



UNIVERSITAT  
POLITÈCNICA  
DE VALÈNCIA

**Department of Biotechnology**

**Ph.D. THESIS**

**Understanding Immune Evasion in  
Neuroblastoma: Tumor Microenvironment  
Heterogeneity and Therapeutic Implications**

Esther Coronado Mondragón

Supervisors: **Dr. Jaime Font de Mora Sainz**

**Dra. Adela Cañete Nieto**

UPV tutor: **Dra. María Adelaida García Gimeno**

January, 2025



## **Agradecimientos**

La realización de esta tesis es el resultado del apoyo y la contribución de muchas personas. En estas líneas me gustaría dar las gracias a algunas de ellas:

En primer lugar, agradecer a la Asociación Española Contra el Cáncer (AECC), pieza indispensable en la investigación oncológica en España. En especial, gracias a la AECC Valencia y al movimiento RunCáncer, por creer en este proyecto. A Silvia Queipo y Puri Aguilar, gracias por vuestro cariño y compromiso social. Espero que podamos seguir trabajando conjuntamente para lograr avances en la investigación de esta enfermedad.

A Jaime Font de Mora, por guiarme hasta aquí. Por inculcarme el análisis crítico y enseñarme a perseguir mis objetivos y a defender lo que creo, incluso cuando desafía las normas establecidas.

A Adela Cañete y Victoria Castel, por sus valiosas aportaciones, por haberme ayudado en todo lo que estaba en sus manos para que llevar a término este proyecto haya sido posible.

A mis compañeros/as, pasados y presentes, del Grupo de Investigación Clínica y Traslacional en Cáncer,

Nuria, María y Alberto, me siento inmensamente afortunada por el equipo que hemos creado. Gracias por vuestra actitud positiva, por acompañarme en momentos de euforia y levantar mi ánimo cuando las cosas se ponían difíciles. Pero, sobre todo, gracias por convertirlos en unos amigos increíbles fuera del laboratorio. Vuestro talento y creatividad han sido un impulso para este trabajo.

A Rebeca, por su enorme generosidad, eres un ejemplo de integridad investigadora. A Eva, por sus aportes críticos y positividad contagiosa.

A las nuevas generaciones, y en especial a Susanne, por su energía y alegría. Tu entusiasmo ha sido un soplo de aire fresco, y me ha mostrado que enseñar también puede ser un gran aprendizaje.

A Elena, Sandra, Amanda, Carol y Carmen, por su apoyo y cariño desde mis primeros pasos hasta ahora, por enseñarme “a andar”.

A Yánia, por el acompañamiento cómplice y sus consejos. En tan solo unos meses me enseñaste algo muy importante que no se aprende en los libros.

*To John M. Maris, for not only welcoming me into his lab but also for his support and inspiring research vision. To Tori and Kate, for making it so easy for me to join the Children's Hospital of Philadelphia. Kya, Emily, Nick, Jarret, and Keelan, working with you has been a constant source of energy and growth. Matteo, grazie mille. Your contagious passion and our discussions have been crucial to completing this thesis.*

*To Frank Westermann and Mario Capasso, thank you for your thoughtful comments and contributions that enriched this work.*

A Rubén, mi suerte al otro lado del océano. Gracias por convertir *Philly* en un hogar.

A mis profesores/as que, desde la infancia hasta ahora, han fomentado mi espíritu crítico y desarrollado mi curiosidad. A Ada, por su interés y consejo.

A mi familia y amigos, gracias por vuestro soporte y por vuestras propias luchas y metas, que son una gran inspiración para mí. A losune y Marta, por estar siempre ahí desde el principio.

A mis padres y a mi hermana, vuestro apoyo ilimitado ha sido la base que me ha traído hasta aquí, gracias por dármelo todo. Siempre os esforzasteis en brindarme todas las oportunidades para que pudiera crecer y aprender libremente. Este trabajo es fruto de ese esfuerzo.

A Gema, mi compañera de batallas y alegrías, gracias por tu amor y tu apoyo incombustible. Tu brillantez y determinación son una fuente de motivación en mi día a día.



## Abstract

High-risk neuroblastoma (HR-NB) remains a clinical challenge due to resistance to current treatments and limited actionable mutations for personalized approaches. Our study focused on the interactions between the tumor immune microenvironment (TIME) and HR-NB subtypes to identify novel therapeutic strategies for enhancing immune responses and overcoming treatment resistance.

We observed that 11q-deleted tumors exhibited an inflammatory profile characterized by increased levels of CD8<sup>+</sup> T cells, resting CD4<sup>+</sup> memory T cells, and tumor-associated macrophages (TAMs), along with heightened immunosuppressive mediators PD-L1, IL-10, TGF- $\beta$ 1, and IDO-1. NB cells consistently polarized macrophages toward an M2 phenotype, promoting tumor proliferation and mesenchymal lineage plasticity. Additionally, we assessed the impact of macrophages on CAR-T cell therapy efficacy, discovering that M2 macrophages impaired PHOX2B CAR-T cell function by downregulating MHC I expression. In contrast, M1 macrophages improved CAR-T efficacy by maintaining or increasing it, enhancing antigen presentation.

11q-deleted HR-NB primary tumors exhibited significant genomic instability, characterized by numerous SCAs. We found a reduced expression of essential DNA damage response genes, including *ATM*, *H2AX*, *CHEK1*, and *MRE11A*, with no detectable second-hit mutations. Importantly, this reduction was independent of promoter methylation. Furthermore, *RASSF1A* hypermethylation emerged as a common event in *MYCN*-amplified and 11q-deleted HR-NB and a negative prognostic marker in these tumors. A specific 52 CpG signature also distinguished 11q-deleted HR-NB from *MYCN*-amplified subtypes, regulating poor-prognosis genes *CSF1R* and *CCR7*, associated with macrophages and lymphocyte recruitment and immune suppression.

In conclusion, our findings underscore the pivotal role of TAMs in mediating resistance mechanisms in NB, supporting its targeting as a precision medicine strategy to strengthen antitumor immunity and improve treatment outcomes in high-risk patients.

### Resumen

El neuroblastoma de alto riesgo (HR-NB) representa un importante desafío clínico debido a la resistencia a los tratamientos actuales y a la escasez de mutaciones accionables que permitan enfoques terapéuticos personalizados. Este estudio se centró en investigar las interacciones entre el microambiente inmune tumoral (TIME) y los subtipos de HR-NB, con el objetivo de identificar estrategias capaces de potenciar la respuesta inmune y superar la resistencia al tratamiento.

Los tumores con delección de 11q presentaron un perfil inflamatorio caracterizado por niveles elevados de células T CD8+, células T CD4+ de memoria en reposo y macrófagos asociados al tumor (TAMs). Además, mostraron altos niveles de los mediadores inmunosupresores PD-L1, IL-10, TGF- $\beta$ 1 e IDO-1. Observamos que las células de NB promovían la polarización de los TAMs hacia un fenotipo M2, favoreciendo así la proliferación tumoral y la plasticidad del linaje mesenquimal. Al evaluar el impacto de los macrófagos en la eficacia de la terapia con CAR-T, descubrimos que los macrófagos M2 reducían la eficacia de las CAR-T dirigidas contra PHOX2B al reducir la expresión de MHC I. Por el contrario, los macrófagos M1 mantenían o incluso aumentaban esta expresión, mejorando la presentación de antígenos y la efectividad de las CAR-T.

Asimismo, los tumores primarios de HR-NB con delección de 11q mostraron una marcada inestabilidad genómica, evidenciada por numerosas aberraciones cromosómicas segmentarias (SCAs). Estos tumores también presentaron una menor expresión de genes clave para la reparación del ADN, como *ATM*, *H2AX*, *CHEK1* y *MRE11A*, sin mutaciones adicionales en dichos genes e independientemente de la metilación de su promotor. Paralelamente, identificamos que la hipermetilación de *RASSF1A* era un evento común tanto en tumores amplificados para *MYCN* como en aquellos con delección de 11q, actuando como un marcador pronóstico negativo. También identificamos una firma epigenética compuesta por 52 sitios CpG que permite distinguir ambos subtipos de HR-NB y regula genes clave como *CSF1R* y *CCR7*, asociados al reclutamiento de macrófagos y linfocitos, la supresión inmune y un pronóstico desfavorable.

En conclusión, nuestros hallazgos subrayan el papel crítico de los TAMs en los mecanismos de resistencia del NB y respaldan el desarrollo de estrategias terapéuticas dirigidas contra estos macrófagos como una vía prometedora para reforzar la respuesta inmune antitumoral y mejorar el pronóstico de los pacientes con HR-NB.

## Resum

El neuroblastoma d'alt risc (HR-NB) continua sent un repte clínic a causa de la resistència als tractaments actuals i l'escassetat de mutacions accionables per a enfocaments personalitzats. Aquest estudi es centra en les interaccions entre el microambient immune tumoral (TIME) i els subtipus de HR-NB per a identificar noves estratègies terapèutiques que milloren les respostes immunitàries i superen la resistència al tractament.

Vam observar que els tumors amb deleció en 11q presentaven un perfil inflamatori caracteritzat per nivells elevats de cèl·lules T CD8+, cèl·lules T CD4+ de memòria en repòs i macròfags associats al tumor (TAMs), juntament amb mediadors immunosupressors alts com PD-L1, IL-10, TGF- $\beta$ 1 i IDO-1. Les cèl·lules de NB polaritzaven de manera consistent els macròfags cap a un fenotip M2, promovent la proliferació tumoral i la plasticitat de llinatge mesenquimal. A més, vam evaluar l'impacte dels macròfags en l'eficàcia de la teràpia CAR-T, descobrint que els macròfags M2 deterioraven la funció de les CAR-T específiques de PHOX2B al reduir l'expressió de MHC I. En canvi, els macròfags M1 milloraven l'eficàcia de les CAR-T al mantindre o augmentar aquesta expressió, potenciant la presentació d'antígens.

Els tumors primaris de HR-NB amb deleció en 11q van mostrar una inestabilitat genòmica significativa, caracteritzada per nombroses aberracions cromosòmiques segmentàries (SCAs). Vam trobar una reducció en l'expressió de gens essencials per a la resposta al dany en l'ADN, com *ATM*, *H2AX*, *CHEK1* i *MRE11A*, sense mutacions addicionals detectables. Cal destacar que aquesta reducció era independent de la metilació del promotor. A més, la hipermetilació de *RASSF1A* va sorgir com un esdeveniment comú en tumors amplificats per a *MYCN* i amb deleció en 11q, així com un marcador pronòstic negatiu en aquests casos. Una signatura específica de 52 llocs CpG va diferenciar els HR-NB amb deleció en 11q dels subtipus amplificats per a *MYCN*, regulant gens de mal pronòstic com *CSF1R* i *CCR7*, relacionats amb el reclutament de macròfags i limfòcits i la supressió immune.

En conclusió, les nostres resultats subratllen el paper fonamental dels TAMs en la mediació dels mecanismes de resistència en el NB, donant suport al seu ús com a objectiu en estratègies de medicina de precisió per a millorar la immunitat antitumoral i els resultats del tractament en pacients d'alt risc.



## Index

|                                                                                            |      |
|--------------------------------------------------------------------------------------------|------|
| i. Tables index .....                                                                      | v    |
| ii. Figures index .....                                                                    | vi   |
| iii. Annexes index .....                                                                   | viii |
| iv. List of abbreviations .....                                                            | ix   |
| 1. Introduction.....                                                                       | 1    |
| 1.1. Neuroblastoma.....                                                                    | 1    |
| 1.1.1. Epidemiology and clinical presentation.....                                         | 1    |
| 1.1.2. Staging and risk classification .....                                               | 4    |
| 1.1.3. Risk-stratified treatments.....                                                     | 8    |
| 1.1.3.1. Low-risk treatment.....                                                           | 9    |
| 1.1.3.2. Intermediate-risk treatment.....                                                  | 9    |
| 1.1.3.3. High-risk treatment.....                                                          | 10   |
| 1.1.3.4. Relapsed or refractory treatment.....                                             | 15   |
| 1.1.4. Pathogenesis and molecular alterations .....                                        | 16   |
| 1.1.4.1. Germline mutations .....                                                          | 16   |
| 1.1.4.2. Recurrent somatic alterations and actionable targets .....                        | 17   |
| 1.1.4.2.1. <i>MYCN</i> amplification and <i>MYC</i> overexpression .....                   | 17   |
| 1.1.4.2.2. <i>ALK</i> amplification and mutations .....                                    | 20   |
| 1.1.4.2.3. Activation of telomere maintenance mechanisms.....                              | 21   |
| 1.1.4.2.4. <i>LIN28B</i> polymorphisms .....                                               | 21   |
| 1.1.4.2.5. Segmental chromosomal aberrations.....                                          | 22   |
| 1.1.4.2.6. Epigenetic modifications.....                                                   | 23   |
| 1.1.4.2.7. MicroRNAs.....                                                                  | 23   |
| 1.1.4.2.8. Neurotrophin receptors .....                                                    | 24   |
| 1.1.4.3. Evolution of genetic alterations in neuroblastoma from diagnosis to relapse ..... | 25   |
| 1.1.5. Neuroblastoma cell of origin: the genesis point .....                               | 26   |
| 1.1.6. Neuroblastoma cell plasticity: regulatory switch in pathogenesis .....              | 29   |

|                                                                             |           |
|-----------------------------------------------------------------------------|-----------|
| 1.1.6.1. Transitioning between adrenergic and mesenchymal cell states ..... | 29        |
| 1.1.6.2. Neuroblastoma plasticity as a driver of resistance .....           | 31        |
| 1.1.7. Immune defense and escape in neuroblastoma .....                     | 32        |
| 1.1.7.1. Immune recognition of tumor cells .....                            | 32        |
| 1.1.7.2. Immune evasion strategies in neuroblastoma .....                   | 34        |
| 1.1.7.2.1. Immunogenicity .....                                             | 34        |
| 1.1.7.2.2. Immune checkpoint molecules .....                                | 35        |
| 1.1.7.2.3. Immunosuppressive myeloid and stromal cells .....                | 35        |
| 1.1.7.2.4. Soluble mediators of immune suppression .....                    | 36        |
| 1.1.7.3. Implications for immunotherapy .....                               | 37        |
| 1.1.8. Emerging immunotherapies in neuroblastoma .....                      | 39        |
| 1.1.8.1. Antibody-based immunotherapies .....                               | 39        |
| 1.1.8.2. Adoptive cellular therapies .....                                  | 41        |
| 1.1.8.2.1. T-cell therapy .....                                             | 41        |
| 1.1.8.2.2. NK-cell therapy .....                                            | 42        |
| 1.1.8.2.3. Vaccines .....                                                   | 43        |
| <b>2. Hypothesis and objectives .....</b>                                   | <b>45</b> |
| <b>3. Materials and methods .....</b>                                       | <b>47</b> |
| 3.1. Patients .....                                                         | 47        |
| 3.2. Cell culture and culture conditions .....                              | 48        |
| 3.2.1. Neuroblastoma cell lines .....                                       | 48        |
| 3.2.2. Neuroblastoma primary cell lines .....                               | 49        |
| 3.2.3. Neuroblastoma cell lines for CAR-T assay .....                       | 49        |
| 3.2.4. Neuroblastoma patient-derived organoids .....                        | 49        |
| 3.2.5. Monocytes cell line and primary human monocytes .....                | 50        |
| 3.3. Monocyte differentiation and macrophage polarization .....             | 51        |
| 3.4. Co-culture system .....                                                | 51        |
| 3.5. CAR-T killing assay .....                                              | 51        |
| 3.6. Cytokine secretion analysis .....                                      | 52        |

|           |                                                                                                      |           |
|-----------|------------------------------------------------------------------------------------------------------|-----------|
| 3.7.      | Single nucleotide polymorphism arrays .....                                                          | 52        |
| 3.8.      | Cell viability assays .....                                                                          | 52        |
| 3.9.      | Gene expression analyses .....                                                                       | 53        |
| 3.9.1.    | RNA isolation and cDNA synthesis.....                                                                | 53        |
| 3.9.2.    | Quantitative PCR .....                                                                               | 54        |
| 3.9.3.    | Droplet digital PCR.....                                                                             | 55        |
| 3.9.4.    | Flow cytometry .....                                                                                 | 56        |
| 3.10.     | Tumor-infiltrating immune cells analysis .....                                                       | 57        |
| 3.10.1.   | High-risk neuroblastoma datasets .....                                                               | 57        |
| 3.10.2.   | Deconvolution of immune cell subsets .....                                                           | 58        |
| 3.11.     | Methylome profiling.....                                                                             | 58        |
| 3.11.1.   | Liquid biopsy sampling and data processing .....                                                     | 58        |
| 3.11.2.   | Annotation of CpG islands.....                                                                       | 59        |
| 3.12.     | miRNA analysis .....                                                                                 | 60        |
| 3.13.     | Transcriptomic and epigenomic datasets.....                                                          | 60        |
| 3.14.     | Statistical analyses .....                                                                           | 62        |
| <b>4.</b> | <b>Results .....</b>                                                                                 | <b>63</b> |
| 4.1.      | Half of high-risk neuroblastoma patients with 11q deletion relapse after anti-GD2 immunotherapy..... | 63        |
| 4.2.      | Immunosuppressive tumor microenvironment in 11q-deleted high-risk neuroblastomas .....               | 67        |
| 4.2.1.    | Increased immune infiltrating cells in 11q-deleted high-risk neuroblastomas                          | 67        |
| 4.2.2.    | Immunosuppressive molecules in 11q-deleted high-risk neuroblastomas..                                | 70        |
| 4.3.      | Intrinsic DNA repair defects in 11q-deleted high-risk neuroblastomas .....                           | 71        |
| 4.4.      | Regulatory Interactions of 11q-Deleted miRNAs .....                                                  | 74        |
| 4.5.      | Neuroblastoma cells induce macrophage polarization towards the M2 phenotype.....                     | 77        |
| 4.6.      | M2 macrophages increase neuroblastoma cell proliferation .....                                       | 82        |

|           |                                                                                                                                       |            |
|-----------|---------------------------------------------------------------------------------------------------------------------------------------|------------|
| 4.7.      | M2 macrophages induce neuroblastoma stemness and mesenchymal phenotype.....                                                           | 84         |
| 4.8.      | Tumor-associated macrophages and their impact on PHOX2B PC-CAR T-cell efficacy in neuroblastoma .....                                 | 87         |
| 4.8.1.    | Enhanced PHOX2B PC-CAR T-cell tumor cell killing by M1 macrophages                                                                    | 87         |
| 4.8.2.    | MHC I suppression by M2 macrophages hinder PHOX2B PC CAR-T cell-mediated killing in neuroblastoma cells .....                         | 94         |
| 4.9.      | Methylome profiling of liquid biopsies from high-risk neuroblastomas.....                                                             | 98         |
| 4.9.1.    | Differential epigenetic signatures distinguish high-risk neuroblastomas with 11q deletion and <i>MYCN</i> amplification.....          | 98         |
| 4.9.2.    | Reduced expression of 11q double-strand break repair genes is independent of their promoter methylation .....                         | 107        |
| <b>5.</b> | <b>Discussion .....</b>                                                                                                               | <b>109</b> |
| 5.1.      | Impact and limitations of anti-GD2 immunotherapy in 11q-deleted high-risk neuroblastoma: insights from the HR-NBL1/SIOPEN trial ..... | 109        |
| 5.2.      | Exploring and targeting the tumor immune microenvironment of 11q-deleted high-risk neuroblastomas.....                                | 111        |
| 5.3.      | Genetic instability of 11q-deleted high-risk neuroblastomas .....                                                                     | 116        |
| 5.4.      | Regulatory landscape of 11q-deleted miRNAs in neuroblastoma .....                                                                     | 117        |
| 5.5.      | Collaboration between M2 macrophages and neuroblastoma cells promotes a tumor-favorable environment .....                             | 119        |
| 5.6.      | Macrophage-induced mesenchymal phenotype: a link to 11q deletion ....                                                                 | 122        |
| 5.7.      | Macrophage polarization: a double-edged sword in CAR-T cell therapy for neuroblastoma .....                                           | 124        |
| 5.8.      | Epigenetic alterations impacting the tumor microenvironment and immune response in 11q-deleted high-risk neuroblastomas .....         | 126        |
| <b>6.</b> | <b>Conclusions .....</b>                                                                                                              | <b>131</b> |
| <b>7.</b> | <b>References .....</b>                                                                                                               | <b>133</b> |
| <b>8.</b> | <b>Annexes .....</b>                                                                                                                  | <b>149</b> |



## i. Tables index

|                                                                                                                                   |     |
|-----------------------------------------------------------------------------------------------------------------------------------|-----|
| Table 1. International Neuroblastoma Staging System (INSS). .....                                                                 | 4   |
| Table 2. Long-term toxicities of neuroblastoma therapy. ....                                                                      | 14  |
| Table 3. Recurrent genetic alterations in neuroblastoma upon diagnosis and relapse. ....                                          | 25  |
| Table 4. Neuroblastoma cell characteristics. ....                                                                                 | 48  |
| Table 5. Primer details used for qPCR. ....                                                                                       | 55  |
| Table 6. Primer details used for ddPCR. ....                                                                                      | 56  |
| Table 7. Antibodies details for flow cytometry analyses. ....                                                                     | 57  |
| Table 8. Transcriptomic and epigenomic datasets. ....                                                                             | 61  |
| Table 9. Clinical characteristics of the Spanish HR-NB cohort enrolled at HR-NBL/SIOPEN trial. ....                               | 64  |
| Table 10. Outcomes after anti-GD2-based immunotherapy compared to isotretinoin alone in high-risk neuroblastoma. ....             | 65  |
| Table 11. Functional annotation of the 52 CpG signature discriminating 11q-deleted and <i>MYCN</i> -amplified high-risk NBs. .... | 101 |

## **ii. Figures index**

|                                                                                                                                                              |    |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| Figure 1. Primary distribution of neuroblastoma in children. ....                                                                                            | 3  |
| Figure 2. COG risk stratification schema version 2. ....                                                                                                     | 8  |
| Figure 3. Outcome analyses based on COG version 2 risk classification system. ....                                                                           | 9  |
| Figure 4. Multimodal therapy for high-risk neuroblastoma. ....                                                                                               | 11 |
| Figure 5. Germline risk of neuroblastoma. ....                                                                                                               | 17 |
| Figure 6. MYCN stabilization mechanisms. ....                                                                                                                | 19 |
| Figure 7. Neural crest and neuroblastoma development. ....                                                                                                   | 27 |
| Figure 8. Cancer immunoediting model. ....                                                                                                                   | 33 |
| Figure 9. Immunosuppression mechanisms and emerging immunotherapies in<br>neuroblastoma. ....                                                                | 40 |
| Figure 10. Improved 3-year EFS and OS in high-risk neuroblastoma patients treated with<br>anti-GD2-based immunotherapy compared to isotretinoin alone. ....  | 66 |
| Figure 11. Tumor-infiltrating immune subsets in high-risk neuroblastoma subgroups:<br>analysis of the Westermann cohort. ....                                | 68 |
| Figure 12. Tumor-infiltrating immune subsets in high-risk neuroblastoma subgroups:<br>analysis of the TARGET cohort. ....                                    | 69 |
| Figure 13. 11q-deleted high-risk neuroblastoma exhibits higher expression of<br>immunosuppressive molecules. ....                                            | 70 |
| Figure 14. Heightened incidence of segmental chromosomal aberrations within the high-<br>risk subgroup with 11q deletion. ....                               | 71 |
| Figure 15. The proportion of differentially expressed genes across chromosomes in 11q-<br>deleted compared to non-11q-deleted high-risk neuroblastomas. .... | 72 |
| Figure 16. Decreased expression of DNA double-strand break repair genes in 11q-<br>deleted high-risk neuroblastomas. ....                                    | 73 |
| Figure 17. Regulatory network orchestrated by 11q-deleted miRNAs. ....                                                                                       | 74 |
| Figure 18. Differential expression of genes targeted by 11q miRNAs between high-risk-<br>neuroblastoma subgroups. ....                                       | 76 |
| Figure 19. Schematic representation of the differentiation and characterization process<br>for generating reference macrophage populations. ....             | 78 |
| Figure 20. Neuroblastoma cells induce monocyte differentiation and polarization towards<br>M2 phenotype. ....                                                | 80 |
| Figure 21. Validation of the effects of neuroblastoma cells on monocyte differentiation<br>and polarization. ....                                            | 81 |
| Figure 22. M2 macrophages increase neuroblastoma cell proliferation. ....                                                                                    | 82 |

|                                                                                                                                                         |     |
|---------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| Figure 23. M2 macrophages increase neuroblastoma expression of stemness and mesenchymal markers.....                                                    | 84  |
| Figure 24. Gene set correlation enrichment analysis of ADRN and MES lineage identities in neuroblastoma cell lines.....                                 | 85  |
| Figure 25. Gene set correlation enrichment analysis of ADRN and MES lineage identities in neuroblastoma primary tumors.....                             | 86  |
| Figure 26. Effect of macrophages on PHOX2B PC-CAR T cell-mediated killing of SK-N-AS cell line.....                                                     | 88  |
| Figure 27. Effect of macrophages on PHOX2B PC-CAR T cell-mediated killing of SK-N-BE(2) HLA-A*23:01 cell line.....                                      | 89  |
| Figure 28. Effect of macrophages on PHOX2B PC-CAR T cell-mediated killing of SK-N-BE(2) HLA-A*24:02 cell line.....                                      | 90  |
| Figure 29. Impact of macrophages on PHOX2B PC-CAR T cell-mediated killing of NB-EBC1 HLA-A*23:01 cell line.....                                         | 91  |
| Figure 30. Impact of macrophages on PHOX2B PC-CAR T cell-mediated killing of NB-EBC1 HLA-A*24:02 cell line.....                                         | 92  |
| Figure 31. MHC CLASS I expression on SK-N-AS cells co-cultured with M1 or M2 macrophages.....                                                           | 95  |
| Figure 32. MHC Class I expression on SK-N-BE(2) co-cultured with M1 or M2 macrophages.....                                                              | 96  |
| Figure 33. MHC Class I expression on NB-EBC1 co-cultured with M1 or M2 macrophages.....                                                                 | 97  |
| Figure 34. Distinct epigenetic patterns differentiate high-risk neuroblastomas with 11q deletion from those with <i>MYCN</i> amplification.....         | 99  |
| Figure 35. Hypermethylation of <i>RASSF1A</i> is an early joint event for high-risk neuroblastoma with <i>MYCN</i> -amplification and 11q-deletion..... | 103 |
| Figure 36. Epigenetic phylogeny of high-risk NB with <i>MYCN</i> -amplification and 11q-deletion.....                                                   | 104 |
| Figure 37. Differential methylation of CpGs with prognostic significance within the 11q subgroup of high-risk neuroblastomas.....                       | 105 |
| Figure 38. Reduced expression of DNA DSB repair genes is independent of their promoter methylation.....                                                 | 107 |
| Figure 39. Immunosuppressive microenvironment in high-risk neuroblastomas with 11q deletion.....                                                        | 114 |

**iii. Annexes index**

|                                                                                                                                                 |     |
|-------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| Annex 1. Clinical characteristics of the Liquidhope cohort.....                                                                                 | 149 |
| Annex 2. Ordinal regression analysis of differential tumor-infiltrating immune populations<br>of Westermann cohort (GSE73517).....              | 150 |
| Annex 3. Ordinal regression analysis of differential tumor-infiltrating immune populations<br>of TARGET cohort. ....                            | 151 |
| Annex 4. Expression of immunosuppressive genes in TARGET cohort. ....                                                                           | 152 |
| Annex 5. Decreased expression of double-strand break DNA repair genes in 11q-deleted<br>high-risk neuroblastomas within the TARGET cohort. .... | 153 |
| Annex 6. Differential expression of targeted genes by the 11q-deleted miRNA in the<br>TARGET cohort. ....                                       | 154 |
| Annex 7. Baseline expression levels of VIM and CDH2 in neuroblastoma cell lines..                                                               | 155 |
| Annex 8. Methylation levels of the promoter region of DSB repair-related genes in the<br>TARGET cohort. ....                                    | 156 |

#### iv. List of abbreviations

|                             |                                               |
|-----------------------------|-----------------------------------------------|
| <b>γΔ T</b>                 | Gamma Delta T                                 |
| <b>13-CRA</b>               | 13-cis-Retinoic Acid                          |
| <b><sup>131</sup>I-MIBG</b> | <sup>131</sup> I- Metaiodobenzylguanidine     |
| <b>ACD</b>                  | Autophagic Cell Death                         |
| <b>ADC</b>                  | Antibody-Drug Conjugate                       |
| <b>ADCC</b>                 | Antibody-Dependent Cell-Mediated Cytotoxicity |
| <b>ADR</b>                  | Adrenergic                                    |
| <b>ALT</b>                  | Alternative Lengthening of Telomeres          |
| <b>AMT</b>                  | Adrenergic to Mesenchymal Transition          |
| <b>ANOVA</b>                | Analysis of Variance                          |
| <b>APC</b>                  | Antigen-presenting cell                       |
| <b>ASCT</b>                 | Autologous Stem Cell Transplantation          |
| <b>ATCC</b>                 | American Type Culture Collection              |
| <b>BCA</b>                  | Bicinchoninic Acid                            |
| <b>BET</b>                  | Bromodomain and Extra-Terminal Domain         |
| <b>BiKEs</b>                | Bispecific Killer Cell Engagers               |
| <b>BiTE</b>                 | Bispecific T-Cell Engager                     |
| <b>BMP</b>                  | Bone Morphogenetic Protein                    |
| <b>BP</b>                   | Base Pair                                     |
| <b>BSA</b>                  | Bovine Serum Albumin                          |
| <b>CARs</b>                 | Chimeric Antigen Receptors                    |
| <b>cCREc</b>                | Candidate Cis-Regulatory Elements             |
| <b>CDCC</b>                 | Complement-Dependent Cellular Cytotoxicity    |
| <b>cDNA</b>                 | Complementary DNA                             |
| <b>cfDNA</b>                | Circulating Free DNA                          |
| <b>CH14.18/CHO</b>          | Chimeric 14.18 Antibody/Choline               |
| <b>CHAS</b>                 | Chromosome Analysis Suite                     |
| <b>CHOP</b>                 | Children's Hospital of Philadelphia           |
| <b>CHR</b>                  | Chromosome                                    |
| <b>CI</b>                   | Confidence Interval                           |
| <b>CM</b>                   | Conditioned Medium                            |
| <b>CMC</b>                  | Complement-Mediated Cytotoxicity              |
| <b>CNA</b>                  | Copy Number Alteration                        |
| <b>CNV</b>                  | Copy Number Variant                           |

## List of abbreviations

|               |                                                                       |
|---------------|-----------------------------------------------------------------------|
| <b>COG</b>    | Children's Oncology Group                                             |
| <b>COJEC</b>  | Cyclophosphamide, Vincristine, Etoposide, and Carboplatin             |
| <b>CPG</b>    | Cytosine-Phosphate-Guanine                                            |
| <b>CRCs</b>   | Core Regulatory Circuitries                                           |
| <b>CTCF</b>   | CCCTC-Binding Factor                                                  |
| <b>CTLs</b>   | Cytotoxic T Lymphocytes                                               |
| <b>DART</b>   | Dual-Affinity Retargeting                                             |
| <b>DCs</b>    | Dendritic Cells                                                       |
| <b>ddPCR</b>  | Digital Droplet PCR                                                   |
| <b>DDR</b>    | DNA Damage Response                                                   |
| <b>DEL</b>    | Deletion                                                              |
| <b>DI</b>     | DNA Index                                                             |
| <b>DMEM</b>   | Dulbecco's Modified Eagle Medium                                      |
| <b>DMSO</b>   | Dimethyl Sulfoxide                                                    |
| <b>DNA</b>    | Deoxyribonucleic Acid                                                 |
| <b>DSB</b>    | Double-Strand Break                                                   |
| <b>EDTA</b>   | Ethylenediaminetetraacetic Acid                                       |
| <b>EFS</b>    | Event Free Survival                                                   |
| <b>EGF</b>    | Epidermal Growth Factor                                               |
| <b>EGTA</b>   | Ethylene Glycol-Bis(B-Aminoethyl Ether)-N, N, N', N'-Tetraacetic Acid |
| <b>EM-SEQ</b> | Enzymatic Methyl-Seq                                                  |
| <b>EMT</b>    | Epithelial-to-Mesenchymal Transition                                  |
| <b>ENCODE</b> | Encyclopedia of DNA Elements                                          |
| <b>ENHd</b>   | Distal Enhancer                                                       |
| <b>ENHp</b>   | Proximal Enhancer                                                     |
| <b>ENQUA</b>  | European Neuroblastoma Quality Assessment                             |
| <b>FA</b>     | Fractional Abundance                                                  |
| <b>FBS</b>    | Fetal Bovine Serum                                                    |
| <b>FDA</b>    | US Food and Drug Administration                                       |
| <b>FDR</b>    | False Discovery Rate                                                  |
| <b>FISH</b>   | Fluorescence In Situ Hybridization                                    |
| <b>GD2</b>    | Disialoganglioside                                                    |
| <b>GEO</b>    | Gene Expression Omnibus                                               |
| <b>GM-CSF</b> | Granulocyte-Macrophage Colony-Stimulating Factor                      |

## List of abbreviations

|               |                                                                         |
|---------------|-------------------------------------------------------------------------|
| <b>GO</b>     | Gene Ontology                                                           |
| <b>GSEA</b>   | Gene Set Enrichment Analysis                                            |
| <b>HDAC</b>   | Histone Deacetylase                                                     |
| <b>HEX</b>    | Hexachlorofluorescein                                                   |
| <b>HLA</b>    | Human Leukocyte Antigen                                                 |
| <b>HR</b>     | Hazard Ratio (Log-Rank Tests)                                           |
| <b>HR-NB</b>  | High-Risk Neuroblastoma                                                 |
| <b>HSA</b>    | Homo Sapiens                                                            |
| <b>ICI</b>    | Immune Checkpoint Inhibitor                                             |
| <b>ID</b>     | Identifier                                                              |
| <b>IL</b>     | Interleukin                                                             |
| <b>IMDM</b>   | Iscove's Modified Dulbecco's Medium                                     |
| <b>INPC</b>   | International Neuroblastoma Pathology Classification                    |
| <b>INRG</b>   | International Neuroblastoma Risk Group                                  |
| <b>INRGSS</b> | International Neuroblastoma Risk Group Staging System                   |
| <b>INSS</b>   | International Neuroblastoma Staging System                              |
| <b>IR</b>     | Intermediate-Risk                                                       |
| <b>ITS</b>    | Insulin-Transferrin-Selenium                                            |
| <b>K4M3</b>   | Dnase-H3k4me3-Based Stratification of Candidate Cis-Regulatory Elements |
| <b>LPS</b>    | Lipopolysaccharides                                                     |
| <b>LR</b>     | Low-Risk                                                                |
| <b>MAB</b>    | Monoclonal Antibody                                                     |
| <b>M-CSF</b>  | Macrophage Colony-Stimulating Factor                                    |
| <b>MDSC</b>   | Myeloid-Derived Suppressor Cells                                        |
| <b>MES</b>    | Mesenchymal                                                             |
| <b>MFI</b>    | Median Fluorescence Intensity                                           |
| <b>MHC</b>    | Major Histocompatibility Complex                                        |
| <b>MiRNA</b>  | Micro RNA                                                               |
| <b>MLPA</b>   | Multiplex Ligation-dependent Probe Amplification                        |
| <b>MNA</b>    | <i>MYCN</i> Amplification                                               |
| <b>Mφ</b>     | Macrophage                                                              |
| <b>mRNA</b>   | Messenger RNA                                                           |
| <b>MS</b>     | Metastatic Special                                                      |
| <b>N</b>      | Number                                                                  |

## List of abbreviations

|                |                                                                     |
|----------------|---------------------------------------------------------------------|
| <b>NA</b>      | Not-Applicable                                                      |
| <b>NB</b>      | Neuroblastoma                                                       |
| <b>NbCDB</b>   | Neuroblastoma Clinical Database                                     |
| <b>NCA</b>     | Numerical Chromosomal Aberrations                                   |
| <b>NCC</b>     | Neural Crest Cell                                                   |
| <b>NK</b>      | Natural Killer                                                      |
| <b>NOS</b>     | Nitric Oxide Synthase                                               |
| <b>NS</b>      | Not Significant                                                     |
| <b>OMIM</b>    | Online Mendelian Inheritance in Man                                 |
| <b>OMS</b>     | Opsoclonus Myoclonus Syndrome                                       |
| <b>ORR</b>     | Overall Response Rate                                               |
| <b>OS</b>      | Overall Survival                                                    |
| <b>PBMC</b>    | Peripheral blood mononuclear cell                                   |
| <b>PBS</b>     | Phosphate Buffered Saline                                           |
| <b>PC-CAR</b>  | Peptide-Centric Chimeric Antigen Receptors                          |
| <b>PCA</b>     | Principal Component Analysis                                        |
| <b>PCR</b>     | Polymerase Chain Reaction                                           |
| <b>PDTO</b>    | Patient-Derived Tumor Organoid                                      |
| <b>PEN</b>     | Penicillin                                                          |
| <b>PMA</b>     | Phorbol 12-Myristate 13-Acetate                                     |
| <b>PROM</b>    | Promoter-like signature                                             |
| <b>PROTACs</b> | Proteolysis-Targeting Chimeras                                      |
| <b>qRT-PCR</b> | Quantitative Reverse Transcription Polymerase Chain Reaction        |
| <b>QVD-OPH</b> | Quinolyl-Valyl-O-Methylaspartyl-[2,6-Difluorophenoxy]-Methyl Ketone |
| <b>R/R</b>     | Relapsed/Refractory                                                 |
| <b>RA</b>      | 13-Cis Retinoic Acid (Isotretinoin)                                 |
| <b>rDHSs</b>   | Repeated Dnase Hypersensitivity Sites                               |
| <b>REMARK</b>  | Reporting Recommendations for Tumor Marker Prognostic Studies       |
| <b>RNA</b>     | Ribonucleic Acid                                                    |
| <b>RPMI</b>    | Roswell Park Memorial Institute                                     |
| <b>RT-qPCR</b> | Real-Time Polymerase Chain Reaction                                 |
| <b>SCA</b>     | Segmental Chromosomal Aberration                                    |
| <b>SD</b>      | Standard Deviation                                                  |



|                         |                                                                      |
|-------------------------|----------------------------------------------------------------------|
| <b>SE</b>               | Super-Enhancer                                                       |
| <b>SE (statistical)</b> | Standard Error                                                       |
| <b>SEM</b>              | Standard Error of the Mean                                           |
| <b>SG</b>               | Sympathetic Ganglia                                                  |
| <b>SIOPEN</b>           | International Society of Paediatric Oncology European Neuroblastoma  |
| <b>SNP</b>              | Single Nucleotide Polymorphism                                       |
| <b>SRO</b>              | Smallest Region of Overlapping Deletion                              |
| <b>STREP</b>            | Streptomycin                                                         |
| <b>TAA</b>              | Tumor-Associated Antigens                                            |
| <b>TAMs</b>             | Tumor-Associated Macrophages                                         |
| <b>TARGET</b>           | Therapeutically Applicable Research to Generate Effective Treatments |
| <b>TCR</b>              | T Cell Receptor                                                      |
| <b>TF</b>               | Transcription Factor                                                 |
| <b>TFH</b>              | T Follicular Helper                                                  |
| <b>TIC</b>              | Tumor-infiltrating immune cells                                      |
| <b>TIL</b>              | Tumor-Infiltrating Lymphocyte                                        |
| <b>TIME</b>             | Tumor Immune Microenvironment                                        |
| <b>TMB</b>              | Tumor Mutational Burden                                              |
| <b>TME</b>              | Tumor Microenvironment                                               |
| <b>TLRs</b>             | Toll-Like Receptors                                                  |
| <b>TREG</b>             | Regulatory T Cells                                                   |
| <b>TRIKEs</b>           | Trispecific Killer Cell Engagers                                     |
| <b>TRIS</b>             | Tris(Hydroxymethyl)Aminomethane                                      |
| <b>TSS</b>              | Transcription Start Site                                             |
| <b>TVD</b>              | Topotecan-Vincristine-Doxorubicin                                    |
| <b>UCSC</b>             | University of California Santa Cruz                                  |
| <b>UT</b>               | Untransduced                                                         |
| <b>VS</b>               | Versus                                                               |
| <b>WGBS</b>             | Whole-Genome Bisulfite Sequencing                                    |



## **1. Introduction**

### **1.1. Neuroblastoma**

Cancer remains one of the leading causes of childhood mortality worldwide. Although survival rates have increased to around 80% in recent decades, the prognosis of some subtypes of solid tumors, especially neuroblastoma (NB), remains very unfavorable, and patients who survive often experience significant long-term sequelae [1, 4, 5]. Pediatric solid tumors have a very different pathophysiology than tumors in adults. They stem from a failure during the development of embryonic tissues. Typically, oncogenic targets involve mutated or overexpressed transcription factors (TFs) and epigenetic alterations, traditionally deemed challenging or nearly impossible to inhibit with drugs [6].

NB, the most prevalent and lethal extracranial solid tumor in childhood, has long captivated and confounded both clinical and basic researchers. These tumors exhibit highly diverse clinical outcomes, ranging from spontaneous regression to fatal disease. Yet, for most children over one-year-old, diagnosis reveals extensive or metastatic disease, resulting in a generally bleak prognosis [4, 5, 7]. Advancements in molecular genetics have shed light on the underlying mechanisms driving distinct clinical behaviors in high-risk patients, paving the way for personalized therapies. However, the low number of mutations in these tumors and the limited understanding of treatment resistance suggest that many pathogenic mechanisms are still waiting to be uncovered [5, 8].

Within the framework of this thesis, we have investigated the role of the tumor microenvironment in NB behavior and its impact on the antitumor immune response. The following section aims to bring together our current understanding of NB biology and the limits of knowledge in developing more effective therapies, with particular attention to immunotherapeutic strategies.

#### **1.1.1. Epidemiology and clinical presentation**

NB is a neuroendocrine tumor that arises in the developing nervous system, resulting in tumors in the adrenal glands or along the sympathetic chain [5]. It presents a very heterogeneous clinical course ranging from spontaneous regression to aggressive metastatic disease. Despite intensive multimodal therapy, some patients face rapid progression with fatal outcomes. The OS for patients with low-risk disease is 85-90%. In contrast, more than half of the children diagnosed with the high-risk subtype will either

## **Introduction**

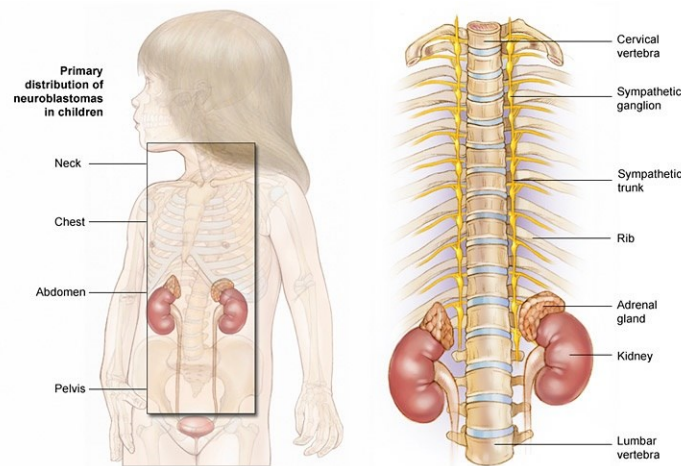
### **Epidemiology and clinical presentation**

---

not respond to current therapies or relapse after treatment, with a post-relapse OS of less than 10-20% [4, 5]. This variable course results from its high biological heterogeneity and clinical characteristics, such as the patient's age and the degree of tumor dissemination. Current protocols aim to identify better response predictors and discover molecular alterations that may represent actionable therapeutic targets [4, 5].

NB typically manifests in early childhood, with a median age at diagnosis of 18 months. Approximately 40% of cases are identified during infancy, and 90% are diagnosed before the age of 10 [9]. Age at diagnosis is a crucial prognostic factor; patients younger than 18 months generally have a more favorable prognosis compared to older children [7, 9]. Adolescents and adults rarely develop NB, accounting for less than 5% of all cases. However, when they do, the disease tends to have a more indolent course and often displays resistance to standard chemotherapy [10].

The clinical presentation of NB is closely tied to the location of the primary tumor and sites of metastasis (Figure 1). Tumors can arise anywhere along the sympathetic nervous system, with over 50% originating in the adrenal medulla [5, 11]. Primary tumors in the adrenal glands are associated with poorer outcomes than those in other locations. While the localized disease may be asymptomatic, large abdominal tumors can lead to symptoms such as hypertension, abdominal distension, and pain. Tumors in the neck may cause Horner syndrome, characterized by ptosis, miosis, enophthalmos, and anhidrosis. Tumors originating from the paravertebral sympathetic ganglia can extend along spinal nerves toward the spinal cord and invade the neural foramina, resulting in spinal cord compression [5, 11].



**Figure 1. Primary distribution of neuroblastoma in children.** NB can develop along the sympathetic nervous system, although it predominantly originates in the adrenal medulla. Metastases from NB commonly spread to nearby regional lymph nodes and the bone marrow through the bloodstream and the sympathetic system. Infiltration of cortical bone by metastatic tumor cells in the marrow is also observed. Additionally, NB can metastasize to the liver, particularly in patients with stage 4S tumors, where involvement may be widespread. Remarkably, complete regression often occurs in stage 4S tumors with no intervention other than supportive care. Source: [5].

Metastasis is identified in roughly half of all cases. NB can metastasize to various sites, including regional lymph nodes, bone marrow, bone, liver, skin, and, rarely, the central nervous system. Metastatic disease may present with constitutional symptoms like bone pain, fever, weight loss, pallor, or bleeding due to anemia or thrombocytopenia. Specific metastatic sites can cause localized symptoms such as bone pain, limping, periorbital bruising, or proptosis [12, 13].

In some cases, NB may occur with associated syndromes. For instance, vasoactive intestinal peptide hypersecretion by tumor cells can lead to profuse, watery diarrhea. Additionally, some patients develop opsoclonus-myoclonus syndrome (OMS), a rare neurological disorder characterized by rapid eye movements, muscle twitching, irritability, and cerebellar ataxia [14].

**1.1.2. Staging and risk classification**

Pediatric cooperative groups have developed classification systems integrating clinical and biological prognostic markers to guide risk-based treatment approaches for NB patients. The primary international cooperative groups leading these efforts are the Children's Oncology Group (COG) in the United States and the International Society of Pediatric Oncology European Neuroblastoma (SIOPEN) in Europe. Their collaborative endeavors have resulted in the development of two central staging systems: the International Neuroblastoma Staging System (INSS) and the International NB Risk Group (INRG) Staging System [7, 15]. The INSS assesses the extent of surgical resection and the presence of metastases at diagnosis (Table 1) [15].

**Table 1.** International Neuroblastoma Staging System (INSS).

| <b>Stage</b>    | <b>Description</b>                                                                                                                                                                                                                                                                |
|-----------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>Stage 1</b>  | Presence of a localized tumor that has been completely surgically removed, without any metastasis to nearby lymph nodes not attached to the tumor.                                                                                                                                |
| <b>Stage 2A</b> | Presence of a localized tumor with incomplete surgical removal and no lymph node involvement.                                                                                                                                                                                     |
| <b>Stage 2B</b> | A localized tumor that may or may not have been completely removed surgically, accompanied by metastasis to nearby lymph nodes on the same side as the tumor, with no spread to lymph nodes on the opposite side.                                                                 |
| <b>Stage 3</b>  | Unresectable tumor located on one side of the body, crossing the midline, along with metastasis to regional lymph nodes. It can also include a localized tumor on one side with metastasis to lymph nodes on the opposite side or a midline tumor with involvement on both sides. |
| <b>Stage 4</b>  | Presence of any primary tumor with distant metastasis to lymph nodes or other organs.                                                                                                                                                                                             |
| <b>Stage 4S</b> | They are also called "special". Applicable to patients under one year old, this stage involves a localized primary tumor (stages 1, 2A, or 2B) with limited metastasis to the skin, liver, or bone marrow (with less than 10% tumor involvement).                                 |

---

Adapted from: [15]

The INRG Staging System is designed to categorize patients into homogeneous risk groups before treatment initiation, facilitating comparisons across clinical trials conducted by the different international cooperative groups [7]. Disease extent is assessed by the presence or absence of image-defined risk factors (IDRFs) and metastatic disease at the time of diagnosis, delineating disease INRG stages into local (L1 and L2) or metastatic (M and MS) categories [16]. Localized disease is defined as L1 when the tumor remains confined to a single body compartment such as the neck, thorax, abdomen, or pelvis without IDRFs, and L2 when it has one or more IDRFs. Metastatic disease is identified as M, indicating widely disseminated disease, or MS, indicating metastatic disease in infants (younger than 18 months of age) restricted to the liver, skin, and bone marrow without cortical bone involvement [16].

The disease stage is integrated alongside additional prognostic factors to delineate pretreatment risk groups, including (I) age at diagnosis, (II) tumor histology, (III) *MYCN* status, (IV) DNA index or ploidy, and (V) segmental chromosome aberrations (SCAs).

- I. **Age at diagnosis:** Older age is associated with poorer outcomes. Recent studies have suggested age cutoffs for risk stratification between 12 and 18 months. Notably, metastatic disease in children under 18 months is not necessarily associated with poor outcomes as in older patients [9, 17]. The reasons behind age-related differences in outcomes remain unclear.
- II. **Tumor histology:** Based on the International Neuroblastoma Pathology Classification (INPC) criteria, histological characterization and grading neuroblastic differentiation are used to classify tumors into favorable and unfavorable groups. The INPC provides a structured framework for categorizing peripheral neuroblastic tumors into four distinct groups [18]:
  - Neuroblastoma: This category includes undifferentiated, poorly differentiated, and differentiating subtypes based on neuroblastic differentiation grade. Most NBs are undifferentiated, characterized by immature small round cells (neuroblasts) with hyperchromatic nuclei and positive staining for neuron-specific enolase, tyrosine hydroxylase, synaptophysin, and NB84 markers.
  - Ganglioneuroblastoma Intermixed: These tumors show partial neural differentiation.
  - Ganglioneuroblastoma Nodular: Like intermixed tumors with more pronounced nodular growth patterns.

## Introduction

### Staging and risk classification

---

- Ganglioneuroma: This category represents the most differentiated tumors, characterized by clusters of mature neurons surrounded by a dense Schwannian cell stroma.

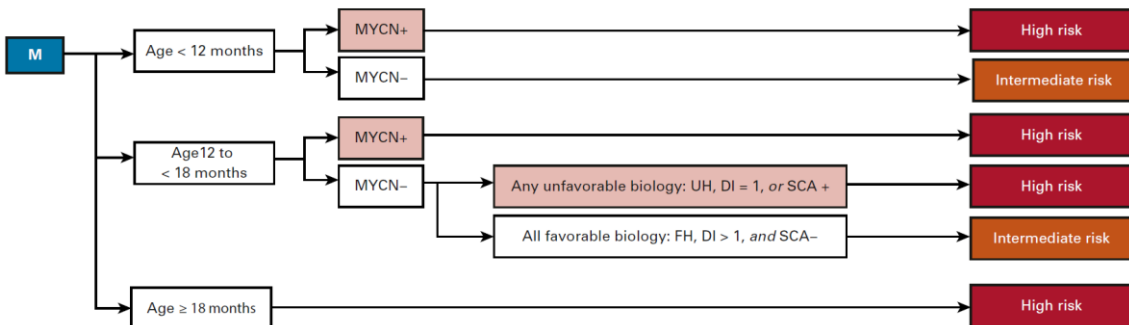
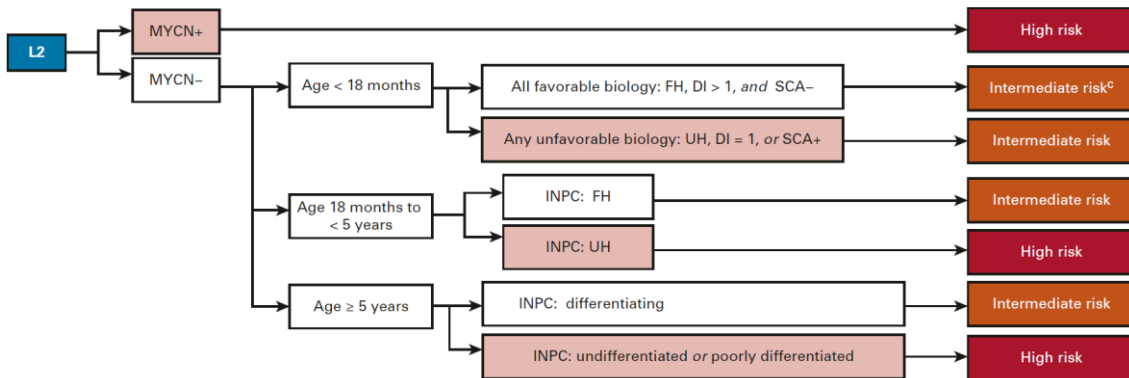
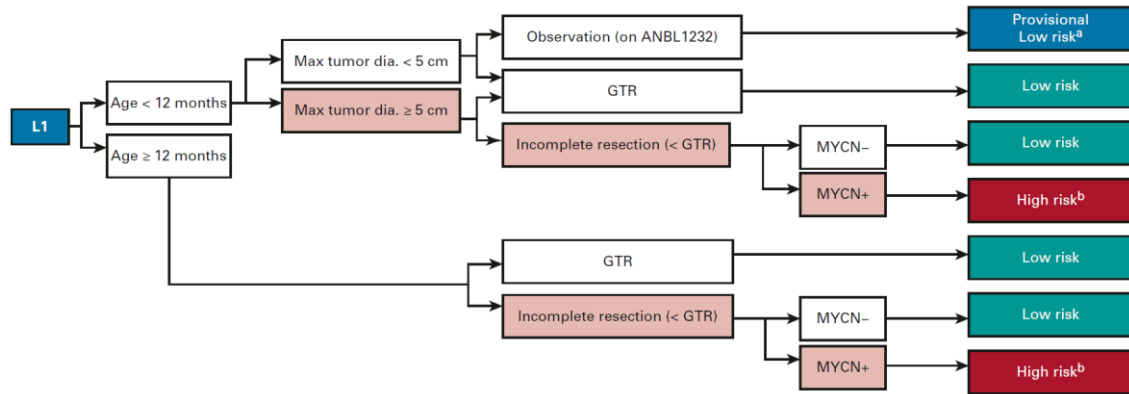
Additional consideration of these stromal Schwannian cells and the mitosis-karyorrhexis index (MKI) further refines the histological assessment [19].

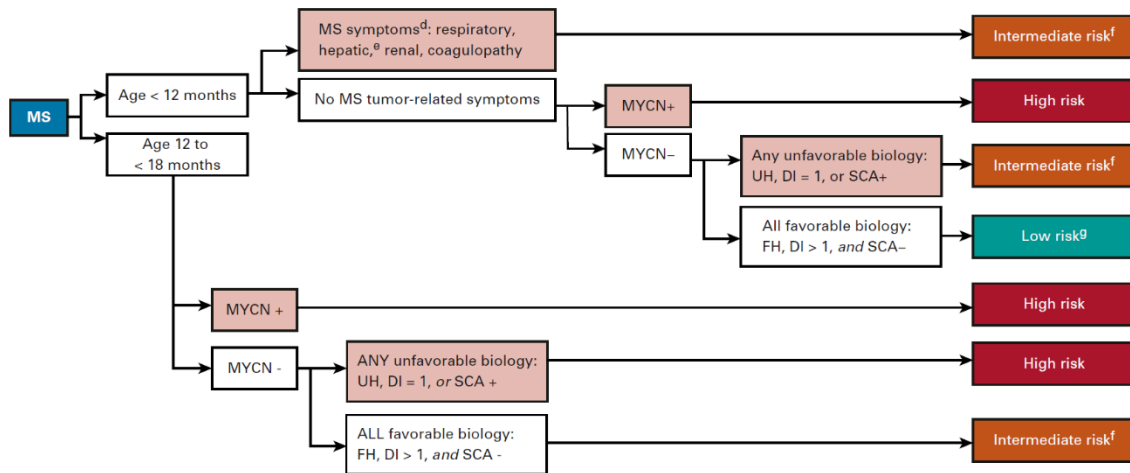
- III. **MYCN status:** *MYCN* amplification is a crucial genetic determinant in NB prognosis. Its discovery in the 1980s marked a significant milestone in cancer genetics, being the first clinically relevant genetic biomarker [20]. Studies have underscored its association with aggressive disease traits, such as advanced tumor stages and diminished progression-free survival rates (further details in section 1.1.4.2.1) [21].
- IV. **DNA index or ploidy:** Chromosomal aberrations can lead to hyperploidy, characterized by a DNA index (DI)  $> 1$  [22]. This prognostic factor is relevant in infants and toddlers lacking *MYCN* amplification, where hyperploidy is linked to a more favorable prognosis than diploidy (DI of 1) [23].
- V. **SCAs:** Numerical chromosome alterations (NCAs) typically indicate favorable survival rates. Conversely, the presence of SCAs is associated with poorer outcomes. Notably, loss of heterozygosity at 1p and 11q have been identified as predictors of reduced survival. 1p loss is linked to *MYCN* amplification, while 11q loss is not. Therefore, 11q status was included in the INRG risk classification as an independent predictor of poor outcome, regardless of *MYCN* status [7].

