEC2B, a marker identified in various cancers and linked to immune response regulation [40], has been shown to act as a protective factor in melanoma [41]. This suggested its potential utility as a therapeutic target, possibly enhancing immune response against tumor cells. The Schlafen (SLFN) gene family, associated with immune cell differentiation and regulation, showed varied impacts across different cancers. For example, high SLFN13 expression correlated with poor prognosis in gastric cancer [42], yet appeared as a low-risk factor in our melanoma studies, potentially due to epigenetic modifications. This indicated the complex role of SLFN genes in cancer and the need for further investigation. OCA2, associated with pigmentation, has been linked to an increased risk of familial melanoma [43] and cutaneous squamous cell carcinoma [44]. This suggested its role in melanoma progression and potential as a therapeutic target. SAMD9 mutations were implicated in various diseases, including myelodysplastic syndrome (MDS), esophageal cancer, and lung cancer. Research indicated that SAMD9 suppression could slow glioblastoma progression, highlighting its role in cancer development and as a potential therapeutic target [45, 46]. Lastly, the neuronal apoptosis inhibitor protein (NAIP), part of the inhibitor of apoptosis protein (IAP) family, was known to suppress apoptosis. Research by Yang et al. showed that tumor suppressor p53 regulates miR-15a to reduce NAIP expression, thereby enhancing apoptosis in breast cancer cells. This finding aligns with earlier studies suggesting that increasing IAP expression can re-sensitize cancer cells to apoptotic signals, offering new avenues for cancer therapy. This highlighted the potential of targeting IAP pathways, including NAIP, as a strategy for inducing cancer cell apoptosis and improving therapeutic outcomes [47-49]. These findings collectively underscored the potential of these genes

Figure 7. The clinical applicability of the prognostic model. (**A-B**) The results of univariate and multivariate Cox regression analysis prove that the risk score has independent predictive value. (**C**) The nomogram was used to calculate the survival rates of 1-, 3-, and 5-years for patients with melanoma. (**D**) Calibration curve for nomogram.



as targets for melanoma treatment, necessitating further studies to fully understand their roles and therapeutic potential in the tumor microenvironment and beyond.

TME consists of tumor cells, immune cells, and cytokines, forming an ecosystem that plays a critical role in tumor development, growth, and metastasis [50]. With the advancement of ICB therapies, the study of immune cells, cytokines, and immune mechanisms within the TME has deepened [51]. In our study, we observed a significant negative correlation between risk scores and the infiltration levels of CD8+ T cells, activated memory CD4+ T cells, M1 macrophages, and plasma cells. Macrophages can be polarized into two types based on their phenotype and secreted cytokines: M1 and M2. M1 macrophages secrete tumor-killing agents such as reactive oxygen species, nitric oxide, IFN-γ, and Fas ligand (FasL), and they also recruit other tumor-specific immune cells through chemokine secretion, playing a key role in anti-tumor responses [52]. Similarly, activated memory CD4+ T helper (Th1) cells and CD8+ T cells are crucial for establishing long-term immune memory, which triggers a rapid cytotoxic response upon re-exposure to tumor cells. These immune cells are essential for the long-term remission of melanoma [53, 54]. A disruption in the balance between tumor cells and the host immune response may lead to the progression of melanoma, contributing to the poorer prognosis seen in high-risk groups. These observations are critical for understanding the molecular underpinnings that differentiate prognostic outcomes in melanoma, providing a basis for targeted therapeutic interventions.

In addition, we evaluated the TME of the high-risk and low-risk groups based on the ESTIMATE algorithm. The results showed that the stromal, immune, and ESTIMATE scores were significantly higher in the low-risk group compared to the high-risk group, suggesting that the low-risk group had better immune defense and response capabilities. However, contrary to our expectations, the TIDE score for the low-risk group was higher, indicating a greater likelihood of immune escape in this group. This apparent paradox underscores the complex and dual-nature role of immune responses in melanoma progression. Melanoma is widely recognized for its high immunogenicity, often generating a substantial number of neoantigens through mechanisms such as chromosomal instability, high mutation burden, and structural variants. These tumor-specific antigens can initiate potent innate and adaptive immune reactions, recruiting lymphocytes and other immune mediators into the tumor bed, which is reflected in the high immune scores observed.However, the very intensity of this immune pressure drives the selection of tumor clones capable of exploiting regulatory pathways to evade destruction. Melanoma cells can engage a variety of resistance mechanisms, including the upregulation of immune checkpoint molecules (e.g., PD-L1, CTLA-4), recruitment of immunosuppressive cells (such as Tregs, MDSCs, or M2 macrophages), and secretion of soluble factors that dampen T-cell function. Therefore, an immune-rich microenvironment may not always correlate with productive cytotoxicity; rather, it can represent a battlefield where immune activation and suppression coexist dynamically. The elevated TIDE score in the context of high immune infiltration may thus reflect this dysfunctional state—a TME characterized by abundant but exhausted or inhibited lymphocytes, and active mechanisms of adaptive immune resistance.In summary, the coexistence of high immune scores and high TIDE scores in the low-risk group illuminates the intricate and often contradictory nature of tumor–immune interactions. It suggests that the low-risk group may be dominated by an "immune-inflamed" but poorly effective phenotype, where the immune response is actively suppressed by escape mechanisms. This insight emphasizes the necessity of combining prognostic signatures with functional biomarkers of immune competence to more

Figure 8. Comparison of TME and TMB between high-risk and low-risk groups. (**A**) The correlation between the number of immune cells infiltrated and the eight prognostic signatures and risk score. (**B**) The TME scores of high-risk and low-risk group. (**C-D**) The TMB of high-risk and low-risk group. (**E-F**) K-M survival analysis based on TMB. (**G**) The TIDE scores of two groups.

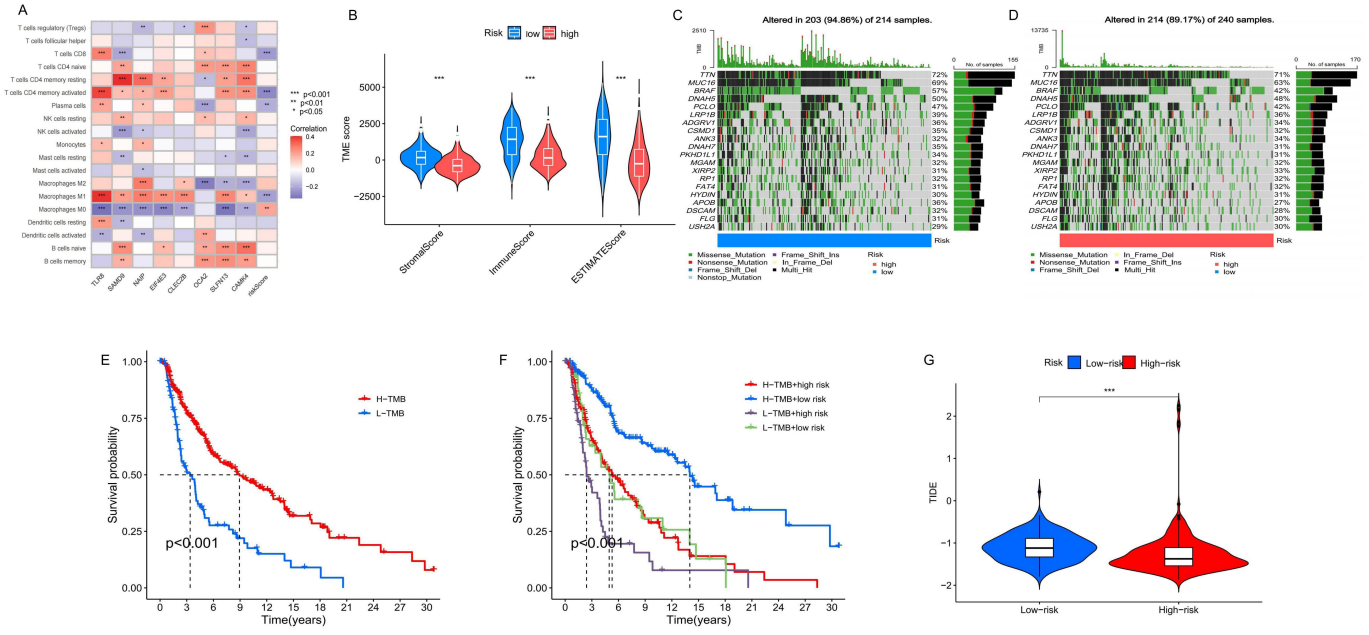
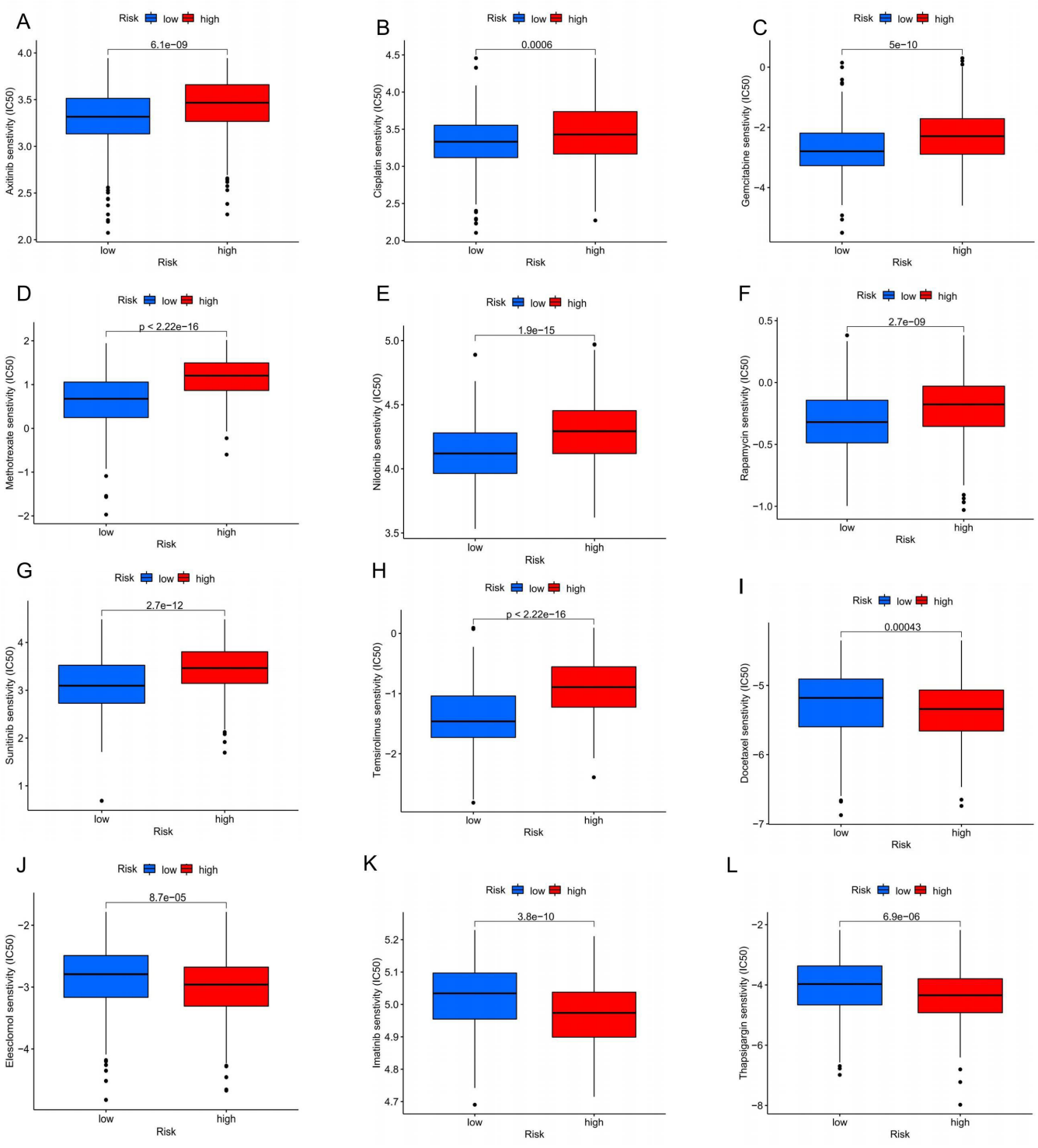


Figure 9. Comparison of sensitivity to chemotherapy or targeted therapy between high-risk and low-risk groups of melanoma patients. (**A**) Axitinib; (**B**) Cisplatin; (**C**) Gemcitabine; (**D**) Methotrexate; (**E**) Nilotinib; (**F**) Rapamycin; (**G**) Sunitinib; (**H**) Temsirolimus; (**I**) Docetaxel; (**J**) Elesclomol; (**K**) Imatinib; (**L**) Thapsigargin.



accurately stratify patients and tailor immunotherapeutic strategies [52].

Despite these findings, our study has some limitations. First, the relationship between CRGs and melanoma development remains unclear and warrants further investigation. Additionally, our analysis is based on retrospective clinical samples, and further prospective studies are needed to validate the clinical utility of our prognostic model.

Conclusion

In conclusion, we identified eight prognostic signatures from differentially expressed genes associated with CRG clusters and developed a prognostic model for melanoma patients. This model offers valuable insights into the immune landscape, prognosis, and potential clinical treatment options, serving as a useful reference for guiding personalized melanoma therapies.

Abbreviations

Area under the curve: AUC; Biological Process: BP; Calmodulin-dependent protein kinase IV: CAMK4; Cuproptosis-related genes: CRGs; Cytotoxic T lymphocyte antigen 4: CTLA4; Copy number variation: CNV; Differentially expressed genes: DEGs; Fas ligand: FasL; Gene set variation analysis: GSVA; Gene Expression Omnibus: GEO; Genomics of Drug Sensitivity in Cancer: GDSC; Immune checkpoint blockade: ICB; Inhibitor of apoptosis protein: IAP; Half-maximal inhibitory concentration: IC50; Kaplan-Meier: K-M; Least absolute shrinkage and selection operator: LASSO; Molecular Function: MF; Myelodysplastic syndrome: MDS; Neuronal apoptosis inhibitor protein: NAIP; Principal component analysis: PCA; Regulated cell death: RCD; Receiver operating characteristic: ROC; Single sample gene-set enrichment analysis: ssGSEA; The Cancer Genome Atlas: TCGA; Tricarboxylic acid: TCA; Tumor microenvironment: TME; Tumor mutation burden: TMB; Tumor immune dysfunction and exclusion: TIDE; Toll-like receptors: TLRs; T helper cell 1: Th1

Author Contributions

Zishen Xia, Nan Gao and Jianwen Wang contributed equally to this work as co-first authors, participating in data curation, formal analysis, methodology development, software implementation, visualization, and manuscript writing. Lizhao Yan, Cong Ma, and Kangwei Wang were responsible for reviewing the conceptual design, writing the article, and proofreading. Yuxiong Weng conceived the study, overseeing the study design, writing, data acquisition, analysis, and interpretation. All authors read and approved the final manuscript.

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Ethics Approval and Consent to Participate

Not Applicable.

Competing Interests

The authors declare that they have no existing or potential commercial or financial relationships that could create a conflict of interest at the time of conducting this study.

Data Availability

The data that support the findings of this study are available in the following repositories: TCGA [http://cancergenome.nih.gov], GEO: [https://www.ncbi.nlm.nih.gov/geo], TIDE [http://tide.dfci.harvard.edu], GDSC [https://www.cancerrxgene.org]. These data were derived from resources available in the public domain and are freely accessible under their respective usage guidelines.

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The Recent Changing Global Landscape of Cancer

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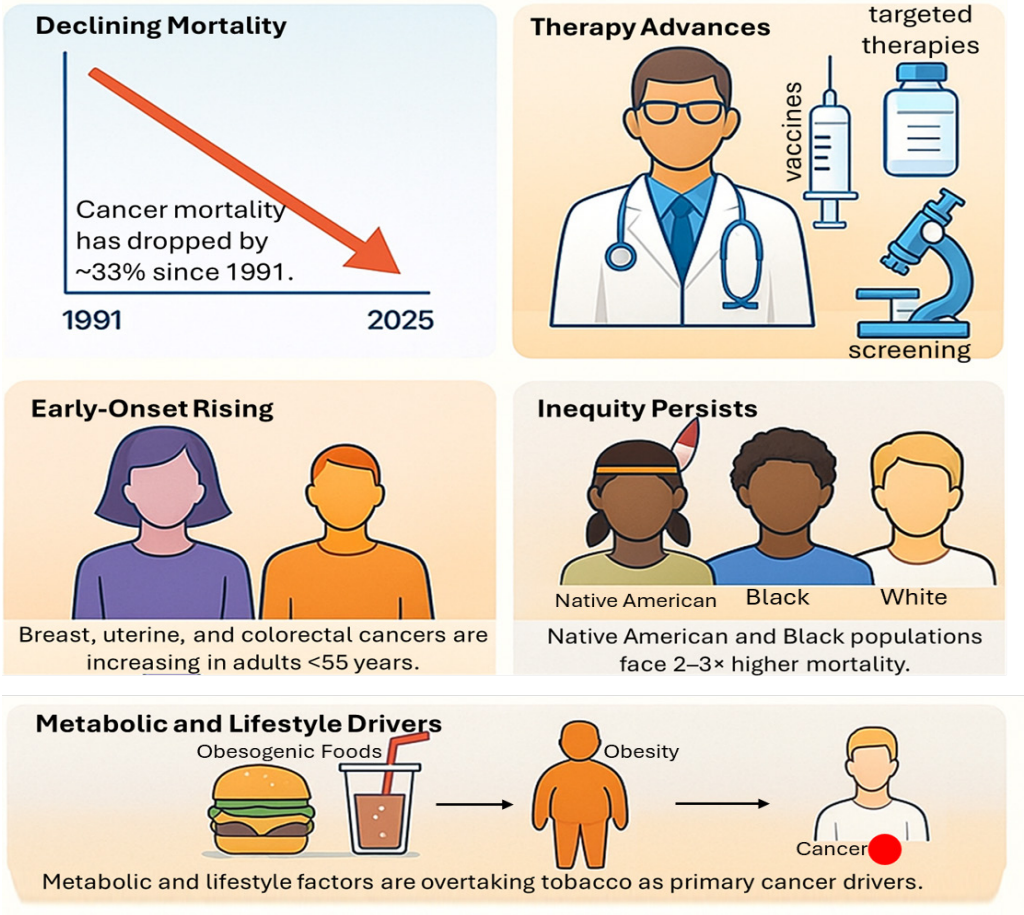
Graphical Abstract



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The Recent Changing Global Landscape of Cancer

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Abstract

The good news, a 33% drop in U.S. cancer deaths since 1991, masks a troubling demographic shift revealed by the American Cancer Society's newest statistics. As overall new cases rose from 1.96 million (2023) to 2.04 million (2025), leading to 618,120 expected deaths, the burden is falling unfairly. We're seeing a rising incidence of breast, uterine corpus, and colorectal cancers in those under 55. For ages 50–64, women now lead men in new diagnoses. Crucially, significant racial gaps persist, with Native American and Black individuals facing two to three times the mortality rate for several cancer types. This evidence points to metabolic and inflammatory environments taking over as the main cancer drivers, moving past tobacco's historical dominance. Our next move must be to fully integrate precision prevention and equitable care access to shift the fight from treatment to early interception.

Keywords: Cancer trends; Early-onset cancer; Prevention.

Over the past three decades, the United States has achieved remarkable progress in reducing cancer mortality. From 1991 to 2022, the age-standardized death rate dropped by roughly 33%, translating to nearly 4.5 million deaths averted [1]. In 2025, approximately 2,041,910 new cancer cases and 618,120 deaths are projected, continuing a long-term downward trend in overall mortality [1]. This success story, however, conceals a more complex picture. The latest *CA: A Cancer Journal for Clinicians series* [1-3] illustrates that the epidemiologic burden of cancer is undergoing a quiet transformation: incidence rates are rising in younger and middle-aged adults, particularly women, even as older populations benefit from better screening, immunization, and targeted therapies. The decline in mortality now coexists with new patterns of inequity and emergent etiologies, signaling that the "war on cancer" has entered a new phase.

Historically, men had far higher cancer rates than women, with a male-to-female incidence ratio of 1.6 in 1992. Yet by 2021, this ratio had narrowed to 1.1, and among individuals aged 50–64, women now surpass men in overall cancer incidence (832.5 vs. 830.6 per 100,000) [1]. Even more striking, women under 50 show an 82% higher incidence rate than their male counterparts (141.1 vs. 77.4 per 100,000), a dramatic increase from the 51% gap observed in 2002 [1]. This reversal is driven by multiple site-specific increases. Breast cancer remains the dominant malignancy in women, with 316,950 new cases expected in 2025, accounting for nearly one-third of female diagnoses [1]. Uterine corpus cancer, once relatively rare, is now among the fastest rising cancers, increasing by 0.6–1% annually since 2015 [2]. Even lung cancer, traditionally male-dominant, has flipped direction: incidence in women under 65 surpassed men for the first time in 2021 (15.7 vs. 15.4 per 100,000) [1]. In the developed world, lifestyle is dethroning tobacco as the key driver of cancer. Obesity, diabetes, and physical inactivity are becoming the dominant, preventable risks. The rise in obesity is particularly concerning; it wreaks havoc on our metabolism, immune system, and hormones, directly driving up rates of endometrial, breast, and colorectal cancers. The central challenge for cancer prevention now is no longer just quitting smoking; it's controlling our metabolism and ensuring equitable health for all.

