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Experimental approaches for modeling and targeting resistant neuroblastoma

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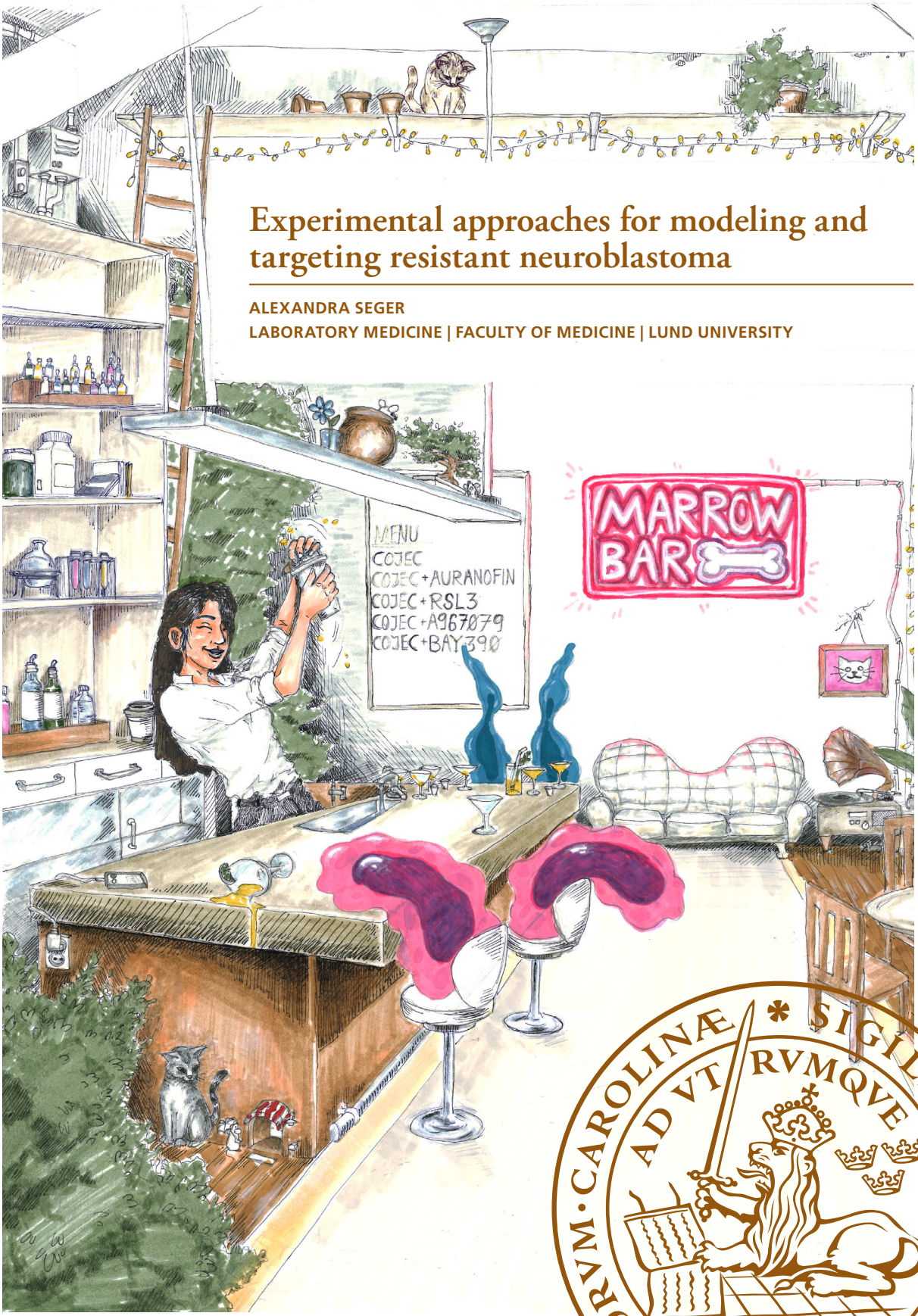

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Experimental approaches for modeling and targeting resistant neuroblastoma

Experimental approaches for modeling and targeting resistant neuroblastoma

Alexandra Seger

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DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Medicine at Lund University to be publicly defended on 14th of November at 09.00 the Auditorium, Medicon Village, Lund

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Abstract: High-risk neuroblastoma (NB) is a rare, solid childhood cancer with poor prognosis due to treatment resistance and metastasis. Improved patient outcome is dependent on the understanding of the resistance mechanisms and subsequent development of novel treatment options.

In Paper I, we established a clinically relevant *in vivo* treatment protocol using current standard-of-care chemotherapy (COJEC). We observed higher genetic diversity in intrinsically resistant NB, mesenchymal-like (MES) phenotype in NB with acquired resistance and an ADRN phenotype in treatment responsive NB. Tumor organoids derived from these treated and resistant NBs retained their phenotype and chemotherapy resistance *in vitro*.

In Paper II we focused on targeting ferroptosis, a therapeutic vulnerability in NB due to upregulation of antioxidant pathways. We highlighted the importance of carefully selecting the mechanisms of action for ferroptosis induction as treatments targeting GPX4 interfered with COJEC. Treatment combinations with COJEC and thioredoxin reductase inhibitor auranofin resulted in reduction of cells with the resistant MES-like phenotype.

In Paper III we investigated TRPA1 as potential treatment target due to its expression in relapsed NB. Inhibition of TRPA1 reduced viability of NB *in vitro*, but not *in vivo*. Pre-treatment using TRPA1 inhibitors resulted in additive or even synergistic effects with COJEC.

In Paper IV we established a novel humanized *in vivo* model using small human derived bones (hOss) and different injection methods to successfully simulate the different stages of the metastatic process. COJEC treatment reduced metastasis and resulted in minimal residual disease (MRD).

Overall, we established a clinically relevant *in vivo* chemotherapy protocol to investigate treatment resistant and relapsed neuroblastoma. By establishing a humanized *in vivo* model of metastatic neuroblastoma, we provided a platform to investigate metastasis treatment resistance and MRD. Uncovering sensitivities of resistant or relapsed NB, we investigated ferroptosis induction and TRPA1-inhibition and their combability to COJEC.

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Original articles and manuscripts

This thesis is based on the following papers:

I. Clinically relevant treatment of PDX models reveals patterns of neuroblastoma chemoresistance.
Adriana Mañas, Kristina Aaltonen, Natalie Andersson, Karin Hansson, Aleksandra Adamska, **Alexandra Seger,** Hiroaki Yasui, Hilda Van Den Bos, Katarzyna Radke, Javanshir Esfandyari, Madhura Satish Bhave, Jenny Karlsson, Diana Spierings, Floris Fojer, David Gisselsson, Daniel Bexell.
Science Advances 2022 Oct 28;8(43):eabq4617.
doi: 10.1126/sciadv.abq4617

II. Targeted ferroptosis induction enhances chemotherapy effect in chemoresistant neuroblastoma.
Adriana Mañas, **Alexandra Seger,** Aleksandra Adamska, Kyriaki Smyrilli, Joachim T. Siaw, Katarzyna Radke, Erick Muciño-Olmos, Oscar C. Bedoya-Reina, Javanshir Esfandyari, Kristina Aaltonen and Daniel Bexell.
npJ Precis Onc. 9,311(2025)
doi: 10.1038/s41698-025-01090-6

III. Evaluation of TRPA1 as a therapeutic target in *MYCN*-amplified high-risk neuroblastoma.
Alexandra Seger, Dora Adamič, Erick Muciño-Olmos, Johannes Nilsson, Sofia Granados-Aparici, Isaac Vieco-Marti, Javanshir Esfandyari, Matilda Engström, Julia Martinez, Adriana Mañas, Samuel Navarro, Rosa Noguera, Kristina Aaltonen and Daniel Bexell
Pediatr Blood Cancer, 2025 Sep;72(9):231875.
doi: 10.1002/pbc.31875

IV. A humanized *in vivo* model of neuroblastoma bone metastasis
Alexandra Seger, Aleksandra Adamska, Javanshir Esfandyari, Paul Bourgine, Kristina Aaltonen and Daniel Bexell
Manuscript in preparation.

Papers not included:

I. Patient-derived models: Advanced tools for precision medicine in neuroblastoma.
Kristina Aaltonen, Katarzyna Radke, Aleksandra Adamska, **Alexandra Seger**, Adriana Mañas, and Daniel Bexell.
Front Oncology 2023 Jan 19,12:1085270
doi: 10.3389/fonc.2022.1085270

Abbreviations

ADR	Adrenergic
AHCT	Autologous hematopoietic stem cell transplant
ALK	Anaplastic lymphoma kinase
ALT	Alternative lengthening of telomerase
BM	Bone marrow
BSO	Buthionine sulfoxamine
CAM	chorioallantoic membrane
CDX	Cell line-derived xenografts
CNA	Copy number alterations
CT	Comupted tomography scan
CTC	Circulating tumor cells
ECM	extracellular matrix
EFS	Event-free survival
EMT	Epithelial to mesenchymal transition
EV	Extracellular vesicles
FISH	Fluorescence in situ hybridization
FPN	Ferroportin
FSP1	Ferroptosis suppressor protein 1
GEMM	Genetically engineered mouse model
GPX4	Glutathione peroxidase 4
GSH	Glutathione
GSSG	oxidized glutathione
HD	High dose
HDAC	Histone deacetylase

HIF	hypoxia-inducible factor
HR	High-risk
IDRF	Imaging-defined risk factors
IHC	Immunohistochemistry
INRG	International Neuroblastoma Risk Group
INSS	International Neuroblastoma Staging System
LD	Low dose
lncRNA	long non-codsing RNA
LOH	Loss of heterozygosity
LR	Low-risk
MES	Mesenchymal
MIBG	^{123}I-metaiodobenzylguanidine
miRNA	microRNA
MRD	Minimal residual disease
MRI	Magnetic resonance imaging
MRP1	Multidrug resistance protein 1
MSC	Mesenchymal stem cell
MSOD-B	Mesenchymal sword of Damocles
NB	Neuroblastoma
NCOA4	Nuclear receptor coactivator 4
ncRNA	non-coding RNA
NCS-1	Neuronal calcium sensor 1
OS	Overall survival
PDO	Patient-derived organoids
PDX	Patient-derived xenografts
PDX-O	Patient-derived xenograft-derived organoids
PHOX2B	Paired-like homeobox 2B
PMN	Premetastatic niche
PUFA	Polyunsaturated fatty acids

RA	irotretinoin
ROS	Reactive oxygen species
scRNA-seq	single-cell RNA sequencing
SIOPEN	International Society of Pediatric Oncology-European Neuroblastoma
siRNA	small interference RNA
TERT	Telomerase reverse transcriptase
TMA	Tumormicroarray
TME	Tumor microenvironment
TRF1	Transferrin receptor 1
TRPA1	Transient receptor potential anakryin 1
Trx	Thrioredoxin
TrxR	Thoredoxin reductase
WES	Whole exome sequencing
WGS	Whole genome sequencing
X_c	Glutamate-cystine antiporter

Popular scientific summary

Neuroblastoma (NB) is a rare childhood cancer with the average patient age of 18 months. Despite its rareness, it accounts for approximately 15% of all childhood cancer related deaths. NB arises from the sympathetic nervous system and is often found close to the kidney in the adrenal gland. At the point of diagnosis, many neuroblastoma patients already have metastasis, meaning that the tumor is no longer just in one location but has spread in the body, most often to the bones. Depending on certain criteria, for example patient age, tumor size and location, NB can be further classified into different risk groups. Specifically for high-risk NB patients, the survival chance is extremely low as the tumors do not respond to treatment or grow back after. Currently, there are a lot of treatment options available such as classical chemotherapy, radiotherapy, surgery and even medication that can help the immune system to identify cancer and fight it. However, many tumors do not respond to treatment (upfront resistance) or stop responding after some time (acquired resistance). To find new therapeutic options, researchers need to understand how and why neuroblastoma is resistant to therapy. For this, we need ways to model it outside of the clinic. Here, we used NB tumor pieces and surgically implanted them into mice (PDX) where the tumor continued to grow. These tumors can also later be removed and made into small pieces (organoids) consisting of a few cells that can grow in the laboratory.

Within research, we often simplify clinical treatment by using only one or two chemotherapies. Neuroblastoma patients, however, receive a mix of five different chemotherapies, making the selection of only one or two not accurate. Within this work, we established a protocol using PDX models and treated them with the five chemotherapies used in the clinic, which is called COJEC. The material we obtained revealed that NB cells that were resistant to treatment expressed a pattern of genes that is referred to as “mesenchymal-like”, while NB cells that were sensitive to treatment expressed “adrenergic-like” genes. These two gene expression patterns are also found during the development of an embryo. Adrenergic cells have a specific role to become the adrenal gland, while the mesenchymal cells can still adapt to changes and do not have a defined fate. When neuroblastoma tumors were treated with COJEC, the cells with the adrenergic-like pattern disappeared, leaving only the mesenchymal-like ones. This suggests that new treatments should either focus on attacking these mesenchymal-like cells that remain after chemotherapy or be able to target both kinds of cells.

Cancer cells have a different metabolism than healthy cells. By re-creating the above-mentioned treatment regime in the laboratory setting, we were able to see how the metabolism of treatment resistant neuroblastoma differs. One of the discoveries was that NB uses a lot of iron. However, if a cell contains too much iron, it can easily become toxic and cause a cell death referred to as ferroptosis. Cancer cells use different ways to keep the balance of enough iron to function but not too much to become toxic. In the second study, we used 17 different therapeutics that are known to interfere with this balance to induce cell death. From these 17, we found two drugs to be the most promising due to their ability to successfully kill neuroblastoma cells from different patients. As our aim is to find a new treatment that can work with the existing therapies, we also evaluated how well they work with the five different chemotherapeutics used in the clinic. As various treatments focus on stopping different survival methods of cancer cells, it can happen that they accidentally stop each other from working instead. This is referred to as inhibition and a major issue as added treatment should never prevent a previously working treatment from having an effect. Within our study, we encountered this problem with one of the two agents we selected. Therefore, we focused on the other agent called Auranofin. Auranofin is a medication that has been used before in settings outside of cancer, making a transition to the clinical use easier, as potential side-effects are already known. When treating NB with Auranofin and COJEC, we observed that less of the chemotherapy resistant MES-like cells survived than when only COJEC was used.

In the third study, we identified that a calcium channel called TRPA1 was more prominent in tumors that had survived treatment and regrew. Additionally, this channel has been shown to be involved in a common side-effect of chemotherapeutic treatment called peripheral neuropathy. Peripheral neuropathy refers to the damage of nerves outside of the brain which is a painful condition that cannot be cured. Using inhibitors to stop the transfer of calcium into the cell via TRPA1, we were able to reduce the NB cell numbers in a laboratory setting. However, when we tried to treat mice with neuroblastoma, we could not observe any effect on the tumor sizes. Currently, companies are developing inhibitors that are more effective and safer for human use, as the ones from this study are not approved for patients and have shown limited effect in other diseases. As combination with COJEC showed that there was a higher amount of cancer cell death than when only using COJEC, future revisiting of this study could benefit patients to prevent damage to the nerves and treat neuroblastoma.

In the last study, we developed a model for neuroblastoma that has spread to bones. Within patients, these cancer cells have shown to be especially resistant to treatment, likely due to environment providing protection from chemotherapy. Currently, there are limited ways to model this, as researchers heavily rely on mouse models. However, cells in mouse bones differ from cells in human bones which limits their ability to communicate. This can be compared to mouse cells speaking a different

language than human cells. To properly study the way human bone and bone marrow cells interact with human tumors, we used small lab grown bones that were transferred into mice. During metastasis, the tumor and the cancer cells undergo changes when traveling to their new location. We mimicked these changes by injecting the cancer cells in different ways. For example, after leaving the original tumor, cells enter the blood stream and travel through the body. To model this behavior, we injected human NB cells directly into the blood stream of mice. Using advanced imaging techniques, we followed the spread of the cancer cells in the mice, like how doctors can image cancer in patients. Overall, we were able to not only model these different stages, but we also again applied the COJEC treatment to cancer cells that were hiding in the small human bones. While we were able to reduce their overall number, we discovered that one of these small bones still contained cancer cells that were not detected by the imaging. This is referred to as minimal residual disease and often goes unnoticed in patients, only to later regrow into a tumor. This showed that our new metastasis model was able to mimic a major problem also seen in the clinic which will provide opportunities to study these remaining cancer cells in the future.