This system allows for classifying patients into distinct risk categories: low-risk, intermediate-risk, and high-risk [7]. In conjunction with the child's stage, these prognostic indicators allow each patient to be assigned their risk group and guide the treatment strategy. A revised COG NB risk classifier was implemented in 2021 (version 2) [4]. This classifier integrates the INRGSS and SCAs and has been adopted to prospectively determine eligibility for COG clinical trials and allocate treatment regimens (Figure 2).



INRGSS stage

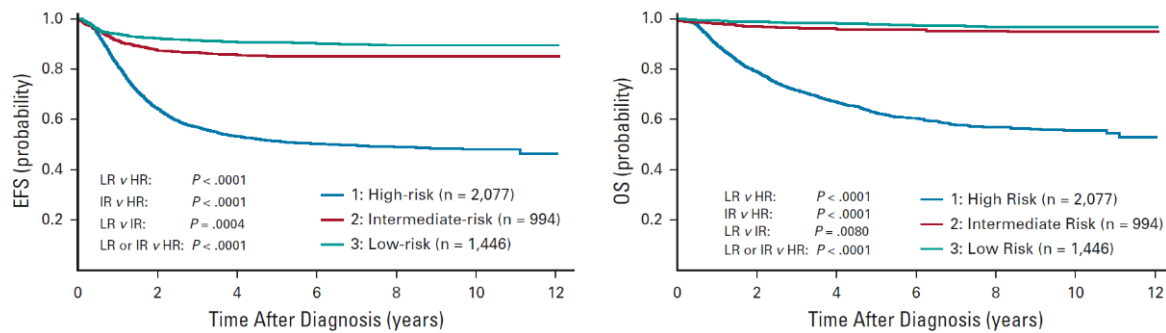




**Figure 2. COG risk stratification schema version 2.** The risk classifier algorithm distinguishes locoregional (L1 and L2) and metastatic (M and MS) tumors. Locoregional tumor classification considers INRG stage, age, resection, biomarkers (*MYCN*, ploidy, SCAs), and INPC. Ages are divided into three categories: <18 months, 18 months to <5 years, and ≥5 years, following the age criteria utilized by INPC. <sup>a</sup>Biopsy/resection is recommended for progressing tumors, followed by biomarker-based reclassification. <sup>b</sup>Complete resection must be considered if feasible. <sup>c</sup>Observation for symptom-free patients is indicated. Metastatic tumor classification involves stage, age, INPC, and biomarkers. <sup>d</sup>Biopsy is contraindicated until the patient is stable. Biomarker results may alter risk class. <sup>e</sup>Hepatomegaly alone is an MS symptom in <3-month-old patients (refer to COG ANBL1232). <sup>f</sup>Response-based therapy must be followed (per COG ANBL1232). <sup>g</sup>MS score-based therapy (per COG ANBL1232). Dia., diameter; FH, favorable histology; GTR, gross-total resection; MYCN+, *MYCN* amplified; MYCN-, *MYCN* not amplified; UH, unfavorable histology. Source: [4].

### 1.1.3. Risk-stratified treatments

Depending on the patient's risk status, NB treatment can range from observation alone for patients with low-risk disease to intensive multimodal therapies for those with high-risk disease [4]. Risk-adapted treatment strategies have successively reduced the intensity of therapy in patients with low- and intermediate-risk disease and incrementally improved the outcomes in those with high-risk disease in prospective cooperative studies [4]. However, despite treatment advances, high-risk patients' outcomes remain dismal (Figure 3).



### 1.1.3.1. Low-risk treatment

Low-risk NBs, comprising localized primary tumors INRG stages L1, L2, and MS with favorable genomic features, represent nearly half of all newly diagnosed NB cases. Their overall prognosis is highly promising, with over 95% achieving survival durations exceeding 5 years [4]. Treatment strategies strive to administer minimal therapy while ensuring excellent patient survival. The treatment strategy hinges on the presence of clinical symptoms. If symptoms are apparent, chemotherapy is initiated, with the number of cycles limited until symptom resolution [7].

In asymptomatic patients presenting with MS or L1 disease, only observation is appropriate if all favorable features are present, as tumors exhibiting these characteristics typically regress spontaneously [24, 25]. Observation entails regular monitoring through physical examinations, urine catecholamine level assessments, and tumor imaging. However, for patients with L1 tumors who are older than 12 months old, consideration of gross total resection is advised. Patients who undergo incomplete resection but show no *MYCN* amplifications can be safely monitored without further intervention due to the relatively low risk of disease recurrence [25]. For individuals with L2 tumors at any site, minimal chemotherapeutic intervention is indicated. Biopsy sampling and metastatic evaluation ensure accurate risk assessment and subsequent management planning [25].

### 1.1.3.2. Intermediate-risk treatment

This cohort encompasses INRG stage L2 tumors without *MYCN* amplification, along with stage M tumors in patients younger than 18 months and MS tumors in those younger

than 12 months with diploid DI or unfavorable histopathological features (Figure 2) [4]. The standard treatment approach for these patients typically involves moderated multi-agent chemotherapy combined with surgical resection. This regimen normally comprises two to eight cycles of carboplatin, cyclophosphamide, doxorubicin, and etoposide, varying doses depending on treatment response. Maximal safe resection of any remaining primary tumors may be conducted, along with differentiation therapy for specific higher-risk subgroups [4, 26]. In cases of MS disease, tumor mass-related symptoms such as hepatomegaly leading to respiratory issues or organ dysfunction are associated with high morbidity and mortality. Thus, prompt initiation of chemotherapy is recommended, while biopsy should be deferred until disease stabilization [4, 26].

Combined surgical resection and chemotherapy have impressive survival rates exceeding 95% [4, 26]. A phase III COG trial is assessing the viability of solely observing asymptomatic patients exhibiting favorable disease characteristics (NCT02176967). However, survival rates dip to around 70% for patients older than 12 months with unresectable disease and unfavorable features. For these patients, consideration of more intensive approaches, such as local radiotherapy, may be warranted [27].

### **1.1.3.3. High-risk treatment**

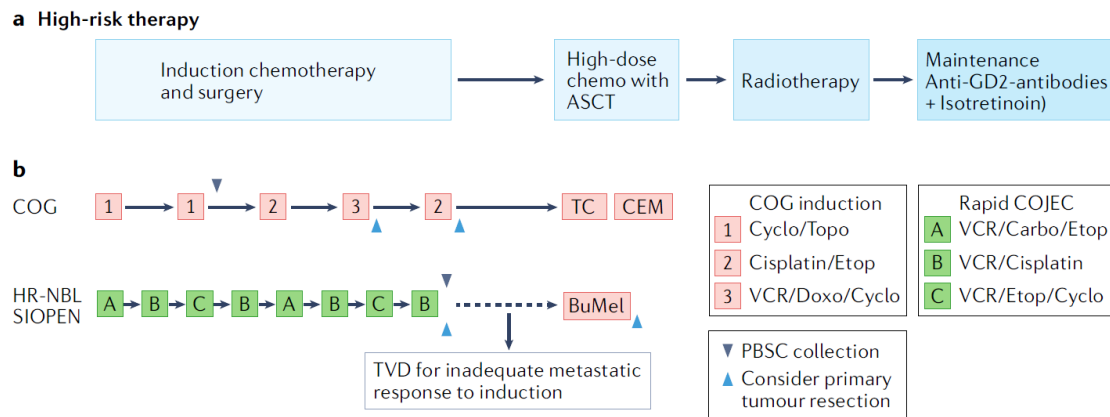
Approximately half of the patients diagnosed with NB are classified as having high-risk disease. This “high-risk” designation often arises from ages exceeding 18 months and the presence of metastatic disease (stage M). However, patients with locally advanced tumors with *MYCN* amplification are also considered high-risk (Figure 2) [4].

The treatment plan includes (1) *induction* chemotherapy, during which the primary tumor is surgically resected, followed by (2) *consolidation* with autologous stem cell transplant (ASCT) and radiotherapy, and finally (3) *post-consolidation* treatment with anti-GD2-based immunotherapy plus isotretinoin (13-cis-retinoic acid). The total upfront treatment duration is approximately 18 months [4]. Next, we will discuss frontline treatment for HR-NB and innovations in therapy for relapsed or refractory patients.

#### **(1) Induction**

Induction protocols vary slightly depending on the geographic location. Patients generally undergo five to eight cycles of intensive chemotherapy comprising platinum, alkylating, and topoisomerase agents. North American regimens follow COG protocols that include vincristine, doxorubicin, cyclophosphamide, cisplatin, and etoposide, adding

topotecan in the first two cycles. On the other hand, the SIOPEN protocol employs the rapid COJEC regimen, entailing eight cycles using combinations of vincristine, carboplatin, etoposide, cyclophosphamide, and cisplatin (Figure 4b) [4, 7, 16]. A randomized trial comparing COJEC with the COG regimen demonstrated similar rates of complete responses, yet COJEC exhibited a lower incidence of toxicities [28].



**Figure 4. Multimodal therapy for high-risk neuroblastoma.** a. Multimodal therapy for HR-NB starts with induction chemotherapy, aimed at reducing metastatic disease burden and improving IDRFs for safe primary tumor resection. b. Recommendations regarding the specific regimen and timing of procedures like autologous peripheral blood stem cell collection and primary tumor resection vary among cooperative groups. COG and HR-NBL/SIOPEN offer standard regimens, with HR-NBL/SIOPEN recommending additional therapy with topotecan, vincristine, and doxorubicin (TVD) before consolidative transplantation for patients with inadequate response to induction chemotherapy. ASCT, autologous stem cell transplant; ALKi, ALK inhibitor; BuMel, busulfan, and melphalan; CEM, carboplatin, etoposide, and melphalan; COJEC, cisplatin, vincristine, carboplatin, etoposide, and cyclophosphamide; Cyclo, cyclophosphamide; Doxo, doxorubicin; Etop, etoposide; TC, thiotepa and cyclophosphamide; Topo, topotecan; VCR, vincristine. Adapted from [3].

Patients undergo stem cell collection throughout induction in anticipation of their ASCT. Stem cells are obtained either peripherally or through bone marrow harvest. In COG protocols, stem cell harvesting occurs after the second induction cycle, while in rapid COJEC, it occurs after the eight induction cycles are completed. Surgery also plays a pivotal role in HR-NB treatment and is usually scheduled toward the conclusion of induction chemotherapy. This timing aims to capitalize on tumor shrinkage to reduce surgical complications [4, 7, 16].

## Introduction

### High-risk treatment

---

Targeted molecular radiotherapy employing iodine-131-labeled meta-iodobenzylguanidine ( $^{131}\text{I}$ -MIBG) has been utilized for induction and consolidation therapy for over 20 years, as well as for patients with relapsed and refractory disease [29, 30].  $^{131}\text{I}$ -MIBG therapy involves targeted radiation delivery to NB cells via human norepinephrine transporter (hNET) uptake. Although hematological toxicities are common, they are manageable with stem cell rescue [31]. Current research is exploring the feasibility and safety of incorporating  $^{131}\text{I}$ -MIBG into induction therapy for high-risk patients (NCT03126916, NCT03165292).

## (2) Consolidation

Following induction, the consolidation period aims to eradicate any remaining minimal disease. This stage comprises two components: high-dose chemotherapy followed by ASCT and radiation therapy. Historically, conditioning regimens for ASCT in high-risk patients have differed between North America and Europe. While the COG typically used the CEM regimen (carboplatin, etoposide, melphalan), European protocols favored Bu/Mel (busulfan/melphalan). In a randomized Phase 3 trial, the utilization of Bu/Mel demonstrated enhanced EFS (five-year EFS: 45% vs. 33%,  $p = 0.0005$ ) and OS (five-year OS: 54% vs. 41%,  $p = 0.001$ ) compared to CEM, with reduced overall toxicity [32].

After ASCT recovery, radiation therapy to the primary tumor bed and metastatic sites is pivotal for local control [33, 34]. Ongoing studies investigate optimizing radiation doses, with novel proton therapy showing the potential to minimize toxicity and maximize normal tissue preservation, though further research is needed due to limited data [35].

## (3) Maintenance

After induction and consolidation treatment regimens, the post-consolidation or maintenance phase was implemented to treat the residual disease. It involves the administration of isotretinoin and the anti-GD2 antibody ch14.18 (dinutuximab) or its European biosimilar ch14.18/CHO [36]. Isotretinoin is an oral non-cytotoxic differentiation agent that has been demonstrated to induce tumor cell differentiation and inhibit the proliferation of NB cells. GD2, on the other hand, is a disialoganglioside expressed in neuroectodermal tumors, including NB [37, 38]. At first, IL-2 was incorporated into maintenance treatment, but the SIOPEN HR-NBL1 trial revealed that IL-2 led to heightened toxicities and did not significantly improve efficacy (3-year EFS 60% vs. 56%; grade 4 non-hematological toxicities in 28% vs. 9%). Therefore, it was discontinued [39].

Despite advancements in treatment, half of the children with HR-NB still face relapse to upfront therapies (Figure 3). Survival rates for relapsed or refractory HR-NB are alarming, with a median EFS of 6.4 months and OS rates of 6% to 20% [4]. This underscores the critical need for more targeted therapeutic approaches. Besides, high-dose therapy with ASCT causes severe acute and chronic organ toxicities and late-onset adverse effects in survivors (Table 2). These effects include hearing impairment, secondary malignancies, endocrine disorders, psychological and cognitive challenges, as well as reproductive health issues [40-47]. Additionally, G2D, apart from expressing in neuroblasts, is expressed in other normal nerve tissue, including pain fibers [48]. While some researchers are studying combining anti-GD2 monoclonal antibodies (MAbs) with chemotherapy, including in frontline induction chemotherapy, others actively seek alternative immunotherapeutic targets with greater tumor specificity and fewer toxicities.

## Introduction

### High-risk treatment

**Table 2.** Long-term toxicities of neuroblastoma therapy.

| Late Effect                             | Therapeutic Exposure                                                                                   | Frequency (%) | Refs     |
|-----------------------------------------|--------------------------------------------------------------------------------------------------------|---------------|----------|
| High-frequency hearing loss             | Platinum-based agents, chemotherapy (cisplatin, carboplatin), ototoxic antibiotics                     | 52            | [44, 45] |
| Endocrine dysfunctions                  | Radiotherapy, <sup>131</sup> I-MIBG, high-dose myeloablative therapy                                   | 28            | [43]     |
| Growth failure                          | High-dose myeloablative therapy, total body irradiation, radiation to the spine or bones, isotretinoin | 50            | [42]     |
| Hypogonadism                            | Radiotherapy to the gonads, alkylating agents, cisplatin                                               | 14            | [43]     |
| Musculoskeletal abnormalities           | Radiotherapy, isotretinoin, intraspinal tumors and surgery                                             | 19            | [45]     |
| Cardiac dysfunction                     | Anthracyclines, radiotherapy to the chest or upper abdomen                                             | 16            | [46]     |
| Secondary malignant neoplasms           | Epipodophyllotoxins, alkylating agents, radiotherapy, anthracyclines                                   | 10            | [47]     |
| Pulmonary dysfunction                   | Busulfan, radiation to the chest or upper abdomen                                                      | 19            | [44]     |
| Renal dysfunction                       | Nephrectomy, platinum-based chemotherapy (cisplatin, carboplatin), radiation to the kidneys            | 9             | [44]     |
| Dental abnormalities                    | Total body irradiation, myeloablative therapy                                                          | 29            | [43, 46] |
| Neurological deficits <sup>1</sup>      | Spinal involvement, radiotherapy                                                                       | 30            | [49]     |
| Learning and psychological difficulties | Radiotherapy, treatment intensity                                                                      | 16            | [41]     |

Frequency among survivors is based on high-risk cohorts unless expressly noted. <sup>1</sup>Late onset impacts on musculoskeletal, neurological, and psychological well-being are evident across all levels of risk. Psychological repercussions are frequently linked to the duration and intensity of treatment. Refs: references.



#### **1.1.3.4. Relapsed or refractory treatment**

Long-term remission in relapsed or refractory NB is uncommon [4]. Standard salvage chemotherapy options include combining temozolomide with irinotecan or topotecan and cyclophosphamide with topotecan, resulting in overall response rates (ORRs) of around 15-20% [40, 50].

The application of targeted therapies in NB has been limited, mainly due to the lack of recurrent mutations at diagnosis, with GD2 immunotherapy being the notable exception [8]. While the promising outcomes of anti-GD2 antibody therapy in relapsed NB have sparked interest in its early inclusion for children with high-risk NB, this approach remains under critical evaluation [51]. St. Jude Children's Research Hospital's phase II trial showcased encouraging outcomes, prompting further evaluation in COG's ANBL17P1 trial (NCT03786783), yet the applicability of these findings remains to be fully explored. There is a growing emphasis on combining  $^{131}\text{I}$ -MIBG with other targeted therapies, but the clinical benefit of these combinations is not yet well-established. Of note, vorinostat, a histone deacetylase (HDAC) inhibitor, has shown promise in preclinical studies by enhancing hNET expression and sensitizing tumors to  $^{131}\text{I}$ -MIBG [52, 53].

A critical emerging trend in NB clinical trial design is the reconsideration of evaluating experimental therapies in very high-risk patients at diagnosis rather than at relapse. This shift is driven by the recognition that there are "*too many targets, not enough patients*," which complicates the rigorous assessment of new treatments in small, severely affected patient cohorts [54]. However, the lack of adequate biomarkers and a comprehensive understanding of the underlying mechanisms of treatment failure and relapse continue to hinder progress [54]. This highlights the urgent need for innovative research to further elucidate the pathogenesis of NB and advance the development of more effective therapeutic strategies. During this thesis, we will delve deeper into the molecular alterations of NB and examine the potential of emerging therapies to bridge these critical gaps.

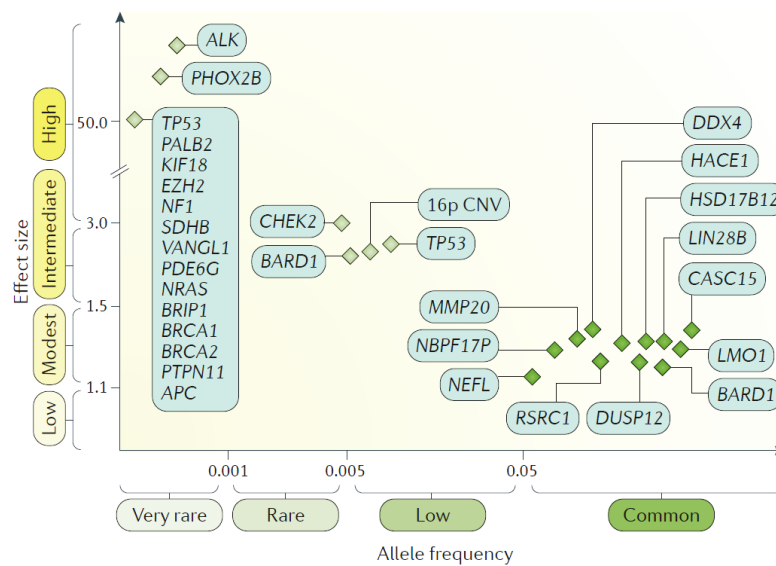
#### **1.1.4. Pathogenesis and molecular alterations**

NB originates from sympathoadrenal cells derived from the neural crest, suggesting that its development is closely linked to aberrant developmental processes [55]. While most NBs are spontaneous, inherited genetic variations can increase the risk. Unlike many solid tumors, NB has a low mutation rate, averaging 0.6 per megabase [8]. This low mutation rate contrasts with the high prevalence of SCAs, which often correlate with poor prognosis [56]. The scarcity of mutations in NB challenges understanding the full spectrum of its pathogenesis. This has led to speculation that other unidentified factors, potentially genetic, epigenetic, or environmental, are critical in driving NB development.

##### **1.1.4.1. Germline mutations**

Familial cases of NB are exceedingly rare and are found in 1-2% of cases. These cases predominantly involve activating mutations in *ALK* (seen in approximately 80% of familial NBs) or inactivating mutations in *PHOX2B* (Figure 5) [57, 58]. Both *ALK* and *PHOX2B* play crucial roles in sympathoadrenal development. Patients with NBs stemming from *PHOX2B* mutations often present with pathological features associated with neural crest disorders, such as Hirschsprung disease and congenital central hypoventilation syndrome [57, 58].

Additionally, genome-wide association studies have identified several lower penetrance polymorphisms that can predispose to NB (Figure 5). Each association has a relatively modest individual effect on disease initiation (with relative risks between 1.5 and 2.5). Still, multiple associations can cooperate in an individual patient to promote malignant transformation during neurodevelopment. These include variations in *LINC00340*, *BARD1*, *LMO1*, *DUSP12*, *DDX4/IL31RA*, *HSD17B12*, *LIN28B*, *HACE1*, and a common inherited CNV at 1q21 [59, 60]. Other genes with damaging mutations in the germline have been identified, including variants in *TP53*, *NRAS*, *CHEK2*, *BRCA1*, and *BRCA2*, but the clinical relevance of these mutations remains to be defined [61, 62].



**Figure 5. Germline risk of neuroblastoma.** Genetic predisposition to NB primarily arises from high-penetrance mutations in *ALK* and *PHOX2B*. These mutations are rare in the population and inherited in an autosomal dominant manner. Other common polymorphisms, though individually having a minor impact on tumor initiation, can collectively contribute to sporadic NB development. Source: [1].

## 1.1.4.2. Recurrent somatic alterations and actionable targets

### 1.1.4.2.1. *MYCN* amplification and *MYC* overexpression

Amplification of the *MYC* family member *MYCN* (2p24) is found in approximately 25% of all cases and is linked with high-risk disease and unfavorable prognoses [21]. Identified as the second *MYC* family member in 1983, *MYCN* amplification has since become one of the first genetic biomarkers in cancer, specifically in HR-NB [20]. Subsequent research has consistently implicated *MYCN* in all stages of tumorigenesis and malignancy [21].

As a critical transcriptional regulator, *MYCN* governs vital processes such as growth, metabolism, and cellular differentiation [20, 63]. During sympathoadrenal development, *MYCN* orchestrates the migration and proliferation of neural crest cells (NCCs), with its expression naturally declining as these cells differentiate into sympathetic neurons (further elaborated in section 1.1.5) [64]. Studies of transgenic mice have shown that *MYCN* overexpression alone can induce neuroblast transformation and NB development [65]. Unfortunately, targeting *MYCN* directly is challenging, yet leading to the absence of *MYCN*-targeted therapy in standard care for *MYCN*-amplified patients [21].

## Introduction

### MYCN amplification and MYC overexpression

---

Apart from NB, *MYCN* amplification plays a significant role in promoting tumor development in other embryonal pediatric malignancies, including rhabdomyosarcoma, medulloblastoma, astrocytoma, meningioma, and retinoblastoma [66].

A subset of HR-NB harbors a rare missense mutation in *MYCN* (P44L) (1%) [8]. Although the prognostic relevance of this alteration is still unknown, data from preclinical studies suggest that this mutation might increase the stability of MYCN [67]. While *MYCN* is typically expressed in developmental and cell type-specific contexts, its closely related counterpart-orthologous gene, *MYC*, exhibits ubiquitous expression and is overexpressed in ~10% of high-risk patients without *MYCN* amplification [68].

Despite the challenge of directly targeting MYCN and MYC, efforts to target MYCN–MYC networks in NB are ongoing [21]. Targeting AURORA-A kinase, which promotes cell-cycle progression and stabilizes MYCN, represents one approach (Figure 6). By interfering allosterically with MYCN interactions, AURORA-A inhibitors like alisertib have shown some efficacy, with a 21% ORR in a phase II clinical trial for relapsed and refractory HR-NB (NCT01601535) [69-71]. A more selective AURORA-A inhibitor, LY3295668 erbumine, is currently under investigation in a phase I study, potentially offering improved toxicity profiles (NCT04106219) [72, 73].

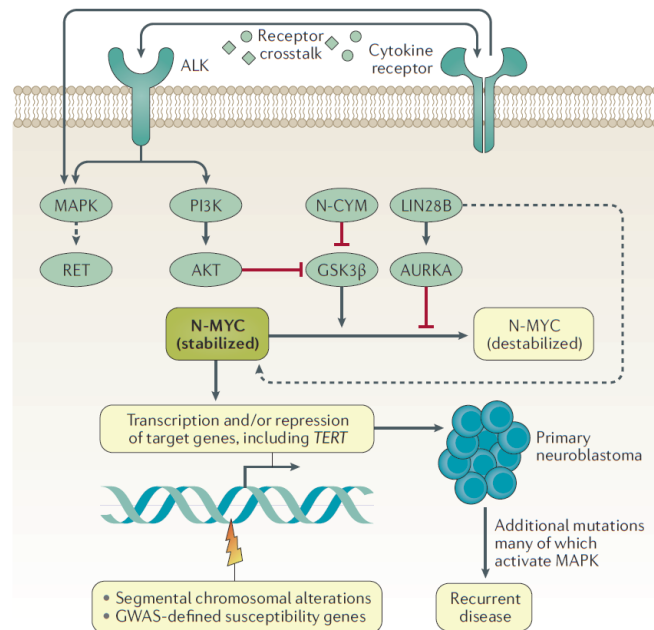
Additionally, innovative strategies like proteolysis-targeting chimeras (PROTACs) and Bromodomain and Extra-Terminal domain (BET) inhibitors are being explored to disrupt MYC/MYCN signaling [74, 75]. An ongoing phase I basket trial with the BETi BMS-986158 is evaluating this strategy in clinical settings (NCT03936465).

Omomyc is the sole compound shown to directly inhibit MYC proteins by disrupting their binding to target promoters, spanning all three forms: MYC, MYCN, and MYCL. Although initially conceived as a proof of principle, the purified Omomyc mini protein showed preclinical effectiveness and progressed into clinical development for adult solid tumors (NCT04808362). The recently completed phase I trial has proved its safety and clinical activity, although still limited [76].

Targeting metabolic pathways downstream of MYCN presents another therapeutic opportunity. Difluoromethylornithine (DFMO), an inhibitor of ornithine decarboxylase crucial in polyamine biosynthesis, has shown promise as a metabolic vulnerability in MYCN-driven cells [77]. Clinical studies have demonstrated the tolerability of single-agent DFMO maintenance therapy in patients with HR-NB in remission, with ongoing

trials investigating DFMO in combination with other agents (NCT03794349, NCT04301843, NCT02679144) [78].

Finally, *MYCN*-altered tumors rely heavily on the cell-cycle checkpoint kinase WEE1 to manage replicative stress, presenting yet another potential target [79]. A phase I trial combining the WEE1 inhibitor adavosertib with irinotecan has yielded encouraging results, with some patients achieving stable disease for up to 11 months [80].



**Figure 6. MYCN stabilization mechanisms.** Diverse mechanisms stabilize MYCN (N-MYC), including the druggable targets ALK, RET, PI3K, and MAPK. Cooperation between ALK and MYCN may stem from ALK activating PI3K. ALK also activates RAS, driving MAPK signaling, which is expected at relapse, and upregulates RET and cholinergic neuronal markers, suggesting developmental roles and potential therapeutic targets in NB. Non-MYCN-driven high-risk disease may involve TERT, ATRX, and MAPK alterations. Solid lines represent direct interactions while dashed lines are indirect. Source [1].

Despite advances, targeting MYCN still faces significant limitations, with strategies like AURORA-A and BET inhibitors limited by specificity, efficacy, and toxicity. The complexity of MYC-related biology suggests that a single-target approach may be insufficient, necessitating a more integrated strategy.

**1.1.4.2.2. ALK amplification and mutations**

*ALK* (2p23) encodes a tyrosine kinase receptor critical in neural lineage tissues, particularly during early sympathoadrenal development, where it protects neuroblasts from nutrient deprivation *in utero* [81, 82]. Germline and somatic mutations in *ALK* are present in 8-10% of NB cases and correlate with poor prognosis. [83]. Activating mutations result in mitogenic signaling via the RAS-MAPK and PI3K-AKT pathways [84].

*ALK* amplifications are reported in 1-2% of HR-NB patients. Due to their proximity on chromosome 2p, *ALK* and *MYCN* frequently undergo co-amplification (97% of *ALK*-amplified cases) [84, 85]. Activating *ALK* mutations are predominantly found in the kinase domain, with frequent hot-spot alterations occurring at F1174, R1275, or F1245 [84, 85]. The most common and aggressive activating mutation is F1174L, which is sufficient to induce tumorigenesis when expressed explicitly in the neural crest in transgenic mice [86]. *ALK* mutations also co-occur with *MYCN* amplifications (44% of cases). Their co-expression synergistically promotes tumor initiation *in vivo*, suggesting a cooperative role in tumorigenesis. The synergy between these two may stem from ALK-PI3K signaling activation, increasing *MYCN* stability (Figure 6) [86].

ALK inhibitors are currently accessible in clinical settings and have demonstrated safety and efficacy in individuals with *ALK* alterations, including amplifications and mutations, in relapsed and refractory NB [83, 87-89]. Crizotinib, a first-generation ALK-ROS-MET inhibitor, showed an ORR of 15%, which, while modest, it indicates some level of activity [90]. Ceritinib, a second-generation ALK inhibitor, achieved an ORR of 20%, suggesting improved response rates compared to crizotinib [87]. Finally, lorlatinib, a third-generation ALK inhibitor, showed superior efficacy across all *ALK* alterations in an ongoing phase I/II trial either alone or in combination with chemotherapy (NCT03107988) [88].

Although ALK inhibitors demonstrate efficacy in NB, ORRs are relatively low and vary among patients with *ALK* alterations, underscoring the need for tailored treatments based on molecular profiles. Anti-ALK antibody-drug conjugates (ADCs) offer a potential alternative therapeutic strategy, which may be beneficial even in *ALK* wild-type disease due to the widespread expression of ALK in NB [89]. Additionally, developing resistance mechanisms over time poses a significant challenge to the long-term effectiveness of ALK inhibitors, a common issue in targeted therapies.

#### 1.1.4.2.3. Activation of telomere maintenance mechanisms

The regulation of telomere length is a critical determinant of NB aggressiveness, with distinct mechanisms at play. Approximately 25% of HR-NB cases exhibit upregulated telomerase activity, primarily through increased expression of TERT. Conversely, about 10% of cases harbor mutations in *ATRX*, a chromatin remodeling RNA helicase, resulting in alternative lengthening of telomeres (ALT) [91, 92].

Notably, TERT alterations and *ATRX* mutations are mutually exclusive, delineating two distinct pathways in telomere length regulation [91, 92]. Moreover, both alterations are typically absent in tumors exhibiting *MYCN* amplification [8]. Since *MYCN* upregulates *TERT*, this suggests that activation of telomere-lengthening mechanisms is essential for the most aggressive forms of NB [93].

Additional chromatin remodeling genes implicated in NB include *ARID1A* and *ARID1B* of the Polycomb complex. While haploinsufficiency for *ARID1A* and *ARID1B* is recurrent in HR-NB, the impact of these mutations on chromatin structure remains undefined [94].

The distinct TERT and *ATRX* pathways offer significant opportunities for targeted therapies in HR-NB. Preclinical evidence highlights EZH2 as a targetable vulnerability in NB harboring *ATRX* in-frame fusions or *MYCN* amplification, as oncogenic transformation downstream of these alterations relies on epigenetic reprogramming [95, 96]. Moreover, HR-NB relies on the histone acetyltransferase EP300 to regulate *MYCN* oncogenic core regulatory transcriptional network, offering a therapeutic vulnerability [97]. In addition to their mentioned roles, BETi hold promise in downregulating TERT expression in NB mouse models [98]. Conversely, ALT-positive tumors may develop chemotherapy resistance due to constitutive activation of the DNA damage checkpoint kinase ATM, which could be countered with ATM inhibitors in preclinical models [99, 100].

#### 1.1.4.2.4. *LIN28B* polymorphisms

*LIN28A* and *LIN28B* regulate the expression of the *let-7* microRNA family during embryogenesis, playing crucial roles in developmental timing and cell growth during the commitment of NCC lineages [101]. Overexpression of *LIN28B* leads to a reduction in *let-7* miRNA levels, promoting the stabilization of *MYCN* and thereby sustaining its tumor-promoting functions (Figure 6) [102]. In transgenic mice, targeted expression of *LIN28B* in the sympathetic adrenergic lineage induced the development of NB [102].

Additionally, LIN28B modulates the activity of the nuclear shuttling by the GTP-binding protein RAN and stabilizes AURORA-A (Figure 6) [91].

#### 1.1.4.2.5. Segmental chromosomal aberrations

Most HR-NBs show recurrent SCAs [56]. Typical SCAs include deletions of chromosomes 1p, 3p, 4p, and 11q, thought to harbor tumor suppressor genes, and gains of chromosomes 1q, 2p, and 17q, believed to contain putative oncogenes [103]. The most frequent SCAs associated with higher-risk disease are 17q gain, 1p, and 11q deletions. 17q gain is detected in 50% of NBs, and 1p deletion is seen in 30% of cases. Both 17q gain and 1p deletion correlate with, but are not exclusive to, *MYCN* amplification and poor prognosis [56].

11q deletion is detected in up to 30-40% of NBs, and most of the cases are diagnosed at an older age in the high-risk group, displaying higher relapse probability and dismal prognosis [99, 104-106]. Interestingly, tumors with 11q deletion typically lack 1p deletions and *MYCN* amplification, suggesting they may represent a distinct tumor category in patients with advanced-stage disease [104, 107]. MiRNAs could be involved in the inverse relationship between *MYCN* and 11q deletions, as evidenced by *let-7a-2*, located on 11q, which has been demonstrated to inhibit *MYCN* mRNA directly [108]. However, *MYCN* has no approved clinical therapeutics yet, and the genetic basis for 11q deletion pathogenesis is unclear.

Despite the unclear genetic drivers behind 11q-deleted NB, genomic instability is a hallmark of this tumor subset, particularly when contrasted with the *MYCN*-amplified subtype [107]. The high frequency of chromosomal breaks in 11q-deleted tumors strongly suggests that defects in DNA repair mechanisms may be at play [99, 107]. This could involve haploinsufficiency or the inactivation of critical DNA repair genes through mutations or epigenetic changes. This hypothesis highlights a potential vulnerability in 11q-deleted NB and opens new avenues for research, potentially focusing on targeting DNA repair pathways.



#### 1.1.4.2.6. Epigenetic modifications

Given the heterogeneous nature of NB and the limited recurrence of genetic mutations, epigenetic modifications may hold crucial insights into understanding NB pathogenesis [8]. Epigenetic mechanisms significantly influence gene expression, embryonic development, chromosomal stability, and tumorigenesis, assuming pivotal roles in physiological and pathological contexts [109]. Major epigenetic modifications include aberrant DNA methylation, disrupted patterns of post-translational histone modifications, alterations in chromatin structure, and dysregulated expression of non-coding RNAs [109].

An early crucial finding revealed *CASP8* inactivation in 63% of *MYCN*-amplified NB and 4% in non-amplified cases and its association with chemotherapy resistance [110]. Subsequent research showed epigenetic silencing of other apoptosis-related genes, including *RASSF1A*, *TMS1*, and the decoy receptors *DCR1* and *DCR2*, linked to their expression profiles [111-113]. Conversely, overexpression of the oncogenic truncated  $\Delta$ Np73 isoform due to DNA demethylation is associated with adverse NB outcomes [114, 115]. Various other apoptosis-related genes, including *HOXA9*, *14.3.3 $\sigma$* , *SCNN1A*, *PRKCDBP*, and *KRT19*, alongside a 58-gene signature, have been linked to prognostic outcomes [111, 116-118]. Intragenic deletions in *ARID1A*, a component of the SWI/SNF chromatin remodeling, were detected in 7% of NB tumors, with additional point mutations in *ARID1A* in another 6% [94]. *ARID1A* and *ARID1B* aberrations correlated with early treatment failure and unfavorable survival outcomes, although functional investigations remain outstanding, as mentioned earlier (section 1.1.4.2.3) [119].

#### 1.1.4.2.7. MicroRNAs

MiRNAs modulate protein translation and mRNA degradation [109]. In addition to let-7, the miRNAs most extensively associated with NB are miR34a and the *miR-17-92* cluster. miR34a is a direct target of p53 and acts as a tumor suppressor by inducing cell cycle arrest and apoptosis through inhibition of cyclins, cyclin-dependent kinases (CDK4, CDK6, cyclin E2), and the anti-apoptotic protein BCL2 [120]. Conversely, the *miR-17-92* cluster, activated by MYC, inhibits P21 (*CDKN1A*), a cyclin-dependent kinase that dampens cell cycle advancement, and BIM, a proapoptotic factor that mediates cell death through interactions with BCL2 [121, 122]. The expanding understanding of miRNAs offers new insights into NB biology, though this research is still emerging.

**1.1.4.2.8. Neurotrophin receptors**

The neurotrophin receptors NTRKA, NTRKB, and NTRKC constitute a family of transmembrane receptor tyrosine kinases pivotal in neurodevelopment [123]. During sympathetic adrenal neuron development, NTRKA and NTRKC exhibit high expression levels correlating with better outcomes [124]. Conversely, elevated levels of NTRKB, associated with drug resistance and angiogenesis, are observed in unfavorable tumors, especially those with *MYCN* amplification [125, 126]. Although NTRK-activating mutations or fusions have not been identified in NB, NTRK inhibitors like lestaurtinib (CEP-701) or the more selective inhibitor AZD6918 have been explored as potential NB therapies [127]. However, none of these preclinical studies have provided sufficient basis for the subsequent clinical study and further use of NTRK as a target in NB therapy. Currently, only the second-generation inhibitor alectinib is under clinical study but based on its ALK inhibition capacity rather than NTRK (NCT05770037).

### 1.1.4.3. Evolution of genetic alterations in neuroblastoma from diagnosis to relapse

Despite the lack of actionable genetic aberrations at diagnosis, recent studies indicate these are more common in relapsed NB, possibly because of clonal expansion, particularly in the *RAS-MAPK* signaling pathway (Table 3) [128, 129]. Early identification of these alterations could enable proactive targeting before clonal expansion occurs. However, this would require deep sequencing of tumor samples or the use of single-cell analysis techniques, along with close tumor monitoring in clinical practice, which is not yet the standard practice.

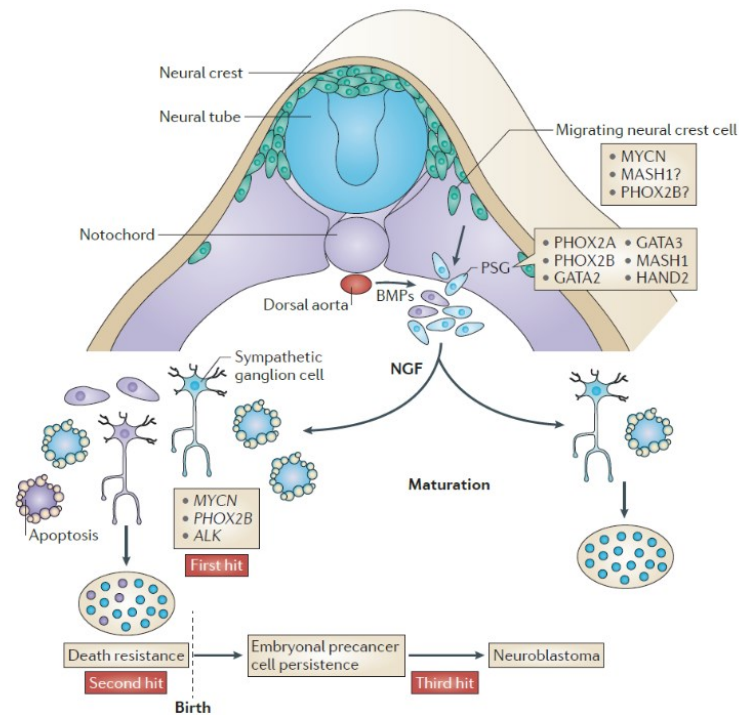
**Table 3.** Recurrent genetic alterations in neuroblastoma upon diagnosis and relapse.

| Alteration                        | Diagnosis frequency (%) (n= 240) [8] | Diagnosis frequency (%) (n= 230) [128] | Relapse frequency (%) (n = 59) [129] |
|-----------------------------------|--------------------------------------|----------------------------------------|--------------------------------------|
| <i>ALK</i> mutation               | 9.2                                  | 14.3                                   | 24.7                                 |
| <i>PTPN11</i> mutation            | 2.9                                  | 1.3                                    | 0                                    |
| <i>ATRX</i> mutation              | 2.5                                  | 1.8                                    | 11.1                                 |
| <i>ATRX</i> deletion              | 7.1                                  | 4.0                                    | 5.6                                  |
| <i>MYCN</i> mutation              | 1.7                                  | 0.9                                    | 3.7                                  |
| <i>MYCN</i> amplification         | 32.0                                 | 25.7                                   | 18.5                                 |
| <i>NRAS</i> mutation              | 0.8                                  | 2.6                                    | 7.4                                  |
| <i>NF1</i> mutation or truncation | 0                                    | 2.2                                    | 5.6                                  |
| <i>KRAS</i> mutation              | 0                                    | 1.7                                    | 1.9                                  |
| <i>FGFR1</i> mutation             | 0                                    | 1.7                                    | 9.3                                  |
| <i>TP53</i> mutation              | 0.8                                  | 3.5                                    | 7.4                                  |

**1.1.5. Neuroblastoma cell of origin: the genesis point**

The origin of NB remains enigmatic owing to the complex nature of neural crest derivatives [6]. Evidence suggests that NB originates during prenatal sympathoadrenal development and transitions through an embryonal precancerous phase [6]. Insight into the developmental niche fostering NB origin carries profound implications for devising pivotal developmental pathways for therapeutical intervention.

Given the common primary sites and the cellular and neurochemical characteristics of NB, it is acknowledged that the cell of origin stems from the sympathoadrenal lineage of the neural crest during embryonic development [5, 6, 130]. The neural crest comprises a transient population of multipotent embryonic progenitors within the developing ectoderm (Figure 7) [131]. During normal sympathoadrenal development, MYCN exhibits high expression levels in the early post-migratory neural crest, orchestrating ventral migration and NCC expansion [132]. Ventrally migrating NCCs respond to bone morphogenetic proteins (BMPs) signaling from the dorsal aorta, initiating a transcriptional program specifying primary sympathetic ganglia's neuronal and catecholaminergic traits. By the fifth week of human development, MASH1 subsequently promotes the expression of PHOX2A and PHOX2B, driving the expression of enzymes crucial for catecholamine biosynthesis. Then, PHOX2B triggers a secondary transcriptional program controlling terminal sympathoadrenal differentiation [132]. As differentiation progresses towards sympathetic neurons, MYCN expression gradually declines [64].



**Figure 7. Neural crest and neuroblastoma development.** During normal development, trunk NCCs migrate to the dorsal aorta (in red), where they differentiate into sympathoadrenal progenitor cells, ultimately contributing to the formation of cells in the peripheral nervous system, including sympathetic ganglia and the adrenal gland, primary sites for NB development. The migration of these neuroblast progenitors is mediated by various factors such as MYCN and BMPs. The fate of these cells, whether they mature into terminal ganglion cells (in blue) or undergo apoptotic cell death, is determined by MYCN, ALK, and PHOX2B, as well as access to NGF. Any mutations or disruptions occurring during these processes can give rise to tumor-initiating neuroblasts. The proposed pathological scenario involves a prenatal proliferative excess (first hit), followed by death resistance of neuroblast precancer cells and embryonal cell persistence postnatally (second hit), which requires resistance to trophic factor withdrawal before transforming NB in early childhood (third hit). Source: [55].

This dynamic shift implies that the maturation of the sympathoadrenal system necessitates minimal or absent MYCN expression [64]. As embryogenesis concludes, MYCN low expression allows cells to transition into a quiescent G0 phase and begin differentiation [133]. Even though MYCN has a short half-life, the elevated MYCN steady-state levels in *MYCN*-amplified cells (over one hundred times higher than normal cells) force cells to be permanently in the G1 phase, unable to step out into the G0 phase [133]. Following the specification and expansion of primary sympathetic ganglia, sympathoadrenal precursor cells diverge into either neuronal or chromaffin cell fates. Differentiation expression patterns suggest that the earliest NB cell of origin arises from a NCCs committed to the sympathoadrenal lineage, yet to receive cues dictating

## **Introduction**

### Neuroblastoma cell of origin: the genesis point

---

neuronal or chromaffin cell identity [64, 133, 134]. Neural precursors undergo apoptotic cell death during the final stages of sympathoadrenal maturation, catalyzed by local nerve growth factor (NGF) deprivation [135].

The development, maintenance, and differentiation of neuronal precursors are intricate processes governed by precisely timed and spatially regulated external signals. Any mutations or disruptions occurring during these processes can give rise to tumor-initiating neuroblasts [132]. In the NB context, mutant PHOX2B proteins appear to act in a dominant-negative manner, promoting inappropriate proliferation in neural precursors. In addition, aberrant MYCN or ALK expression or function, along with the dysregulated function of select neurodevelopmental regulators, are prime candidates for initiating neuroblast precancer [68, 134]. The exact sequence of events and intrauterine factors leading to neuroblast precancer remain unknown, alongside other unresolved questions regarding NB origin. Are all NBs derived from a common precursor, or do favorable and unfavorable NBs arise independently? Despite these uncertainties, recent findings highlight the significant influence of lineage-specific developmental programs on NB pathogenesis.

### 1.1.6. Neuroblastoma cell plasticity: regulatory switch in pathogenesis

#### 1.1.6.1. Transitioning between adrenergic and mesenchymal cell states

NB cells display diverse morphological and biochemical properties [136]. Seminal studies observed that NB cells could switch between two distinct phenotypes, N-type (neuroblast) and S-type (substrate adherent), with an intermediate I-type state characterized by a mixed and more aggressive phenotype called "malignant neuroblastoma stem cells" [136, 137]. The mechanisms driving this transdifferentiation have remained elusive for a long time. In 2017, two fundamental studies offered novel insights into these subtypes by analyzing NB cell lines' transcriptomic and epigenetic landscapes [138, 139].

Groningen and colleagues illustrated that most NBs consist of two genetically identical cellular categories: lineage-committed adrenergic (ADRN) and undifferentiated mesenchymal (MES) cells, each displaying markedly distinct phenotypes and gene expression profiles driven by different super-enhancers (SEs) and autoregulatory transcriptional loops, known as core regulatory circuitries (CRCs) [139]. Boeva and her team referred to the more undifferentiated identity as NCC-like [138]. We will use the term MES since it carries broader implications within the oncology field.

CRCs are interconnected self-regulatory circuitries formed by a group of core TFs. These core TFs collectively regulate gene expression by binding to their own SEs and other core TFs. Cancer cells frequently acquire SEs at genes that promote tumorigenesis [140]. SEs thus provide a program for signaling pathways to regulate genes that control cell identity during development and tumorigenesis [140, 141]. In normal human development, CRCs drive the early differentiation of sympathetic neuronal progenitors from migratory NCC during embryogenesis. However, in NB, this process is disrupted, resulting in neuroblasts failing to differentiate and instead progressing toward a malignant cell state [132].

Two primary CRC transcriptional networks were reported to drive NB tumors. The ADRN CRC, including the TFs *HAND2*, *GATA3*, *ISL1*, *PHOX2B*, *TBX2*, *ASCL1*, and *TFAP2B*, closely associated with committed progenitors of the sympathoadrenal cell lineage; and the MES state, driven by a CRC formed by *NOTCH2*, *BACH1*, *ID1*, *EGR3*, *FLI1*, *CBFB*, and *STAT3* [139]. Emerging data is creating a more detailed picture of the CRCs in defining NB cell identity. So far, 18 MES and 20 ADRN SE-associated TFs have been identified to govern the lineage identity of each cell type [138, 139, 142].

## Introduction

### Transitioning between adrenergic and mesenchymal cell states

---

ADRN and MES cell types can spontaneously interconvert, providing the tumor with transcriptional flexibility. Groningen and colleagues demonstrated that inducing PRRX1 expression can transform ADRN cells into a MES state [139], with NOTCH3 identified as a critical regulator of this transition [143]. Interestingly, inhibiting TERT led to a reversible shift from ADRN to MES phenotypes without impacting telomere length, indicating a role for TERT beyond the above-mentioned telomere maintenance functions [144]. Additionally, TOP2B was found crucial for maintaining the ADRN state by suppressing MES-like profiles [145]. A regulatory polymorphism in the LMO1 gene was also discovered, promoting ADRN states and accelerating tumor progression when overexpressed with MYCN [146, 147].

This ability to shift between cell states is known as *tumor plasticity*, a concept now recognized as a novel “emerging hallmark of cancer” [148].

Yet, the existence of a pure MES phenotype in NB cells *in vivo* remains uncertain. Although single-cell RNA sequencing (scRNA-seq) studies from three independent cohorts of primary tumors failed to detect MES-like cells [6, 149, 150], this finding should be approached with caution. The absence of MES cells could reflect a strong influence of the TME, which might promote ADRN identity *in vivo* [151]. It is also plausible that the scarcity of MES cell detection results from sampling biases, such as spatial or temporal variability in the tumor landscape. This raises the possibility that we might be missing critical insights due to limitations in the current sampling methods.

ScRNA-seq studies have also shed light on the cell of origin of NB, revealing that NB cells share similarities with normal fetal adrenal neuroblasts [6, 149, 150]. Interestingly, some cases contained ADRN tumor cells with MES traits [150], Schwann-cell precursors, bridge cells [152], or post-natal progenitor cells [153], suggesting a more complex scenario. These findings imply that NB may not arise from a single cell type but from multiple potential origins, each contributing to the tumor's plasticity and heterogeneity. This challenges the traditional paradigm and opens new avenues for research into NB cells' diverse cellular origins and dynamic nature.



**1.1.6.2. Neuroblastoma plasticity as a driver of resistance**

NB plasticity also plays a crucial role in treatment resistance, with MES cells exhibiting greater resistance to cisplatin, doxorubicin, and etoposide compared to ADRN cells [139]. This resistance is further supported by immunohistochemical analyses, which show an increased proportion of PRRX1+ cells in tumor biopsies following chemotherapy and in tumors after relapse, indicating a selective enrichment of MES cells during and after treatment [139].

Additionally, a scRNA-seq study has revealed that drug exposure induces distinct transcriptome profiles in NB cells, correlating with resistance to cisplatin or etoposide [154]. This resistance was associated with upregulating DNA repair genes, including *BARD1*, *BRCA1*, and *PARP1*. Cisplatin-resistant cells showed enrichment in ADRN genes, while etoposide-resistant cells exhibited comparable enrichment in both ADRN and MES genes, suggesting heightened plasticity potential [154].

Further research by Westerhout *et al.* found a strong SE at the *ALK* locus, which was present in ADRN but absent in MES cells. This loss of ALK SE correlated with reduced ALK expression and increased resistance to the ALK inhibitor lorlatinib, both *in vitro* and *in vivo* [155].

Of note, some studies have identified ADRN tumor cells exhibiting MES-like features. This population of transitional cells expressed genes associated with sympathoadrenal development and rapid tumor proliferation, indicating a potentially more aggressive phenotype [151]. These findings suggest a potential need to shift our focus from solely eradicating NB cells to reprogramming them into non-malignant states for a more effective approach. In this sense, therapeutic approaches targeting CRC TFs or MES-specific may be relevant to make tumor cells more sensitive to chemotherapy.

Overall, the findings indicate that intratumoral diversity in NB is not merely a consequence of mutational events and stochastic processes but comprises two distinct cell populations with consistent epigenetic features and gene expression profiles.

#### 1.1.7. Immune defense and escape in neuroblastoma

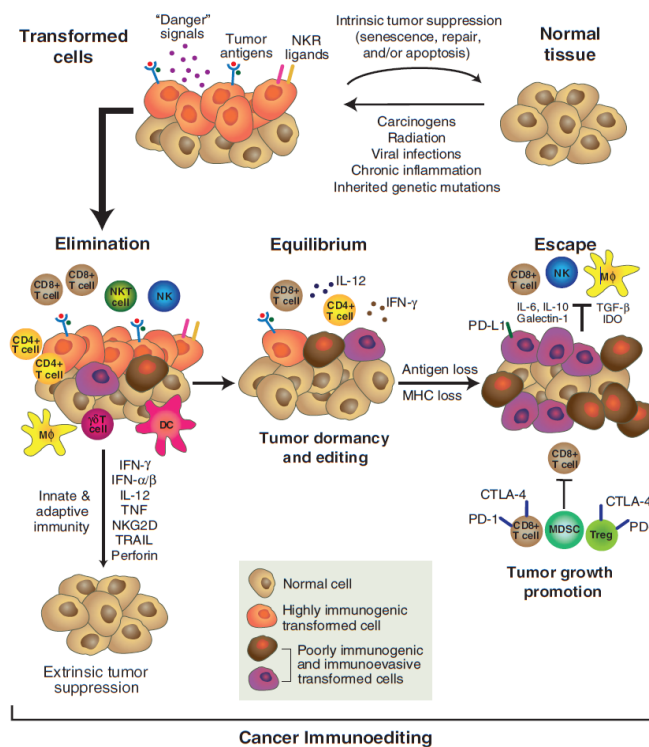
Incorporating immunotherapy into standard treatment has significantly improved survival rates for HR-NB [4, 5]. However, survival rates for these patients remain dismal and cause severe immunotherapy-related toxicities [45-47]. T cell-engaging therapies have shown limited efficacy in NB, primarily attributed to its low immunogenicity and inadequate immune cell infiltration into the TME [156]. Overcoming these challenges is crucial for achieving effective antitumor responses. This section delves into the composition and function of tumor-infiltrating immune cells (TICs), the immune evasion tactics employed by NB, and the opportunities for advancing NB immunotherapy to improve treatment outcomes.

##### 1.1.7.1. Immune recognition of tumor cells

In an ideal scenario of effective tumor recognition and killing, the following steps occur [157]:

- I. **Recognition Phase:** Antigens derived from tumor cells are presented by antigen-presenting cells (APCs), including dendritic cells (DCs), B-lymphocytes, and macrophages. These tumor-associated antigens (TAAs) or neoantigens are recognized by T cell receptors (TCRs) on cytotoxic T lymphocytes (CTLs) or by natural killer (NK) cell receptors. Innate immune cells are also recruited to the tumor site through inflammatory signals released during tumor-induced stromal remodeling.
- II. **Activation Phase:** APCs provide co-stimulatory signals to T cells, leading to their activation and proliferation. Cytokines such as interleukin-12 (IL-12) and interferon-gamma (IFN- $\gamma$ ) further activate and promote the expansion of CTLs.
- III. **Effector Phase:** Activated CTLs and NK cells exert cytotoxic effects on tumor cells by releasing perforin, granzymes, TNF-related apoptosis-inducing ligands (TRAILs), and reactive oxygen species.
- IV. **Memory Phase:** Some activated T cells differentiate into memory T cells, providing long-lasting immune surveillance against tumor recurrence. Memory T cells respond more rapidly and robustly upon re-exposure to tumor antigens, facilitating rapid tumor clearance.