Perhaps the most alarming trend is the steady rise of early-onset cancers, diagnoses in adults younger than 50 years. Colorectal cancer, once the domain of the elderly, is now the leading cause of cancer death in men under 50 and second in women [2]. Incidence in this group increased 1–2% annually during 2015–2019, even as overall rates fell in older adults. The etiology remains multifactorial: Westernized diets, gut

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microbiome dysbiosis, antibiotic overuse, and early-life exposures have all been implicated. The implications are profound. A 2025 modeling analysis cited by Siegel et al. estimated that 4,000–7,000 excess colorectal cancer deaths may occur by 2040, depending on how rapidly screening recovers from pandemic disruptions [1]. This "younger shift" in cancer biology calls for a re-examination of screening paradigms. Lowering the starting age for colorectal screening to 45 has been a critical step, yet evidence suggests that even this may not fully capture high-risk populations, especially in underrepresented ethnic groups where screening uptake remains low. A precision-prevention approach, integrating genomic risk scores and lifestyle biomarkers, may soon become essential.

Despite epidemiologic progress, racial disparities remain stark and largely unchanged. The 2025 report highlights that Native American populations experience cancer mortality rates 2–3 times higher than White populations for kidney, liver, stomach, and cervical cancers [1]. Similarly, Black Americans continue to face two-fold higher mortality for prostate, stomach, and uterine corpus cancers. Such inequities are not solely biological, they reflect the cumulative effect of structural determinants: healthcare access, screening availability, and socioeconomic deprivation. For example, while mortality from cervical cancer has plummeted by 65% among vaccinated young women [3], unvaccinated and rural groups remain vulnerable. As the benefits of precision medicine expand, these disparities risk becoming even more entrenched unless prevention, screening, and treatment resources are equitably distributed.

Between 1995 and 2021, the proportion of cancer cases in adults aged ≥65 declined from 61% to 59%, whereas cases in the 50–64 age group rose from 25% to 29% [1]. This demographic shift parallels the "post-war cohort effect," in which individuals born after 1950 experienced greater lifetime exposure to processed foods, environmental toxins, and sedentary lifestyles. The transition of cancer burden to the middle-aged demographic carries enormous implications for workforce productivity and healthcare cost. Unlike elderly patients, middle-aged individuals are more likely to live long enough to face secondary malignancies and therapy-induced chronic conditions. The need for longitudinal survivorship infrastructure, including cardiovascular monitoring, metabolic management, and psychosocial care, will only intensify.

We used to rely on the American Cancer Society's long-term tracking as the definitive measure, but the 2025 data is a wake-up call: focusing only on lower mortality isn't cutting it. The problem has shifted: incidence rates are climbing right where the impact will be hardest felt, among young people, women, and minority populations. True progress now demands a three-pronged approach: preventing more cancers, closing those stubborn racial gaps, and managing the ongoing health and financial fallout for survivors. Moving forward, cancer control hinges on weaving prevention, genomics, and health equity into a single policy strategy that respects the social and environmental context as much as the molecular one.

Cancer mortality in the U.S. continues to decline, a testament to decades of public health, screening, and therapeutic advances. Yet the battlefront is shifting. Rising incidence in younger and female populations, persistent racial disparities, and the growing influence of metabolic and lifestyle factors all demand a paradigm shift, from cure to early interception and equitable prevention. As Siegel et al. remind us, "continued progress will require investment in cancer prevention and access to equitable treatment, especially for Native American and Black individuals" [1]. The next decade will determine whether we can turn these insights into structural change, or whether success in mortality will mask a new generation of preventable cancers.

Abbreviations

Not Applicable.

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Competing Interests

The authors declare that the paper was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data Availability

Not applicable.

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Review Article

Life Conflux

The cGAS-STING Pathway: Insights into Regulatory Mechanisms, Disease Dysregulation, and Therapeutic Development

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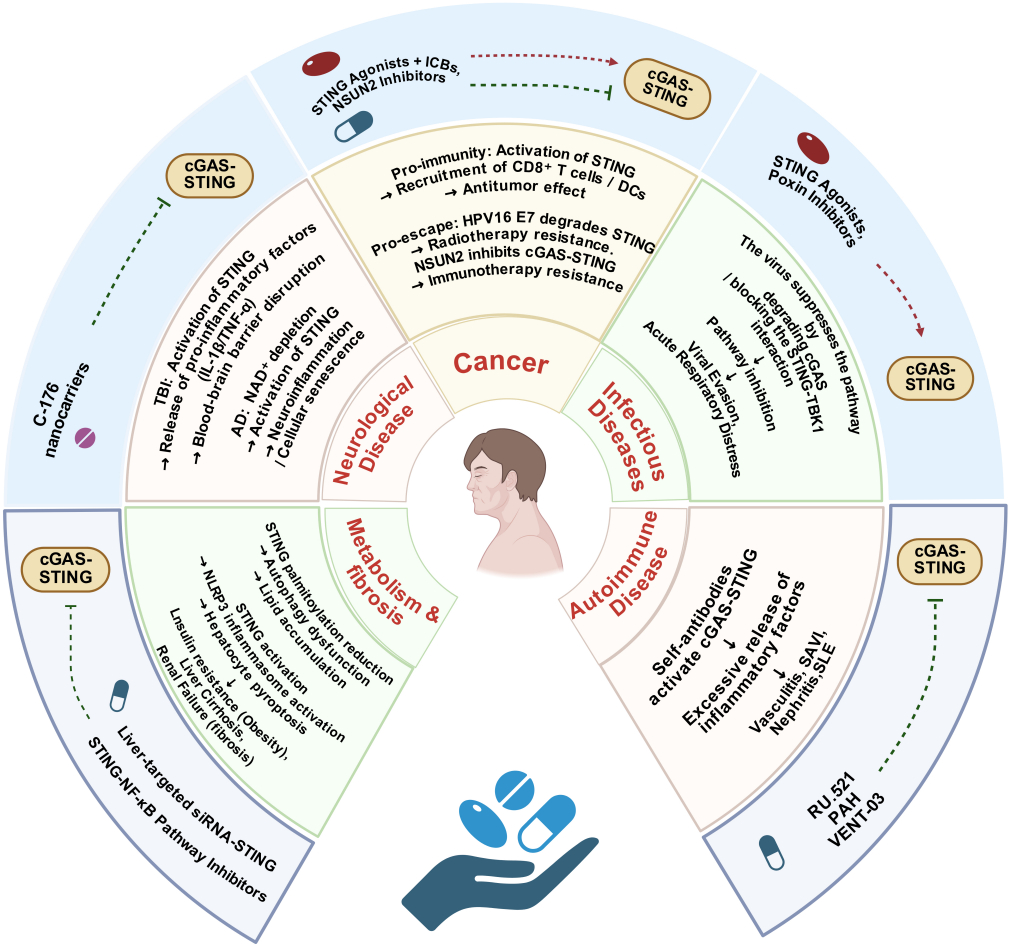
Graphical Abstract



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Review Article

Life Conflux

The cGAS-STING Pathway: Insights into Regulatory Mechanisms, Disease Dysregulation, and Therapeutic Development

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Abstract

The cyclic GMP-AMP synthase-stimulator of interferon genes (cGAS-STING) pathway, a central hub of the innate immune system, is a key mediator of immune surveillance against abnormal cytoplasmic dsDNA: cGAS recognizes such dsDNA to synthesize 2'3'-cGAMP, which activates STING and downstream signaling to drive IFN-I and proinflammatory cytokine expression for the maintenance of homeostasis. This mechanism enables the pathway to exert multidimensional roles in physiology and pathology. Its activity is fine-tuned by post-translational modifications and non-coding RNAs. Given its critical role in linking innate immunity to disease progression, it has become a promising therapeutic target. This review summarizes the pathway's regulatory mechanisms and pathological implications, detailing its roles in immune activation, disease dysregulation, and therapeutic development. It also addresses existing challenges and proposes future directions, aiming to provide new insights for precision therapy against cGAS-STING-associated diseases.

Keywords: cGAS; STING; Inflammation; Tumor Immunity; Diseases therapy

Introduction

The innate immune system is the first line of defense against external pathogens, playing a crucial role in immune responses. As a major component of the immune system, the innate immune system not only provides timely defense responses at the onset of infection but also triggers a series of immune reactions by recognizing exogenous pathogens and endogenous damage signals, thereby maintaining bodily homeostasis [1]. Unlike adaptive immunity, the innate immune system does not rely on prior immunological memory. Instead, it directly recognizes pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) through a wide array of pattern recognition receptors (PRRs), which rapidly activate inflammatory responses, initiate antiviral mechanisms, and regulate immune balance [2-3].

In innate immune responses, the cGAS-STING pathway has become a research hotspot in recent years and has been shown to play a critical role in various immune responses [4]. The cGAS-STING signaling pathway is a central immune signaling pathway in both the innate immune system and intracellular signaling. It serves as a key "DNA-sensing" pathway, deeply involved in the host's immune response to exogenous pathogens (such as viruses and bacteria) and responses to endogenous damage [5-7]. As a DNA sensor in this pathway, cGAS recognizes double-stranded DNA (dsDNA) that is abnormally present in the cytoplasm. Under physiological conditions, there is no exogenous DNA in the cell, but when viral infections or cellular damage occur, exogenous DNA appears. At this point, cGAS synthesizes the cGAMP (cyclic GMP-AMP) dimer molecule. STING, located on the membrane of the endoplasmic reticulum (ER), serves as a receptor for cGAMP. Upon binding with cGAMP, STING undergoes a conformational change and translocates to the Golgi apparatus, where it activates downstream signaling molecules such as TBK1 and IRF3. This activation ultimately triggers the expression of type I interferons, pro-inflammatory cytokines, and other immune-related genes, helping the body resist pathogen invasion, eliminate damaged cells, and exert antiviral, antitumor, and immune regulatory effects [8].

The cGAS-STING pathway is not only a critical component of antiviral immunity but also plays a significant role in regulating various physiological and pathological processes, including cell death, tumor immunity, and anti-inflammatory responses. Therefore, understanding the molecular mechanisms underlying this pathway and elucidating its functions in different cells and tissues is crucial for the development of novel immunotherapies. Despite the increasing recognition of the role of the cGAS-STING pathway in disease defense, current research still faces several bottlenecks. These bottlenecks primarily

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include an incomplete understanding of the fine-tuned regulatory mechanisms that activate the pathway and challenges in developing targeted therapeutics [9]. Issues such as multiple variations of the STING receptor, the stability of cGAMP, and the complexity of the interaction between cGAS and STING limit the clinical application potential of this pathway [10].

It is important to note that the role of the cGAS-STING pathway extends beyond immune defense. It is also closely associated with the development and progression of various diseases, including cancer and autoimmune diseases. Therefore, research into the regulatory mechanisms of this pathway holds significant theoretical and clinical value in immunology, oncology, and other related fields.

This review aims to summarize the mechanisms of the cGAS-STING pathway in different physiological and pathological states and to analyze the challenges and bottlenecks it faces in clinical applications. By providing a comprehensive biological analysis of the cGAS-STING pathway, this paper seeks to offer new insights and targets for drug development in related diseases and to provide a theoretical foundation for the optimization of future therapeutic strategies. It is hoped that this review will inspire new breakthroughs and directions in the field of disease treatment, particularly in immunotherapy for cancer, autoimmune diseases, and viral infections.

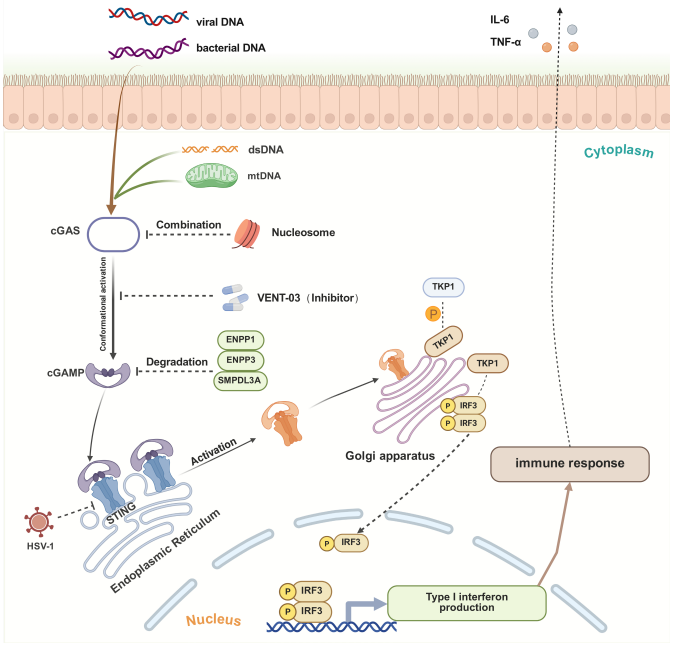
Activation and Inhibition of the cGAS-STING Pathway

When the chromosomal DNA of a virus or cancer cell enters the cytoplasm and binds to cGAS, cGAS catalyzes the production of 2'3'-cGAMP. On the ER, STING undergoes a conformational change upon sensing 2'3'-cGAMP, causing the cGAS-STING signaling pathway to transition from an inactive (closed) state to an active (open) state. This transition promotes the assembly of the TBK1-IRF3 complex, ultimately triggering type I interferon responses and the release of other inflammatory cytokines [11-12]. As a sentinel for pathogen invasion, cGAMP ensures the pathway remains activated until the virus or pathogen is cleared. Once eliminated, the cGAS-STING pathway reverts to its closed state. Studies have shown that cGAMP could be degraded by several enzymes, such as ENPP1, ENPP3, and SMPDL3A, to limit cGAS-STING signaling and maintain systemic inflammatory homeostasis [13].

Under physiological conditions, the activation and inhibition of the cGAS-STING pathway are tightly regulated. However, some viruses possess mechanisms to suppress this pathway. For instance, the HSV-1 virus inactivates STING, thereby inhibiting the cGAS-STING pathway to evade the innate antiviral immune response [14]. Additionally, methyltransferase PRMT6 has been shown to impair the TBK1-IRF3 signaling cascade, weakening the innate antiviral immune response [15]. These findings emphasize the crucial role of the cGAS-STING pathway in defending against viral invasion and activating the innate immune system.

In certain pathological conditions, such as systemic lupus erythematosus (SLE), Stimulator of IFN Genes-Associated Vasculopathy with Onset in Infancy (SAVI), and systemic sclerosis, the accumulation of abnormal DNA could activate the cGAS-STING pathway, leading to sustained activation of downstream immune signaling and exacerbating inflammation-mediated damage [16]. The use of cGAS-specific small molecule inhibitors effectively suppresses interferon expression triggered by dsDNA, mitigating inflammation [17]. Examples of such inhibitors include RU.521 [18], PAH [19], and VENT-03 [20]. Notably, the VENT-03 inhibitor has entered Phase I clinical trials, representing a novel therapeutic approach for autoimmune diseases (Figure 1).

Figure 1. Fundamental Activation and Inhibition Mechanisms of the cGAS-STING Pathway.



Crosstalk Between the cGAS-STING Pathway and Subcellular Organelles

Regulation of STING by the ER

STING is primarily localized within the ER, where it can activate the NF-κB and IRF3 transcriptional pathways, thereby inducing the expression of type I interferons (e.g., IFN-α and IFN-β), which subsequently promote an effective antiviral state upon expression [21]. There is a close anatomical and functional connection between the ER and mitochondria, which communicate through calcium ions and reactive oxygen species (ROS), facilitating inter-organelle signaling. STING is highly localized in the ER-mitochondria-associated membrane (MAM) regions, a unique position that allows it to respond acutely to cellular organelle stress, such as the leakage of mitochondrial DNA (mtDNA) [22]. The ER plays a crucial role in protein folding, lipid synthesis, and calcium storage, serving as an important platform for STING synthesis, modification, and residence. The interaction between STING and the ER forms the core of the cGAS-STING pathway, making the ER a key hub in the regulation of STING signaling [23].

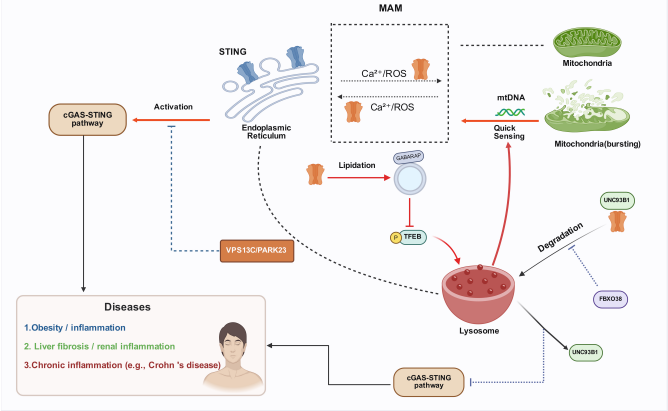
Crosstalk Between Lysosomes and STING Signaling

Studies have shown that there is a reciprocal regulatory relationship between the cGAS-STING pathway and lysosomes. During pathogen invasion, the cGAS-STING pathway activates the transcription factor TFEB, which promotes lysosomal biogenesis and accelerates the clearance of cytosolic DNA and

invading pathogens [24]. This process highlights that inducing lysosomal biogenesis is another important function of the cGAS-STING pathway. In a mouse model of HSV-1 infection, UNC93B1 targets STING to promote the autophagy-lysosome degradation pathway, which in turn reduces the activity of the cGAS-STING signaling pathway [25]. Furthermore, the absence of the T-cell immune-related FBXO38 protein leads to lysosome-dependent STING degradation, inhibiting the activation of the STING pathway [26].

Moreover, research indicates that the ER-lysosome lipid transporter VPS13C/PARK23 could inhibit abnormal mtDNA-dependent STING signaling [27]. Recent studies show that STING induces the lipidation of GABARAP on single-membrane vesicles, specifically inhibiting mTORC1-mediated phosphorylation of TFEB. Subsequently, TFEB translocates to the nucleus to regulate the expression of lysosome-related genes. STING-activated lysosomes not only efficiently clear cytosolic DNA but also enhance the clearance of bacteria (e.g., Salmonella Typhimurium) and viruses (e.g., HSV-1) [24]. These studies underscore the significant role of STING in inter-organelle interactions (Figure 2).

Figure 2. Crosstalk Mechanisms between the cGAS-STING Pathway and Subcellular Organelles (ER / Mitochondria / Lysosomes).



Non-Coding RNA Regulatory Networks

miRNA Regulation of STING

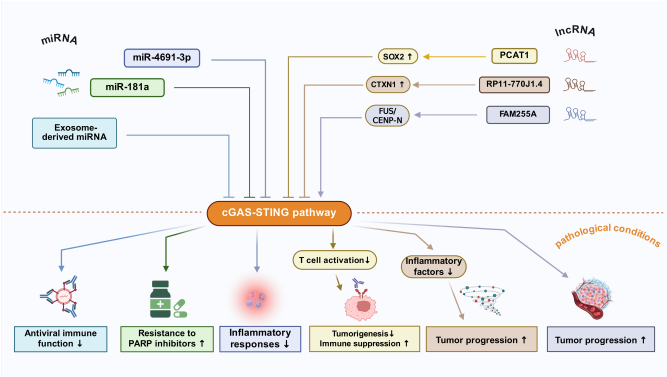
MicroRNAs (miRNAs) are non-coding RNA molecules, typically around 20-24 nucleotides in length, that play a crucial role in regulating STING gene expression [28]. Studies have shown that STING is a direct target of miR-4691-3p, which inhibits STING expression and negatively regulates the cGAS-STING pathway, thereby suppressing inflammatory responses [29]. Additionally, miR-181a can target STING to inhibit the production of pro-inflammatory factors, promoting resistance to PARP inhibitors in triple-negative breast cancer (TNBC) and ovarian cancer (OVCA) [28]. Similarly, in multiple myeloma (MM), exosome-derived miRNA secretion suppresses the antiviral immune function of the cGAS-STING pathway [30].

lncRNA Regulation of STING

Studies have shown that long non-coding RNAs (lncRNAs) are closely associated with the activation of the cGAS-STING pathway and play significant regulatory roles in both physiological and pathological processes [31]. In non-small cell lung cancer (NSCLC), lncRNA PCAT1 could inhibit T cell activation mediated by the cGAS-STING signaling pathway through the activation of SOX2, thereby promoting tumorigenesis and immune suppression [32]. In glioma, the inhibition of lncRNA RP11-770J1.4 downregulates the expression of the downstream protein CTXN1, activates the cGAS-STING pathway, and induces the secretion of related inflammatory factors [33]. In nasopharyngeal carcinoma (NPC), lncRNA FAM255A regulates the expression of CENP-N through interaction with FUS, affecting the cGAS-STING pathway. Specifically, the activation of the FUS/CENP-N/cGAS-STING signaling pathway promotes tumor progression, while the suppression of lncRNA FAM255A expression weakens the malignant characteristics of tumor cells [34].

These studies highlight the close regulatory association between non-coding RNAs and STING, suggesting that th ey may serve as upstream regulatory genes for STING and potential molecular targets for therapeutic interventions (Figure 3).

Figure 3. Regulation of the cGAS-STING pathway by non-coding RNAs (miRNA/lncRNA).



Inflammation-Related Diseases

cGAS-STING Pathway Regulation of Inflammation-Related Mechanisms

The cGAS-STING pathway activates the expression of pro-inflammatory factors such as LPS, IL-6, and IL-1β through the non-classical NF-κB pathway, thereby promoting the exacerbation of the inflammatory response [35]. Studies have shown that the gut microbiota can initiate a systemic antiviral immune response through the cGAS-STING-IFN-I axis [36]. Additionally, autophagy regulates the cGAS-STING pathway negatively by clearing cytosolic DNA. Defects in this process may lead to the development of chronic inflammatory diseases such as Crohn's disease [37]. At the molecular level, the TBK1-activated p62/SQSTM1- mediated autophagy pathway effectively weakens the transmission of cGAS-STING signals [38], thus modulating the intensity and duration of immune responses and preventing immune dysregulation and chronic inflammation caused by excessive activation. Meanwhile, STING activation also suppresses the secretion of the anti-inflammatory factor IL-10, further exacerbating the inflammatory response [39-40]. However, there is controversy regarding STING's regulation of IL-10. Some studies suggest that STING activation can promote IL-10 secretion in certain inflammatory environments, especially in intestinal inflammation [41]. This suggests that the immunoregulatory role of STING may depend on specific physiological and pathological states.