Overall, this work improved our understanding of neuroblastoma that is resistant to chemotherapeutic treatment used on patients by unveiling how resistant and responsive cells differ in their gene expression. In addition, we created a new model that allows researchers to study neuroblastoma cells hiding in the bone and bone marrow to understand why these escape treatments. We further tested new therapeutics to target the resistant neuroblastoma cells and assessed the potential of combining them with the already existing chemotherapeutic treatment.

Populärvetenskaplig sammanfattning

Neuroblastom är en sällsynt barncancer. Patienternas medelålder är 18 månader. Trots att neuroblastom är mycket ovanligt står denna cancer för ungefär 15 procent av dödsfallen i barncancer. Neuroblastom uppstår från celler i det sympatiska nervsystemet och hittas ofta i binjurarna. Vid diagnos är sjukdomen ofta redan långt framskriden och har spridit sig i kroppen, något som kallas metastasering. Dessa metastaser finns oftast i skelettet eller i levern. Beroende på tumörens storlek, dess placering i kroppen, patientens ålder och några andra faktorer kan patienter delas in i olika riskgrupper. Särskilt de patienter som hamnar i högriskgruppen har låg chans att överleva, eftersom cancern ofta redan är resistent mot olika behandlingsalternativ.

Standardbehandlingar innefattar kemoterapi, kirurgi för att avlägsna tumören, strålbehandling och/eller läkemedel som stimulerar immunförsvaret att bekämpa tumören. Trots dessa olika möjligheter svarar många patienter inte på behandling. Denna resistens kan delas in i två kategorier: primär resistens, som finns från början, och förvärvad resistens, som oftast uppstår när tumören växer tillbaka efter behandling. För att forskare ska kunna studera dessa mekanismer och hitta sätt att kringgå eller förhindra dem, behövs modeller som kan efterlikna sjukdomen under kontrollerade former. I vårt arbete har vi använt så kallade PDX-modeller, där en bit av en patients tumör opereras in i en mus, där den kan fortsätta växa. Tumören kan senare delas upp i mindre enheter, som vi kallar "organoider", i laboratoriet.

Inom forskning används ofta förenklade versioner av kliniska behandlingar. Till exempel testas bara en eller två kemoterapier, trots att patienter i kliniken får en kombination av fem olika läkemedel. För att bättre återspegla verkligheten utvecklade vi i vår första studie ett protokoll där alla fem kemoterapier ingår. Denna kombination kallas COJEC. Med hjälp av detta protokoll kunde vi undersöka resistens och såg att bara vissa cancerceller dödades. De celler som svarade på behandlingen kallas ofta "adrenerga", eftersom de liknar celler i embryonalutvecklingen som senare utvecklas till binjuren. De celler som var resistenta hade däremot egenskaper som liknar mesenkymala stamceller och kallas därför mesenkymala neuroblastomceller. Dessa celler är mer flexibla och inte låsta till en bestämd utvecklingsväg, vilket gör dem mer motståndskraftiga och svåra att behandla. Slutsatsen är att nya terapier behöver riktas mot båda typerna av neuroblastomceller.

Cancerceller skiljer sig från normala kroppsceller på flera sätt; en viktig skillnad är ämnesomsättningen. Särskilt neuroblastomceller har ett ökat behov av järn. Samtidigt kan för höga järnnivåer vara giftiga och orsaka en särskild form av celldöd, ferroptos. För att hålla balansen använder cancerceller olika skyddsmekanismer. I vår andra studie testade vi 17 läkemedel som kan rubba denna balans och framkalla celldöd. Två av dessa läkemedel var särskilt effektiva mot cancerceller från olika patienter, och vi gick vidare med dessa. Vårt mål var att hitta nya terapier som kan kombineras med standardbehandlingen COJEC. Det är dock viktigt att de inte motverkar varandra. Vi upptäckte att ett av läkemedlen hämmade effekten av COJEC, men det andra, auranofin, gjorde det inte. När vi kombinerade auranofin med COJEC såg vi att färre resistenta cancerceller överlevde än med COJEC ensamt.

I vår tredje studie fokuserade vi på en annan aspekt av cellens ämnesomsättning. Vi fann att en kalciumkanal på cellens yta, TRPA1, fanns i högre grad hos resistenta neuroblastomceller. TRPA1 är dessutom kopplad till en vanlig biverkan av kemoterapi, perifer neuropati, som innebär nervskador i armar och ben och är smärtsam. När vi blockerade TRPA1 med läkemedel såg vi en minskning av celler i våra organoider. Däremot kunde vi inte se en tumörminskning när vi testade samma läkemedel i möss. Två läkemedelsföretag utvecklar just nu nya TRPA1-hämmare, eftersom de vi använde i vår studie inte är godkända för människor och inte har visat tillräcklig effekt i andra studier. Kombinationen av en TRPA1-hämmare med COJEC gav dock bättre resultat i organoider än COJEC ensamt, vilket gör det intressant att upprepa studien med mer relevanta substanser.

I vår sista studie fokuserade vi på metastaserad sjukdom. Metastaserade cancerceller "gömmer sig i skelettet" och är ofta orsaken till återfall, eftersom de är resistenta mot behandling. Det finns idag få modeller för att studera detta i laboratoriet, och de flesta modeller bygger på möss. Problemet är att musceller inte är identiska med människoceller, vilket leder till brister i kommunikationen mellan cellerna – ungefär som om de talade olika språk. Därför utvecklade vi en modell där vi odlade små mänskliga ben i laboratoriet och transplanterade dem till möss. På så sätt kunde vi ge mänskliga cancerceller en mer naturlig miljö. Genom att injicera neuroblastomceller i vener kunde vi återskapa processen där cellerna sprider sig via blodet till det mänskliga benet. Med hjälp av avancerad avbildning kunde vi följa cellerna och studera hur de reagerade på behandling. Precis som i kliniken kunde många av cellerna dödas med kemoterapi, men några överlevde och var osynliga i avbildningen. Detta kallas ”minimal residual disease” (MRD) och är ett stort problem även hos patienter. Vår modell gör det möjligt att studera detta vidare.

Sammanfattningsvis har vårt arbete förbättrat förståelsen av kemoterapiresistenta neuroblastom. Vi har visat hur resistenta och känsliga celler skiljer sig på genuttrycksnivå, utvecklat en ny modell för att studera metastaser i ben, och testat nya terapier mot resistenta celler. Vi har också undersökt hur dessa kan kombineras

med befintlig behandling för att på sikt förbättra prognosen för barn med neuroblastom.

Populärwissenschaftliche Zusammenfassung

Das Neuroblastom ist ein seltener Kinderkrebs. Das Durchschnittsalter der Patienten beträgt 18 Monate. Obwohl Neuroblastome sehr selten sind, ist dieser Krebs für ungefähr 15 Prozent der Kinderkrebstode verantwortlich. Neuroblastome entstehen aus Zellen des sympathischen Nervensystems, sie sind häufig an der Nebenniere aufzufinden. Zum Zeitpunkt der Diagnose ist der Krebs allerdings schon sehr oft fortgeschritten und hat sich im Körper verteilt, was metastatisch genannt wird. Diese Metastasen sind meistens im Knochen oder in der Leber aufzufinden. Je nach Größe des Tumors, dessen Lage im Körper, dem Alter der Person und einigen anderen Kriterien können Patienten in verschiedene Risikogruppen unterteilt werden. Speziell Patienten, die der höchsten Risikogruppe haben eine niedrige Überlebenschance, da der Krebs häufig bereits gegen die verschiedenen Behandlungsmöglichkeiten resistent ist.

Als Standardtherapie werden oft Chemotherapie, Operationen zum Entfernen des Tumors, Strahlentherapie oder sogar Medikamente, die das Immunsystem dazu anregen, den Tumor zu bekämpfen, eingesetzt. Trotz dieser unterschiedlichen Möglichkeiten sprechen viele Patienten nicht auf die Behandlung an. Diese Resistenz kann in zwei Kategorien unterteilt werden: die Resistenz im Vorfeld, also eine Resistenz von Anfang an, oder eine erworbene Resistenz, die meistens dann entsteht, wenn der Tumor nach der Therapie wieder zurückwächst. Damit Wissenschaftler genau diese Resistenzen erforschen können und Möglichkeiten finden, diese zu umgehen oder zu verhindern, brauchen sie ein Modell, um den Krebs in einem kontrollierten Feld nachzuahmen. In dieser Arbeit haben wir so genannte PDX-Modelle verwendet, bei denen ein Stück des Patiententumors in eine Maus eingesetzt wird, in der dieser Tumor weiterhin wachsen kann. Der Tumor kann später in einem Labor in kleinere Einheiten geteilt werden, die wir hier „Organoide“ nennen.

In der Forschung werden die klinischen Therapiemöglichkeiten oft vereinfacht eingesetzt. Zum Beispiel werden nur ein oder zwei der Chemotherapien angewendet, obwohl Patienten in der Klinik eine Kombination von fünf verschiedenen Therapien erhalten. Um dies besser darzustellen, haben wir in unserer ersten Studie ein Protokoll entwickelt, bei dem wir alle fünf Chemotherapien einsetzen. Diese Chemotherapie-Kombination wird auch als

COJEC bezeichnet. Mit diesem Protokoll war es uns möglich, Aspekte der Resistenz zu erforschen, die uns gezeigt haben, dass nur bestimmte Krebszellen abgetötet werden. Diese Krebszellen, die auf die Chemotherapie ansprechen, werden oft auch als „adrenergisch“ bezeichnet, da sie Zellen in der Embryonalentwicklung gleichen, die später einmal zur Nebenniere werden. Jene Krebszellen, die Resistenz zeigen, entsprechen in ihren Eigenschaften mesenchymalen Stammzellen und werden daher oft auch als mesenchymale Neuroblastomzellen bezeichnet. Mesenchymale Stammzellen haben Eigenschaften, durch die sie flexibler sind und noch keinem bestimmten Pfad der Entwicklung entsprechen. Ähnlich wie diese, zeigen mesenchymale Neuroblastomzellen Eigenschaften, die sie resistenter machen, wodurch sie selbst nach der Chemotherapie noch bestehen. Daraus können wir den Schluss ziehen, dass neue Therapien darauf abzielen sollten, entweder beide Gruppen an Neuroblastomzellen zu attackieren oder sich zumindest auf die mesenchymalen Neuroblastomzellen fokussieren sollten.

Krebszellen unterscheiden sich von normalen Körperzellen in mehreren Aspekten; einer davon ist der Metabolismus der Zellen. Krebszellen, insbesondere die von Neuroblastomen, haben einen erhöhten Bedarf an Eisen. Allerdings kann eine zu hohe Konzentration von Eisen auch giftig für die Zellen sein und zu einem Zelltod führen, der Ferroptose genannt wird. Damit die Krebszellen nicht zu viel oder zu wenig Eisen aufnehmen, verlassen sie sich auf verschiedene Sicherheitsmaßnahmen, die das Eisen in Balance halten. In unserer zweiten Studie haben wir 17 Medikamente getestet, die genau diese Balance stören und zu einem Zelltod führen. Von diesen 17 haben wir zwei weiter getestet, da sie bei Krebszellen von verschiedenen Patienten effektiv waren. Eines unserer Ziele war, neue Therapien zu finden, die mit der derzeitigen Standard-Chemotherapie COJEC zusammen verabreicht werden kann. Da die einzelnen Therapien auf verschiedene Überlebensmethoden der Zellen abzielen, kann es vorkommen, dass sie miteinander agieren und einander gegenseitig hemmen. Diese Hemmung wollen wir verhindern, da eine neue Therapie nicht eine alte, bereits funktionierende stoppen sollte. In dieser Studie entdeckten wir, dass eines unserer Medikamente eine derartige Hemmung ausgelöst hatte, und daher fokussierten wir uns auf das andere. Dieses Medikament heißt Auranofin. Es wurde früher bereits bei anderen Erkrankungen eingesetzt und kann daher leicht für Krebspatienten verwendet werden, da potenzielle Nebenwirkungen bereits bekannt sind. Als wir Auranofin und COJEC kombinierten, konnten wir feststellen, dass weniger Chemotherapie-resistente Krebszellen überlebten, als wenn wir nur die COJEC-Chemotherapien verwendeten.

In der dritten Studie haben wir uns auf einen anderen Aspekt des Metabolismus der Krebszelle fokussiert. Wir konnten feststellen, dass ein Kalzium-Kanal auf der Zellenoberfläche, auch TRPA1 genannt, vermehrt auf resistenten Neuroblastomzellen vorkommt. TRPA1 wurde außerdem mit einer häufigen

Nebenwirkung von Chemotherapie in Verbindung gebracht, bei der die Nerven in Armen oder Beinen geschädigt werden. Diese Erkrankung wird als periphere Neuropathie bezeichnet. Sie ist relativ schmerzhaft. Mit Hilfe von Medikamenten, die die Aufnahme von Kalzium durch TRPA1 blockieren, konnten wir in unseren Organoiden eine Verminderung der Zellen feststellen. Als wir die Medikamente in einem Experiment in Mäusen mit Tumoren einsetzten, konnten wir jedoch keine Verkleinerung des Tumors feststellen. Derzeit gibt es zwei Pharmafirmen, die an neuen TRPA1-Inhibatoren arbeiten, da die in dieser Arbeit verwendeten nicht für Menschen zugelassen sind und in Studien von anderen Wissenschaftlern ebenfalls keine großen Effekte erzielen konnten. Die Kombination des Medikaments mit dem Chemotherapie-Cocktail COJEC erzielte ein besseres Resultat in der Krebszellenreduktion von Organoiden, als wenn nur COJEC angewendet wurde. Daher könnte eine Wiederholung unserer Studie mit relevanteren Medikamenten von Bedeutung sein, speziell mit dem Fokus auf die Verhinderung von peripherer Neuropathie.

In unserer letzten Studie haben wir uns vor allem auf die metastatische Erkrankung fokussiert. Diese Krebszellen, die sich sozusagen „in den Knochen verstecken“, sind meist der Grund für ein Wiederaufflammen der Krebserkrankung; sie sind resistent gegenüber Behandlungen. Derzeit gibt es nicht sehr viele Wege, diese Erkrankung im Labor nachzustellen, und die meisten Modelle verlassen sich sehr stark auf Mäuse. Das Problem ist, dass Zellen von Mäusen nicht vollkommen denen von Menschen gleichen und daher einige Aspekte der Kommunikation verloren gehen – in etwa so, als ob die Zellen verschiedene Sprachen sprechen würden. Daher haben wir ein Modell entwickelt, bei dem wir kleine menschliche Knochen im Labor gezüchtet haben, die dann in Mäuse eingesetzt wurden. Dadurch konnten wir den von Menschen entnommenen Krebszellen ein Umfeld geben, das ebenfalls von Menschen kommt. Wenn Metastasen entstehen, müssen Krebszellen des originalen Tumors in die Blutbahn gelangen und zu dem neuen Ort reisen. Hierfür haben wir verschiedene Injektionswege genutzt, um diesen Prozess nachzustellen. Zum Beispiel wurden Neuroblastomzellen in die Venen gespritzt, sodass sie durch die Blutbahnen zu ihrem neuen Zuhause, dem menschlichen Knochen in der Maus, reisen konnten. Mit Hilfe von fortschrittlichen Bildgebungstechniken konnten wir die Krebszellen verfolgen und sehen, wo sie sich befinden. Wir haben außerdem auch getestet, was passiert, wenn wir Neuroblastomzellen, die sich in diesen menschlichen Knochen verstecken, mit Chemotherapie behandeln: Ähnlich wie in der Klinik wurden viele der Krebszellen abgetötet. Allerdings mussten wir feststellen, dass wir in einem Knochen Krebszellen hatten, die diese Behandlung überlebten und auf der Bildgebung nicht auftauchten. Dies ist ein Problem, das häufig auch bei Patienten auftritt und als minimale Resterkrankung bezeichnet wird. Diese Erkenntnis bestätigte, dass wir ein Problem in der Klinik in unserem Modell nachstellen konnten, wodurch wir hoffentlich in der Zukunft genau diese resistenten Krebszellen erforschen können.