However, the immune system plays a dual role in cancer: it can suppress tumor growth but also promote tumor progression by selecting more resilient tumor cells or creating conditions favoring tumor growth within the TME. This dual role is referred to as "cancer immunoediting" (Figure 8). It consists of three phases: *elimination*, also known as immunosurveillance, in which innate and adaptive immunity ideally eradicates developing tumors; *equilibrium*, in which the cancer cells that are not destroyed enter and are kept in a state of dormancy by T cell-dependent immune mechanisms (CD4+ and CD8+ TCR- $\alpha\beta$  T cells, IFN- $\gamma$ , and IL-12). In this phase, immunogenicity takes over, resulting in the selection of immune-resistant variants that enter the *escape* phase and lead to clinically apparent disease [157].



**Figure 8. Cancer immunoediting model.** Cancer immunoediting is a mechanism that suppresses tumors after cellular transformation and when intrinsic mechanisms have failed. It consists of three phases: elimination, equilibrium, and escape. During the elimination phase, innate and adaptive immunity collaborate to destroy developing tumors before they become clinically detectable. Successful completion results in a cancer-free state. In the equilibrium phase, surviving cancer cell variants are dormant. T cells, IL-12, and IFN- $\gamma$ , are crucial for maintaining this dormancy, indicating a reliance on adaptive immunity rather than innate immunity. Under constant immune selection pressure, genetically unstable tumor cells may evade recognition or suppression and enter the escape phase. Source: [157].

**1.1.7.2. Immune evasion strategies in neuroblastoma****1.1.7.2.1. Immunogenicity**

NB is particularly challenging to target due to its low immunogenicity, characterized by decreased expression of major histocompatibility complex class I (MHC-I) and a reduced tumor mutational burden (TMB). The low immunogenicity and neoantigens render NB cells undetectable to surveilling T cells (Figure 9a) [8, 158]. This phenomenon is further exacerbated in tumors with *MYCN* amplification, which exhibit even lower MHC-I expression than non-amplified NB [159]. This suggests that the low immunogenicity observed in *MYCN*-amplified tumors may result from MHC-I suppression. A promising approach to counteract this immunosuppression lies in the treatment with IFN- $\gamma$ , which has demonstrated the ability to enhance MHC-I expression and strengthen CTL-mediated killing [160].

NB can also disrupt T cell priming by DCs through the expression of pro-apoptotic FAS ligands, a phenomenon correlated with the *MYCN*-dependent status of advanced stages [161, 162]. Furthermore, NB-secreted gangliosides impair the function of DCs by altering antigen presentation and co-stimulation, which leads to reduced levels of critical molecules, including HLA-DR, CD40, CD80, CD83, and CD86, decreased secretion of IL-12 and TNF $\alpha$ , and elevated expression of IL-6 and IL-10. As a result, DCs exposed to NB cannot effectively activate T cells, leading to decreased proliferation, activation, Th1 polarization, and diminished production of IFN- $\gamma$  [163-165].

While NB cells may evade CTLs recognition due to low MHC-I expression, they theoretically remain susceptible to NK cell-mediated lysis. However, NB tumors often exhibit reduced expression of key ligands such as PVR, NECTIN2, MICA, MICB, and ULBPs, essential for activating the DNAM-1 and NKG2D receptors on NK cells. This diminished ligand expression significantly impairs NK cell activation and cytotoxicity. Furthermore, the upregulation of *MYCN* can exacerbate this issue by further reducing NK cell-mediated lysis [166-168].

#### **1.1.7.2.2. Immune checkpoint molecules**

Immune checkpoints significantly influence TILs. PD-L1, the most studied checkpoint molecule, binds to its receptor PD-1 on effector T cells, enabling tumors to evade immune surveillance [169]. Blocking this interaction has shown significant success in various adult cancers by restoring T-cell reactivity [170]. However, the status of PD-L1 expression in NB remains contradictory. Some studies associate high PD-L1 expression with poor survival and increased recurrence, but these findings are inconsistent across all research, highlighting the need for more investigation [171-174].

NB cells exploit other various immune checkpoint molecules to evade immune surveillance. Overexpression of CD200 in NB cells has been linked to decreased levels of CD4<sup>+</sup> and CD8<sup>+</sup> TILs, alongside reduced production of key cytokines such as IFN- $\gamma$  and TNF- $\alpha$  [175].

B7-H3, another immune checkpoint protein encoded by the CD276 gene, is highly expressed in NB and correlates with poor EFS. Blocking B7-H3 enhances NK cell-mediated killing of NB cells [176, 177]. B7-H3 has been the target of ADCs conjugated with the genotoxic drug pyrrolobenzodiazepine, an approach with *in vivo* preclinical efficacy [178]. More recently, chimeric antigen receptor (CAR) T-cell therapy targeting B7-H3 resulted in controlled growth of NB *in vitro* and in xenograft mouse models, which included patient-derived xenografts, further supporting B7-H3 as a feasible target and the clinical development of B7-H3 CAR-Ts [179].

TIGIT (T cell immunoreceptor with Ig and ITIM domains) is a crucial immune checkpoint protein that significantly regulates immune responses by interacting with PVR and NECTIN2. It is expressed in T cells, NK cells, and Tregs. Recent studies have identified the TIGIT/NECTIN2 interaction as a critical factor in T/NK cell dysfunction within NB tumors [180]. Blocking TIGIT, particularly in combination with PD-L1 inhibitors, has shown remarkable effectiveness in experimental models, including those resistant to chemotherapy [180].

#### **1.1.7.2.3. Immunosuppressive myeloid and stromal cells**

Myeloid-derived suppressor cells (MDSCs) represent a significant population of myeloid cells within the TME, capable of exerting profound suppression on lymphocyte responses [181, 182]. These MDSCs contribute to immunosuppression and tumor growth by secreting reactive oxygen species (ROS), arginase-1, and TGF- $\beta$  [183].

## Introduction

### Soluble mediators of immune suppression

---

In the context of NB, studies in mouse models have shown that treatment with polyphenol E reduces the number of MDSCs, consequently restoring T cell proliferation and mitigating tumor growth [184]. Additionally, *in vitro* and *in vivo* investigations have revealed that stromal cells and monocytes actively suppress NK cell-mediated antibody-dependent cellular cytotoxicity (ADCC) against NB when targeted with anti-GD2 therapy [185]. Notably, the depletion of stromal and endothelial cells along with monocytes has been shown to restore NK cell activity, highlighting the immunosuppressive role of the myeloid and stromal compartments within the TME [185].

#### 1.1.7.2.4. Soluble mediators of immune suppression

NB cells release a spectrum of immunosuppressive factors, including TGF- $\beta$ , galectin-1, MIF, soluble GD2 (sGD2), and arginase-2, all of which impair the function of CTLs and NK cells [186-188]. In NB models, TGF- $\beta$  has been observed to influence NK cell function directly. At the same time, galectin-1 or MIF level reductions have been linked to enhanced T-cell-mediated cytotoxicity and improved tumor rejection rates [187, 189, 190]. Also, heightened tumoral TGF- $\beta$  levels have been associated with shorter EFS [191].

Soluble galectin-1 and MIF have been found to induce apoptosis in T cells, inhibit proliferation, and hinder DC maturation, further exacerbating immunosuppression [187, 192, 193]. Moreover, elevated tumoral MIF levels correlate with decreased abundance of CTLs, NK cells, B cells, and DCs and are linked to poor prognosis in stage 4 NB, irrespective of *MYCN* status [194].

GD2, whether on the cell surface or in soluble form, suppresses immune responses by binding to the myeloid checkpoint Siglec-7 on macrophages and NK cells [195]. Arginase-2 reduces arginine levels, impairing lymphocyte function and correlating with worse patient outcomes. Additionally, catecholamines derived from NB cells contribute to immune modulation [196]. Soluble forms of MICA and B7-H6 released by NB cells inhibit immune responses by downregulating receptors on NK cells, leading to reduced NK cell-mediated cytotoxicity, chemoresistance, and an increased risk of metastasis [168, 197].

HLA-E, found abundantly in NB, particularly in stage 4 tumors, inhibits NK cell activity and stimulates the secretion of IL-10 and TGF- $\beta$  [198]. Other immunosuppressive factors such as soluble HLA-E (sHLA-E), soluble HLA-G (sHLA-G), IL-10, and HMGB1 have

been identified in NB. These factors impact NK cell and CTL function and are associated with disease progression and relapse [199-201].

### **1.1.7.3. Implications for immunotherapy**

The restricted immunogenicity of NB and the arsenal of immunosuppressive mechanisms described above impede the activity of TILs (Figure 9a). Combining treatments to overcome these barriers is crucial for an effective antitumoral response.

Various strategies are currently under investigation. Arginase inhibition in NB cell lines has proved to restore CAR T cell functionality [202], and TGF- $\beta$  blockade overcame resistance to anti-PD-1 [203]. In murine NB models, immune checkpoint inhibitors (ICIs) alone were ineffective; however, combined with other treatments, they significantly enhanced anti-tumor immunity. These supplementary interventions included integrating ICIs with anti-GD2 [204], vaccination [205], Treg depletion [206], or myeloid inhibition [195]. Additionally, galunisertib, a small-molecule inhibitor of TGF- $\beta$ R1, counteracted the immunosuppressive effects induced by TGF- $\beta$  on the direct cytotoxicity and anti-GD2 ADCC of human *ex vivo* propagated and activated NK (aNK) cells against NB cells *in vitro* [207].

Epigenetic therapies have emerged as a valuable tool for targeting somatic alterations contributing to abnormal sympathoadrenal development, blocking differentiation, and promoting tumorigenesis [208]. Of note, HDAC inhibitors like entinostat not only improved the cytotoxic activity of T and NK cells but also induced a shift towards a more mesenchymal cell lineage [209]. Significantly, entinostat facilitated the essential prerequisite for CTCs activation: surface expression of MHC-I [209].

Combining radiotherapy with immunotherapy has also demonstrated significant antitumor activity in preclinical models, suggesting a potential strategy to overcome the immunosuppressive TME. Phase I studies are evaluating combinations of  $^{131}\text{I}$ -MIBG with dinutuximab (anti-GD2) or dinutuximab- $\beta$  plus nivolumab (anti-PD-1) (NCT03332667, NCT02914405). This combination is believed to synergize radiation-inducing acute inflammation and innate antitumor immunity. At the same time, PD-1 inhibition enhances the ADCC functions of NK cells and the antitumor activity of T cells infiltrating the radiation-treated area. Furthermore, vorinostat has been found to increase infiltrating myeloid effector cells and decrease MDSCs, resulting in synergy with anti-GD2 antibodies [210].

## Introduction

### Implications for immunotherapy

---

Other epigenetic drugs are under investigation for their capacity to enhance current immunotherapy. Pharmacologic inhibition of the histone methyltransferase EZH2 resulted in epigenetic rewiring of mesenchymal NB cells and reexpression of enzyme ST8SIA1, restoring surface expression of GD2 and sensitivity to anti-GD2 antibody [211]. Furthermore, targeting YAP-mediated transcriptional repression of ST8SIA1 revealed a mechanism for NB transition to a mesenchymal state and pointed to YAP as another therapeutic target to augment GD2 immunotherapy responses [212].

Integrating these approaches with conventional treatments like chemotherapy and radiation therapy could yield synergistic anti-tumor effects and improved patient outcomes. However, this approach requires careful scrutiny. For instance, while combining ICIs with anti-GD2 therapy shows potential, the efficacy of these combinations can be inconsistent, as evidenced by variable responses in preclinical models and early-phase trials. Moreover, the use of HDAC inhibitors like entinostat to enhance T-cell and NK-cell cytotoxicity may shift NB cells toward a more aggressive mesenchymal state [209], which could paradoxically increase tumor plasticity and resistance [139, 148].



### **1.1.8. Emerging immunotherapies in neuroblastoma**

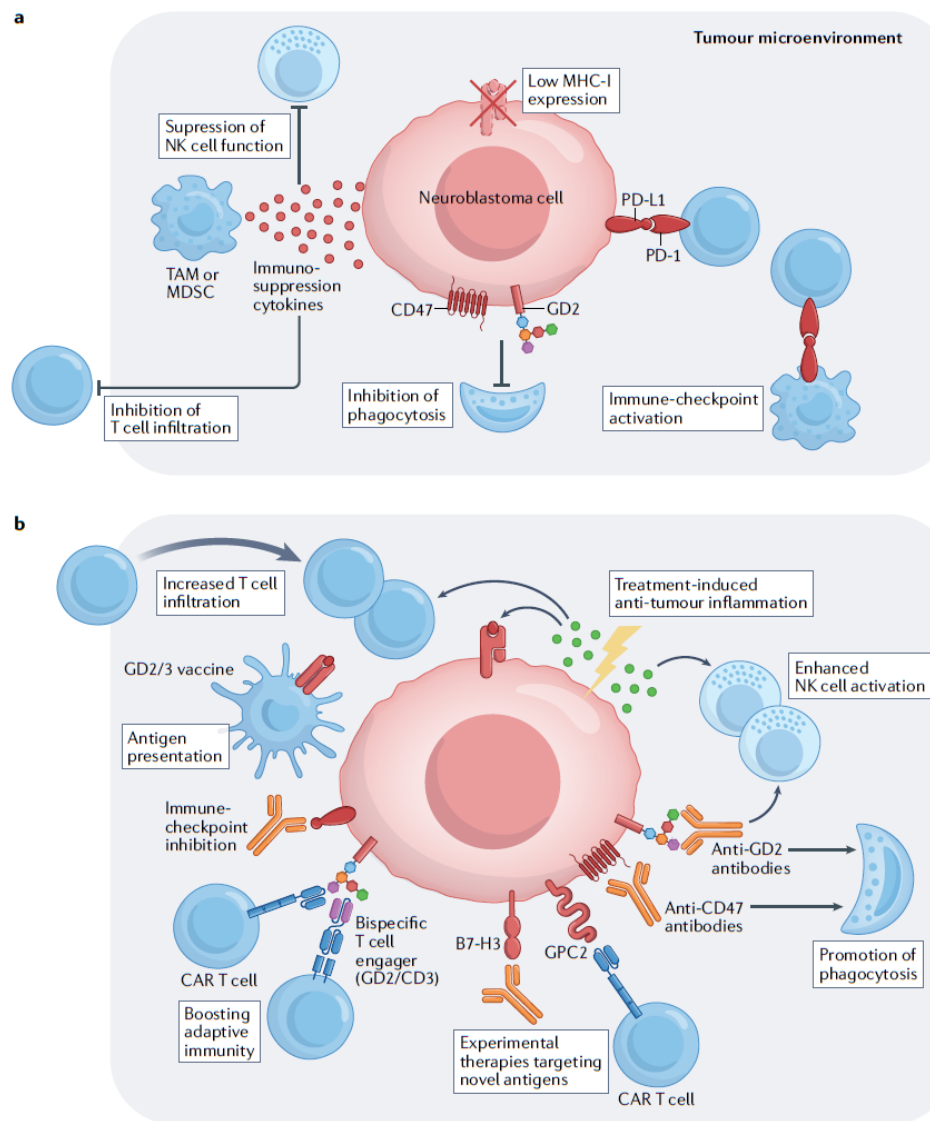
Due to the limitations of current immunotherapies, it becomes increasingly important to explore new therapeutic avenues. Emerging immunotherapies harnessing the adaptive immune system offer promise for durable remission in therapy-resistant patients. The following section will delve into these innovative strategies, emphasizing their potential to reshape the TIME (Figure 9b).

#### **1.1.8.1. Antibody-based immunotherapies**

MAbs target unique tumor surface antigens, triggering various killing pathways, including complement-mediated cytotoxicity (CMC), NK- and neutrophil-mediated ADCC, complement-dependent cellular cytotoxicity (CDCC), and antibody-dependent cell-mediated phagocytosis (ADCP) [213, 214]. Building on these mechanisms, emerging antibody-based therapies, including bispecific antibodies (bsAbs) and ADCs, are being developed to enhance the precision and efficacy of immunotherapy.

BsAbs like bispecific T-cell engager (BiTE) and dual-affinity retargeting (DART) antibodies link tumor-specific MAbs to anti-CD3 MAbs, activating T cells for tumor destruction. ADCs consist of MAbs conjugated to cytotoxic payloads, delivering potent drugs directly to cancer cells expressing specific surface antigens. Though effective for B-cell acute lymphoblastic leukemia, their efficacy against solid tumors like NB remains challenging [215]. Other constructs, such as BiKEs (targeting CD16) and TriKEs, aim to activate NK cells and incorporate cytokines, respectively. However, these have yet to match *in vitro* success in patients [216].

Apart from GD2, other overexpressed molecules on NB cells are being explored as targets for antibody-based immunotherapies. Preclinical research has shown promising antitumor activity with ADCs targeting ALK, B7-H3, glypican-2 (GPC2), NCAM (CD56) and L1-CAM (CD171) [89, 217-221]. However, ALK and GPC2's relatively low expression density on NB cells, in contrast to the highly expressed GD2, might hinder their effectiveness [222, 223]. Lorvotuzumab mertansine, an ADC targeting NCAM (CD56) and conjugated to mertansine (a potent microtubule toxin), has shown good tolerability in a phase II trial with pediatric patients having relapsed and refractory solid tumors, including NB [217]. Additionally, a clinical trial is evaluating the efficacy of B7-H3-targeted antibody enoblituzumab (MGA271) with results pending publication (NCT02982941) [224].



**Figure 9. Immunosuppression mechanisms and emerging immunotherapies in neuroblastoma.** (a) Mechanisms employed by NB to evade immune response include low MHC class I expression by NB cells, expression of immune checkpoint ligands, and immunosuppressive cytokines that suppress effector cell function and suppress tumor cell phagocytosis (b) Various immunotherapies show promise in enhancing antitumor immune function in NB. Cytokine-based therapies, radiotherapy, and immunogenic chemotherapies can potentially promote antitumor inflammation, MHC class I expression, and T and NK cell infiltration. Anti-GD2 antibodies possess intrinsic antitumor activity and enhance NK cell-dependent ADCC while promoting tumor cell phagocytosis. Similarly, anti-CD47 antibodies may enhance antitumor phagocytosis. Although single-agent ICIs have shown limited activity in NB patients, combining ICIs with therapies that promote T cell infiltration and antitumor inflammation may enhance their effectiveness. Additional mechanisms for boosting adaptive immunity include bispecific antibodies, tumor vaccines, and CAR T cell therapy or invariant NK T cell therapy. Source: [3].

Macrophages within the TME offer another promising target for enhancing antitumor effects in NB. Tumor cells express CD47, a macrophage checkpoint that acts as a "don't eat me" signal, thereby inhibiting phagocytosis by macrophages [225]. Preclinical studies have demonstrated that combining anti-CD47 with anti-GD2 antibodies enhances synergistic efficacy, potentially through disruption of the GD2-Siglec-7 interaction (Figure 9. Immunosuppression mechanisms and emerging immunotherapies in neuroblastoma. [195]. This integrated approach has been translated into a first-in-child/first-in-human trial investigating the combined use of anti-GD2 and anti-CD47 antibodies in treating relapsed NB (NCT04751383).

### **1.1.8.2. Adoptive cellular therapies**

#### **1.1.8.2.1. T-cell therapy**

CAR T cell therapies have remarkably succeeded in treating hematological malignancies, particularly those expressing CD19. However, their application in solid tumors, including NB, has faced significant challenges [226, 227]. These challenges include difficulties in maintaining T-cell survival and expansion, T-cell exhaustion, activation-induced cell death (AICD), as well as complications like trogocytosis, fratricide, antigen loss, and the risk of unintended gene transduction into tumor cells [228, 229].

New CAR T cell receptor designs are being developed to address these obstacles. These innovations focus on incorporating alternative costimulatory domains, enhancing cytokine secretion, improving *in vivo* persistence, and tackling antigen heterogeneity. Additionally, these designs aim to minimize adverse effects such as cytokine release syndrome and neurotoxicity, making CAR T cell therapy a more viable option for solid tumors [228, 229].

Ongoing research is expanding the range of antigens targeted for NB therapy, including the promising candidates GPC2 and B7-H3 (CD276) [219, 230]. Preclinical studies of anti-GPC2 CAR T cell therapy have shown encouraging results, demonstrating significant tumor regression and indicating its potential as a novel treatment option [231]. However, the effectiveness of systemic antibody therapy targeting B7-H3 is limited by hepatic toxicities, which represent a challenge to its clinical application and require strategies to mitigate these side effects. To address this, intrathecal delivery of radiolabeled B7-H3 (<sup>131</sup>I-8H9) has been employed in CNS-relapsed NB patients, demonstrating favorable tolerability in a proof-of-principle study [232]. Ongoing clinical

trials are exploring CAR T cells targeting L1CAM (NCT02311621), B7-H3 (NCT04483778), EGFR (NCT03618381), and GD2 (multiple trials). Of particular interest is GD2-targeted CAR T-cell therapy, which has recently been demonstrated to achieve a sustained antitumor effect in HR-NB. Although treatment-related toxic effects were observed, the activation of a suicide gene effectively controlled these side effects, demonstrating its feasibility and safety (NCT03373097) [233].

Even though actionable tumor neoantigens are limited in NB patients, they possess targetable MHC-associated tumor antigens at low levels in specific normal tissues, including cancer-testis antigens and embryonic/differentiation antigens [234]. Notably, oncofetal proteins like PHOX2B, a transcriptional factor and part of NB's recently identified CRC, are promising targets for T cell-based immunotherapies. Preclinical proof-of-concept studies have demonstrated the potential of CAR T cells engineered to target a PHOX2B-specific epitope presented by MHC, highlighting a novel avenue for treating NB [235].

Other potential genetic modifications involve attracting T-cells through chemokine receptors and decreasing Treg interference by enhancing the Akt pathway [236, 237]. Similarly, strategies for adoptive cell therapy with NK cells and NKT cells involving their *ex vivo* expansion using engineered IL-15 or IL-21 are under investigation [238, 239]. Expanding TILs *ex vivo* for reinfusion therapy has been successfully demonstrated. However, these TILs did not exhibit direct reactivity against NB; instead, they produced IFN- $\gamma$  and displayed migration toward NB cell lines [240].

#### **1.1.8.2.2. NK-cell therapy**

CARs are also being integrated into NK T cells (CAR-NKT), which recognize glycolipids presented via CD1d and exhibit an enhanced ADCC and cytokine release capacity compared to CD8+ CAR T cells [241]. CAR-NKT can kill target cells independently of MHC, providing an alternative to the autologous restriction of CAR T cells and implying broader potential for off-the-shelf use [242].

A traditional drawback of NK cell therapy was its inability to generate immunological memory, posing a risk of relapse in patients with residual disease [243]. Nevertheless, recent results have shown that NK cells exhibit characteristics akin to adaptive immunity and can develop immunological memory comparable to T and B cells [244]. V $\alpha$ 24-invariant-natural killer T (iNKT) cells are a promising CAR-T system, demonstrating

efficient trafficking to tumors like NBs and evoking anti-tumor immunity by targeting CD1d-expressing TAMs [191]. Clinical trials administering CAR-NKT cells to refractory NB patients have shown persistence *in vivo*, tumor trafficking, and induced objective responses and metastasis regression (NCT03294954, NCT00877110) [245, 246].

#### **1.1.8.2.3. Vaccines**

Despite extensive research and testing of vaccines employing whole tumor cells, peptides, lysates, and proteins in various pediatric tumors, the clinical benefits of these approaches remain uncertain [156].

A GD2/GD3 vaccine tested in relapsed NB patients showed promising results, with detectable serum anti-GD2 antibodies correlating with improved EFS and OS (NCT00911560) [247]. The vaccine was well-tolerated, with minimal adverse effects. However, interpreting survival data is complicated by subsequent therapies received by patients [247]. Besides, the observed correlation between immune response and clinical outcomes is unusual, given the disparity between immune response evidence in laboratories and actual clinical benefits observed in vaccine trials. This discrepancy may arise due to irrelevant biomarkers, inadequate response, or advanced tumor bulk [156]. While there's strong evidence that T-cell vaccines utilizing these antigens can be effective in syngeneic mouse models, their clinical success has been limited [248, 249].



## 2. Hypothesis and objectives

Despite advancements in multimodal therapies, HR-NB continues to pose significant challenges in terms of prognosis and treatment-related comorbidities. As the field of cancer medicine evolves, there is a notable shift towards developing personalized and molecularly directed therapies for each tumor subtype. However, the lack of identified actionable mutations complicates the development of tailored treatments for specific subtypes of NB.

This research is based on the central hypothesis that the TIME is a crucial orchestrator of immune modulation within NB, thus significantly impacting therapy resistance and immune evasion. We propose that understanding the complex interactions between different subtypes of NB and the TIME will enable us to discover novel therapeutic strategies to enhance immune response and overcome therapeutic resistance in NB patients.

### **Main objective:**

Investigate the TIME of HR-NB to identify key immune evasion mechanisms that enhance precision medicine strategies.

### **Subspecific objectives:**

1. Assess the outcomes of HR-NB subtypes after standard anti-GD2 immunotherapy in the Spanish cohort of HR-NBL/SIOPEN trial.
2. Characterization of HR-NB TIME:
  - 2.1. Investigate the heterogeneity of immune cell populations within the TIME across different HR-NB subtypes.
  - 2.2. Identify and characterize the molecular and cellular mechanisms by which NB cells evade immune surveillance within the TIME.
  - 2.3. Explore the epigenetic mechanisms contributing to immune evasion in NB.
3. Assess implications for precision medicine strategies:
  - 3.1. Determine the impact of identified immune evasion mechanisms on the effectiveness of CAR-T cell therapy in NB cell lines.





### 3. Materials and methods

#### 3.1. Patients

To evaluate the effectiveness of anti-GD2 immunotherapy in HR-NB patients, we conducted a retrospective analysis using the Spanish cohort enrolled in the HR-NBL/SIOPEN trial between 2007 and 2015 ( $n = 83$ ). Patient staging was performed according to the INRG classification system [7]. Biological analyses of patients involved evaluating *MYCN* status using Fluorescence in Situ Hybridization (FISH) and 11q status using Multiplex Ligation-dependent Probe Amplification (MLPA). FISH and MLPA techniques were utilized for samples collected between 2008 and 2012. From 2013 to 2015, CytoScan HD array (ThermoFisher Scientific, see 3.7) was employed to analyze 11q status and verify *MYCN* status, following the European Neuroblastoma Quality Assessment (ENQUA) guidelines.

Clinical data, including treatment regimens and patient outcomes, were retrieved from the Spanish Neuroblastoma Clinical Database (NbCDB). The study followed the Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) and the principles outlined in the Declaration of Helsinki. Ethical approval for the study protocol was obtained from La Fe Hospital Research Ethics Committee. Informed consent for sample and data management was obtained from parents or legal guardians of participating patients.

We conducted a comparative analysis of treatment outcomes in HR-NB patients who received anti-GD2 antibody ch14.18/CHO (dinutuximab beta) in combination with isotretinoin (13-cis-retinoic acid) ( $n = 50$ ) vs. those who received isotretinoin alone ( $n = 33$ ) during maintenance therapy. The cohort treated with isotretinoin alone received their treatment regimen before 2010 when the immunotherapy arm was activated in Spain. All patients included in the study had undergone induction therapy, consisting of either rapid COJEC or modified N7 protocols, with or without two additional cycles of TVD (topotecan-vincristine-doxorubicin), followed by surgery, myeloablative treatment, and radiotherapy. The interval between the start of induction chemotherapy and the initiation of high-dose chemotherapy, followed by ASCT, consistently remained below 9 months. Of note, the survival analysis begins at the post-consolidation phase, excluding patients who did not respond to induction chemotherapy, failed to reach consolidation, or succumbed to complications.

## Materials and methods

### Cell culture and culture conditions

## 3.2. Cell culture and culture conditions

### 3.2.1. Neuroblastoma cell lines

SK-N-BE(2) and SH-SY5Y cell lines were purchased from the American Type Culture Collection (ATCC). R. Versteeg kindly provided IMR-5 cells. F. Spelemann kindly provided SK-N-AS cells. SK-N-BE(2), SH-SY5Y, IMR-5, and SK-N-AS cells were maintained in high-glucose Dulbecco's Modified Eagle Medium (DMEM) (ThermoFisher Scientific) supplemented with 10% fetal bovine serum (FBS) (Gibco), 100U/mL penicillin, 100 mg/mL streptomycin (1% Pen/Strep) (ThermoFisher Scientific), and 2mM L-glutamine (Gibco). J. M. Maris kindly provided KELLY and NBL-S. These were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (ThermoFisher Scientific), 10% FBS, 1% Pen/Strep, and 2 mM L-Glutamine. Cells were detached using 0.05% trypsin-EDTA (ThermoFisher Scientific) and maintained in a humidified 5% CO<sub>2</sub> air atmosphere at 37°C. All cells were routinely checked for mycoplasma with MycoAlert Detection Kit (Lonza). Cell line characteristics are provided in Table 4.

**Table 4.** Neuroblastoma cell characteristics.

| Cell line            | Sex | <i>MYCN</i> | 11q                  |
|----------------------|-----|-------------|----------------------|
| SH-SY5Y <sup>1</sup> | F   | Non-amp     | Non-del              |
| SK-N-BE(2)           | M   | Amp         | Non-del              |
| SK-N-AS              | F   | Non-amp     | Del                  |
| IMR-5                | M   | Amp         | Del                  |
| NBL-S                | M   | Non-amp     | Del                  |
| KELLY                | F   | Amp         | Del                  |
| CHP-134              | M   | Amp         | Non-del              |
| NB-EBc1              | M   | Non-amp     | Del                  |
| COG-N-415            | F   | Amp         | Non-del              |
| NB-69                | M   | Non-amp     | Non-del <sup>2</sup> |
| LAFE-NB1             | M   | Amp         | Non-del              |
| LAFE-NB2             | M   | Amp         | Non-del              |
| LAFE-NB3             | M   | Non-amp     | Non-del              |
| LAFE-NB4             | M   | Amp         | Del                  |
| LAFE-NB5             | M   | Amp         | Non-del              |

<sup>1</sup>SH-SY5Y is a triple-cloned derivative of the bone marrow biopsy-derived cell line SK-N-SH.

<sup>2</sup>cnLOH q23.1, q23.3. Del: deleted; Amp: amplified. Non-amp: non-amplified.

### **3.2.2. Neuroblastoma primary cell lines**

CHP-134, COG-N-415, NB-EbC1, and NB-69 cell lines were obtained from the J. M. Maris laboratory repository (Table 4). Cell passages ranged from 1 to 3. COG-N-415 cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) (Thermo Fisher Scientific) supplemented with 20% FBS, 4 mM L-glutamine, 0.5% Pen/Strep, 1X ITS (5 µg/mL insulin, 5 µg/mL transferrin, 5 ng/mL selenous acid) (Gibco) at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. CHP-134, NB-EBc1, and NB-69 cell lines were maintained in RPMI 1640 medium, 10% FBS, 1% Pen/Strep, 2 mM L-Glutamine and detached using 0.02% Versene (Gibco). Cell lines were tested for mycoplasma regularly with MycoAlert kit (Lonza).

### **3.2.3. Neuroblastoma cell lines for CAR-T assay**

To investigate macrophages' impact on CAR-T cells' killing potential, we utilized the PHOX2B-specific PC-CAR 10LH, which targets the unmutated PHOX2B peptide QYNPIRTTF [235]. PHOX2B PC-CAR T cell has a high affinity for the PHOX2B peptide when presented by HLA-A\*24:02 or HLA-A\*23:01 molecules [235]. We used the human NB cell lines SK-N-AS, SK-N-BE(2), and NB-EBc1 for this assay. These cell lines present the following HLA alleles: SK-N-AS (HLA-A\*24:02 and HLA-A\*26:01), NB-EBc1 (HLA-A\*02:01:01 and HLA-A\*02:01:18), and SK-N-BE(2) (HLA-A\*01:01:03 and HLA-A\*30:02:01). To ensure presentation of the PHOX2B peptide, NB-EBc1 and SK-N-BE(2) were transduced with HLA-A\*24:02 and HLA-A\*23:01 GFP vectors. PC-CAR 10LH and transduced NB cell lines were provided by John M. Maris's laboratory. Parental NB cell lines and untransduced T cells served as negative controls in the experiments. All NB cell lines were cultured in RPMI medium supplemented with 10% FBS, 2 mM L-glutamine, and 1% Pen/Strep. Cultures were maintained at 37°C in a humidified incubator with 5% CO<sub>2</sub>.

### **3.2.4. Neuroblastoma patient-derived organoids**

Tissue samples were obtained from HR-NB patients undergoing surgical resection at La Fe Hospital, following the guidelines approved by La Fe Research Ethics Committee. Informed consent was obtained from parents or legal guardians before sample collection. Upon arrival, tumor specimens were immediately processed under the supervision of a pathologist, who selected the tumor section for subsequent procedures. To establish NB

## Materials and methods

### Cell culture and culture conditions

---

patient-derived tumor organoids (PDTOs), we utilized a modified protocol based on the method outlined by Bate-Eya and colleagues [250]. Tissue dissociation was performed by mechanical disruption with scalpels, followed by enzymatic digestion of minced tissue using 2 mg/mL collagenase/dispase (Roche) and 0,25 µg/mL DNase I (Roche). Enzymatic digestion ranged from 45 minutes to 2 hours, depending on the degree of tissue disaggregation observed under visualization. Following digestion, the resulting cell suspensions were filtered through a 70 µm cell strainer (ThermoFisher Scientific) to obtain single cells.

Single cells were embedded in Matrigel (Corning) and, once solidified, plated in organoid culture medium consisting of Neurobasal-A medium (ThermoFisher Scientific) supplemented with 1X B-27 Supplement minus vitamin A (ThermoFisher Scientific), 1% N-2 Supplement (ThermoFisher Scientific), 0.5% Pen/Strep (ThermoFisher Scientific), 40 ng/mL epidermal growth factor (EGF) (ThermoFisher Scientific), and 20 ng/mL fibroblast growth factor-basic (FGF-2) (ThermoFisher Scientific). A 10 µM concentration of ROCK inhibitor Y-27632 (STEMCELL Technologies) was included in the medium for the initial two weeks to prevent dissociation-induced apoptosis (anoikis). After this period, Y-27632 was removed from the medium.

The medium was refreshed every week until visible signs of growth were observed. Organoids were maintained in a humidified incubator at 37°C with 5% CO<sub>2</sub> in hypoxic conditions (2% O<sub>2</sub>) and dissociated using Accutax (ThermoFisher Scientific). Mycoplasma was regularly tested using the MycoAlert kit (Lonza). NB PDTOs characteristics are summarized in Table 4.

#### 3.2.5. Monocytes cell line and primary human monocytes

Primary human monocytes were obtained from peripheral blood samples from healthy donors at the Immunology Core of Perelman School of Medicine (University of Pennsylvania, Philadelphia). Monocyte subsets were enriched by negative selection using RosetteSep Kit (STEMCELL Technologies) and cultured in RPMI 1640 medium supplemented with 10% FBS and 1% Pen/Strep.

The human monocytic THP-1 cell line was obtained from the ATCC and maintained in RPMI 1640 medium supplemented with 10% FBS, 1% Pen/Strep, and 2 mM L-glutamine at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. Cell lines were routinely tested for mycoplasma with MycoAlert kit (Lonza).

### **3.3. Monocyte differentiation and macrophage polarization**

To differentiate THP-1 cells into M0 macrophages, monocytes at  $5 \times 10^5$  cells/mL density were treated with 100 nM phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) for 24 hours. Subsequently, they were allowed to rest in complete RPMI medium without PMA for an additional 24 hours. Primary monocytes isolated from healthy donor peripheral blood were cultured at  $1 \times 10^6$  cells/mL in complete RPMI medium plus 25 ng/mL human recombinant macrophage colony-stimulating factor (M-CSF) (Peprotech) for 7 days. Peripheral blood mononuclear cells (PBMCs) were differentiated within 24 hours of collection.

Following differentiation, both M0 macrophages from THP-1 cells and PBMCs were polarized into M1 macrophages by incubating them in complete RPMI medium with 50 ng/mL recombinant human IFN- $\gamma$  (Peprotech) and 50 ng/mL lipopolysaccharides (LPS) (Sigma-Aldrich) for 48 hours. M2 macrophages were generated by incubating them with 25 ng/mL recombinant human IL-4 (Peprotech) and IL-13 (Peprotech) for 48 hours. Confirmation of macrophage differentiation and polarization was achieved through light microscopy adherence visualization and analysis of specific marker expression using droplet digital PCR (ddPCR) or quantitative PCR (qPCR).

### **3.4. Co-culture system**

Transwell inserts with 0.4  $\mu$ m pore polycarbonate membrane inserts (Corning) were utilized for co-culture experiments in two-phase independent chambers where cells were maintained in non-contact crosstalk. Monocyte and macrophage populations were seeded into the receiver wells, while NB cells were seeded into the upper chamber (2:1 monocytes or macrophages to NB cells ratio). In the case of PDTOs, dissociated organoids were embedded in Matrigel and incubated for 24 hours to allow for organoid formation before being co-cultured with monocytes or macrophages. Following 72 hours of co-culture, cells were harvested for subsequent analysis.

### **3.5. CAR-T killing assay**

Human T cells were thawed, activated for 24 hours in the presence of 5 ng/mL IL-7 and 5 ng/mL IL-15 (Invitrogen), as previously described [235], and co-incubated with  $2 \times 10^4$

NB cells at 1:1 and 5:1 effector to target (E:T) ratios. Cells were monitored over 48 hours, with Caspase-3/7 activity tracked using Incucyte Caspase-3/7 Dye (Sartorius) as an apoptosis marker. Images were captured every 2 hours via the Incucyte S3 system (Sartorius), and apoptotic activity was quantified by measuring Caspase-3/7 integrated intensity per image, normalized to the time of T cell addition.

### **3.6. Cytokine secretion analysis**

After 24 hours of co-culture, supernatants were collected from each experimental condition, including both macrophage-polarized and control samples. Supernatants were stored at -80°C until further analysis to ensure cytokine stability. Levels of IFN- $\gamma$  and IL-2 were measured using DuoSet ELISA kits (R&D Systems), following the manufacturer's protocol. Each condition was analyzed in triplicate.

### **3.7. Single nucleotide polymorphism arrays**

High-resolution Single Nucleotide Polymorphism (SNP) array analysis was conducted on 41 metastatic NB patients. Of these, 29 were classified as stage M, and 12 as stage MS. Whole genome SCAs were analyzed using CytoScan HD array (Thermo Fisher Scientific). Genomic DNA was extracted from frozen tumor tissues with DNeasy Tissue kit (QIAGEN). The quantity and quality of DNA were assessed using NanoDrop spectrophotometer absorbance (ThermoFisher Scientific), gel electrophoresis, and second quantification after PCR amplification. Isolated DNA underwent fragmentation via Nsp I (New England Biolabs) digestion, followed by adaptor ligation and PCR amplification. Analysis of SNP array data was performed using Chromosome Analysis Suite (ChAS) Software v3.1 (ThermoFisher Scientific), using the annotation version of the human reference sequence GRCh37 (hg19).

### **3.8. Cell viability assays**

Two methods were used to assess cell viability: CyQuant XTT assay (ThermoFisher Scientific) and CellTiter Glo 2.0 luminescent assay (Promega). For CyQuant XTT assay, cells were seeded at a density ranging from 3 to  $5 \times 10^3$  cells per well in 96-well plates, each containing a final volume of 100  $\mu$ L. After exposure to experimental conditions, CyQuant XTT reagent was prepared per the manufacturer's guidelines, combining 1 mL

of Electron Coupling Reagent with 6 mL of XTT Reagent, followed by vortexing. Subsequently, 70  $\mu$ L of the working solution was added to each well, and plates were incubated at 37°C for 4 hours in a CO<sub>2</sub> incubator. Absorbance measurements were then taken at 450 nm 660 nm wavelengths to account for background signals. To determine the specific absorbance of the sample, the following formula was employed: Specific Absorbance = [Abs 450 nm (Test) - Abs 450 nm (Blank)] - Abs 660 nm (Test), with the blank wells containing only medium without cells, serving as a reference.

Similarly, for measuring cell viability with CellTiter Glo 2.0 luminescent assay, cells were seeded at densities ranging from 3 to 5×10<sup>3</sup> cells/well in white opaque 96-well plates (white opaque plates aimed to minimize well-to-well crosstalk and reduce background luminescence). After conducting the experiments, the CellTiter Glo 2.0 reagent was prepared following the manufacturer's instructions. Before its addition, the plates were equilibrated at room temperature for 30 minutes. Subsequently, CellTiter-Glo 2.0 reagent was added to each well, matching the volume of cell culture medium present (i.e., 100  $\mu$ L). The plate contents were mixed for 2 minutes on an orbital shaker to induce cell lysis, facilitating the release of intracellular contents, and incubated for 10 minutes at room temperature to stabilize the luminescent signal. Luminescence measurements were taken in GloMax Discover microplate reader (Promega) at an integration time of 1 second per well. Control wells containing only medium were included to record background luminescence.

### **3.9. Gene expression analyses**

#### **3.9.1. RNA isolation and cDNA synthesis**

Total RNA was extracted from cultured cells using RNeasy Mini Kit (Qiagen), following the manufacturer's instructions. Briefly, cells were lysed with lysis buffer supplemented with  $\beta$ -mercaptoethanol to ensure RNA stability. The lysate was then processed through RNeasy spin columns. On-column DNA digestion was incorporated during the RNA purification to eliminate genomic DNA contamination. An extra initial process involving Matrigel depolymerization was conducted to isolate RNA from PDOs. PDO cultures were incubated with Cell Recovery solution (Corning) for 20-30 minutes at 4°C, followed by PBS washes and centrifugation to eliminate Matrigel excess. This step was repeated until no Matrigel residue was visible.

RNA concentration and purity were determined using NanoDrop 2000 spectrophotometer (ThermoFisher Scientific). RNA samples with 260/280 ratios within the range of approximately 1.8 to 2 and 260/230 ratios around 2 to 2.2 were considered acceptable. Otherwise, RNA was repurified by ethanol precipitation until good quality values were achieved [251]. All RNA samples were used immediately or stored at -80°C until further analysis. 500 ng of RNA were reverse transcribed using the High-Capacity cDNA Reverse Transcription kit (ThermoFisher Scientific) according to the manufacturer's instructions. Thermal cycling conditions for cDNA synthesis were 10 minutes at 25°C for initial denaturation, followed by reverse transcription at 37°C for 120 minutes, and reaction termination by heating at 85°C for 5 minutes. Finally, samples were either maintained at 4°C for immediate use or stored at -20°C for future use.

### **3.9.2. Quantitative PCR**

To analyze mRNA expression levels, cDNA samples underwent quantitative PCR (qPCR) with TaqMan Fast Advanced Master Mix assay (ThermoFisher Scientific). For qPCR reactions, 5 µL cDNA template (1:100 diluted retrotranscribed cDNA) and 300 nM of primers were used. Primer details are found in Table 5. Reactions were performed in triplicate in optical-grade 96-well PCR plates (Applied Biosystems) and run on ViiA 7 Real-Time PCR System (ThermoFisher Scientific). Thermal cycling conditions involved an initial denaturation at 95°C for 3 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, and annealing/extension at the primer-specific temperature for 30 seconds. Melting curve analysis was conducted to confirm the specificity of amplification products. Negative controls, without cDNA template, were incorporated to monitor potential contamination or primer-dimer formation. Ct values were obtained from ViiA 7 Software (ThermoFisher Scientific) and normalized using three housekeeping genes as references: *GAPDH*, *IPO8*, and *UBC*. Relative gene expression was determined using the comparative  $2^{(-\Delta\Delta Ct)}$  method [252], utilizing Ct values obtained from triplicates.



**Table 5.** Primer details used for qPCR.

| Gene Symbol         | Assay ID      | Supplier                |
|---------------------|---------------|-------------------------|
| <i>HLA-DRB1</i>     | Hs04192464_mH | ThermoFisher Scientific |
| <i>MRC1 (CD206)</i> | Hs07288635_g1 |                         |
| <i>CD86</i>         | Hs01567026_m1 |                         |
| <i>CD163</i>        | Hs00174705_m1 |                         |
| <i>GAPDH</i>        | Hs02786624_g1 |                         |
| <i>IPO8</i>         | Hs00183533_m1 |                         |
| <i>UBC</i>          | Hs00824723_m1 |                         |

### 3.9.3. Droplet digital PCR

To measure mRNA levels by ddPCR, ddPCR Gene Expression Assays protocol by Bio-Rad Laboratories was followed. Reaction was prepared in a 20  $\mu$ L sample volume comprising 10  $\mu$ L of ddPCR Supermix no dUTP, 300 nM FAM-labeled target probe, 150 nM HEX-labeled housekeeping-probe (*ACTB*), and 5  $\mu$ L of template DNA (5 ng/ $\mu$ L), with nuclease-free water added to adjust the sample to the final volume. Probes details are provided in Table 6.

The reaction mix was added to a DG8 Cartridge, followed by the injection of 70  $\mu$ L of Droplet Generation Oil for Probes. The DG8 Cartridge was transferred to a QX200 Droplet Generator. Generated droplets were carefully transferred to a 96-well PCR plate, heat-sealed, and placed in the C1000 Touch thermal cycler. The PCR conditions involved one cycle at 95°C for 10 min, followed by 40 cycle steps at 94°C for 30 sec and 55°C for 1 min, with a final denaturation at 98°C for 10 min for enzyme deactivation.

Upon completion of the PCR, the 96-well plate was inserted into the QX200 droplet reader to measure droplet fluorescence. The generated ddPCR data were then analyzed using QuantaSoft analysis software version 1.7.4. The ratio and fractional abundance (FA) for each sample were calculated using QuantaSoft software. The ratio is defined as the number of copies/ $\mu$ L obtained in the FAM channel (A) divided by the copies/ $\mu$ L in the HEX channel (B): A/B. The FA of the target gene was obtained by dividing the number of copies/ $\mu$ L of the target gene (FAM) by the total copies/ $\mu$ L of the reference gene (HEX) plus the target gene (FAM):  $[A / (A + B)]$ . The determination of the number of target copies per droplet (number of copies of target molecule) was adjusted with the software to fit a Poisson distribution model with a 95% confidence level.

**Table 6.** Primer details used for ddPCR.

| Gene Symbol         | Assay ID       | Supplier             |
|---------------------|----------------|----------------------|
| <i>TGFB1</i>        | dHsaCPE5055188 | Bio-Rad Laboratories |
| <i>MRC1 (CD206)</i> | dHsaCPE5193017 |                      |
| <i>HLA-DRA</i>      | dHsaCPE5190585 |                      |
| <i>POU5F1</i>       | dHsaCPE5191335 |                      |
| <i>ACTB</i>         | dHsaCPE5190200 |                      |
| <i>CD68</i>         | dHsaCPE5027628 |                      |
| <i>CD163</i>        | dHsaCPE5050636 |                      |
| <i>IDO1</i>         | dHsaCPE5036376 |                      |
| <i>CD274</i>        | dHsaCPE5058502 |                      |
| <i>IL10</i>         | dHsaCPE5036966 |                      |
| <i>SOX2</i>         | dHsaCPE5030244 |                      |
| <i>SNAI1</i>        | dHsaCPE5192683 |                      |
| <i>VIM</i>          | dHsaCPE5032314 |                      |
| <i>TWIST1</i>       | dHsaCPE5035640 |                      |
| <i>CDH1</i>         | dHsaCPE5032390 |                      |
| <i>CDH2</i>         | dHsaCPE5052922 |                      |
| <i>SNAI2</i>        | dHsaCPE5049912 |                      |
| <i>NANOG</i>        | dHsaCPE5191855 |                      |

#### 3.9.4. Flow cytometry

NB cells from the co-culture system were harvested after 5 days and detached using 0.2% versene (EDTA solution). Approximately  $5 \times 10^5$  cells per condition were used for staining with viability dyes (LIVE/DEAD Fixable Aqua Dead Cell Stain, Invitrogen) and primary antibodies. Cells were stained at 4°C for 30 minutes, washed with PBS, and immediately analyzed using a CytExpert flow cytometer (Beckman Coulter). Details on the antibodies and corresponding IgG isotype controls are provided in Table 7. Data analysis was performed using FlowJo software (FlowJo LLC), and results were quantified as the percentage of MHC-positive cells and median fluorescence intensity (MFI). Median was selected for its accuracy in representing marker expression levels, as it minimizes the impact of outliers.

**Table 7.** Antibodies details for flow cytometry analyses.

| Antibodies                                                                                                 | Supplier  |
|------------------------------------------------------------------------------------------------------------|-----------|
| Anti-HLA-DR, DP, DQ Mouse Monoclonal Antibody (FITC (Fluorescein Isothiocyanate)) [clone: Tü39]            | BioLegend |
| Anti-IgG2a, k Isotype Ctrl Mouse Monoclonal Antibody (FITC (Fluorescein Isothiocyanate)) [clone: MOPC-173] |           |
| Anti-HLA-A, B, C Mouse Monoclonal Antibody (PE (Phycoerythrin)) [clone: W6/32]                             |           |
| Anti-IgG2a, k Isotype Ctrl Mouse Monoclonal Antibody (PE (Phycoerythrin)) [clone: MOPC-173],               |           |

### 3.10. Tumor-infiltrating immune cells analysis

#### 3.10.1. High-risk neuroblastoma datasets

To analyze the tumor-infiltrating immune cells across HR-NB subtypes, we employed CIBERSORT algorithm ([cibersortx.stanford.edu/](http://cibersortx.stanford.edu/)) [253]. This computational tool allowed us to estimate the relative abundance of immune cell subsets within the TME by deconvoluting gene expression profiles. Two publicly available primary HR-NB cohorts were utilized in this study. The first cohort, referred to as the discovery cohort (GEO ID GSE73517), was derived from Westermann and colleagues [254]. The second dataset, designated as the validation cohort (GEO ID GSE131189), is part of the Therapeutically Applicable Research to Generate Effective Treatments (TARGET) initiative [255].

Only HR-NB patients with available expression and SCAs data were included. Patients within each cohort were categorized into three subgroups based on their genetic characteristics: 11q-deleted, *MYCN*-amplified, and 11q-normal without *MYCN* amplification, labeled as "others." The Westermann cohort (n = 55) comprised 18 patients with 11q-deletion, 27 *MYCN*-amplified, and 5 without either alteration. Five patients with both alterations were excluded from the study. The TARGET cohort (n = 215) included 95 patients with 11q-deletion, 56 *MYCN*-amplified, and 60 without either alteration. Four patients with both alterations were excluded from the analysis.

### **3.10.2. Deconvolution of immune cell subsets**

Gene expression data from Westermann and TARGET cohorts were input as a mixture file. LM22 signature matrix was used to distinguish 22 immune cell types, including naïve B cells, memory B cells, plasma cells, CD8<sup>+</sup> T cells, naïve CD4<sup>+</sup> T cells, resting CD4<sup>+</sup> memory T cells, activated CD4<sup>+</sup> memory T cells, follicular helper T cells (T<sub>fh</sub>), Tregs, gamma delta T cells ( $\gamma\delta$  T cells), resting NK cells, activated NK cells, monocytes, M0 macrophages, M1 macrophages, M2 macrophages, resting DCs, activated DCs, resting mast cells, activated mast cells, eosinophils, and neutrophils.

Bulk-mode batch correction was applied to remove variances between platforms employed by Westermann and TARGET cohorts. Expression data were quantile normalized. Permutations were set to 1000, and the algorithm was run in absolute mode, facilitating comparisons between samples and different cell types. Samples with a deconvolution P-value < 0.05 were deemed acceptable for further analysis. The resulting scores represent the absolute proportion of each cell type within the mixture. In cases where gene symbols were duplicated, the gene with the highest mean expression across samples was selected to prioritize the most consistently expressed variant.

## **3.11. Methylome profiling**

### **3.11.1. Liquid biopsy sampling and data processing**

Whole-genome bisulfite sequencing (WGBS) was performed to examine cell-free DNA (cfDNA) methylation patterns derived from HR-NB patients. CfDNA was extracted from plasma samples obtained through 1 mL liquid biopsies. The investigation involved a retrospective cohort of 64 patients treated at La Fe Hospital between 2007 and 2019, designated as the Liquidhope cohort. Plasma samples from 20 of these patients who experienced relapse were also incorporated into the study. Detailed clinical summaries are available in Annex 1. Blood-derived cfDNA was isolated using QIAamp circulating nucleic acids kit (QIAGEN). The quality and quantity of cfDNA were assessed by electrophoresis in 2200 TapeStation (Agilent). The study adhered to REMARK guidelines and the Declaration of Helsinki and was approved by La Fe Research Ethics Committee. Informed consent was obtained from parents or legal guardians for sample and data management.

Libraries were prepared using 10 ng of cfDNA using the NEBNext Enzymatic Methyl-seq (EM-seq) method from New England Biolabs. Libraries were sequenced on a NovaSeq 6000 (Illumina) platform with 2x150 bp paired-end reads. Image analysis, base calling, and quality scoring were performed using the manufacturer's Real-Time Analysis software (RTA 3.3.3), followed by generating FASTQ sequence files. EM-seq sequencing reads were processed using the gemBS pipeline version 4.03 with GRCh38 as the reference genome [256]. Methylome data is deposited under the accession ID GSE221317. For analysis validation, we used two external independent cohorts, TARGET (GEO ID: GSE131189) [255] and Westermann (GEO ID: GSE73515) [254], which were profiled using Illumina's 450 K arrays.

WGBS patterns of human early embryos were acquired from publicly available datasets encompassing human oocytes, sperm, and blastocysts (NBDC Human Database ID JGAS000006) [257], as well as fetal brain and adrenal gland samples (GEO ID: GSE54721) [258]. DNA methylation data from hematopoietic samples were sourced from the Blueprint Epigenome Project [259].

### **3.11.2. Annotation of CpG islands**

The genomic coordinates of each CpG in the human genome (GRCh38 assembly) were retrieved from the UCSC Genome Browser ([genome.ucsc.edu/](http://genome.ucsc.edu/)). Annotation included the distance to the transcription start site (TSS) and overlaps with promoters, introns, exons, and regulatory elements. Promoters were defined as the 4000-bp region flanking both sides [260]. Thus, CpG islands within  $\pm 4000$  bp of the TSS were annotated as promoter-associated.

To identify potential regulatory elements within or in proximity to the CpG islands, candidate cis-regulatory elements (cCREs) and TF CHIP-seq data were obtained from the ENCODE (Encyclopedia of DNA Elements) Project Consortium database ([encodeproject.org](http://encodeproject.org)). cCREs denote representative DNase hypersensitive sites supported by histone modifications (H3K4me3, H3K27me3, and H3K27ac) and CCCTC-binding factor (CTCF) data [261]. These cCREs were categorized according to their regulatory signatures, including prom (promoter-like signature), enhP (proximal enhancer-like signature), enhD (distal enhancer-like signature), K4m3 (DNase-H3K4me3, promoter-like signature that is not within 200 bp of an annotated TSS), and CTCF (CTCF-only) [261].

### **3.12. miRNA analysis**

The genes targeted by 11q-located miRNAs were identified using TargetScan [262]. miRNA sequences and annotation data, including precursor miRNA (MIID) and location, were obtained from the miRbase database (mirbase.org) [263]. A search was conducted to identify conserved 8mer, 7mer, and 6mer sites matching the seed region of each miRNA [264]. Predictions with poorly conserved sites were excluded. Additionally, sites with mismatches in the seed region, compensated by conserved 3' pairing, and centered sites were included [265, 266]. Predictions were ranked based on the predicted efficacy of targeting, calculated using cumulative weighted context ++ scores of the sites [262]. A threshold predictive value of 0.9 or higher was used to ensure the highest prediction accuracy in the miRNA-mRNA interaction. The algorithm for identifying targeted genes integrated data from TargetScan (conserved site context scores, version 7.1), miRDB (mirdb.org) (5.0 release), and validated information from miRTarBase (version 7.0).

The expression levels of predicted genes regulated by the miRNAs were further analyzed using Westermann (GSE73517) and TARGET datasets (GSE131189). Gene Set Enrichment Analysis (GSEA) of genes targeted by 11q miRNAs was conducted using the PGSEA R package from Bioconductor (bioconductor.org/biocLite.R) [267]. Cytoscape 3.7.1 was employed to integrate and visualize the results obtained from the GSEA.