Systemic Autoimmune Diseases

Gain-of-function mutations in STING lead to excessive activation of the cGAS-STING pathway, triggering an overactive inflammatory response. For example, mutations in the TMEM173 gene (such as N154S and V155M) result in sustained STING activation, causing systemic autoimmune vasculitis and pulmonary fibrosis, known as SAVI, which typically manifests in infancy [42-43]. Research has shown that interferon-stimulated genes (ISGs) are persistently upregulated in SAVI patients, closely associated with the overactivation of the JAK-STAT signaling pathway. This abnormal activation not only promotes enhanced immune responses but may also worsen the inflammatory process of autoimmune diseases, highlighting the central role of the cGAS-STING pathway in the pathogenesis of SAVI [44-45].

Studies have indicated that genetic STING gain-of-function mutations are critical factors in familial inflammatory syndromes with lupus-like symptoms, revealing an important link between STING and SLE [46-47]. It has been reported that self-antibodies in SLE patients continually activate the cGAS-STING pathway, resulting in the release of numerous inflammatory factors and further exacerbating the condition [48].

In lupus nephritis (LN), STING activation promotes ferroptosis and inflammation through the TBK1/NF-κB signaling pathway, advancing disease progression [49]. However, studies in mouse models have found that the cGAS-STING pathway does not promote autoimmune responses in SLE mouse models [50]. This finding suggests that directly applying mouse model findings to human diseases may present challenges due to the complex genetic background of SLE patients.

Organ-Specific Inflammation

In the pathogenesis of alcoholic hepatitis, leakage of mitochondrial DNA (mtDNA) is considered a key trigger of immune responses. mtDNA activates the cGAS-STING pathway, thereby stimulating downstream IRF3 and NF-κB signaling pathways. The activation of these pathways leads to the excessive secretion of pro-inflammatory cytokines such as IL-6 and TNF-α, further aggravating the liver's inflammatory response. mtDNA leakage is closely related to alcohol-induced hepatocyte damage, and the cGAS-STING pathway plays a key role in the immune dysregulation and inflammatory response in alcoholic hepatitis [51-52].

In LN, STING activation induces NLRP3 inflammasome activation and promotes necroptosis of kidney macrophages, thereby worsening the formation of proteinuria [53]. However, STING deficiency can alleviate symptoms of glomerulonephritis [54]. Mechanistically, STING upregulation enhances TBK1 expression and activates NF-κB signaling, which triggers ferroptosis and intensifies renal inflammation [49]. Therefore, regulating STING activity may represent a potential strategy for treating LN.

Studies have shown that under oxidative stress conditions, STING accelerates retinal pigment epithelial cell senescence through the NF-κB/HIF-1α signaling pathway [55]. In blood flow patterns, oscillatory shear stress (OSS) activates the ROS-STING axis, leading to endothelial cell senescence and promoting the development of atherosclerosis [56]. Furthermore, it has been shown that in aging endothelial cells, the cGAS-STING pathway is activated, further damaging vascular dilation function, while inhibition of cGAS-STING expression helps protect vascular function [57]. In Alzheimer's disease (AD), NAD+ depletion activates the cGAS-STING pathway, exacerbating neuroinflammation and accelerating cellular senescence. Supplementing NAD+ has been shown to alleviate cellular senescence effectively [58]. This suggests that the STING signaling pathway plays a critical role in the progression of age-related chronic diseases.

Future research directions include developing tissue-specific STING inhibitors, such as targeting kidney-specific nanoparticles to deliver H-151 inhibitors, to improve treatment targeting and efficacy [59]. Additionally, analyzing the structure-activity relationship of STING mutants (e.g., SAVI-related variants) will aid in the design of highly selective allosteric modulators to precisely regulate the cGAS-STING pathway, thereby improving the therapeutic outcomes for related diseases. These studies will provide new treatment strategies for clinical applications.

Infection and Immune Response

Antiviral Immunity

The cGAS-STING pathway activates intracellular immune responses through the recognition of viral DNA (such as HSV-1), triggering the secretion of IFN-I [60]. This process depends on cGAS recognizing viral DNA to generate cGAMP, which activates the STING protein. The activated STING interacts with TBK1 to promote its phosphorylation, further activating the transcription factor IRF3, which ultimately induces the expression of IFN-I [61-62].

RNA viruses, such as SARS-CoV-2 [63] and respiratory viruses (RVs) [64], induce mitochondrial dysfunction upon infection, leading to the release of mtDNA into the cytoplasm. Released mtDNA is recognized as a danger signal, further activating the cGAS-STING pathway. Following STING activation, a phosphorylation cascade involving TBK1 and IRF3 induces the production of a large amount of type I interferons and inflammatory factors, triggering a cytokine storm [65].

Studies have shown that HSV-1 escapes host immune surveillance by targeting the cGAS-STING pathway, inhibiting the immune response to the virus. STING is an important recognition molecule in the immune system that senses intracellular DNA damage or infection signals, activating downstream interferon responses and initiating antiviral immunity. HSV-1 effectively suppresses this immune response by disrupting the cGAS-STING pathway, facilitating its survival within the host [61]. Therefore, key regulation of the cGAS-STING pathway for oncolytic virus therapy may become an important strategy to improve efficacy. Optimizing cGAS-STING pathway activation or blocking its evasion mechanisms may enhance the immune effects of oncolytic viruses, highlighting the value of precise regulation of the cGAS-STING pathway in this therapeutic context [66].

However, some viruses can suppress host antiviral immune responses through various specific immune evasion strategies. For instance, the UL41 protein of herpesvirus degrades cGAS or blocks the binding of STING with TBK1, inhibiting IFN activation and thereby evading immune surveillance [67]. HPV11 targets STING for ubiquitin-mediated degradation via the E7 protein, reducing the expression of IFN-I in epithelial cells and further evading host immune defense [68]. However, adenoviruses have a minimal impact on this immune evasion mecha-

nism, indicating that different viruses exhibit variability in their immune escape strategies [69].

In addition to collaborating with RIG-I to recognize RNA viruses, STING interacts with other PRRs to coordinate immune responses. For example, TBK1 recruits STING to activate IRF3 and NF-κB, mediating immune defense against tumors and viral infections [70]. Moreover, STING and RIG-I activate the IFN-I cascade via the mitochondrial adapter protein MAVS and TBK1, demonstrating the central role of STING in regulating host immune responses [71]. In summary, the cross-regulation between STING and PRR pathways amplifies the host's immune response to viruses and optimizes antiviral defense mechanisms.

Bacterial and Parasitic Infections

The cGAS-STING pathway recognizes the DNA or metabolic products of Legionella, inducing the production of IFN-I and pro-inflammatory cytokines to restrict bacterial replication. However, the HAQ-STING variant significantly weakens this immune response, increasing the host's susceptibility to Legionella [72]. Additionally, during the later stages of the developmental cycle, Chlamydia trachomatis activates STING through the CTL0390 protein, the key molecule that connects C. trachomatis to STING and mediates the 'STING-dependent lysis process.' This activation regulates the translocation of STING to the Golgi apparatus. Subsequent to the activation of STING, which leads to lytic expulsion, ultimately aiding the release of the pathogen from the host cell [73].

In sepsis-induced acute lung injury (ALI), the cGAS-STING pathway significantly enhances inflammation by activating the PARP-1/NLRP3 signaling pathway, leading to pathological damage in lung tissue, pulmonary edema, and exacerbated inflammation [74]. Furthermore, research indicates that STING deficiency aggravates Gram-negative bacterial infections, suggesting a complex bidirectional regulatory role for STING in immune responses [72]. Thus, the expression levels of STING may play a key "balancing" role in different pathological states, potentially promoting disease progression or, in some cases, inhibiting pathological processes.

Plasmodium infections activate the host immune response via the cGAS-STING pathway, inducing the production of IFN-I [75]. This process enhances the expansion of Treg cells, exerting immune-suppressive effects and limiting excessive inflammation and pathological damage. The activation of the cGAS-STING pathway not only enhances IFN-I expression but also promotes the proliferation of Treg cells, playing an important role in the immune regulation of Plasmodium infections [76].

Immune Evasion Mechanisms

Studies indicate that the NS4B protein of HCV can directly bind to STING and inhibit RIG-I-mediated IFN-I expression, helping the virus evade host immune responses [77]. Additionally, flaviviruses could activate the RIG-I-STING pathway, causing neuronal death and triggering inflammatory responses. This mechanism underscores the key role of this pathway in viral infections of the nervous system [78]. Moreover, poxviruses suppress IFN-I responses induced by dsDNA via the cGAS-STING pathway, inhibiting host immune responses by blocking STING activation, thereby regulating the cGAS-STING pathway to promote viral survival [79-80].

STING's function is significantly influenced by genetic polymorphisms, such as R232, H232, and HAQ variants. For example, the H232 variant exhibits impaired function, resulting in increased susceptibility to DNA viruses like HSV-1 and MVA, while the HAQ and R232 variants maintain normal function [81]. Additionally, STING deficiency weakens monocyte differentiation and antigen-presenting capacity, affecting immune responses. In HIV-infected individuals, the HAQ/HAQ STING variant is associated with lower chronic immune activation and slower disease progression [82], suggesting that STING genetic variants may modulate immune responses and influence host susceptibility to viral infections.

Moreover, chronic viral infections, such as HIV, are often accompanied by prolonged immune activation. Although antiretroviral therapy (ART) effectively suppresses the virus, it may lead to long-term STING activation, thereby triggering autoimmune responses. In this context, the expression of STING and cGAS genes is significantly downregulated, while autoantibody production increases, indicating the important role of STING in maintaining immune tolerance [83]. Furthermore, activating mutations in STING1 can lead to SAVI, which presents with early-onset systemic inflammation, skin vasculopathy, and interstitial lung disease (ILD). Although the clinical features of SAVI are relatively well defined, its specific molecular mechanisms remain unclear and require further investigation to reveal STING's potential role in autoimmunity and related diseases [42].

Future research could focus on two main areas: first, the screening of broad-spectrum antiviral compounds, particularly those targeting the pathogen-STING interaction interface, such as poxin inhibitors from poxviruses, which could provide new strategies for antiviral therapy [84]. Secondly, a deeper understanding of the impact of STING allele polymorphisms (e.g., R232, H232, and HAQ) on susceptibility to infections across global populations will help elucidate the relationship between individual immune response differences and disease susceptibility, offering more efficient approaches for personalized immunotherapy [85].

Metabolism and Fibrosis

Metabolic Abnormalities

Obesity is widely regarded as a risk factor for various cancers and is closely associated with chronic inflammation. In adipocytes, mitochondrial dysfunction leads to mtDNA leakage, activating the cGAS-STING pathway, which reduces fat accumulation by promoting autophagy in adipocytes [86-87]. Studies have shown that palmitoylation of STING plays a key role in the development of obesity. Fatty acid oxidation significantly inhibits the antiviral activity of STING by reducing its palmitoylation, a critical modification for activating its downstream signaling pathways. This inhibitory effect on fatty acid oxidation may impair STING's normal function by lowering palmitoylation [88]. Palmitoylation typically occurs in the Golgi apparatus, and its levels are significantly reduced in obesity models, resulting in abnormal binding between STING and TBK1, which suppresses its normal autophagic function, thereby exacerbating fat accumulation and promoting the development of obesity [89]. Additionally, the cGAS-STING pathway plays an anti-inflammatory role in adipocytes by promoting mitophagy, thus inhibiting excessive activation of the inflammatory response. Inhibition

of STING expression leads to a reduction in autophagosome numbers, disrupting the balance of fat metabolism [86]. This process may exacerbate inflammation in adipocytes and further increase the risk of cancer associated with obesity. Therefore, STING may play a crucial role in the link between obesity and cancer, regulating metabolic and inflammatory responses in adipocytes. This suggests that dysregulated palmitoylation of STING could be an important mechanism in obesity-related metabolic disorders, and it indicates a close connection between lipid metabolism and immune responses. This provides a potential therapeutic target for future treatments targeting the cGAS-STING pathway.

Glucose could regulate the cGAS-STING pathway. Studies have shown that high glucose concentrations can induce STING activation, promoting macrophage polarization to the M1 type, thereby inhibiting wound healing in diabetic patients [90]. In type 2 diabetes models, reducing STING expression can improve peripheral insulin resistance and correct glucose intolerance abnormalities [91]. During tumor development, NSUN2 acts as a glucose sensor and inhibits the cGAS-STING signaling pathway, thereby promoting tumor progression and increasing immune therapy resistance. In contrast, inhibiting NSUN2 activity activates the cGAS-STING pathway, not only curbing tumor growth but also enhancing the effectiveness of immune therapy [92].

Organ Fibrosis

In liver fibrosis research, the cGAS-STING pathway, as a DNA sensor located in the cytoplasm, has attracted significant attention. Studies have found that STING is expressed in non-parenchymal liver cells, particularly in macrophages [93]. In patients with non-alcoholic fatty liver disease (NAFLD), the expression of STING in monocyte-derived macrophages is closely related to the worsening of liver inflammation and fibrosis [94]. Furthermore, STING activates the NLRP3 inflammasome, inducing pyroptosis in hepatocytes and thereby exacerbating the progression of liver fibrosis [95]. These findings suggest that dysregulated STING expression may be a key driver of liver fibrosis progression.

Pulmonary fibrosis is a progressive and ultimately life-threatening lung disease. Studies have shown that abnormal activation of cGAS-STING participates in and promotes the development of fibrotic lung diseases. Polystyrene microplastics (PS-MPs) can promote ferroptosis in alveolar epithelial cells through cGAS-STING pathway, thereby triggering pulmonary fibrosis [96-97]. However, other studies have pointed out that in idiopathic pulmonary fibrosis (IPF), STING has a protective effect on lung fibrosis, with its reduced expression exacerbating the fibrotic process [98].

Renal fibrosis is a common lesion leading to end-stage renal failure. Studies have shown that activation of the STING/ACSL4 pathway promotes ferroptosis and inflammation, further advancing chronic kidney disease (CKD) [99]. Additionally, mitochondrial damage and activation of the cGAS-STING pathway exacerbate kidney inflammation and fibrosis progression [100]. Butyrate, through modulation of the STING/NF-κB/p3 pathway, can affect NLRP65-mediated pyroptosis, thereby alleviating kidney fibrosis symptoms in CKD patients [101].

Future research could focus on developing tissue-specific regulation strategies for the STING pathway, such as using liver-targeted lipid nanoparticles (LNPs) to deliver siRNA-STING, to explore their effects on liver metabolism and fibrosis [102]. Additionally, establishing multi-omics integration platforms to analyze the dynamic network of the metabolism-fibrosis-immune axis could provide a more comprehensive understanding of the mechanisms, help identify new therapeutic targets, and promote the application of precision medicine in metabolic diseases [65, 103]. These studies are expected to reveal the role of the cGAS-STING pathway in various diseases and provide new ideas for clinical interventions.

cGAS-STING Pathway and the Nervous System

cGAS-STING Pathway Regulates Neuroinflammation

Studies have shown that under hypoxic conditions, glioblastoma (GBM) cells release extracellular vesicles (EVs) carrying miR-25/93 to macrophages, thereby inhibiting the cGAS-STING pathway, reducing type I interferon secretion (e.g., IFN-β), lowering the expression of M1 polarization-related genes (e.g., Cxcl9, Cxcl10, Il12b), and weakening macrophage anti-tumor immunity and T cell activation, which further fosters an immunosuppressive tumor microenvironment (TME) [104]. Traumatic brain injury (TBI) triggers the activation of the cGAS-STING pathway, which exacerbates neuroinflammatory responses through type I interferons (IFN-α/β) and pro-inflammatory factors (e.g., TNF-α, IL-1β, and IL-6), while also inducing autophagy dysfunction (e.g., abnormal LAMP2). Studies have shown that STING gene knockout ($STING^{-/-}$) could reduce the release of inflammatory factors, decrease lesion volume, and restore autophagic function, suggesting that STING exacerbates neuroinflammatory damage by enhancing type I interferon signaling [105].

In a mouse spinal cord injury model, STING interacts with TBK1 to enhance TBK1 phosphorylation, activating downstream NF-κB and MAPK signaling pathways that amplify the inflammatory response of microglial cells, whereas suppressing STING expression reduces the activation of these pathways and alleviates the inflammatory response, thereby facilitating spinal cord injury repair [106-107]. This suggests that STING may play a role in spinal cord injury by regulating inflammatory responses.

In HSV-1 encephalitis, neurons promote the secretion of IFN-λ via the activation of the cGAS-STING pathway, which aids in antiviral immunity and suppresses viral spread [61, 108]. However, a study indicates that excessive activation of STING may trigger an overactive inflammatory response, leading to blood-brain barrier disruption, thus exacerbating neuronal damage and disease progression [109]. Therefore, the regulation of the cGAS-STING pathway needs to be finely balanced to ensure defense against viral infections while preventing damage to the blood-brain barrier.

Non-Classical Regulation of Neuronal Function by STING

In addition to its regulatory functions through the classical cGAS-STING pathway, STING could regulate neuronal functions through non-classical pathways. Research suggests that intestinal neuroglial cells may employ alternative signaling mechanisms or express STING solely under certain disease conditions, with studies also revealing potential pathways for neuroglial cell-microbe communication within the intestinal

nervous system [110-111]. In a multiple sclerosis (MS) model, STING is activated in neurons and triggers the non-classical STIM1-STING signaling pathway, leading to the autophagic degradation of glutathione peroxidase 4 (GPX4) and causing ferroptosis [112]. This initiates inflammatory stress responses and cell death in neurons. STING is indirectly regulated by the biological clock gene BMAL1 through the LINE1-cGAS-STING pathway. When BMAL1 is deficient, heterochromatin stability is reduced, LINE1 is aberrantly activated, and the cGAS-STING pathway is triggered, leading to type I interferon responses and the senescence-associated secretory phenotype (SASP) [113-114]. This suggests that BMAL1, through its non-classical chromatin regulatory function, suppresses the LINE1-STING axis, maintains cellular homeostasis and delays aging.

STING Interaction with GBM

Research indicates that in GBM models, the activation of STING induces a strong immune response, mediates the NK cell-mediated tumor regression, and contributes to TME remodeling [115]. Additionally, preclinical studies have found that activation of the cGAS-STING pathway in the neuro-GBM immune microenvironment plays a positive role in therapy and achieves anti-tumor effects [116]. However, other studies have shown that in high-risk, recurrent-grade gliomas, STING expression is significantly upregulated, which may reflect the tumor cells' resistance to its effects [117].

Future research may explore the development of STING inhibitors that can cross the blood-brain barrier, such as utilizing nano-carriers for delivering C-176 analogs, to precisely regulate cGAS-STING pathway activity and mitigate or slow the progression of neurodegenerative diseases [66]. Additionally, exploring the role of STING in regulating various neurological diseases could open avenues for its application in personalized treatment. Another key direction is the study of combination therapies, such as combining STING modulation with immune agents, to explore synergistic effects in tumor suppression [118]. Furthermore, STING, as a potential biomarker for neurodegenerative diseases, warrants further exploration for early diagnosis and monitoring of these diseases [119].

Cancer and Immunotherapy

cGAS-STING Pathway and Tumor Immunity

As the global incidence of cancer increases, tumors have become one of the leading causes of death, so timely diagnosis and intervention are crucial for improving cure rates and enhancing patients' quality of life [120-121]. The cGAS-STING pathway serves as a crucial immune surveillance mechanism, inducing the production of IFN-I and various chemokines. These factors play a vital role in the recruitment and activation of CD8+ T cells [122]. In the TME, STING activation enhances immune responses, particularly the infiltration of T cells, contributing to its antitumor effects [123-124]. Activation of the cGAS-STING pathway effectively suppresses the expression of immune checkpoint molecules such as PD-L1, thereby alleviating the immune suppression of tumor cells on T cells and promoting tumor immune clearance [125].

In certain subtypes of gastric cancer, such as dMMR/MSI-H gastric cancer, high STING expression has been confirmed to be closely associated with T cell infiltration. Studies show that patients with dMMR/MSI-H gastric cancer exhibit stronger immune responses and higher levels of T cell infiltration upon activation of the STING pathway, suggesting that these patients may benefit more from immune checkpoint inhibitor therapies [126-127]. Based on this, STING emerges as a potential therapeutic target, capable of significantly enhancing the efficacy of immunotherapy and improving patient survival by enhancing immune responses and remodeling the TME [128].

cGAS-STING Pathway and Synergy with Immune Cells

The synergy between STING and innate immune cells plays a crucial role in tumor immunity. Dendritic cells (DCs), upon activation of STING, promote cross-presentation of tumor antigens through the cGAS-STING pathway, thereby enhancing antitumor immune responses [129]. This process not only enhances the immunogenicity of DCs but also strengthens the activation and functionality of T cells, facilitating the effective recognition and elimination of tumor cells. Research indicates that STING activation plays a key role in DC maturation, cytokine release, and the inhibition of immune escape, providing important support for the development of cancer immunotherapy [130-131].

However, some studies have shown that in pancreatic cancer, STING agonists could inhibit NK cell antitumor activity by activating Breg cells to release IL-35, revealing the limitations of STING agonist monotherapy [132].