Insgesamt hat diese Arbeit unser Verständnis von Chemotherapie-resistenten Neuroblastomen verbessert: Sie hat aufgezeigt, wie sich resistente und nicht resistente Neuroblastomzellen auf der Genexpressionsebene unterscheiden. Ein Gen ist wie ein Rezept in einem Kochbuch. Die Genexpressionsebene zeigt, wie oft und wie viel nach diesem Rezept gekocht wird. Darüber hinaus haben wir ein neues Modell entwickelt, mit dem wir Neuroblastomzellen, die sich im Knochen verstecken, untersuchen können. So wollen wir verstehen, warum diese Zellen der Behandlung entkommen. Außerdem haben wir neue Therapien getestet, die auf resistente Krebszellen abzielen. Dabei haben wir die Möglichkeiten bewertet, diese neuen Therapien mit der bereits bestehenden chemotherapeutischen Behandlung zu kombinieren.

Neuroblastoma

Neuroblastoma (NB) is the most common extracranial childhood tumor with an average age of 18 months at diagnosis, and originates from the sympathetic nervous system (1,2). Despite being a rare disease, approximately 7% of all childhood cancers, it accounts for 15 % pediatric cancer related deaths (3). NB is a highly heterogeneous disease with approximately 50% of patients being classified as "high-risk" (HR) which often present with wide-spread metastasis and large genetic aberrations (4). One of the big challenges in the field of NB is the high rate of relapses, metastasis and resistance to standard treatment.

Diagnosis

Neuroblastoma can arise anywhere along the sympathetic nervous system causing clinical symptoms to vary between locations. Most commonly, patients present with an abdominal distension or mass. Further, loss of appetite, weight loss, constipation, fever, anemia, hypertension, paralysis and general irritability are common symptoms. Especially with metastasis, bone pain, pancytopenia and bruising or swelling around the eyes, so-called "racoon eyes", can occur (5).

Initial diagnosis is performed by contrast computed tomography scan (CT), magnetic resonance imaging (MRI) for paraspinal lesions, and ^{123}I-metaiodobenzylguanidine (MIBG) scan for metastatic lesions. On occasion, ultrasound imaging can detect NB prenatally (6–10). In addition, urine tests can be performed as metabolites of noradrenaline and adrenaline are increased in about 75% of patients (11). Biopsies are performed to provide samples for histologic analysis, genetic assays, and chemical profiling. NB demonstrates as small, round and blue, undifferentiated cells in histological assessments and expresses proteins such as PHOX2B, synaptophysin and chromogranin A, which extinguishes it from other tumor types (5,12,13). Together with genetic assessment, these tests are needed to make accurate risk stratifications and treatment plans.

Staging

The first international staging for Neuroblastoma, International Neuroblastoma Staging System (INSS), was developed in 1986 based on the extend of surgical excision (14,15). Over the years, this lead to difficulties when analyzing the outcome of clinical studies based on INSS staging, as the same tumor could be classified as stage 1 or 3, depending on the extend of surgical removal (16).

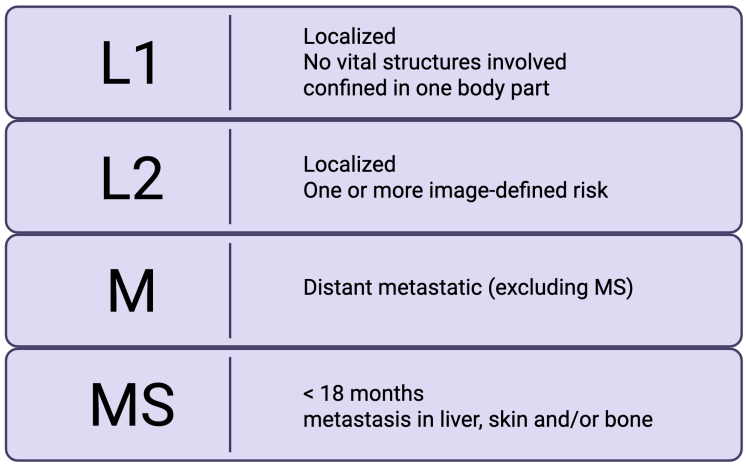

Figure 1 The International Neuroblastoma Risk group classification system.
Patients are divided into four subgroups based on imaging-defined risk factors and tumors locations.

Therefore a new classification system was developed, based on survival tree regression analysis of data from 8,800 patients, to provide clearly defined pretreatment cohorts (17). A survival tree regression is a non-parametric test used to predict the time until an event occurs or to identify risk factors that are connected to the event (18). The International Neuroblastoma Risk group (INRG) classification system is based on tumor imaging-defined risk factors (IDRFs), as these are more robust and can be uniformly assessed (Figure 1) (17). Based on the location for the tumor, the risks can include the tumor encasing the aorta, mesenteric artery or other large vessels, the tumor extending withing two body compartments or adjacent organs. Patients classified by this can be placed in one of four groups: L1, L2, M or MS. L1 are tumors that are localized and are not involving vital structures defined in the IDRFs and confined in one body compartment. L2 includes tumors that have one or more IDRFs. M is defined as tumors with distant metastatic disease that do not fall under MS.

MS is a special subgroup of metastatic disease where the patient is under 18 months with metastasis that are confined to liver, bone marrow (less than 10% of cells

replaced by tumor cells) and skin (17). Despite having widespread metastasis, these patients have a favorable outcome after surgical removal of the primary tumor and only need minimal supportive care as these tumors are known to spontaneously regress as long as no other genetic risk factors are present (19–21). While it is not yet clear why MS staged tumors tend to regress; studies have shown that epigenetically modifications or telomerase shortening could be playing a key role here (22).

Risk Factors

Besides staging patients based on their surgical risk factors, they can be further classified by age, genetic aberrations, favorable biological features, tumor size and symptoms (Figure 2). For example, L1 patients with an age above 12 months, that had a total resection are considered "low-risk" (LR), while patients with an incomplete resection are further divided by their *MYCN* amplification status. This subclassification is based on prognostic outcomes, such as *MYCN* amplification or histology, with clinical and biological factors in mind. Over 5,000 patient outcomes were statistically analyzed with a focus of recent diagnosis to incorporate the modern-day treatment and therapies, as these have made a major impact on survival (23).

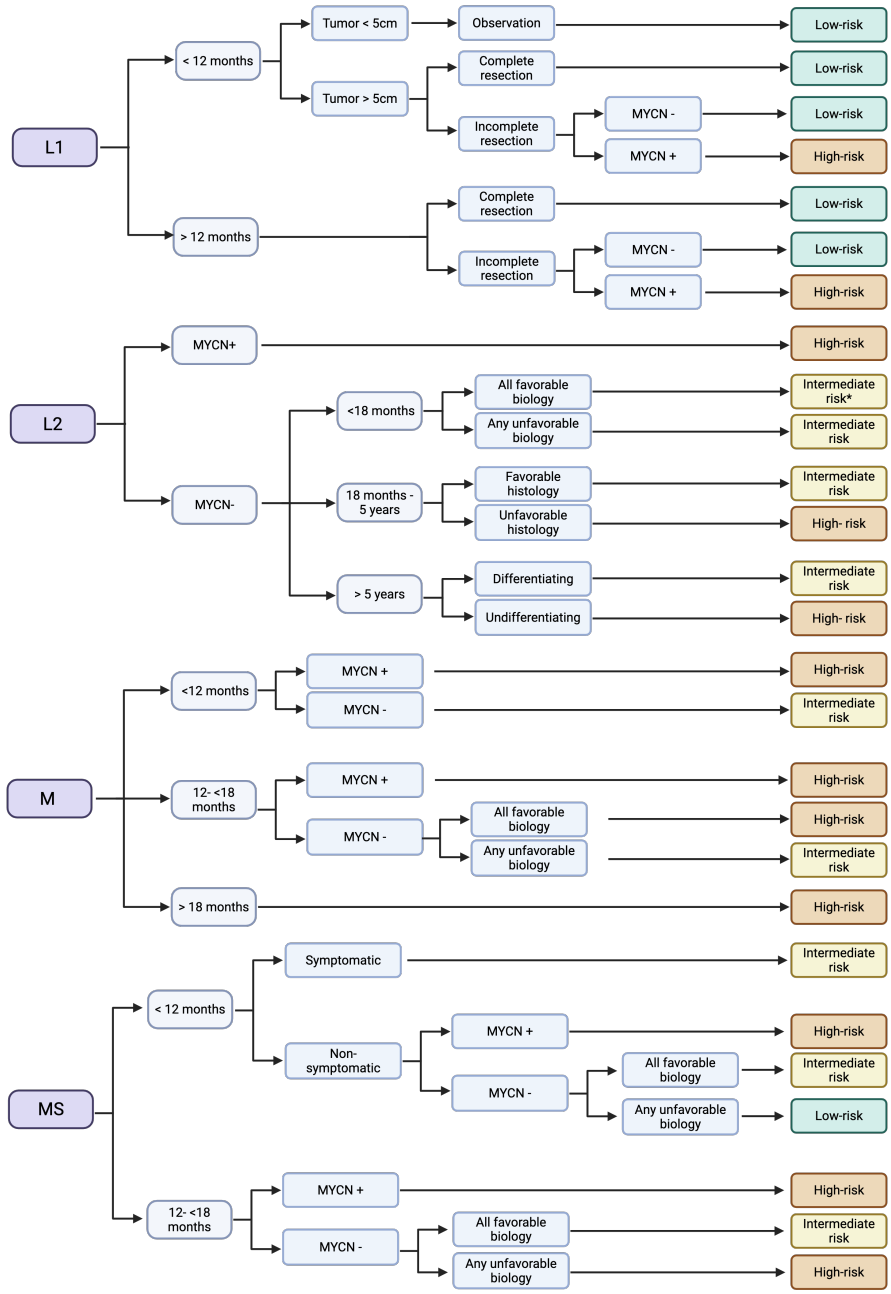

Figure 2 Simplified Risk Classification System.
Patient groups defined by surgical and imaging-defined risk factors on the left (L1, L2, M, MS) can be further divided into risk categories based on the genetical abberations, tumor size, age and histology. The resulting risk category is used for treatment decisions. Intermediate* indicates observation as primary treatment.

Screening

In many other cancers, screening methods have been developed and successfully reduced mortality rate by catching malignancies early. Several studies have been performed in the 80ies (24), 90ies and even during the early 2000s (25,26) where infants were screened for NB using urine tests. Analysis 20 years post-study showed that there was no decrease in stage 4/M stage diagnosis and no reduction in mortality, rendering the large screening as ineffective (27,28).

Cell of origin

Neuroblastoma has been extensively studied in regard to its molecular mechanisms, genetic aberrations, transcriptomic status and epigenetic changes (29). Körber et al. found that neuroblastoma develops in the first trimester of pregnancy due to aberrant mitoses. Their study also suggested that based on the length of tumor evolution prior to clonal expansion, the aggressiveness of the disease differs (30).

Neuroblastoma is often found in the adrenal gland, sympathetic ganglia, abdominal area, neck, thoracic and pelvic, all being along the sympathetic nervous system (31). Based on the location of NB, different biological and clinical features relating to higher malignancies have been identified, suggesting that there are different cells of origin depending on location (32). This classification has also been present in the *in vitro* setting where neuroplastic (N-type), non-neuronal Schwann cell-like (S-type) and morphologically intermediate (I-type) cell lines have been identified (33). Single-cell RNA sequencing (scRNA-seq) studies of patient tumors have identified several subpopulations that resemble either cells of the sympathoadrenal lineage or cells of the neuronal-crest derived precursors (34–36). Jansky et al. further compared neuroblastomas to normal human developing adrenal glands at varying stages of fetal and embryonic development. Contrary to the above-mentioned findings, they could not identify Schwann-cell precursors in the patient tumors. However, they could conclude that NB transcriptionally resembled developing adrenal neuroblasts, further suggesting that the differentiation state of NB along the normal neuroblast differentiation is connected to prognosis (37).

Genetics

Unlike adult cancer, pediatric cancers are prominently caused by large chromosomal aberrations and copy number alterations. Using whole genome sequencing (WGS) and whole exome sequencing (WES), Gröbner et al. have shown that pediatric cancers on average had 14 times lower mutational frequencies than adult cancers.

Further, from the 47% of pediatric cancers with at least one significantly mutated genes, only 30% overlapped with adult cancer-related significantly mutated genes (38).

Familial Neuroblastoma

Familial neuroblastoma is a rare occurrence of approximately 1-1.5% (39). Most cases of familiar neuroblastoma have been linked to mutations in the anaplastic lymphoma kinase (*ALK*) gene or the paired-like homeobox 2B (*PHOX2B)* gene. (40,41). Using WGS, missense mutations in the tyrosine kinase domain of *ALK* were found as a common denominator in neuroblastoma cases in eight separate families (41). Similarly, missense mutations in the homeobox of *PHOX2B* have been uncovered in several families (40,40,42).

Sporadic Neuroblastoma

Sporadic neuroblastoma represents the bulk of cases and often occurs with several genetic alterations.

Segmental chromosomal aberrations

Segmental chromosomal aberrations are defined as change in large chromosomal areas, usually identified by fluorescence in situ hybridization (FISH) or loss of heterozygosity (LOH) analysis. Aberrations or deletions in either entire or sections of chromosomal arms are some of the common forms in neuroblastoma. For example, LOH of *1p, 3p,4p* and *11q,* as well as gain of *1q, 2p, 12q* and *17q* is related to poor prognosis or outcome (43–49). Specifically, LOH of *1p* and gain of *17q* are strongly correlating with *MYCN* amplification, thereby classified as high-risk (44,48,50,51). Interestingly, LOH of *11q* is not correlated to *MYCN* amplification, but connected to chromosomal instability due to high numbers of segmental aberrations (50,52). *17q* gain also co-occurs with other segmental chromosome aberrations and is associated, either by itself or with *MYCN* amplification, with increase in reactive oxygen species (ROS) signature (53). WIP1, whose gene *PPM1D* is located on *17q,* has also been highlighted as an important factor in the negative regulation of p53 activity, influencing thereby cell cycle, apoptosis and DNA repair mechanisms (54). A less common form of segmental genetic aberration, chromothripsis, occurs in approximately 18% of stage 3 or stage 4 neuroblastoma (55). Chromothripsis describes genetic alterations where catastrophic breakage occurs resulting in 10s to 100s DNA fragments. Attempted repair of these chromosomes results in mixing of these fragments where parts are often lost to the cell (56).

ALK mutations

Normally, ALK is involved in the fetal development of the nervous system. Expression usually decreases after birth and is restricted mainly to the brain, small intestines and testis (57–59). While *ALK* knockout mice had no obvious defects or malformations, their brain showed similarities to a brain after long-term antidepressant use. These mice were reported to have an improvement in performance when it came to object location tests (58), suggesting that while ALK is not necessary for survival, it plays an important role in correct brain development. Within neuroblastoma, *ALK* mutations or amplifications are one of the common forms of aberrations, accumulating to approximately 18% of sporadic neuroblastoma cases (60). While the percentage is lower in primary tumors (between 10- 12%), it increases to 25% at the relapse point (61). This is also reflected in patient survival data, as changes in *ALK* correlate to poor outcome in primary neuroblastoma, but turn fatal in relapses (62). Mutations of *ALK* are commonly located in the kinase domain and occur as point mutations (59). As ALK has several downstream targets, such as ERK, PI3K and JAK/STAT, it helps to keep cells in an undifferentiated, proliferation state (57,63). Targeted therapy studies focusing on the inhibition of ALK, using lorlatinib, showed promising results, with 63% of patients responding when combined with chemotherapy (64). These results encouraged testing ALK inhibitors as a frontline treatment in newly diagnosed patients in a phase 3 study (65).

MYCN

MYCN is a transcription factor with many targets, regulating important cellular processes such as proliferation, metabolism, apoptosis and differentiation (66–70). During early development, *MYCN* expression is found in tissues such as brain, kidney, lung, heart and limbs (71–75). MYCN is further highly active during the development of the sympathetic nervous system, and controls both the migration and growth of neuronal crest cells. When cells mature to sympathetic neurons, becoming either chromaffin cells or nerve cells, MYCN reduces, suggesting that it is no longer needed at these steps. From experiments in zebrafish, we know that normal MYCN expression at this stage blocks chromaffin cell development, suggesting that missing or abnormal signaling can form early lesions that further turn into neuroblastoma. As MYCN regulates growth and apoptosis as a transcription factor, continuing expression of MYCN prevents removal of these excess or abnormal precursor cells (76,77). Further research has shown that MYCN plays a crucial role as a modulator in the p53/MDM2/p14^{ARF} negative feedback loop, thereby preventing the cells from undergoing apoptosis and resulting in a more chemoresistant disease (78).