### **3.13. Transcriptomic and epigenomic datasets**

A range of transcriptomic and epigenomic datasets were utilized to explore the molecular characteristics of NB and compare these features with normal and other developmental tissues (Table 8).

**Table 8.** Transcriptomic and epigenomic datasets.

| ID              | Type of data                        | Tissue                                | Source |
|-----------------|-------------------------------------|---------------------------------------|--------|
| GSE73517        | Expression                          | NB                                    | [254]  |
| GSE131189       | Expression/Methylation              | NB                                    | [255]  |
| EGAD00001008345 | Expression                          | Normal fetal adrenal gland            | [149]  |
| GSE221317       | Expression                          | NB cell lines                         | [99]   |
| GSE90805        | Expression<br>(MES/ADRN signatures) | NB                                    | [139]  |
| GSE73515        | Methylation                         | NB                                    | [254]  |
| JGAS000006      | Methylation                         | Human oocytes, sperm, and blastocysts | [257]  |
| GSE54721        | Methylation                         | Fetal brain and adrenal glands        | [258]  |
| EGAC00001000135 | Methylation                         | Hematopoietic samples                 | [259]  |

MES: mesenchymal; ADRN: adrenergic.

### **3.14. Statistical analyses**

Univariate OS and EFS analyses were performed using Cox proportional hazards regression for survival analysis. The primary endpoints were 3-year OS and EFS. EFS was defined as the interval from post-consolidation therapy to the first relapse, death, second neoplasm, or until the last follow-up. OS was calculated from the initiation of post-consolidation therapy to death from any cause or until the last follow-up.

For methylation analysis, Kaplan-Meier curves associated with each CpG were generated by dividing patients into two groups based on the methylation value of that CpG. The threshold for defining the groups was selected to maximize survival differences. This was determined by identifying the grouping term with the lowest P-value from the Wald test.

GSEA was performed to assess the ADRN and MES lineage identities of NB cell lines. The gene set lists for evaluating ADRN and MES cell states were obtained from Groningen and colleagues [139]. Gene expression data for the cell lines were analyzed using Pearson's correlation coefficient to evaluate the relationship between gene sets associated with ADRN and MES lineages.

Depending on the number of independent variables, appropriate analysis of variance (ANOVA) methods was used to compare SCA profiles, proliferation rates, methylation, and gene expression levels. Further details for each statistical analysis performed are provided in the caption of each figure or table presented. Gene expression levels were normalized to log2 values before analysis. An ordinal regression model assessed immune cell population proportions variances among patient subgroups. This model was applied exclusively to samples with a deconvolution P-value less than 0.05.

Statistical analyses and graphs were performed using R or GraphPad Prism v9 (GraphPad Software Inc.). P-values < 0.05 were considered statistically significant. Significance codes are denoted as follows: \*P ≤ 0.05; \*\*P < 0.01; \*\*\*P < 0.001; \*\*\*\*P < 0.0001, ns: no significant.



## 4. Results

### 4.1. Half of high-risk neuroblastoma patients with 11q deletion relapse after anti-GD2 immunotherapy

NB treatment strategies are tailored to each patient's risk status [4]. Although this approach has successfully reduced treatment intensity while maintaining or improving outcomes for LR- and most IR-risk patients, the prognosis for HR-NB cases continues to be unfavorable [4].

In recent years, it has become increasingly clear that the forefront of cancer medicine lies in developing molecularly targeted therapies for each tumor subtype. HR-NB patients, accounting for about half of all NB patients, are broadly subdivided into two primary cohorts: patients whose tumors show *MYCN* amplification, 1p deletion, and *TERT* overexpression and tumors harboring 11q deletion, frequently occasionally along with other recurrent SCAs, and maintaining chromosome ends via ALT mechanism [4]. While targeting *MYCN* remains challenging, novel approaches like BET inhibitors [74, 75], PROTACs [74], and Omomyc [268], which directly inhibit MYC proteins, are under development. However, issues with specificity, efficacy, and toxicity persist, highlighting the need for more integrated treatment strategies. Additionally, the genetic basis of 11q deletion in NB is not well understood, and it's unclear whether these or other genomic alterations impact anti-GD2 efficacy or contribute to immune escape [269].

To address these gaps, we retrospectively analyzed 83 HR-NB patients from the Spanish cohort enrolled in the HR-NBL/SIOPEN trial between 2007 and 2015 (Table 9). We compared the 3-year EFS and OS rates of patients who received anti-GD2 antibody ch14.18/CHO (dinutuximab beta) in combination with isotretinoin (n = 50) to those who received isotretinoin therapy alone (n = 33).

## Results

Half of high-risk neuroblastoma patients with 11q deletion relapse after anti-GD2 immunotherapy

**Table 9.** Clinical characteristics of the Spanish HR-NB cohort enrolled at HR-NBL/SIOPEN trial.

| Characteristics        | N (%)         |           |           |
|------------------------|---------------|-----------|-----------|
|                        | Immunotherapy | RA alone  | Total     |
| <b>Treatment group</b> | 50 (60.2)     | 33 (39.8) | 83 (100)  |
| <b>Sex</b>             |               |           |           |
| Male                   | 30 (60)       | 17 (51.5) | 47 (56.6) |
| Female                 | 20 (40)       | 16 (48.5) | 36 (43.4) |
| <b>INRG stage</b>      |               |           |           |
| M                      | 50 (100)      | 33 (100)  | 83 (100)  |
| <b>MYCN status</b>     |               |           |           |
| Amplified              | 14 (28)       | 11 (33.3) | 25 (30.1) |
| Non amplified          | 36 (72)       | 22 (66.7) | 58 (69.9) |
| <b>11q status</b>      |               |           |           |
| Deleted                | 24 (48)       | 11 (33.3) | 35 (42.2) |
| Non deleted            | 26 (52)       | 22 (66.7) | 48 (57.8) |
| <b>Relapse</b>         |               |           |           |
| Yes                    | 31 (62)       | 25 (75.8) | 56 (67.5) |
| No                     | 19 (38)       | 8 (24.2)  | 27 (32.5) |
| <b>Patient status</b>  |               |           |           |
| Dead                   | 25 (50)       | 24 (72.7) | 49 (59)   |
| Alive                  | 25 (50)       | 9 (27.3)  | 34 (41)   |

RA: 13-cis retinoic acid (isotretinoin); N: number; INRG: International NB Risk Group.

In the complete patient pool, without subgroup categorization, individuals administered anti-GD2 plus isotretinoin exhibited notably enhanced 3-year EFS rates, with 50% (95% CI 38 - 66) compared to 30% in patients receiving isotretinoin alone (95% CI 18 - 51) ( $P = 0.033$ ) (Table 10). Similarly, 3-year OS rates were higher in patients receiving anti-GD2 immunotherapy at 72% (95% CI 61 - 86) compared to 52% in patients receiving conventional treatment (95% CI 37 - 72), confirming the efficacy of anti-GD2 immunotherapy in HR-NB patients. However, the significance value was borderline ( $P = 0.047$ ) (Table 10).

**Table 10.** Outcomes after anti-GD2-based immunotherapy compared to isotretinoin alone in high-risk neuroblastoma.

| Subgroup            | Treatment group  | N  | 3-year EFS (95% CI) | HR (95% CI)          | P     | 3-year OS (95% CI) | HR (95% CI)          | P     |
|---------------------|------------------|----|---------------------|----------------------|-------|--------------------|----------------------|-------|
| <b>All patients</b> | ch14.18/CHO + RA | 50 | 50<br>(38-66)       | 0.53<br>(0.30- 0.94) | 0.028 | 72<br>(61-86)      | 0.49<br>(0.24-1)     | 0.047 |
|                     | RA               | 33 | 30<br>(18-51)       |                      |       | 52<br>(37-72)      |                      |       |
| <b>11q-deleted</b>  | ch14.18/CHO + RA | 24 | 50<br>(34-75)       | 0.32<br>(0.14-0.76)  | 0.006 | 71<br>(55-92)      | 0.46<br>(0.15- 1.37) | 0.152 |
|                     | RA               | 10 | 9<br>(1-59)         |                      |       | 46<br>(24-87)      |                      |       |

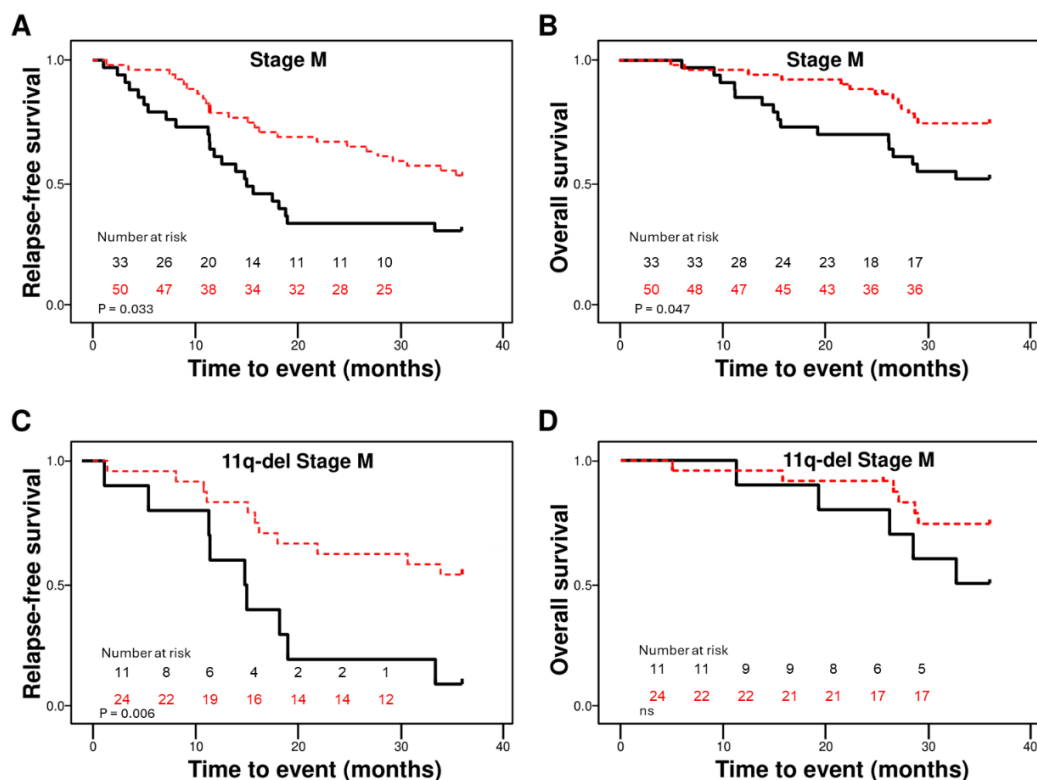
RA: 13-cis retinoic acid (isotretinoin); HR: hazard ratio (log-rank tests); CI: confidence interval. N: number; P: P value; ch14.18/CHO: chimeric 14.18 monoclonal antibody produced in Chinese hamster ovary cells.

We then examined the specific response of the 11q-deleted NB subgroup to anti-GD2 immunotherapy. The 3-year EFS rate was notably higher for 11q-deleted patients treated with anti-GD2 immunotherapy at 50% (95% CI 34 - 75) compared to 9% (95% CI 1 - 59) for those receiving conventional treatment ( $p = 0.006$ ). A notable decrease in the hazard ratio (HR) for EFS was also observed among patients with 11q-deletion who received immunotherapy (68% vs. 47%). This reduction indicates the potential of anti-GD2 immunotherapy in postponing relapse in patients with 11q-deletion (Table 10).

The 3-year OS was also higher, at 71% (95% CI 55 - 92) for 11q-deleted patients treated with anti-GD2 immunotherapy, compared to 46% (95% CI 24 - 87) for the conventional treatment group (Figure 10). While the p-value for the 3-year OS was not significant ( $p = 0.152$ ), comparable survival rates were observed across the entire population, suggesting potential limitations attributable to sample size constraints. The wide CI range in the 11q-deleted subgroup receiving isotretinoin points to significant variability in treatment response. This variability may be due to the small sample size or the heterogeneity in the study population (Table 10).

## Results

Half of high-risk neuroblastoma patients with 11q deletion relapse after anti-GD2 immunotherapy



**Figure 10. Improved 3-year EFS and OS in high-risk neuroblastoma patients treated with anti-GD2-based immunotherapy compared to isotretinoin alone.** Kaplan-Meier curves illustrating the 3-year EFS (A) and OS (B) in patients treated with anti-GD2-based immunotherapy plus isotretinoin vs. those receiving conventional maintenance treatment with isotretinoin alone. (C) 3-year EFS and (D) OS in HR-NB subgroup with 11q-deletion treated with anti-GD2-based immunotherapy plus isotretinoin vs. those receiving conventional maintenance treatment with isotretinoin alone. The red dotted lines represent the immunotherapy arm, while black lines denote conventional treatment with isotretinoin alone. Censored cases are indicated by crosses along the plots. The number of patients at risk is displayed in each panel after excluding censored patients from the denominator. Log-rank P values were used to compare curves between subgroups. EFS: event-free survival. OS: overall survival. Ns: no significance ( $P > 0.05$ ).

Despite improved outcomes after anti-GD2-based immunotherapy for 11q-deleted HR-NB, survival rates remain at 50-70% (Table 10, Figure 1). These results highlight the need for continued research to improve the efficacy of immunotherapy in this high-risk subgroup, focusing on understanding NB resistance mechanisms and identifying alternative immunotherapeutic targets.

## **4.2. Immunosuppressive tumor microenvironment in 11q-deleted high-risk neuroblastomas**

### **4.2.1. Increased immune infiltrating cells in 11q-deleted high-risk neuroblastomas**

To deepen our comprehension of the factors impacting the outcome after anti-GD2 immunotherapy, we analyzed the immune infiltrating cells within the TME of HR-NB patients. The analysis was conducted using RNA-seq data from two primary NB cohorts: Westermann's, used as the discovery cohort (GSE73517, n = 55) [254] and TARGETs, serving as validation cohort (GSE131189, n = 215) [255].

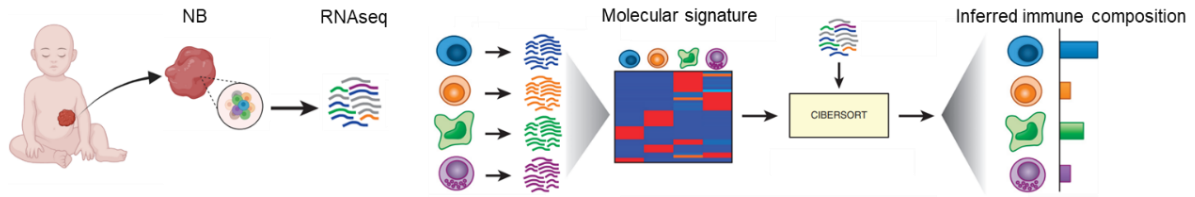
We employed CIBERSORTx for the deconvolution of gene expression data. This methodology enabled us to quantitatively assess the variations in immune cell composition within the TME of HR-NB. Patients were divided into three subgroups based on their distinct genetic alterations: (a) 11q-deleted, (b) *MYCN*-amplified, and (c) 11q normal without *MYCN* amplification (here referred to as "others"). Patients with both 11q deletion and *MYCN* amplification were excluded as the independent effect of these alterations could not be assessed in these cases.

The analysis revealed significant differences in the immune landscape among HR-NB subgroups (Figure 2). *MYCN*-amplified tumors showed an almost immune-excluded TME, whereas those HR-NB with 11q-deletion showed a more inflamed TME. Specifically, compared to *MYCN*-amplified NB, 11q-deleted tumors exhibited significantly higher proportions of CD8<sup>+</sup> T cells, Tregs, M0, M1, and M2 macrophages, being M2 more abundant than M1 macrophages (Figure 2). Moreover, 11q-deleted HR-NBs demonstrated elevated resting CD4<sup>+</sup> memory T cells and activated NK cells compared to *MYCN*-amplified NB and tumors without any of the two alterations ( $P < 0.05$ ) (Figure 2). The immunesurveillant  $\gamma\delta$  T cells and the APC DCs were nearly absent across all subgroups (Annex 2).

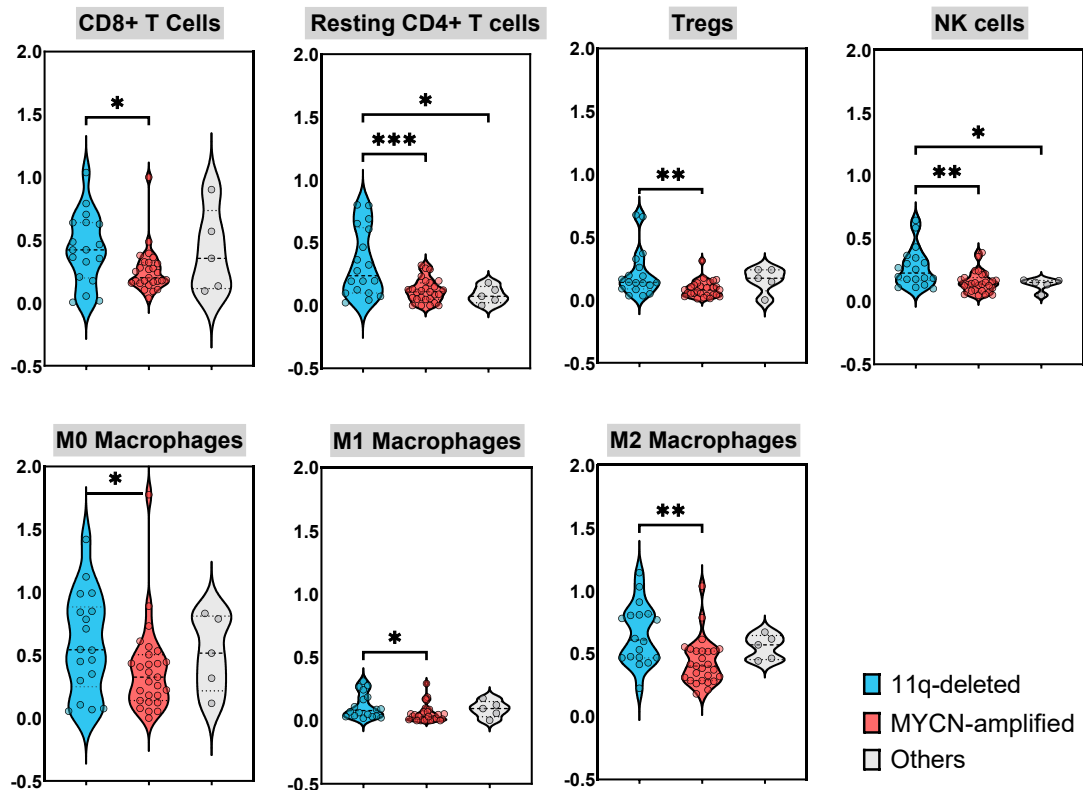
## Results

### Immunosuppressive tumor microenvironment in 11q-deleted high-risk neuroblastomas

**A**



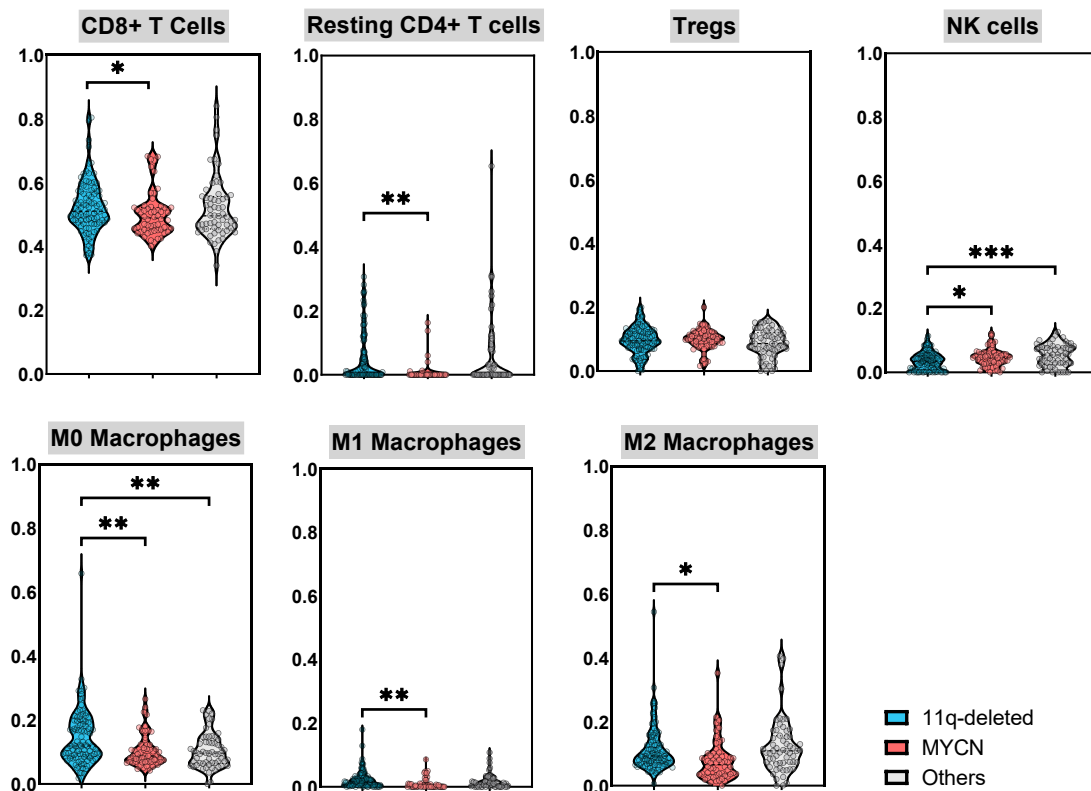
**B**



**Figure 11. Tumor-infiltrating immune subsets in high-risk neuroblastoma subgroups:**

**analysis of the Westermann cohort.** (A) Schematic representation of immune landscape analysis. The workflow begins with inputting gene expression profiles and signature matrix files, followed by the inference of abundance of immune cell populations using CIBERSORTx deconvolution. (B) Inferred composition of immune cell subsets from the HR-NB RNAseq Westermann dataset (GSE73517). Violin plots depict absolute proportions (scores) of tumor-infiltrating immune populations in HR-NB with 11q deletion (n = 18; blue violins), MYCN-amplified (n = 27; red violins), and other HR-NB (n = 5; grey violins). 11q-deleted HR-NB exhibited higher proportions of CD8+ T cells, Tregs, M0, M1, and M2 macrophages compared to MYCN-amplified NB, and increased resting CD4+ memory T cells and NK cells compared to MYCN-amplified subgroup and NB tumors lacking both alterations (P < 0.05). Ordinal regression was employed to assess the differences among the three subgroups. Significance codes: \*\*\*0.001; \*\*0.01; \*0.05. Only significant p values are indicated.

We used the TARGET independent cohort to confirm our findings. Analysis revealed consistently elevated proportions of CD8+ T cells, resting CD4+ memory T cells, M0, M1, and M2 macrophages in 11q-deleted HR-NB compared to *MYCN*-amplified tumors (Figure 12). However, discrepancies arose regarding the presence of NK cells and Tregs. Specifically, no significant differences in Tregs were observed in the validation cohort. Concerning activated NK cells, although the discovery dataset indicated a significantly higher proportion of NK cells in 11q-deleted HR-NBs, this trend was not replicated in the validation cohort (Figure 12).



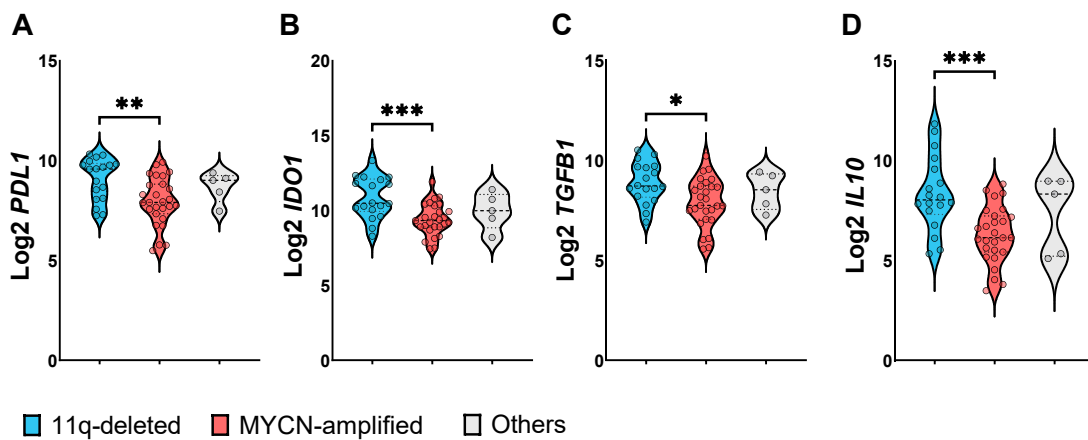
**Figure 12. Tumor-infiltrating immune subsets in high-risk neuroblastoma subgroups: analysis of the TARGET cohort** Inferred composition of immune cell subsets from HR-NB RNAseq data sourced from the TARGET cohort (GSE131189). Violin plots show absolute proportions of tumor-infiltrating immune populations in HR-NB with 11q deletion (n = 95; blue violins), *MYCN*-amplified (n = 56; red violins), and other HR-NB (n = 60; grey violins). 11q-deleted tumors showed higher proportions of CD8+ T cells, resting memory CD4+ T cells, M0, M1, and M2 macrophages compared to *MYCN*-amplified tumors. Contrary to the discovery cohort, NK cells are significantly lower in 11q-deleted NB than in other high-risk NB subgroups. Significance codes: \*\*\*0.001; \*\*0.01; \*0.05. Only significant p values are indicated.

## Results

### Immunosuppressive tumor microenvironment in 11q-deleted high-risk neuroblastomas

#### 4.2.2. Immunosuppressive molecules in 11q-deleted high-risk neuroblastomas

Next, we analyzed the differential expression between 11q-deleted, *MYCN*-amplified, and HR-NB patients without *MYCN* amplification or 11q deletion (Figure 13). Interestingly, 11q-deleted patients showed significantly higher levels of the immune checkpoint PD-L1 (Figure 13A), as well as the immunosuppressive molecules IL-10 (Figure 13B), TGF- $\beta$ 1 (Figure 13C), and IDO1 (Figure 13D), compared to *MYCN*-amplified NB. These results were further validated in the TARGET dataset (Annex 4).

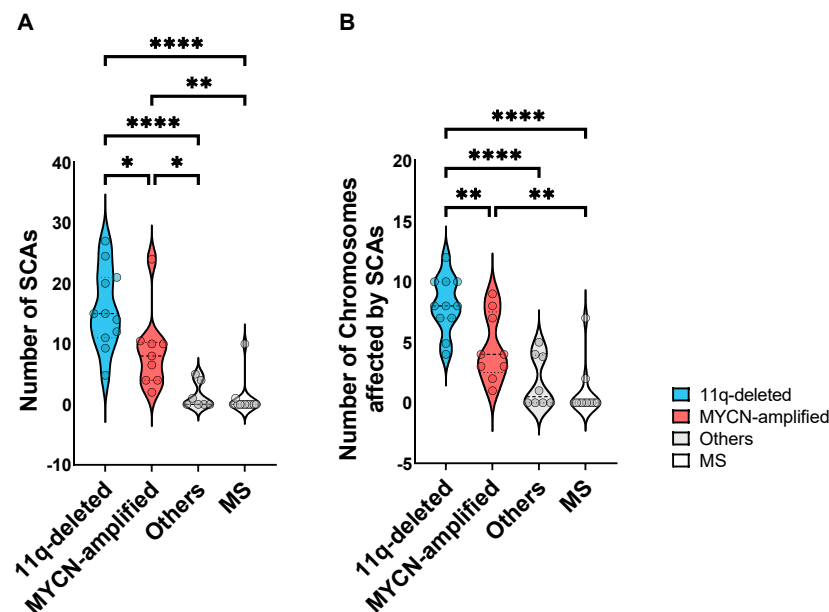


**Figure 13. 11q-deleted high-risk neuroblastoma exhibits higher expression of immunosuppressive molecules.** Differential RNA expression analysis was conducted on samples from 50 primary HR-NB cases (GSE73517). The expression levels of (A) *PD-L1*, (B) *IDO1* (C) *TGFβ*, and (D) *IL10* are compared in 11q-deleted HR-NB (n = 18; blue), *MYCN*-amplified (n = 27; red), and other HR-NB cases (n = 5; grey). Gene expression levels were normalized to log2. One-way ANOVA was used to test the statistical differences in gene expression. Significance levels are denoted as \*P ≤ 0.05; \*\*P < 0.01; \*\*\*P < 0.001, \*\*\*\*P < 0.0001. Only significant p values are indicated.



### 4.3. Intrinsic DNA repair defects in 11q-deleted high-risk neuroblastomas

While NB tumors display a scarcity of recurrent somatic mutations, they frequently exhibit NCAs and SCAs [56]. We conducted a SNP array analysis on 41 HR-NB primary cases, confirming a notably higher total SCA count in the 11q-deleted subgroup than in other high-risk subgroups (Figure 14A). While MS cases are not classified as high-risk, we included them in our analysis to highlight differences in SCAs compared to high-risk groups, regardless of metastatic status. The study revealed that this subgroup exhibited the lowest average number of SCAs among metastatic NB patients, with only two MS cases presenting SCAs (Figure 14A). Likewise, the 11q-deleted subgroup exhibited a notably higher prevalence of SCA-affected chromosomes, reflecting an intrinsic genetic instability mechanism (Figure 14B).

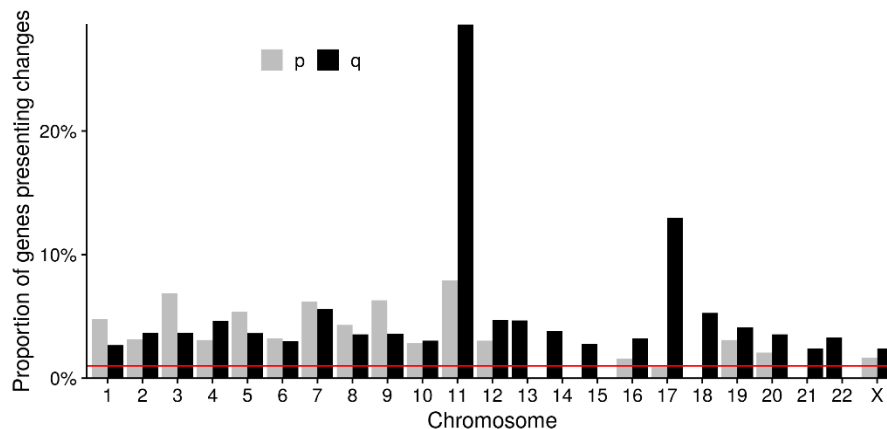


**Figure 14. Heightened incidence of segmental chromosomal aberrations within the high-risk subgroup with 11q deletion.** The violin plots depict (A) the number of SCAs and (B) the number of SCA-affected chromosomes among NB subgroups: 11q-deleted (in blue), *MYCN*-amplified (in red), others (in grey) (M stage), and MS without *MYCN*-amplification or 11q deletion (in white). A greater prevalence of SCAs and affected chromosomes within the 11q-deleted subgroup is observed. One-way ANOVA was used to test the statistical differences between subgroups. Significance levels are denoted as \* $P \leq 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ . MS: metastatic special; SCAs: segmental chromosomal aberrations.

## Results

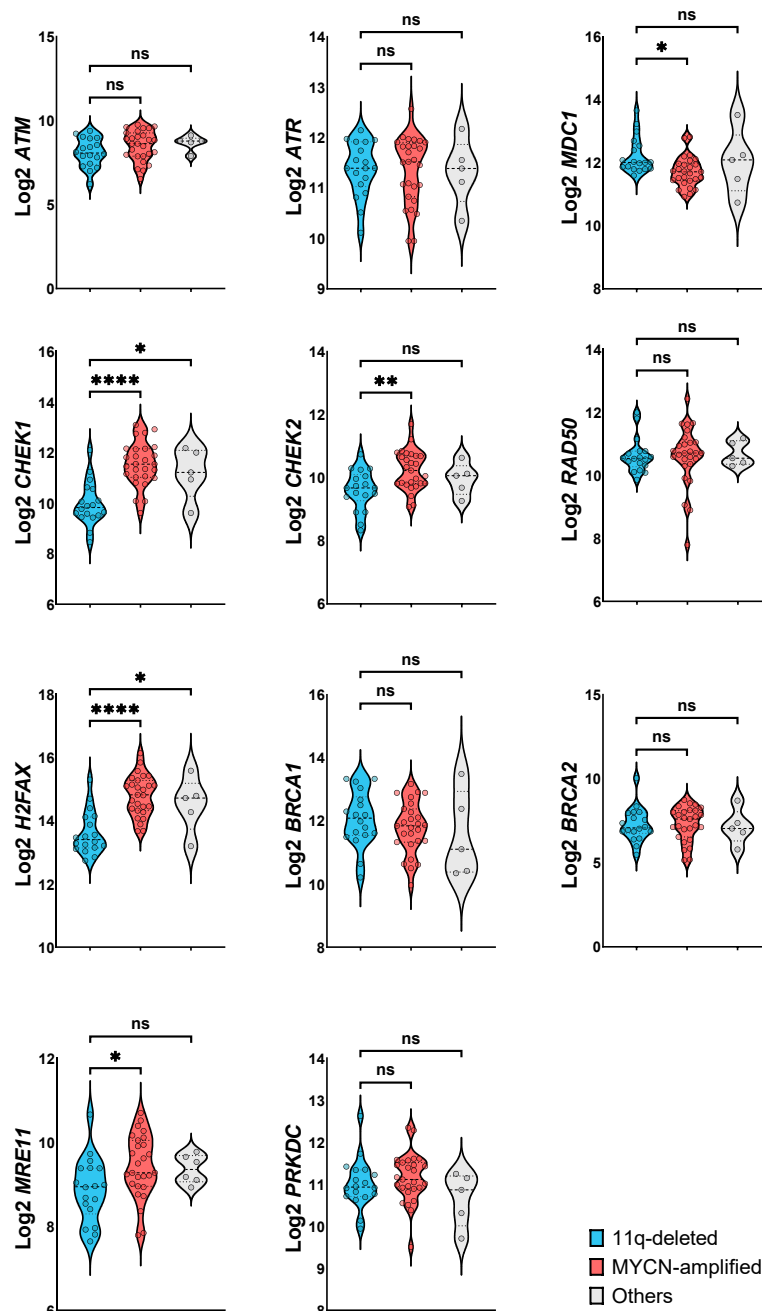
### Intrinsic DNA repair defects in 11q-deleted high-risk neuroblastomas

We further explored the genomic landscape of HR-NB by depicting the percentage of significantly altered genes based on their chromosomal location. This approach offered a comprehensive view of genomic alterations associated with each tumor subgroup. Remarkably, the highest percentage of differentially expressed genes was identified in the 11q region, followed by 17q (Figure 15). This observation confirms that the loss of expression in 11q leads to significant changes in the expression of genes within the 11q region.



**Figure 15. The proportion of differentially expressed genes across chromosomes in 11q-deleted compared to non-11q-deleted high-risk neuroblastomas.** The primary HR-NB cohort (GSE131189) was examined to determine the proportion of genes showing karyotype-based differential expression with and without 11q deletion, while adjusting for *MYCN* amplification. The false discovery rate (FDR) was set to 0.01, indicated by a red line. Black bars represent the q chromosomal region, and grey bars represent the p region.

The high frequency of SCAs observed in 11q-deleted tumors suggests potential defects in the DNA repair machinery. With this premise in mind, we analyzed the differential expression of DNA DSB repair genes in both the Westermann and TARGET HR-NB cohorts. In the Westermann cohort, we observed significant downregulation of *H2AX*, *CHEK1*, and *MRE11A*, all located within the 11q-deleted region, compared to other subgroups (Figure 16). Additionally, we noted a marked decrease in the expression of *CHEK2*, located in 22q (Figure 16). No significant differences in the expression levels of other DNA DSB repair genes were observed (Figure 16). The analysis in the TARGET cohort confirmed a notable decrease in *H2AX*, *CHEK1*, and *MRE11A* expression levels in the 11q-deleted subgroup compared to other HR-NB cases. Additionally, the downregulation of *ATM* and *RAD50* was also observed (Annex 5).



**Figure 16. Decreased expression of DNA double-strand break repair genes in 11q-deleted high-risk neuroblastomas.** Comparison of gene expression profiles of genes associated with DNA DSB repair among Westermann HR-NB cohort (GSE73517). The analysis compares patients with 11q deletion (highlighted in blue) against those with *MYCN* amplification (in red) and the Others subgroup (in grey). One-way ANOVA was used to test the statistical differences in gene expression. Significance levels are denoted as \* $P \leq 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ , ns: not significant.

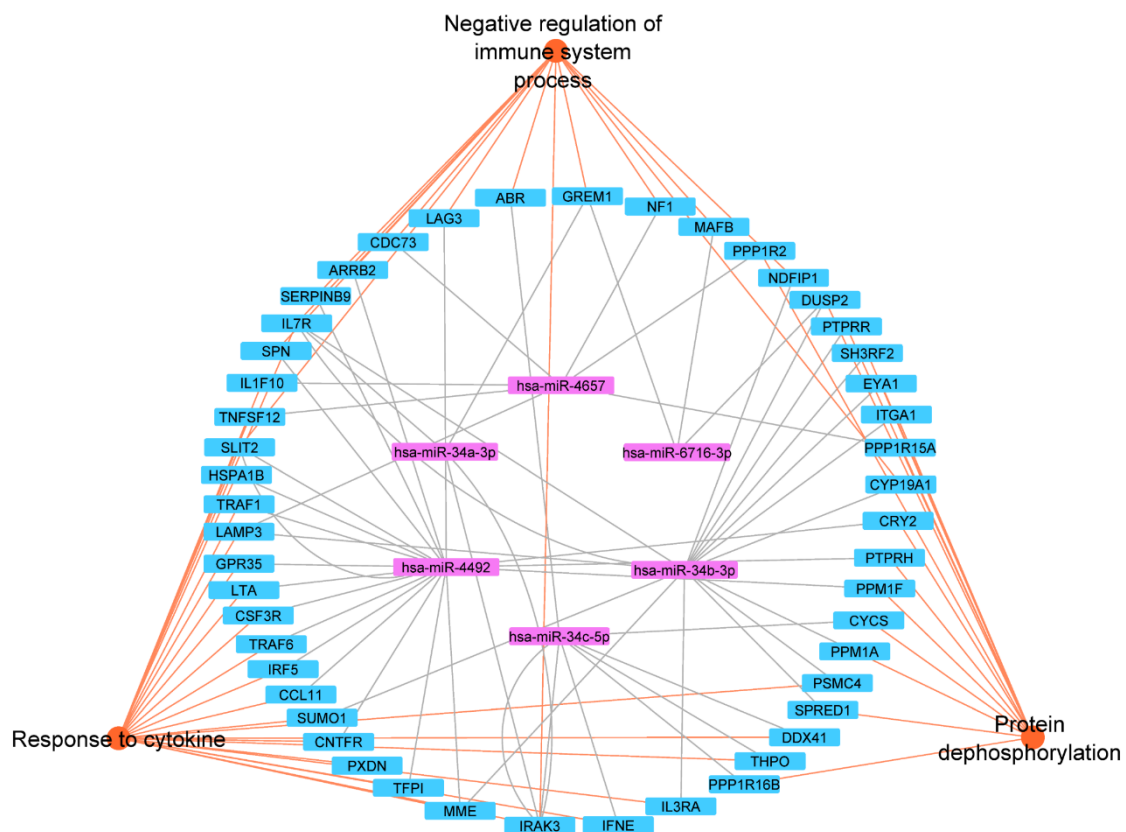
## Results

### Regulatory Interactions of 11q-Deleted miRNAs

#### 4.4. Regulatory Interactions of 11q-Deleted miRNAs

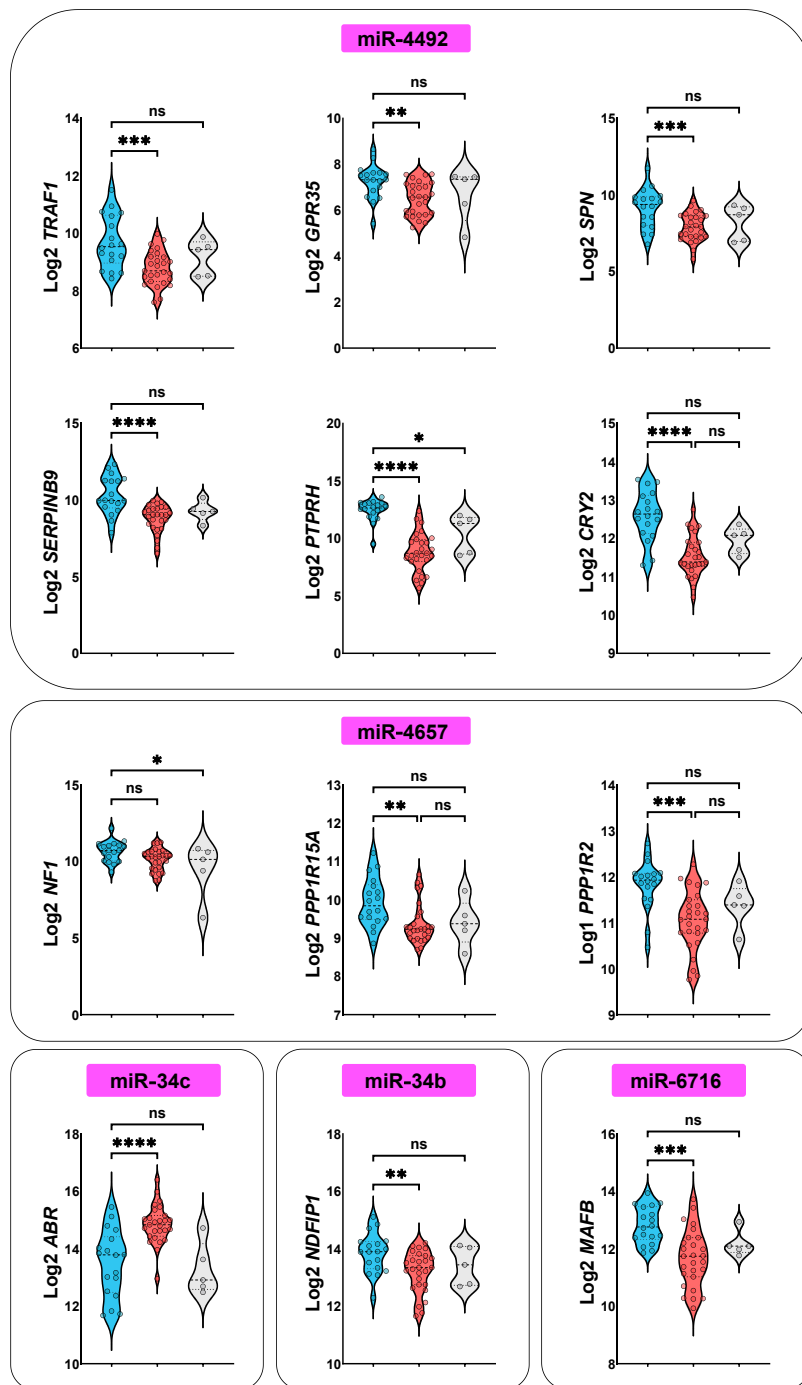
miRNAs have emerged as potential contributors to NB aggressiveness by activating effector cells or inhibiting regulatory cells [270]. Within the 11q smallest region of deletion overlap (SRO) lie six miRNAs: *miR-4491*, *miR-34b*, *miR-34c*, *miR-4301*, *miR-6716*, and *miR-4492* [99].

To better understand the regulatory interactions between 11q-located miRNAs and their target genes, we created an 11q miRNA-mRNA regulatory network comprising the 11q set of miRNAs and their respective targets. We combined validated targets from miRTarBase [271] with our bioinformatics-based predictions to map out the biological pathways influenced by the 11q-deleted miRNA set (Figure 17). Notably, the affected processes included *response to cytokines* (GO:0034097), *negative regulation of immune system processes* (GO:0002683), and *protein dephosphorylation* (GO:0006470) (Figure 17).



**Figure 17. Regulatory network orchestrated by 11q-deleted miRNAs.** Purple nodes within the inner circle depict the miRNAs localized in the 11q SRO. In contrast, blue nodes in the outer circle represent known or predicted targeted genes by these miRNAs. Orange nodes indicate the enriched biological processes. Lines connecting nodes represent interactions linking miRNAs with genes and biological processes. miR: microRNA; hsa: Homo sapiens.

To further explore the regulatory potential of these miRNAs, we analyzed the expression of the identified target genes in Westermann and TARGET HR-NB datasets (Figure 18, Annex 6). *miRNA-4492* emerged as a central player in regulatory interactions, orchestrating most gene expression changes. Notably, *TRAF1*, *GPR35*, *SPN*, *SERPINB9*, *PTPRH*, *PPP1R15A*, and *CRY2* were significantly upregulated in 11q-deleted HR-NB compared to *MYCN*-amplified tumors (Figure 18). Additionally, *PPP1R15A* and *PPP1R2* predicted targets of *miR-4657* exhibited significant upregulation in 11q-deleted HR-NB. The loss of *miR-34c* correlated with the downregulation of *ABR*, while the absence of *miR-34b* was associated with the upregulation of *NDFIP1*. Similarly, upregulation of *MAFB*, the target of *miR-6716*, was observed in 11q-deleted HR-NB (Figure 18).



**Figure 18. Differential expression of genes targeted by 11q miRNAs between high-risk-neuroblastoma subgroups.** The expression levels of individual genes targeted by the 11q miRNA set was analyzed in HR-NB (GSE73517) and compared among 11q-deleted (blue), *MYCN*-amplified (red), and Other (grey) subgroups. One-way ANOVA was used to test the statistical differences in gene expression. Significance levels are denoted as \* $P \leq 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ . Ns: not significant.

#### **4.5. Neuroblastoma cells induce macrophage polarization towards the M2 phenotype**

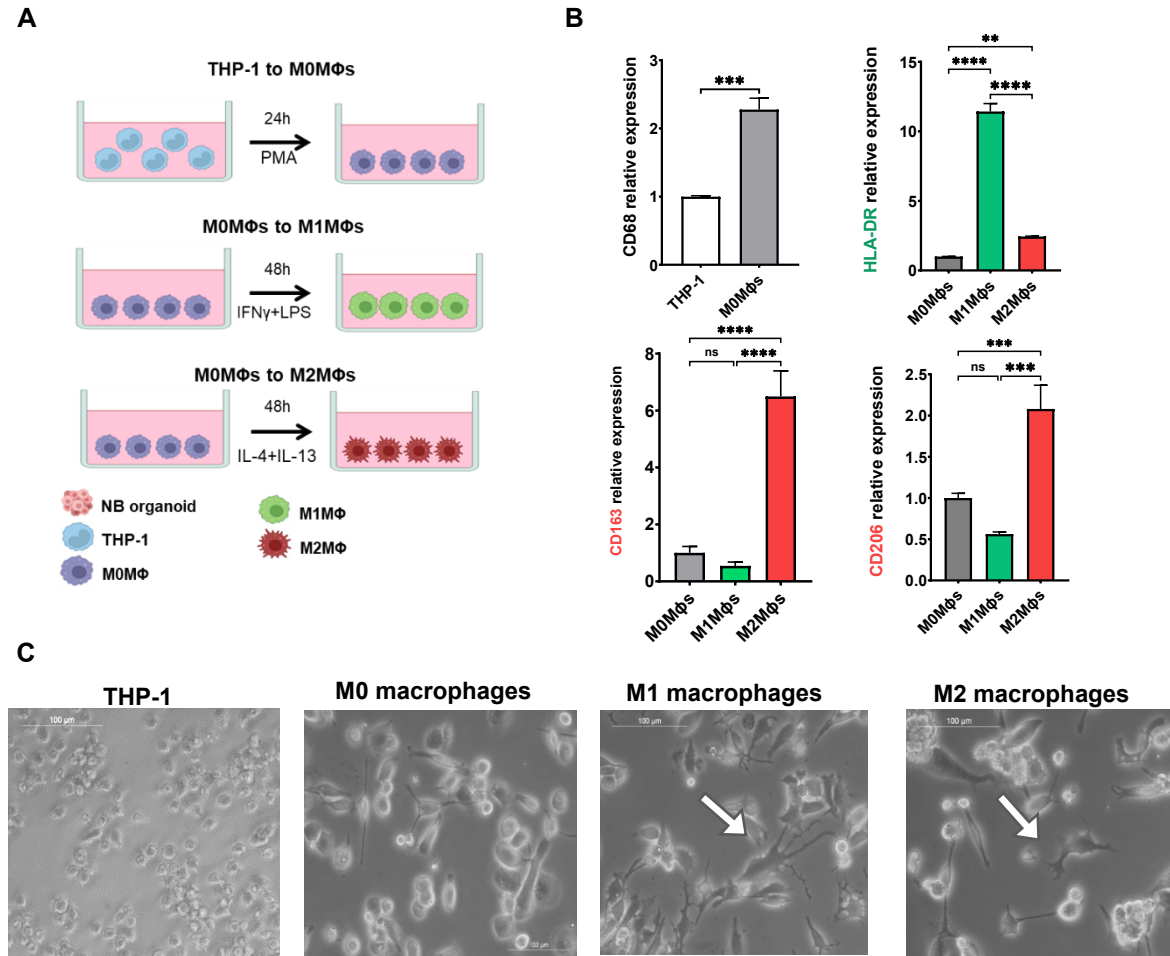
Our investigation into the TIME of HR-NB subgroups unveiled distinct immune infiltration patterns: while *MYCN*-amplified tumors tend to exhibit an almost immune-exclusive TME, 11q-deleted tumors frequently display more immune infiltration, notably characterized by increased macrophages' presence. Although the higher presence of effector cells in the 11q-deleted subgroup may initiate an adaptive immune response, the dominance of the M2 phenotype suggests a potential barrier to effective antitumor responses. Consequently, we investigated how NB cells with different genetic backgrounds influenced macrophages' behavior and, vice versa, how these macrophages affected NB cells' growth and resistance to treatment.

To evaluate the influence of NB cells on macrophage phenotype, THP-1 monocytes were co-cultured in transwell inserts with a diverse array of NB cell lines and PDOs (Table 4). This approach allowed us to explore NB's influence across a diverse biological landscape, encompassing established cell lines with known genetic alterations like *MYCN* amplification and 11q deletion and PDOs reflecting the intricate heterogeneity of original tumors. Transwell inserts with 0,4  $\mu\text{m}$  pore size membrane facilitated co-culture in two distinct phases, allowing molecular exchange between cell populations while maintaining physical separation and preventing cell migration.

After 3-day co-culture, we examined the expression profiles of specific markers for each macrophage's subpopulation. These profiles were compared with those of macrophages pharmacologically polarized towards classical M1 or alternative M2 phenotypes. Initially, monocytes were differentiated into M0 macrophages (non-polarized) via a 24-hour incubation with PMA. Then, M0 macrophages were polarized into M1 macrophages and M2 macrophages by exposure to IFN- $\gamma$  plus LPS and IL-4 plus IL-13, respectively (Figure 19A). Accurate chemical differentiation and polarization were confirmed by assessing specific markers within the macrophage's populations. Macrophages adherence and ramification processes resulting from differentiation and polarization were evaluated by visual inspection using light microscopy (Figure 19B-C).

## Results

### Neuroblastoma cells induce macrophage polarization towards the M2 phenotype



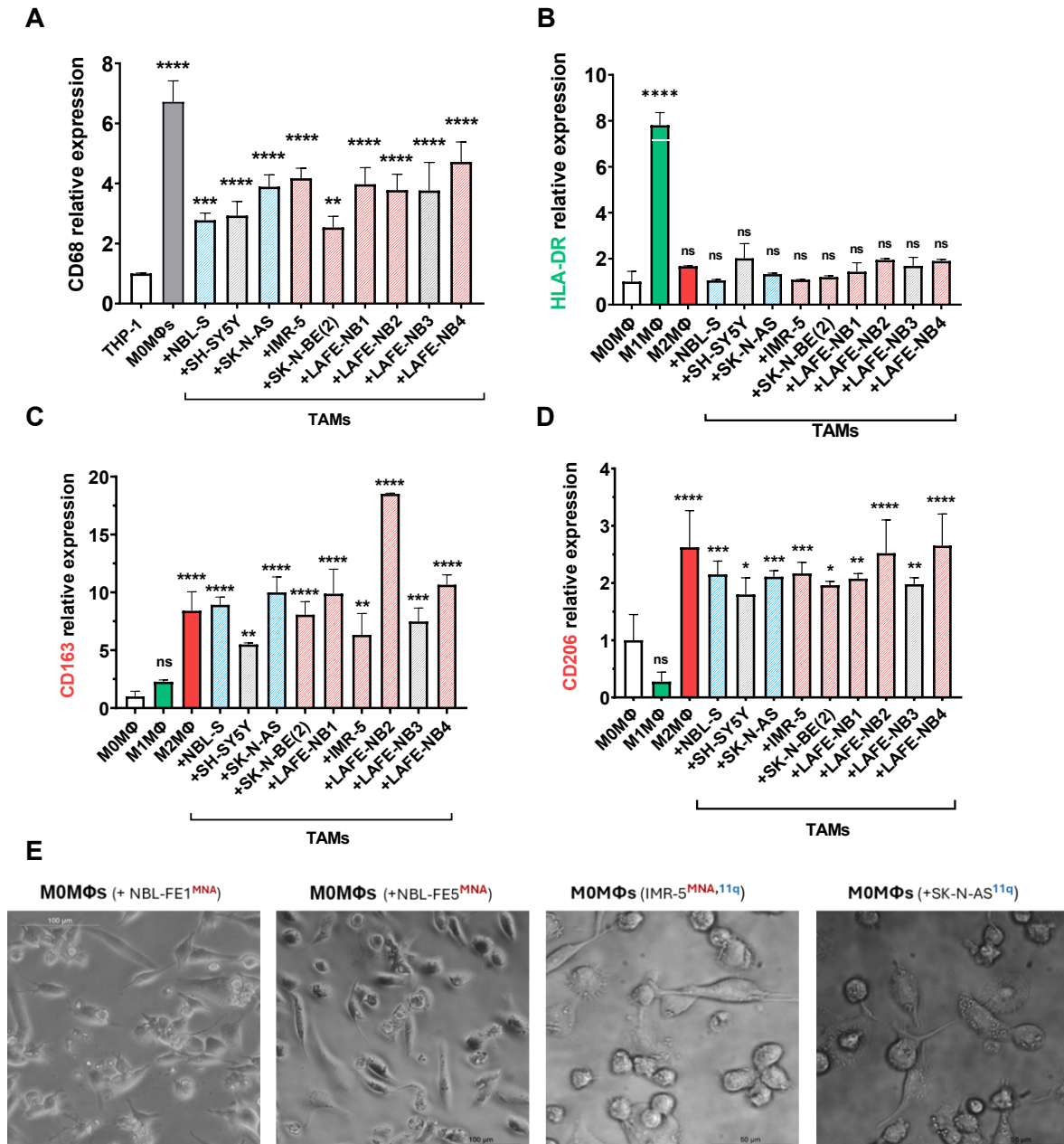
**Figure 19. Schematic representation of the differentiation and characterization process for generating reference macrophage populations.** (A) THP-1 monocytes were induced into M0 macrophages differentiation via 24-hour incubation with 100 nM PMA, followed by a 24-hour rest period in RPMI medium without it. M0 macrophages were polarized into M1 macrophages with 50 ng/mL IFN- $\gamma$  and LPS for 48 hours, while M2 macrophages were generated with 25 ng/mL IL-4 and IL-13 for the same duration. (B) Monocyte differentiation and polarization were validated through the assessment of specific macrophage markers using ddPCR: CD68 (M0 macrophages marker), HLA-DR (M1 macrophages marker), CD163 and CD206 (M2 macrophages markers). Results are presented as relative expression normalized to *ACTB*. Error bars represent  $\pm$  SD derived from three independent experiments conducted in triplicate. One-way ANOVA was used to test the statistical differences in gene expression. Significance levels are denoted as \* $P \leq 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ . Ns: not significant. (C) Macrophages adherence and ramification processes resulting from differentiation and polarization were assessed through visual inspection using light microscopy (pointed with arrows in the images). M $\phi$ : macrophage.