STING Suppression and Resistance to Targeted Therapy

STING suppression is a significant mechanism of resistance to targeted cancer therapies. HPV16 E7 inhibits STING by promoting its degradation, thereby blocking the IFN-I signaling pathway and suppressing the antitumor immune response in cervical cancer cells. This enhances tumor resistance to radiotherapy, allowing tumor cells to evade host immune surveillance and increasing resistance to radiation therapy [133-134]. Furthermore, in triple-negative breast cancer (TNBC), ARAh silences STING through epigenetic mechanisms, thereby inhibiting its immune response activation and diminishing the effectiveness of PARP inhibitors [135-136]. These findings underscore the pivotal role of STING suppression in tumor immune evasion and therapy resistance, emphasizing the need for targeted therapies to modulate the STING pathway.

cGAS-STING Pathway and Oncogenic Signaling

The cGAS-STING pathway plays a double-edged sword role in cancer development. mtDNA leakage caused by mitochondrial damage activates the cGAS-STING pathway, triggering intracellular inflammation and promoting chromosomal instability (CIN) [137-138]. This process supports tumor cell survival and accelerates tumor progression via an IL-6-dependent pathway. In BRCA1-deficient ovarian cancer, the STING-mediated inflammatory microenvironment further promotes immune evasion and weakens the antitumor immune response. Studies have shown that PARP inhibitors significantly reverse this immune escape phenomenon, aiding the immune system in recognizing and clearing tumor cells, thereby improving therapeutic outcomes [139]. Activation of the STING-TBK1 axis promotes the expression of ATP-citrate lyase (ACLY), enhancing fatty acid synthesis and driving macrophages toward M2 polarization by remodeling lipid metabolism. This chromatin-regulated process further affects immune cell metabolism and function

[140-141].

Dual Roles of STING in Cancers

STAT3 deficiency disrupts the cGAS-STING-IFN pathway, thereby impairing the inhibitory effect of NK and NKT cells on SCLC metastasis and dissemination. This process can be restored through the overexpression of IRF7 or exogenous supplementation of IFN, thereby improving the prognosis of SCLC patients [142]. However, a study suggests that STING, through the TBK1-NF-κB pathway, contributes to the formation of an inflammatory microenvironment that promotes bone metastasis in cervical cancer [143]. Moreover, in recurrent gliomas, overexpression of STING correlates with IDH1 mutations, suggesting that STING may serve as an independent prognostic marker for glioma progression [144-145]. These studies reveal the complex role of STING signaling in different cancer types.

Future research should focus on understanding the spatiotemporal activation mechanisms of STING in both tumor and immune cells. Single-cell spatial transcriptomics could particularly reveal its role within the TME [146]. Additionally, exploring STING agonists in combination with immune checkpoint inhibitors (ICIs) or IL-35 in combination therapies will help improve the effectiveness of antitumor immune responses, providing more effective treatment options in clinical therapy [132, 147].

Concluding Remarks

The cGAS-STING pathway is a crucial intracellular immune signaling pathway, primarily involved in the host's immune response to exogenous pathogens (such as viruses and bacteria) and responses to endogenous damage. Recent studies have shown that, in response to viral and pathogen invasion, STING plays a pivotal regulatory role in immune responses through various post-translational modifications (PTMs). The development of specific probes targeting different PTM states of STING could precisely regulate its activity, thereby enhancing the activation of the downstream TBK1-IRF3 pathway. For example, TMED2, in combination with the MITA signaling mediator, can further enhance IRF3 activation, improving the efficiency of antiviral immune responses [148].

As a key immune response regulatory mechanism, the cGAS-STING pathway is involved in the regulation of several subcellular organelles, such as the ER and lysosomes, and interacts with other intracellular signaling pathways to form a complex regulatory network. These organelles play an increasingly important role in cellular immune responses, inflammatory reactions, and pathogen defense, and their dysfunction is often closely associated with the development of various diseases. Therefore, exploring how to regulate the functions of these subcellular organelles via the cGAS-STING pathway offers new perspectives and possibilities for developing nanotechnology-based disease therapies. Nanotechnology can precisely target these intracellular structures, and by activating or inhibiting the cGAS-STING pathway, it can influence immune responses, opening up new frontiers in disease treatment.

On the other hand, non-coding RNAs (ncRNAs), which have been a focal point of research in recent years due to their significant roles in gene expression regulation, genome stability maintenance, and cellular physiological functions, have attracted widespread attention. NcRNAs play crucial roles in biological development and health maintenance and are also closely linked to the onset of various diseases. Increasing evidence indicates that ncRNAs regulate the activation and inhibition of the cGAS-STING pathway through direct or indirect interactions at various levels. For instance, certain microRNAs and long non-coding RNAs can modulate the expression or stability of cGAS or STING through interactions, thereby affecting the strength and duration of downstream immune responses. These findings provide a theoretical basis for regulating the cGAS-STING pathway via ncRNAs and offer new insights for the development of novel disease treatment strategies, such as gene therapy and immunotherapy. With the integration of nanotechnology, future approaches may precisely regulate the cGAS-STING pathway through targeting ncRNAs, achieving more refined therapeutic outcomes for diseases.

In recent years, the role of the cGAS-STING pathway in autoimmune diseases has increasingly attracted the attention of researchers. However, excessive or abnormal activation of the cGAS-STING pathway has been found to be closely associated with the onset of various autoimmune diseases, such as SLE, Sjögren's syndrome, and rheumatoid arthritis. Studies have shown that when the activation of the cGAS-STING pathway becomes uncontrolled, it may lead to the loss of immune tolerance, triggering autoimmune responses that result in tissue damage and inflammation. Notably, in certain autoimmune disease patients, abnormal activation of the cGAS-STING pathway can promote the excessive secretion of inflammatory cytokines, thereby exacerbating the clinical symptoms of the disease.

To address this issue, researchers have been developing inhibitors of the cGAS-STING pathway as potential therapeutic strategies. Inhibitors such as VENT-03 and PAH have been found to effectively suppress the abnormal activation of the cGAS-STING pathway. Notably, the oral drug VENT-03 has completed its Phase I clinical trial and is scheduled to initiate Phase II clinical trials soon; it is expected to be used in the treatment of patients with SLE in the future. By inhibiting the cGAS-STING pathway, these inhibitors can significantly alleviate inflammation caused by excessive immune responses and reduce tissue damage, thereby mitigating the symptoms of autoimmune diseases. Particularly in the treatment of diseases such as SLE, inhibition of the cGAS-STING pathway is considered a promising strategy, as the abnormal activation of this pathway plays a key role in the pathogenesis of these diseases.

In cancer treatment, activating the cGAS-STING pathway to counter tumor progression has become a theoretically feasible approach. However, studies have found that the efficacy of using cGAS-STING agonists to combat tumor progression is suboptimal [149], and some research even suggests that it may further promote tumor progression, highlighting the limitations of cGAS-STING agonist monotherapy [132, 150]. Activation of the cGAS-STING pathway is crucial for initiating the initial anti-tumor immune response. Recent studies have shown that persistent activation of the cGAS-STING pathway in tumors could induce an immune-suppressive TME, promoting the survival and metastasis of cancer cells. Additionally, activation of the cGAS-STING pathway in myeloid-derived suppressor cells (MDSCs) has been shown to participate in the recruitment of MDSCs and enhance their immunosuppressive activity, thereby promoting TME remodeling [151-153]. Furthermore, while the cGAS-STING pathway may play an anti-tumor

role in the early stages of cancer, tumor cells exhibit strong immune evasion capabilities. In the later stages of tumor development, the pathway could evolve into a chronic inflammatory state. Persistent inflammation could induce immune tolerance through mechanisms such as immune cell exhaustion, the expansion of regulatory cells, and clonal anergy, thus driving tumor progression [154-155]. This suggests that using cGAS-STING agonists alone to combat tumor progression may not be the optimal approach. Furthermore, the mechanisms by which sustained activation of the cGAS-STING pathway promotes tumor progression remain a major research question. Additionally, current research on the cGAS-STING pathway in non-tumor cells of the TME is limited, and the role of this pathway in non-tumor cells remains unclear. These gaps hinder our deeper understanding of tumor mechanisms.

The rise of immunotherapy has opened a new chapter in cancer treatment, and the use of cGAS-STING agonists in combination with ICIs may represent a novel therapeutic approach in cancer therapy. A thorough investigation of the mechanisms underlying the cGAS-STING pathway in cancer therapy is crucial for advancing personalized treatment approaches, which holds significant clinical implications for deepening our exploration of cancer treatments [156-157].

Moreover, in certain diseases, aberrant activation or inhibition of the cGAS-STING pathway is not only closely associated with the onset of the disease but also plays a crucial role in disease progression. For instance, in some autoimmune and chronic inflammatory diseases, excessive activation of the cGAS-STING pathway may lead to an overactive immune response, resulting in tissue damage and pathological changes. In certain viral infections, defects or inhibition of the cGAS-STING pathway could impair the host's immune defenses, enabling persistent viral presence. Therefore, the precise modulation of the cGAS-STING pathway using specific agonists or inhibitors has become a critical strategy in treating these diseases. By regulating the activity of the cGAS-STING, it is possible to maintain immune defense while avoiding the side effects of excessive immune responses, thereby effectively treating autoimmune or chronic inflammatory diseases.

In conclusion, as a central pathway in the innate immune system, the cGAS-STING pathway plays an irreplaceable role not only in combating exogenous pathogens but also in cancer immunity, infections, inflammation, and autoimmune diseases. In-depth studies on the role of the cGAS-STING pathway in various diseases will lead to more precise targeted therapies for clinical treatment, promote the development of personalized medicine, and provide patients with additional treatment options. Therefore, investigating how to regulate the cGAS-STING pathway across diverse disease contexts will be a pivotal direction in future therapeutic research.

Abbreviations

Acute Lung Injury: ALI; Alzheimer's Disease: AD; ATP-Citrate Lyase: ACLY; Cyclic GMP-AMP: cGAMP; Cyclic GMP-AMP Synthase: cGAS; Dendritic Cells: DCs; Double-Stranded DNA: dsDNA; Endoplasmic Reticulum: ER; Extracellular Vesicles: EVs; Glioblastoma: GBM; Hepatitis C Virus: HCV; Herpes Simplex Virus Type 1: HSV-1; Human Papillomavirus Type 11: HPV11; Hypoxia-Inducible Factor-1α: HIF-1α; Immune Checkpoint Inhibitors: ICBs; Interferon Regulatory Factor 3: IRF3; Interferon-Stimulated Genes: ISGs; Lupus Nephritis: LN; Long Non-Coding RNA: lncRNA; Lipid Nanoparticle: LNP; Mitochondrial DNA: mtDNA; Myeloid-Derived Suppressor Cells: MDSCs; Non-Alcoholic Fatty Liver Disease: NAFLD; Nuclear Factor Kappa-B: NF-κB; NOD-Like Receptor Pyrin Domain-Containing Protein 3: NLRP3; Non-Small Cell Lung Cancer: NSCLC; Ovarian Cancer: OVCA; Programmed Death-Ligand 1: PD-L1; Regulatory T Cell: Treg; Stimulator of Interferon Genes: STING; Stimulator of Interferon Genes-Associated Vasculopathy with Onset in Infancy: SAVI; Systemic Lupus Erythematosus: SLE; TANK-Binding Kinase 1: TBK1; Traumatic Brain Injury: TBI; Tumor Microenvironment: TME; Triple-Negative Breast Cancer: TNBC; Type I Interferon: IFN-I.

Author Contributions

Yunyong Wang: Conceptualization, Writing -- original draft; Xiaohang Lu: Writing -- original draft; Jinna Tan: Writing -- review & editing; Jiaqian He: Writing -- review & editing; Hui Yin: Writing -- review & editing; Peipei Chen: Visualization; Hemeng Wu: Visualization; Yuzhen Luo: Visualization; Mingfen Li: Supervision.

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Ethics Approval and Consent to Participate

Not Applicable.

Competing Interests

The authors declare that they have no existing or potential commercial or financial relationships that could create a conflict of interest at the time of conducting this study.

Data Availability

All data generated or analyzed during this study are included in this published article and its supplementary information files.

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Review Article

Life Conflux

New Insights into Influenza Virus-Host Interactions, Immune Responses, and Vaccine Development at Single-Cell Resolution

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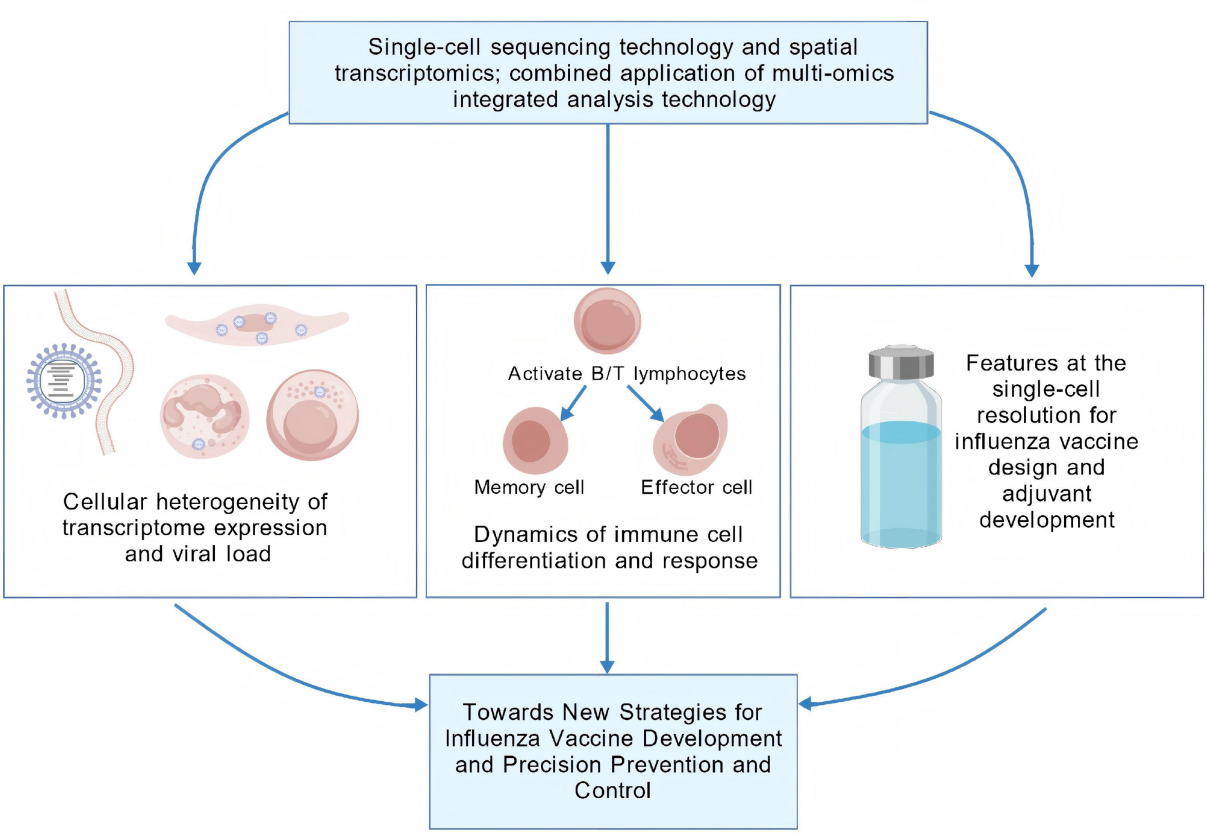
Graphical Abstract



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Review Article

Life Conflux

New Insights into Influenza Virus-Host Interactions, Immune Responses, and Vaccine Development at Single-Cell Resolution

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Abstract

The integration of single-cell sequencing with spatial transcriptomics and multi-omics analyses has enabled a paradigm shift in biomedical research, thereby expanding its applicability and scientific impact. In the context of influenza virus studies, this technology has been instrumental in dissecting cellular heterogeneity, as demonstrated by its capacity to analyze differential transcriptomic profiles and reconstruct differentiation trajectories at the single-cell level following viral infection. These advances have provided mechanistic insights and a holistic understanding of influenza pathogenesis, surpassing the limitations of bulk-level analyses. This review provides a comprehensive dissection of cutting-edge applications of single-cell sequencing in elucidating influenza virus infection mechanisms, immune cell heterogeneity, and vaccine development. By highlighting the single-cell resolution of virus–host interactions and vaccine efficacy studies, this work offers novel perspectives for designing precision-targeted antiviral interventions.

Keywords: Single-cell sequencing technology; Influenza virus; Cellular heterogeneity; Immune cell subset differentiation; Influenza vaccine

Introduction

The genetic material of the influenza virus exhibits a high propensity for mutation, and genetic recombination can readily give rise to novel viral variants, leading to the emergence of new influenza strains [1]. This significantly hinders in-depth research on specific strains, thereby intensifying the challenges of vaccine development and drug screening [2]. Furthermore, influenza virus infection is prone to induce secondary infections and polymicrobial co-infections, the mechanisms of which are highly complex and multifaceted [3]. The severity and clinical outcomes of influenza virus infections are determined by a combination of viral and host factors. The multifactorial nature of the host immune response significantly influences the progression and severity of influenza virus infections, thereby substantially increasing the complexity of related research [4-5]. Influenza viruses employ a diverse array of strategies to evade host innate and adaptive immune responses. The multifaceted nature of their immune evasion mechanisms poses significant challenges in achieving a comprehensive understanding of the intricate virus–host immune system interactions [6-7]. Viral infections involve complex interactions with the structural and functional components of host cells, encompassing a multitude of molecular and cellular mechanisms [8]. In conclusion, addressing the aforementioned research challenges requires the implementation of high-precision experimental techniques and interdisciplinary collaborations.

Previous research methodologies have demonstrated limitations in elucidating the infection process and pathogenic mechanisms of influenza virus, particularly in accurately analyzing cellular heterogeneity and the complexity of virus–host interactions. For instance, conventional bulk sequencing techniques are incapable of resolving cellular heterogeneity in influenza virus infections; they also fail to detect rare cell subpopulations, gene expression variability, or mutational diversity, resulting in the loss of critical biological information [9]. Moreover, it is incapable of tracing the continuous trajectory of cellular state transitions, such as differentiation, development, or disease progression, rendering it unsuitable for rare cell populations (e.g., circulating tumor cells, early embryonic cells) or minimal clinical samples (e.g., needle biopsies) [10]. Moreover, mixed-cell sequencing fails to distinguish cell type- or subpopulation-specific gene expression patterns or mutational profiles, and is incapable of unbiased identification of novel cellular subpopulations [11]. In response to these challenges, the emergence of single-cell sequencing (SCS) technology has provided an effective solution by delivering high-resolution cellular-level data, thereby facilitating a comprehensive understanding of the impact of influenza virus infection on host cells [12]. The integration of multi-omics technologies has significantly advanced our understanding of gene regulatory networks during influenza virus infection, offering critical insights into how the virus evades immune sur-

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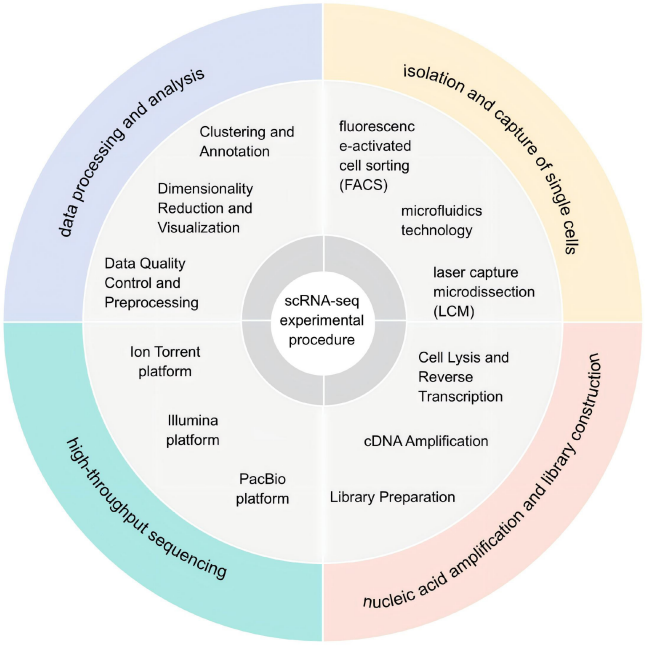
veillance and enhances viral replication by modulating the host cell transcriptomic profile [13-14]. Furthermore, compared to conventional bulk sequencing methodologies, SCS technology enables the identification of intercellular heterogeneity, which is crucial for elucidating the mechanisms of viral transmission and replication across diverse cell types [14]. This paper aims to systematically summarize the advancements in the application of SCS technology in influenza virus research, elucidate the significance of SCS technology in investigating infection mechanisms, reveal the single-cell resolution features of virus–host interactions and vaccine development, and offer novel perspectives on precision-based antiviral strategies.

Single-cell sequencing technology

The integration of SCS technology with multi-omics approaches facilitates a comprehensive analysis of the genome, transcriptome, and epigenome at the single-cell level, allowing for a more holistic understanding of cellular function and regulation [15]. Traditional sequencing techniques are performed at the multicellular level, producing averaged signal outputs across a cell population, and consequently masking variations in cellular heterogeneity (i.e., differences among individual cells). In influenza virus research, single-cell RNA sequencing (scRNA-seq) is widely employed as a key component of SCS technology, primarily used to investigate transcriptomic expression profiles at the individual cell level [16-17]. The experimental protocol consists of the following sequential steps: single-cell isolation, cell lysis and nucleic acid extraction, reverse transcription for cDNA synthesis, whole transcriptome amplification, library preparation, high-throughput sequencing, and bioinformatics analysis, including quality control, alignment, gene expression quantification, and cell clustering, to characterize cellular heterogeneity [18] (Figure 1).

Figure 1. The experimental workflow of scRNA-seq technology involves single-cell isolation and capture, followed by nucleic acid amplification and library construction, selection of an appropriate high-throughput sequencing platform, and concludes with data processing and bioinformatics analysis.