In neuroblastoma, amplification of *MYCN* occurs in approximately 40% of high-risk cases and 25% of all neuroblastoma cases (79). High expression of *MYCN* has been identified to be a driver of neuroblastoma (77). Due to this, several researchers

have attempted to target and downregulate MYCN using for example RNA interference, DNA antisense oligomers or anti-gene peptide nucleic acid (80–84). Results commonly showed a cell cycle arrest in the G1 phase, morphological differentiation or apoptosis (70,82–84). Recently, Volegova et al. used an amidino-rocaglate to inhibit MYCN, by increasing eukaryotic translation initiation factor 4A1 affinity to polypurine-rich 5' UTRs which led to growth inhibition *in vivo* without major side effects (85). This suggests that MYCN plays a major role in the upkeep of an undifferentiated, proliferating state.

ATRX and TERT alteration

Another genetic alteration that is associated with poor overall survival is *ATRX* (53). ATRX is a protein that participates in chromatin remodeling at genomic sites such as telomeres and is mutually exclusive with *MYCN* amplifications (53,86). It is found in approximately 10% of neuroblastoma patients and leads to the activation of the Alternative Lengthening of Telomerases (ALT) mechanism. This process sustains the length of telomers, enabling proliferation without telomerase (87). While *ATRX* alterations are found more in older patients, alterations in another telomer lengthening protein, telomerase reverse transcriptase (TERT), is found in younger patients (53,87). *TERT* and *ATRX* alterations are mutually exclusive, possibly due to their similar function (53). Previously, *TERT* rearrangements and *MYCN* amplifications were found to be mutually exclusive, but newer studies identified patients harboring both modifications (53,88). Importantly to note, *TERT* expression has still be found upregulated in tumors without *TERT* alterations, as it is one of the targets of the transcription factor MYCN, but tumors with *TERT* rearrangements tend to have higher expressions (88).

Epigenetics

In general, malignancies such as cancer involve both genetic and epigenetic components. The epigenome is maintained by various mechanisms such as DNA methylation, histone modifications and non-coding RNAs. Changes in this balance can promote cancer progression, plasticity and drug sensitivity by altering transcriptional programs (89–93).

DNA methylation

DNA methylation regulates gene expression by adding or removing methyl groups to the C5 position of the cytosine (94). This process is done by DNA methyltransferases. Changes in the DNA methylation thus can alter the genes accessible to the cell and give rise to diseases such as cancer (89). Studies focusing on the DNA methylation in neuroblastoma have uncovered that a loss of caspase 8, which is involved in apoptosis, was associated with increased DNA methylation

levels and could be reversed by a demethylating drug called 5-aza (89,95). However, emerging studies connected the use of 5-aza to infertility (96), giving rise to concerns about its use. Other DNA methyltransferase inhibitors have been explored in combination chemotherapy, which resulted in an increased therapy response in NB cell lines, showcasing the potential of therapies targeting the epigenome (97). DNA methylation is not only necessary in cell proliferation but also important during development. Studies comparing the spontaneously regressing 4S tumors to other stages showed hypermethylation of genes involved in differentiation and neural crest development (98).

Histone modification and chromatin remodeling

Posttranslational modifications of histone tails can control chromatin configuration which in turn influence gene activity. If the chromatin structure is relaxed, it allows for genes to become activated while condensed chromatin causes genes to be silenced (99). Studies have shown that MYCN interacts and upregulates specific histone deacetylases (HDAC), which in turn promote expression of MYCN (100). Waldeck et al. were able to treat TH-MYCN transgenic mice with a pan-HDAC inhibitor, achieving fast tumor regression with no regrow (101). Combination of chemotherapeutics with such inhibitors further showed a synergistic effect on neuroblastoma cell lines (102).

Non-coding RNAs

Non-coding RNAs (ncRNAs), have been shown to negatively regulate gene expression by degrading mRNA or preventing translation (103). ncRNAs can be further divided into categories based on their size. Short RNAs are RNAs smaller than 200 nucleotides which include for example small interfering RNAs (siRNAs) and microRNAs (miRNAs). Long non-coding RNAs (lncRNAs) are longer than 200 nucleotides (104). ncRNAs have been widely studied as biomarkers, in the context of therapy resistance and development (89,105,106). In recent years, several studies showing the indirect influence of lncRNAs to chemosensitivity in neuroblastoma have been described (104). One example is the lncRNA *NDM29*, neuroblastoma differentiation marker 29, which suppresses the multidrug resistance protein 1 (MRP1), thereby leading to increased sensitivity to cisplatin (104,107,108).

Tumor heterogeneity and cellular plasticity

Cellular plasticity is an essential process during development, but a hinderance when it comes to treatment of cancer, as tumor cells can change their phenotype to survive (109). In addition, neuroblastoma is highly heterogeneous both within the tumor and between tumors. This intra-tumoral and inter-tumoral heterogeneity is a challenge as it influences metastasis, therapy response and prognosis (35,110–112).

Genetic heterogeneity

Intratumoral heterogeneity is a large factor contributing to treatment resistance in the clinic. Schmelz et al. followed tumoral evolution via sequencing patient's tumor samples from several locations within a tumor and during several time points. Their results showed that mutations were timepoint specific and changed during the disease and treatment, confirming high temporal heterogeneity in NB. Interestingly, mutations such as specific *ALK* mutations, that can be targeted with treatments, were observed to be found in only some locations of the tumor, indicating further a large spatial heterogeneity (113). In addition, they found that the same mutation that was found in abundance at diagnosis was lost after chemotherapy treatment (113). Karlsson et al. performed similar studies investigating tumor samples from different locations and timepoints. Their results confirmed as well that mutations that were found prior to chemotherapeutic treatment were replaced by novel mutations. Interestingly, these findings were not tumor location specific, as they could be observed in primary and metastatic samples after treatment. However, the overall mutational burden was similar in pre- and post-treatment samples. When investigating the heterogeneity of subclones, they found it to be higher after chemotherapy than what they observed in the primary tumor (114). Interestingly, both studies found that metastatic clones diverged from primary tumor clones at an early stage, prior to the evolution of the detected primary subclones (113,114). Taken together, this suggests that sampling plays a crucial role for treatment decisions, as potential targets could be missed if only one location, within the tumor and the patient, is selected. Further, these studies show that tumors undergo evolution not just under treatment pressure, but also already prior, contributing to the high heterogeneity of NB.

Cellular plasticity

The most known form of cellular plasticity is the epithelial to mesenchymal transition (EMT) which happens during the metastatic process. Here, cancer cells undergo reprogramming to become more mesenchymal, which increases their mobility and alters their metabolism to increase survival during the metastatic process (115). As previously mentioned, subclassifications in NB cell types in the

in vitro setting have been reported as early as 1987, namely the neuroplastic (N-type), non-neuronal Schwann cell-like (S-type) and morphologically intermediate (I-type) (33,116). In 2017, similar phenotypes were identified in additional cell lines and novel epigenetic and transcriptional signatures were defined. Instead of the previously established names, these were referred to as adrenergic-like (ADRN) instead of the N-type, mesenchymal-like (MES) instead of the S-type, and mixed/intermediate instead of the I-type (36,117).

Adrenergic and mesenchymal cell states

The ADRN and MES cell states were defined based on membrane markers, epigenetic and transcriptional profiles. Like the N-type, ADRN cells are described to resemble cells differentiated along the adrenergic lineage while MES cells resemble undifferentiated cells such as neuronal-crest driven precursor cells, similar to the S-type (35,36,118–120). The first two publications starting the new era of ADRN/MES phenotypes were published in 2017 by van Groningen et al. and Boeva et al. (35,36). Van Groningens definition was based on cell line derived data using ChIP-sequencing, while Boevas further included patient derived samples (35,36). Both publications however reached the same conclusion – neuroblastoma consists of at least two phenotypically different cell states with plasticity between them. Further, treatment and relapse was associated with an increase in the MES cell state, suggesting it is involved in resistance (35,36). Van Groningen et al. followed up their first publication with functional studies in 2019, showing that NOTCH signaling in ADRN cells induced transcriptional reprogramming to the MES state (121). Since then, a variety of studies, using either the van Groningen, Boeva or their own signatures, investigated the different cell states in NB. Nevertheless, the genes included in the respective signatures are an ongoing discussion in the field, as results vary between material (cell lines, PDXs, patient tumors) and methods (ChIP-seq, single cell sequencing, multiomics approaches, etc) used (35,36,119–126). Particularly when comparing ADRN and MES signatures, the ADRN signature has been more consistently defined due to the cells similarity to the cells in the adrenergic development, but the MES signatures vary vastly, with some researchers not being able to identify it in their data cohort at all (127,128). As Durbin and Versteeg summarized in their 2024 review, MES-like states found in human samples bulk RNA sequencing data should be carefully reviewed, as RNA from infiltrating stroma cells can skew the data. However, evidence has shown that MES cells might be localized in regions of the tumor with higher inflammation and hypoxia, as suggested by their increased inflammatory signaling (128). This suggests that the lack of MES cells in some studies might be due to sampling location in the tumor.

Intermediate state

Besides the ADRN and MES cell states, a few publications have identified an intermediate state, sometimes also referred to as mixed or even Schwann cell precursor like state (36,129,130). While Olsen et al. referred to their findings of a Schwann cell precursor like state as an intermediate state in their pre-print paper (130), their paper from 2024 suggested that it a small subpopulation was instead a precursor state for the ADRN cell state (122).

Polar extremes, gradient or spectrum?

Due to the intermediate state observed by some researchers and the overlap of signatures between states and studies presented, an ongoing discussion about the plasticity of neuroblastoma cells was started. There is a hypothesis that instead of extreme binary states, there is either a gradient of cell states or a spectrum of distinct cells states. These additional distinct cell states may be induced by therapy or tumor microenvironmental influence (128). For example, the addition of retinoids to NB revealed a retino-sympathetic cell state caused by reprogramming of the super-enhancer states (131). Considering that NBs are highly heterogeneous tumors, a spectrum of cell states seems more likely, with the possibility to shift between states depending on therapeutic, genetic or micro-environmental pressure.

Implications for treatment resistance and relapse

Currently, most studies focusing on the MES cell's role in resistance and relapse are based on cell lines and confirmation using patient derived samples are not always available (128). However, these studies suggest that MES cells are a key factor in NB aggressiveness and resistance. For example, serial *in vivo* passaging of SH-SY-5Y cells revealed an increase in tumorigenesis and expression of drug resistance proteins (132). Recent investigation of a limited number of matched primary and relapsed samples revealed that patient tumors that seem to consist of mostly ADRN cells, had an increased MES-like signature at the relapse point. These findings are in accordance with several of the previous reported results found in cell lines and *in vivo* studies (35,118,124,125,128). Interestingly, results from a preprint further suggest that MES cells are resistant to treatment with retinoic acid, which is used in the clinic to differentiate NB cells. Instead of the desired effect, MES cells use it for proliferation and were even shown to synthesize it themselves (133). Further, studies on cell lines showed that MES cells seem to reduce the expression of GD-2, thereby becoming resistant to clinically used GD-2 immunotherapy (134). However similar experiments using primary derived NB cells suggested that GD-2 expression was independent of cell state and the authors instead indicated that GD-2 expression varies due to the heterogenous nature of NB (135).

In summary, literature suggests that cells in the ADRN state are responsive to chemotherapeutic treatment compared to MES and intermediate state cells (35,36,121,123,126,136), and chemotherapeutic treatment might target ADRN

cells, thereby selecting for the more resistant MES cell state (35,36). Opinions vary between whether MES-like cells or intermediate state cells show more resistance, as Yuan et al. for example identified the in-between state cells to have worse prognosis with a more aggressive disease (129). Taking it together, current data points to the need of treatments that target all cell states to eradicate neuroblastoma.

Current treatment of neuroblastoma

The treatment of neuroblastoma varies between patients and is based on risk factors and staging. Further, there are differences between the European standard, defined by the International Society of Pediatric Oncology-European Neuroblastoma (SIOPEN), and the United States standard therapy.

Low risk tumors

The treatment for LR tumors of choice is surgery or observation, as these have excellent outcomes with event-free survival (EFS) of 90% and overall survival (OS) of 99%. For cases where the tumor is unresectable due to unfavorable location or when the disease is symptomatic, chemotherapy can be used. Radiotherapy in this staging group is only used as emergency therapy (120–122). Observation is performed by imaging using MRI or ultrasound for imaging and measurement of catecholamine metabolite levels in the urine (140) and decided upon by the risk classification discussed in the chapter before.

Intermediate risk tumors

Treatment options for intermediate risk patients include chemotherapy with or without surgery, radiation therapy and surgery with following observation. Clinical trials have been performed to find the optimal number of cycles of chemotherapy needed with the aim of reducing long term side-effects. As the intermediate risk group contains stages L2, M and MS, all without *MYCN* amplification, the approaches are tailored to the individual patient and their risk factors (137,140,141).

High risk tumors

As previously discussed, high-risk patients face a low survival rate, with 50% or less. Due to this, the treatment for this patient group has evolved rapidly in the past

years and is a focus point for ongoing research. The current treatment lasts about 18 months and is separated into three sections (140,142).

Induction therapy and surgery

Induction therapy consists of an intensive multiagent chemotherapy, followed by surgical resection of the primary tumor, with the aim to reduce tumor burden, and peripheral blood stem cell harvest. Peripheral blood stem cell harvest is performed as preparation for the autologous hematopoietic stem cell (AHCT) rescue during consolidation therapy (143).

Around 80% of patients have a partial response to induction, but still only 20% have complete response (144). Chemotherapeutic treatment differs between North America and Europe. In North America, the regiment is given in five cycles using topotecan and cyclophosphamide in cycles 1 and 2, etoposide and cisplatin for cycles 3 and 5, and doxorubicin, vincristine and cyclophosphamide for cycle 4 (144–146). In Europe, the focus lies on treatment intensity, as was established by a randomized trial that compared standard timed treatment in 3 week cycles to a version with 10 day cycles (147). Comparison to the North American regiment revealed a reduced rate of acute grade nonhematologic toxicity but similar rates of partial, complete and OS survival rate (144,148). Chemotherapy in Europe consists therefore of rapid courses of vincristine, carboplatin and etoposide, followed by vincristine and cisplatin, and then vincristine, etoposide and cyclophosphamide, in 10-day cycles. This regiment is also referred to as rapid COJEC (147).

Even with rapid COJEC, approximately one quarter of patients require additional treatment to achieve treatment goals to proceed with surgery or consolation therapy. These additional treatments can consist of added chemotherapy (149), immunotherapy (150) and radiopharmaceutical therapy (151).

After induction chemotherapy, which aims to reduce tumor size and elimination for metastatic sites, surgical removal is attempted as this increases survival (152). While complete surgical resection is the main aim, unfavorable biology, such as involvement of large blood vessels, can limit success. Still, studies have shown that larger extent of resection correlated with higher EFS (152).

Consolidation therapy

Consolidation therapy is given with the aim to remove any remaining cancer cells, such as minimal residual disease (MRD), and to prevent relapse. Routinely, myeloablative chemotherapy, AHCT, followed by radiotherapy, is given (144).

In clinical studies, treatment with busulfan and melphalan instead of normal carboplatin, etoposide and melphalan consolidation therapy treatment achieved

reduced adverse effects and increased EFS of 50% versus 38% (153). North America performed clinical trials using a tandem transplant strategy where an initial stem cell transplant was performed, with thiotepa and cyclophosphamide treatment, followed by a second transplant with treatment of reduced doses of carboplatin, etoposide and melphalan. Patients assigned to this group had an increased 3-year EFS of 61.6% versus the normal treated patient group with 48.3% (144,154). European studies are now comparing this new North American therapy regime to the previously mentioned busulfan and melphalan treatment (155).

As previously mentioned, hematopoietic stem cell transplants are performed as part of the consolidation therapy treatment to restore bone marrow function. This involves harvesting and sorting blood from the patients after which they are infused back. This procedure does not come without its risks, as transplant-associated acute and chronic organ toxicities have been associated with ACST in neuroblastoma (156–158). Due to these adverse effects, a novel study explored the comparison of a group of patients who received standard COJEC + myeloablative chemotherapy with ACST to a group without those treatments. Instead, these patients were treated with naxitamab, a GD2-antibody, and GM-CSF. The results showed that there were no adverse effects and for the group of patients with local disease, a 100% EFS was observed (159), promising a potential adaptation of future consolidation therapy.

Radiation therapy is another component of consolidation therapy. Here, primary and residual secondary locations receive radiation, which has improved local control of MRD (160). Although clinical trials have been performed to assess if increased radiotherapy would increase local control, prospective evaluations showed that there was no evidence for it and additional approaches are needed (161).