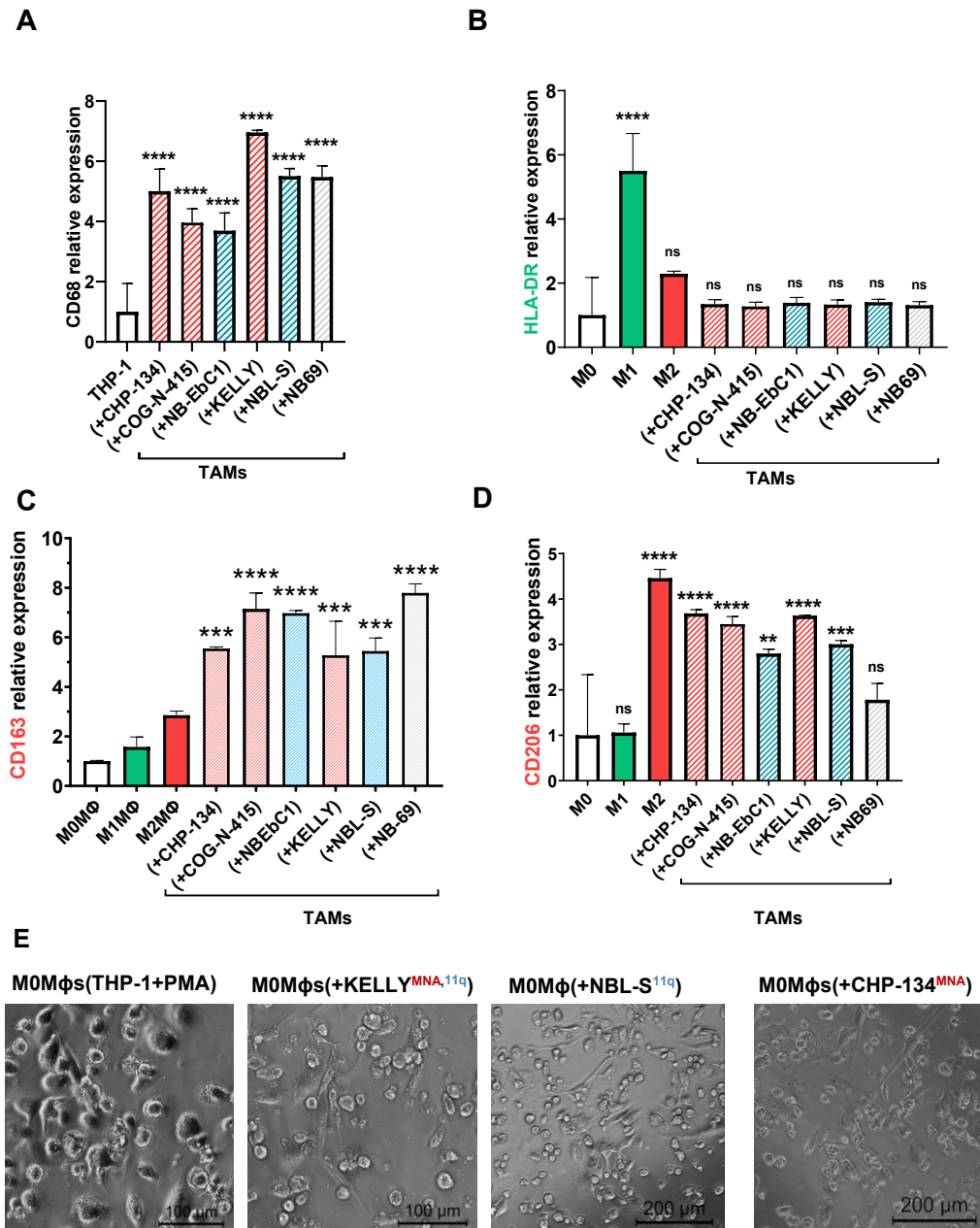
Several key observations were made following the co-culture of NB cell lines and PDTOs with THP-1 monocytes. Firstly, NB cells triggered the differentiation of monocytes into M0 macrophages, evidenced by a marked increase in the CD68 marker compared to reference cells (Figure 20A). Additionally, an augmented adherence of monocytic cells to the cell surface was noted (Figure 20E), a morphological change associated with the organization of microvilli and filopodia formation. Furthermore, NB cells directed M0 macrophages towards the M2 phenotype, as evidenced by a significant rise in the expression of CD163 and CD206 compared to reference cells, regardless of their genetic alterations. Importantly, no significant alteration was observed in the expression of the HLA-DR marker typically associated with M1 macrophages (Figure 20B-D). A second set of established NB cell lines and primary NB cell lines were used (Figure 21), yielding consistent results that reinforce the previous findings.

## Results

### Neuroblastoma cells induce macrophage polarization towards the M2 phenotype



**Figure 20. Neuroblastoma cells induce monocyte differentiation and polarization towards M2 phenotype.** Monocyte differentiation and polarization were confirmed via ddPCR by analyzing the expression of macrophage markers CD68 (M0 macrophages), HLA-DR (M1 macrophages), and CD163/CD206 (M2 macrophages). Expression levels were normalized to *ACTB* and presented as the mean ratio relative to control populations: non-co-cultured monocytes (A) and M0 macrophages (B-D). Error bars represent  $\pm$  SD derived from three independent experiments conducted in duplicate. One-way ANOVA was used to test the statistical differences in gene expression. Significance levels are denoted as \* $P \leq 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ . Ns: not significant. (E) Microscopic analysis revealed morphological changes in macrophage populations after co-culture with NB cells. NB cells with *MYCN* amplification alone, or with both *MYCN* amplification and 11q deletion, are shaded in red, and those with only 11q deletion are shaded in blue. Cell lines with neither alteration are shown in grey.



**Figure 21. Validation of the effects of neuroblastoma cells on monocyte differentiation and polarization.** Monocyte differentiation and polarization were confirmed by qPCR analysis of specific macrophage markers. Results are presented as the mean ratio relative to control populations: non-co-cultured monocytes (A) and M0 macrophages (B-D). Expression levels were normalized to the mean of *GAPDH*, *IPO8*, and *UBC*  $\pm$  SD of three independent experiments conducted in triplicate. One-way ANOVA was used to test the statistical differences in gene expression. Statistical codes: \* $p \leq 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ ; ns: not significant. (E) Microscopic analysis revealed morphological changes in macrophage cell populations after co-culture with NB cell lines. NB cells with *MYCN* amplification alone, or with both *MYCN* amplification and 11q deletion, are shaded in red, and those with only 11q deletion are shaded in blue. Cell lines with neither alteration are shown in grey.

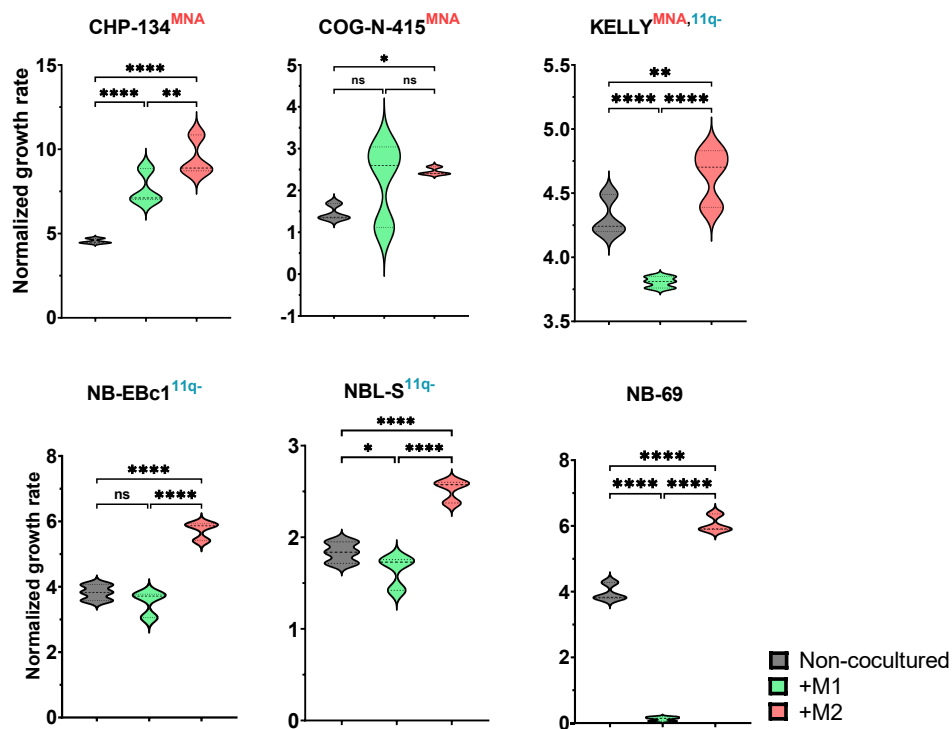
## Results

### M2 macrophages increase neuroblastoma cell proliferation

#### 4.6. M2 macrophages increase neuroblastoma cell proliferation

Based on the previous observations, we proposed that M2 macrophages and NB cells collaborate to create an environment that supports tumor growth. To gain insights into the impact of macrophages on NB cell proliferation, we compared the growth rate of NB cells co-cultivated with THP1-derived M1 and M2 macrophages.

Following a 3-day incubation period, we evaluated the growth rates of each NB cell population for 5 days. Our findings revealed a significant increase in NB proliferation in the presence of M2 macrophages, contrasting with co-cultures involving M1 macrophages, which did not exhibit such an enhancement, except for CHP-134 (Figure 22). However, CHP-134 cells co-cultured with M2 macrophages displayed a significantly higher growth rate than co-culture with M1 macrophages.



**Figure 22. M2 macrophages increase neuroblastoma cell proliferation.** M0 macrophages were induced to M1 macrophages through 48-hour incubation with IFN- $\gamma$  and LPS. M2 macrophages were polarized via 48-hour incubation IL-4 and IL-13. NB cells were co-cultured for 3 days under three conditions: alone (grey), with M1 macrophages (green), or with M2 macrophages (red) using Transwell inserts. Proliferation rate was measured after 5 days using Cell Titer Glo 2.0 assay. Results are expressed as the mean ratio normalized to day 0 luminiscent measurement. One-way ANOVA was used to test the statistical differences in growth rate. Statistical significance levels are denoted as: \*p < 0.01; \*\*p < 0.01; \*\*\*p < 0.001; \*\*\*\*p < 0.0001; ns: not significant.

This increase in proliferation resulting from M2 macrophage crosstalk was consistent across all NB cell lines, regardless of their genetic profiles. Notably, co-culture with M1 macrophages significantly reduced the proliferation rate of the KELLY, NBL-S, and NB-69 cell lines.

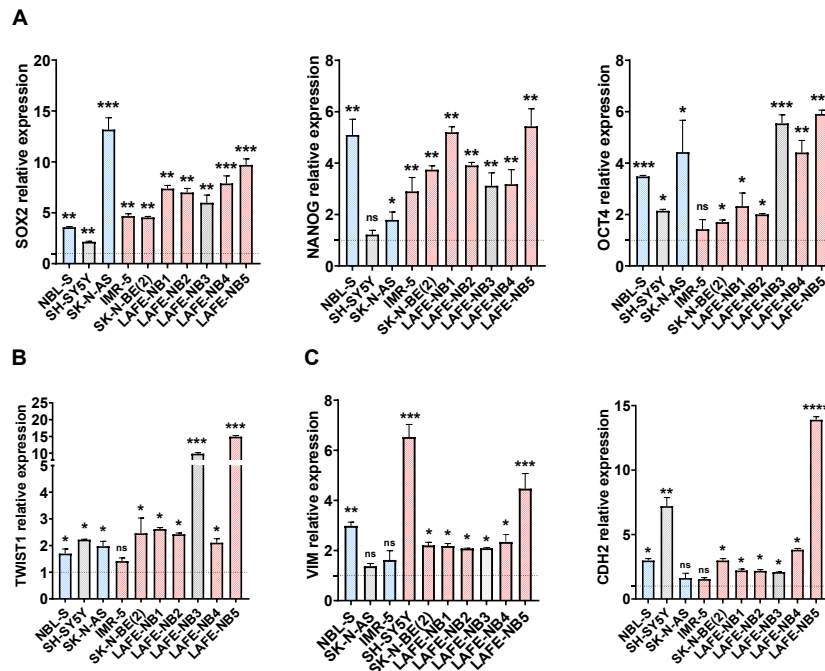
## Results

### M2 macrophages induce neuroblastoma stemness and mesenchymal phenotype

#### 4.7. M2 macrophages induce neuroblastoma stemness and mesenchymal phenotype

NB therapy resistance remains poorly understood, but recent studies have identified two genetically identical cell types: lineage-committed ADRN and undifferentiated MES cells [138, 139], with MES cells showing higher chemoresistance [139, 155]. These cell types can interconvert, but the mechanisms of this transition remain unclear. Understanding the ADRN-to-MES shift is critical to addressing NB drug resistance.

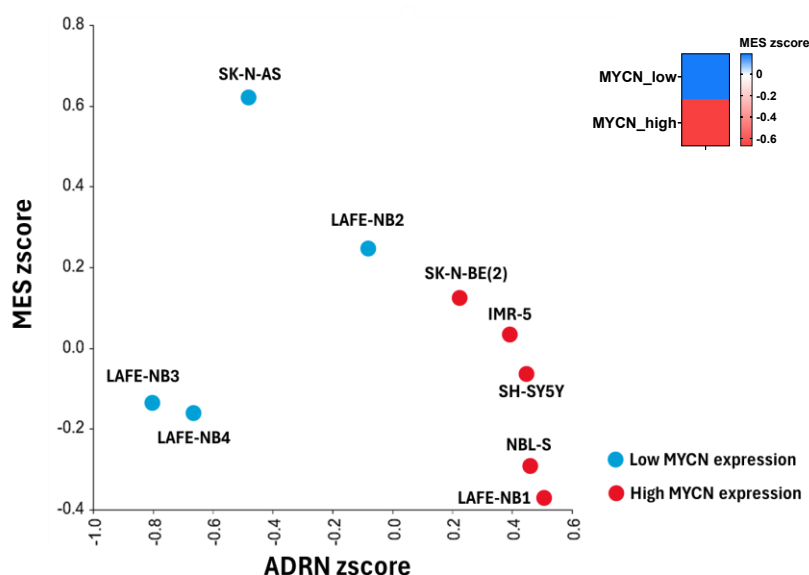
We conducted a comparative expression analysis by ddPCR between NB cell lines before and after non-contact crosstalk in two-phase co-culture with THP-1-derived M1 and M2 macrophages. Notably, co-culture with M2 macrophages resulted in a significant increase in the expression of the stemness markers OCT4, SOX2, and NANOG (Figure 23A). Additionally, there was an increased expression of the mesenchymal transition effector TWIST1 (Figure 23B), and a notable upregulation of MES lineage markers VIM and CDH2 (Figure 23C).



**Figure 23. M2 macrophages increase neuroblastoma expression of stemness and mesenchymal markers.** (A) Stemness markers OCT4, SOX2, and NANOG are significantly upregulated in NB cells upon two-phase co-culture with pharmacologically polarized M2 macrophages, indicating enhanced pluripotency and self-renewal potential. (B) MES lineage markers VIM and CDH2, characteristic of a mesenchymal phenotype, show increased expression in NB cells in response to M2 macrophages. (C) The MES transition effector TWIST1 is upregulated in NB cells following co-culture with M2 macrophages, further supporting mesenchymal transition induction. Expression levels were normalized to ACTB and presented as

the mean ratio relative to each NB cell line basal expression values. Red-filled bars represent NB cell lines with MYCN amplification, blue-filled bars represent those with 11q deletion, and grey-filled bars represent cell lines without either alteration. Statistical significance levels are denoted as follows: \* $p < 0.01$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ ; ns: not significant.

SK-N-AS and IMR-5 were the exception, not showing a significant increase in mesenchymal markers VIM and CDH2 when co-cultured with M2 macrophages (Figure 23C). We conducted a gene set correlation enrichment analysis to explore this further and assess our NB cell lines' ADRN and MES lineage identities. The gene set lists for evaluating ADRN and MES cell states were obtained from Groningen and colleagues [139]. The analysis revealed that the SK-N-AS cell line has the most robust mesenchymal phenotype (Figure 24), suggesting that mesenchymal transition had already peaked in these cells. In contrast, IMR-5 showed a moderate MES z-score but also displayed a high ADRN profile (Figure 24). Furthermore, SK-N-AS and IMR-5 exhibited the highest baseline expression levels of CDH2, with SK-N-AS also showing elevated levels of VIM (Annex 7).

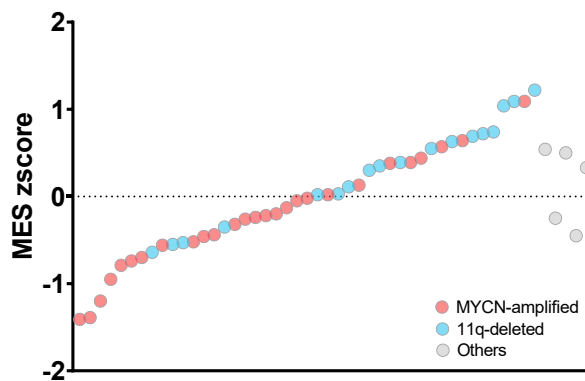


**Figure 24. Gene set correlation enrichment analysis of ADRN and MES lineage identities in neuroblastoma cell lines** Correlation plot illustrating the ADRN and MES profiles of NB cell lines, categorized by MYCN expression using the median MYCN level as a cutoff. Blue dots represent cell lines with low MYCN expression, which show higher correlation with the MES lineage, indicated by elevated MES z-scores. Red dots represent cell lines with high MYCN expression, correlating more with the ADRN phenotype. The heatmaps on the right display MES z-scores, with a color scale ranging from red (negative correlation or lower association with the MES signature) to deep blue (positive correlation or stronger association).

## Results

### M2 macrophages induce neuroblastoma stemness and mesenchymal phenotype

Interestingly, when we categorized our NB cell lines based on MYCN expression, we observed that those with low MYCN expression were associated with a mesenchymal profile (Figure 24). This led us to analyze the lineage identities of primary NB tumors (GSE73517) to determine whether the 11q-deleted subtype was linked to a more mesenchymal profile. The analysis revealed that 11q-deleted NB samples correlate strongly with the MES lineage, while *MYCN*-amplified NB samples correlate more strongly with the ADRN phenotype (Figure 25).



**Figure 25. Gene set correlation enrichment analysis of ADRN and MES lineage identities in neuroblastoma primary tumors.** (B) Correlation and distribution of primary HR-NB samples (GSE73517) between MES and ADRN gene sets. Blue dots indicate 11q-deleted NB samples, which show a higher correlation with the MES lineage, reflected by elevated z-scores and mesenchymal solid characteristics. Red dots represent *MYCN*-amplified NB samples, correlating more with the ADRN phenotype. Gray dots denote other NB samples not classified as 11q-deleted or *MYCN*-amplified. Pearson's correlation coefficients between ADRN and MES gene sets were analyzed using Fisher's Z transformation, revealing a significant difference ( $p = 0.0182$ ).



#### **4.8. Tumor-associated macrophages and their impact on PHOX2B PC-CAR T-cell efficacy in neuroblastoma**

##### **4.8.1. Enhanced PHOX2B PC-CAR T-cell tumor cell killing by M1 macrophages**

CAR T-cell therapy represents a groundbreaking approach in immunotherapy, using genetically engineered T cells to target and destroy cancer cells [272]. While effective in some hematologic malignancies [226, 227], its success in solid tumors, including NB, has been limited [272]. The TME can hinder CAR T-cell efficacy in solid tumors by affecting T-cell function and causing its exhaustion [272]. As previously described, TAMs are among the most predominant immune cells in NB's TME; thus, understanding how they impact CAR T cell killing could provide novel insights into improving treatment efficacy.

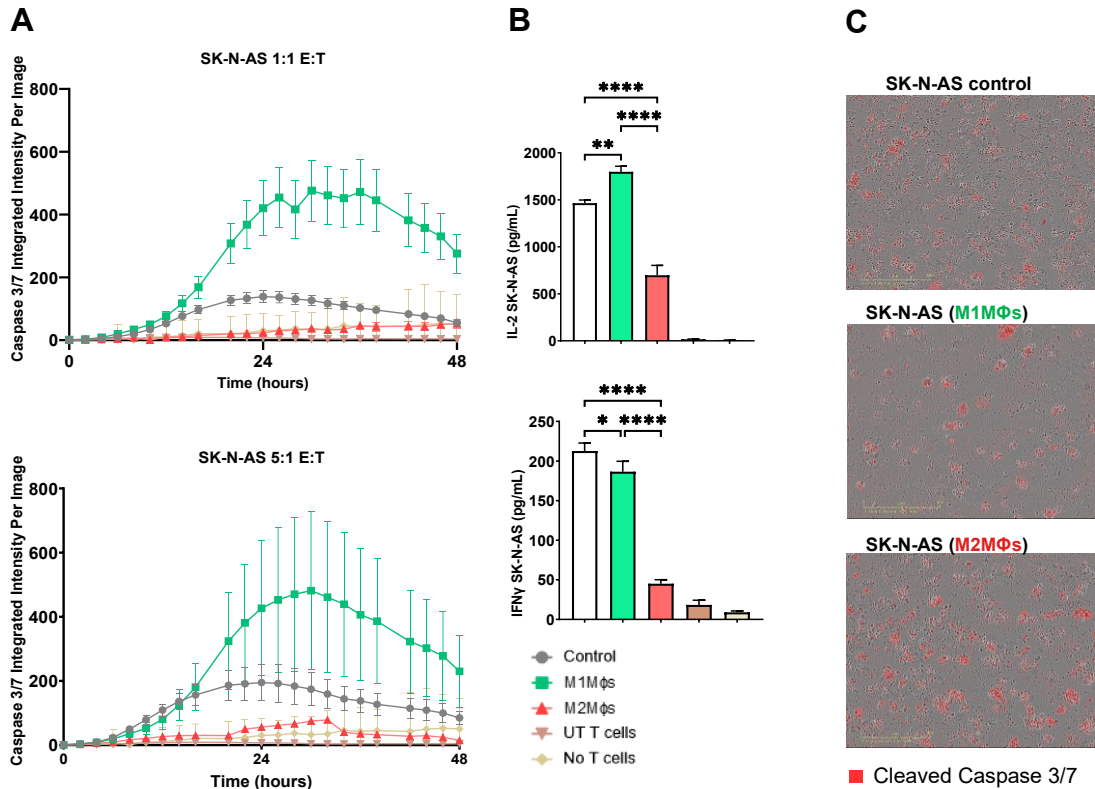
To investigate how TAMs affect CAR T-cell therapy, we employed the recently developed PHOX2B PC-CAR 10LH, which targets the unmutated PHOX2B peptide QYNPIRTTF, commonly expressed in NB cells [235]. PHOX2B PC-CARs have a high affinity for the PHOX2B peptide when presented by HLA-A\*24:02 or HLA-A\*23:01 molecules and have shown effective NB killing [235]. For this study, we used the NB cell lines SK-N-AS, SK-N-BE(2), and NB-EBc1. SK-N-AS, having the haplotype HLA-A\*24:02 and high PHOX2B antigen expression, served as a positive control of PC-CAR 10LH efficacy [235]. SK-N-BE(2) and NB-EBc1 have alleles different from HLA-A\*24:02 and HLA-A\*23:01. To be recognized by PC-CAR 10LH, these cell lines were engineered in the John M. Maris laboratory to express the HLA-A\*24:02 or HLA-A\*23:01 and tagged with green fluorescent protein (GFP). Parental SK-N-BE(2) and NB-EBc1 cell lines, as well as untransduced T cells, were used as negative controls in the killing assay.

We co-cultured the NB cell lines with PBMC-derived M1 and M2 macrophages. After 5 days, we added the PC-CAR 10LH and monitored caspase 3/7 activity as a measure of apoptosis for an additional 2 days. Our findings demonstrate that macrophages significantly influence the response of NB cells to PHOX2B PC-CAR T cell therapy. In SK-N-AS cells, co-culture with M1 macrophages markedly enhanced the cytotoxic effect of PC-CAR 10LH cells, as evidenced by significantly elevated caspase-3/7 activity compared to both control and M2 macrophages co-cultures (Figure 26A). In contrast, caspase-3/7 activity was substantially reduced in SK-N-AS cells co-cultured with M2 macrophages, indicating a weakened apoptotic response (Figure 26A). This trend was

## Results

### Tumor-associated macrophages and their impact on PHOX2B PC-CAR T-cell efficacy in neuroblastoma

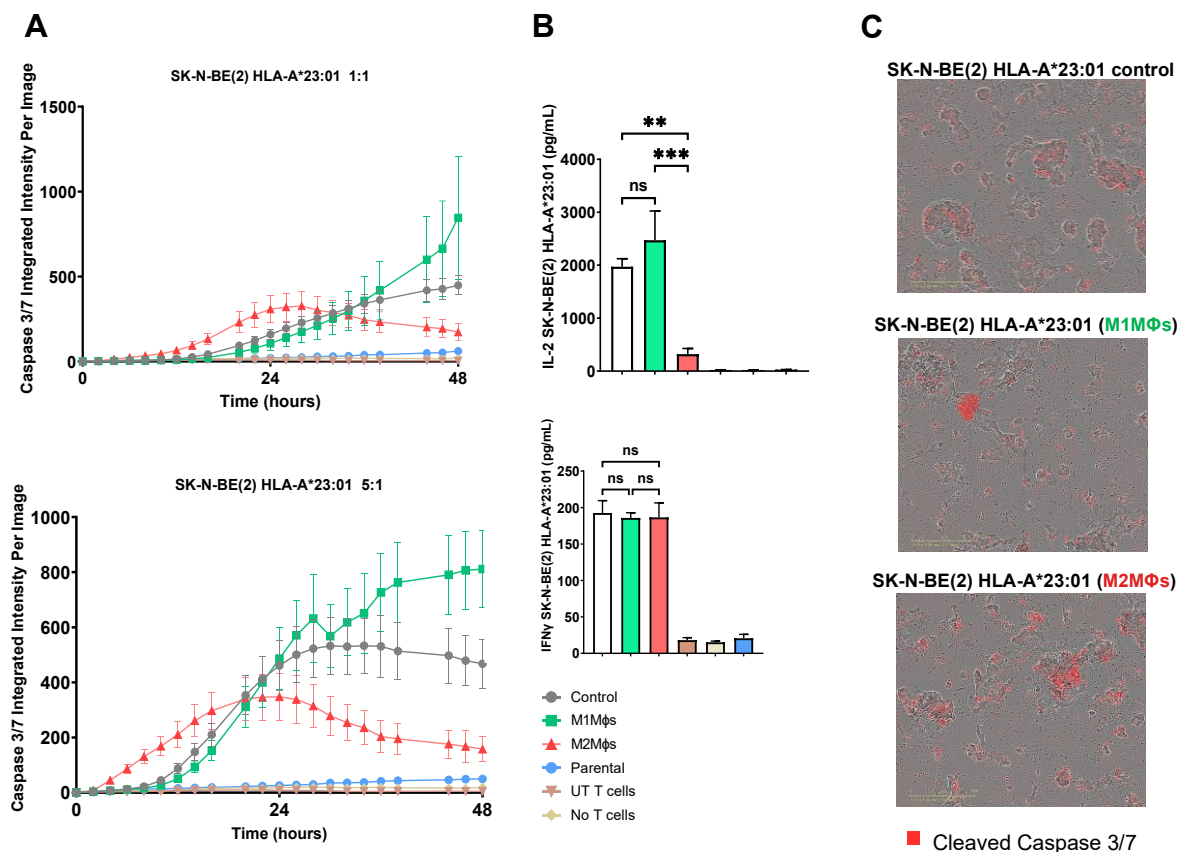
consistently observed at both 1:1 and 5:1 E:T ratios. Increased caspase activity was also accompanied by significantly higher levels of IFN- $\gamma$  and IL-2 in control and M1 macrophage conditions (Figure 26B), signaling robust T cell activation and function.



**Figure 26. Effect of macrophages on PHOX2B PC-CAR T cell-mediated killing of SK-N-AS cell line.** (A) The left panel displays caspase 3/7 cleavage activity in SK-N-AS cells co-cultured with M1 or M2 macrophages alongside control conditions without macrophages. Both untransduced T (UT) cells and wells without T cells were included as negative controls. Apoptosis was quantified by the total integrated intensity of caspase 3/7 cleavage, normalized to the T cell addition time. The upper graph shows apoptosis at a 1:1 E:T ratio, while the lower graph depicts results at a 5:1 E:T ratio (B) IFN- $\gamma$  and IL-2 cytokine levels were measured by ELISA 24 hours after T cell at 1:1 E.T ratio conditions. Assays were conducted in triplicate, and data represent mean  $\pm$  SD. One-way ANOVA was used to test the statistical differences between conditions. Statistical significance levels are denoted as follows: \* $p < 0.01$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ ; ns: not significant. The graph does not indicate significance for the conditions of no T cells and UT T cells ( $p < 0.0001$ ). (C) Cytotoxicity was visualized using a cleaved caspase 3/7 fluorescent marker. Cells were monitored over 48 hours, and images were captured every 2 hours using the IncuCyte Live-Cell analysis system. Representative images at 24 hours are shown.

In SK-N-BE(2) HLA-A\*23:01, apoptosis in control cells initially matched M1 conditions but declined after 48 hours, while M1-induced caspase-3/7 activity remained elevated (Figure 27A). M2 macrophages initially increased apoptosis, but this effect quickly

diminished. This explains why we observed caspase 3/7 red signal and IFN- $\gamma$  levels comparable to control and M1 macrophages conditions at 24h post T cell addition (Figure 27B-C). This trend was consistent at both 1:1 and 5:1 E:T ratios.

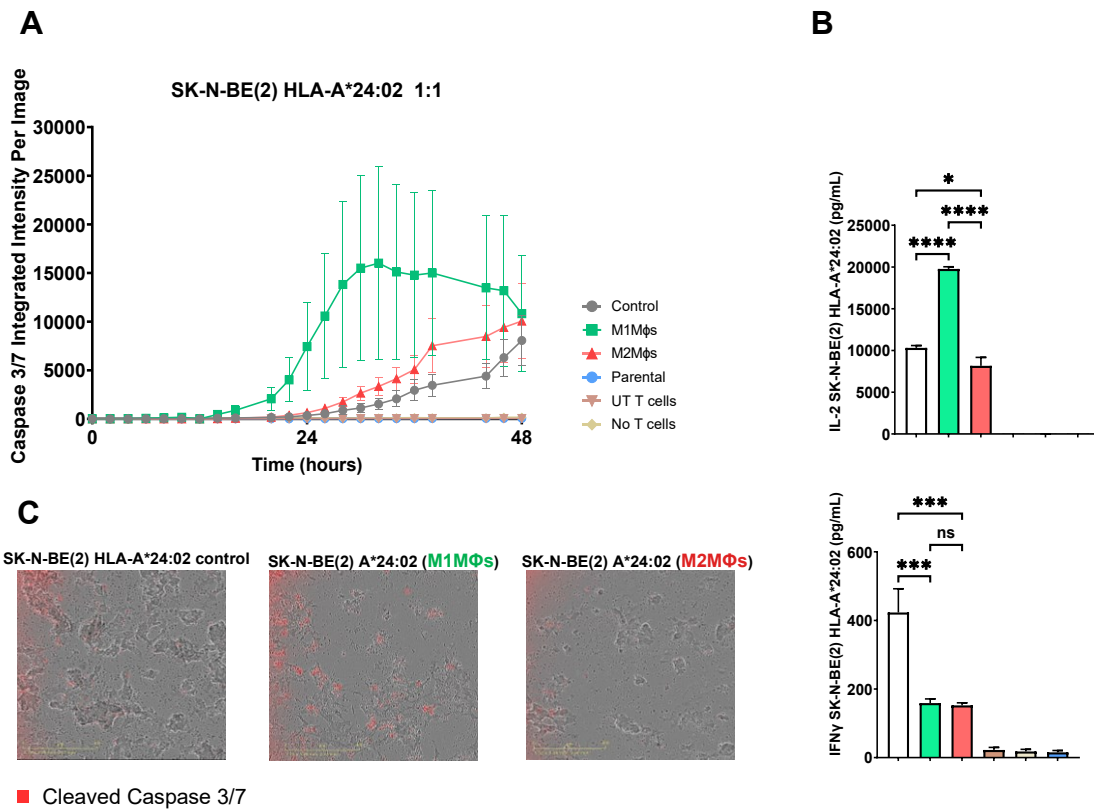


**Figure 27. Effect of macrophages on PHOX2B PC-CAR T cell-mediated killing of SK-N-BE(2) HLA-A\*23:01 cell line.** (A) The left panel shows caspase 3/7 cleavage activity in SK-N-BE(2) HLA-A\*23:01 cells co-cultured with M1 or M2 macrophages compared to controls without macrophages. Parental SK-N-BE(2), UT cells, and wells without T cells served as negative controls. Apoptosis, normalized to T cell addition, is presented for 1:1 and 5:1 E:T ratios. (B) IFN- $\gamma$  and IL-2 levels were measured via ELISA 24 hours after T cell addition at 1:1 E:T ratio. Data represent mean  $\pm$  SD from triplicate assays. One-way ANOVA was used to test the statistical differences between conditions. Statistical significance levels are denoted as follows: \* $p < 0.01$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ ; ns: not significant. Significance values for the conditions Parental, no T cells, and UT T cells are not shown in the graph ( $p < 0.0001$ ). (C) Cytotoxicity was tracked using cleaved caspase 3/7 fluorescence. Images were taken every 2 hours for 48 hours, with representative images at 24 hours shown.

## Results

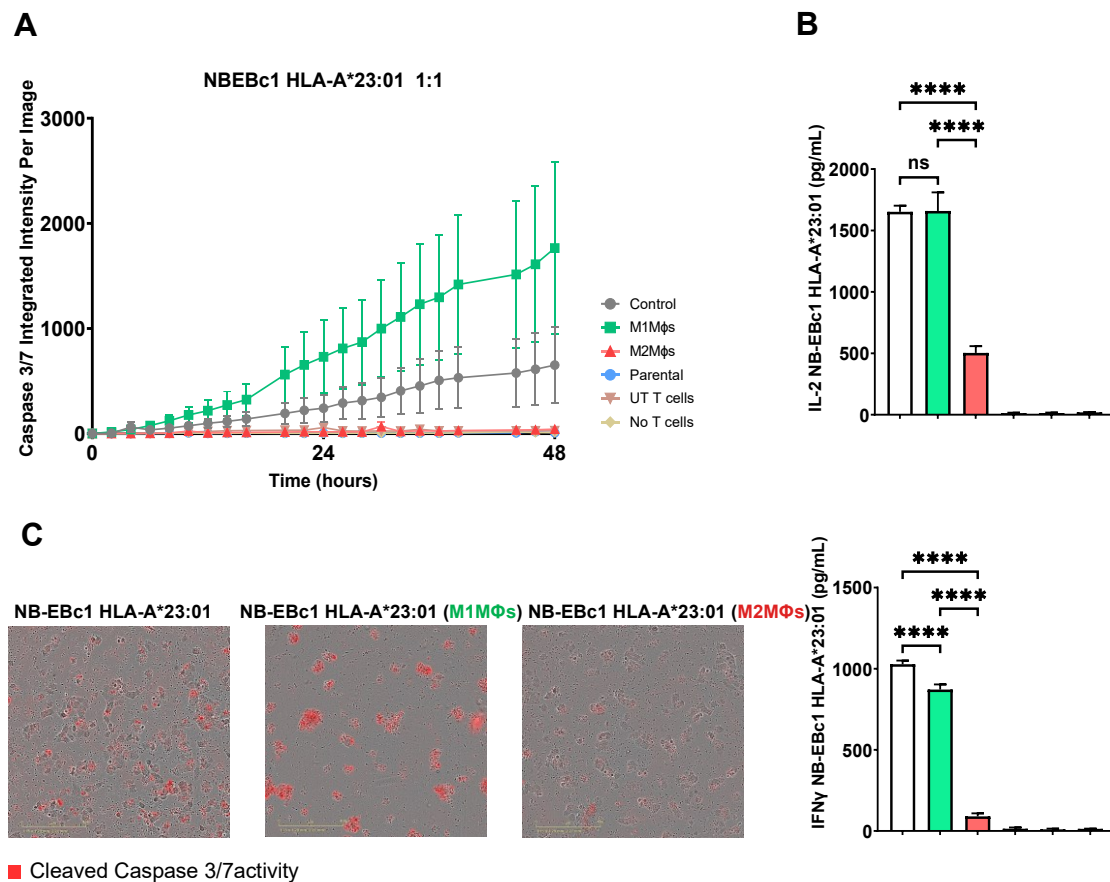
### Tumor-associated macrophages and their impact on PHOX2B PC-CAR T-cell efficacy in neuroblastoma

In SK-N-BE(2) HLA-A\*24:02, co-culturing with M1 macrophages resulted in high-intensity caspase-3/7 activity, peaking at 24 hours (Figure 27A). Control cells showed delayed killing compared to M1 macrophage conditions, and co-culturing with M2 macrophages reduced caspase-3/7 activity, indicating an inhibitory effect on 10LH CAR-T cell-mediated killing (Figure 27A). Only the 1:1 ratio was tested for SK-N-BE(2) HLA-A\*24:02. Co-culture with M1 macrophages resulted in elevated IL-2 levels compared to the M2 macrophage condition, with levels comparable to the control condition (Figure 27B). However, no significant increase in IFN- $\gamma$  was observed across the conditions (Figure 27B).



**Figure 28. Effect of macrophages on PHOX2B PC-CAR T cell-mediated killing of SK-N-BE(2) HLA-A\*24:02 cell line.** (A) Caspase 3/7 cleavage activity in SK-N-BE(2) HLA-A\*24:02 cells co-cultured with M1 or M2 macrophages compared to controls without macrophages. Parental SK-N-BE(2), UT cells, and wells without T cells served as negative controls. Apoptosis, normalized to T cell addition time, is shown for 1:1 E:T ratio. (B) IFN- $\gamma$  and IL-2 levels were quantified by ELISA 24 hours after T cell addition at a 1:1 E:T ratio. Data are mean  $\pm$  SD from triplicate assays. One-way ANOVA was used to test the statistical differences between conditions. Statistical significance levels are denoted as follows: \* $p < 0.01$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ ; ns: not significant. The graph does not show parental, no T cells, and UT T cells' significance values ( $p < 0.0001$ ). (C) Cytotoxicity was tracked via cleaved caspase 3/7 fluorescence with representative images at 24 hours.

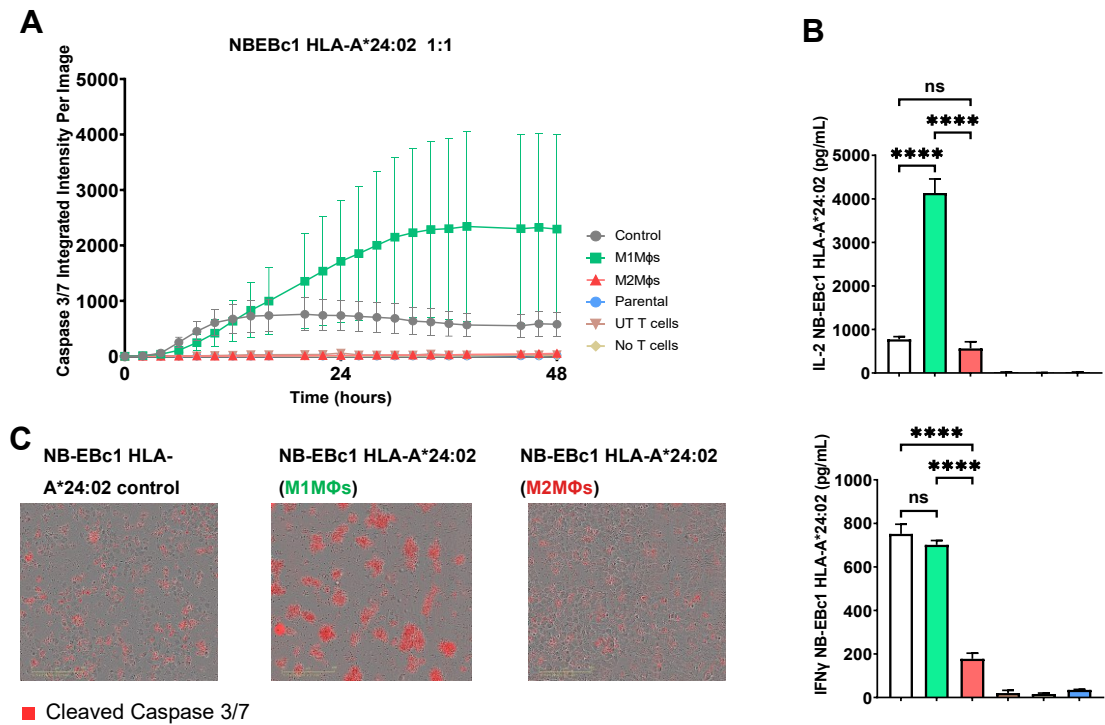
The interaction of NB-EBc1 HLA-A\*23:01 cells with M1 macrophages significantly enhanced the apoptotic response following the introduction of PC-CAR 10LH (Figure 29A) This heightened apoptotic signal was evident earlier and with greater intensity compared to the control condition, which displayed a similar apoptotic pattern but with a delayed onset and lower magnitude.



**Figure 29. Impact of macrophages on PHOX2B PC-CAR T cell-mediated killing of NB-EBc1 HLA-A\*23:01 cell line.** (A) Caspase 3/7 cleavage activity in NB-EBc1 HLA-A\*23:01 cells co-cultured with M1 or M2 macrophages compared to controls without macrophages. Parental cells, UT T cells, and wells without T cells served as negative controls. Apoptosis is shown for a 1:1 E:T ratio. (B) IFN- $\gamma$  and IL-2 levels were measured by ELISA 24 hours post T cell addition. Data are mean  $\pm$  SD from triplicates. One-way ANOVA tested statistical differences between conditions. Statistical significance levels are denoted as follows: \* $p < 0.01$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ ; ns: not significant. Significance for the conditions Parental, no T cells, and UT T cells are not indicated in the graph ( $p < 0.0001$ ). (C) Cytotoxicity was tracked via cleaved caspase 3/7 fluorescence over 48 hours, with representative images at 24 hours.

## Results

### Tumor-associated macrophages and their impact on PHOX2B PC-CAR T-cell efficacy in neuroblastoma



**Figure 30. Impact of macrophages on PHOX2B PC-CAR T cell-mediated killing of NB-EBc1 HLA-A\*24:02 cell line.** (A) Caspase 3/7 cleavage in NB-EBc1 cells co-cultured with M1 or M2 macrophages compared to controls without macrophages. Parental cells, UT T cells, and wells without T cells served as negative controls. Apoptosis at 1:1 E:T ratio. (B) IFN- $\gamma$  and IL-2 levels measured by ELISA 24 hours post T cell addition. Data are mean  $\pm$  SD from triplicates. One-way ANOVA tested significance between conditions. Statistical significance levels are denoted as follows: \* $p < 0.01$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ ; ns: not significant. Significance for the conditions Parental, no T cells, and UT cells are not indicated in the graph ( $p < 0.0001$ ). (C) Cytotoxicity tracked via cleaved caspase 3/7 fluorescence over 48 hours, with representative 24-hour images.

Finally, in NB-EBc1 HLA-A\*24:02, co-culture with M1 macrophages significantly boosted the apoptotic signal following PC-CAR 10LH addition (Figure 30A). The control condition showed a similar apoptotic trend, albeit slightly delayed compared to the M1 macrophages condition. In the control condition, caspase 3/7 activity reached a plateau about 24h post T cell addition, whereas M1 macrophages co-culture resulted in continued tumor killing. This was reflected by a significantly higher IL-2 release in M1 macrophage wells than in control and M2 macrophage wells. In both haplotypes, co-culture with M2 macrophages resulted in almost no killing, indicating a robust inhibitory effect on CAR-T cell efficacy.

In summary, across all NB cell lines, M1 macrophages consistently enhanced the efficacy of PHOX2B PC-CAR T, resulting in significantly higher levels of apoptosis. Conversely, co-culture with M2 macrophages led to lower caspase-3/7 activity, demonstrating a protective and tumor-protective role of M2 macrophages. Of note, unlike SK-N-AS and NB-EBc1 HLA-A\*23:01 and HLA-A\*24:02, which showed minimal or no caspase 3/7 activity when co-cultured with M2 macrophages, SK-N-BE(2) HLA-A\*23:01 and HLA-A\*24:02 exhibited an initial caspase-3/7 activity in M2 conditions (Figure 27A, Figure 28A). However, this activity rapidly decreased compared to the stable or enhanced activity observed in M1 macrophages.



## Results

### Tumor-associated macrophages and their impact on PHOX2B PC-CAR T-cell efficacy in neuroblastoma

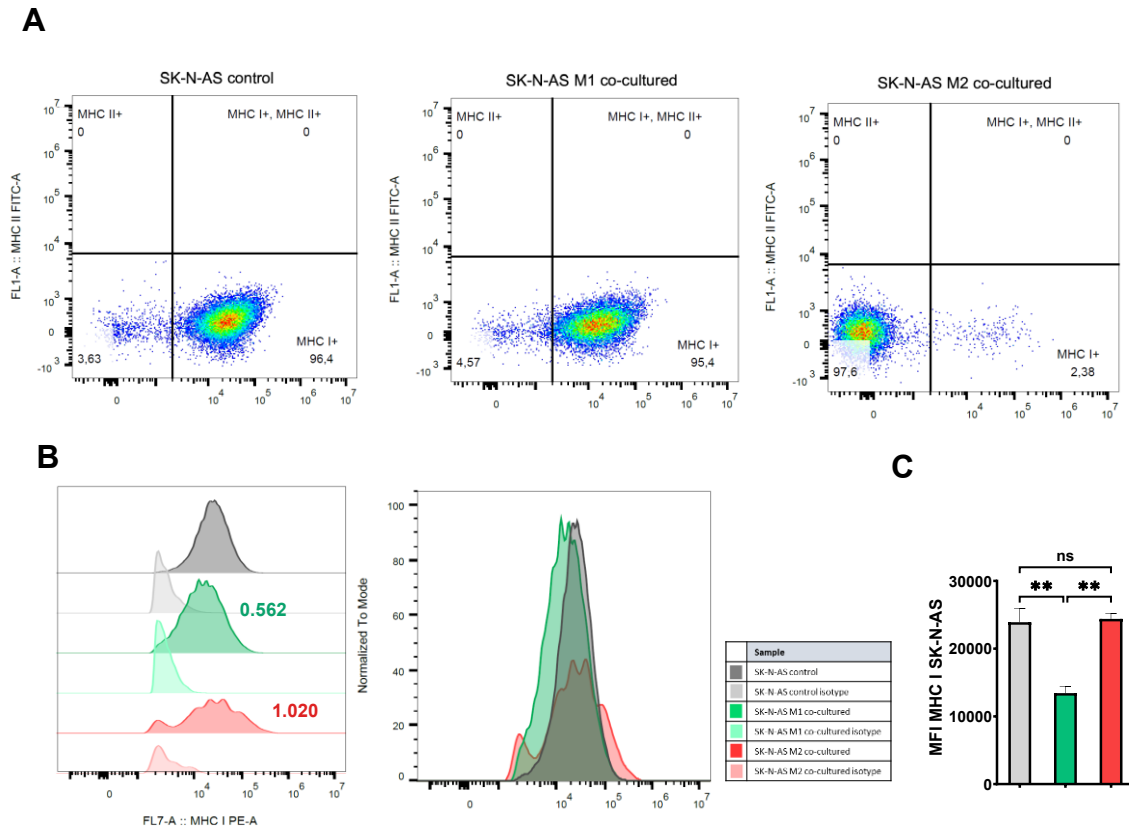
---

#### **4.8.2. MHC I suppression by M2 macrophages hinder PHOX2B PC CAR-T cell-mediated killing in neuroblastoma cells**

We hypothesized that macrophages could influence MHC I expression in NB cells. Specifically, we proposed that M1 macrophages would upregulate MHC I, thereby enhancing the presentation of the PHOX2B antigen and improving CAR T cell recognition and cytotoxicity. In contrast, we expected M2 macrophages to downregulate MHC I expression, reducing CAR T cell recognition and activity. We analyzed the expression of MHC I before and after co-culturing parental NB cell lines with M1 or M2 macrophages for five days. Flow cytometry analysis confirmed distinct patterns of MHC I expression on the NB cells upon co-culture with both macrophage populations.

In the SK-N-AS cell line, control cells exhibited a high baseline expression of MHC I, with 96.4% of the cells being MHC I-positive (Figure 31A). Co-culture with M1 macrophages had a minimal effect on this expression, with 95.4% of the cells remaining MHC I-positive. However, the MFI of MHC I in the M1 macrophage condition decreased to 0.6 times that observed in the control condition (Figure 31B-C). This reduction in MFI, despite the high percentage of MHC I-positive cells, raised questions about technical issues, such as antibody saturation, rather than a true biological downregulation. The slight decrease in MHC I expression may not fully explain this condition's enhanced CAR-T cell activity. Still, it aligns with the preservation of antigen presentation, which is critical for effective immune recognition. Conversely, M2 macrophages had a profound suppressive effect on MHC I expression in SK-N-AS cells, with only 2.38% of the cells expressing MHC I (Figure 31A). This sharp decrease in MHC I+ cells coincided with the observed loss of caspase-3/7 activity (Figure 26A) highlighting how M2 macrophages may facilitate immune evasion by diminishing MHC I expression and impairing CAR-T cell-mediated killing.





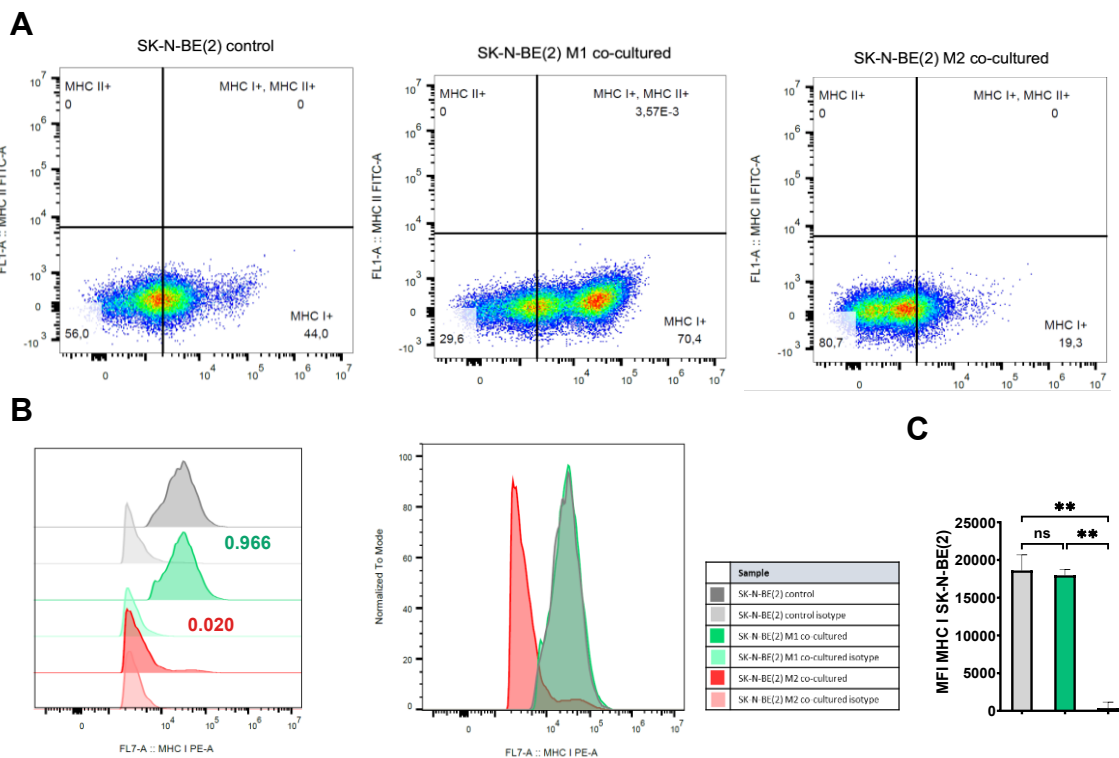
**Figure 31. MHC CLASS I expression on SK-N-AS cells co-cultured with M1 or M2 macrophages.** (A) Dot plots illustrate MHC class I and class II expression on SK-N-AS cells under control conditions and in co-culture with M1 and M2 macrophages. (B) Histograms depict MHC class I fluorescence intensity for primary antibodies and their respective isotypes. Fold-change values are shown for M1 macrophages (green) and M2 macrophages (red) co-cultures relative to control conditions. The MFI fold change was calculated as:  $\frac{[MFI(\text{condition}) - MFI(\text{condition isotype})]}{[MFI(\text{control}) - MFI(\text{control isotype})]}$ . (C) Bar graph shows MFI of MHC class I across conditions. Statistical differences between MFIs were assessed using one-way ANOVA, with significance indicated as: \*\* $p < 0.01$ ; ns, not significant.

In SK-N-BE(2) cells, the baseline MHC I expression was lower, with only 44.0% of cells positive for MHC I (Figure 32A). However, co-culture with M1 macrophages substantially increased the percentage of MHC I-positive cells to 70.4% (Figure 32A), suggesting that M1 macrophages actively promote MHC I expression, potentially enhancing immune surveillance and CAR-T cell efficacy. Interestingly, the MFI in the M1 co-culture was comparable to that of the control (Figure 32B-C), indicating that while M1 macrophages increased the number of MHC I-positive cells, they did not significantly alter the expression levels within already positive cells. On the other hand, M2 macrophages dramatically reduced MHC I expression to 19.3%, along with a significant 50-fold

## Results

### Tumor-associated macrophages and their impact on PHOX2B PC-CAR T-cell efficacy in neuroblastoma

decrease in MFI (Figure 32A). This downregulation was consistent with the reduced caspase-3/7 activity in SK-N-BE(2) cells, demonstrating the immune-suppressive role of M2 macrophages in these cells (Figure 27, Figure 28).