Sample Preparation and Single-Cell Isolation and Capture

Depending on the research objectives, cells are isolated from tissues, cell lines, or body fluid. After dissociation into a single-cell suspension, automated microscopy or flow cytometry is used for cell quantification, along with a comprehensive evaluation of cell viability and quality parameters [19]. Subsequently, single-cell isolation is performed using conventional methods such as serial dilution, micromanipulation, and laser capture microdissection [20-22]. Currently, the most widely used techniques are Fluorescence-Activated Cell Sorting (FACS) and microfluidic-based platforms. FACS plays a crucial role in single-cell B-cell receptor/T-cell receptor sequencing (scBCR-seq/scTCR-seq) protocols, particularly when prior selection of specific cell types is necessary. This technology allows for the enrichment of defined B/T cell subpopulations and improves sample quality, serving as an essential tool in advanced immunological research [23-24]. Microfluidic technology enables precise manipulation of small liquid volumes via microchannels and microfluidic control components, leveraging microstructures such as micropores, microvalves, or droplet generators to compartmentalize cells into isolated reaction units (e.g., droplets or microchambers). This approach achieves specific functional outcomes through controlled droplet generation and the deliberate design of micropore architectures [25-26]. Droplet-based microfluidics technology, including methodologies such as Droplet-based single-cell RNA sequencing (Drop-seq) and 10x Genomics, integrates single-cell isolation and labeling into a streamlined workflow, enabling the parallel processing of thousands of cells. This advanced technique allows for precise control and facilitates the accurate separation and manipulation of individual cells [27-29]. Micro-well array technology (such as Sequencing Well) utilizes micro-well structures to enable the precise physical isolation of single cells, exhibiting comparable performance in accurate cell separation. In contrast, droplet-based microfluidics is better suited for applications that require ultra-high-throughput processing [30].

Nucleic Acid Amplification and Library Preparation

Following the capture of RNA from individual cells, reverse transcription and amplification are performed to generate sufficient cDNA for downstream sequencing analysis [28]. Several reverse transcription initiation strategies are commonly employed, including the Poly(A) Tailing method, the Template-Switching (TS) approach, and random primer-based methods designed for non-poly(A) RNA capture (e.g., SUPeR-seq, snHH-seq, and snRandom-seq) [31-34]. Subsequently, the cDNA is subjected to pre-amplification, during which Multiple Annealing and Tailing-based Quantitative Single-cell RNA-seq (MATQ-seq) utilizes a multiplex annealing and tailing strategy to substantially reduce amplification noise, thereby improving sensitivity and quantitative accuracy [35]. The integration of barcodes and Unique Molecular Identifiers (UMIs) before or during amplification (e.g., Single-Cell RNA Barcoding and sequencing (SCRB-seq), MULTIplexing using lipid-Tagged Indices for single-cell RNA sequencing (MULTI-seq)) allows for the correction of quantification errors caused by PCR dupli-

cates [18, 36]. Subsequently, the amplified cDNA is subjected to a series of processing steps, including fragmentation, end repair and tailing, adapter ligation, and library PCR enrichment, ultimately yielding libraries customized for specific research objectives.

Post-High-Throughput Sequencing Data Processing and Analysis

Single-cell libraries are sequenced using high-throughput sequencing platforms (short-read platforms: Illumina, Ion Torrent, MGI; long-read platforms: PacBio, Oxford Nanopore) to generate raw sequencing data (reads) [37-42]. Raw data files generated by sequencing instruments are converted into a standardized FASTQ format.

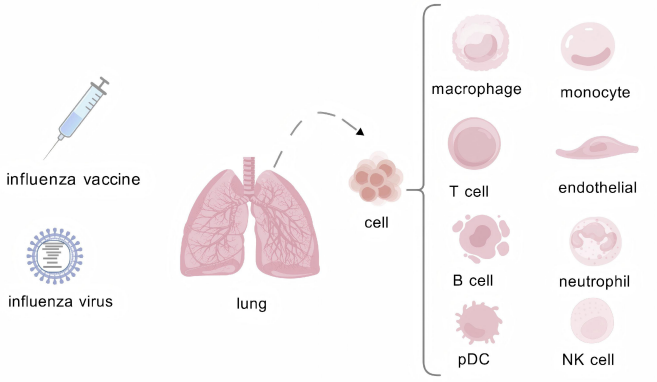
In the context of data quality control, tools such as FastQC are used to evaluate the quality of raw FASTQ files, focusing on metrics such as base quality distribution, sequence length distribution, Guanine-Cytosine content, and adapter contamination. Subsequently, Trimmomatic or Cutadapt is employed to remove low-quality bases ("quality trimming") and adapter sequences ("adapter trimming"). Quality control reports generated by these tools, along with those from downstream processes, are consolidated using MultiQC [19, 43-44]. Clean reads are aligned to the reference genome using STAR, Kallisto, or Cell Ranger to generate a cell-by-gene expression matrix containing gene's UMI counts. Quality control and filtering of the expression matrix (Expression Matrix QC) are performed to remove abnormal cells based on predefined thresholds. Background contamination was subsequently corrected using SoupX to adjust for ambient RNA, and low-abundance genes expressed in fewer than a certain number of cells [45].

In the context of data preprocessing, normalization procedures are applied to mitigate technical biases such as differences in sequencing depth and cell capture efficiency, thereby ensuring comparability of gene expression levels across distinct cell populations. Specifically, the scran package (used for estimating cell size factors) and the Seurat::NormalizeData/SCTransform methods were utilized to remove variations in sequencing depth [46]. For the selection of highly variable genes, informative genes were identified using Seurat::FindVariableFeatures or scran::modelGeneVar. Subsequently, Seurat::ScaleData (centering and scaling) was applied to the highly variable genes for normalization. After gene normalization, Principal Component Analysis (PCA)-based dimensionality reduction was performed. Batch effects were then integrated into the PCA space (as opposed to the gene expression matrix) using Harmony or MNNCorrect. The preprocessed data are saved as Seurat or AnnData objects and stored in universal file formats, such as h5ad, for downstream analysis, which can include cellular clustering and subpopulation identification, differential expression analysis, data visualization, and interpretation [47-48].

Application of Single-Cell Sequencing Technology in Influenza Virus Infection Research

SCS technology has been widely applied to investigate cellular heterogeneity profiles following infection with the influenza virus. Cells were categorized based on immune function into immune and non-immune cells, and further classified according to infection status into infected cells, bystander cells, and uninfected cells. Cluster analysis has identified key cellular subpopulations that, based on differentially expressed genes, provide novel insights into the interactions between influenza viruses and various cell types. A systematic review of recent applications of SCS technology in influenza virus research is shown (Figure 2 and Table 1).

Figure 2. illustrates that research on the SCS technology in influenza viruses primarily centers on analyzing differentially expressed genes (DEG) across various cell types and characterizing immune cell differentiation following infection. (Created with BioGDP.com)



Cellular Heterogeneity During Viral Infection

Heterogeneous Distribution of Influenza Viral mRNA

The replication of the influenza virus within host cells involves multiple stages, during which genomic replication triggers a series of changes in host cell gene expression [71]. Upon infecting cells or in mice, the influenza virus exploits host factors and proteins to support its replication and proliferation. By integrating UMI and cellular barcode information, the dynamic abundance of influenza viral mRNA within cells can be accurately quantified [72-73]. Following viral infection of A549 cells, most cells exhibited minimal or undetectable levels of viral mRNA. Over time, progressive accumulation of viral mRNA was observed in the infected cells. Analysis of viral mRNA dynamics indicated the activation of viral clearance mechanisms approximately one week post-infection [49-50]. Analysis of viral mRNA abundance in the lung tissues of infected mice revealed a higher proportion of infected cells, with epithelial cells exhibiting the highest infection rate and T cells the lowest. Additionally, the infection rate of non-immune cells was higher than that of immune cells [51]. Compared to A549 cells, the pulmonary microenvironment in murine lung tissue is more complex, especially with regard to tissue specificity and cellular interactions, highlighting the need for more in-depth investigations [74]. Additionally, studies have identified an intriguing phenomenon in which most infected cells show minimal viral mRNA presence, whereas a minority of infected cells exhibit viral mRNA comprising over half of the cellular transcriptome [49, 75]. In-depth analysis suggests two potential explanations: first, the absence or varying expression ratios of viral genomic mRNA fragments may contribute to the elevated viral mRNA abundance observed in a subset of cells [49, 58]. For instance, during influenza virus infection, viral replication shifts from the transcription phase to the replication phase. The NS2 protein, efficiently synthesized through early transcription, interacts with the RNA-dependent RNA polymerase (RdRp) to facilitate the transition of viral RNA from transcrip-

tion to replication. When NS2 protein expression decreases, viral replication levels decline. Cell-to-cell variations in NS2 expression can lead to differences in viral mRNA abundance [76-79]. Second, this phenomenon may also be attributed to variations in the number of cellular receptors, as the distribution of sialic acid receptors is heterogeneous across different cell types, and even within the same cell type, heterogeneity exists depending on differentiation status or cell cycle phase [80]. The study conducted by Ni Z et al. showed that after the viral hemagglutinin (HA) protein binds to metabotropic glutamate receptor 2 (mGluR2), mGluR2 interacts with the calcium-activated potassium channel (KCa1.1), thereby participating in the initiation and completion of clathrin-mediated endocytosis (CME) of influenza viruses. The expression level of mGluR2 di-

Table 1. Applications, advantages, and limitations of different single-cell sequencing technologies in influenza research

technology	Influenza research	advantage	boundedness
scRNA-seq	Differences in viral RNA load and distribution across cell types [49]; Key cellular sources of inflammatory factors [50]; Cellular heterogeneity between infected and bystander cells [51]; Comparative immune characteristics of cross-species infections [52-53]; Comparative analysis of IAV and COVID-19 infections, including co-infection with Streptococcus pneumoniae [54-57]; Macrophage polarization and its association with viral susceptibility [58]; Peripheral immune profiles across infected populations [59]; Development of porcine animal models [60]; Lymphatic system remodeling during infection [61]; Impact of environmental humidity on infection dynamics [62]; Memory CD8+ T cell responses following primary infection and reinfection [63]; Differentiation trajectories of plasmacytoid dendritic cells (pDCs) [64]; Vaccine-induced transcriptional responses, dynamic shifts in !immune cell subsets, and adjuvant research [65-66]	High-throughput, capable of revealing cellular heterogeneity and enabling simultaneous analysis of host and polyA-tailed viral transcriptomes	Viral transcripts exhibit low expression levels, the method is unsuitable for analyzing viral RNAs lacking polyA tails, and technical noise is substantial.
	Combined with spatial transcriptomics, this study aims to elucidate spatial differences in the lungs of young versus old mice following infection, as well as the influence and underlying mechanisms by which age affects B cell differentiation trajectories [67]	Preserves spatial location information and enables direct correlation between infected areas and the immune microenvironment	The resolution fails to achieve true single-cell level, as most spots contain multiple cells; the cost is prohibitively high, and data analysis is complex
scBCR-seq/ scTCR-seq	Transcriptional differences and high diversity of memory B cells in the lungs and lymphoid organs [68]; Functional regulation of tissue-resident memory B and T cells [115-116, 122]	High-throughput analysis of adaptive immune responses, discovery of neutralizing antibody clones, and elucidation of immune memory mechanisms	It is primarily limited to lymphocytes and cannot directly provide full transcriptome information, often requiring combination with single-cell RNA sequencing (scRNA-seq)
mudRapp-seq	Interconnections between viral RNA (vRNA and mRNA) and cellular heterogeneity [69]	Direct RNA detection without reverse transcription, high sensitivity, high specificity, and preservation of spatial information	There are relatively few application cases, probe design is complex, and the approach requires dedicated instruments and specific operational procedures
scATAC-seq	The epigenomic and transcriptional landscapes of human immunity to seasonal and pandemic influenza vaccines [70]	Uncover the upstream regulatory mechanisms—such as transcription factor activity—that drive transcriptional heterogeneity; integrate with single-cell RNA sequencing (scRNA-seq) to construct a comprehensive gene regulatory network	Direct detection of virus-related changes is not feasible, the data are sparse and analysis is complex, and integration with single-cell RNA sequencing (scRNA-seq) is required to obtain a comprehensive understanding

rectly influences the efficiency of influenza virus cellular entry. Variations in viral mRNA content across cells may correlate with differences in mGluR2 expression levels in those cells [81]. The extreme heterogeneity in viral mRNA distribution not only reflects differences in replication efficiency but also shows a significant association with host cell cycle regulation (e.g., downregulation of G2-M checkpoint genes) and oxidative stress responses (e.g., activation of the Nrf2 pathway). These findings suggest that the virus may exploit host transcriptome remodeling as a mechanism for immune evasion. Recent technological advancements highlight the potential of mudRapp-seq in elucidating viral replication mechanisms. By analyzing the heterogeneity of viral mRNA abundance across different cell types, this approach deepens our understanding of viral infections [69].

Dynamics of Pro-inflammatory Factors after Influenza Infection

Single-cell analysis of DEGs revealed significant upregulation of antiviral-related signaling pathways in infected cells, such as the IFN and IRF7 signaling pathways. Simultaneously, the principal components involved in antiproliferative and inflammatory processes is also elevated. The expression levels of key transcriptional regulators of host immune responses, such as STAT3, NFKB1, and REL, are upregulated. Additionally, several cytokines with incompletely characterized functions in influenza virus infection, including CHD1, BCLAF1, and PHF3, also show increased expression [51-56, 59-60]. Each immune cell subset plays distinct or overlapping roles and engages in intercellular interactions. The antiviral effects are evident across consistent cell types—including NK cells, B cells, T cells, and neutrophils—irrespective of their specimen source. This was demonstrated in samples from PR8 H1N1-infected mice (lungs and spleens), individuals with confirmed IAV infection (peripheral whole blood from children, adults, and pregnant women), and children with severe H5N6 avian influenza (PBMC samples). Although immune responses induced by different influenza viruses are broadly similar, infection with the H5N6 avian influenza virus tends to be more severe [50-51, 53, 59, 67-68]. The research team provided an overview of the current key findings (Table S1), with specific emphasis on pro-inflammatory factors. Neutrophils are a key cell type involved in influenza infection [56-57]. Zhang et al. performed an analysis demonstrating that the release of pro-inflammatory factors induced by influenza virus infection occurs in two distinct phases [50]. Kasmani MY et al. propose that the incidence of pulmonary inflammation rises with advancing age, and by applying spatial sequencing technology and scRNA-seq data, they have identify temporal and age-associated changes in neutrophil populations [67]. Initial clustering and Gene Ontology (GO) enrichment analyses were performed, followed by the identification of cell types expressing pro-inflammatory factors based on single-cell-specific marker genes. PD-L1-high neutrophils were identified as the primary contributors to the first wave of proinflammatory factor release. Subsequent transcriptional profiling of inflammatory responses in the second wave of cell populations, combined with Pearson correlation analysis, revealed that Pf4-high macrophages are the main source of the second wave of pro-inflammatory factors [50]. In young mice post-infection, the expression levels of neutrophil inflammatory regulatory genes, including IL-1α, CCL3, CXCL3, and CXCL1, were elevated, with CXCL chemokine signaling notably exceeding the release levels observed in neutrophils from aged mice. Single-cell DGE data revealed a contrasting pattern compared to the generally more severe inflammatory condition observed in older mice. Some studies attribute this discrepancy to the masking effect of neutrophil quantity on functional quality. Kulkarni U et al. confirmed that aged mice exhibit higher neutrophil counts following influenza infection [67, 82]. The analysis of pro-inflammatory factors should be expanded to include the inflammatory regulatory network to provide a more comprehensive understanding of the host response following infection. Single-cell differential gene expression DGE data can be employed to examine mitochondrial-related and coagulation-associated genes. Furthermore, such data also enables the comparison of how varying humidity levels affect influenza virus infection [62, 67]. The aforementioned analysis primarily aims to elucidate the origins of pro-inflammatory factors during influenza infection, thereby enabling a more comprehensive integration of both global and local perspectives in the conceptualization of the infection process.

Dynamic Profiling of Host Immune Cell Populations During Infection

The cellular heterogeneity of virus-infected cells is reflected in the dynamic responses of host immune cells. Following viral infection, this heterogeneity among immune cell populations is predominantly characterized by the activation or suppression of specific signaling pathways, which arise from differential gene expression across distinct subpopulations or are modulated by various regulatory factors. The differentiation patterns of various immune cell types following infection, as well as the interrelationships among their respective subsets, are analyzed using single-cell sequencing of immune cells. Future investigations should focus on exploring the cellular heterogeneity of B and T cells in response to influenza virus infection.

Heterogeneity and dynamic regulation of T-cell subsets

Following the presentation of viral antigens by dendritic cells (DCs), CD8+ T cells are activated and differentiate into cytotoxic effector T cells (CTLs). These CTLs mediate their cytotoxic effects through two distinct mechanisms: direct cytolysis of infected cells via the granzyme/perforin pathway or induction of target cell apoptosis through the Fas/FasL signaling pathway [83-85]. They secrete IFN-γ to inhibit viral replication and mediate antiviral and immunomodulatory effects through cytokines, such as TNF-α [86-87]. CD4+ T cells primarily differentiate into Th1 and Tfh subsets and can also give rise to other subsets, such as Th17 cells, which play a critical role in the immune response to influenza virus infection [88]. CD4+ T cells play a central role in enhancing the functionality of CTLs, promoting antibody production by B cells, and supporting mucosal defense mechanisms [89]. Following pathogen clearance, a subset of effector T cells differentiates into heterogeneous memory T cell populations, including central memory T cells (Tcm) located in lymphoid tissues, stem cell-like memory T cells (Tscm), effector memory T cells (Tem) found in peripheral tissues, and tissue-resident memory T cells (Trm) that permanently reside in respiratory mucosal tissues [90-91]. Memory cells can rapidly respond to reinfection at local sites and efficiently migrate to infection foci via circulatory pathways, thereby mediating immunological functions [92]. Regulatory

T cells (Tregs) play a critical role in modulating excessive immune responses and maintaining immune homeostasis during influenza virus infections [93]. During influenza virus infection, T cells may undergo exhaustion, characterized by functional impairment, which can impair viral clearance and delay disease recovery [94-95].

SCS technology facilitates a more comprehensive exploration of underlying mechanisms. Through clustering and visualization analysis, multiple CD4+ and CD8+ T cell subsets can be identified, which are significantly enriched in biological processes associated with oxidative stress and cell death [53, 67]. Furthermore, the analysis of CD4+T cell subsets can reveal the regulatory factors governing each subset through pseudotime trajectory analysis, thereby identifying the subsets involved in immune and inflammatory pathways [53]. Similar methodologies have been applied in studies of memory CD8+T cells, where tissue-resident memory T cells (CD8+Trm) accumulate in the lungs following infection and concurrently exhibit sustained high expression of CD49a for up to 90 days. The upregulated DEGs in CD8+ Trm cells are predominantly enriched in the FoxO signaling pathway, apoptosis, PD-L1 expression, PD-1 checkpoint pathway, and adherens junctions. According to the KEGG database. Following reinfection, both effector memory T cells (CD8+ Tem) and CD8+Trm cells show enrichment in the PI3K-Akt-mTOR and type I interferon signaling pathways [63]. In T cell research, it is essential to obtain comprehensive data that elucidate the cellular heterogeneity of naïve, effector, and memory T cells following influenza virus infection. Such data would significantly contribute to drug development and deepen our understanding of immune mechanisms.

Organ-specific differentiation of B cells during influenza infection

In influenza virus infection, B cells play a pivotal role through a multi-stage differentiation process. Initially, their surface B cell receptors (BCRs) specifically recognize viral antigens, such as HA, and are activated with T cell help. Subsequently, they enter the germinal center (GC), where they undergo somatic hypermutation (SHM) and affinity maturation. This process involves the random introduction of BCR mutations via activation-induced cytidine deaminase (AID), ultimately leading to the selection of high-affinity clones [96-99]. Simultaneously, class switch recombination (CSR) leads to the production of IgG or IgA antibodies. Mucosal IgA antibodies can prevent viral invasion of epithelial cells, whereas IgG antibodies exert their effects by neutralize the virus or recruit immune effector cells [100-101]. The integration of scRNA-seq and scBCR-seq allows for the precise characterization of B cell immune responses and the functional analysis of memory B cells following influenza virus infection. Using the Immcantation pipeline and scRepertoire, BCR sequence data can be systematically integrated into comprehensive multi-omics analyses [102-104]. Cell clustering analysis based on genes associated with specific differentiation states revealed that genes related to IgA antibody secretion and B cell receptor expression were significantly enriched in HA-specific memory B cells (HA-Bmems) and plasma blasts (PBs) across all examined organs. Notably, IgA-secreting cells showed preferential enrichment in these two cell populations. HA-positive B cell clusters display a high degree of organ specificity and lack temporal specificity [68]. To decipher the single-cell-level differentiation patterns of memory B cells (Bmems), trajectory analysis was performed using Slingshot, and RNA velocity analysis was performed with scVelo, revealing the distinct transcriptional profile of pulmonary Bmems marked by activation and tissue-residency features [68, 105-106]. Distinct microenvironments provided by different tissues and organs promote B cell differentiation. These analytical findings demonstrate a high degree of inter-organ dissemination of GC-derived HA-Bmems [68].

In addition to B and T cells, the cellular heterogeneity of other cell types following influenza virus infection highlights their influence on the infection microenvironment. As key components of the innate immune system, macrophages display diverse immune functions and are defined by distinct polarization states, underscoring their complex heterogeneity [107-108]. Yu et al. elucidated the influence of macrophages with distinct polarization states on T cell responses following influenza infection; utilizing time-resolved single-cell sequencing and metabolic RNA labeling techniques [58]. Influenza infection triggers a substantial increase in pulmonary lymphatic vessel density, accompanied by extensive proliferation of lymphatic endothelial cells (LECs) in the lungs, and a novel PD-L1-expressing subpopulation was identified that persists during viral infection and suppresses LEC differentiation and/or proliferation [61, 109]. Plasmacytoid dendritic cells (pDCs), a rare subset of the innate immune system, have a distinctive ability to produce large amounts of type I interferons. During influenza virus infection, these cells shift from being specialized cytokine hyperproducers to adopting antigen-presenting cell (APC)-like features, thereby exhibiting transcriptional diversity [64, 110-111]. The study of cellular heterogeneity, enabled by the integration of multiple advanced technologies, has substantially advanced our understanding of host cell responses to influenza virus infection.