Maintenance therapy

Maintenance therapy is used to improve EFS and consists, in Europe, of 13-cis-retinoic acid, also called isotretinoin (RA), and dinutuximab beta, given in 5 cycles. Isotretinoin is used as a differentiation agent in the setting of maintenance therapy. While clinical studies vary in their results about increased OS using only isotretinoin, its combination with GD2 antibodies has shown greatly improved OS (162–165).

Dinutuximab beta is an antibody targeting GD2, a commonly expressed cell surface marker on neuroblastoma (166,167). Anti-GD2 antibodies induce phagocytosis, apoptosis and indirect cell death via cell toxicity mechanisms (168). Its introduction to the therapy after consolidation therapy has greatly increase the survival of patients (167). Dinutuximab beta is a recloned version of dinutuximab, which is given together with IL-2 in the North American maintenance therapy (144). Usage of dinutuximab beta with IL-2 showed increase toxicity and prevented patients from finishing their cycles, causing the recommendation of using dinutuximab beta only

in combination with RA (169). Despite the success in improving survival using GD2-antibodies, its use is currently limited to economically developed nations due to its associated costs (170).

Late side effects

Given the harsh treatments given to NB patients, late side effects are not uncommon. The most prevalent one is hearing loss (between 85 – 95%), associated to platin based chemotherapeutics used in treatment (171–173). Endocrinopathies resulting in growth failure, thyroid disease, delayed or abnormal puberty and maturation, and insulin resistance are the second most common group of issues (172,174). Part of these issues have been contributed to the former use of total body irradiation during treatment (175). Recent results showed that even in absence of total body irradiation, the short statue is still prevalent (172). Reason for this could be the introduction of RA into the clinic, as RA advances bone aging and can cause premature physeal closure (176,177). Further common later side effects are dental problems, cataracts, gonadal failure, and renal, cardiac or pulmonary diseases (171,172). Throughout their life, NB survivors are at risk of developing second malignancies, especially acute myeloid leukemia (178,179). Aspects of mental health and learning abilities are also affected by treatments. Compared to siblings, former NB patients have an increase in anxiety and depression, higher attention deficit, more conflict with peers and are more antisocial or withdrawn. Regarding their learning abilities, survivors relied more on special education services than their siblings (180).

Treatment in the context of resistance neuroblastoma

Despite these harsh, multimodal treatments, half of the children with HR NB still relapse or even fail to respond to treatment. Survival for children with relapsed NB is less than 20%, in terms of 4-year survival, and the average progression free time is 6.4 months (144).

Relapsed and refractory tumors

Relapsed NB presents with a higher mutational burden than the primary tumor, for example in the RAS/MAPK pathway (181–183). Additionally, *ALK* mutations have found to be more frequent (62,182,184). With the emergence of these targetable mutations, a molecular guided therapy approach in refractory and relapsed NB has been found feasible in clinical studies (185,186).

Therapy approaches for this relapsed and refractory patient group originally included mainly additional chemotherapeutic treatment or ^{131}I-MIBG, the latter having increased response-rates to a mean of 30% (187). Clinical studies investigated the potential of combining ^{131}I-MIBG with other agents, namely irinotecan and vincristine or vorinostat. Combination with vorinostat, an HDAC inhibitor, had an improved response rate of 32% (188). In Europe, the ongoing LuDO-N trial investigates ^{177}Lu-DOTATATE as a replacement for ^{131}I-MIBG with the aim to asses treatment related toxicity and long term survival (189,190). ^{177}Lu-DOTATATE targets somatostatin receptors and has previously been used in other adult neuroendocrine tumors which resulted in higher quality of life than their standard treatment (191). Additionally, previous and ongoing studies are focusing on the optimization of chemotherapeutics used for this patient group. One of these clinical studies investigated the combination of irinotecan-temozolomide and dasatinib-rapamycin in relapsed or refractory neuroblastoma. Results in 2024 showed that, while not statistically significant, the overall survival of patients with this treatment was increased substantially (192).

In addition, clinical trials trying to improve immunotherapy for this patient group has been a focus in recent years. A phase I/II trial investigated the use of GD2-

CART cells. Overall, the response rate was 63%, with approximately half of the patients having a partial response and half a complete response. Out of the 27 patients, 74% experienced cytokine release syndrome, albeit 95% were mild. Taken together, GD2-CART cell treatment seems like a feasible option for this patient group (193). Another immunomodulatory treatment that was studied in a clinical trial is the use of dinutuximab beta as a long-term infusion. Overall, the aim was to establish an acceptable pain to toxicity routine while still achieving immunomodulation. Unlike maintenance therapy, IL-2 was included in this study, as the previous study has only explored the combination in newly diagnosed patients. Results showed an impressive 2-yeal EFS of 56% and OS of 75% (194). Interestingly, statistical analysis of survival showed that relapsed patients had a worse outcome than refractory disease patients and the authors suggested that these patient groups should be separated in future studies (194). Currently, an active phase I/phase II study for patients with a focus specifically on relapsed HR NB is investigating the efficacy and safety of either adding dinuximab beta to the chemotherapy regiment consisting of irinotecan and temozolomide or adding bevacizumab to the same chemotherapy regiment (195).

Clinical studies are not just focusing on the management of relapsed or refectory neuroblastoma, but also on novel detection methods for the clinic. The MONALISA study specifically aims to investigate whether minimally invasive liquid biopsies can be used to detect NB cells. Further, they are developing an aid for clinicians with the interpretation of the data by implementing a digital decision-support tool. Using a remote patient reporting app, they will assess the effect of the liquid biopsies upon the patient's quality of life (196).

Treatment resistance

Despite these multimodal treatment approaches, treatment resistance remains an issue in the clinic. High risk neuroblastoma often presents with upfront resistance or acquired multi-drug resistance after long, repetitive treatments (197). While these resistance mechanisms can be mostly separated into non-genetic and genetic mechanisms, it is important to note that they are not mutually exclusive and can occur together (198). Furthermore, treatment resistance of tumors can occur by selection of specific clones which then in turn repopulate the tumors. Three different main paths of selection process have been proposed so far: Darwinian selection (Pre-existing resistance), gradual Darwinian selection (Acquired resistance) and Lamarkian induction (Acquired resistance) (Figure 3) (198).

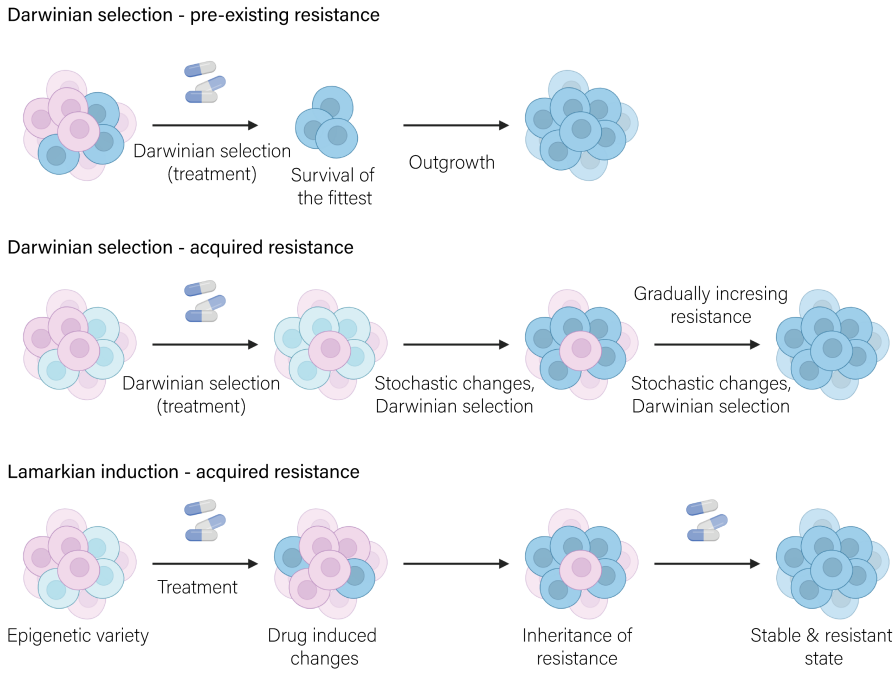

Figure 3 Overview of proposed selection processes with the focus on resistance.
Darwinian selection describes pre-existing resistances by survival of cells who already had a benefitial genetical or non-genetical changes. These cells then repopulate the now resistant tumors. Acquired darwinian resistance happens gradually during multiple steps of selecetion. Cells undergo stochastic changes, meaning random changes in genetics (mutations) etc. The darwinian selection selects clones with these random changes that have survival advantages until a resistant tumor forms. Lamarkin induction describes non-genetic, inheritable changes that give cells an advantage at surviving treatments. These changes are inherited by daughter cells and the tumor is repopulated by resistant cells.

Darwinian selection refers to what is also called “survival of the fittest”. Different clones compete over resources, whereas only the clone with the most beneficial genetical changes survives. In the context of treatment resistance, this means that only clones with pre-existing beneficial genetical changes survive the applied treatment to then repopulate the tumors. The gradual Darwinian selection and Lamarkian induction focus on acquired resistances. For the gradual Darwinian selection, random modifications of an organism guarantee its survival by surrounding, ongoing selection pressure for the “fittest clones”. The Lamarkian induction focusses on the inheritance of acquired non-genetic resistances, for example to treatment, to a new generation of cells. This, over a longer period of time, makes cells adapt to treatment pressure (198,199).

Genetic resistance

NB cells can adapt to treatment by undergoing genetic alterations. Genetic alterations such as on-target mutation or gain-of function mutations, down- or upstream, and activation of alternative signaling pathways can result in acquired resistance to therapy (200). On-target mutations can be tackled by developing additional inhibitors for these variants, as has been done for ALK-driven neuroblastoma. Point mutations on the *ALK* gene lead to resistance to crizotinib, which was circumvented with the novel inhibitor PF-06463922 in preclinical studies (201). Additionally, co-treatment with inhibitors targeting up- and downstream mutations or targeting alternative pathways can circumvent those acquired resistances (200).

Non-genetic resistance

Non-genetic drug resistances are driven by altered metabolism, epigenetic changes and/or selection processes (198). The most common non-genetic mode of resistance is the upregulation of efflux pumps. In NB, the multidrug resistance protein 1 (MRP1) has been shown to directly influence drug resistance as several of the first line treatments are MRP1 substrates (197). MRP1, and many other drug efflux pumps, are upregulated via the transcription factor MYCN (197).

Plasticity is another example that cancer cells can take for non-genetic drug resistance. While the nature of plasticity is still fairly unknown, researchers have pinpointed that chromatin remodeling and thereby epigenetic changes are required (198). Plasticity and selection often go hand in hand, as not all cells survive the treatment process, resulting in selection for cells with the capability to adapt. As previously mentioned, three cell states have been identified, ADRN, MES and intermediate. Investigation into NB cells post chemotherapeutic treatment revealed that the bulk of remaining NB cells exhibited a MES-like signature. These MES cells then repopulated the tumor with the higher proliferative ADRN state via the intermediate state. This resulted in a lower phenotype diversity of the regrown population, but selected for lineages with higher plasticity (202). In the case of ADR and MES cell states, cells undergo Darwinian selection as MES cells have a pre-existing resistance to chemotherapy (202). For future acquired resistances, it has been theorized that cells undergo gradual increased resistance, selecting for stable non-genetic alterations with the cost of losing their plasticity (198).

Another major influence in non-genetic resistance is the tumor microenvironment (TME). A more detailed discussion about the TME will follow in a later section. In the context of non-genetic resistance, it has been shown that cells within the TME such as immune cells, epithelial cells and fibroblasts interact with cancer cells via so-called extracellular vesicles (EV). EVs are secreted by the cells in the TME and can contain for example proteins, miRNA or lncRNA. These EVs can alter the TME

to an immunosuppressive state, increasing the resistance to immunotherapy, or transfer miRNAs from monocytes to NB cells which has been identified to increase resistance to cisplatin (197).

Targeted Therapies

Traditional chemotherapies target all fast-proliferating cells, making no distinction between healthy cells and tumor cells. Targeted therapies define therapies that affect specific pathways, receptors or mutations acquired by the cancer cells and can overcome limitations encountered by standard chemotherapy. GD-2 immunotherapy and ALK inhibitors are examples of successful targeted therapy currently in use in the clinic. As summarized in a review from 2025, there are a few targets under investigation for targeted therapies to overcome treatment resistance and/or relapse in neuroblastoma (203).

As just mentioned, ALK is one of the focus points currently. Although ALK inhibitors are already used in the clinic, new generations of ALK inhibitors are under investigations as *de-novo* mutations render the first generation ALK inhibitor crizotinib ineffective (203,204). Another interesting target is MYCN due to its abundant presence in high-risk NB. MYCN however is harder to target than ALK due to the lack of binding pockets. Indirect targeting by inhibition of pathways that activate MYCN has shown promise in clinical trials for various cancers including neuroblastoma. Additionally, degradation of MYCN using small molecules resulted in anti-tumor effects in clinical trials (203), giving hope for future therapies to target MYCN.

However, not just molecular targets are of interest. Targeted therapy can also be used to trigger alternative cell death pathways from apoptosis. As previously mentioned, approximately 40% of high-risk neuroblastoma cases have amplifications in *MYCN* which was shown to prevent apoptosis by modulating the p53/MDM2/p14^{ARF} negative feedback loop (78,79). As neuroblastoma has been shown to be highly addicted to iron and cysteine, triggering ferroptosis (an alternative, iron depending form of cell death) has become a novel target of interest (205). Ferroptosis in the context of neuroblastoma will be discussed in detail in a later chapter.

Not all targeted therapies are effective on their own. Approximately 78% of relapsed neuroblastoma have mutations within the RAS/MAPK signaling cascade, making it an interesting target (203). While preclinical data showed that MEK inhibitors were effectively reducing tumor burden, clinical testing resulted in disappointing results, due to PI3K/AKT signaling compensating for the loss of MEK. Combination of other agents with MEK inhibitors are currently under investigation to overcome this issue (203) and increase the effectiveness of these targeted agents.

Treatment combination strategy

Combination of one or more therapies can be used to target different cell states, pathways, or resistance mechanisms and has been a staple in the treatment of cancer for years. The first successful combination strategy was found in 1965 for acute lymphoid leukemia patients by administering methotrexate, vincristine, 6-MP and prednisone (206,207). While clinical results were positive for chemotherapeutic agents, the underlying mechanisms on how these worked were discovered years later (207). Until the 1970ies, the development of new chemotherapeutics was steady and improved clinical outcomes in various patient groups. By the early 1980ies, the market was saturated, and novel chemotherapies were only marginally better than the older generations, but more expensive. With the new understanding of signaling pathways and cell biology, in healthy and cancerous cells, targeted therapies were developed (207). This second wind in the cancer therapy development led us to the new strategies we use in the clinic. Still, our understanding of cancer and its treatment options is not enough to fully cure all patients, as resistance to agents persists. With the addition of many targeted therapies, new ways of pre-selecting agents to test in clinical trials emerged to reduce drug interactions and decrease toxicity. One of these strategies is synergy testing (208).

Drug synergy testing

Synergy testing refers to the investigation of interaction between two or more therapeutics. It is important to distinguish between efficacy and synergy, as efficacy refers to the phenotypic response to treatment, while synergy purely looks at the drug interactions (209). Synergy can be classified into roughly three categories: synergistic, additive or antagonistic, and is used to find combinations that have higher efficacy with reduced toxicity. The assumption behind it is that a combination of two or more drugs has no interactions. If the experimental response differs from this null hypothesis, either positively, or negatively, it can be classified as synergistic or antagonistic (208,209). Over time, different models have been developed, such as the Bliss Independence model, the Loewe additivity model and the ZIP model.

Bliss Independence model

The Bliss Independence model is based on probability and functions on the hypothesis that two therapies act independently to achieve the same effect. This translates to the therapies not interacting with each other but working together to achieve the result of, for example, reduced viability in cancer cells. If the combined effect on, in this case viability of the cells, is higher than expected, the model predicts synergy. If the combined effect is lower than expected, it predicts an antagonistic effect (210,211).

Loewe additivity model

The Loewe additivity model is based on a "sham experiment" where the drug A and drug B are combined to themselves, so drug A + drug A and drug B + drug B. These individual combinations are then compared to the combination of drug A + drug B. If there is a positive difference between the "sham experiment" and the "real" combination, there is synergy. The Lowe additivity works based on the assumption that there is no interaction within the drugs, and that the drugs are equivalent to each other based on two doses achieving the same effect, thereby assuming that they can be replaced by each other (212,213).