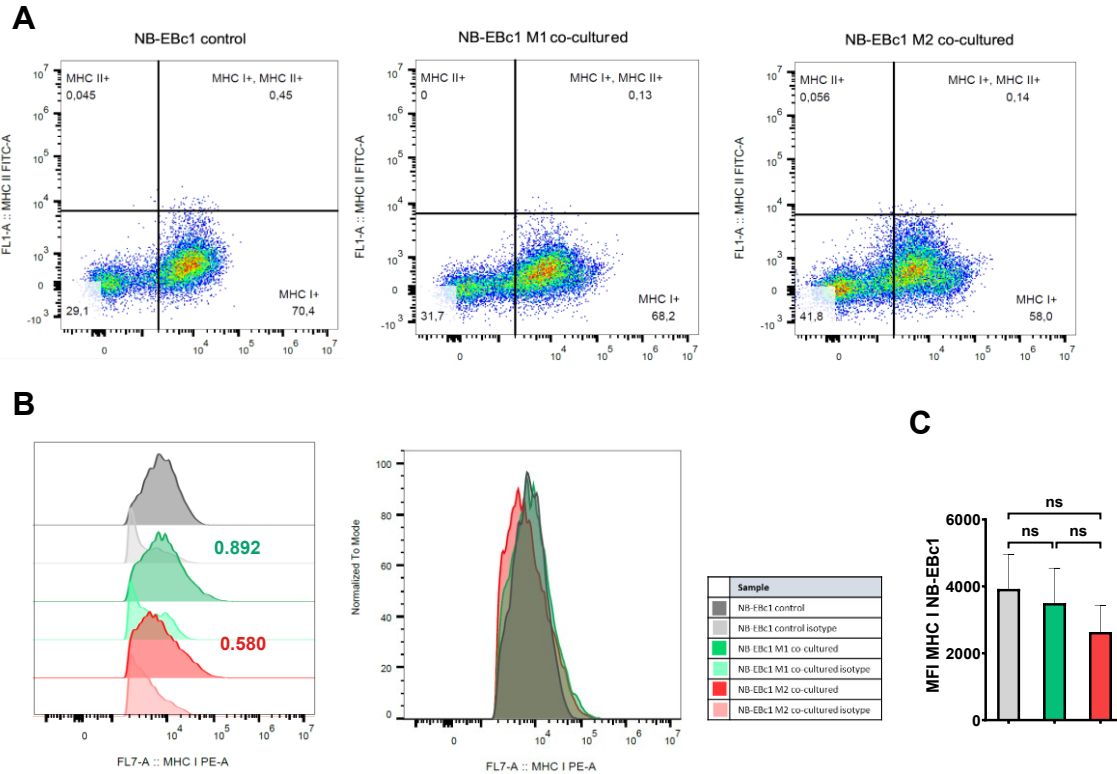


**Figure 32. MHC Class I expression on SK-N-BE(2) co-cultured with M1 or M2 macrophages.**

(A) Dot plots showing MHC class I and II expression on SK-N-BE(2) under control conditions and co-culture with M1 or M2 macrophages. (B) Histograms of MHC class I fluorescence intensity for primary antibodies and isotypes. Fold-change values for M1 (green) and M2 (red) macrophages are shown relative to the control. MFI fold change is calculated as  $\text{MFI fold change} = \frac{[\text{MFI}(\text{condition}) - \text{MFI}(\text{isotype condition})]}{[\text{MFI}(\text{control}) - \text{MFI}(\text{isotype control})]}$ . (C) Bar graph shows the quantification of MFI for MHC class I across conditions. Statistical significance was assessed by one-way ANOVA: \*\* $p < 0.01$ ; ns, not significant.

In the NB-EBc1 cell line, MHC I expression in control conditions was relatively high, with 70.4% of the cells expressing MHC I (Figure 33A). Co-culture with M1 macrophages had little effect on this frequency, with 68.2% of cells remaining MHC I-positive (Figure 33A) and MFI values similar to the control condition (Figure 33B-C). This suggests that M1 macrophages have a more modest effect on enhancing MHC I expression in NB-EBc1 cells. This finding aligns with the T cell killing assay results, which showed only a slight delay in apoptosis in control conditions compared to M1 co-culture (Figure 29). In contrast, M2 macrophages caused a 10% decrease in MHC I-positive cells, reducing the frequency to 58.0% (Figure 33A) and a ten-fold decrease in MFI (Figure 33B-C). The

reduction in MHC I+ cells and MFI in the M2 condition correlated with the almost complete absence of CAR-T cell-mediated killing (Figure 29), further confirming the suppressive effect of M2 macrophages on immune activity.



**Figure 33. MHC Class I expression on NB-EBc1 co-cultured with M1 or M2 macrophages.** (A) Dot plots showing MHC class I and II expression on NB-EBc1 under control conditions and in co-culture with M1 or M2 macrophages. (B) Histograms of MHC class I fluorescence intensity for primary antibodies and isotypes. Fold-change values for M1 (green) and M2 (red) macrophages are shown relative to control. MFI fold change is calculated as:  $\text{MFI fold change} = \frac{[\text{MFI}(\text{condition}) - \text{MFI}(\text{isotype condition})]}{[\text{MFI}(\text{control}) - \text{MFI}(\text{isotype control})]}$ . (C) Bar graph shows the quantification of MFI for MHC class I across conditions. Statistical significance was assessed by one-way ANOVA; ns, not significant.

In conclusion, M1 macrophages maintained or increased MHC I expression across all NB cell lines tested, thus potentially impacting CAR-T cell-mediated cytotoxicity. In contrast, M2 macrophages consistently downregulated MHC I expression, correlating with impaired CAR-T cell efficacy.

## **4.9. Methylome profiling of liquid biopsies from high-risk neuroblastomas**

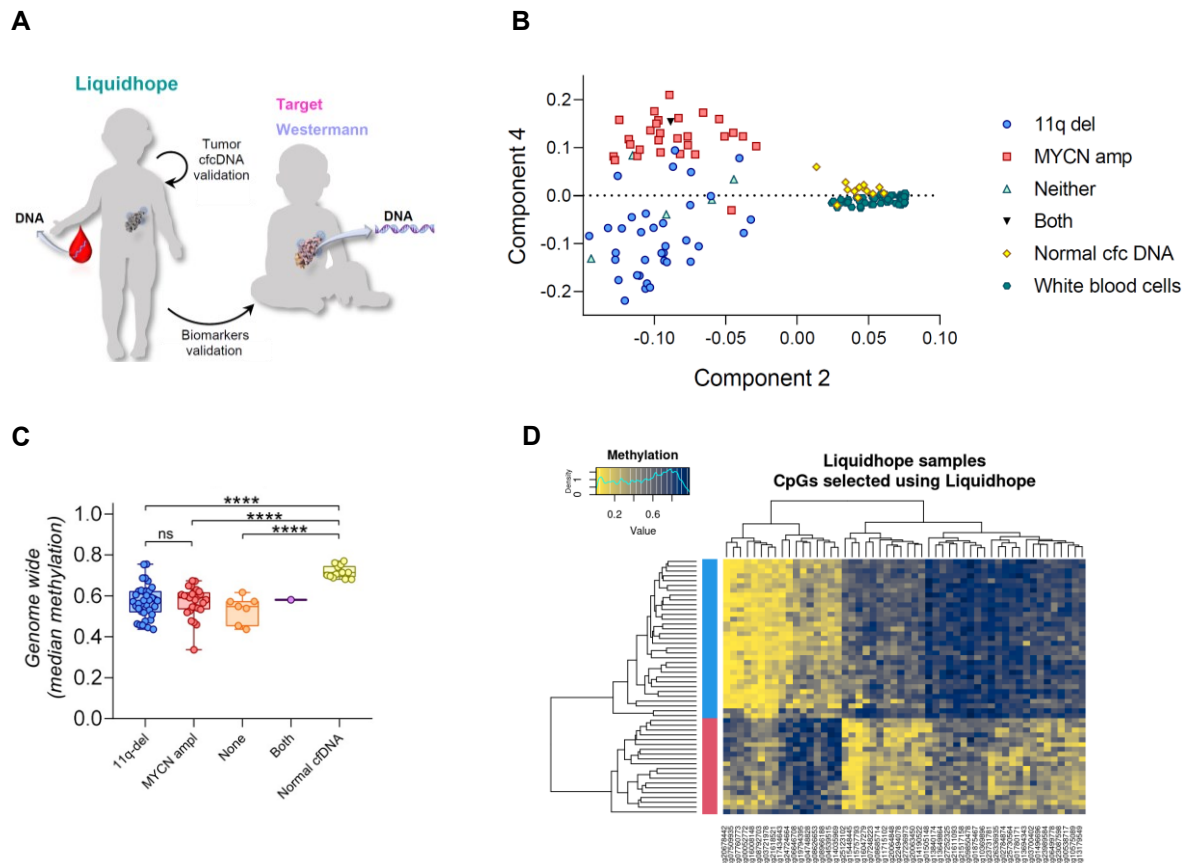
### **4.9.1. Differential epigenetic signatures distinguish high-risk neuroblastomas with 11q deletion and *MYCN* amplification**

Given NB's heterogeneity and low mutation rate, exploring epigenetic alterations could provide deeper insights into immune evasion mechanisms. To investigate this, we conducted WGBS on plasma-derived cfDNA from a HR-NB cohort, which we named Liquidhope.

The Liquidhope cohort included 64 samples from HR-NB patients at diagnosis and 20 at relapse. As cfDNA samples can often be contaminated with normal DNA, we first discriminated tumor-derived cfDNA from non-tumor sources, followed by validation in HR-NB using the TARGET and Westermann external cohorts. (Figure 34A). First, to select samples with tumor DNA, we conducted a PCA using the methylome data of the 84 sequenced samples and 80 healthy control samples from hematopoietic cells collected as part of the Blueprint project [259]. Thirteen samples clustered with the non-tumor controls, indicating the absence of tumor DNA or masking by non-tumor DNA released during sample handling (Figure 34B). Significantly, PCA distinctly separated NB samples with 11q deletion from those with *MYCN* amplification, suggesting distinct epigenetic signatures associated with each genetic alteration. Samples lacking both alterations tended to fall between the two subgroups (Figure 34B).

In addition, *MYCN*-amplified and 11q-deleted tumors exhibited reduced methylation levels compared to normal controls, suggesting a prevalent global hypomethylation pattern across high-risk NB types, consistent with an open chromatin state and active transcriptional regulation (Figure 34C).

From the patient cohort, 53 samples with only *MYCN* or 11q alterations were chosen for further analysis, ensuring the identification of only tumor-specific epigenetic signatures. The analysis was validated using the external independent cohorts, TARGET (GEO ID: GSE221317) and Westermann (GEO ID: GSE73515). For comparability with these



**Figure 34. Distinct epigenetic patterns differentiate high-risk neuroblastomas with 11q deletion from those with *MYCN* amplification.** (A) Tumor cfDNA was obtained from plasma samples from HR-NB patients. Self-validation within the Liquidhope cohort ensured the tumor DNA presence in each sample, followed by results validation in HR-NB Target and Westermann cohorts. (B) PCA discriminated between *MYCN* amplification, 11q deletion, and samples with undetectable tumor cfDNA (normal cfDNA), which cluster alongside reference DNA from white blood cells. (C) Median methylation levels were calculated for each sample within the Liquidhope cohort and compared across subtypes and with 13 samples containing normal cfDNA. Paired t-tests were used to assess differences in methylation levels. Statistical significance levels are denoted as follows: \* $p < 0.01$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ ; ns: not significant. (D) The heatmap depicts the 52 CpG signature, categorizing samples based on 11q or *MYCN* status. This signature was identified using an elastic net regression model. The color code in the upper left corner represents the degree of methylation. CfcDNA: cell-free circulating DNA. Adapted from [2].

## Results

### Methylome profiling of liquid biopsies from high-risk neuroblastomas

---

cohorts, CpGs from the Liquidhope WGBS data were selected based on their presence in Illumina's 450K arrays, the technique used by Westermann and TARGET cohorts.

By elastic net penalized linear regression modeling, we identified a 52 CpG signature that accurately clustered samples based on 11q or *MYCN* status. These findings suggest distinct epigenetic pathways between 11q-deleted and *MYCN*-amplified HR-NB, pointing to different origins for these subtypes.

Notably, the model misclassified three samples in the TARGET cohort, while in the Westermann cohort, only one was misclassified, highlighting its prediction efficiency. This indicates that, despite differences in biological sources and methods for 5-methylcytosine conversion and analysis, liquid biopsy cfDNA accurately reflects the tumor's epigenomic signature.

Understanding the functional significance of the 52 CpGs could reveal abnormal DNA methylation pathways in HR-NB, providing new insights into the molecular mechanisms driving NB pathogenesis and contributing to the observed tumor heterogeneity. Thus, we performed the functional annotation of the CpG signature to understand their biological significance and clinical implications better.

We analyzed the distance to the nearest TSS, affected genes and their function, and the presence of regulatory elements, including cCREs and TFs binding sites [261]. We categorized cCREs into promoter-like, enhancer-like, DNase-H3K4me3, and CTCF-only groups. This classification relied on signals from representative DNase hypersensitivity sites (rDHSs) supported by H3K4me3, H3K27ac, and CTCF-binding data [261]. The 52 CpGs overlapped with cCREs alongside multiple TFs binding sites (Table 11), indicating their potential regulatory role in the nearby gene expression.

**Table 11.** Functional annotation of the 52 CpG signature discriminating 11q-deleted and *MYCN*-amplified high-risk NBs.

| Loci       | CpG Position (GRCh38/hg38) | cCREs <sup>(1)</sup> | Gene                | Genomic region <sup>(2)</sup> | Distance to TSS (bp) |
|------------|----------------------------|----------------------|---------------------|-------------------------------|----------------------|
| cg00538717 | chr6:34155767              |                      | <i>GRM4</i>         | Promoter                      | 146                  |
| cg01496696 | chr7:158019172             |                      | <i>PTPRN2</i>       | Intron 10                     | 568651               |
| cg01780171 | chr2:235192044             | K4m3                 | <i>SH3BP4</i>       | Intergenic                    | 542090               |
|            |                            |                      | <i>AGAP1</i>        | Intergenic                    | 302063               |
| cg13649864 | chr9:98502417              |                      | <i>GABBR2</i>       | Intron 3                      | 206518               |
| cg14190522 | chr9:121581942             | enhD                 | <i>DAB2IP</i>       | Intron 1                      | 16326                |
| cg21611093 | chr3:134794114             |                      | <i>EPHB1</i>        | Promoter                      | 1146                 |
| cg25730564 | chr2:235192107             | K4m3                 | <i>SH3BP4</i>       | Intergenic                    | 240090               |
|            |                            |                      | <i>AGAP1</i>        | Intergenic                    | 302000               |
| cg08626653 | chr7:100484699             | enhP                 | <i>NYAP1</i>        | Intron 1                      | 772                  |
| cg00052772 | chr6:33,278,027            | enhP                 | <i>B3GALT4</i>      | Exon 1                        | 798                  |
| cg02784874 | chr17:41572710             |                      | <i>KRT9</i>         | Promoter                      | 651                  |
| cg03721978 | chr6:33277929              | enhP                 | <i>B3GALT4</i>      | Exon1                         | 806                  |
| cg08966188 | chr17:41529660             | enhP                 | <i>KRT19</i>        | Promoter                      | 1352                 |
| cg10369896 | chr7:1709130               |                      | <i>ELFN1</i>        | Exon 2                        | 43385                |
| cg13179549 | chr17:41612923             | prom                 | <i>KRT16</i>        | Promoter                      | 156                  |
| cg13840174 | chr1:18366968              |                      | <i>IGSF21</i>       | Intron 6                      | 259170               |
| cg15757793 | chr17:79220943             | K4m3                 | <i>RBFOX3</i>       | Intron 3                      | 295205               |
| cg20063450 | chr17:41464958             | enhD                 | <i>KRT32</i>        | Intron 3                      | 2428                 |
| cg21618521 | chr6:33277993              | enhP                 | <i>B3GALT4</i>      | Exon 1                        | 832                  |
| cg22494078 | chr1:156680117             |                      | <i>NES</i>          | Promoter                      | 2710                 |
|            |                            |                      | <i>CRABP2</i>       | Intergenic                    | 25699                |
| cg23087598 | chr1:165349119             |                      | <i>LMX1A</i>        | Intron 3                      | 7596                 |
| cg23989584 | chr11:120167813            |                      | <i>TRIM29</i>       | Intergenic                    | 29700                |
| cg25123102 | chr20:46251084             |                      | <i>CDH22</i>        | Exon 2                        | 57414                |
| cg26336935 | chr17:41612961             | prom                 | <i>KRT16</i>        | Promoter                      | 194                  |
| cg27236973 | chr17:41625745             | enhP                 | <i>KRT17</i>        | Promoter                      | 1170                 |
| cg17434643 | chr20:63173715             |                      | <i>MIR124-3</i>     | Intergenic                    | 4785                 |
|            |                            |                      | <i>RP5-963E22.4</i> | Intron 1                      | 7188                 |
| cg01875467 | chr5:150113445             | prom                 | <i>CSF1R</i>        | Promoter                      | 73                   |
| cg04539515 | chr1:220895452             |                      | <i>HLX</i>          | Intergenic                    | 16009                |
| cg07248223 | chr17:40561023             | prom                 | <i>CCR7</i>         | Intron 1                      | 4449                 |
| cg08792703 | chr11:61012194             |                      | <i>CD6</i>          | Intron 6                      | 40514                |
| cg14035969 | chr1:220895081             | enhD                 | <i>HLX</i>          | Intergenic                    | 15638                |

## Results

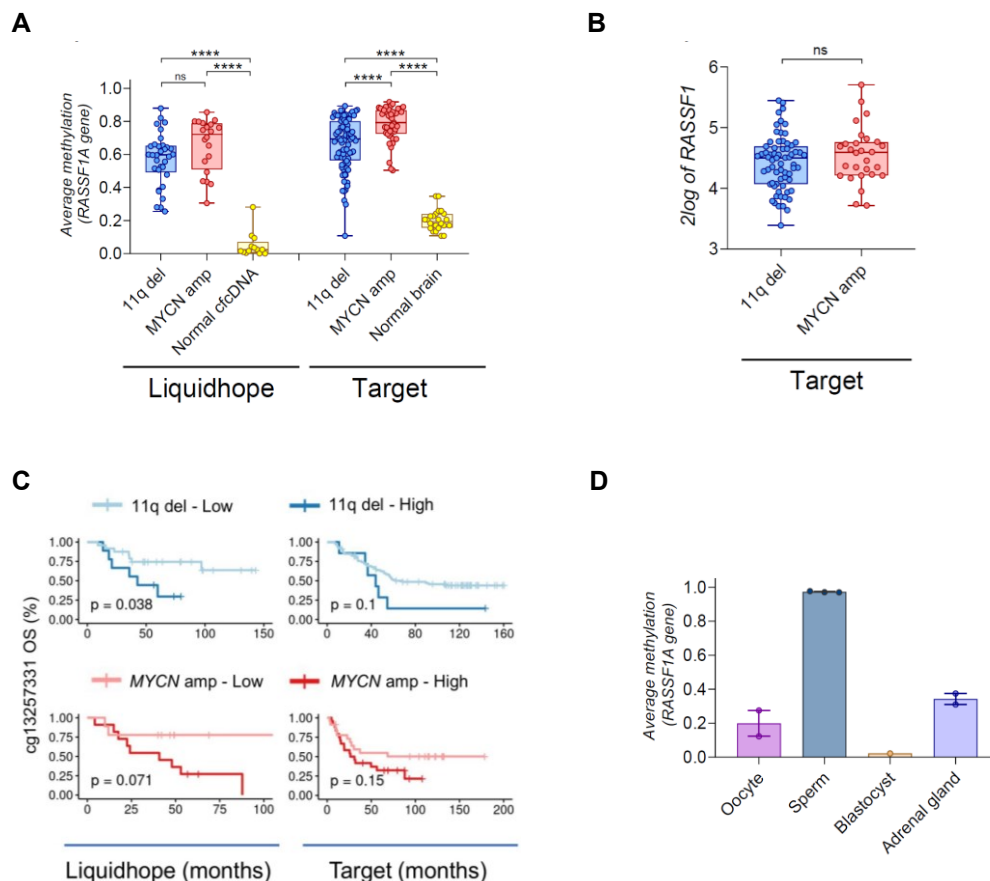
### Methylome profiling of liquid biopsies from high-risk neuroblastomas

|                   |                 |      |                              |            |        |
|-------------------|-----------------|------|------------------------------|------------|--------|
| <b>cg15448445</b> | chr12:108339531 | enhP | <i>CMKLR1</i>                | Promoter   | 218    |
| <b>cg16008148</b> | chr1:220889477  |      | <i>HLX</i>                   | Intergenic | 10034  |
| <b>cg16047279</b> | chr17:40560990  | prom | <i>CCR7</i>                  | Intron 1   | 4482   |
| <b>cg07509935</b> | chr14:24310961  | enhP | <i>CIDEB</i>                 | Promoter   | 461    |
|                   |                 |      | <i>LTB4R</i>                 | Promoter   | 541    |
|                   |                 |      | <i>LTB4R2</i>                | Exon 1     | 810    |
| <b>cg07760773</b> | chr14:24310753  | enhP | <i>CIDEB</i>                 | Promoter   | 669    |
|                   |                 |      | <i>LTB4R</i>                 | Promoter   | 749    |
|                   |                 |      | <i>LTB4R2</i>                | Exon 2     | 602    |
| <b>cg20064848</b> | chr1:160068087  | enhD | <i>KCNJ10</i>                | Intron 1   | 2073   |
| <b>cg20678442</b> | chr1:244730201  | enhP | <i>DES12</i>                 | Intergenic | 77075  |
| <b>cg21517158</b> | chr17:82931817  | enhD | <i>TBCD</i>                  | Intron 34  | 179752 |
| <b>cg13694343</b> | chr17:78880124  |      | <i>TIMP2</i>                 | Intron 1   | 45263  |
| <b>cg03700402</b> | chr16:85108207  |      | <i>FAM92B/CIBAR2</i>         | Promoter   | 4183   |
| <b>cg19794395</b> | chr12:57764510  |      | <i>CYP27B1</i>               | Exon 6     | 2568   |
| <b>cg09850478</b> | chr22:43425023  | enhD | <i>MPPED1</i>                | Exon 2     | 13827  |
| <b>cg04748828</b> | chr2:74959181   |      | <i>POLE4</i>                 | Intron 1   | 538    |
| <b>cg06499778</b> | chr11:130635269 | enhD | <i>LINC02873</i>             | Intergenic | 37687  |
| <b>cg10575089</b> | chr17:58529181  |      | <i>SEPT4/SEPTIN4</i>         | Exon 1     | 15147  |
|                   |                 |      | <i>SEPT4-AS1/SEPTIN4-AS1</i> | Intron 2   | 9344   |
| <b>cg11715102</b> | chr10:123970340 |      | <i>CPXM2</i>                 | Intergenic | 78533  |
|                   |                 |      | <i>CHST15</i>                | Intergenic | 123258 |
| <b>cg06646708</b> | chr6:18122993   | enhP | <i>NHLRC1</i>                | Promoter   | 316    |
| <b>cg01505148</b> | chr19:55659251  |      | <i>U2AF2</i>                 | Exon 2     | 4216   |
| <b>cg23731781</b> | chr1:156908178  |      | <i>PEAR1</i>                 | Exon 9     | 14460  |
| <b>cg08685714</b> | chr1:156908399  |      | <i>PEAR1</i>                 | Intron 9   | 14681  |
| <b>cg24724664</b> | chr2:172080655  |      | <i>METAP1D</i>               | Exon 10    | 80702  |
| <b>cg27252325</b> | chr12:49351623  |      | <i>DNAJC22</i>               | Exon 4     | 4735   |

<sup>1</sup>CCREs were labeled according to the ENCODE Registry as follows [261]: prom (promoter-like signature), enhP (proximal enhancer-like signature), enhD (distal enhancer-like signature), K4m3 (DNase-H3K4me3), and CTCF (CTCF-only). <sup>2</sup>Genomic regions are defined as exon, intron, promoter (<4000bp from TSS) or intergenic (>4000bp from TSS) [260]. Chr: chromosome; cCREs: candidate cis-regulatory elements; TSS: transcription start site; Bp: base pairs.



*RASSF1A* is known to be involved in various tumor types, including NB, and its hypermethylation has been associated with poor outcomes [113]. We specifically investigated its methylation status across high-risk subtypes and found that *RASSF1A* was hypermethylated in HR-NB with either *MYCN* amplification or 11q deletion (Figure 35A). In addition, higher methylation levels were significantly associated with worse prognosis, particularly in the 11q-deleted subgroup of the Liquidhope cohort (Figure 35C). A trend towards worse prognosis was observed in the *MYCN*-amplified subgroup (Figure 35C).

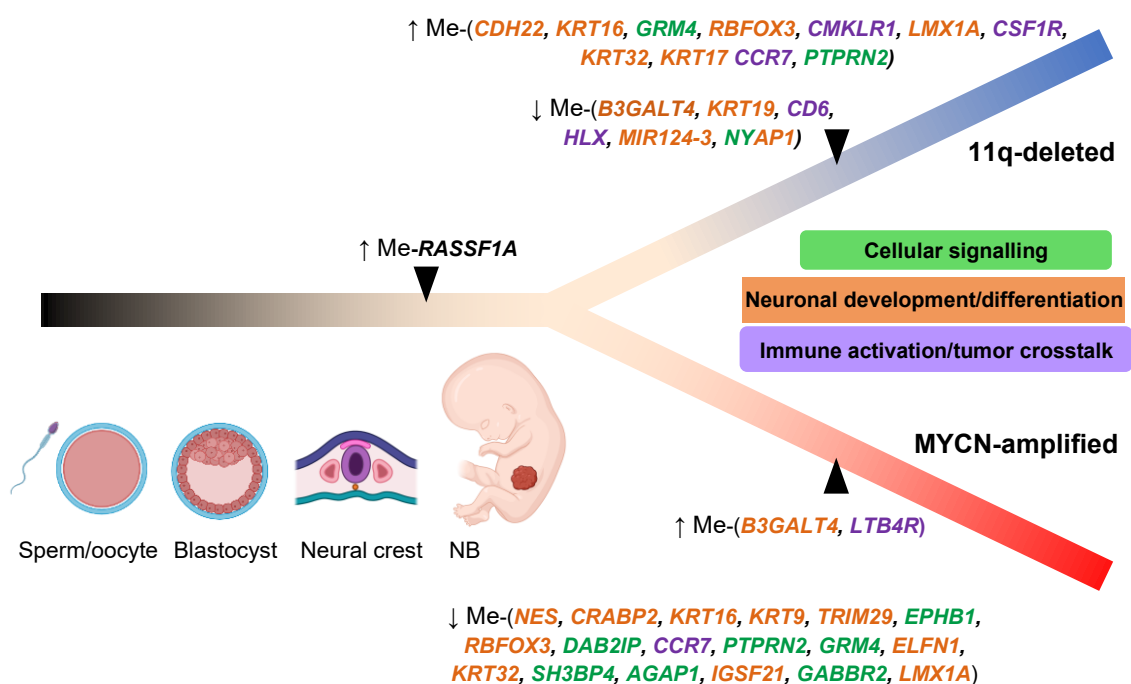


**Figure 35. Hypermethylation of *RASSF1A* is an early joint event for high-risk neuroblastoma with *MYCN*-amplification and 11q-deletion.** (A) Methylation levels of *RASSF1A* were compared between 11q-deleted (in blue) and *MYCN*-amplified subgroups (in red) within Liquidhope and TARGET cohorts and with normal cfDNA and normal brain samples (in yellow). (B) *RASSF1A* expression levels were assessed in paired methylation/expression samples from the TARGET cohort. (C) OS with samples categorized into two groups based on methylation values. The chosen threshold for grouping maximized the evidence of survival differences between the groups (lower Wald test p-value). (D) *RASSF1A* average methylation in the oocytes, sperms, blastocysts, and adrenal glands. Statistical significance for each CpG site was determined using paired T-tests ( $p > 0.05$ , ns;  $p < 0.05$ , \*;  $p < 0.01$ , \*\*;  $p < 0.001$ , \*\*\*;  $p < 0.0001$ , \*\*\*\*). Source [2].

## Results

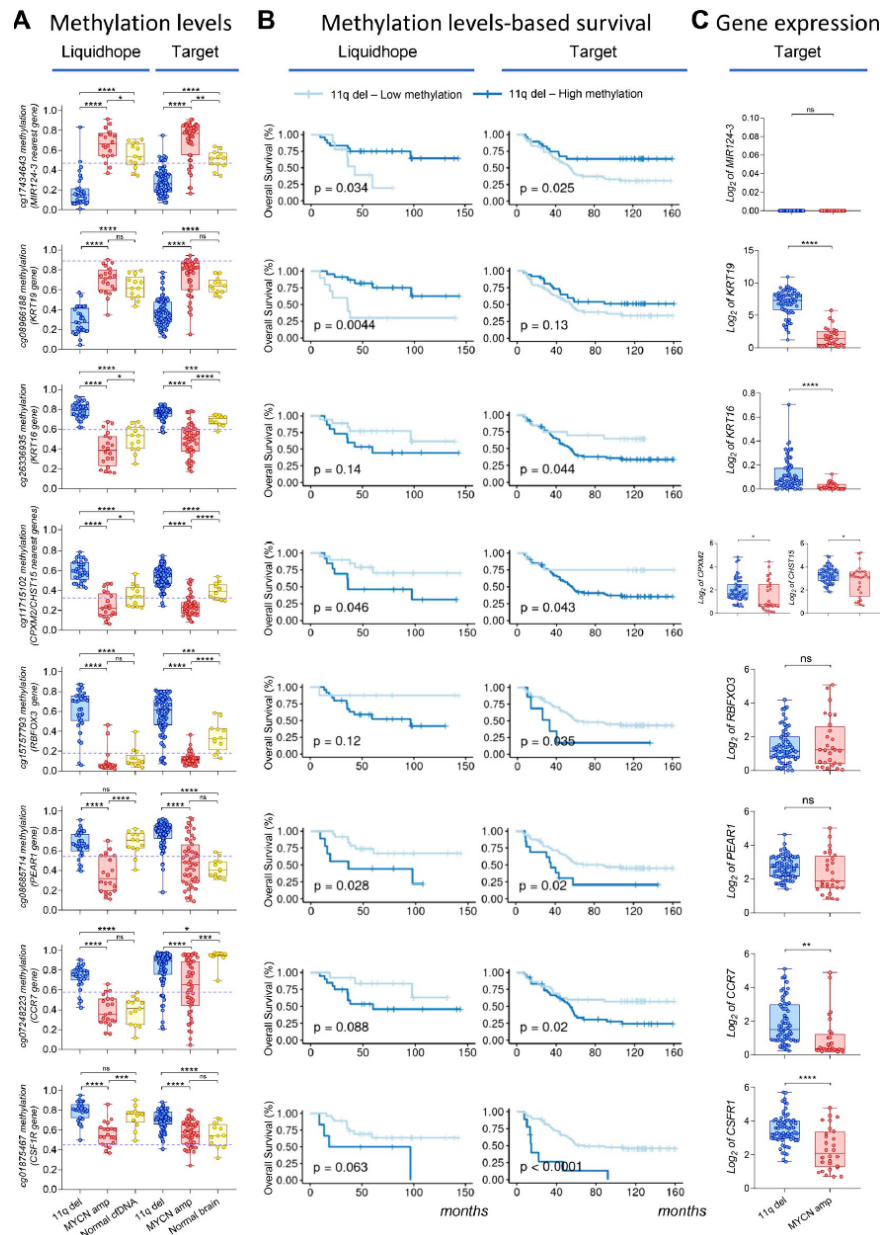
### Methylome profiling of liquid biopsies from high-risk neuroblastomas

To determine the timing of *RASSF1A* hypermethylation, we compared the levels of DNA methylation with those of normal cfDNA and early embryonic tissues (Figure 35D). This comparison involved integrating existing DNA methylation data from human oocytes, sperm, and blastocysts [257], fetal brain, and adrenal glands [258]. Our findings indicated that *RASSF1A* becomes hypermethylated, suggesting defects in DNA methyltransferase overactivation rather than DNA demethylase inactivation (Figure 35D). We next compared the DNA methylation levels of the 52 CpGs from tumor samples to construct an epigenetic phylogeny of HR-NB development (Figure 36).



**Figure 36. Epigenetic phylogeny of high-risk NB with *MYCN*-amplification and 11q-deletion.** The phylogeny represents the DNA methylation changes of HR-NB subgroups characterized by 11q deletion and *MYCN* amplification. The phylogeny depicts distinct methylation patterns influencing cellular signaling (green), neuronal development and differentiation (orange), and immune activation and tumor interaction (purple) pathways. Additionally, hypermethylation of *RASSF1* is identified as a common event to 11q deletion and *MYCN* amplification, and thus, as an early event in HR-NB progression. Adapted from [2].

We subsequently evaluated the prognostic implications of the 52 CpGs and identified eight within genes that showed significant relevance: *MIR124-3*, *KRT19*, *KRT16*, *CPXM2/CHST15*, *RBFOX3*, *PEAR1*, *CCR7*, and *CSF1R* (Figure 37).



**Figure 37. Differential methylation of CpGs with prognostic significance within the 11q subgroup of high-risk neuroblastomas.** (A) Methylation levels of the 8 prognostic CpGs were compared between HR-NB subgroups: 11q-deleted (blue boxplots) and *MYCN*-amplified (red boxplots). Normal cfDNA and brain samples (yellow boxplots) served as references. This analysis was replicated with the TARGET cohort. (B) Kaplan-Meier survival curves were plotted for patients from the Liquidhope and TARGET cohorts, stratified by methylation values, with group thresholds optimized for survival differences and assessed using the Wald test. (C) Gene expression levels associated with each CpG were assessed in paired methylation/expression samples from the TARGET cohort. Statistical significance ns: not significant, \*:  $p < 0.05$ , \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.001$ , \*\*\*\*:  $p < 0.0001$ ). Source [2].

## Results

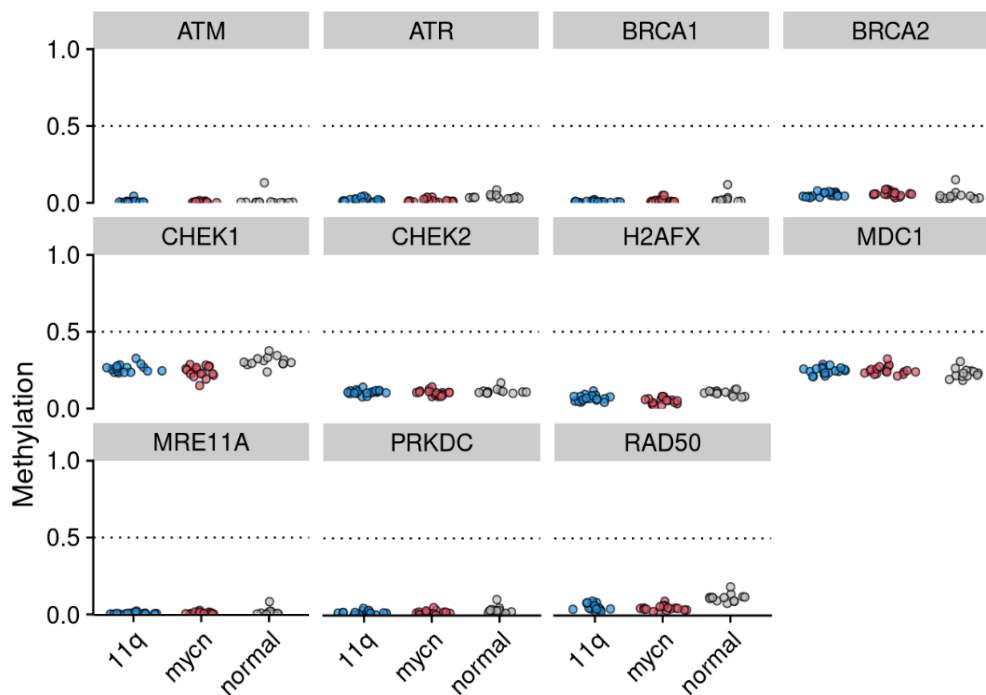
### Methylome profiling of liquid biopsies from high-risk neuroblastomas

---

Of note, these CpGs correlated with poorer survival outcomes in 11q-deleted HR-NB (Figure 37B). In addition, comparative analysis with reference cohorts underscored more pronounced variations in median methylation levels for these 8 CpGs in 11q-deleted compared to *MYCN*-amplified HR-NB, along with notable differences in gene expression (Figure 37C).

#### 4.9.2. Reduced expression of 11q double-strand break repair genes is independent of their promoter methylation

Based on our previous findings regarding impaired DNA DSB repair in 11q-deleted HR-NB, we turned our attention to investigating the methylation patterns of these genes. In earlier results, we observed significantly lower expression levels of the genes *ATM*, *H2AX*, *CHEK1*, and *MRE11A* in 11q-deleted HR-NB compared to the *MYCN*-amplified subgroup (Figure 16). We next analyzed the promoter methylation profiles of these genes in the Liquidhope (Figure 38) and TARGET cohorts (Annex 8). No significant differences were found between the average methylation promoters of 11q-deleted, *MYCN*-amplified tumors and normal reference samples (Figure 38). This result suggests that the methylation of their promoters does not account for the observed reduced expression of DNA repair genes located in 11q.



**Figure 38. Reduced expression of DNA DSB repair genes is independent of their promoter methylation.** DNA methylation analysis was conducted on the promoter region of DNA DSB-related genes, comparing HR-NB subgroups within the Liquidhope cohort. Samples from HR-NB with 11q deletion are depicted in blue, those with *MYCN* amplification in red, and reference cohorts (normal) in grey.



## 5. Discussion

### 5.1. Impact and limitations of anti-GD2 immunotherapy in 11q-deleted high-risk neuroblastoma: insights from the HR-NBL1/SIOPEN trial

HR-NB patients with 11q deletion exhibit reduced responsiveness to induction therapies [40], yet their response to maintenance immunotherapy has not been defined. In our study, we aimed to elucidate the influence of 11q deletion on the effectiveness of anti-GD2-based immunotherapy.

The validation of anti-GD2 immunotherapy as maintenance therapy for HR-NB was primarily driven by the pivotal ANBL0032 trial conducted by COG [36]. In this trial, dinutuximab anti-GD2 antibody, combined with GM-CSF and IL-2, resulted in significant improvements in 2-year EFS (66% vs. 46%,  $p = 0.01$ ) and OS rates (86% vs. 75%,  $p = 0.02$ ) in HR-NB patients. Subsequent trials by SIOPEN explored the variant ch14.18/CHO (dinutuximab beta) alongside IL-2 and isotretinoin, demonstrating comparable benefits to the ANBL0032 trial [39]. However, the addition of IL-2 increased toxicity without improving outcomes, leading SIOPEN to discontinue its use.

Adding anti-GD2-based immunotherapy into maintenance treatment has shown promise in enhancing HR-NB prognosis, but the response to the therapy varies significantly [4]. This highlights the importance of identifying specific molecular subsets within HR-NB to develop more effective immunotherapeutic or other anti-cancer strategies. We analyzed anti-GD2-based immunotherapy in the Spanish cohort from the HR-NBL1/SIOPEN trial. Our analysis in the broader study population, without categorization into subgroups, aligns with previous findings, confirming the benefits of dinutuximab beta for HR-NB patients (Figure 10, Table 10). When focusing on the specific response of 11q-deleted HR-NB patients, we also observed improved survival rates compared to patients receiving isotretinoin alone. Notably, the analysis reveals a noteworthy reduction in relapse risk among patients with 11q deletion undergoing immunotherapy, with a 68% decrease in risk compared to those receiving conventional treatment. This reduction is substantially more significant than the 47% observed in the overall population, underscoring the potential of anti-GD2 immunotherapy to delay relapse, particularly in patients with 11q deletion.

The findings of this study mark the first evidence indicating that including dinutuximab beta in the HR-NB treatment regimen enhances survival outcomes in high-risk patients with 11q deletion (Figure 10). Although we must acknowledge the limitations in

## Discussion

### Impact and limitations of anti-GD2 immunotherapy in 11q-deleted high-risk neuroblastoma: insights from the HR-NBL1/SIOPEN trial

---

comparing with historical data, the results from the COG ANBL0032 trial excluded a randomized comparison of immunotherapy, as withholding potentially beneficial treatment was considered unethical [36]. In addition, the survival analysis does not encompass all patients in the initial high-risk cohort. Specifically, it excludes those who did not respond to induction chemotherapy, could not proceed to consolidation therapy, or succumbed to treatment complications. As such, the analysis inherently focuses on a selected, more favorable subgroup of high-risk patients.

Despite the promising potential of anti-GD2 immunotherapy in delaying relapse among 11q-deleted HR-NB patients, survival outcomes remain poor, with a 3-year EFS rate of 50% and an OS rate of 71% (Figure 10, Table 10). Moreover, it is essential to consider the intensive treatments preceding immunotherapy, including high-dose chemotherapy, surgery, and radiotherapy, which lead to significant late-effect organ toxicities [40-47]. Additionally, neuropathic pain resulting from GD2 expression on peripheral nerve fibers raises significant concerns [48]. This underscores the critical importance of continued research to optimize immunotherapy efficacy for HR-NB patients. Such efforts involve exploring novel combination approaches or investigating alternative immunotherapeutic targets with enhanced specificity.



## 5.2. Exploring and targeting the tumor immune microenvironment of 11q-deleted high-risk neuroblastomas

The interplay between infiltrating immune cells in the TME and the tumor has emerged as a central determinant of immunotherapy efficacy across diverse cancer types [273]. However, their impact on NB subgroups remains largely unknown. This study aimed to fill this gap by investigating potential factors affecting the outcome of 11q-deleted HR-NB patients.

By analyzing two independent transcriptomic datasets of HR-NB, we discovered significant differences in immune cell composition between tumors with 11q-deletion and those without it. Tumors harboring 11q deletion showed higher proportions of CD8+ and resting CD4+ memory T cells, resting M0 macrophages, and polarized M1 and M2 macrophages. In contrast, HR-NB tumors without 11q deletion, which were predominantly characterized by *MYCN* amplification. In contrast, HR-NB tumors without 11q deletion, which were predominantly characterized by *MYCN* amplification, had lower proportions of these immune cell populations (Figure 11, Figure 12).

Our findings highlighting distinct immune subsets between HR-NB subtypes suggest that 11q-deleted NBs may have a greater capacity than *MYCN*-amplified tumors to recruit an immunosuppressive TIME. However, our co-culture experiments do not fully support this hypothesis, where cell lines with either 11q deletion or *MYCN* amplification similarly promoted M2 polarization. Alternatively, *MYCN*-amplified tumors typically present at an earlier age [105] and exhibit a much faster growth rate compared to HR-NB tumors with 11q deletion that show a more progressive pattern of growth, with a longer lag period before clinical symptoms become apparent [274]. This accelerated growth allows less time for interaction with the TIME. Conversely, 11q-deleted tumors, due to their slower progression, may interact with the TIME for a longer period, facilitating the development of tolerance and immunosuppression. Additionally, patients with 11q-deleted HR-NB experience more frequent relapses compared to those with rapidly progressing *MYCN*-amplified tumors, indicating a failure of immunosurveillance and the onset of immunosuppression.

Macrophages originate from monocytes in the bone marrow and differentiate into tissue-resident macrophages within the tumor TME, then referred to as TAMs [275]. TAMs can be broadly classified into two main types based on their functions: M1 macrophages and M2 macrophages [276]. M1 macrophages produce pro-inflammatory cytokines, including

## Discussion

Exploring and targeting the tumor immune microenvironment of 11q-deleted high-risk neuroblastomas

---

IL-12, TNF- $\alpha$ , CXCL-10, and IFN- $\gamma$ , along with increasing nitric oxide synthase (NOS) levels. They aid in fighting infections and are associated with tumor-suppressive functions [275, 276]. Conversely, M2 macrophages secrete anti-inflammatory cytokines, including IL-4, IL-10, and TGF- $\beta$  [275, 276]. While M2 macrophages play a crucial role in maintaining tissue homeostasis by promoting an anti-inflammatory environment, their presence in the TME has been linked to tumor progression and immunosuppression [276]. Thus, the higher proportion of M2 TAMs, IL-10, and TGF- $\beta$  in the 11q-deleted subgroup (Figure 11 - Figure 13) suggests a conducive environment for tumor growth and immune evasion.

Our analysis also revealed elevated resting CD4<sup>+</sup> memory T cells in the TME of 11q-deleted HR-NB (Figure 11). When exposed to TGF- $\beta$ , resting CD4<sup>+</sup> memory T cells can undergo reprogramming into Tregs [277, 278]. Tregs, vital for self-tolerance, can hinder protective immunosurveillance and antitumor responses by inhibiting APCs via CTLA-4, consuming IL-2, and producing immunosuppressive cytokines [279]. However, while Tregs were notably more prevalent in 11q-deleted HR-NB in the discovery cohort, this pattern did not persist in the validation cohort (Figure 11, Figure 12).

One plausible explanation for this variation could be the inherent differences in the clinical characteristics and tumor evolution dynamics between the two cohorts. Emerging evidence suggests that age-related variations influence Treg abundance, with studies demonstrating that older mice exhibit reduced differentiation of CD4<sup>+</sup> T cells into Tregs compared to their younger counterparts [280]. Remarkably, the age at diagnosis significantly differed between cohorts. There was a higher proportion of high-risk patients under 18 months in the discovery cohort compared to a much smaller percentage in the validation cohort (28% of patients vs. 1,9%) [254, 255].

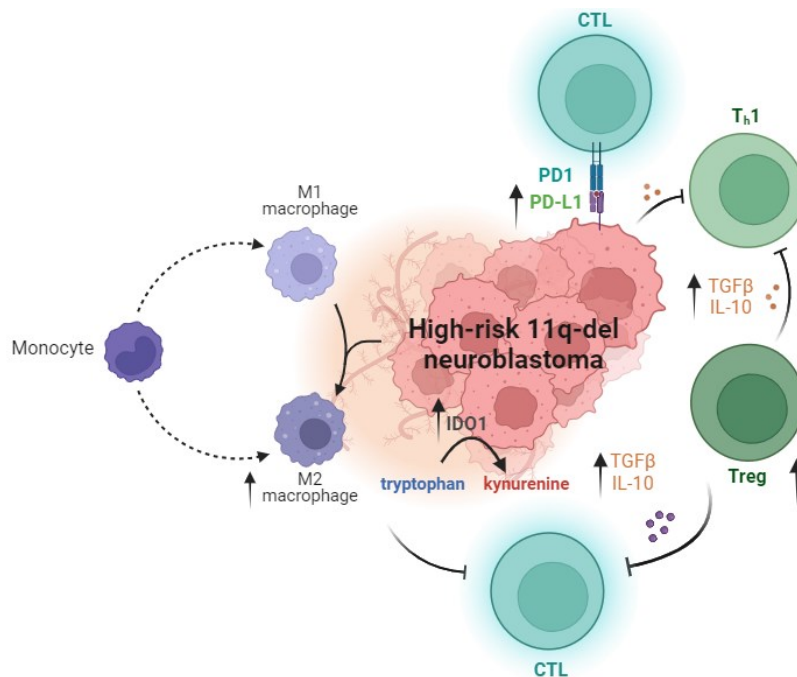
Discordance was also observed regarding NK cells, with the discovery dataset showing significantly higher proportions of activated NK cells in the 11q-deleted subgroup, whereas this was not replicated in the validation cohort. Reduced activation of NK in the *MYCN*-amplified subgroup aligns with recent discoveries suggesting that *MYCN* downregulates key NK cell-activating ligands, including PVR, NECTIN2, MICA, MICB, and ULBPs. These receptors are crucial for triggering DNAM-1 and NKG2D activating receptors on NK cells [166-168]. Given that the efficacy of anti-GD2-mediated tumor killing hinges primarily on NK cells, the effectors of ADCC [281], this contradiction prompts further investigation into whether the low levels of activated NK cells are a

hallmark of 11q-deleted NB tumors. Such findings could have significant implications for treatment strategies focused on immunomodulation.

Our results corroborate and extend the existing literature, reinforcing that MYCN fosters an immunosuppressive TME primarily attributed to MHC-I downregulation and often renders HR-NB a "cold" tumor [21, 282]. In addition, our study reveals a notable deviation from this paradigm, demonstrating that HR-NB patients with 11q deletion possess immune effector cells within the TME capable of eliciting an adaptive immune response. This challenges the conventional view of NB as a predominantly "cold" tumor [282] and underscores the potential for personalized immunotherapeutic strategies to improve outcomes in these patients. However, the interaction among M2 macrophages, Tregs, and the upregulation of immunosuppressive factors observed in 11q-deleted HR-NB can hinder the activation of CD8<sup>+</sup> T cells and impede efficient priming, ultimately compromising the functions of NK cells [169, 283]. This complex interplay implies the existence of a challenging immunosuppressive TME within 11q-deleted HR-NB that must be circumvented to achieve an effective antitumoral response (Figure 39).

## Discussion

### Exploring and targeting the tumor immune microenvironment of 11q-deleted high-risk neuroblastomas



**Figure 39. Immunosuppressive microenvironment in high-risk neuroblastomas with 11q deletion.** The figure illustrates the intricate network of immunosuppressive factors contributing to tumor evasion in 11q-deleted HR-NB. M2 macrophages and Tregs orchestrate immunosuppression by inhibiting anti-tumor immune responses. TGF- $\beta$  promotes immune evasion by suppressing effector cell function and inducing Treg differentiation, while IL-10 acts as a potent immunosuppressive cytokine, dampening anti-tumor immunity. PD-L1 on tumor cells engages with T cell PD-1 receptors, producing T cell exhaustion. IDO-1-mediated tryptophan depletion and kynurenine production further contribute to immune tolerance. Collectively, these mechanisms orchestrate an immunosuppressive milieu that facilitates tumor progression in 11q-deleted HR-NB. Image created with BioRender.

While this study contributes to our understanding of the immune evasion mechanisms in 11q-deleted HR-NB, it also unveils a promising avenue for therapeutic intervention. One strategy to combat tumor evasion involves using ICIs to block inhibitory factors like PD-1 and PD-L1, which impede the host's immune response [169]. This approach has demonstrated therapeutic efficacy in adult cancers by restoring T-cell reactivity [170]. However, pediatric cancers, including NB, often present challenges due to a low TMB and limited TILs, rendering them resistant to ICIs [54].

Elevated PD-L1 expression is associated with an improved response to PD-L1/PD-1 pathway blockade [170]. However, the status of PD-L1 expression in NB remains inconclusive [171-174]. Our analysis indicates increased PD-L1 expression in 11q-deleted HR-NB compared to non-11q-deleted cases (Figure 13). Thus, combining

immunotherapy to alleviate PD-1/PD-L1 axis blockade could enhance the anti-GD2 response, particularly benefiting 11q-deleted NB patients.

In mouse NB models, ICI monotherapy showed limited efficacy but significantly boosted anti-tumor immunity when combined with anti-GD2 therapy, vaccination, Treg depletion, or myeloid inhibition [195, 204-206]. This underscores the importance of combination therapies to maximize treatment effectiveness. The ongoing phase I trial (NCT02914405) investigating the combination of dinutuximab beta with nivolumab (anti-PD-1) will provide crucial insights into the potential efficacy of this approach.

Alternative clinical strategies, such as IDO-1 inhibition and CD25-targeted agents for Tregs depletion, are being explored for their potential efficacy in overcoming immunosuppression. While promising in other cancers, whether they have a therapeutic potential on 11q-deleted tumors has yet to be determined [284-286].

### **5.3. Genetic instability of 11q-deleted high-risk neuroblastomas**

Numerous investigations, as outlined in [269], have centered on the genetic imbalances in 11q, seeking to unravel this NB subtype's clinical implications and underlying causes. While the clinical presentation of this subgroup is now well-defined (cases are typically diagnosed at later stages among the high-risk category, with heightened relapse rates and dismal prognoses [99, 104, 106]) the causative genes of this tumor subtype remain unknown. Since the clinical significance of 11q deletion was recognized, numerous studies have focused on pinpointing the specific SRO containing its associated pathogenic genes. The narrower SRO spanned from 11q23.1 to 11qter [287-289]. Our recent SNP array analysis efforts narrowed it to 11q22.3-11q23.3 (chr11:104,934,658-119,216,493, hg19) [99].

Despite the ambiguity surrounding the genetic origins of 11q-deleted NB, it is evident that genomic instability is a defining feature of this subset. Our SNP array analysis confirmed a significant difference in the number of SCAs between *MYCN*-amplified and 11q-deleted NB (Figure 14), indicating a phenotype characterized by significant chromosomal instability predominantly associated with the 11q region (Figure 15).

We analyzed the expression levels of DNA repair genes within the 11q SRO to identify the responsible gene or genes. We observed a significant decrease in *ATM*, *H2AX*, *CHEK1*, and *MRE11A* in 11q-deleted HR-NB compared to the rest of HR-NB (Figure 16). Notably, previous studies in the laboratory detected rare allele variants in *ATM* in only half of the 11q-deleted cases [99], suggesting that there are other causes in its etiology other than *ATM* oncogenic variants. No second-hit mutations have been found in *H2AX*, *MRE11A*, or *CHEK1* [8, 99]. Therefore, the reduced expression of DNA repair genes, frequently combined with the inactivation of the remaining *ATM* allele, and the high number of SCAs in 11q-deleted HR-NB supports a potential role of combined haploinsufficiency of DNA repair genes in the pathogenesis of 11q deletions.

Interestingly, while 11q deletions are prevalent among children with mental retardation and craniofacial abnormalities, they rarely lead to NB [290]. Moreover, no mouse model of 11q deletion has been reported to induce NB. This implies that 11q deletions alone are insufficient to cause NB and might necessitate additional genetic events.

#### 5.4. Regulatory landscape of 11q-deleted miRNAs in neuroblastoma

In recent years, miRNAs have emerged as critical regulators of NB growth and progression [270]. The network of miRNAs located within the 11q region remains largely unexplored. We used computational methods to analyze this network's complexity to identify the interactions between miRNAs within the 11q deleted region and their target genes. Our analysis unveiled the significant involvement of 11q miRNAs in regulating immune responses, with *miR-4492* and *miR-34b/c* emerging as critical players in orchestrating the crosstalk among this pathway (Figure 17).

Although the function of *miR-4492* remains unclear, our analysis identified *TRAF1* as one of its targets (Figure 17), with elevated expression in 11q-deleted HR-NB (Figure 18). *TRAF1*, belonging to the tumor necrosis factor receptor (TNFR) family, shows low expression in resting monocytes and lymphocytes but increases upon activation via the NF- $\kappa$ B pathway triggered by toll-like receptors (TLRs) and TNF signaling [291]. Its overexpression is implicated in lymphoid malignancies, sustaining NF- $\kappa$ B signaling and promoting tumor progression [292-294]. Dysregulation of NF- $\kappa$ B signaling in NB has been associated with tumor development and aggressive phenotypes, although its constitutive activation mechanism is unknown [295]. Consequently, targeting *TRAF1* might be a potential strategy to disrupt NF- $\kappa$ B activation in HR-NB, yet challenges arise due to its critical roles in innate immunity, hematopoiesis, and immune surveillance [291].

*miR-34b/c* was also a pivotal mediator coordinating the 11q miRNAs regulatory network. The *miR-34* family comprises three transcripts encoded by two distinct loci. While *miR-34b* and *miR-34c* share a primary transcript (*miR-34b/c*), *miR-34a* has its own transcript at the 1p36 location, also frequently deleted in NB [106]. These three miRNAs are transcriptionally regulated by p53 in response to DNA damage, and their dysregulation has been widely implicated in cancer, including NB [270, 296]. As a result, *miR-34*-based therapies are currently under clinical evaluation and have entered phase I clinical trials [297].

Of note, our study identified *IL-7R* as a target gene of *miR-34b/c*. Interestingly, previous research has noted upregulation of IL-7 in 11q-deleted NB [298]. Given the critical role of the *IL-7/IL-7R* axis in regulating the survival and development of memory CD4 cells [299], dysregulation of this pathway may contribute to immune dysfunction, implying a potential therapeutic target within this HR-NB subgroup.

This study sheds light on the regulatory landscape of 11q-deleted miRNAs in NB, uncovering novel interactions and therapeutic targets within this subgroup. *miR-4492*

## Discussion

### Regulatory landscape of 11q-deleted miRNAs in neuroblastoma

---

and *miR-34b/c* were highlighted as critical regulators of immune responses, with *TRAF1* and *IL-7R* identified as potential targets to disrupt aberrant pathways implicated in tumor development and immune dysfunction in 11q-deleted HR-NB.



### **5.5. Collaboration between M2 macrophages and neuroblastoma cells promotes a tumor-favorable environment**

Thus far, we have underscored a crucial difference between *MYCN*-amplified and 11q-deleted HR-NB regarding immune cell infiltration within the TME. While *MYCN*-amplified tumors typically exhibit immune exclusion, patients with 11q deletion often display immune effector cells in the TME, hinting at the potential for adaptive immune responses. Nevertheless, addressing the observed immunosuppressive TME remains vital for achieving effective anti-tumoral responses.

Among the numerous factors influencing immunotherapeutic response in HR-NB, our investigation has particularly emphasized the role of macrophages. Expanding on these findings, we proposed a hypothesis implicating M2 macrophages and 11q-deleted NB in fostering a pro-tumorigenic TME.

Our investigation aimed to evaluate how NB cells with different genetic backgrounds influence macrophages' behavior and *vice versa*, how these macrophages, particularly M2 macrophages, affect NB cell growth and resistance to treatment. We utilized a co-culture system employing THP-1 monocytes and PBMC from healthy donors and a diverse array of NB cell lines and PDOs. This approach allowed us to explore the influence of NB cells across a spectrum of biological contexts, ranging from established cell lines with known genetic alterations to PDOs reflecting the intricate heterogeneity of original tumors.