The impact of aging on T and B cell susceptibility to influenza virus infection

Aging profoundly reshapes the host's immune response to influenza viruses through mechanisms of immunosenescence and chronic inflammation [112]. In the rhesus monkey model, the numbers of alveolar macrophages and infiltrating macrophages were significantly increased in the elderly group, while the number of T cells decreased concurrently [113]. The major coding genes differ between the young and the elderly. Aging leads to dysfunction of key cytolytic and memory functions in T cells, and the expression of multiple T-cell exhaustion markers shows an upward trend [67]. Wang et al. discovered in children that a subset of B cells exhibits a potentially protective cytotoxic effect, which diminishes with age [114]. The differentiation of B cells following influenza virus infection is influenced by age. Elderly individuals' B cells tend to differentiate into plasma cells rather than memory cells, which contributes to reduced vaccine efficacy [67]. Alice R. Burton et al. discovered that hemagglutinin-specific memory B cells formed in young individuals exhibit an FcRL5+ atypical phenotype, potentially originating from pre-GC precursor cells, and show evidence of somatic hypermutation and positive selection. In contrast, these features are less pronounced in the elderly population, confirming impairments in the germinal center reaction and memory B cell response following vaccination [115]. After vaccination, young individuals exhibit a stronger clonal response compared to the elderly. The proportion of plasmab-

lasts is reduced in older adults. Differential abundance analysis has also identified a greater number of vaccine-responsive cells that do not participate in expanded clones, a feature particularly prominent in the elderly population [116]. These findings not only elucidate the mechanisms underlying the heightened susceptibility to influenza and reduced vaccine efficacy in the elderly, but also provide critical scientific foundations for developing novel adjuvants and immune-targeted interventions for this population, as well as for the advancement of universal vaccine research.

Molecular Mechanisms Underlying Vaccine Development

The development of influenza vaccines has continually faced several major challenges: the rapid mutation of influenza viruses via antigenic drift and antigenic shift necessitates annual vaccine updates and hinders the attainment of broad-spectrum efficacy, the existence of substantial variations in vaccine-induced immune responses across different population groups, and ongoing evaluations of the safety profile of live attenuated vaccines [117-121]. SCS technology enables researchers to construct a comprehensive immunome atlas at single-cell resolution following influenza vaccination, thereby facilitating in-depth analysis of cellular heterogeneity and molecular regulatory networks in immune responses. The integration of multiple technologies and methodologies has substantially advanced influenza vaccine research. The authors summarize the common applications of scRNA-seq in vaccine studies (Table 2), offering novel insights into vaccine-induced innate immune training, lymphocyte differentiation, and the formation of tissue-resident memory T cells.

One week after vaccination, a significant increase in the relative proportion of plasma cells was observed, which was associated with the production of protective neutralizing antibodies. Monocytes, DCs, CD8+T cells, natural killer (NK) cells, and γδ T cells showed a decreasing trend, all of which recovered by the second week, revealing dynamic changes in immune cell populations following vaccination [123]. Following vaccination, transcriptomic alterations were observed across various immune cell populations, displaying distinct patterns dependent on cell type specificity. These changes are primarily associated with activation processes, clonal expansion induction, and antiviral response mechanisms [123-124]. Research on the safety of influenza vaccination warrants attention. A comprehensive safety assessment must consider the immune status and underlying conditions of diverse populations. At the population level, inactivated influenza vaccines are generally safe for older adults and immunocompromised individuals. However, immunosenescence in the former may slightly increase the risk of non-specific adverse events such as fever and fatigue, while the latter requires long-term monitoring due to potential risks associated with immune activation [125-127]. For pregnant women, inactivated vaccines serve as a crucial protective measure and have been proven safe throughout all stages of pregnancy [128]. At the technical level, the type of vaccine directly determines differences in risks such as inflammation and thrombosis [129]. Research on molecular mechanisms provides a profound explanation for this: inactivated vaccines do not upregulate platelet aggregation or pro-inflammatory genes, confirming their safety advantages. In contrast, adenovirus-vector vaccines, which mimic natural infections, generate a distinct pulmonary inflammatory environment. Understanding these differences is crucial for vaccine design [123, 130]. Safety serves as the cornerstone of vaccine development. On this foundation, novel vaccines—such as cHA vaccines—designed to elicit broad-spectrum and long-lasting immune responses, together with strategies combining adjuvants like AS03, are advancing into a new stage of enhancing the balance between immunity and tolerance through coordinated epigenomic regulation [70, 122]. Intranasal vaccines display distinct characteristics compared to other vaccine types, as they promote the secretion of IgA antibodies [131].To elucidate the origin of IgA in the pulmonary cavity, the phenotype, residency, and function of IgA-secreting B cells in the lung were analyzed using scBCR-seq, confirming that tissue-resident memory B cells are the primary source of IgA [100]. Simultaneously, a rare subpopulation was identified among keratinocyte nasal immune interaction front epithelium (KNIIFE) cells, which showed a concurrent increase in tissue-resident memory T-like cells. The presence of the CXCL16-CXCR6 axis in these populations has substantially contributed to the comprehensive mapping of nasal infection dynamics [132]. Adjuvants have been shown to enhance vaccine immunogenicity. However, in a comparative study between the self-amplifying mRNA vaccine (SAM-H1/CNE) for influenza A (H1N1) virus and the MF59-adjuvanted monovalent influenza vaccine, the SAM-based vaccine demonstrated superior efficacy in inducing stronger and more robust CD8+ T cell responses [133-135]. The first investigation of influenza vaccines utilizing SCS technology was conducted in a llama model, providing a unique perspective for vaccine and antibody development, thus advancing innovative research in this field [136]. Based on single-cell sequencing data, researchers have developed a vaccine response prediction model, highlighting the urgent need for integrating additional predictive models and artificial intelligence technologies [137]. The safety and efficacy of influenza vaccines remain central research priorities. Heterogeneous cellular data have provided new insights into this field, laying a critical foundation for the development of next-generation vaccines.

Conclusion and Future Directions

The SCS technology has significantly advanced influenza virus research by leveraging the heterogeneous responses of distinct cellular subpopulations to overcome these limitations. This approach has enabled a qualitative leap in our understanding of immune cell population differentiation and dynamics following infection. Through the analysis of single-cell differential expression profiles, researchers have gained deeper insights into the regulatory networks activated after viral infection. Furthermore, the establishment of a specialized single-cell database for influenza viruses is now feasible. Such a database would enable refined analysis of host cell subpopulations by integrating multi-timepoint and multi-tissue data, for instance, from the spleen and lung. Additionally, a cross-species single-cell database encompassing avian, swine, and human hosts should be developed to identify key cellular targets involved in viral cross-species transmission. Moreover, the dynamic expansion patterns of virus-specific T/B cell clones can be systematically monitored. To realize this vision and enhance its scientific value, a standardized pipeline has been implemented for rigorous quality control, batch correction,

and automated cell annotation to ensure data comparability and accuracy. A model combining annual major releases with quarterly incremental updates is recommended, along with the sharing of pre-trained analysis models by referencing the scvi-hub framework. In this way, the database can evolve from a static archive into an intelligent platform capable of continuously supporting dynamic analysis and hypothesis generation [138]. Based on the single-cell characteristics of lung tissues from severe influenza patients, integrated with metabolic differences in host genes such as CES derived from drug-sensitive and drug-resistant patient data, this study adopts a multidimensional and multi-perspective approach to offer systematically oriented strategic insights into the infection mechanisms and therapeutic strategies of the influenza virus [139]. In the field of vaccine and drug development, this single-cell database facilitates the elucidation of B cell cross-reactivity to conserved epitopes within the HA stem region, reveals the differential activation mechanisms of adjuvants across dendritic cell subsets (cDC1/cDC2), and offers a comprehensive understanding of the regulatory networks through which viruses hijack host metabolic pathways, as revealed by multi-omics integrated sequencing [140-142]. Consequently, enhancing the depth and breadth of existing databases is essential. The development of these databases requires standardized frameworks to improve comparability, and dynamic updates to continuously incorporate data on emerging strains. Furthermore, fostering interdisciplinary collaboration across fields, such as virology, computational biology, and clinical medicine, is essential to enhance the utility and coverage of databases. The integration of artificial intelligence, particularly through deep learning approaches, to predict virus-host interaction networks will significantly accelerate the development of anti-influenza strategies [143].

Multi-omics integration represents a key strength and an emerging direction in SCS technology. By leveraging bioinformatics analysis to capture cellular heterogeneity, it can be synergistically integrated with spatial transcriptomics, immune cell sorting, mass spectrometry, and other advanced techniques to generate comprehensive datasets; thereby enabling more precise and in-depth research outcomes [144]. For instance, spatial transcriptomics technologies (e.g., 10x Visium and Slide-seq) preserve the spatial localization of cells within tissues, thereby elucidating the spatial distribution of distinct immune cell subpopulations. The integration of spatiotemporal information enables the reconstruction of spatiotemporal trajectories during cell differentiation. The combined use of spatial transcriptomics and single-cell sequencing compensates for the loss of in situ tissue information; thereby resolving the spatial distribution of virus-infected cells and immune cell infiltration patterns in lung tissue [145-148]. The integration of multi-omics analysis with single-cell sequencing technologies enables a comprehensive understanding of infection-induced

Table 2. The combined application of single-cell RNA sequencing (scRNA-seq) with multiple technologies in influenza vaccine research

technical combination		research problem	main discovery
scRNA-seq +	scBCR-seq	Vaccines exhibit a relatively weaker protective effect in the elderly, and age may influence the quantity or type of B cells	The immune response following vaccination declines with age, and the magnitude of plasmablast expansion is greater in younger individuals than in older adults [116]
		Characteristics of B cell specificity, function, and subsets induced by chimeric hemagglutinin (cHA) vaccines	The cHA vaccine enriches stem-binding B cells within the memory B cell compartment one year after vaccination [122]
	EpiTOF, scATAC-seq	At the single-cell level, there remains a gap in the comprehensive map of the human epigenome during the immune response process	After influenza vaccination, chromatin accessibility at the AP-1 site in myeloid cells decreases; the AS03 adjuvant enhances accessibility in the IRF/STAT binding region [70]
	Indexed Flow Sorting (IFS), scBCR-seq	How does aging impact the memory B cell response	After vaccination, the elderly exhibit deficiencies in both the germinal center (GC) response and the memory B cell response [115]
	scBCR-seq, Fluorescence Activated Cell Sorting (FACS)	The cell types responsible for IgA production in the lower respiratory tract following intranasal vaccination remain unclear	This study investigated the phenotypic characteristics, residency status, and functional roles of IgA-secreting B cells in the lungs and confirmed that the development of these cells depends on CXCR3 signaling [100]
	Protein Microarray (PM)	Explore the mechanism by which adjuvants enhance the breadth of cross-reactivity	Adjuvants enhance the magnitude and durability of the antibody response to vaccines [65]
	Longitudinal Antibody Repertoire Sequencing (LAR-Seq)	Analyze the molecular and cellular characteristics of the antibody response following influenza vaccination	Numerous antibody clones induced by vaccination do not bind to the vaccine antigen and instead activate non-specific bystander antibodies through the bystander effect [66]

immune regulatory mechanisms and the identification of precise therapeutic targets. This includes epigenomic profiling (single-cell ATAC-seq for chromatin accessibility, scCOOL-seq for chromatin state and DNA methylation), proteomic analysis (CITE-seq for surface protein detection and CyTOF), and metabolomic characterization (single-cell metabolic mass spectrometry imaging). When combined with in vivo dynamic tracking and lineage tracing technologies (CRISPR barcoding, fluorescent reporter systems), these omics approaches, together with SCS technology, facilitate in-depth analysis of dynamic epigenetic modifications in memory cells following influenza virus infection, thereby identifying key molecular determinants of cell fate [149-156]. Furthermore, the integration of computational biology with artificial intelligence can accelerate the development of SCS technologies, facilitating large-scale data mining, cross-species comparisons, and the organization of clinical information, among other applications[157].

Certainly, there is still room for improvement in Single-Cell SCS technology. For instance, the preparation of single-cell suspensions may result in the loss of microenvironmental information from tissues. Given the low abundance of influenza virus mRNA in most infected cells, single-cell sequencing may fail to detect certain viral signals. Additionally, low-abundance viral genes might be masked by highly expressed host genes (e.g., interference from mitochondrial transcripts) [158-159]. In the future, there is a need to develop virus-specific primer enrichment technologies or to optimize data analysis algorithms, constructing a comprehensive training dataset integrating multi-dimensional features such as sequence k-mer frequency, alignment quality scores (e.g., MAPQ), coverage depth uniformity, and sequence context embedding; Employ convolutional neural networks (CNNs) or Transformer models to automatically learn deep sequence patterns and features within viral genomes. By incorporating weighted loss functions and transfer learning strategies, effectively address the challenges of extreme class imbalance—where viral reads constitute an extremely low proportion—and sparse annotated data [160-162]. Furthermore, developing conserved sequence capture probes for influenza viruses is feasible, and their integration with single-cell RNA sequencing can significantly enhance the sensitivity of viral gene detection. Alternatively, utilizing PacBio or Oxford Nanopore Technologies (ONT) platforms enables the direct capture of complete viral genomes, thereby facilitating a comprehensive analysis of quasispecies diversity [163]. Single time point sequencing is unable to capture dynamic processes; therefore, it is critical to employ tools such as Monocle3 to construct infection progression models that simulate the continuum from viral entry and replication to host cell apoptosis [164]. Furthermore, developing advanced tools such as Viral-Track is essential to automatically segregate host and viral reads, enabling simultaneous analysis of host gene expression and viral genomic variations. This approach overcomes the limitations of existing algorithms, which are predominantly designed for single-species analysis [165]. Although single-cell sequencing technology has become a powerful tool for influenza virus research, its high sequencing costs and complex data analysis workflows remain barriers to its widespread adoption in certain studies.Future efforts should focus on refining technical protocols, reducing sequencing costs, and developing more efficient bioinformatics tools to fully harness the potential of this technology. This study presents the first systematic integration of single-cell sequencing in three-dimensional applications, including investigations into influenza virus infection mechanisms, immune cell dynamics, and vaccine development. It highlights the pivotal role of technological convergence in advancing future research, aiming to provide novel insights for the development of precise antiviral strategies.

Abbreviations

Activation-induced cytidine deaminase: AID; Antigen-presenting cell: APC; Assay for Transposase-Accessible Chromatin with sequencing: ATAC-seq; B cell receptors : BCRs; Cellular Indexing of Transcriptomes and Epitopes by sequencing: CITE-seq; Clathrin-mediated endocytosis: CME; Convolutional neural networks: CNNs; Clustered Regularly Interspaced Short Palindromic Repeats: CRISPR; Class switch recombination: CSR; Cytotoxic effector T cells: CTLs; Cytometry by Time-Of-Flight: CyTOF; Dendritic cells: DCs; Differentially expressed genes: DEG; Droplet-based single-cell RNA sequencing: Drop-seq; Fluorescence-Activated Cell Sorting: FACS; Germinal center: GC; Gene Ontology: GO; Hemagglutinin: HA; Confirmed influenza A virus: IAV; Indexed Flow Sorting: IFS; Keratinocyte nasal immune interaction front epithelium: KNIIFE; Longitudinal Antibody Repertoire Sequencing: LAR-Seq; Lymphatic endothelial cells: LECs; Multiple Annealing and Tailing-based Quantitative Single-cell RNA-seq: MATQ-seq; Metabotropic glutamate receptor 2: mGluR2; MULTIplexing using lipid-Tagged Indices for single-cell RNA sequencing: MULTI-seq; Natural killer: NK; Oxford Nanopore Technologies: ONT; Peripheral Blood Mononuclear Cell: PBMC; Principal Component Analysis: PCA; Plasmacytoid dendritic cells: pDCs; Protein Microarray: PM; RNA-dependent RNA polymerase: RdRp; Single-cell B-cell receptor/T-cell receptor sequencing: scBCR-seq/scTCR-seq; Single-Cell RNA Barcoding and sequencing: SCRB-seq; Single-cell RNA sequencing: scRNA-seq; Single-cell sequencing: SCS; Somatic hypermutation: SHM; high-throughput and high-sensitivity single-nucleus total RNA sequencing: snHH-seq; Single nucleus Random-seq: snRandom-seq; Single-cell Universal Poly(A)-independent RNA sequencing: SUPeR-seq; Central memory T cells: Tcm; Effector memory T cells: Tem; Regulatory T cells: Tregs; Tissue-resident memory T cells: Trm; Template Switching: TS; Stem cell-like memory T cells: Tscm; Unique Molecular Identifiers: UMIs

Authors Contributions

Xingting Li : Data curation, Formal analysis, Writing : original draft; Lingxi Gao : Conceptualization, Methodology, Writing : review & editing, Supervision.

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Ethics Approval and Consent to Participate

Not Applicable.

Competing Interests

The authors declare that they have no existing or potential commercial or financial relationships that could create a conflict of interest at the time of conducting this study.

Data Availability

Not Applicable (this is a review article and no new data were generated).

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Trends and Cross-Country Inequalities of Melanoma and Non-Melanoma Skin Cancer from 1990 to 2021 and Predictive Trends from 2022 to 2044: A Global Burden of Disease Study

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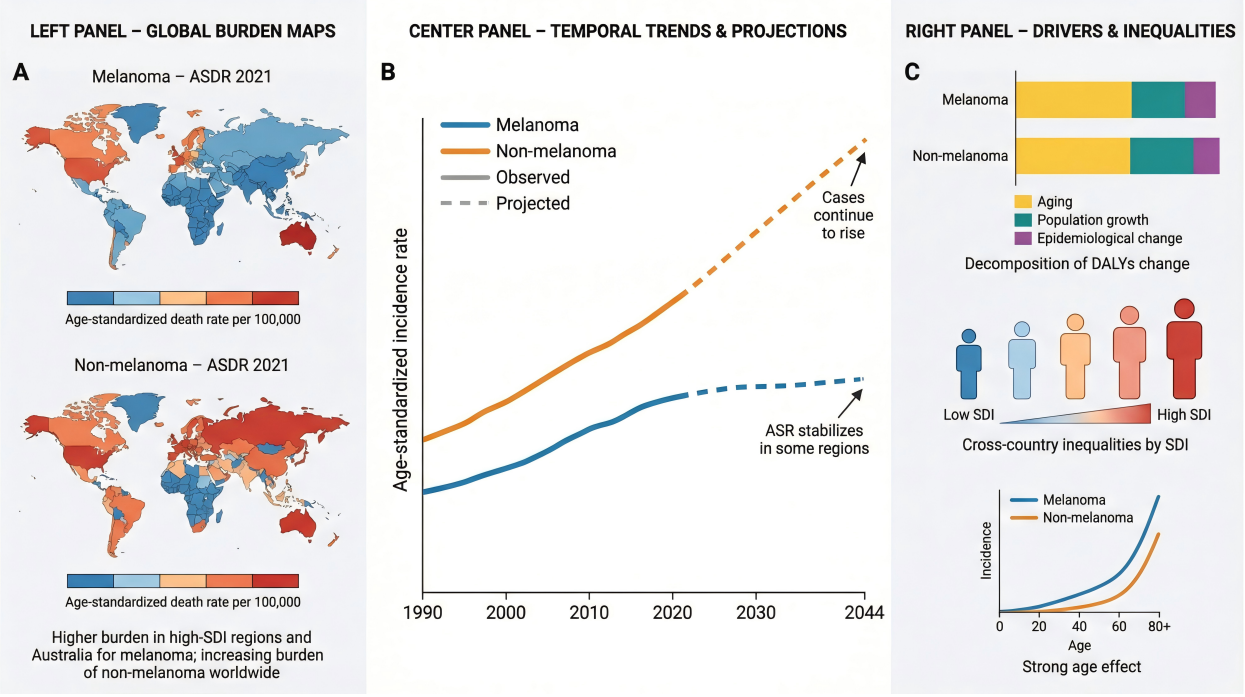
Graphical Abstract



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Research Article Life Conflux

Trends and Cross-Country Inequalities of Melanoma and Non-Melanoma Skin Cancer from 1990 to 2021 and Predictive Trends from 2022 to 2044: A Global Burden of Disease Study

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Abstract

Background: Malignant skin melanoma (MSM) and non-melanoma skin cancer (NMSC) have imposed a significant health and economic burden globally. This study aims to explore the disease burden and temporal trends of MSM and NMSC to inform evidence-based prevention and control strategies.

Methods: Data were derived from the Global Burden of Diseases (GBD) database, covering deaths, disability-adjusted life years (DALYs), incidence, and prevalence. Joinpoint regression, inequality analysis, decomposition analysis and age-period-cohort analysis identified trends and revealed the causes of burden changes from 1990 to 2021. Nordpred package in R projected the future trends of MSM and NMSC from 2022 to 2044.

Results: From 1990 to 2021, global trends showed an increase in the number of deaths, DALYs, incidence, and prevalence for MSM, squamous cell carcinoma (SCC), and basal cell carcinoma (BCC). The disease burden was highest in high Socio-Demographic Index (SDI) regions, predominantly concentrated in New Zealand, Australia, and the United States. Among MSM, SCC and BCC, SCC experienced the most substantial increases in age-standardized incidence (AAPC = 1.62 [95% CI: 1.51 to 1.73]) and prevalence (AAPC = 1.90 [95% CI: 1.78 to 2.02]). The primary drivers of changes in DALYs were identified as population aging and population growth. Persistent health inequalities continue to exist in the global burden of skin cancer. In the future, the deaths, DALYs, incidence, and prevalence cases of MSM and NMSC may continue to increase.