ZIP model

The ZIP model focusses on the comparison of the individual drug-response curves versus the combined drug-response curve. The null hypothesis here is that minimal changes in the combined drug-response is equivalent to the drugs not interacting. The benefit of this model is that the changes are calculated in percentage change to the expected outcome, providing a quantifiable outcome that can be used to compare results to other combinations (214).

Ferroptosis

High-risk neuroblastoma is resistant to apoptosis via several mechanisms, including MYCN inhibiting apoptosis via the p53/MDM2/p14^{ARF} negative feedback loop (78). Additionally, amplifications of *17q* containing the gene for Survivin, another anti-apoptotic protein, and hypermethylation of the caspase 8 gene *CASP8* contribute to neuroblastomas apoptosis resistance (76,95,215,216). Recently, *MYCN* amplified high risk neuroblastoma has been shown to be highly addicted to iron and cysteine, the latter being important for the glutathione pathway. By targeting said glutathione pathway, Floros et al. could show that neuroblastoma is sensitive to ferroptosis (205).

Ferroptosis is a form of peroxidation-driven, iron dependent cell death. Unlike apoptosis and necrosis, it is not a programmed cell death and does not rely on their regulators. Instead, free cellular iron interacts with lipids containing polyunsaturated fatty acid (PUFA) and oxygen. This leads to accumulation of lipid peroxides in the membrane, swelling of the cell and rupture of the membrane. Naturally, cells have developed a careful balance to regulate cellular sensitivity to ferroptosis (217–219) (Figure 4).

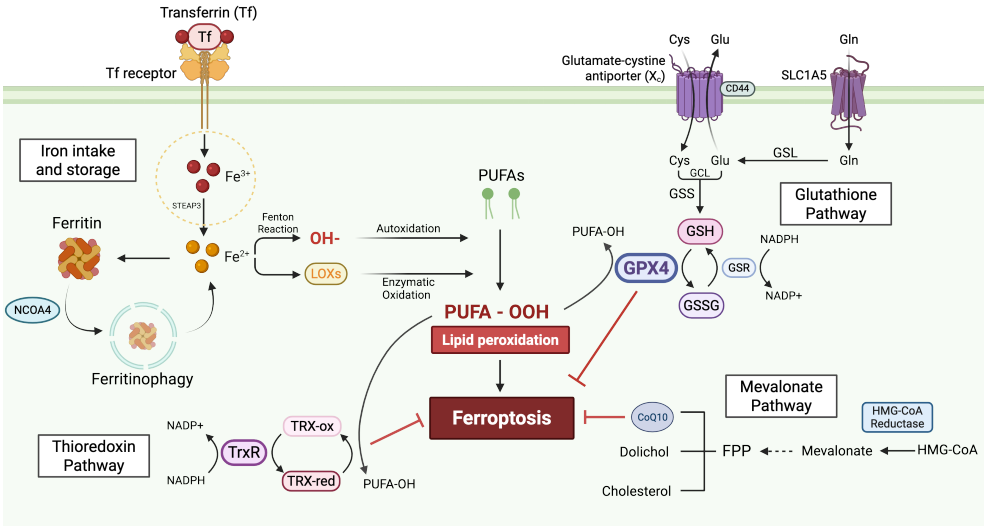

Figure 4 Ferroptosis pathways.
Overview of iron intake and storage and three different ferroptosis related pathways: Glutathione pathway, thioredoxin pathway and the mevalonate pathway.

Iron intake and storage

Within the cell, iron exists in different oxidative states: Ferric iron (Fe^{3+}) and ferrous iron (Fe^{2+}). Ferric iron is water insoluble and needs binding to a protein called transferrin to be transported. In the ferric state, iron is redox-inactive and harmless. Ferrous iron on the other hand is water soluble and redox active. An overload of ferrous iron can cause cell damage by producing reactive oxygen species (ROS) through the Fenton reaction (217,220). Within the cells, iron is stored in unstable iron pools called ferritin. When the cell needs iron, a process called ferritinophagy is performed where ferritin is transported to lysosomes via the nuclear receptor coactivator 4 (NCOA4) and degraded to release the stored iron (221). Another way for the cell to increase iron uptake is via the expression of transferrin receptors (TRF1), which increases the binding of transferrin and import of iron. Ferroportin (FPN) is used to export iron from the cells if intracellular iron levels need to be reduced (217). Iron itself is needed by cells to maintain proliferation, specifically during DNA replication. Therefore, fast replicating cells such as cancer cells, have a higher need for iron and many iron homeostasis related protein are increased in cancers to meet the metabolic need (222).

Glutathione pathway

As previously mentioned, cancer cells require a higher load of iron to meet their metabolic needs. A side-effect of this increased cellular iron is the production of ROS via the Fenton reaction. The glutathione peroxidase 4 (GPX4) is a cellular ROS scavenger that uses glutathione (GSH) to prevent lipid peroxidation (205,223). Specifically with the focus on neuroblastoma, MYCN has been shown to upregulate the intake of cysteine due to high GSH synthesis (205,224). Cysteine and glutamate are taken up via Glutamate-cystine antiporter (X_c) and form one of the building stones of GSH via formation of γ-glutamylcysteine. γ-glutamylcysteine interacts with glycine, imported into the cell via SLC1A5, and forms GSH (225). While reducing the lipid peroxides to non-toxic alcohol, GPX4 converts GSH to oxidized glutathione (GSSG) (226). Interestingly, the relation of GSH:GSSG has been found as a reliable marker for oxidative stress in pediatric patients (227). Flores et al. showed in their work that inhibition of GPX4 sensitized neuroblastoma to ferroptosis (205).

Thioredoxin pathway

Another important pathway keeping ROS at bay is the thioredoxin pathway. It is composed of thioredoxin (Trx), thioredoxin reductase (TrxR) and NAPDH. By providing electrons to the thiol-depending peroxidases, it removes ROS from the cells (228). Interestingly, the Trx system has been shown to be involved in angiogenesis as it stimulates HIF-1 and VEGF expression. Further, it has been suspected that a functional Trx system is required for tumor growth (229).

Mevalonate pathway

The mevalonate pathway results in Q10, an antioxidant coenzyme that is essential for the ferroptosis suppressor protein FSP1 function (230,231). To protect cells from lipid peroxidation, FSP1 reduces Q10 in an NADPH-dependent manner. Although the mevalonate pathway contributes to the selenoprotein synthesis, similar to the glutathione pathway, research has shown that the ferroptosis protective function of Q10 and FSP1 is independent and parallel to GPX4 (230–232).

Calcium in neuroblastoma

Calcium is ubiquitous in healthy and malignant cells and has been connected to various cellular functions. In the focus of neuroblastoma, calcium has been identified to be vital for example for differentiation, proliferation and apoptosis (233,234). Studies as early as 1986 showed that manipulation of the calcium homeostasis had an effect on morphological differentiation (235). Despite the discovery of intriguing data pointing towards the calcium metabolism as a potential target in therapy, our understanding of the channels, receptors and signaling pathways involved is still rather limited (234). The concentration of calcium in a resting cell is somewhere between 10 and 100 nM. This concentration can rise by releasing cellular calcium from stores or by influx via calcium channels, and reduced via efflux mechanism or by active transport mechanism to cellular storages (233). These mechanisms are altered in cancer and can lead to increased stemness and resistance to chemotherapeutic drugs (236).

Studies revealed that differentiation of NB cells is mediated by calcium increase post calcium release from internal storages, and is not dependent on specific receptors (233). Instead, it depends on the NB subtype. Experiments using retinoic acid revealed that S-type NB changed their morphology to a flattened state, resembling a glial morphology. N-type cells showed an increase in neurites and neuronal related morphology. Upon removal of RA, S-type cells remained in their differentiated morphology, while N-type cells reverted to their undifferentiated state. Besides the morphology, RA also affected store operated calcium entry, which remained altered in S-types as well (234,237).

Besides retinoic acid, neuroblastoma cells have shown to react with altered calcium levels to chemotherapeutics. For example, cisplatin has been shown to increase calcium in NB cell lines (236). Theories around the interaction between cisplatin and calcium resulting in treatment resistance have not been fully proven. However, it is suspected that long-term cisplatin exposure alters the expression of calcium homeostasis proteins and channels. This further activates calcium dependent anti-apoptotic pathways as well as cancer stem cell related transcription factors. These in turn promote proliferation and treatment resistance (236).

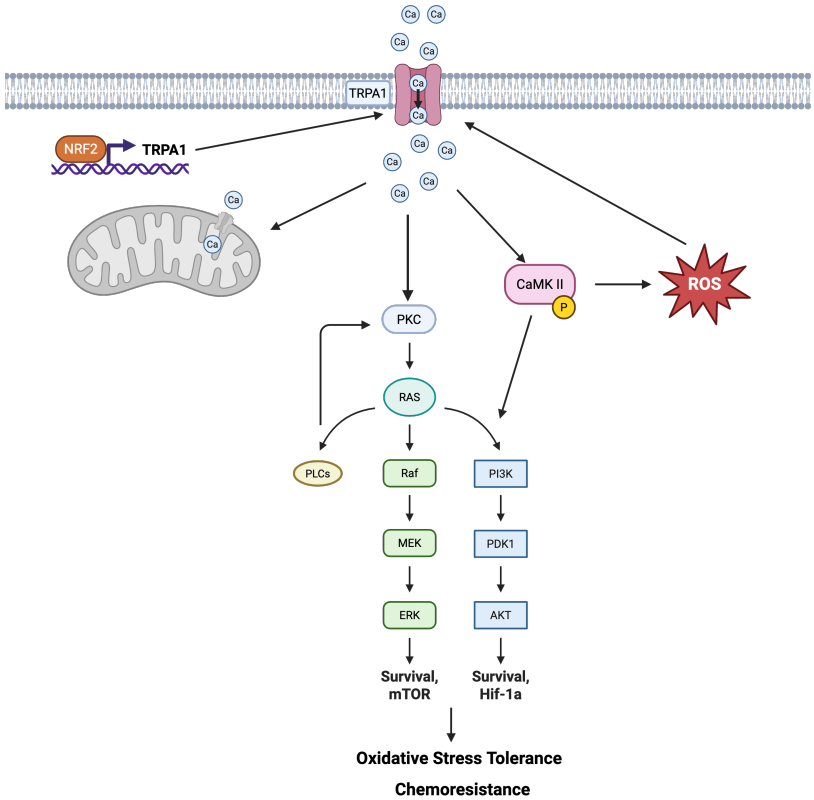

Figure 5 TRPA1 singaling pathway.
Upregulation of TRPA1 leads to increased upteake of calcium. This in turn upregulated downstream pathways that are pro-survival, increase oxidative stress tolerance and increase chemoresistance.

One of the channels linked to treatment resistance is the transient receptor potential ankyrin 1 (TRPA1). A publication in 2018 proved the interaction between the platin-based chemotherapeutic carboplatin and TRPA1 via ROS in breast cancer. Chemotherapy related increase in ROS upregulated the uptake of calcium via the ion channel TRPA1. This in turn upregulated the ERK pathway, PI3K/AKT pathway, mTOR-pathway and MCL-1, involving anti-apoptotic and pro-survival pathways (Figure 5). By inhibiting TRPA1, chemosensitivity to carboplatin could be restored (238). A similar concept was shown in 2023 while studying metastatic colorectal carcinoma (239). TRPA1 has further been shown to be involved in chemotherapy-induced neuropathy together with NCS-1, the neuronal calcium sensor 1 (240,241). Interestingly, NCS1 was shown to modulate the functionality of TRPA1 using a NB cell line (241).

Metastasis

Neuroblastoma is a highly metastatic disease, with 70% of patients presenting with metastasis at diagnosis (242). The most common sites for neuroblastoma metastasis are the bone and bone marrow followed by liver, lymph nodes, skin and, less often, lung and brain (243). Relapsed neuroblastoma, especially at the distant metastatic site, is a challenge in the clinic, as survival for patients with relapses drops from 50% to less than 10% (244,245). The process for cancer to move from the primary tumor to a new metastatic site is a dynamic, multistep process which involves the invasion of the circulatory blood or lymphatic system, exiting said system and establishing as micro metastatic site (246).

Metastatic process

Invasion is the first step in the metastatic process. During invasion, cell-cell adhesion is lost, leading to the dissociation of tumor cells from the primary tumor. Through changes in cell-matrix interaction, cells can invade the surrounding stoma by secreting substances to degrade the extracellular matrix (ECM) and increasing motility related proteins (247). One hallmark of cancer is the increase of angiogenesis to elevate nutrient transport to the fast-growing mass (248). These novel blood vessels give the cancer cells access to the circulatory system which allows them to travel to new locations.

This part of the metastatic process is referred to as circulation. During circulation, the circulating tumor cells (CTCs) experience sheer stress, metabolic reprogramming and immune reactions, causing their numbers to get reduced. Within the circulatory system, CTCs can get arrested in small capillaries or exit the circulation via extravasation (249).

Extravasation is the process of CTCs exiting the bloodstream by interacting with endothelial cells. As previously mentioned, cancer cells can get arrested in capillaries, which suggests that attachment to the endothelial cells follows the physical restriction due to capillary size. In certain locations however, such as the liver, CTCs can proliferate within the blood vessels first and then invade the tissue later (250–253). After attaching to the endothelial cells, CTCs secret factors to loosen endothelial junctions and disrupt basement membranes to transmigrate

through the endothelial layer. This migratory process can occur either between cells (paracellular) or through cells (transcellular) (253). While the majority of *in vitro* and *in vivo* research data shows that CTCs prefer paracellular extravasation, *in vitro* studies on colorectal cancer cells showed that these were able to migrate transcellular (253,254).

The premetastatic niche

As explained above, metastasis occurs over several steps, ending with the engraftment into the distant location. The microenvironment of the future metastatic site has suggested to undergo changes enabling metastasis (255). This phenomenon of a remodeled secondary location that enables tumor cells to disseminate and engraft is referred to as the premetastatic niche (PMN). For example, in lung cancer it has been shown that VEGFR1+ expressing hematopoietic progenitor cells aggregated in the bone marrow niche prior to the arrival of cancer cells. Under inhibition of VEGFR+, metastasis was suppressed. Literature mainly suggests that the remodeling of the PMN is done by the primary tumor via excretion of secretory factors and extracellular vessels which cause vascular leakages, suppress the immune system and remodel the ECM, prior to cells metastasizing (256–258).

Metastasis in neuroblastoma

In high-risk neuroblastoma, patients often present with metastasis already at diagnosis. Research on patient tumors showed that the clones that are found at the metastatic site already separated from the primary tumor at an early stage and only share a distant ancestor (113,114,259). As metastasis is a big challenge in the field of neuroblastoma, scientists have been trying to better understand the process and details of it for years. On a molecular level, several of the already discussed genetic alterations have been connected to metastasis. For example, MYCN has been identified as an enhancer of metastasis by interacting with regulatory partners involved in mechanisms such as motility, ECM degradation and angiogenesis (260). But also, other factors, such as the tumor microenvironment and specific chemokines attracting NB cells to the bone marrow niche, have been researched.

The premetastatic niche in neuroblastoma

In neuroblastoma, EVs have been shown to promote the change of mesenchymal stroma cells (MSCs) to a more pro-metastatic phenotype, specifically in the bone marrow (BM) niche. These pro-metastatic MSCs increased their production in IL-

6, IL-8 and VEGF (261,262). IL-6, a factor expressed by MSCs to activate osteoclasts, has further been identified to increase proliferation in NB cells (263), creating a pro-survival environment for NB. Similarly, injection of hypoxic EV in zebrafish aided NB proliferation at metastatic sites (264), indicating that several factors are necessary to create the perfect NB TME at metastatic sites. Interestingly, it has been suggested that chemotherapeutic treatment results in increased EV secretion containing proteins that accelerated metastasis (265). However, further research is needed to fully understand the process of priming the metastatic site for disseminating NB cells.