Our findings revealed several key observations: NB cells induced the differentiation of monocytes into M0 macrophages, as evidenced by a significant increase in the CD68 marker compared to reference cells, accompanied by enhanced adherence of monocytic cells to the cell surface (Figure 19). Furthermore, NB cells directed M0 macrophages towards the M2 phenotype, as indicated by elevated expression of CD163 and CD206, without significant changes in HLA-DR expression, which is typically associated with M1 macrophages (Figure 19). The effect on Macrophages was consistent across a range of established and primary NB cell lines, reinforcing the notion that NB cells can modulate macrophages phenotype toward an immunosuppressive state (Figure 20).

The innate capacity of macrophages to adapt in response to microenvironmental changes, such as variations in nutrient levels, metabolites, or oxygen availability within tumors, results in a notable diversity of TAMs across types of cancer and within individual tumors [300]. While our understanding of macrophage diversity is still evolving, using

## Discussion

Collaboration between M2 macrophages and neuroblastoma cells promotes a tumor-favorable environment

---

M1-M2-associated markers provides a practical approach for characterizing TAMs in our current research [301].

Building on our earlier findings indicating a heightened presence of M2 macrophages in 11q-deleted HR-NB (Figure 11, Figure 12), we expected that these tumors would more effectively polarize macrophages than other NB subgroups. However, contrary to our earlier hypothesis, we consistently observed M2 polarization across all tested cell lines, irrespective of their genetic backgrounds, which implies a common mechanism for macrophages polarization (Figure 19, Figure 20). While the genetic background can influence macrophages' phenotype, other factors, such as epigenetic modifications, can be behind these observations. Following our previous discussion in 5.2, *MYCN*-amplified tumors tend to be larger and have distinct imaging features compared to non-amplified tumors [302]. Hence, one hypothesis is that the lower tumor size of 11q-deleted HR-NB reflects a lower growth rate that enables them more time and higher capacity to recruit and interact with immune cells in the microenvironment, resulting in a hot type of tumor, in contrast with those with *MYCN* amplification. Even though both tumor subgroups can induce such effects, the implications may not be clinically relevant if the TME remains immune-excluded, as observed in *MYCN*-amplified HR-NB (Figure 11, Figure 12). One additional explanation is that this exclusion might be linked to *MYCN*'s immunosuppressive functions [21, 159, 282].

Next, we compared the impact of M1 macrophages and M2 macrophages on NB cell proliferation. The significant increase in NB cell proliferation in the presence of M2 macrophages underscored the pro-tumorigenic properties of these polarized macrophages (Figure 22). This enhancement in NB cell growth was also consistent across NB cell lines and independent of their genetic characteristics. Conversely, M1 macrophages generally did not promote proliferation and even reduced the growth rate in certain NB cell lines (KELLY, NBL-S, and NB69). The exception of the CHP-134 cell line, where both M1 and M2 macrophages enhanced proliferation but with a more substantial effect from M2 macrophages, further underscores the complex and context-dependent interactions between NB cells and TAMs (Figure 22).

By demonstrating that NB cells direct monocytes toward an M2-like phenotype, our study highlights their potential to shape the immune landscape in favor of tumor progression. This underscores the importance of developing combination therapies targeting tumor cells and the immune microenvironment to achieve optimal treatment outcomes in HR-NB.

Current research is investigating two primary strategies to modify TAMs behavior: depletion and reprogramming [303]. Depletion involves using CSF1R inhibitors like BLZ945 and PLX7486 to reduce TAMs, though most of the research has been in adult cancers [303]. Reprogramming, on the other hand, aims to shift TAMs from a tumor-promoting M2 (or M2-like) phenotype to a tumor-suppressing M1 phenotype. This approach includes using PI3K $\gamma$  inhibitors, which target the PI3K $\gamma$  pathway, an innate immune checkpoint, to suppress inflammation and promote anti-tumor immunity [304]. Following this line of reasoning to reprogram TAMs, HDAC inhibitors, like TMP195, modulate gene expression in macrophages by altering the expression of chemokines such as CCL1 and CCL2, driving monocyte differentiation into anti-tumoral macrophages [305]. In addition to these strategies, targeting functional molecules like arginase 1, which suppresses the production of pro-inflammatory cytokines, and Fc receptors, involved in macrophage-mediated ADCC, represents another promising strategy [306]. These strategies show potential in various solid tumors, with ongoing clinical trials [303]. However, their application in pediatric cancers, including NB, remains underdeveloped and warrants further research.

### **5.6. Macrophage-induced mesenchymal phenotype: a link to 11q deletion**

The mechanisms underlying NB therapy resistance remain largely elusive. Recent studies, however, have demonstrated that NB consists of two phenotypically distinct but genetically identical cell types: lineage-committed ADRN cells and undifferentiated MES cells [138, 139]. Notably, MES cells have demonstrated increased resistance to cisplatin, doxorubicin, and etoposide than ADRN cells [139], as well as to the ALK inhibitor lorlatinib [155] and GD2-immunotherapy [211]. ADRN and MES cell types can spontaneously interconvert [138, 139], but the mechanism for this process remains mainly unknown. Unraveling the mechanisms of ADRN-to-MES transition (AMT) is a pivotal step for overcoming therapy resistance in NB. Our results help bridge this gap by highlighting the role of M2 macrophages in driving mesenchymal traits in NB cells, thus offering a potential explanation for the increased resistance of MES-like NB cells to therapy.

We conducted a comparative expression analysis between NB cell lines before and after co-incubation with M2 macrophages. Compared to their basal expression levels, M2 macrophages significantly elevated the expression of the stemness markers OCT4, SOX2, and NANOG (Figure 23A) [307], essential TFs for maintaining the characteristics of pluripotent embryonic stem cells [308]. Additionally, there was an upregulation of the mesenchymal transition effector TWIST1 (Figure 23B) and MES lineage markers VIM and CDH2 (Figure 23C) [309], associated with heightened tumor cell migration and invasion capabilities [310].

We performed a gene set correlation enrichment analysis to evaluate the ADRN and MES lineage identities of our NB cell line panel, using the gene sets from Groningen and colleagues [139]. Upon categorizing the cell lines by MYCN expression, we found that low MYCN expression was associated with a mesenchymal profile (Figure 24). This prompted further analysis of a larger cohort of primary NB tumors (GSE73517), to identify if the mesenchymal phenotype was indeed a characteristic of 11q-deleted NBs. Our results revealed that 11q-deleted tumors were more correlated with the MES lineage, while *MYCN*-amplified tumors aligned showed a negative correlation (Figure 25).

These results are consistent with our earlier observation that the TIME of 11q-deleted NB is enriched in M2 macrophages (Figure 11, Figure 12), likely driving the observed lineage plasticity and resistance. They also align with recent findings showing that ADRN tumors are less immunogenic, while MES tumors enhance T cell infiltration by secretion

of inflammatory cytokines. This raises important questions: could targeting M2 macrophages reverse mesenchymal transition in NB? If successful, addressing macrophage-driven MES traits might not only help predict drug resistance, but it could also transform our treatment strategies, potentially unveiling new therapeutic approaches that target both the TME and the tumor cells.

Importantly, Kendsersky *et al.* have recently shown that most immunotherapeutic targets under development for NB are predominantly expressed in ADRN cells, with limited expression in MES-like cells [311]. This indicates that therapies aimed at ADRN-specific antigens may be ineffective against MES-dominant tumors, which are often more resistant to treatment, highlighting the need for tailored treatments that address lineage heterogeneity of NB. Pan-NB targets such as CD276 (B7-H3) and L1CAM, which are expressed across both ADRN and MES subtypes [311], offer promising alternatives for broader therapeutic applications. Additionally, receptor tyrosine kinases (RTKs) such as AXL, EGFR, PDGFRA, and EPHA2 were specifically enriched in MES cells, providing potential therapeutic targets for these resistant states [311].

In our study, we employed a widely accepted gene signature approach, utilizing 485-gene MES and 369-gene ADRN signatures to characterize NB cell states. However, Kendsersky *et al.*, in the same study, pointed out that only 75 of the top 1,000 differentially expressed genes matched the published gene signatures, suggesting that there are additional, yet uncharacterized, features that define these phenotypes [311]. This emphasizes the need for ongoing refinement of the genetic and molecular markers used to delineate ADRN and MES states, as relying on limited gene sets may oversimplify the complexity of NB heterogeneity.

## Discussion

### Macrophage polarization: a double-edged sword in CAR-T cell therapy for neuroblastoma

---

#### **5.7. Macrophage polarization: a double-edged sword in CAR-T cell therapy for neuroblastoma**

The promise of CAR-T cell therapy has been demonstrated predominantly in hematologic malignancies, where engineered T cells can effectively target and eliminate cancer cells [226, 227, 272]. Solid tumors present a more complex and hostile TME, characterized by the presence of a dense extracellular matrix and immunosuppressive cells [272]. NB poses additional hurdles due to its low mutation rate, which results in a limited number of neoantigens available for targeting by CAR-T cells [8]. In addition, most oncogenic drivers are intracellular proteins, making them hard to target with immunotherapies, which typically rely on mutated peptides or neoantigens presented by HLA molecules [272]. Yamarkovich's development of PC-CARs that target intracellular unmutated oncogenic drivers offered a novel solution to this challenge [235].

Based on our previous findings, we hypothesize that TAMs could significantly impact the effectiveness of CAR-T cells. We explored the influence of M1 and M2 macrophages on the cytotoxic efficacy of PHOX2B-specific PC-CAR T cells (10LH PC-CAR), which binds with high affinity to PHOX2B peptide-HLA-A\*24:02 or HLA-A\*23:01 complex [235]. The term "10LH" refers to the specific scFv used in the study, engineered to bind to the peptide-MHC complex QYNPIRTTF [235].

Our data demonstrate that M1 macrophages consistently enhanced the efficacy of PHOX2B PC-CAR T cells across all NB cell lines tested. This was evidenced by the increased caspase-3/7 activity and higher levels of IL-2 and IFN- $\gamma$  cytokine secretion, critical cytokines for T-cell activation and function. These findings indicate that M1 macrophages create a more favorable immune environment for CAR-T cell-mediated killing.

In the SK-N-BE(2) HLA-A\*23:01 cell line, IL-2 and IFN- $\gamma$  levels did not significantly increase by the presence of M1 macrophages, remaining comparable to control conditions (Figure 27B). The integrated caspase-3/7 activity displayed a temporal delay in cytotoxicity under these conditions (Figure 27A) suggesting that the short-term cytokine measurements at 24 hours may not fully capture the long-term effects. Additional measurements at 48 hours could provide further insights into the sustained impact of M1 macrophages. Conversely, M2 macrophages markedly suppressed apoptosis and reduced CAR-T cell activity, highlighting their role in immune evasion and tumor promotion (Figure 26 - Figure 30).

Interestingly, SK-N-BE(2) transduced to express HLA-A\*24:02, exhibited a transient initial response to M2 macrophages co-culture, with slight caspase-3/7 activity in the early phase of CAR-T cell addition (Figure 27A). However, this quickly diminished, suggesting that while M2 macrophages may temporarily prime NB cells for immune recognition, their overall long-term impact is predominantly suppressive. This phenomenon could be linked to the rapid exhaustion of CAR-T cells in solid tumors [272].

Our results support the idea that regulating MHC I expression on NB cells by macrophages could explain the impact of CAR-T cell efficacy. Co-culture with M1 macrophages consistently maintained or slightly increased MHC I expression in our NB cell lines, potentially enhancing antigen presentation and promoting superior immune surveillance by CAR-T cells (Figure 31 - Figure 33). Conversely, M2 macrophages significantly suppressed MHC I expression across all NB cell lines, with the most pronounced effect seen in SK-N-AS, where MHC I positive cells dropped to 2.38% (Figure 31). This reduction in MHC I expression corresponds to the diminished apoptosis observed in the cytotoxicity assays, suggesting that M2 macrophages foster an immune-evasive TME by downregulating antigen presentation.

Notably, in SK-N-AS and NB-EBc1, while M1 macrophages had minimal impact on MHC I-positive cells, they still played a crucial role in enhancing CAR-T cell activity. This could be due to their ability to create a more conducive environment for T-cell action and facilitate immune responses through mechanisms beyond antigen presentation.

This study brings forward several pivotal questions. First, by what mechanisms do M2 macrophages suppress MHC I expression, and could targeting this pathway restore CAR-T cell function? Furthermore, it is critical to evaluate the durability of CAR-T cell responses within the highly immunosuppressive TME, as sustained CAR-T activity may trigger compensatory mechanisms that hinder efficacy. For instance, prolonged IFN- $\gamma$  release from CAR-T cells could drive IDO1 expression in tumor and stromal cells, compromising CAR-T persistence and promoting Treg expansion [277, 278]. Additionally, tumor-expressed inhibitory ligands like PD-L1, along with soluble factors such as TGF- $\beta$  and IL-10 within the TME, actively inhibit T-cell responses [169, 283]. Testing these dynamics *in vivo* within more complex models would yield essential insights into therapeutic potential and resistance mechanisms.

## Discussion

Epigenetic alterations impacting the tumor microenvironment and immune response in 11q-deleted high-risk neuroblastomas

---

### 5.8. Epigenetic alterations impacting the tumor microenvironment and immune response in 11q-deleted high-risk neuroblastomas

NB displays significant heterogeneity at both the cellular and prognostic levels, which cannot be solely attributed to its low mutation rate [8]. This indicates that studying epigenetic changes could provide valuable insights into NB's complexity, leading to a better understanding of its origins and developing new therapeutic strategies. To investigate this further, we conducted methylome profiling on cfDNA liquid biopsy samples from HR-NB patients.

Our study yielded several insights into the epigenetic landscape of HR-NB. Firstly, hierarchical clustering based on 11q or *MYCN* status indicated distinct methylation profiles associated with each genetic alteration (Figure 34B). By logistic regression analysis, we identified a 52 CpG signature capable of accurately clustering samples based on 11q or *MYCN* status (Figure 34D). The model showed high accuracy when applied to independent external cohorts, indicating its potential for reliable clinical application.

The prognostic evaluation of the 52 CpG signature highlighted 8 CpGs whose methylation status correlated with worse survival in 11q-deleted HR-NB (Figure 37). The associated genes with these CpGs were: *MIR124-3*, *KRT19*, *KRT16*, *CPXM2/CHST15*, *RBFOX3*, *PEAR1*, *CCR7*, and *CSF1R*. Among them, *CSF1R* and *CCR7* are particularly noteworthy due to their established pharmacological profiles, offering potential avenues for targeted intervention for 11q-deleted HR-NB patients.

The CC chemokine receptor *CCR7* is a pivotal coordinator in directing cancer and immune cells toward lymphatic organs [312, 313]. This guidance is facilitated by its two ligands, *CCL19* and *CCL21*, constitutively expressed within lymphoid tissues [312, 313]. The two CpG sites within the *CCR7* promoter, cg07248223, and cg16047279, were hypermethylated in HR-NB with 11q deletion. Significantly, hypermethylation was associated with increased *CCR7* expression and lower OS in these patients (Figure 37).

*CCR7* overexpression has also been reported in hematologic cancers, promoting tumor lymphatic invasion and metastasis [312, 313]. In addition, *CCL21/CCR7* signaling has been shown to promote microglia and macrophage recruitment in glioblastoma [314]. Thus, targeting the *CCR7* axis may represent a promising therapeutic approach to modulate tumor-immune interactions and inhibit metastatic spread in HR-NB. Preclinical evidence supports its ability to impede cancer spread and overcome treatment



resistance [314-316]. Notably, the anti-hCCR7 antibody CAP-100 is currently being evaluated in a phase I clinical trial for patients with chronic lymphocytic leukemia (NCT04704323).

Likewise, the increased methylation of cg01875467 within the *CSF1R* promoter in 11q-deleted HR-NB was accompanied by a notably elevated expression and reduced OS in this subgroup (Figure 37). *CSF1R*, also known as macrophage colony-stimulating factor receptor (*M-CSFR*), plays a crucial role in regulating the differentiation of myeloid progenitors and shaping macrophages polarization towards M1 and M2 phenotypes [317]. These findings are consistent with prior research indicating that elevated *CSF1R* gene expression in NB is associated with an unfavorable prognosis [318]. *CSF1R* also regulates the differentiation of neural progenitor cells, with its expression decreasing from neonatal to adult brain [319]. The exact role of *CSF1R* in NB is not fully elucidated, but a higher expression in 11q-deleted NB suggests an increased undifferentiation state and plasticity.

Hypermethylation and increased expression of *CSF1R* in 11q-deleted tumors align with the higher infiltration of macrophages we observed in their TIME (Figure 2, Figure 12). Interestingly, the *CSF1R* signaling inhibitor pexidartinib has demonstrated remarkable efficacy in blocking the polarization of M2 macrophages and has shown encouraging results in phase I trials for other pediatric solid tumors (NCT02390752). Given the elevated expression of *CSF1R* specifically observed in 11q-deleted HR-NB, targeted inhibition of *CSF1R* holds significant potential as a tailored therapeutic approach for this subgroup of patients.

Notably, recent research by Valind and colleagues has revealed that chemotherapy can enhance M2 macrophages infiltration in *MYCN*-amplified NB, with no such trend observed in non-*MYCN* amplified NB [320]. They observed that blocking macrophage recruitment with anti-*CSF1R* antibody prevented the regrowth of *MYCN*-amplified NB post-chemotherapy [320]. The process by which macrophages invaded the TME after treatment in *MYCN*-amplified tumors involved the upregulation of colony-stimulating factor (*CSF1*) and the chemokine ligand 2 (*CCL2*) [320]. Our findings support the idea that *CSF1R* inhibition may primarily benefit 11q-deleted patients. One explanation for Valind's results is that the non-*MYCN*-amplified group likely included a mix of risk levels, with a higher proportion of younger patients (under 2 years old), who generally have better prognoses [9, 17].

## Discussion

### Epigenetic alterations impacting the tumor microenvironment and immune response in 11q-deleted high-risk neuroblastomas

---

We then examined the DNA methylation dynamics during early embryogenesis to gain insights into HR-NB development and progression. Our analysis uncovered that *RASSF1A* hypermethylation was a joint event in *MYCN*-amplified and 11q-deleted high-risk tumors (Figure 35A, B). Interestingly, the study of methylation patterns during developmental stages revealed that *RASSF1A* becomes hypermethylated following neural crest formation. This finding suggests potential deficiencies in DNA methyltransferase pathways rather than DNA demethylases (Figure 35D).

Furthermore, our results demonstrated a significant correlation between higher methylation levels of *RASSF1A* and poorer prognosis, particularly within the 11q-deleted subgroup (Figure 35C). This finding is consistent with existing literature associating *RASSF1A* hypermethylation with a worse prognosis in NB [113]. Our study extends prior research by suggesting that *RASSF1A* hypermethylation may be an early event in NB development, potentially preceding other epigenetic and genetic alterations. This discovery has significant implications for early detection and intervention strategies. As a precursor to further molecular changes, *RASSF1A* hypermethylation could help identify high-risk patients at earlier stages, enabling timely and more effective treatments, ultimately improving patient outcomes.

Importantly, *RASSF1A* acts as a negative regulator of the NF- $\kappa$ B pathway by binding to TBK1 [321]. This finding provides a mechanistic explanation for the elusive constitutive NF- $\kappa$ B activation observed in NB [295], offering potential alternatives for targeted therapeutic interventions. However, targeting NF- $\kappa$ B has been challenging due to its wide-ranging biological functions [322, 323], leading to significant side effects, except in the case of bortezomib, used in multiple myeloma [324]. As such, current efforts concentrate on testing the feasibility of targeting upstream and downstream NF- $\kappa$ B effectors [291, 322].

Notably, NF- $\kappa$ B is a central regulator of TAMs, often driving their polarization toward an M2-like phenotype [322, 325]. *RASSF1A* hypermethylation has been shown to result in constitutive NF- $\kappa$ B activation in tumor cells Zhang X. et al., 2016, which could promote the recruitment and polarization of TAMs toward the M2 phenotype, as observed in NB cell lines (Figure 20). This suggests a potential link between *RASSF1A* hypermethylation in NB cells and the immunosuppressive environment fostered by M2-like TAMs.

Finally, building upon our prior research that highlighted deficiencies in the DNA DSB repair machinery in 11q-deleted HR-NB, we investigated the methylation patterns of these genes. Contrary to our expectations, our analysis of the promoter methylation of

these genes revealed no significant differences between 11q-deleted and *MYCN*-amplified NBs (Figure 38). This suggests that the observed expression disparities in *ATM*, *H2AX*, *CHEK1*, and *MRE11A*, genes located within the 11q region, may be attributed to factors such as gene dosage alterations or other epigenetic modifications such as histone acetylation and methylation rather than DNA methylation. Notably, no second-hit mutations have been identified in these genes except for *ATM* [99]. These findings highlight the complexity of the molecular mechanisms underlying 11q-deletion in HR-NB and emphasize further exploration to elucidate the precise mechanisms driving DSB repair deficiencies in 11q-deleted tumors.



## 6. Conclusions

1. Anti-GD2 immunotherapy significantly enhances 3-year EFS and OS in 11q-deleted HR-NB patients, though survival rates remain suboptimal.
2. 11q-deleted HR-NB display a more inflamed TIME with increased CD8<sup>+</sup> T cells, resting CD4<sup>+</sup> memory T cells, and TAMs.
3. Elevated levels of PD-L1, IL-10, TGF- $\beta$ 1, and IDO-1 are observed in 11q-deleted tumors compared to *MYCN*-amplified tumors.
4. 11q-deleted tumors exhibit higher genomic instability and a greater number of SCAs, particularly affecting the 11q region.
5. NB cells, regardless of genetic alterations, promote the polarization of macrophages towards the M2 phenotype, as indicated by increased expression levels of CD163 and CD206 while maintaining low levels of M1-associated marker HLA-DR.
6. Co-culturing NB cells with M2 macrophages significantly increases tumor cell proliferation across various NB cell lines, highlighting a cooperative interaction that supports tumor progression.
7. M2 macrophages drive NB mesenchymal phenotype, promoting lineage plasticity and drug resistance.
8. 11q-deleted NBs are strongly associated with the MES phenotype, while *MYCN*-amplified tumors are predominantly enriched in ADRN signatures.
9. M2 macrophages drastically reduce the efficacy of PHOX2B PC-CAR T cells, while M1 macrophages significantly enhance their antitumor activity.
10. M1 macrophages help sustain or increase MHC I expression, enhancing CAR-T cell recognition, while M2 macrophages significantly downregulate MHC I levels, impairing CAR-T cell activity and promoting tumor immune evasion.
11. *RASSF1A* hypermethylation is a joint early event in HR-NB, correlating with poor prognosis, particularly in 11q-deleted HR-NB.
12. A 52 CpG signature distinguishes *MYCN*-amplified from 11q-deleted HR-NBs. These CpGs regulate, among other genes, *CSF1R* and *CCR7*, which are linked to worse survival, especially in 11q-deleted tumors.
13. Reduced expression of *ATM*, *H2AX*, *CHEK1*, and *MRE11A* in 11q-deleted HR-NB is not linked to promoter methylation.



## 7. References

1. Matthay, K.K., et al., *Neuroblastoma*. Nat Rev Dis Primers, 2016. **2**: p. 16078.
2. Trinidad, E.M., et al., *Liquidhope: methylome and genomic profiling from very limited quantities of plasma-derived DNA*. Brief Bioinform, 2023. **24**(1).
3. Qiu, B. and K.K. Matthay, *Advancing therapy for neuroblastoma*. Nat Rev Clin Oncol, 2022. **19**(8): p. 515-533.
4. Irwin, M.S., et al., *Revised Neuroblastoma Risk Classification System: A Report From the Children's Oncology Group*. J Clin Oncol, 2021. **39**(29): p. 3229-3241.
5. Maris, J.M., *Recent advances in neuroblastoma*. N Engl J Med, 2010. **362**(23): p. 2202-11.
6. Dong, R., et al., *Single-Cell Characterization of Malignant Phenotypes and Developmental Trajectories of Adrenal Neuroblastoma*. Cancer Cell, 2020. **38**(5): p. 716-733 e6.
7. Cohn, S.L., et al., *The International Neuroblastoma Risk Group (INRG) classification system: an INRG Task Force report*. J Clin Oncol, 2009. **27**(2): p. 289-97.
8. Pugh, T.J., et al., *The genetic landscape of high-risk neuroblastoma*. Nat Genet, 2013. **45**(3): p. 279-84.
9. London, W.B., et al., *Evidence for an age cutoff greater than 365 days for neuroblastoma risk group stratification in the Children's Oncology Group*. J Clin Oncol, 2005. **23**(27): p. 6459-65.
10. Cheung, N.K., et al., *Association of age at diagnosis and genetic mutations in patients with neuroblastoma*. JAMA, 2012. **307**(10): p. 1062-71.
11. Vo, K.T., et al., *Clinical, biologic, and prognostic differences on the basis of primary tumor site in neuroblastoma: a report from the international neuroblastoma risk group project*. J Clin Oncol, 2014. **32**(28): p. 3169-76.
12. Taggart, D.R., et al., *Prognostic value of the stage 4S metastatic pattern and tumor biology in patients with metastatic neuroblastoma diagnosed between birth and 18 months of age*. J Clin Oncol, 2011. **29**(33): p. 4358-64.
13. DuBois, S.G., et al., *Metastatic sites in stage IV and IVS neuroblastoma correlate with age, tumor biology, and survival*. J Pediatr Hematol Oncol, 1999. **21**(3): p. 181-9.
14. Hero, B. and G. Schleiermacher, *Update on pediatric opsoclonus myoclonus syndrome*. Neuropediatrics, 2013. **44**(6): p. 324-9.
15. Brodeur, G.M., et al., *Revisions of the international criteria for neuroblastoma diagnosis, staging, and response to treatment*. J Clin Oncol, 1993. **11**(8): p. 1466-77.
16. Monclair, T., et al., *The International Neuroblastoma Risk Group (INRG) staging system: an INRG Task Force report*. J Clin Oncol, 2009. **27**(2): p. 298-303.
17. Schmidt, M.L., et al., *Favorable prognosis for patients 12 to 18 months of age with stage 4 nonamplified MYCN neuroblastoma: a Children's Cancer Group Study*. J Clin Oncol, 2005. **23**(27): p. 6474-80.
18. Shimada, H., et al., *The International Neuroblastoma Pathology Classification (the Shimada system)*. Cancer, 1999. **86**(2): p. 364-72.
19. Teshiba, R., et al., *Age-dependent prognostic effect by Mitosis-Karyorrhexis Index in neuroblastoma: a report from the Children's Oncology Group*. Pediatr Dev Pathol, 2014. **17**(6): p. 441-9.
20. Seeger, R.C., et al., *Association of multiple copies of the N-myc oncogene with rapid progression of neuroblastomas*. N Engl J Med, 1985. **313**(18): p. 1111-6.
21. Huang, M. and W.A. Weiss, *Neuroblastoma and MYCN*. Cold Spring Harb Perspect Med, 2013. **3**(10): p. a014415.

## References

---

22. Look, A.T., et al., *Cellular DNA content as a predictor of response to chemotherapy in infants with unresectable neuroblastoma*. N Engl J Med, 1984. **311**(4): p. 231-5.
23. George, R.E., et al., *Hyperdiploidy plus nonamplified MYCN confers a favorable prognosis in children 12 to 18 months old with disseminated neuroblastoma: a Pediatric Oncology Group study*. J Clin Oncol, 2005. **23**(27): p. 6466-73.
24. Nuchtern, J.G., et al., *A prospective study of expectant observation as primary therapy for neuroblastoma in young infants: a Children's Oncology Group study*. Ann Surg, 2012. **256**(4): p. 573-80.
25. Strother, D.R., et al., *Outcome after surgery alone or with restricted use of chemotherapy for patients with low-risk neuroblastoma: results of Children's Oncology Group study P9641*. J Clin Oncol, 2012. **30**(15): p. 1842-8.
26. Twist, C.J., et al., *Maintaining Outstanding Outcomes Using Response- and Biology-Based Therapy for Intermediate-Risk Neuroblastoma: A Report From the Children's Oncology Group Study ANBL0531*. J Clin Oncol, 2019. **37**(34): p. 3243-3255.
27. Kohler, J.A., et al., *Treatment of children over the age of one year with unresectable localised neuroblastoma without MYCN amplification: results of the SIOPEN study*. Eur J Cancer, 2013. **49**(17): p. 3671-9.
28. Kushner, B.H., et al., *Reduction from seven to five cycles of intensive induction chemotherapy in children with high-risk neuroblastoma*. J Clin Oncol, 2004. **22**(24): p. 4888-92.
29. French, S., et al., *<sup>131</sup>I-MIBG followed by consolidation with busulfan, melphalan and autologous stem cell transplantation for refractory neuroblastoma*. Pediatr Blood Cancer, 2013. **60**(5): p. 879-84.
30. Gaze, M.N., et al., *Optimization of molecular radiotherapy with [<sup>131</sup>I]-meta iodobenzylguanidine for high-risk neuroblastoma*. Q J Nucl Med Mol Imaging, 2013. **57**(1): p. 66-78.
31. Matthay, K.K., et al., *Phase II study on the effect of disease sites, age, and prior therapy on response to iodine-131-metaiodobenzylguanidine therapy in refractory neuroblastoma*. J Clin Oncol, 2007. **25**(9): p. 1054-60.
32. Gaze, M.N., et al., *Results of a quality assurance review of external beam radiation therapy in the International Society of Paediatric Oncology (Europe) Neuroblastoma Group's High-risk Neuroblastoma Trial: a SIOPEN study*. Int J Radiat Oncol Biol Phys, 2013. **85**(1): p. 170-4.
33. Mazloom, A., et al., *Radiation therapy to the primary and postinduction chemotherapy MIBG-avid sites in high-risk neuroblastoma*. Int J Radiat Oncol Biol Phys, 2014. **90**(4): p. 858-62.
34. Casey, D.L., et al., *Local Control With 21-Gy Radiation Therapy for High-Risk Neuroblastoma*. Int J Radiat Oncol Biol Phys, 2016. **96**(2): p. 393-400.
35. Hill-Kayser, C.E., et al., *Outcomes After Proton Therapy for Treatment of Pediatric High-Risk Neuroblastoma*. Int J Radiat Oncol Biol Phys, 2019. **104**(2): p. 401-408.
36. Yu, A.L., et al., *Anti-GD2 antibody with GM-CSF, interleukin-2, and isotretinoin for neuroblastoma*. N Engl J Med, 2010. **363**(14): p. 1324-34.
37. Nazha, B., C. Inal, and T.K. Owonikoko, *Disialoganglioside GD2 Expression in Solid Tumors and Role as a Target for Cancer Therapy*. Front Oncol, 2020. **10**: p. 1000.
38. Reynolds, C.P., et al., *Response of neuroblastoma to retinoic acid in vitro and in vivo*. Prog Clin Biol Res, 1991. **366**: p. 203-11.
39. Ladenstein, R., et al., *Interleukin 2 with anti-GD2 antibody ch14.18/CHO (dinutuximab beta) in patients with high-risk neuroblastoma (HR-NBL1/SIOPEN): a multicentre, randomised, phase 3 trial*. Lancet Oncol, 2018. **19**(12): p. 1617-1629.



40. Bagatell, R., et al., *Phase II study of irinotecan and temozolomide in children with relapsed or refractory neuroblastoma: a Children's Oncology Group study*. J Clin Oncol, 2011. **29**(2): p. 208-13.
41. Zheng, D.J., et al., *Long-term psychological and educational outcomes for survivors of neuroblastoma: A report from the Childhood Cancer Survivor Study*. Cancer, 2018. **124**(15): p. 3220-3230.
42. Friedman, D.N. and T.O. Henderson, *Late Effects and Survivorship Issues in Patients with Neuroblastoma*. Children (Basel), 2018. **5**(8).
43. Geurten, C., et al., *Endocrine consequences of neuroblastoma treatment in children: 20 years' experience of a single center*. J Pediatr Endocrinol Metab, 2019. **32**(4): p. 347-354.
44. Laverdiere, C., et al., *Long-term outcomes in survivors of neuroblastoma: a report from the Childhood Cancer Survivor Study*. J Natl Cancer Inst, 2009. **101**(16): p. 1131-40.
45. Gurney, J.G., et al., *Hearing loss, quality of life, and academic problems in long-term neuroblastoma survivors: a report from the Children's Oncology Group*. Pediatrics, 2007. **120**(5): p. e1229-36.
46. Cohen, L.E., et al., *Late effects in children treated with intensive multimodal therapy for high-risk neuroblastoma: high incidence of endocrine and growth problems*. Bone Marrow Transplant, 2014. **49**(4): p. 502-8.
47. Applebaum, M.A., et al., *Second malignancies in patients with neuroblastoma: the effects of risk-based therapy*. Pediatr Blood Cancer, 2015. **62**(1): p. 128-33.
48. Svennerholm, L., et al., *Gangliosides and allied glycosphingolipids in human peripheral nerve and spinal cord*. Biochim Biophys Acta, 1994. **1214**(2): p. 115-23.
49. Norsker, F.N., et al., *Neurologic disorders in long-term survivors of neuroblastoma - a population-based cohort study within the Adult Life after Childhood Cancer in Scandinavia (ALiCCS) research program*. Acta Oncol, 2020. **59**(2): p. 134-140.
50. London, W.B., et al., *Phase II randomized comparison of topotecan plus cyclophosphamide versus topotecan alone in children with recurrent or refractory neuroblastoma: a Children's Oncology Group study*. J Clin Oncol, 2010. **28**(24): p. 3808-15.
51. Mody, R., et al., *Irinotecan, Temozolomide, and Dinutuximab With GM-CSF in Children With Refractory or Relapsed Neuroblastoma: A Report From the Children's Oncology Group*. J Clin Oncol, 2020. **38**(19): p. 2160-2169.
52. DuBois, S.G., et al., *Phase I Study of Vorinostat as a Radiation Sensitizer with <sup>131</sup>I-Metaiodobenzylguanidine (<sup>131</sup>I-MIBG) for Patients with Relapsed or Refractory Neuroblastoma*. Clin Cancer Res, 2015. **21**(12): p. 2715-21.
53. More, S.S., et al., *Vorinostat increases expression of functional norepinephrine transporter in neuroblastoma in vitro and in vivo model systems*. Clin Cancer Res, 2011. **17**(8): p. 2339-49.
54. Fletcher, J.I., et al., *Too many targets, not enough patients: rethinking neuroblastoma clinical trials*. Nat Rev Cancer, 2018. **18**(6): p. 389-400.
55. Marshall, G.M., et al., *The prenatal origins of cancer*. Nat Rev Cancer, 2014. **14**(4): p. 277-89.
56. Janoueix-Lerosey, I., et al., *Overall genomic pattern is a predictor of outcome in neuroblastoma*. J Clin Oncol, 2009. **27**(7): p. 1026-33.
57. Amiel, J., et al., *Polyalanine expansion and frameshift mutations of the paired-like homeobox gene PHOX2B in congenital central hypoventilation syndrome*. Nat Genet, 2003. **33**(4): p. 459-61.
58. Trochet, D., et al., *Germline mutations of the paired-like homeobox 2B (PHOX2B) gene in neuroblastoma*. Am J Hum Genet, 2004. **74**(4): p. 761-4.
59. Diskin, S.J., et al., *Common variation at 6q16 within HACE1 and LIN28B influences susceptibility to neuroblastoma*. Nat Genet, 2012. **44**(10): p. 1126-30.

## References

---

60. Oldridge, D.A., et al., *Genetic predisposition to neuroblastoma mediated by a LMO1 super-enhancer polymorphism*. *Nature*, 2015. **528**(7582): p. 418-21.
61. Bosse, K.R. and J.M. Maris, *Advances in the translational genomics of neuroblastoma: From improving risk stratification and revealing novel biology to identifying actionable genomic alterations*. *Cancer*, 2016. **122**(1): p. 20-33.
62. Manolio, T.A., et al., *Finding the missing heritability of complex diseases*. *Nature*, 2009. **461**(7265): p. 747-53.
63. Brodeur, G.M., et al., *Amplification of N-myc in untreated human neuroblastomas correlates with advanced disease stage*. *Science*, 1984. **224**(4653): p. 1121-4.
64. Zimmerman, K.A., et al., *Differential expression of myc family genes during murine development*. *Nature*, 1986. **319**(6056): p. 780-3.
65. Weiss, W.A., et al., *Targeted expression of MYCN causes neuroblastoma in transgenic mice*. *EMBO J*, 1997. **16**(11): p. 2985-95.
66. Rickman, D.S., J.H. Schulte, and M. Eilers, *The Expanding World of N-MYC-Driven Tumors*. *Cancer Discov*, 2018. **8**(2): p. 150-163.
67. Liu, Y., et al., *The genomic landscape of pediatric and young adult T-lineage acute lymphoblastic leukemia*. *Nat Genet*, 2017. **49**(8): p. 1211-1218.
68. Zimmerman, M.W., et al., *MYC Drives a Subset of High-Risk Pediatric Neuroblastomas and Is Activated through Mechanisms Including Enhancer Hijacking and Focal Enhancer Amplification*. *Cancer Discov*, 2018. **8**(3): p. 320-335.
69. Brockmann, M., et al., *Small molecule inhibitors of aurora-a induce proteasomal degradation of N-myc in childhood neuroblastoma*. *Cancer Cell*, 2013. **24**(1): p. 75-89.
70. Gustafson, W.C., et al., *Drugging MYCN through an allosteric transition in Aurora kinase A*. *Cancer Cell*, 2014. **26**(3): p. 414-427.
71. DuBois, S.G., et al., *Phase II Trial of Alisertib in Combination with Irinotecan and Temozolomide for Patients with Relapsed or Refractory Neuroblastoma*. *Clin Cancer Res*, 2018. **24**(24): p. 6142-6149.
72. Du, J., et al., *Aurora A-Selective Inhibitor LY3295668 Leads to Dominant Mitotic Arrest, Apoptosis in Cancer Cells, and Shows Potent Preclinical Antitumor Efficacy*. *Mol Cancer Ther*, 2019. **18**(12): p. 2207-2219.
73. Buchel, G., et al., *Association with Aurora-A Controls N-MYC-Dependent Promoter Escape and Pause Release of RNA Polymerase II during the Cell Cycle*. *Cell Rep*, 2017. **21**(12): p. 3483-3497.
74. Adhikari, B., et al., *PROTAC-mediated degradation reveals a non-catalytic function of AURORA-A kinase*. *Nat Chem Biol*, 2020. **16**(11): p. 1179-1188.
75. Puissant, A., et al., *Targeting MYCN in neuroblastoma by BET bromodomain inhibition*. *Cancer Discov*, 2013. **3**(3): p. 308-23.
76. Garraalda, E., et al., *MYC targeting by OMO-103 in solid tumors: a phase 1 trial*. *Nat Med*, 2024. **30**(3): p. 762-771.
77. Hogarty, M.D., et al., *ODC1 is a critical determinant of MYCN oncogenesis and a therapeutic target in neuroblastoma*. *Cancer Res*, 2008. **68**(23): p. 9735-45.
78. Sholler, G.L.S., et al., *Maintenance DFMO Increases Survival in High Risk Neuroblastoma*. *Sci Rep*, 2018. **8**(1): p. 14445.
79. Hirai, H., et al., *Small-molecule inhibition of Wee1 kinase by MK-1775 selectively sensitizes p53-deficient tumor cells to DNA-damaging agents*. *Mol Cancer Ther*, 2009. **8**(11): p. 2992-3000.
80. Cole, K.A., et al., *Phase I Clinical Trial of the Wee1 Inhibitor Adavosertib (AZD1775) with Irinotecan in Children with Relapsed Solid Tumors: A COG Phase I Consortium Report (ADVL1312)*. *Clin Cancer Res*, 2020. **26**(6): p. 1213-1219.
81. Reiff, T., et al., *Midkine and Alk signaling in sympathetic neuron proliferation and neuroblastoma predisposition*. *Development*, 2011. **138**(21): p. 4699-708.

82. Cheng, L.Y., et al., *Anaplastic lymphoma kinase spares organ growth during nutrient restriction in Drosophila*. Cell, 2011. **146**(3): p. 435-47.
83. Bresler, S.C., et al., *ALK mutations confer differential oncogenic activation and sensitivity to ALK inhibition therapy in neuroblastoma*. Cancer Cell, 2014. **26**(5): p. 682-94.
84. O'Donohue, T., et al., *Differential Impact of ALK Mutations in Neuroblastoma*. JCO Precis Oncol, 2021. **5**.
85. Bellini, A., et al., *Frequency and Prognostic Impact of ALK Amplifications and Mutations in the European Neuroblastoma Study Group (SIOPEN) High-Risk Neuroblastoma Trial (HR-NBL1)*. J Clin Oncol, 2021. **39**(30): p. 3377-3390.
86. Heukamp, L.C., et al., *Targeted expression of mutated ALK induces neuroblastoma in transgenic mice*. Sci Transl Med, 2012. **4**(141): p. 141ra91.
87. Fischer, M., et al., *Ceritinib in paediatric patients with anaplastic lymphoma kinase-positive malignancies: an open-label, multicentre, phase 1, dose-escalation and dose-expansion study*. Lancet Oncol, 2021. **22**(12): p. 1764-1776.
88. Infarinato, N.R., et al., *The ALK/ROS1 Inhibitor PF-06463922 Overcomes Primary Resistance to Crizotinib in ALK-Driven Neuroblastoma*. Cancer Discov, 2016. **6**(1): p. 96-107.
89. Sano, R., et al., *An antibody-drug conjugate directed to the ALK receptor demonstrates efficacy in preclinical models of neuroblastoma*. Sci Transl Med, 2019. **11**(483).
90. Bresler, S.C., et al., *Differential inhibitor sensitivity of anaplastic lymphoma kinase variants found in neuroblastoma*. Sci Transl Med, 2011. **3**(108): p. 108ra114.
91. Schnepf, R.W., et al., *A LIN28B-RAN-AURKA Signaling Network Promotes Neuroblastoma Tumorigenesis*. Cancer Cell, 2015. **28**(5): p. 599-609.
92. Valentijn, L.J., et al., *TERT rearrangements are frequent in neuroblastoma and identify aggressive tumors*. Nat Genet, 2015. **47**(12): p. 1411-4.
93. Hertwig, F., M. Peifer, and M. Fischer, *Telomere maintenance is pivotal for high-risk neuroblastoma*. Cell Cycle, 2016. **15**(3): p. 311-2.
94. Sausen, M., et al., *Integrated genomic analyses identify ARID1A and ARID1B alterations in the childhood cancer neuroblastoma*. Nat Genet, 2013. **45**(1): p. 12-7.
95. Qadeer, Z.A., et al., *ATRX In-Frame Fusion Neuroblastoma Is Sensitive to EZH2 Inhibition via Modulation of Neuronal Gene Signatures*. Cancer Cell, 2019. **36**(5): p. 512-527 e9.
96. Chen, L., et al., *CRISPR-Cas9 screen reveals a MYCN-amplified neuroblastoma dependency on EZH2*. J Clin Invest, 2018. **128**(1): p. 446-462.
97. Durbin, A.D., et al., *EP300 Selectively Controls the Enhancer Landscape of MYCN-Amplified Neuroblastoma*. Cancer Discov, 2022. **12**(3): p. 730-751.
98. Chen, J., et al., *Targeted Therapy of TERT-Rearranged Neuroblastoma with BET Bromodomain Inhibitor and Proteasome Inhibitor Combination Therapy*. Clin Cancer Res, 2021. **27**(5): p. 1438-1451.
99. Sanmartin, E., et al., *Deletion of 11q in Neuroblastomas Drives Sensitivity to PARP Inhibition*. Clin Cancer Res, 2017. **23**(22): p. 6875-6887.
100. Koneru, B., et al., *ALT neuroblastoma chemoresistance due to telomere dysfunction-induced ATM activation is reversible with ATM inhibitor AZD0156*. Sci Transl Med, 2021. **13**(607).
101. Rybak, A., et al., *A feedback loop comprising lin-28 and let-7 controls pre-let-7 maturation during neural stem-cell commitment*. Nat Cell Biol, 2008. **10**(8): p. 987-93.
102. Molenaar, J.J., et al., *LIN28B induces neuroblastoma and enhances MYCN levels via let-7 suppression*. Nat Genet, 2012. **44**(11): p. 1199-206.
103. Schleiermacher, G., et al., *Segmental chromosomal alterations have prognostic impact in neuroblastoma: a report from the INRG project*. Br J Cancer, 2012. **107**(8): p. 1418-22.

## References

---

104. Attiyeh, E.F., et al., *Chromosome 1p and 11q deletions and outcome in neuroblastoma*. N Engl J Med, 2005. **353**(21): p. 2243-53.
105. Juan Ribelles, A., et al., *Clinical Features of Neuroblastoma With 11q Deletion: An Increase in Relapse Probabilities In Localized And 4S Stages*. Sci Rep, 2019. **9**(1): p. 13806.
106. Spitz, R., et al., *Loss in chromosome 11q identifies tumors with increased risk for metastatic relapses in localized and 4S neuroblastoma*. Clin Cancer Res, 2006. **12**(11 Pt 1): p. 3368-73.
107. Caren, H., et al., *High-risk neuroblastoma tumors with 11q-deletion display a poor prognostic, chromosome instability phenotype with later onset*. Proc Natl Acad Sci U S A, 2010. **107**(9): p. 4323-8.
108. Powers, J.T., et al., *Multiple mechanisms disrupt the let-7 microRNA family in neuroblastoma*. Nature, 2016. **535**(7611): p. 246-51.
109. Peng, Y. and C.M. Croce, *The role of MicroRNAs in human cancer*. Signal Transduct Target Ther, 2016. **1**: p. 15004.
110. Teitz, T., et al., *Caspase 8 is deleted or silenced preferentially in childhood neuroblastomas with amplification of MYCN*. Nat Med, 2000. **6**(5): p. 529-35.
111. Alaminos, M., et al., *Clustering of gene hypermethylation associated with clinical risk groups in neuroblastoma*. J Natl Cancer Inst, 2004. **96**(16): p. 1208-19.
112. van Noesel, M.M., et al., *Tumor-specific down-regulation of the tumor necrosis factor-related apoptosis-inducing ligand decoy receptors DcR1 and DcR2 is associated with dense promoter hypermethylation*. Cancer Res, 2002. **62**(7): p. 2157-61.
113. Yang, Q., et al., *Association of epigenetic inactivation of RASSF1A with poor outcome in human neuroblastoma*. Clin Cancer Res, 2004. **10**(24): p. 8493-500.
114. Casciano, I., et al., *Expression of DeltaNp73 is a molecular marker for adverse outcome in neuroblastoma patients*. Cell Death Differ, 2002. **9**(3): p. 246-51.
115. Casciano, I., et al., *Role of methylation in the control of DeltaNp73 expression in neuroblastoma*. Cell Death Differ, 2002. **9**(3): p. 343-5.
116. Caren, H., et al., *Identification of epigenetically regulated genes that predict patient outcome in neuroblastoma*. BMC Cancer, 2011. **11**: p. 66.
117. Decock, A., et al., *Methyl-CpG-binding domain sequencing reveals a prognostic methylation signature in neuroblastoma*. Oncotarget, 2016. **7**(2): p. 1960-72.
118. Decock, A., et al., *Genome-wide promoter methylation analysis in neuroblastoma identifies prognostic methylation biomarkers*. Genome Biol, 2012. **13**(10): p. R95.
119. Shain, A.H. and J.R. Pollack, *The spectrum of SWI/SNF mutations, ubiquitous in human cancers*. PLoS One, 2013. **8**(1): p. e55119.
120. Cole, K.A., et al., *A functional screen identifies miR-34a as a candidate neuroblastoma tumor suppressor gene*. Mol Cancer Res, 2008. **6**(5): p. 735-42.
121. Fontana, L., et al., *Antagomir-17-5p abolishes the growth of therapy-resistant neuroblastoma through p21 and BIM*. PLoS One, 2008. **3**(5): p. e2236.
122. Schulte, J.H., et al., *MYCN regulates oncogenic MicroRNAs in neuroblastoma*. Int J Cancer, 2008. **122**(3): p. 699-704.
123. Brodeur, G.M., et al., *Trk receptor expression and inhibition in neuroblastomas*. Clin Cancer Res, 2009. **15**(10): p. 3244-50.
124. Nakagawara, A., et al., *Association between high levels of expression of the TRK gene and favorable outcome in human neuroblastoma*. N Engl J Med, 1993. **328**(12): p. 847-54.
125. Ho, R., et al., *Resistance to chemotherapy mediated by TrkB in neuroblastomas*. Cancer Res, 2002. **62**(22): p. 6462-6.
126. Eggert, A., et al., *Expression of neurotrophin receptor TrkA inhibits angiogenesis in neuroblastoma*. Med Pediatr Oncol, 2000. **35**(6): p. 569-72.
127. Norris, R.E., et al., *Preclinical evaluation of lestaurtinib (CEP-701) in combination with retinoids for neuroblastoma*. Cancer Chemother Pharmacol, 2011. **68**(6): p. 1469-75.

128. Chmielecki, J., et al., *Genomic Profiling of a Large Set of Diverse Pediatric Cancers Identifies Known and Novel Mutations across Tumor Spectra*. *Cancer Res*, 2017. **77**(2): p. 509-519.
129. Padovan-Merhar, O.M., et al., *Enrichment of Targetable Mutations in the Relapsed Neuroblastoma Genome*. *PLoS Genet*, 2016. **12**(12): p. e1006501.
130. Anderson, D.J., et al., *Antibody markers identify a common progenitor to sympathetic neurons and chromaffin cells in vivo and reveal the timing of commitment to neuronal differentiation in the sympathoadrenal lineage*. *J Neurosci*, 1991. **11**(11): p. 3507-19.
131. Dupin, E., S. Creuzet, and N.M. Le Douarin, *The contribution of the neural crest to the vertebrate body*. *Adv Exp Med Biol*, 2006. **589**: p. 96-119.
132. Huber, K., *The sympathoadrenal cell lineage: specification, diversification, and new perspectives*. *Dev Biol*, 2006. **298**(2): p. 335-43.
133. Wartiovaara, K., et al., *N-myc promotes survival and induces S-phase entry of postmitotic sympathetic neurons*. *J Neurosci*, 2002. **22**(3): p. 815-24.
134. Hansford, L.M., et al., *Mechanisms of embryonal tumor initiation: distinct roles for MycN expression and MYCN amplification*. *Proc Natl Acad Sci U S A*, 2004. **101**(34): p. 12664-9.
135. Yuan, J. and B.A. Yankner, *Apoptosis in the nervous system*. *Nature*, 2000. **407**(6805): p. 802-9.
136. Biedler, J.L., L. Helson, and B.A. Spengler, *Morphology and growth, tumorigenicity, and cytogenetics of human neuroblastoma cells in continuous culture*. *Cancer Res*, 1973. **33**(11): p. 2643-52.
137. Ciccarone, V., et al., *Phenotypic diversification in human neuroblastoma cells: expression of distinct neural crest lineages*. *Cancer Res*, 1989. **49**(1): p. 219-25.
138. Boeva, V., et al., *Heterogeneity of neuroblastoma cell identity defined by transcriptional circuitries*. *Nat Genet*, 2017. **49**(9): p. 1408-1413.
139. van Groningen, T., et al., *Neuroblastoma is composed of two super-enhancer-associated differentiation states*. *Nat Genet*, 2017. **49**(8): p. 1261-1266.
140. Hnisz, D., et al., *Super-enhancers in the control of cell identity and disease*. *Cell*, 2013. **155**(4): p. 934-47.
141. Whyte, W.A., et al., *Master transcription factors and mediator establish super-enhancers at key cell identity genes*. *Cell*, 2013. **153**(2): p. 307-19.
142. Upton, K., et al., *Epigenomic profiling of neuroblastoma cell lines*. *Sci Data*, 2020. **7**(1): p. 116.
143. van Groningen, T., et al., *A NOTCH feed-forward loop drives reprogramming from adrenergic to mesenchymal state in neuroblastoma*. *Nat Commun*, 2019. **10**(1): p. 1530.
144. Yu, E.Y., et al., *Reciprocal impacts of telomerase activity and ADRN/MES differentiation state in neuroblastoma tumor biology*. *Commun Biol*, 2021. **4**(1): p. 1315.
145. Khazeem, M.M., et al., *TOP2B Is Required to Maintain the Adrenergic Neural Phenotype and for ATRA-Induced Differentiation of SH-SY5Y Neuroblastoma Cells*. *Mol Neurobiol*, 2022. **59**(10): p. 5987-6008.
146. Weichert-Leahey, N., et al., *Genetic predisposition to neuroblastoma results from a regulatory polymorphism that promotes the adrenergic cell state*. *J Clin Invest*, 2023. **133**(10).
147. Zhu, S., et al., *LMO1 Synergizes with MYCN to Promote Neuroblastoma Initiation and Metastasis*. *Cancer Cell*, 2017. **32**(3): p. 310-323 e5.
148. Hanahan, D., *Hallmarks of Cancer: New Dimensions*. *Cancer Discov*, 2022. **12**(1): p. 31-46.
149. Kildisiute, G., et al., *Tumor to normal single-cell mRNA comparisons reveal a pan-neuroblastoma cancer cell*. *Sci Adv*, 2021. **7**(6).
150. Jansky, S., et al., *Single-cell transcriptomic analyses provide insights into the developmental origins of neuroblastoma*. *Nat Genet*, 2021. **53**(5): p. 683-693.

## References

---

151. Thirant, C., et al., *Reversible transitions between noradrenergic and mesenchymal tumor identities define cell plasticity in neuroblastoma*. Nat Commun, 2023. **14**(1): p. 2575.
152. Kameneva, P., et al., *Single-cell transcriptomics of human embryos identifies multiple sympathoblast lineages with potential implications for neuroblastoma origin*. Nat Genet, 2021. **53**(5): p. 694-706.
153. Bedoya-Reina, O.C., et al., *Single-nuclei transcriptomes from human adrenal gland reveal distinct cellular identities of low and high-risk neuroblastoma tumors*. Nat Commun, 2021. **12**(1): p. 5309.
154. Avitabile, M., et al., *Single-cell transcriptomics of neuroblastoma identifies chemoresistance-associated genes and pathways*. Comput Struct Biotechnol J, 2022. **20**: p. 4437-4445.
155. Westerhout, E.M., et al., *Mesenchymal-Type Neuroblastoma Cells Escape ALK Inhibitors*. Cancer Res, 2022. **82**(3): p. 484-496.
156. Casey, D.L. and N.V. Cheung, *Immunotherapy of Pediatric Solid Tumors: Treatments at a Crossroads, with an Emphasis on Antibodies*. Cancer Immunol Res, 2020. **8**(2): p. 161-166.
157. Schreiber, R.D., L.J. Old, and M.J. Smyth, *Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion*. Science, 2011. **331**(6024): p. 1565-70.
158. Chen, D.S. and I. Mellman, *Elements of cancer immunity and the cancer-immune set point*. Nature, 2017. **541**(7637): p. 321-330.
159. Wei, J.S., et al., *Clinically Relevant Cytotoxic Immune Cell Signatures and Clonal Expansion of T-Cell Receptors in High-Risk MYCN-Not-Amplified Human Neuroblastoma*. Clin Cancer Res, 2018. **24**(22): p. 5673-5684.
160. Reid, G.S., et al., *Interferon-gamma-dependent infiltration of human T cells into neuroblastoma tumors in vivo*. Clin Cancer Res, 2009. **15**(21): p. 6602-8.
161. Chen, X., et al., *Neuroblastoma cells inhibit the immunostimulatory function of dendritic cells*. J Pediatr Surg, 2003. **38**(6): p. 901-5.
162. Gross, N., et al., *Expression of Fas (APO-1/CD95) and Fas ligand (FasL) in human neuroblastoma*. Med Pediatr Oncol, 2001. **36**(1): p. 111-4.
163. Walker, S.R., R.E. Redlinger, Jr., and E.M. Barksdale, Jr., *Neuroblastoma-induced inhibition of dendritic cell IL-12 production via abrogation of CD40 expression*. J Pediatr Surg, 2005. **40**(1): p. 244-9; discussion 249-50.
164. Shurin, G.V., et al., *Neuroblastoma-derived gangliosides inhibit dendritic cell generation and function*. Cancer Res, 2001. **61**(1): p. 363-9.
165. Harada, K., et al., *Soluble factors derived from neuroblastoma cell lines suppress dendritic cell differentiation and activation*. Cancer Sci, 2019. **110**(3): p. 888-902.
166. Brandetti, E., et al., *MYCN is an immunosuppressive oncogene dampening the expression of ligands for NK-cell-activating receptors in human high-risk neuroblastoma*. Oncoimmunology, 2017. **6**(6): p. e1316439.
167. Veneziani, I., et al., *The BET-bromodomain inhibitor JQ1 renders neuroblastoma cells more resistant to NK cell-mediated recognition and killing by downregulating ligands for NKG2D and DNAM-1 receptors*. Oncotarget, 2019. **10**(22): p. 2151-2160.
168. Raffaghello, L., et al., *Downregulation and/or release of NKG2D ligands as immune evasion strategy of human neuroblastoma*. Neoplasia, 2004. **6**(5): p. 558-68.
169. Freeman, G.J., et al., *Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation*. J Exp Med, 2000. **192**(7): p. 1027-34.
170. Shen, X. and B. Zhao, *Efficacy of PD-1 or PD-L1 inhibitors and PD-L1 expression status in cancer: meta-analysis*. BMJ, 2018. **362**: p. k3529.