Conclusion: The disease burden associated with MSM and NMSC remains substantial. Primary prevention for the elderly should be given priority. In the prevention of skin cancer, particular attention should be directed toward SCC. Global medical resources should be appropriately tilted towards skin cancer.

Keywords: Trend; Inequality; Global burden of disease; Melanoma; Non-melanoma skin cancer

Introduction

Skin cancer primarily includes malignant skin melanoma (MSM) and non-melanoma skin cancer (NMSC), the latter mainly comprising squamous cell carcinoma (SCC) and basal cell carcinoma (BCC) [1]. MSM originates from melanocytes, which function to produce melanin, thereby protecting the skin against ultraviolet radiation [2]. SCC mainly derives from epidermal keratinocytes [3], while BCC originates from basal cells of the skin [4]. Once these cells become cancerous, the body may have pain and itching symptoms, and the psychology may produce anxiety and fear [5-7]. If skin cancer is not detected and treated early, it may metastasize and become life-threatening [8-9]. MSM and NMSC represent a significant public health challenge, imposing substantial burdens on healthcare systems worldwide [10]. Australia, New Zealand and the United States have the highest health system costs due to skin cancer [11]. In the United States, the overall estimated annual cost of skin cancer treatment was $8.9 billion between 2016 and 2018 [12]. With changing lifestyles and a growing elderly population, skin cancer is becoming an escalating threat across the globe [13]. Therefore, MSM and NMSC should be recognized as critical public health priorities.

Currently, studies have analyzed the burden of MSM and NMSC [14-18]. A study analyzed the burden of MSM and NMSC in the United States from 1990 to 2019 [14]. Two studies used the Global Burden of Diseases database (GBD) 2019 and Global Cancer 2022 (GLOBOCAN) databases respectively [15-16]. The GLOBOCAN 2022 database lacked DALYs indicators and had no data on the major subtypes of NMSC. The remaining studies either only focused on the elderly population or had incomplete indicators [17-18]. As a result, there is currently no

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latest and comprehensive global burden evaluation of MSM and NMSC.

We utilized the latest GBD database of 2021 to depict the disease burden of MSM, SCC and BCC. Temporal trends of the three types of skin cancer were analyzed from multiple dimensions. Decomposition analysis was employed to identify the factors contributing to disability-adjusted life years (DALYs). Socioeconomic health inequalities were compared, and future trends in disease burden were projected. Understanding the current disease burden and epidemiological trends is essential for the formulation of effective early prevention and control strategies.

Methods

Study Design and Population

The GBD 2021 database includes data on 371 diseases and injuries across multiple regions and countries. The study retrieved the estimates along with 95% uncertainty intervals (UI) for deaths, DALYs, incidence, and prevalence for MSM and NMSC. As deaths data for BCC were unavailable in the GBD 2021 dataset, analyses of this indicator were excluded. The GBD database does not include deaths data for BCC, primarily due to its extremely low fatality rate and structural limitations in the global data collection system. The Socio-Demographic Index (SDI) measures the development of a country or region by assessing income levels, education levels and health status [19].

Statistical Analysis

To comprehensively understand the burden of MSM, SCC and BCC, we conducted a descriptive analysis. The number and age-standardized rates (ASR) of global deaths, DALYs, incidence and prevalence were visually displayed [20-21]. We compared the burden of MSM, SCC, and BCC in 2021. We utilized Joinpoint regression to investigate the change trend of three types of skin cancer [22-23]. The age-period-cohort model was employed to analyze the underlying trends in incidence among different ages, periods, and birth cohorts [24-25]. We conducted decomposition analysis on the potential factors driving the DALYs of MSM, SCC and BCC [26]. The distribution of health inequalities related to MSM, SCC, BCC burden across countries was evaluated [27]. To forecast the future burden of MSM, SCC, and BCC for the next 23 years, the Norpred package in R was used [28-29]. These methods were described in detail in Supplementary Methods.

Results

Descriptive analysis of MSM and NMSC in 2021

From 1990 to 2021, the global burden of MSM, SCC, and BCC demonstrated a significant upward trend in the numbers of deaths, DALYs, incidence and prevalence (Figure S1). In 2021, MSM exhibited the highest age-standardized death rate (ASDR) (0.73 [95% UI, 0.65, 0.79]) and age-standardized DALYs rate (ASDALYs) (19.63 [95% UI, 17.25, 21.50]). BCC had the highest age-standardized incidence rate (ASIR) (51.71 [95% UI, 45.70, 57.58]), while SCC presented the highest age-standardized prevalence rate (ASPR) (26.85 [95% UI, 22.77, 31.77]) (Tables S1-S3). The high SDI region had the highest ASR of deaths, DALYs, incidence, and prevalence. (Figure S3). Among the 21 GBD regions, Western Europe exhibited the highest burden for MSM in numbers of deaths, DALYs, incidence and prevalence (Table S4). High-income North America had the highest incidence and prevalence of BCC (Table S5). East Asia had the highest numbers of deaths, DALYs and prevalence of SCC (Table S6). The ASRs for deaths, DALYs, incidence, and prevalence of the three types of skin cancer varied significantly between countries around the world. Specifically, New Zealand had the highest ASDR, ASDALYs, ASIR and ASPR for MSM, while the United States of America exhibited the highest ASIR and ASPR for SCC and BCC (Figure 1, Figure S2). In addition, the disease burden of skin cancer was mainly concentrated in older adults and males (Figure S4).

Trends in MSM and NMSC using joinpoint analysis

From 1990 to 2021, the trend in ASDR (AAPC = -0.48 [95% CI: -0.37 to -0.59]) and ASDALYs (AAPC = -0.71 [95% CI: -0.60 to -0.82]) for MSM showed a decline (Table S7). Conversely, the ASIR and ASPR for MSM increased over time with AAPC values of 0.56 (95% CI: 0.33 to 0.79) and 0.87 (95% CI: 0.68 to 1.06) (Table S7). For SCC, the AAPC values for ASDR, ASDALYs, ASIR and ASPR were 0.14 (95% CI: 0.06 to 0.22), 0.06 (95% CI: 0.03 to 0.08), 1.62 (95% CI: 1.51 to 1.73) and 1.90 (95% CI: 1.78 to 2.02), respectively (Table S7). The ASDR and ASDALYs of SCC showed a downward trend since 2015. During the period from 2000 to 2004, ASIR and ASPR of SCC had the fastest growth rates, where the annual percentage change values were 14.23 (95% CI: 13.75 to 14.71) and 14.66 (95% CI: 14.16 to 15.16), respectively. From 2007 to 2021, the ASIR and ASPR of SCC remained at a relatively high level (Figure 2). The ASIR and ASPR of BCC were similar to that of SCC (Figure S5).

Age-period-cohort analysis of incidence in MSM and NMSC

The age effect curve shows that the risk of skin cancer increases with aging (Figure 3, Figure S6-S7). Among the SDI regions, high SDI countries consistently demonstrated the highest incidence across all age groups, with male showing a higher incidence than female. Regarding period effects, the overall trend of SCC and BCC presented an upward tendency. Globally, the upward trends were more prominent among male than female. For MSM and SCC, the incidence increased globally between 1992 and 2011, but decreased between 2011 and 2021 (Figure S6-S7). For MSM, the global cohort effects peaked in the 1942 to 1951 birth cohort (Figure S6). Among post-1952 birth cohorts, an improvement in the burden of MSM was only observed in high SDI regions. A significant gender-based difference in cohort effects between male and female was noted in high SDI regions for individuals born around 1945. For SCC and BCC, the birth cohort effects were on the rise overall, globally and five SDI regions, reaching a maximum in the 1992 to 2001 birth cohort with no significant difference between male and female (Figure 3).

Decomposition analysis regarding DALYs in MSM and NMSC

From 1990 to 2021, there was a significant increase in DALYs for three types of skin cancer globally, with the largest increase for MSM and BCC in high SDI regions and the largest increase for SCC in middle SDI regions (Figure 4). For the three types of skin cancer, the overall increase was larger in males

than females. For MSM, the contribution of aging, population growth, and epidemiological changes to the global increase in DALYs was 36.23%, 112.88%, and -49.12%, respectively (Table S8). Population growth emerged as the most influential factor driving DALYs increases across all SDI regions, while epidemiological changes had a negative impact, particularly in high SDI regions. For SCC, aging, population growth, and epidemiological changes contributed 31.95%, 66.51%, and 1.54% to the global increase in DALYs, respectively (Table S8). In high and high-middle SDI regions, aging and population factors had nearly equal impacts on the rise in DALYs, while in other SDI regions, population growth played the dominant role. For BCC, aging, population growth and epidemiological changes contributed 21.72%, 43.58%, and 34.70% to the global increase in ASDALYs, respectively (Table S8). Notably, epidemiological changes had the largest impact on DALYs in high SDI regions, whereas population growth emerged as a critical determinant of DALYs in low SDI regions.

Cross-country inequality analysis regarding incidence in MSM and NMSC

The absolute inequality in the burden of the three types of skin cancer associated with SDI increased over time. A higher proportion of incidence was observed in countries characterized by advanced socio-demographic development (Figure 5). In 1990, the slope index of incidence for MSM stood at 3.06 (95 %UI 2.34, 3.78) per 100,000 people, and it increased to 9.53 (95 %UI 7.36, 11.70) in 2021, showing that the wealthiest country had an incidence rate approximately 9.53 per 100,000 individuals higher than the poorest country (Table S9). The slope index of SCC and BCC showed a similar growth trend to that of MSM, but the increase in BCC was more pronounced.

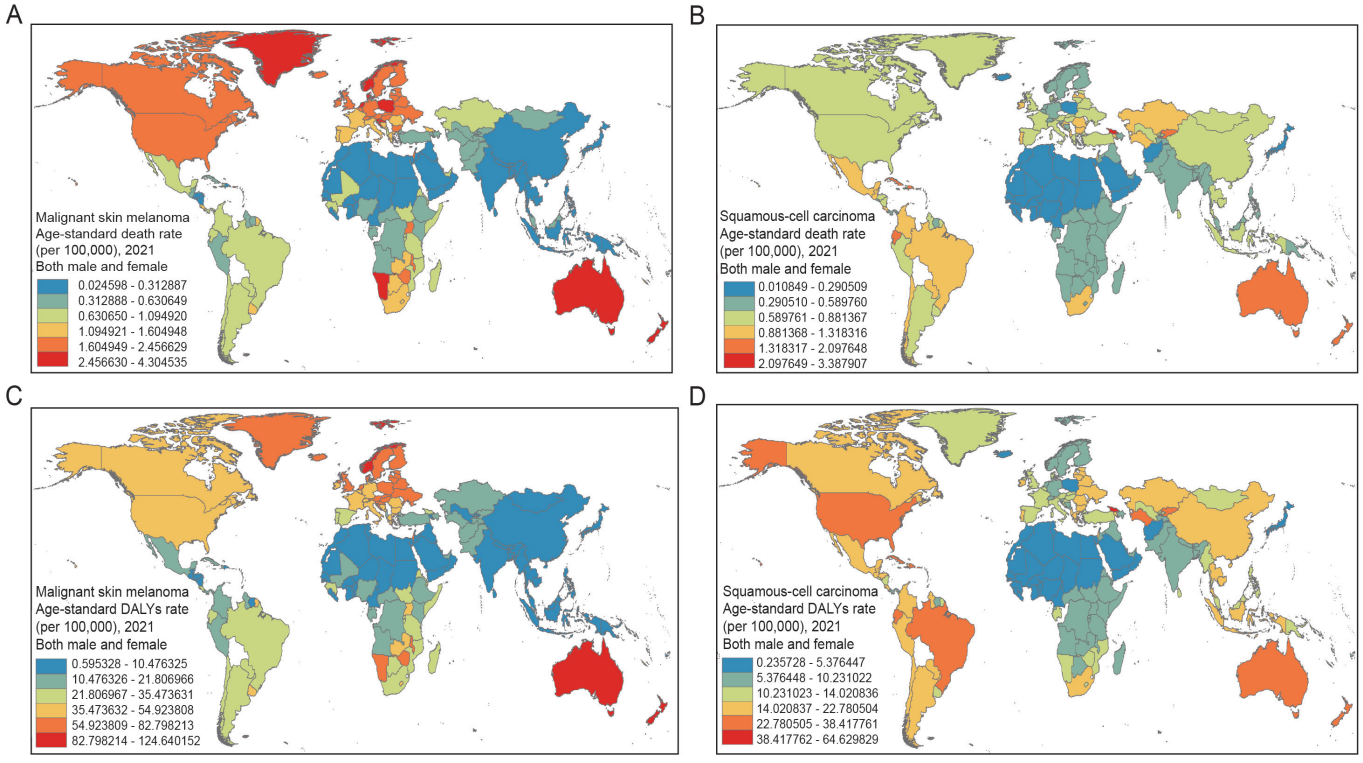
Predictive analysis in MSM and NMSC until 2044

From 2022 to 2044, the incidence numbers for MSM, SCC and BCC were predicted to increase from 303,431, 1,928,413, and 4,376,687 to 351,863, 3,429,869 and 7,362,321 (Table S10), with increase of approximately 15.96%, 77.86% and 68.22%, respectively. In recent years, the gap of number of deaths between SCC and MSM has been progressively narrowing. It is predicted that by 2025, the number of deaths attributable to SCC will surpass that of MSM. Over the next 23 years, the numbers of deaths and DALYs, and the prevalence for the three types of skin cancer will all increase (Figure 6, Figure S8). By 2044, the ASR for deaths, DALYs, incidence, and prevalence of MSM will decline to 0.55 per 100,000 people, 14.79, 2.47, and 17.03 respectively (Table S11). The changing trends of SCC and BCC were similar to those of MSM, but the declines were relatively slight (Figure 6, Figure S8).

Discussion

The study utilized the latest data to analyze the global trends of deaths, DALYs, incidence, and prevalence for the three types of skin cancer from 1990 to 2021. Globally, the numbers of deaths, DALYs, incidence and prevalence for the three types of skin cancer showed upward trends. The disease burden in Western Europe and High-income North America was relatively more severe. Males exhibited higher burden of the three types of skin cancer compared to females. Compared to previous similar studies, there are many new findings. (1) In 2021, MSM

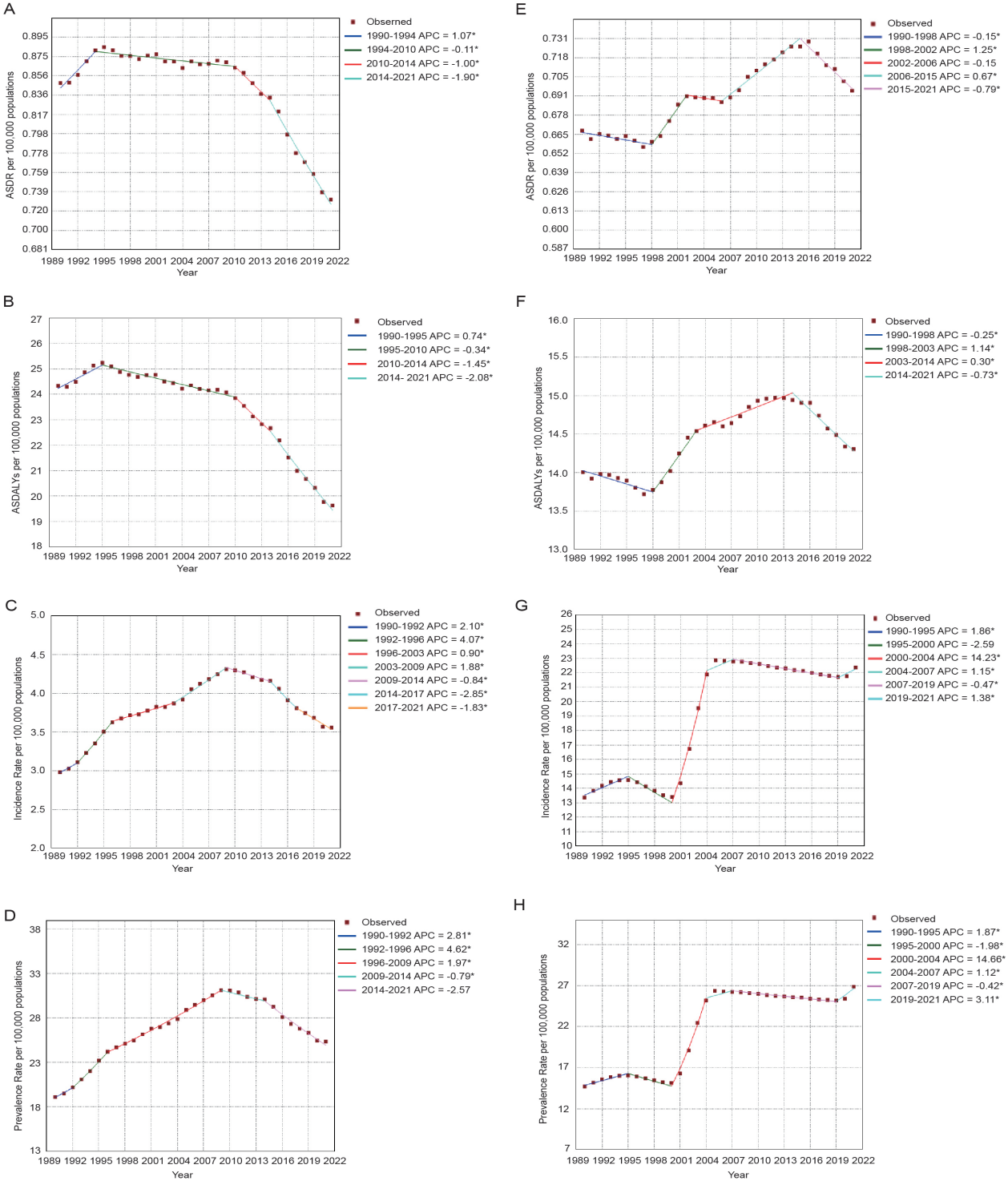
Figure 1. Age-standardized rates of deaths and DALYs attributed to MSM and SCC in 2021. (**A**, **B**) death. (**C**, **D**) DALYs. DALYs = disability-adjusted life years. MSM = malignant skin melanoma. SCC = squamous cell carcinoma.



exhibited the highest ASDR and ASDALYs. BCC had the highest ASIR, and SCC had the highest ASPR. (2) Among the three types of skin cancer, SCC displayed the most pronounced upward trends in ASIR and ASPR from 2000 to 2004. (3) Age and birth cohort showed an upward trend of the three skin cancers with time. (4) Decomposition analysis showed that aging and population growth were the primary contributors to the increase of DALYs. (5) The incidence rate was higher in high SDI countries, and the inequality intensified over time. (6) While the ASR for deaths, DALYs, incidence, and prevalence for the three types of skin cancer was expected to decline until 2044, the absolute number for these metrics was projected to rise.

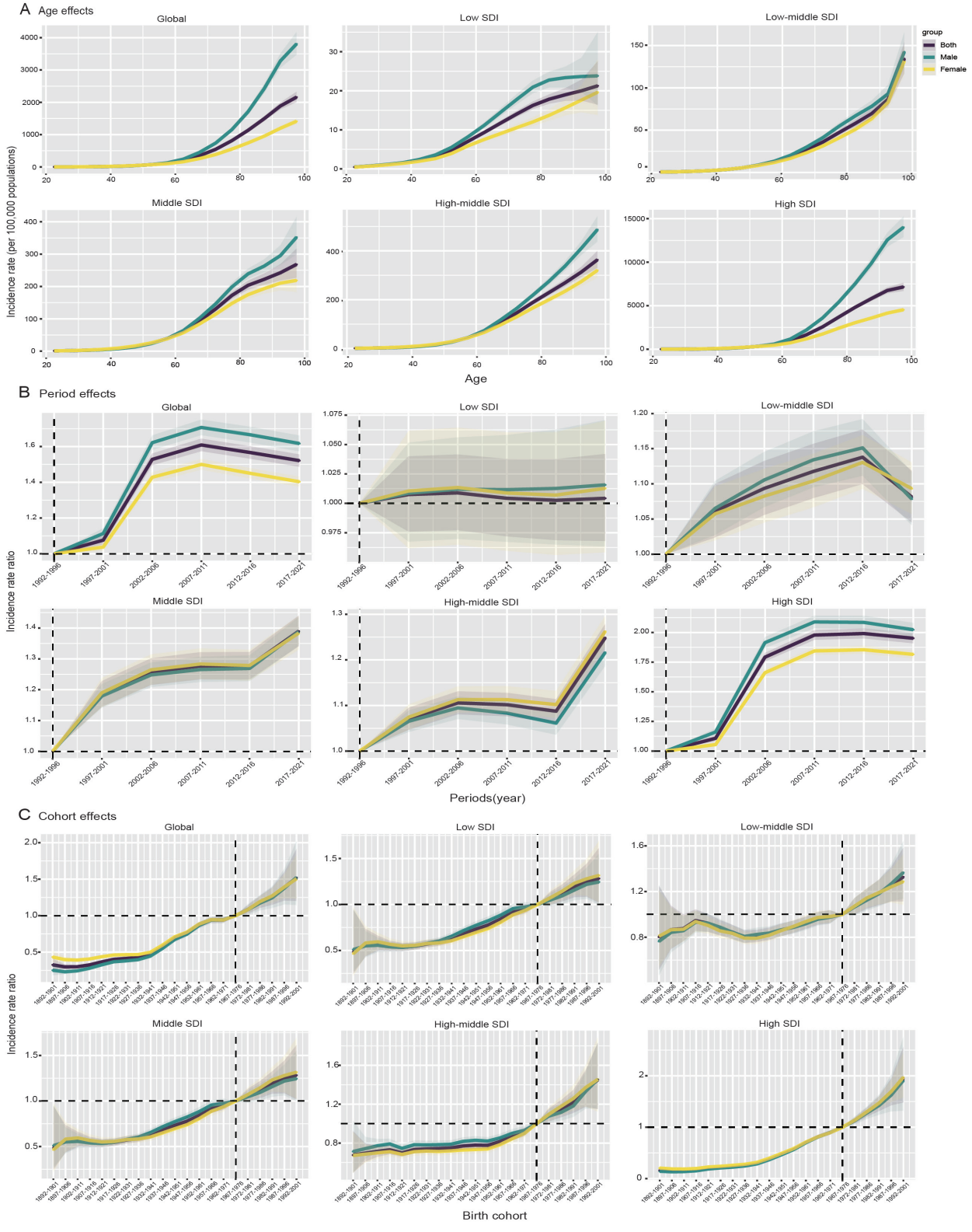
In 2021, MSM exhibited the highest ASDR and ASDALYs. BCC had the highest ASIR, while SCC showed the highest ASPR.