The bone and bone marrow niche in neuroblastoma

In the healthy population, the bone marrow is home to hematopoietic cells and several non-hematopoietic cells such as MSCs, adipocytes, chondroblasts and osteoblasts. Normally, the BM homeostasis is balanced by interactions between these cell types. Upon NB cells entering the BM niche, their interactions inevitably alter the homeostasis towards NB cell survival (266). Specifically, studies using single-cell transcriptomics and epigenomics on BM samples, from NB patients with and NB patients without metastasis, revealed a distinct enrichment in cell types that formed an immune-suppressive microenvironment as well as a decrease in myeloid cells in these BM niches (267,268). Additionally, researchers have identified that there is a presence of a distinct MSC subtype, found only in patients with confirmed bone metastasis. While the population was reduced during treatment, it re-emerged in several of the relapsed cases, indicating a connection to resistance (269). As mentioned in the premetastatic section, BM MSCs release factors that activate osteoclasts and can support NB proliferation. NB cells as well has been shown to release RANKL, another osteoclast activating factor, to promote osteolytic activity, increasing the space for the proliferating NB cells (266).

Minimal residual disease in the bone

Minimal residual disease is defined by a small number of cancer cells that survived treatment and can give rise to treatment-resistant tumors. NB patients with such relapsed, resistant disease have a very low survival chance of less than 10%. In the clinic, MRD can be detected by specific NB associated mRNAs in the patients' peripheral blood and bone marrow. Some of these mRNAs have even been shown to correlate with patients prognosis and were detectable already at diagnosis and during treatment (270,271). However, current treatments are not successful in eradicating MRD, and the mechanism of their resistance is not yet understood. Further research in this field is needed to improve treatment efficacy and patient survival.

Preclinical models

Pre-clinical research relies on ways to model the disease either in culture or in animals. While these methods have their restrictions and ethical concerns, usage of these has contributed vastly to the understanding of neuroblastoma as a disease and the possibility to quickly screen for potential therapies. In this chapter, some of the models used to research neuroblastoma will be discussed.

In vitro models

In vitro models are the oldest form of pre-clinical research. Starting with HeLa cells, derived from Henrietta Lacks in 1951 who died of an aggressive adenocarcinoma (272). Over the years, more complex models have been developed to better represent the disease, as will be discussed in the following chapter.

Cell lines

Cell lines are the oldest established method of researching cancer. When it comes to neuroblastoma derived cell lines, usages in research are not just limited to cancer. Using differentiation agents, cell lines are also used in the study of neuronal differentiation, neurodegeneration and neurotoxicity (273,274). Over 100 neuroblastoma cell lines have been characterized and used in research (275), with the most commonly used ones being SH-SY5Y and the parental line SK-N-SH (276). The usage of cell lines is highly advantageous as they are immortalized and genetically stable. While most cell cultures are a homogeneous culture, neuroblastoma cell lines have been shown to contain cells with different phenotypical presentation, as previously discussed (275,276). The advantage of these cell cultures is that their maintenance is relatively cost effective.

A high disadvantage with conventional cell lines is their growth as a 2D monolayer. This monolayer does not represent the natural structure of the tumor tissue, as cell-ECM and cell-cell signaling are missing. These connections play an important role in proliferation, drug metabolism and differentiation (277). Further, the exposure to the standard 21% oxygen in incubators does not recapitulate the physiological (2%-5%) or even hypoxic (1%) pressure cells undergo in tumors (278). Tumor cells

undergoing hypoxia activate hypoxia-inducible factors (HIF)-1 and HIF-2 to survive in oxygen lacking environments. Expression of these factors in tumors correlated to patient outcome, adding to the need of recapitulating these situations *in vitro* (279). In addition, the usage of serum in neuroblastoma cell culture has been shown to highly influence differentiation. Traditionally, cell lines are established in serum containing media to provide a big range of growth factors, which seems to select for more aggressive and fast proliferating cells. The derived culture therefore does not fully recapitulate the tumor population (280,281). Specifically for neuroblastoma, research has shown that serum containing media induced reduction of *MYCN* expression and differentiation to a sympathetic neuronal phenotype (281).

3D cultures

Development of 3D cultures has improved our ability of mimicking physiological conditions of tumors *in vitro*. Compared to conventional 2D culturing, cells in 3D culture build tight cell-cell adhesions, necrotic cores and have varying proliferation profiles depending on location in the structure (277,282). Two examples of 3D cultures are spheroids and organoids. Spheroids often consist of cell clusters, most commonly from cell lines, that are either free floating or held in shape by matrix. Organoids, however, are often defined as dense cluster of self-organizing cells that have the ability to differentiate and reflect the properties of an organ (283,284). This definition mainly comes from developmental and organ focused research, where the organoids are used to investigate function of tissues and cell organization (283). Therefore, they are often separated by their ability to mimic the organ on histological level, which is more accurately represented in organoids than spheroids (284).

Definition of 3D cultures in cancer research

In the context of cancer research, opinions in the nomenclature of 3D cultures differ vastly. Some argue that tumor derived 3D cultures should be referred to as (tumor) spheroids, as these cultures often lack tumor microenvironmentally derived cells and only consist of cancer cells. This goes against the definition of "typical" organoids, where different cell populations of an organ are reflected. In the context of neuroblastoma, it has been shown that these NB tissue derived cultures retain their tumorigenic and metastatic capability while also retaining patient-specific genomic aberrations (281). Together with the fact that NB tissue derived 3D cultures consist of different cancer cells, this author argues that they should be referred to as tumor organoids, as they more accurately represent the tumor. 3D structures engineered from cell lines, however, should be referred to as spheroids. In general, tumor organoids and spheroids can be established using three different preparation categories: 1) free floating in suspension, 2) cultured in gel-like substances and 3) cultured in scaffolds (277). In the field of NB, 3D cultures have been used for

important studies including high-throughput drug screens (285,286), co-cultures with for example fibroblasts and endothelial cells (283,287–290) and as an assessment tool for personalized immunotherapies (291).

Patient-derived organoids

Patient-derived organoids (PDO) are derived directly from patient tissue. While these PDOs have been shown to retain many of the patient tumor characteristics, establishment and long-term culturing has been challenging (285,292–294). While the usage of Matrigel showed the most promising results for establishment, its influence on cells to differentiate is similarly discussed as serum (292,295). In addition, the high batch to batch variability of Matrigel leads to varying success in organoid establishment (296). Novel approaches using 10-20% of human plasma in replacement for serum or Matrigel has showed success in establishment of PDOs, but overall success rate is still low (297).

Patient-derived xenograft-derived organoids

Patient-derived xenograft-derived organoids (PDX-O) are cultures established after expanding the tumor tissue *in vivo*. Like PDOs, PDX-Os retain genotypical and phenotypical features, as well as the metastatic potential and treatment response (112,126,298). Due to the often small amount of tumor sample obtained, expansion *in vivo* can increase the rate of establishment (285). One drawback of PDOs and PDX-Os is that the high heterogeneity of neuroblastoma is not represented in its full capacity. While the heterogeneity of tumor organoids is higher than in conventional cell lines, tumor organoids are established from only one biopsy taken of one small subsection of the tumor.

In vivo models

In vivo models are often used to recapitulate cancer in the context of a whole biological system. In the case of NB, *in vivo* models can be used to study the origin of NB, potential novel treatment options and enrich our understanding in the NB cancer biology. Unlike current standard *in vitro* systems, *in vivo* models include additional cell types and can recapitulate their influence. Additionally, *in vivo* models are essential for treatment studies to evaluate efficacy, potential toxicity and distribution of drugs before clinical testing can begin.

The 3Rs of animal research

The 3Rs are composed of Reduction, Replacement and Refinement, and are a fundamental aspect of research using animal models. The concept was first

published in 1959 with the aim to improve the laboratory animals' welfare and reduce unnecessary distress by focusing on varying aspects. Reduction encases the idea of decreasing the number of animals used while still retaining statistically relevant data. Replacement is often defined as replacing animal experiments where possible with non-animal experiments, for example *in vitro* experiments (299–301). Franco et al. discussed in their editorial piece the possibility of replacing, or at least reducing, the number of animals needed in the education by using virtual reality and interactive learning tools (302). The last R, Refinement, discusses the topic of reducing the amount of pain, distress and other adverse effects that are inflicted on animals. This for example can contain enrichment in their cages, alternative handling and pain medication (299–301).

Genetically engineered mouse models

Genetically engineered mouse models (GEMM) are used to study the initiating steps and progression of NB. GEMM tumors arise spontaneously and use immunocompetent mice, allowing for treatment studies including immunotherapy. Due to the genetic engineering, the role of specific driver oncogenes can be studied (303), which can also be a drawback, as they do not fully represent the genetic alterations of patient tumors (304).

The most common GEMM used are the ones involving *MYCN*. Specifically, transgenic TH-MYCN mice as these model tumors that resemble the human form of NB in their heterogeneity. The lack of tumors found in the adrenal gland and limited metastatic spread are large a shortcoming of this model. Metastatic spread is limited to the lungs in contrast to the bone, which is the preferred human equivalent (304). Recent advances in the use of the TH-MYCN models revealed that tumor cells derived from TH-MYCN mice are transplantable. Using intra-venous injection of such derived cells resulted in disseminated tumors, overcoming the previous metastasis limitation (305). Further adaptations of the TH-MYCN model, for example the TH-*MYCN*^{CPM32} model, are able to recapitulate chemoresistant NB with spontaneous dissemination to the bone marrow (306). Despite their drawbacks, genetically engineered mouse models have contributed to the understanding and treatment research for NB (304,306–309). With novel genetic engineering tools such as CRISPR/Cas9, refined GEMM are expected to be established, as has been done in other cancers (310,311). However, a strong limitation of this model is that both tumors and microenvironment are murine.

Zebrafish models

Another model used in NB research is zebrafish. Due to their low cost, simplicity in imaging and possibility of being genetically altered, they have become a popular model alternative for GEMM when it comes to tumor development studies. Their

translucent bodies allow observation and imaging without the need of euthanizing or expensive CT/MRI equipment, making it possible to follow NB from early onset of the disease in real-time. While a different species, zebrafish have corresponding organs to humans, allowing the study of metastasis to functionally similar organs (312). In recent years, zebrafish PDXs have been established as a cheaper and faster method (313) and tested as a high-throughput model for promising novel treatment targets and compounds (314).

A big disadvantage of zebrafish as a model environment for NB is that they are not mammals and therefore the tumor microenvironment differs. In addition, the water's optimal temperature for zebrafish is approximately 29 °C, while human cells prefer temperatures around 37 °C. When it comes to treatment studies, the metabolism and drug distribution in zebrafish is not widely explored (313). Additionally, therapeutics are limited to water-soluble compounds as they need to be added to the water tank (294).

Chick embryo model

Chick embryos have a potential as a NB model with the focus of angiogenesis using the chorioallantoic membrane (CAM). Mouse and human derived NB can be implanted on the CAM as the immune system of the chick is not yet fully developed (315). Further, spontaneous metastasis modelling using human derived cells have been successfully performed (316). Like zebrafish, an advantage of the chick embryo model over the mouse model is the possibility of monitoring the tumor development in real-time, which also progresses faster in this model, needing only between 2 and 5 days. A major drawback of this model is that the cancer cells cannot disseminate to other organs and establish colonies there (315). While the establishment of tumors is faster, the overall time limit for experiments is shorter as well (316).

Xenograft mouse models

Xenograft mouse models describe a model where human derived cells are implanted into mice can be separated into two categories, cell line derived and patient-derived xenografts (298,317).

Cell line derived

Cell line derived xenografts (CDX) use conventional cell lines injected into immunocompromised mice, either subcutaneously, intra-venously or orthotopically. CDX have limitations similarly to their *in vitro* counterpart such as their inability to recapitulate the original tumor in drug response or histologically (318,319). Orthotopic injection of CDX, which in the case of neuroblastoma means

the adrenal gland, yields better results. These tumors demonstrate better vascularization and the ability to metastasize, resulting in relevant tumor biology compared to their subcutaneous counterparts (320,321). To circumvent the lack of metastasis in CDX models, researchers use intra-cardiac or intra-venous injections of cell lines to mimic the circulation of tumor cells (322,323).

Patient-derived

Patient-derived xenografts (PDX) are established by implanting a piece of tumor or tumor cells derived directly from patients into immunocompromised mice. Tumors arising with this method closely resemble the patients tumor by retaining molecular and functional features (112,298). Orthotopically implanted PDXs retain their invasive growth and metastatic pattern to distinct organs such as livers and bone marrow. Cells from these PDXs can be brought back into culture and used for future PDX implantations (298). Importantly, PDXs retain clinically important factors such as the tumors geno- and phenotype, such as expression or markers, differentiation status, and mutational profile. Even angiogenic profiles are mimicked and the treatment response correlates to the patients history (112,126,324). However, while the tumor microenvironment retains human derived cells in the beginning, these are replaced by murine cells over time, limiting the potential of studying the human TME-NB interaction (324). Like PDX-Os, PDXs are established from a small subset of the patient tumor, thereby only reflecting one portion of the tumor due to neuroblastomas high heterogeneity. Engraftment in murine models is successful only in highly aggressive tumors. A selection is likely happening in the engraftment process where less aggressive subclones are lost, enriching in cells able to adapt to the new microenvironment (126,325). Another limitation of PDX and CDX is the lack of reduced immune system which is needed to allow engraftment of the tumors. Ongoing work on including human hematopoietic stem cells into radiated mice could, in the future, circumvent this restriction (326).

Applications

Applications of PDXs reach from identification of treatment resistance mechanisms and biomarkers, validation of potential novel therapies to approaches in precision medicine. As treatment resistance is an ongoing challenge in the clinic, studies have used PDXs to identify novel mechanisms involved in resistance (126). PDX are used as proof-of-concept in preclinical drug testing. A large variety of drugs and small molecules have been identified in recent years thanks to PDXs. Still, high throughput testing as is done *in vitro* is not feasible using PDXs as their establishment and maintenance are costly. Therefore, screening of drugs and targets using *in vitro* is used to limit the amount of animals needed (285).

Humanized mice models

Humanized mouse models are a novel attempt to overcome limitations of conventional PDX models. MISTRG mice have been developed to allow co-transplantation of human hematopoietic cells and NB cells. Using this model, Nguyen et al. were able to study the immunological response to NB engraftment and apply treatment (327). Another method to mimic the human immune system includes sub-lethally irradiating mice and transplanting HSCs from human donors (326). Humanized mouse models are not limited to immunology studies but can also be used for metastasis studies. Recent advances in development of small human bones, so called ossicles or hOss, can provide a humanized bone marrow niche for neuroblastoma (328). Humanized bone marrow ossicles originated from studying human hematopoietic neoplasms, as transplanting these into mice largely remained unsuccessful. While it is not clear why, researchers suspect that the lack of cross-reactivity for environmental cues and specific factors are to blame. Factors such as secreted human cytokines, chemokines and growth factors as well as interaction of hematopoietic and progenitor cells with the mesenchymal cells are required to properly recapitulate hematopoietic diseases (329). Studies focusing on bone marrow metastasis in NB showed a similar need for interaction between mesenchymal stem cells and NB cells (330). Grigoryan et al. recently used human derived mesenchymal sword of Damocles (MSOD-B) cells to establish ossicles for solid tumors to thrive in the bone marrow niche. These ossicles were transplanted subcutaneously into NSG mice and injected directly with PDX cells. In comparison to standard intra-femoral injections, intra-ossicle injected PDX cells engrafted quicker with similar or higher engraftment rate. Additionally, ossicles with NB engraftment showed reduced bone volume due to osteoclast activity, a mechanism often observed in the clinic (328). Limitation to these models is the need of mesenchymal derived stem cells derived from bone marrow, as previous research showed that easier accessible stem cells from adipose tissue and skin had lower success in establishment of endochondral ossification and ectopic hematopoietic niche formation (331). Specifically, with the focus on NB, a limitation is the age of the donor. MSCs are often derived from adult donors, resulting in higher adipose tissue formation in ossicles than observed in young patients (332). Future work using MSCs derived from younger donors could circumvent this problem.

The present investigation

Aim

The aim of this investigation was to 1) mimic clinically relevant treatment of neuroblastoma to establish a model of treatment resistance, 2) find and characterize novel therapeutic options to treat resistant neuroblastoma and 3) model metastatic NB.

The specific aims of each paper were:

Paper I – Establishment of a clinically relevant chemotherapy protocol for PDX models which mimics treatment in patients, and to investigate mechanisms of treatment resistance and relapse.

Paper II – To investigate ferroptosis inducing agents as a treatment option in neuroblastoma.

Paper III – To investigate the potential of inhibiting the calcium channel TRPA1 as treatment for NB.

Paper IV – Establishment of a humanized mouse model using small, humanized bones to mimic metastatic disease in NB.