171. Saletta, F., et al., *Programmed Death-Ligand 1 Expression in a Large Cohort of Pediatric Patients With Solid Tumor and Association With Clinicopathologic Features in Neuroblastoma*. JCO Precis Oncol, 2017. **1**: p. 1-12.
172. Zuo, S., et al., *Potential role of the PD-L1 expression and tumor-infiltrating lymphocytes on neuroblastoma*. Pediatr Surg Int, 2020. **36**(2): p. 137-143.
173. Melaiu, O., et al., *PD-L1 Is a Therapeutic Target of the Bromodomain Inhibitor JQ1 and, Combined with HLA Class I, a Promising Prognostic Biomarker in Neuroblastoma*. Clin Cancer Res, 2017. **23**(15): p. 4462-4472.
174. Majzner, R.G., et al., *Assessment of programmed death-ligand 1 expression and tumor-associated immune cells in pediatric cancer tissues*. Cancer, 2017. **123**(19): p. 3807-3815.
175. Xin, C., et al., *CD200 is overexpressed in neuroblastoma and regulates tumor immune microenvironment*. Cancer Immunol Immunother, 2020. **69**(11): p. 2333-2343.
176. Gregorio, A., et al., *Small round blue cell tumours: diagnostic and prognostic usefulness of the expression of B7-H3 surface molecule*. Histopathology, 2008. **53**(1): p. 73-80.
177. Castriconi, R., et al., *Identification of 4Ig-B7-H3 as a neuroblastoma-associated molecule that exerts a protective role from an NK cell-mediated lysis*. Proc Natl Acad Sci U S A, 2004. **101**(34): p. 12640-5.
178. Kendersky, N.M., et al., *The B7-H3-Targeting Antibody-Drug Conjugate m276-SL-PBD Is Potently Effective Against Pediatric Cancer Preclinical Solid Tumor Models*. Clin Cancer Res, 2021. **27**(10): p. 2938-2946.
179. Du, H., et al., *Antitumor Responses in the Absence of Toxicity in Solid Tumors by Targeting B7-H3 via Chimeric Antigen Receptor T Cells*. Cancer Cell, 2019. **35**(2): p. 221-237 e8.
180. Wienke, J., et al., *Integrative analysis of neuroblastoma by single-cell RNA sequencing identifies the NECTIN2-TIGIT axis as a target for immunotherapy*. Cancer Cell, 2024. **42**(2): p. 283-300 e8.
181. Maier, B., et al., *A conserved dendritic-cell regulatory program limits antitumour immunity*. Nature, 2020. **580**(7802): p. 257-262.
182. Veglia, F., M. Perego, and D. Gabrilovich, *Myeloid-derived suppressor cells coming of age*. Nat Immunol, 2018. **19**(2): p. 108-119.
183. Bianchi, G., et al., *ATP/P2X7 axis modulates myeloid-derived suppressor cell functions in neuroblastoma microenvironment*. Cell Death Dis, 2014. **5**(3): p. e1135.
184. Santilli, G., et al., *Polyphenon [corrected] E enhances the antitumor immune response in neuroblastoma by inactivating myeloid suppressor cells*. Clin Cancer Res, 2013. **19**(5): p. 1116-25.
185. Wu, H.W., et al., *Anti-CD105 Antibody Eliminates Tumor Microenvironment Cells and Enhances Anti-GD2 Antibody Immunotherapy of Neuroblastoma with Activated Natural Killer Cells*. Clin Cancer Res, 2019. **25**(15): p. 4761-4774.
186. Abe, R., et al., *Regulation of the CTL response by macrophage migration inhibitory factor*. J Immunol, 2001. **166**(2): p. 747-53.
187. Soldati, R., et al., *Neuroblastoma triggers an immunoevasive program involving galectin-1-dependent modulation of T cell and dendritic cell compartments*. Int J Cancer, 2012. **131**(5): p. 1131-41.
188. Thomas, D.A. and J. Massague, *TGF-beta directly targets cytotoxic T cell functions during tumor evasion of immune surveillance*. Cancer Cell, 2005. **8**(5): p. 369-80.
189. Buchel, G., et al., *Immune response modulation by Galectin-1 in a transgenic model of neuroblastoma*. Oncoimmunology, 2016. **5**(5): p. e1131378.
190. Zhou, Q., et al., *Expression of macrophage migration inhibitory factor by neuroblastoma leads to the inhibition of antitumor T cell reactivity in vivo*. J Immunol, 2008. **181**(3): p. 1877-86.

## References

---

191. Song, L., et al., *Valpha24-invariant NKT cells mediate antitumor activity via killing of tumor-associated macrophages*. J Clin Invest, 2009. **119**(6): p. 1524-36.
192. Perillo, N.L., et al., *Apoptosis of T cells mediated by galectin-1*. Nature, 1995. **378**(6558): p. 736-9.
193. Yan, X., R.J. Orentas, and B.D. Johnson, *Tumor-derived macrophage migration inhibitory factor (MIF) inhibits T lymphocyte activation*. Cytokine, 2006. **33**(4): p. 188-98.
194. Cavalli, E., et al., *Overexpression of Macrophage Migration Inhibitory Factor and Its Homologue D-Dopachrome Tautomerase as Negative Prognostic Factor in Neuroblastoma*. Brain Sci, 2019. **9**(10).
195. Theruvath, J., et al., *Anti-GD2 synergizes with CD47 blockade to mediate tumor eradication*. Nat Med, 2022. **28**(2): p. 333-344.
196. Flierl, M.A., et al., *Catecholamines-crafty weapons in the inflammatory arsenal of immune/inflammatory cells or opening pandora's box?* Mol Med, 2008. **14**(3-4): p. 195-204.
197. Semeraro, M., et al., *Clinical impact of the NKp30/B7-H6 axis in high-risk neuroblastoma patients*. Sci Transl Med, 2015. **7**(283): p. 283ra55.
198. Zhen, Z., et al., *Involvement of IL-10 and TGF-beta in HLA-E-mediated neuroblastoma migration and invasion*. Oncotarget, 2016. **7**(28): p. 44340-44349.
199. Morandi, F., et al., *Human neuroblastoma cells trigger an immunosuppressive program in monocytes by stimulating soluble HLA-G release*. Cancer Res, 2007. **67**(13): p. 6433-41.
200. Morandi, F., et al., *Soluble HLA-G and HLA-E Levels in Bone Marrow Plasma Samples Are Related to Disease Stage in Neuroblastoma Patients*. J Immunol Res, 2016. **2016**: p. 7465741.
201. Vanichapol, T., et al., *Secretory High-Mobility Group Box 1 Protein Affects Regulatory T Cell Differentiation in Neuroblastoma Microenvironment In Vitro*. J Oncol, 2018. **2018**: p. 7946021.
202. Mussai, F., et al., *Neuroblastoma Arginase Activity Creates an Immunosuppressive Microenvironment That Impairs Autologous and Engineered Immunity*. Cancer Res, 2015. **75**(15): p. 3043-53.
203. Martin, C.J., et al., *Selective inhibition of TGFbeta1 activation overcomes primary resistance to checkpoint blockade therapy by altering tumor immune landscape*. Sci Transl Med, 2020. **12**(536).
204. Siebert, N., et al., *PD-1 blockade augments anti-neuroblastoma immune response induced by anti-GD(2) antibody ch14.18/CHO*. Oncoimmunology, 2017. **6**(10): p. e1343775.
205. Williams, E.L., et al., *Immunomodulatory monoclonal antibodies combined with peptide vaccination provide potent immunotherapy in an aggressive murine neuroblastoma model*. Clin Cancer Res, 2013. **19**(13): p. 3545-55.
206. Rigo, V., et al., *Combined immunotherapy with anti-PDL-1/PD-1 and anti-CD4 antibodies cures syngeneic disseminated neuroblastoma*. Sci Rep, 2017. **7**(1): p. 14049.
207. Tran, H.C., et al., *TGFbetaR1 Blockade with Galunisertib (LY2157299) Enhances Anti-Neuroblastoma Activity of the Anti-GD2 Antibody Dinutuximab (ch14.18) with Natural Killer Cells*. Clin Cancer Res, 2017. **23**(3): p. 804-813.
208. Zeineldin, M., A.G. Patel, and M.A. Dyer, *Neuroblastoma: When differentiation goes awry*. Neuron, 2022. **110**(18): p. 2916-2928.
209. Cornel, A.M., et al., *Epigenetic modulation of neuroblastoma enhances T cell and NK cell immunogenicity by inducing a tumor-cell lineage switch*. J Immunother Cancer, 2022. **10**(12).
210. van den Bijgaart, R.J.E., et al., *Anti-GD2 antibody and Vorinostat immunocombination therapy is highly effective in an aggressive orthotopic neuroblastoma model*. Oncoimmunology, 2020. **9**(1): p. 1817653.



211. Mabe, N.W., et al., *Transition to a mesenchymal state in neuroblastoma confers resistance to anti-GD2 antibody via reduced expression of ST8SIA1*. Nat Cancer, 2022. **3**(8): p. 976-993.
212. Pilgrim, A.A., et al., *The yes-associated protein (YAP) is associated with resistance to anti-GD2 immunotherapy in neuroblastoma through downregulation of ST8SIA1*. Oncoimmunology, 2023. **12**(1): p. 2240678.
213. Ferris, R.L., E.M. Jaffee, and S. Ferrone, *Tumor antigen-targeted, monoclonal antibody-based immunotherapy: clinical response, cellular immunity, and immunoescape*. J Clin Oncol, 2010. **28**(28): p. 4390-9.
214. Cheung, N.K. and M.A. Dyer, *Neuroblastoma: developmental biology, cancer genomics and immunotherapy*. Nat Rev Cancer, 2013. **13**(6): p. 397-411.
215. Shanshal, M., et al., *T-Cell Engagers in Solid Cancers-Current Landscape and Future Directions*. Cancers (Basel), 2023. **15**(10).
216. Valleria, D.A., et al., *NK-Cell-Mediated Targeting of Various Solid Tumors Using a B7-H3 Tri-Specific Killer Engager In Vitro and In Vivo*. Cancers (Basel), 2020. **12**(9).
217. Geller, J.I., et al., *ADV1522: A phase 2 study of lorvotuzumab mertansine (IMGN901) in children with relapsed or refractory wilms tumor, rhabdomyosarcoma, neuroblastoma, pleuropulmonary blastoma, malignant peripheral nerve sheath tumor, or synovial sarcoma-A Children's Oncology Group study*. Cancer, 2020. **126**(24): p. 5303-5310.
218. Hoefnagel, C.A., et al., *A comparison of targeting of neuroblastoma with mIBG and anti L1-CAM antibody mAb chCE7: therapeutic efficacy in a neuroblastoma xenograft model and imaging of neuroblastoma patients*. Eur J Nucl Med, 2001. **28**(3): p. 359-68.
219. Bosse, K.R., et al., *Identification of GPC2 as an Oncoprotein and Candidate Immunotherapeutic Target in High-Risk Neuroblastoma*. Cancer Cell, 2017. **32**(3): p. 295-309 e12.
220. Krytska, K., et al., *Evaluation of the DLL3-targeting antibody-drug conjugate rovalpituzumab tesirine in preclinical models of neuroblastoma*. Cancer Res Commun, 2022. **2**(7): p. 616-623.
221. Pascual-Pasto, G., et al., *GPC2 antibody-drug conjugate reprograms the neuroblastoma immune milieu to enhance macrophage-driven therapies*. J Immunother Cancer, 2022. **10**(12).
222. Heitzeneder, S., et al., *GPC2-CAR T cells tuned for low antigen density mediate potent activity against neuroblastoma without toxicity*. Cancer Cell, 2022. **40**(1): p. 53-69 e9.
223. Walker, A.J., et al., *Tumor Antigen and Receptor Densities Regulate Efficacy of a Chimeric Antigen Receptor Targeting Anaplastic Lymphoma Kinase*. Mol Ther, 2017. **25**(9): p. 2189-2201.
224. Loo, D., et al., *Development of an Fc-enhanced anti-B7-H3 monoclonal antibody with potent antitumor activity*. Clin Cancer Res, 2012. **18**(14): p. 3834-45.
225. Jaiswal, S., et al., *CD47 is upregulated on circulating hematopoietic stem cells and leukemia cells to avoid phagocytosis*. Cell, 2009. **138**(2): p. 271-85.
226. Maude, S.L., et al., *Chimeric antigen receptor T cells for sustained remissions in leukemia*. N Engl J Med, 2014. **371**(16): p. 1507-17.
227. Maude, S.L., et al., *Tisagenlecleucel in Children and Young Adults with B-Cell Lymphoblastic Leukemia*. N Engl J Med, 2018. **378**(5): p. 439-448.
228. Shah, N.N. and T.J. Fry, *Mechanisms of resistance to CAR T cell therapy*. Nat Rev Clin Oncol, 2019. **16**(6): p. 372-385.
229. Brown, C.E. and C.L. Mackall, *CAR T cell therapy: inroads to response and resistance*. Nat Rev Immunol, 2019. **19**(2): p. 73-74.
230. Li, N., et al., *CAR T cells targeting tumor-associated exons of glypican 2 regress neuroblastoma in mice*. Cell Rep Med, 2021. **2**(6): p. 100297.

## References

---

231. Majzner, R.G., et al., *CAR T Cells Targeting B7-H3, a Pan-Cancer Antigen, Demonstrate Potent Preclinical Activity Against Pediatric Solid Tumors and Brain Tumors*. Clin Cancer Res, 2019. **25**(8): p. 2560-2574.
232. Kramer, K., et al., *Compartmental intrathecal radioimmunotherapy: results for treatment for metastatic CNS neuroblastoma*. J Neurooncol, 2010. **97**(3): p. 409-18.
233. Del Bufalo, F., et al., *GD2-CART01 for Relapsed or Refractory High-Risk Neuroblastoma*. N Engl J Med, 2023. **388**(14): p. 1284-1295.
234. Haen, S.P., et al., *Towards new horizons: characterization, classification and implications of the tumour antigenic repertoire*. Nat Rev Clin Oncol, 2020. **17**(10): p. 595-610.
235. Yarmarkovich, M., et al., *Cross-HLA targeting of intracellular oncoproteins with peptide-centric CARs*. Nature, 2021. **599**(7885): p. 477-484.
236. Craddock, J.A., et al., *Enhanced tumor trafficking of GD2 chimeric antigen receptor T cells by expression of the chemokine receptor CCR2b*. J Immunother, 2010. **33**(8): p. 780-8.
237. Sun, J., et al., *T cells expressing constitutively active Akt resist multiple tumor-associated inhibitory mechanisms*. Mol Ther, 2010. **18**(11): p. 2006-17.
238. Cho, D., et al., *Cytotoxicity of activated natural killer cells against pediatric solid tumors*. Clin Cancer Res, 2010. **16**(15): p. 3901-9.
239. Denman, C.J., et al., *Membrane-bound IL-21 promotes sustained ex vivo proliferation of human natural killer cells*. PLoS One, 2012. **7**(1): p. e30264.
240. Olle Hurtado, M., et al., *Tumor infiltrating lymphocytes expanded from pediatric neuroblastoma display heterogeneity of phenotype and function*. PLoS One, 2019. **14**(8): p. e0216373.
241. Godfrey, D.I., et al., *NKT cells: what's in a name?* Nat Rev Immunol, 2004. **4**(3): p. 231-7.
242. Lv, Z., F. Luo, and Y. Chu, *Strategies for overcoming bottlenecks in allogeneic CAR-T cell therapy*. Front Immunol, 2023. **14**: p. 1199145.
243. O'Sullivan, T.E., J.C. Sun, and L.L. Lanier, *Natural Killer Cell Memory*. Immunity, 2015. **43**(4): p. 634-45.
244. Paust, S. and U.H. von Andrian, *Natural killer cell memory*. Nat Immunol, 2011. **12**(6): p. 500-8.
245. Heczey, A., et al., *Anti-GD2 CAR-NKT cells in patients with relapsed or refractory neuroblastoma: an interim analysis*. Nat Med, 2020. **26**(11): p. 1686-1690.
246. Modak, S., et al., *Adoptive immunotherapy with haploidentical natural killer cells and Anti-GD2 monoclonal antibody m3F8 for resistant neuroblastoma: Results of a phase I study*. Oncoimmunology, 2018. **7**(8): p. e1461305.
247. Cheung, I.Y., et al., *Survival Impact of Anti-GD2 Antibody Response in a Phase II Ganglioside Vaccine Trial Among Patients With High-Risk Neuroblastoma With Prior Disease Progression*. J Clin Oncol, 2021. **39**(3): p. 215-226.
248. Fest, S., et al., *Survivin minigene DNA vaccination is effective against neuroblastoma*. Int J Cancer, 2009. **125**(1): p. 104-14.
249. Jing, W., et al., *Depletion of CD25(+) T cells from hematopoietic stem cell grafts increases posttransplantation vaccine-induced immunity to neuroblastoma*. Blood, 2011. **117**(25): p. 6952-62.
250. Bate-Eya, L.T., et al., *Newly-derived neuroblastoma cell lines propagated in serum-free media recapitulate the genotype and phenotype of primary neuroblastoma tumours*. Eur J Cancer, 2014. **50**(3): p. 628-37.
251. Rio, D.C., et al., *Ethanol precipitation of RNA and the use of carriers*. Cold Spring Harb Protoc, 2010. **2010**(6): p. pdb prot5440.
252. Livak, K.J. and T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method*. Methods, 2001. **25**(4): p. 402-8.

253. Newman, A.M., et al., *Determining cell type abundance and expression from bulk tissues with digital cytometry*. Nat Biotechnol, 2019. **37**(7): p. 773-782.
254. Henrich, K.O., et al., *Integrative Genome-Scale Analysis Identifies Epigenetic Mechanisms of Transcriptional Deregulation in Unfavorable Neuroblastomas*. Cancer Res, 2016. **76**(18): p. 5523-37.
255. Lopez, G., et al., *Somatic structural variation targets neurodevelopmental genes and identifies SHANK2 as a tumor suppressor in neuroblastoma*. Genome Res, 2020. **30**(9): p. 1228-1242.
256. Merkel, A., et al., *gemBS: high throughput processing for DNA methylation data from bisulfite sequencing*. Bioinformatics, 2019. **35**(5): p. 737-742.
257. Okae, H., et al., *Genome-wide analysis of DNA methylation dynamics during early human development*. PLoS Genet, 2014. **10**(12): p. e1004868.
258. Gomez, S., et al., *DNA methylation fingerprint of neuroblastoma reveals new biological and clinical insights*. Epigenomics, 2015. **7**(7): p. 1137-53.
259. Martens, J.H. and H.G. Stunnenberg, *BLUEPRINT: mapping human blood cell epigenomes*. Haematologica, 2013. **98**(10): p. 1487-9.
260. Carninci, P., et al., *Genome-wide analysis of mammalian promoter architecture and evolution*. Nat Genet, 2006. **38**(6): p. 626-35.
261. Consortium, E.P., *An integrated encyclopedia of DNA elements in the human genome*. Nature, 2012. **489**(7414): p. 57-74.
262. Agarwal, V., et al., *Predicting effective microRNA target sites in mammalian mRNAs*. Elife, 2015. **4**.
263. Kozomara, A. and S. Griffiths-Jones, *miRBase: annotating high confidence microRNAs using deep sequencing data*. Nucleic Acids Res, 2014. **42**(Database issue): p. D68-73.
264. Lewis, B.P., C.B. Burge, and D.P. Bartel, *Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets*. Cell, 2005. **120**(1): p. 15-20.
265. Friedman, R.C., et al., *Most mammalian mRNAs are conserved targets of microRNAs*. Genome Res, 2009. **19**(1): p. 92-105.
266. Shin, C., et al., *Expanding the microRNA targeting code: functional sites with centered pairing*. Mol Cell, 2010. **38**(6): p. 789-802.
267. Subramanian, A., et al., *Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles*. Proc Natl Acad Sci U S A, 2005. **102**(43): p. 15545-50.
268. Beaulieu, M.E., et al., *Intrinsic cell-penetrating activity propels Omomyc from proof of concept to viable anti-MYC therapy*. Sci Transl Med, 2019. **11**(484).
269. Mlakar, V., et al., *11q deletion in neuroblastoma: a review of biological and clinical implications*. Mol Cancer, 2017. **16**(1): p. 114.
270. Stallings, R.L., et al., *Therapeutic targeting of miRNAs in neuroblastoma*. Expert Opin Ther Targets, 2010. **14**(9): p. 951-62.
271. Huang, H.Y., et al., *miRTarBase update 2022: an informative resource for experimentally validated miRNA-target interactions*. Nucleic Acids Res, 2022. **50**(D1): p. D222-D230.
272. Sadelain, M., R. Brentjens, and I. Riviere, *The basic principles of chimeric antigen receptor design*. Cancer Discov, 2013. **3**(4): p. 388-98.
273. de Visser, K.E. and J.A. Joyce, *The evolving tumor microenvironment: From cancer initiation to metastatic outgrowth*. Cancer Cell, 2023. **41**(3): p. 374-403.
274. Luttikhuis, M.E., et al., *Neuroblastomas with chromosome 11q loss and single copy MYCN comprise a biologically distinct group of tumours with adverse prognosis*. Br J Cancer, 2001. **85**(4): p. 531-7.
275. Ginhoux, F. and M. Guillemins, *Tissue-Resident Macrophage Ontogeny and Homeostasis*. Immunity, 2016. **44**(3): p. 439-449.
276. Christofides, A., et al., *The complex role of tumor-infiltrating macrophages*. Nat Immunol, 2022. **23**(8): p. 1148-1156.

## References

---

277. Freudenberg, K., et al., *Critical Role of TGF-beta and IL-2 Receptor Signaling in Foxp3 Induction by an Inhibitor of DNA Methylation*. Front Immunol, 2018. **9**: p. 125.
278. Olkhanud, P.B., et al., *Tumor-evoked regulatory B cells promote breast cancer metastasis by converting resting CD4(+) T cells to T-regulatory cells*. Cancer Res, 2011. **71**(10): p. 3505-15.
279. Togashi, Y., K. Shitara, and H. Nishikawa, *Regulatory T cells in cancer immunosuppression - implications for anticancer therapy*. Nat Rev Clin Oncol, 2019. **16**(6): p. 356-371.
280. Carpentier, M., et al., *Extrathymic induction of Foxp3(+) regulatory T cells declines with age in a T-cell intrinsic manner*. Eur J Immunol, 2013. **43**(10): p. 2598-604.
281. Zeng, Y., et al., *Anti-neuroblastoma effect of ch14.18 antibody produced in CHO cells is mediated by NK-cells in mice*. Mol Immunol, 2005. **42**(11): p. 1311-9.
282. Wienke, J., et al., *The immune landscape of neuroblastoma: Challenges and opportunities for novel therapeutic strategies in pediatric oncology*. Eur J Cancer, 2021. **144**: p. 123-150.
283. Farhood, B., M. Najafi, and K. Mortezaee, *CD8(+) cytotoxic T lymphocytes in cancer immunotherapy: A review*. J Cell Physiol, 2019. **234**(6): p. 8509-8521.
284. Liu, X., et al., *Selective inhibition of IDO1 effectively regulates mediators of antitumor immunity*. Blood, 2010. **115**(17): p. 3520-30.
285. Nayak-Kapoor, A., et al., *Phase Ia study of the indoleamine 2,3-dioxygenase 1 (IDO1) inhibitor navoximod (GDC-0919) in patients with recurrent advanced solid tumors*. J Immunother Cancer, 2018. **6**(1): p. 61.
286. Onda, M., K. Kobayashi, and I. Pastan, *Depletion of regulatory T cells in tumors with an anti-CD25 immunotoxin induces CD8 T cell-mediated systemic antitumor immunity*. Proc Natl Acad Sci U S A, 2019. **116**(10): p. 4575-4582.
287. Guo, C., et al., *Allelic deletion at 11q23 is common in MYCN single copy neuroblastomas*. Oncogene, 1999. **18**(35): p. 4948-57.
288. Maris, J.M., et al., *Allelic deletion at chromosome bands 11q14-23 is common in neuroblastoma*. Med Pediatr Oncol, 2001. **36**(1): p. 24-7.
289. Schleiermacher, G., et al., *Chromosomal CGH identifies patients with a higher risk of relapse in neuroblastoma without MYCN amplification*. Br J Cancer, 2007. **97**(2): p. 238-46.
290. Feenstra, I., et al., *European Cytogeneticists Association Register of Unbalanced Chromosome Aberrations (ECARUCA); an online database for rare chromosome abnormalities*. Eur J Med Genet, 2006. **49**(4): p. 279-91.
291. Aggarwal, B.B., *Signalling pathways of the TNF superfamily: a double-edged sword*. Nat Rev Immunol, 2003. **3**(9): p. 745-56.
292. Zapata, J.M., et al., *TNFR-associated factor family protein expression in normal tissues and lymphoid malignancies*. J Immunol, 2000. **165**(9): p. 5084-96.
293. Munzert, G., et al., *Tumor necrosis factor receptor-associated factor 1 gene overexpression in B-cell chronic lymphocytic leukemia: analysis of NF-kappa B/Rel-regulated inhibitors of apoptosis*. Blood, 2002. **100**(10): p. 3749-56.
294. Wang, C.Y., et al., *NF-kappaB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation*. Science, 1998. **281**(5383): p. 1680-3.
295. Zhi, Y., et al., *NF-kappaB signaling pathway confers neuroblastoma cells migration and invasion ability via the regulation of CXCR4*. Med Sci Monit, 2014. **20**: p. 2746-52.
296. Hermeking, H., *The miR-34 family in cancer and apoptosis*. Cell Death Differ, 2010. **17**(2): p. 193-9.
297. Rupaimoole, R. and F.J. Slack, *MicroRNA therapeutics: towards a new era for the management of cancer and other diseases*. Nat Rev Drug Discov, 2017. **16**(3): p. 203-222.

298. McArdle, L., et al., *Oligonucleotide microarray analysis of gene expression in neuroblastoma displaying loss of chromosome 11q*. Carcinogenesis, 2004. **25**(9): p. 1599-609.
299. Kondrack, R.M., et al., *Interleukin 7 regulates the survival and generation of memory CD4 cells*. J Exp Med, 2003. **198**(12): p. 1797-806.
300. Donadon, M., et al., *Macrophage morphology correlates with single-cell diversity and prognosis in colorectal liver metastasis*. J Exp Med, 2020. **217**(11).
301. Jayasingam, S.D., et al., *Evaluating the Polarization of Tumor-Associated Macrophages Into M1 and M2 Phenotypes in Human Cancer Tissue: Technicalities and Challenges in Routine Clinical Practice*. Front Oncol, 2019. **9**: p. 1512.
302. Ghosh, A., et al., *Whole-tumour apparent diffusion coefficient (ADC) histogram analysis to identify MYCN-amplification in neuroblastomas: preliminary results*. Eur Radiol, 2022. **32**(12): p. 8453-8462.
303. Cassetta, L. and J.W. Pollard, *Targeting macrophages: therapeutic approaches in cancer*. Nat Rev Drug Discov, 2018. **17**(12): p. 887-904.
304. Lanahan, S.M., M.P. Wymann, and C.L. Lucas, *The role of PI3Kgamma in the immune system: new insights and translational implications*. Nat Rev Immunol, 2022. **22**(11): p. 687-700.
305. Guerriero, J.L., et al., *Class IIa HDAC inhibition reduces breast tumours and metastases through anti-tumour macrophages*. Nature, 2017. **543**(7645): p. 428-432.
306. Li, F. and J.V. Ravetch, *Inhibitory Fcgamma receptor engagement drives adjuvant and anti-tumor activities of agonistic CD40 antibodies*. Science, 2011. **333**(6045): p. 1030-4.
307. Veschi, V., F. Verona, and C.J. Thiele, *Cancer Stem Cells and Neuroblastoma: Characteristics and Therapeutic Targeting Options*. Front Endocrinol (Lausanne), 2019. **10**: p. 782.
308. Mu, P., et al., *SOX2 promotes lineage plasticity and antiandrogen resistance in TP53- and RB1-deficient prostate cancer*. Science, 2017. **355**(6320): p. 84-88.
309. Gautier, M., et al., *Plasticity in Neuroblastoma Cell Identity Defines a Noradrenergic-to-Mesenchymal Transition (NMT)*. Cancers (Basel), 2021. **13**(12).
310. Dongre, A. and R.A. Weinberg, *New insights into the mechanisms of epithelial-mesenchymal transition and implications for cancer*. Nat Rev Mol Cell Biol, 2019. **20**(2): p. 69-84.
311. Kendersky, N.M., et al., *Lineage-dependence of the neuroblastoma surfaceome defines tumor cell state-dependent and independent immunotherapeutic targets*. bioRxiv, 2024.
312. Forster, R., A.C. Davalos-Misslitz, and A. Rot, *CCR7 and its ligands: balancing immunity and tolerance*. Nat Rev Immunol, 2008. **8**(5): p. 362-71.
313. Shields, J.D., et al., *Autologous chemotaxis as a mechanism of tumor cell homing to lymphatics via interstitial flow and autocrine CCR7 signaling*. Cancer Cell, 2007. **11**(6): p. 526-38.
314. Geraldo, L.H., et al., *CCL21-CCR7 signaling promotes microglia/macrophage recruitment and chemotherapy resistance in glioblastoma*. Cell Mol Life Sci, 2023. **80**(7): p. 179.
315. Cuesta-Mateos, C., et al., *CCR7 as a novel therapeutic target in t-cell PROLYMPHOCYTIC leukemia*. Biomark Res, 2020. **8**: p. 54.
316. Mastini, C., et al., *Targeting CCR7-PI3Kgamma overcomes resistance to tyrosine kinase inhibitors in ALK-rearranged lymphoma*. Sci Transl Med, 2023. **15**(702): p. eabo3826.
317. Stanley, E.R. and V. Chitu, *CSF-1 receptor signaling in myeloid cells*. Cold Spring Harb Perspect Biol, 2014. **6**(6).

## References

---

- 318. Mao, Y., et al., *Targeting Suppressive Myeloid Cells Potentiates Checkpoint Inhibitors to Control Spontaneous Neuroblastoma*. Clin Cancer Res, 2016. **22**(15): p. 3849-59.
- 319. Nandi, S., et al., *The CSF-1 receptor ligands IL-34 and CSF-1 exhibit distinct developmental brain expression patterns and regulate neural progenitor cell maintenance and maturation*. Dev Biol, 2012. **367**(2): p. 100-13.
- 320. Valind, A., et al., *Macrophage infiltration promotes regrowth in MYCN-amplified neuroblastoma after chemotherapy*. Oncoimmunology, 2023. **12**(1): p. 2184130.
- 321. Zhang, X., et al., *Analysis of Liver Tumor-Prone Mouse Models of the Hippo Kinase Scaffold Proteins RASSF1A and SAV1*. Cancer Res, 2016. **76**(9): p. 2824-35.
- 322. Hoesel, B. and J.A. Schmid, *The complexity of NF-kappaB signaling in inflammation and cancer*. Mol Cancer, 2013. **12**: p. 86.
- 323. Perkins, N.D., *The diverse and complex roles of NF-kappaB subunits in cancer*. Nat Rev Cancer, 2012. **12**(2): p. 121-32.
- 324. Goy, A., et al., *Bortezomib in patients with relapsed or refractory mantle cell lymphoma: updated time-to-event analyses of the multicenter phase 2 PINNACLE study*. Ann Oncol, 2009. **20**(3): p. 520-5.
- 325. Cornice, J., et al., *NF-kappaB: Governing Macrophages in Cancer*. Genes (Basel), 2024. **15**(2).

## 8. Annexes

### Annex 1. Clinical characteristics of the Liquidhope cohort.

| Clinical Parameters                | INRG stage   |              |              |
|------------------------------------|--------------|--------------|--------------|
|                                    | M            | L2           | Total        |
| Number of patients                 | 59           | 5            | 64           |
| <b>Age at diagnosis (months)</b>   |              |              |              |
| Median                             | <b>41.9</b>  | <b>17.8</b>  | <b>41.2</b>  |
| Range                              | [10.7 - 105] | [9.5 - 74.1] | [9.5- 105]   |
| <b>Sex</b>                         |              |              |              |
| Female                             | 21 (35.6)    | 2 (40)       | 23 (36)      |
| Male                               | 38 (64.4)    | 3 (60)       | 41 (64)      |
| <b>MYCN status</b>                 |              |              |              |
| Amplified (%)                      | 18 (30.5)    | 4 (80)       | 22 (37.3)    |
| Non-amplified (%)                  | 40 (67.8)    | 1 (20)       | 41 (69.5)    |
| Non-determined (%)                 | 1 (1.7)      | 0 (0)        | 1 (1.7)      |
| <b>11q</b>                         |              |              |              |
| Deleted (%)                        | 34 (57.6)    | 0 (0)        | 34 (53.1)    |
| Normal (%)                         | 23 (39)      | 5 (100)      | 28 (43.8)    |
| Non-determined (%)                 | 2 (3.4)      | 0 (0)        | 2 (3.1)      |
| <b>Relapse</b>                     |              |              |              |
| Yes (%)                            | 30 (50.9)    | 3 (60)       | 33 (51.6)    |
| No (%)                             | 29 (49.2)    | 2 (40)       | 31 (48.4)    |
| Median time to relapse (months)    | <b>17.12</b> | <b>24.73</b> | <b>17.47</b> |
| <b>Death</b>                       |              |              |              |
| Yes (%)                            | 24 (40.7)    | 3 (60)       | 27 (42.2)    |
| No (%)                             | 35 (59.3)    | 2 (40)       | 37 (57.8)    |
| Median time to death (months)      | <b>23.48</b> | <b>41.07</b> | <b>24.17</b> |
| <b>Plasma samples at diagnosis</b> | 59           | 5            | 64           |
| <b>Plasma samples at relapse</b>   | 18           | 2            | 20           |

**Annex 2. Ordinal regression analysis of differential tumor-infiltrating immune populations of Westermann cohort (GSE73517).**

| Immune Cell Population        | 11q-deleted vs. | Odds ratio                  | SE    | Z value | P value  |
|-------------------------------|-----------------|-----------------------------|-------|---------|----------|
| Naïve B Cells                 | MYCN-amplified  | 0.492                       | 0.573 | -1.238  | 0.216    |
|                               | Others          | 0.659                       | 0.885 | -0.472  | 0.637    |
| Memory B Cells                | MYCN-amplified  | 0.441                       | 0.569 | -1.440  | 0.15     |
|                               | Others          | 0.399                       | 0.979 | -0.939  | 0.348    |
| Plasma Cells                  | MYCN-amplified  | 0.49                        | 0.54  | -1.325  | 0.185    |
|                               | Others          | 0.668                       | 0.843 | -0.479  | 0.632    |
| CD8+ T Cells                  | MYCN-amplified  | 0.233                       | 0.585 | -2.486  | 0.0129*  |
|                               | Others          | 0.557                       | 1.008 | -0.581  | 0.5613   |
| Naïve CD4+ T Cells            | MYCN-amplified  | 0.767                       | NA    | NA      | NA       |
|                               | Others          | 5.637E-09                   | NA    | NA      | NA       |
| Resting CD4+ Memory T Cells   | MYCN-amplified  | 0.171                       | 0.595 | -2.963  | 0.003**  |
|                               | Others          | 0.101                       | 0.912 | -2.510  | 0.012*   |
| Activated CD4+ Memory T Cells | MYCN-amplified  | 1.13                        | 0.665 | 0.184   | 0.854    |
|                               | Others          | 2.798                       | 0.927 | 1.111   | 0.266    |
| Follicular Helper T Cells     | MYCN-amplified  | 0.283                       | 0.578 | -2.184  | 0.029*   |
|                               | Others          | 0.318                       | 0.833 | -1.377  | 0.168    |
| T Regulatory Cells            | MYCN-amplified  | 0.19                        | 0.578 | -2.883  | 0.004**  |
|                               | Others          | 1.184                       | 0.88  | 0.193   | 0.847    |
| Γδ T Cells                    | MYCN-amplified  | 0.1                         | 1.145 | -2.009  | 0.045*   |
|                               | Others          | 0.564                       | 1.215 | -0.472  | 0.637    |
| Resting NK Cells              | MYCN-amplified  | Underrepresented            | NA    | NA      | NA       |
|                               | Others          | The model does not converge | NA    | NA      | NA       |
| Activated NK Cells            | MYCN-amplified  | 0.157                       | 0.593 | -3.127  | 0.002**  |
|                               | Others          | 0.146                       | 0.875 | -2.200  | 0.028*   |
| Monocytes                     | MYCN-amplified  | 0.53                        | 0.529 | -1.201  | 0.23     |
|                               | Others          | 0.436                       | 0.971 | -0.855  | 0.392    |
| M0 macrophages                | MYCN-amplified  | 0.308                       | 0.569 | -2.071  | 0.0384*  |
|                               | Others          | 0.727                       | 0.866 | -0.368  | 0.713    |
| M1 macrophages                | MYCN-amplified  | 0.229                       | 0.556 | -2.649  | 0.008**  |
|                               | Others          | 0.851                       | 0.846 | -0.191  | 0.84869  |
| M2 macrophages                | MYCN-amplified  | 0.129                       | 0.602 | -3.405  | 0.000*** |
|                               | Others          | 0.585                       | 0.815 | -0.658  | 0.51     |
| Resting Dendritic Cells       | MYCN-amplified  | 0.588                       | 0.629 | -0.846  | 0.398    |
|                               | Others          | 0.353                       | 1.202 | -0.867  | 0.386    |
| Activated Dendritic Cells     | MYCN-amplified  | Underrepresented            | NA    | NA      | NA       |
|                               | Others          | The model does not converge | NA    | NA      | NA       |
| Resting Mast Cells            | MYCN-amplified  | 0.578                       | 0.63  | -0.875  | 0.382    |
|                               | Others          | 3.09                        | 0.893 | 1.263   | 0.207    |
| Activated Mast Cells          | MYCN-amplified  | 1.246                       | 0.543 | 0.405   | 0.686    |
|                               | Others          | 0.237                       | 0.965 | -1.491  | 0.136    |
| Eosinophils                   | MYCN-amplified  | 0.772                       | 0.555 | -0.467  | 0.64     |
|                               | Others          | 0.607                       | 0.871 | -0.573  | 0.567    |
| Neutrophils                   | MYCN-amplified  | 3.203                       | NA    | NA      | NA       |
|                               | Others          | 2.261E-08                   | NA    | NA      | NA       |

Significance codes: \*\*\*0.001; \*\*0.01; \*0.05. Vs.: versus. SE: standard error. NA: Not applicable

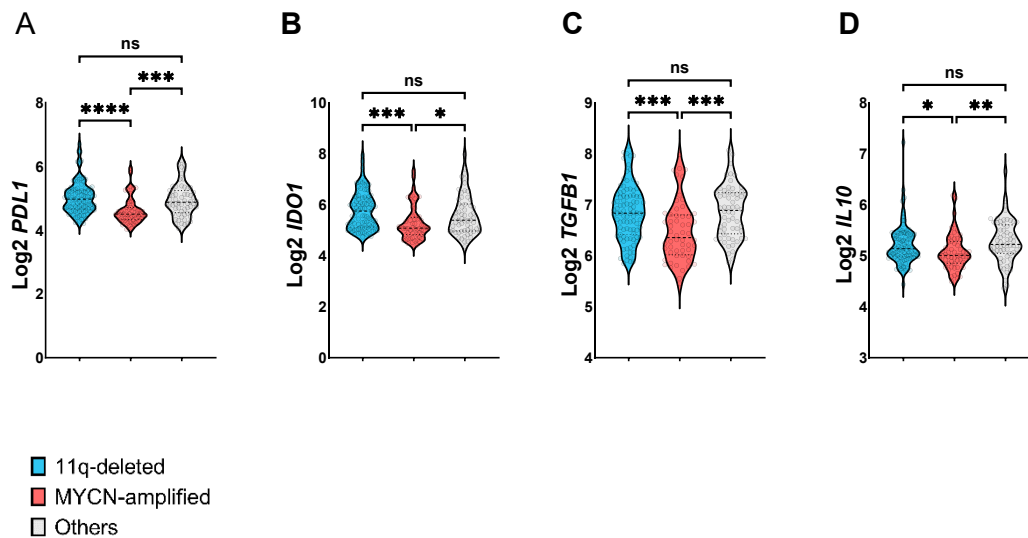


**Annex 3. Ordinal regression analysis of differential tumor-infiltrating immune populations of TARGET cohort.**

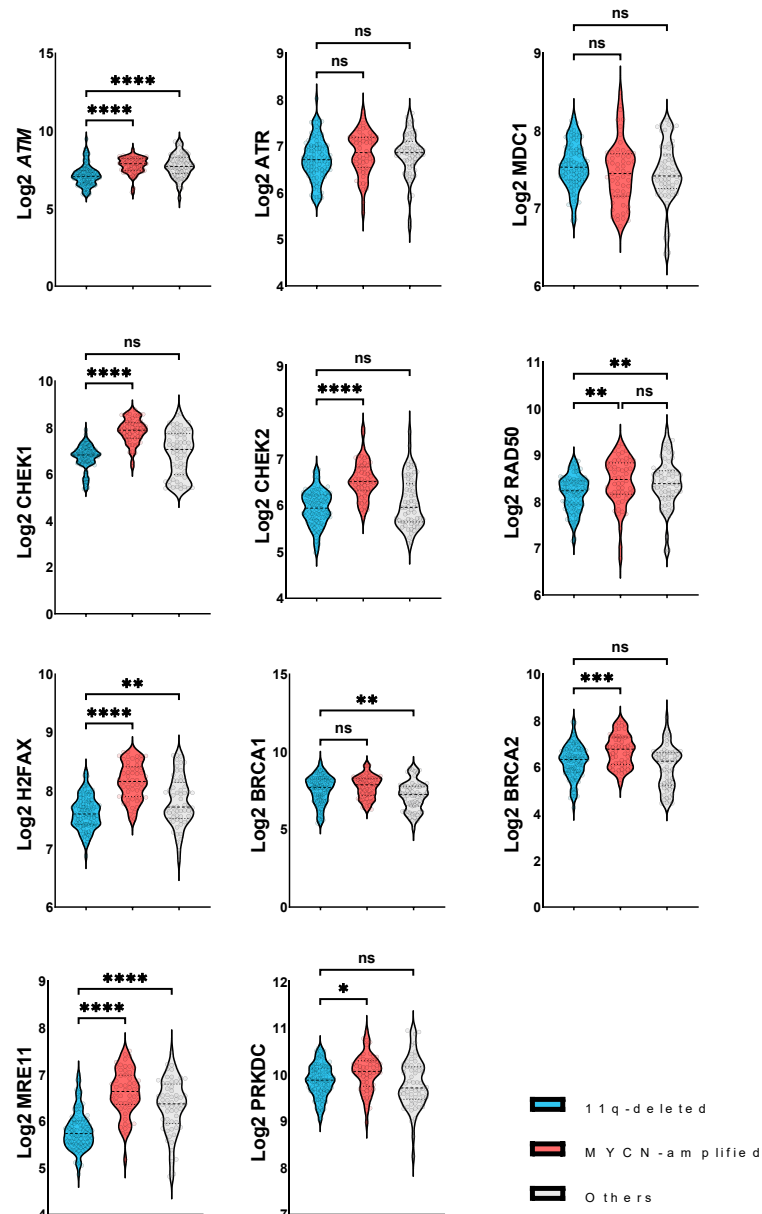
| Immune Cell Population        | 11q-deleted vs. | Odds ratio | SE    | Z value | P value      |
|-------------------------------|-----------------|------------|-------|---------|--------------|
| Naïve B Cells                 | MYCN-amplified  | 0.494      | 0.293 | -2.403  | 0.016*       |
|                               | Others          | 0.836      | 0.288 | -0.624  | 0.532        |
| Memory B Cells                | MYCN-amplified  | 0.741      | 0.340 | -0.881  | 0.379        |
|                               | Others          | 1.060      | 0.320 | 0.181   | 0.856        |
| Plasma Cells                  | MYCN-amplified  | 0.735      | 0.287 | -1.072  | 0.284        |
|                               | Others          | 1.034      | 0.293 | 0.113   | 0.910        |
| CD8+ T Cells                  | MYCN-amplified  | 0.550      | 0.290 | -2.406  | 0.040*       |
|                               | Others          | 0.757      | 0.292 | -0.955  | 0.340        |
| Naïve CD4+ T Cells            | MYCN-amplified  | 0.560      | 0.387 | -1.499  | 0.134        |
|                               | Others          | 0.524      | 0.397 | -1.629  | 0.103        |
| Resting CD4+ Memory T Cells   | MYCN-amplified  | 0.156      | 0.427 | -4.353  | 1.340e-05*** |
|                               | Others          | 0.847      | 0.311 | -0.535  | 0.592        |
| Activated CD4+ Memory T Cells | MYCN-amplified  | 0.286      | 0.398 | -3.145  | 0.002**      |
|                               | Others          | 1.370      | 0.316 | 0.997   | 0.319        |
| Follicular Helper T Cells     | MYCN-amplified  | 0.714      | 0.296 | -1.138  | 0.255        |
|                               | Others          | 0.731      | 0.285 | -1.099  | 0.272        |
| T Regulatory Cells            | MYCN-amplified  | 1.077      | 0.283 | 0.262   | 0.793        |
|                               | Others          | 0.665      | 0.296 | -1.378  | 0.168        |
| T $\delta$ T Cells            | MYCN-amplified  | 0.000      | NA    | NA      | NA           |
|                               | Others          | 1.637      | NA    | NA      | NA           |
| Resting NK Cells              | MYCN-amplified  | 0.746      | 0.286 | -1.026  | 0.305        |
|                               | Others          | 0.466      | 0.297 | -2.571  | 0.010*       |
| Activated NK Cells            | MYCN-amplified  | 1.983      | 0.286 | 2.397   | 0.017*       |
|                               | Others          | 2.851      | 0.305 | 3.440   | 0.000***     |
| Monocytes                     | MYCN-amplified  | 0.567      | 0.290 | -1.956  | 0.051        |
|                               | Others          | 1.119      | 0.292 | 0.386   | 0.700        |
| M0 macrophages                | MYCN-amplified  | 0.402      | 0.293 | -3.119  | 0.002**      |
|                               | Others          | 0.384      | 0.296 | -3.236  | 0.001**      |
| M1 macrophages                | MYCN-amplified  | 0.187      | 0.317 | -5.291  | 1.220e-07*** |
|                               | Others          | 0.590      | 0.286 | -1.842  | 0.065        |
| M2 macrophages                | MYCN-amplified  | 0.297      | 0.308 | -3.949  | 7.850e-05*** |
|                               | Others          | 1.195      | 0.287 | 0.620   | 0.535        |
| Resting Dendritic Cells       | MYCN-amplified  | 0.962      | 0.311 | -0.125  | 0.900        |
|                               | Others          | 1.521      | 0.303 | 1.383   | 0.167        |
| Activated Dendritic Cells     | MYCN-amplified  | 0.777      | 0.290 | -0.870  | 0.384        |
|                               | Others          | 1.064      | 0.300 | 0.208   | 0.835        |
| Resting Mast Cells            | MYCN-amplified  | 0.659      | 0.288 | -1.447  | 0.148        |
|                               | Others          | 2.287      | 0.298 | 2.772   | 0.006**      |
| Activated Mast Cells          | MYCN-amplified  | 1.822      | 0.365 | 1.644   | 0.100        |
|                               | Others          | 0.876      | 0.395 | -0.335  | 0.738        |
| Eosinophils                   | MYCN-amplified  | 2.060      | 0.583 | 1.240   | 0.215        |
|                               | Others          | 1.326      | 0.629 | 0.448   | 0.654        |
| Neutrophils                   | MYCN-amplified  | 1.847      | 0.331 | 1.853   | 0.064        |
|                               | Others          | 1.275      | 0.337 | 0.723   | 0.470        |

Significance codes: \*\*\*0.001; \*\*0.01; \*0.05. Vs: *versus*. SE: standard error. NA: Not applicable

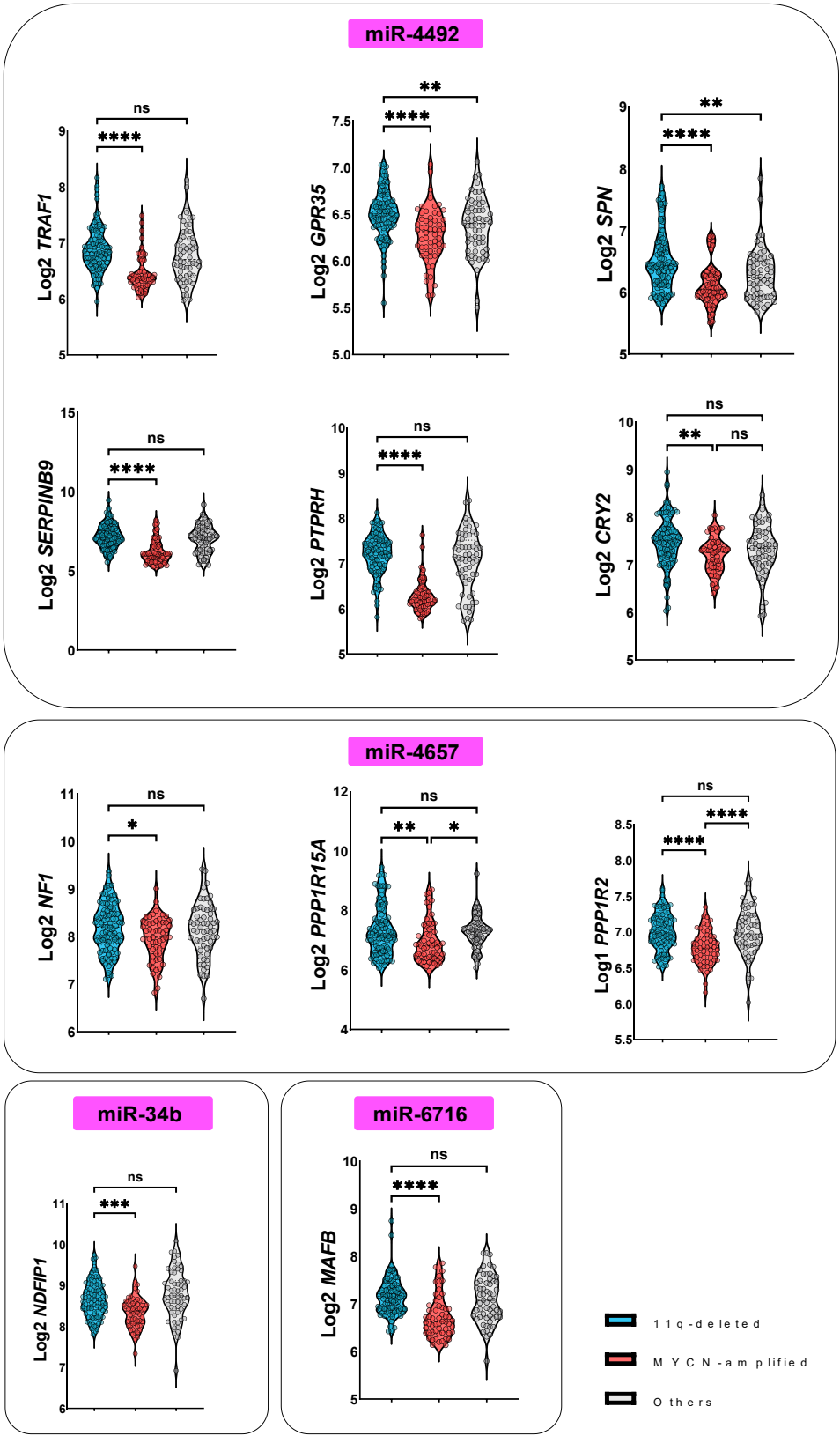
**Annex 4. Expression of immunosuppressive genes in TARGET cohort.** Differential RNA expression analysis was conducted on samples from 50 primary HR-NB cases (GSE131189, n = 215). The expression levels of (A) *CD274* (PD-L1), (B) *IL-10*, (C) *TGFB1* (TGF- $\beta$ 1), and (D) *IDO1* were examined in 11q-deleted high-risk (n = 95; blue), *MYCN*-amplified (n = 56; red), and other HR-NB (n = 60; grey) cases. One-way ANOVA was used to test the statistical differences in gene expression. \*P  $\leq$  0.05; \*\*P < 0.01; \*\*\*P < 0.001; NS: no significance.

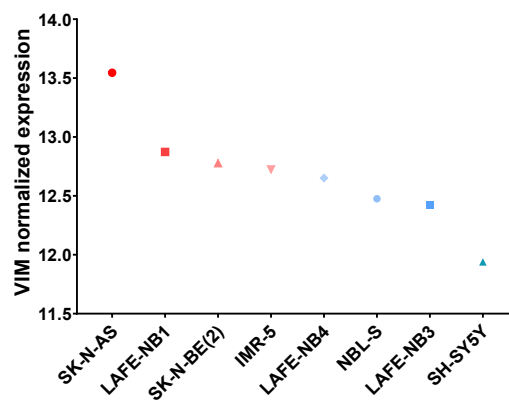
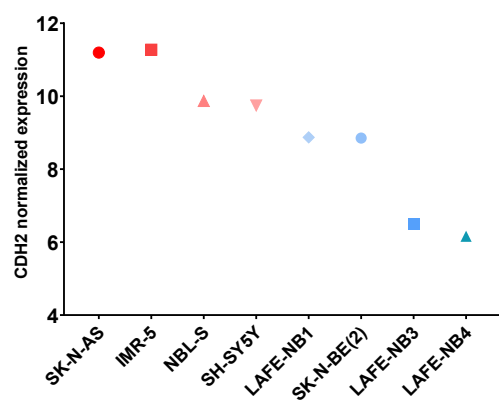


**Annex 5. Decreased expression of double-strand break DNA repair genes in 11q-deleted high-risk neuroblastomas within the TARGET cohort.** The analysis compared the gene expression profiles of DSB DNA repair genes across HR-NB subgroups within the TARGET cohort (GSE131189). Patients with 11q deletion are highlighted in blue, those with *MYCN* amplification are depicted in red, and the *others* subgroup is represented in grey. Statistical differences in gene expression were evaluated using one-way ANOVA, with significance levels indicated as \* $P \leq 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ , and "ns" indicating non-significant differences.



Annex 6. Differential expression of targeted genes by the 11q-deleted miRNA in the TARGET cohort.



**Annex 7. Baseline expression levels of VIM and CDH2 in neuroblastoma cell lines.**

**Annex 8. Methylation levels of the promoter region of DSB repair-related genes in the TARGET cohort.** We examined the methylation levels of the promoter region of genes involved in repairing DNA DSBs. HR-NB patients with 11q deletion are colored in blue, whereas those with MYCN amplification are in red. The reference group, referred to as normal, is depicted in grey. Statistical significance was determined using paired t-tests.