Figure 2. Global temporal trends in ASDR, ASDALYs, ASIR, and ASPR attributed to MSM and SCC based on the joinpoint regression analysis (1990-2021). (**A-D**) MSM. (**E-H**) SCC. (**A**, **E**) ASDR. (**B**, **F**) ASDALYs. (**C**, **G**) ASIR. (**D**, **H**) ASPR. *Indicates that the annual percent change is significantly different from zero at the alpha = 0.05 level.



Although MSM accounted for only 3% of all skin cancers, its mortality accounts for 65% of all skin cancers [30]. MSM had the highest ASDR among the three types of skin cancer because it is a highly aggressive skin tumor. The global health system should establish a special scientific research fund for MSM, encourage global scientific research institutions to collaborate with medical institutions, to deeply explore the pathogenesis and the patterns of invasion and metastasis, and accelerate the research and development of innovative treatment technologies. The incidence of BCC and SCC was higher than

Figure 3. The age–period–cohort analysis of BCC in global and five SDI regions. (**A**) Longitudinal age curve. (**B**) Period rate ratio. (**C**) Cohort rate ratio.



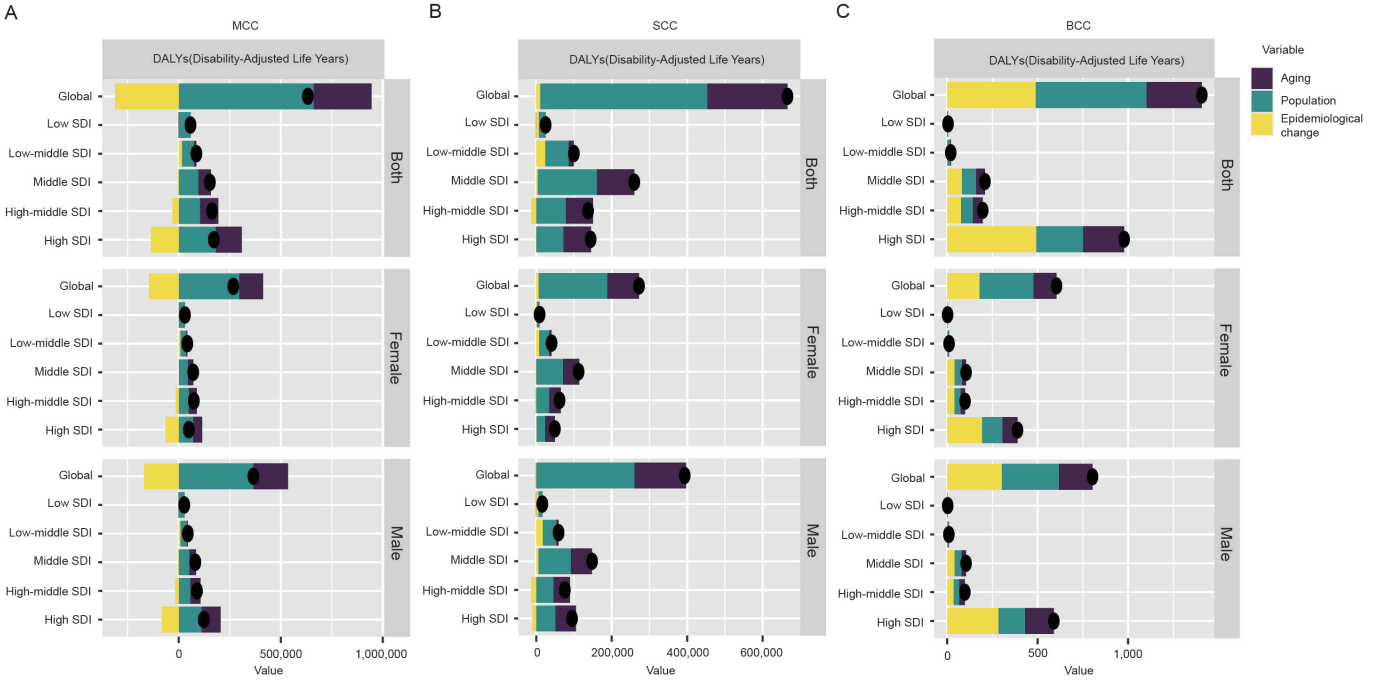
that of MSM. This result indicated that in the formulation of relevant health strategies, the prevention of NMSC should be placed in the primary position.

Significant variations in the burden of the three types of skin cancer were observed across different countries and regions. While East Asia accounts for the highest absolute numbers of SCC deaths and DALYs globally, high-SDI regions exhibit the highest standardized rates of these outcomes. This may be related to the following reasons. First, the huge population base in East Asia directly increases the absolute number of SCC deaths and DALYs. Second, East Asia is experiencing rapid aging, with a large absolute number of elderly people, further magnifying the overall scale of SCC deaths and DALYs [31]. Third, the core purpose of age standardization is to eliminate the differences in age structure between different regions. The higher standardized rate in high SDI regions may be related to risk exposure factors in this region, such as longer ultraviolet (UV) exposure time and higher skin cancer screening rates [32]. In view of the differences of skin cancer in different regions, a dynamic resource allocation mechanism for skin cancer should be established. At the same time, efforts should be made to promote the sharing of medical resources, and narrow the gap in the diagnosis and treatment levels of skin cancer between regions through means such as telemedicine and expert rounds.

In 2021, gender-stratified analysis revealed that males bore a higher disease burden than females across three skin cancer types. It is important to clarify that the "male" and "female" categories referenced in this study align with the biological sex classifications defined by the GBD Study, reflecting inherent biological traits rather than social gender identities or gender roles. The observed sex differences in SCC burden are not attributable to essentialist notions of inherent vulnerability or resistance to the disease, but instead are mediated by key behavioral and occupational factors [33-34]. A study revealed that the ASDR of MSM among males was 30% higher than that among females [35]. This difference could be explained by the following perspectives. In some outdoor jobs, males constitute the majority of practitioners [36-37]. Differences in sunscreen use and attitudes to asymptomatic illness may explain the difference [38]. Therefore, all countries should make full use of this gender difference and formulate targeted prevention and intervention strategies. Regulations should be established to limit the working hours of outdoor workers in policies and build sun-protection areas for them.

Joinpoint regression method is a highly effective tool in data analysis and trend research. It divides the overall trend precisely into multiple distinct phases. We found that among the three types of skin cancer, the increasing trends in ASIR and ASPR of SCC and BCC were the most significant. In addition, the most rapid increase occurred between 2000 and 2004. The following reasons may account for this result. Skin detection technology has improved. With the development of optical technology, dermoscopy technology has gradually emerged [39]. Entering the 21st century, the integration of computer and imaging technology further facilitated the development of more efficient detection methods. Additionally, the increasing aging of the population has contributed to the rising burden of skin cancer, especially for SCC and BCC [40]. Since 2010, the ASIR and ASPR of the three types of skin cancer have shown a downward trend. This can be attributed to increased public awareness of sun protection. Primary prevention is the most effective measure for preventing skin cancer [41]. Therefore, it is necessary to formulate a national health education plan

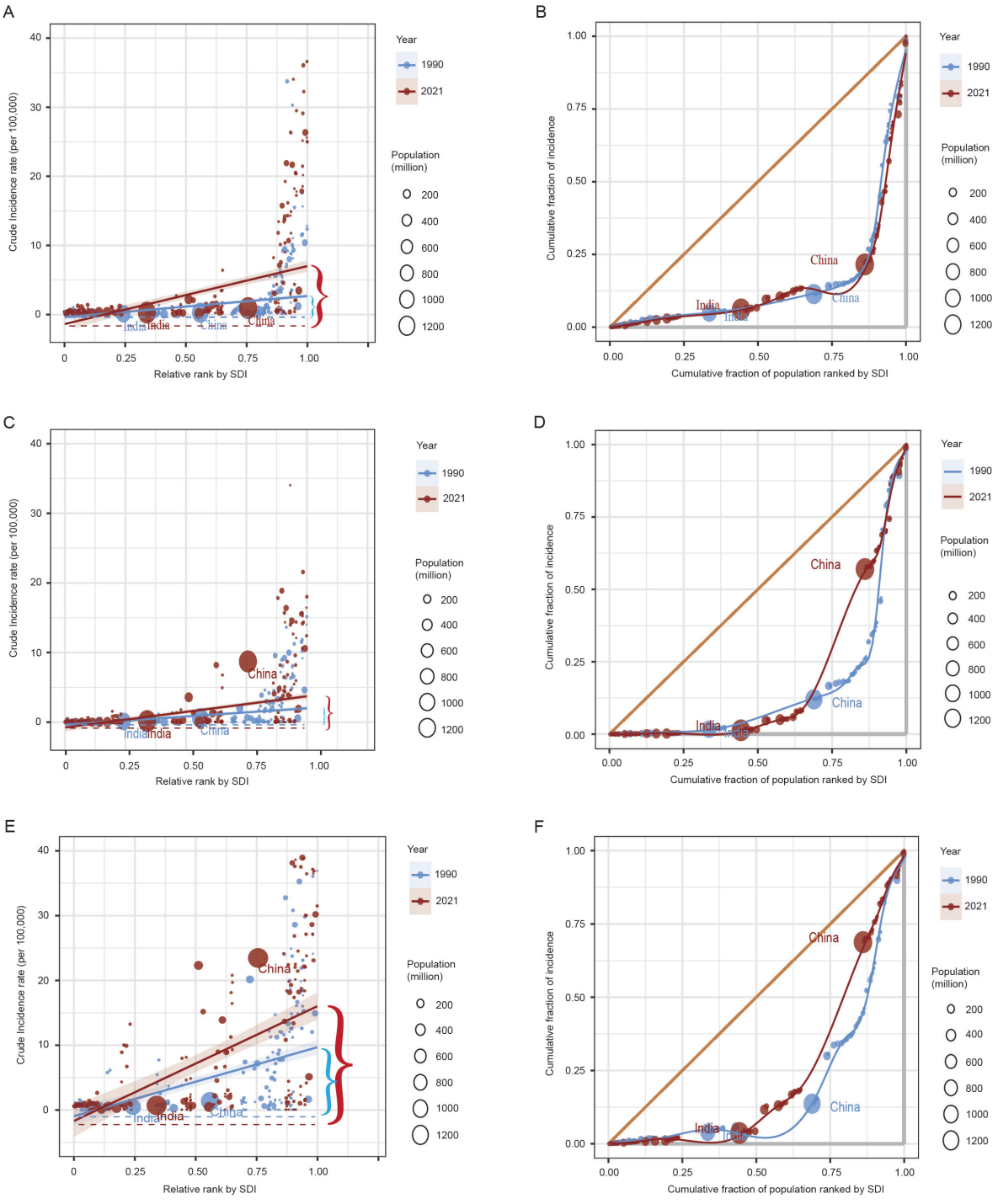
Figure 4. Changes in DALYs of MSM, SCC, BCC according to aging, population growth and epidemiological change from 1990 to 2021 at global level by SDI regions and sexes. The black dot denotes the overall value of the change resulting from all three components. For each component, the magnitude of a positive value suggests a corresponding increase in DALYs attributed to the component; the magnitude of a negative value suggests a corresponding decrease in DALYs attributed to the component.



for skin cancer and carry out publicity of skin cancer prevention knowledge. In Australia and the United States of America, preventive initiatives including sun protection education and increased use of sunscreen have been implemented [42].

We explored the trends of skin cancer across three temporal dimensions. The incidence rates of the three types of skin cancer increase with age, and the increasing speed became even faster starting from the age range of 50 to 60. With increasing age, the self-repair ability of the skin declines, and it is difficult to repair DNA damage in a timely manner after being damaged by carcinogenic factors [43-44]. The relative incidence risk trended upward, suggesting that later-born individuals faced higher risks. Consequently, effective preventive and management measures need to be taken for people in this age group. For the elderly and newly born population, regular skin examination and screening programs should be strengthened. For these two groups, it is particularly important to promote the use of safe protective supplies.

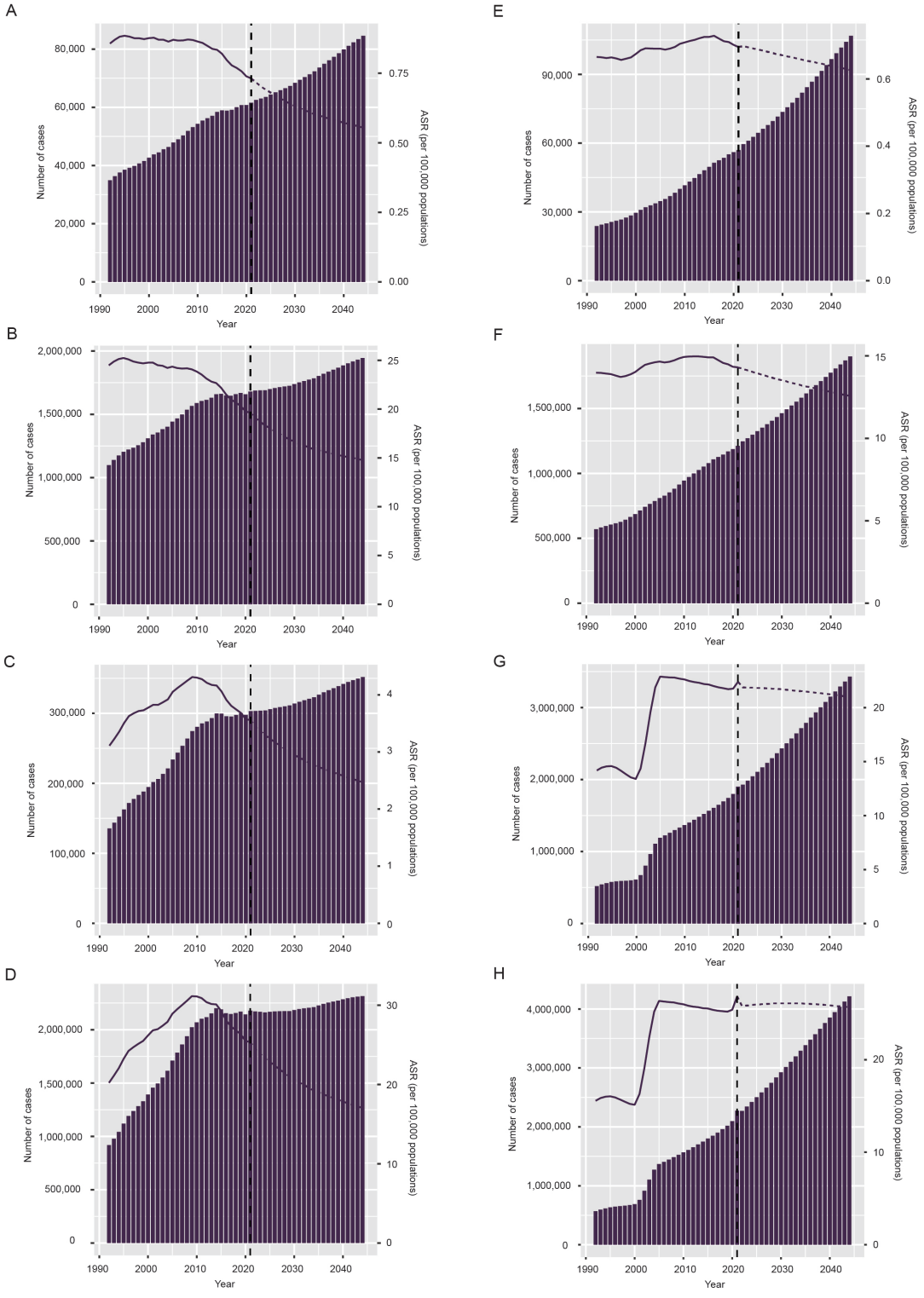
Figure 5. Health inequality regression curves and concentration curves for the incidence of MSM, SCC and BCC worldwide, 1990 and 2021. (**A**, **B**) MSM. (**C**, **D**) SCC. (**E**, **F**) BCC.



Cross-country inequality analysis revealed that the gap in health inequality between low and high SDI regions is expanding. This growing inequality may be the result of economic uncertainty and inadequate public health policies. This indicated that a transnational skin cancer research and monitoring network should be established, where countries could share data, including incidence rates, risk factors, treatment strategies, and prognostic outcomes. Such a network would facilitate a deeper understanding of the evolving trends in inequality and provide a strong foundation for future intervention measures and optimal resource allocation.

While the ASDR, ASDALYs, ASIR, and ASPR for MSM and

Figure 6. Trends in the number and age-standardized rates of deaths, DALYs, incidence, and prevalence attributed to MSM and SCC globally in the next 23 years (2022 - 2044). (**A - D**) MSM. (**E - H**) SCC. (**A**, **E**) deaths. (**B**, **F**) DALYs. (**C**, **G**) incidence. (**D**, **H**) prevalence.



NMSC were forecasted to decline, the absolute numbers for these four indicators were forecasted to rise. This distinction is not only essential for the accurate interpretation of epidemiological indicators but also critical for effective public health messaging [45-46]. The decline in ASIR reflects the success of recent primary prevention efforts for SCC, including widespread sun protection education, improved occupational UV exposure controls, and enhanced early detection initiatives—demonstrating that disease risk at the population level has been substantially reduced. Nevertheless, the projected rise in absolute case numbers should not be interpreted as a failure of these interventions; instead, it is primarily driven by demographic dynamics such as population growth and aging, which increase the total number of susceptible individuals. The rise in numbers was attributable to alterations in population structures, lifestyle and living environments. Decomposition analysis showed that population growth and aging were the main causes of the increase in global DALYs for skin cancer. The process of global population ageing is accelerating continuously. The continuous increase in the global population and the acceleration of the aging process have formed the basic framework for the increase in the disease burden [47]. In addition, the popularity of sun-worshipping culture has led to a significant increase in the time spent on outdoor leisure activities. Therefore, policymakers should seize the opportunity of changes in population structure and lifestyle to improve the healthcare system, consequently enhancing the efficiency and quality of medical services.

Based on the analysis of the regional and population characteristics of the disease burden of SCC, the research results can be translated into the following primary prevention levers, and differentiated strategies should be implemented in combination with the differences in resource endowments and disease drivers between high and low SDI regions. In high SDI regions, (1) Early-life photoprotection: Strengthen structured sun-protection policies and ensure early access to effective photoprotection; (2) Occupational UV exposure: Enforce UV-safety regulations and improve protective measures for outdoor workers; (3) Older-adult screening: Expand routine skin examinations and promote early recognition of lesions; (4) SCC focus: Prioritize surveillance of high-risk groups and streamline diagnostic pathways. In low SDI regions, (1) Early-life photoprotection: Promote low-cost protective behaviors to maintain currently low exposure levels; (2) Occupational UV exposure: Provide basic UV-safety education and simple protective solutions for outdoor laborers; (3) Older-adult screening: Utilize opportunistic screening and enhance lesion recognition in primary care; (4) SCC focus: Improve basic awareness and diagnostic capacity to prevent future burden increases.

Our study had the following limitations. First, regarding the inherent bias of secondary data, although the GBD 2021 data provided support for the integrated analysis of the global burden of skin cancer, its core limitation lies in the heterogeneity of the data sources. Second, the underreporting of skin cancer cases in low-income and middle-income countries is driven by a combination of factors, including limited healthcare access, fragmented or underdeveloped tumor registry systems, and patient-level cognitive barriers. Third, due to the lack of internal data for each country, it is difficult to conduct a detailed analysis of the internal trends within countries.

Conclusions

The disease burden of MSM and NMSC is substantial and is expected to increase in the coming years. The global health inequality in the disease burden of MSM and NMSC persists over time. Globally, there is a need to increase the allocation of medical resources for MSM and NMSC, particularly targeting the elderly population.

Abbreviations

age-standardized rates: ASR; age-standardized death rate: ASDR; age-standardized DALYs rate: ASDALYs; age-standardized incidence rate: ASIR; age-standardized prevalence rate: ASPR; basal cell carcinoma: BCC; disability-adjusted life years: DALYs; Global Burden of Diseases: GBD; Malignant skin melanoma: MSM; Non-melanoma skin cancer: NMSC; squamous cell carcinoma: SCC; Socio-Demographic Index: SDI.

Author Contributions

Zhan Li: Writing – review & editing, Writing – original draft. Yuqin Tang: Data curation, Formal analysis. Yuan Chen: Writing – review & editing, Validation. Yuxin Wang:Conceptualization. Yaping Feng:Writing – review & editing. Yue Li:Writing – review & editing. Yingying Yu: Data curation, Formal analysis. Mingyi Zhang:Data curation, Formal analysis. Fang Wang:Validation, Funding acquisition, Data curation.

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Ethics Approval and Consent to Participate

Not Applicable.

Competing Interests

The authors declare that they have no existing or potential commercial or financial relationships that could create a conflict of interest at the time of conducting this study.

Data Availability

Not Applicable.

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