Key material and methods

PDX models

As mentioned in the chapter about preclinical research models, patient derived xenograft models are established by expanding the tumor tissue in mice. This model is especially useful in research where the tumor material is small or scarce to receive. A disadvantage is that the implantation and culture in mice creates an evolutionary bottleneck where potentially only the most aggressive tumor cells get selected and thereby only reflect a small subset of the original tumor (325). Still, research has shown that PDX models retain important patient tumor characteristics, such as phenotypes, differentiation status, genotypes, transcriptional profiles, genetic aberrations, expression of markers, metastatic potential and treatment response, making them ideal model to test novel therapies (112,298,324). In our group, we have previously established PDX models from several different patients. In this work, we are focusing on the use of LU-NB-1, LU-NB-2 and LU-NB-3 (298) (Table 1).

Table 1:Summary of the general characteristics of PDX models used.

Name	Tumor Type	Site	Stage	Differentiation Status	Patient Genomics	Patient response to COJEC
LU-NB-1	Primary	Adrenal Gland	IV	Undifferentiated	*MYCN* amp., 1p-, 17q+	Refractory
LU-NB-2	Metastasis	Brain	IV	Undifferentiated	*MYCN* amp., 1p-, 17q+	Responsive
LU-NB-3	Primary	Adrenal Gland	III	Poorly differentiated	*MYCN* amp., 1p-, 17q+	Responsive

In **Paper I**, we further established LU-NB-3R, a relapse from LU-NB-3 which was treated with a clinically relevant chemotherapy regiment *in vivo*. LU-NB-3R was subsequently used in Paper II and Paper III. For papers I-IV, PDXs were generated from tumor organoids which were injected into immunocompromised NSG mice.

Animal Experiments

Paper I

Most preclinical research is performed using one or two chemotherapeutics, which does not reflect all mechanisms targeted in the clinically used regiment. To better understand the interactions of potential novel treatments with the current standard of care in a complex biological setting, a treatment model was established

incorporating the five different chemotherapeutics used in the clinic (cisplatin, carboplatin, vincristine, etoposide and cyclophosphamide).

While *in vitro* systems are gradually improving in replicating *in vivo* situations, to this date, no perfect replacement for animal experiments is established. While animal testing is suggested to be phased out (333), it is at the moment still a corner stone in neuroblastoma research. With the limited number of patients, clinical trials take a long time to enroll enough patients, which reduces the number of treatments that can be tested.

One could argue that the establishment of this clinically relevant treatment protocol both *in vivo* and *in vitro* goes in accordance with two of the 3Rs of animal research: **Replacement** and **Reduction**. **Replacement:** as part of this paper, we also established a COJEC *in vitro* treatment that can be used to screen potential drugs for interactions and efficacy before moving on to animal trials. Thereby replacing the need of animal to test all combinations. Further, the usage of the *in vitro* protocol allows for further mechanistical studies that can replace studies using mice. **Reduction:** Going hand in hand with the previous argument, establishing a protocol where we can use all five chemotherapies reduces the number of mice needed to test drug interactions with the five individual chemotherapies. Pre-screening of interactions with promising targets in combination with COJEC can reveal inhibitory effects, meaning the number of treatments that move on to *in vivo* testing is reduced.

Paper II and Paper III

For these papers, animal models were used to confirm *in vitro* findings *in vivo* as a proof-of-concept and to test bioavailability of the additional drugs. In accordance with the aim to reduce animal testing, drugs were screened *in vitro* to find the most promising agents before moving on to evaluating their efficacy *in vivo*. While the use of mice is not perfectly translatable to humans, it is still important to evaluate the bioavailability of the drugs.

Paper IV

Paper IV focusses heavily on mimicking a state of disease, metastasis, which is not well understood in the field of NB. This model was established to give opportunities to study metastasis and test novel therapeutics. An alternative to this would be the development of an *in vitro* model using a bioreactor. Several different systems are currently in development to achieve this (334–339), but to date, there are issues that need to be overcome before it can replace the *in vivo* version. The main issue within the NB research is that the differentiation of MSCs to bone tissue relies on serum (328). As previously stated, NB cells differentiate under media conditions that contain serum, therefore an adaptation of the protocol would be needed (281). Further, to fully differentiate the chondrogenic tissue into bone tissue, the current protocol heavily relies on the implantation into mice (328). While there are

promising approaches to mimic metastatic processes *in vitro*, the use of a humanized mouse is currently superior.

Tumor organoid culture

PDX-derived tumor organoids (PDX-Os) were used in all four papers. Organoids from LU-NB-1, LU-NB-2 and LU-NB-3 had previously been established by harvesting PDX tumors. Through mechanical and enzymatical dissociation, the tumor cells formed organoids. PDX-Os were kept in serum free media conditions, as previous research showed that the addition of serum, such as fetal bovine serum, can lead to differentiation (281). In Paper I, we additionally established tumor organoids from a relapsed LU-NB-3 tumor. In short, mice which were originally injected with LU-NB-3 underwent a chemotherapeutic treatment schedule mimicking COJEC and underwent surgery. One of the tumors that relapsed was used to establish LU-NB-3R.

As previously mentioned in the section about preclinical *in vitro* models, PDX-Os are derived from PDXs instead of directly from the patient. This system was chosen for LU-NB-1, LU-NB-2 and LU-NB-3, mainly because direct *in vitro* establishment from the patient tumor was unsuccessful. Passaging through the mouse enabled the expansion of tumor material and allowed for a higher success rate. As LU-NB-3R is a relapse of a treated PDX, the PDX-O could only be derived from the PDX. Research on our own PDX and PDX-O models showed that they retained a lot of the patient tumor characteristics (112,126,298), making it the optimal model to test therapeutics on. Additionally, while the PDX-Os only represent a small subsection of the tumor where the sample was taken and cells that engrafted in the tumor, it still has improved heterogeneity over 2D cell cultures.

In vitro treatment studies and synergy testing

For Papers II and III, we performed synergy testing to see whether the therapies we investigated were suitable to be used with the current standard of care treatment COJEC. In short, we seeded dissociated tumor organoids into 96-well plates and waited 48 hours to allow organoid formation. Then, treatment was applied in a matrix with increasing dosage. After another 48h, cell viability and cell death were measured using Cytotox Glo. Measurements were performed in triplicates and then the average was used to calculate synergy using SynergyFinder (https://synergyfinder.fimm.fi).

We used the Bliss Independence model to evaluate drug synergy as it directly quantifies the expected combined effect under the assumption of independent action. On the other hand, ZIP models are intended for data with excess zeros, which did not match our experimental setup. The Loewe additivity assumes that drugs act

through similar mechanisms and can be substituted for each other. While we discovered that in **Paper II,** we had two compounds with the same target, the original assumption was that they were independent, fitting the criteria for Bliss.

Ossicle differentiation and transplantation

In Paper IV, we use a protocol established by our collaborator to differentiate MSOD-B cells to hOss via *in vitro* and *in vivo* steps (274) (Figure 6).

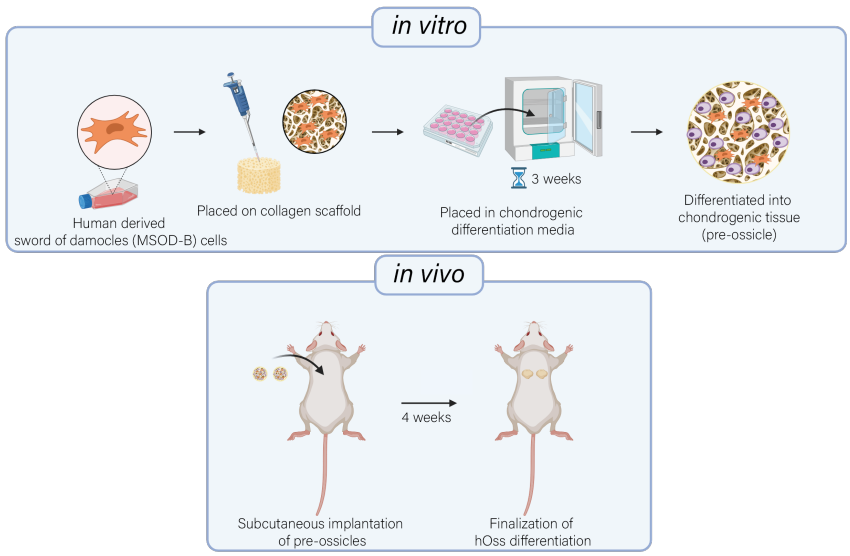

Figure 6 Overview of hOss differentiation.
MSDO-B cells were placed onto a collagene sponge and differentiated into pre-ossicles. These pre-ossicles were then transplanted into NSG mice where they finished their differentiation into hOss. Using these, three different injection methods for LU-NB PDX cells were used. Growth of the resulting tumors was observed using the *in vivo* live imaging system IVIS.

In short, MSOD-B cells were placed on a collagen scaffold and differentiated into chondrogenic tissue (pre-ossicle). These were then transplanted into NSG mice where they finalized their differentiation into hOss. We then injected luciferin labeled NB PDX cells via one of three different injection methods: Intra-hOss, orthotopically into the adrenal gland or intra-venously into the tail vein. Tumor growth was then observed using bioluminescent *in vivo* live imaging (IVIS).

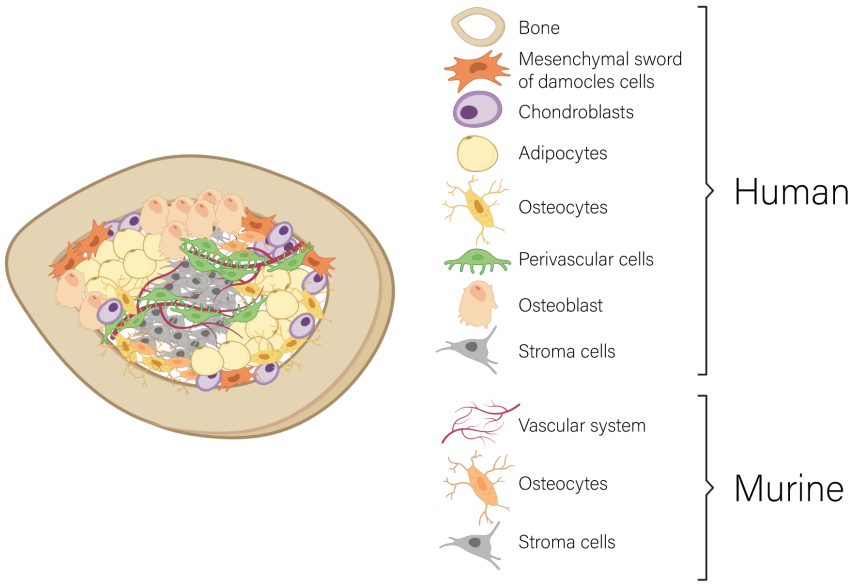

Figure 7 Schematic of a differentiated hOss.
After differentiation *in vivo*, some human derived cells and some murine cells can be found in hOss. The human derived ones stem from MSOD cells, while the murine cells migrate via the vascular system.

Within the differentiated hOss, there are various cell types either derived from human or from mouse (Figure 7). Human derived cells stem from the differentiated MSOD cells and include for examples: chondroblasts, adipocytes, osteocytes, perivascular cells, osteoblasts and stroma. Due to the vascular system provided from the mouse, some stroma cells and osteocytes can migrate into the hOss (340).

This is not the only protocol currently available to create hOss, as Reinisch et al. for example published theirs in 2017 using direct injection of MSCs with a Matrix solution into mice (329). Methods to mimic human bone in mice have been developed since the early 2000, using for examples scaffolds made from ceramics, hydrogels or commercially available ECM (341,342). With the variety of options available, selection of the method can be overwhelming with several factors to consider.

For Paper IV we chose the method developed by our collaborators due to the stable growth and easy expansion of MSOD-B cells. Many protocols rely on extraction of MSC from the bone marrow of healthy donors (342). While the use of primary MSCs is tempting, large donor-to-donor variations have been observed (343). The MSCs for our chosen protocol, in contrast, were derived from a healthy donor and immortalized by hTERT transduction (344). Additionally, BMP-2 overexpression was introduced to increase the MSODs chondrogenic differentiation potential (345). BMP-2 protein is involved in the repair and development of the skeleton and

sometimes supplemented in other hOss protocols (342,345). Thereby, these MSCs, referred to as MSOD-B cells, achieved a stable growth and reliably differentiated into chondrogenic tissue (345). This circumvents the common issue of MSC batch-to-batch variation (343). However, it has been shown that BMP-2 has influence on NBs sensitivity to RA (346) and its transport via EV could lead to alteration of the NB cells in the BM niche due to the increased expression of MSOD-B cells.

A limitation of this method is the fact that MSOD-B cells were derived from an adult donor. As previously mentioned, pediatric bone has a lower adipocyte population than adult bone (332). Therefore, adaptations of the method to include MSCs derived from pediatric donors could be beneficial.

Summary of papers

Paper I - Clinically relevant treatment of PDX models reveals patterns of neuroblastoma chemoresistance

In this study we sought to develop a treatment protocol to mimic the treatment patients receive in the clinic using PDX models. Using this treatment protocol, we investigated COJEC treatment resistance on genomic and transcriptomic level.

Establishment of a treatment mimicking COJEC

Using the previously mentioned PDX-derived organoids LU-NB-1, LU-NB-2 and LU-NB-3, mice were injected subcutaneously. Once tumors reached approximately 500 mm^3, mice were randomly assigned to one of three groups: control, cisplatin, COJEC. The control group was treated with saline, the cisplatin group was used to represent the single agent usage in research, and the COJEC-like group received cisplatin, vincristine, etoposide, cyclophosphamide and carboplatin in a cycled manner (**Manuscript Figure 1**).

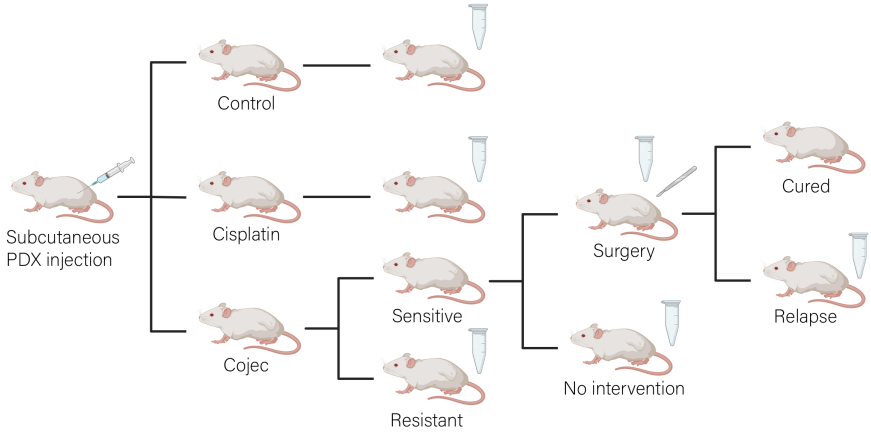

Figure 8 Schematic of *in vivo* COJEC treatment groups for LU-NB-3.
Mice were seperated into three different treatment groups, control, cisplatin treatment and COJEC treatment. Mice with LU-NB-3 tumors that responded to COJEC tretment were further seperated into surgery and no surgery groups. Eppendorf tubes represent samples taken from tumors for sequencing.

For LU-NB-1 and additional COJEC group received a higher dose referred to as COJEC-HD, while the standard dose is referred to as COJEC. Additionally, two mice strains were used, nude and NSG mice. Treatment response showed no difference between the strains, but COJEC-HD significantly increased survival.

LU-NB-2 showed a clear response to both cisplatin and COJEC, although COJEC had an increased survival compared to cisplatin, resulting in one mouse with complete response.

In mice with LU-NB-3 tumors, COJEC yielded the best response, with significantly reduced tumor sizes and increased survival, mirroring the patient's response. For a subgroup of LU-NB-3 tumor bearing mice, as shown in Figure 8, surgery was performed to remove the tumors once they reached a size smaller than 200 mm^3. Mice did not receive any more chemotherapy after surgery which was curative for most mice. However, two mice developed local relapses.

Histological analysis of COJEC treated tumors

All PDX tumors post COJEC treatment were positive for the NB marker PHOX2B. Additionally, staining revealed that LU-NB-1 showed the least differentiation while LU-NB-3, which additionally underwent surgery, showed clear morphological differentiation. Increased cell death was observed only in the LU-NB-1 COJEC-HD group and LU-NB-3 COJEC + surgery group. In general, a strong staining for the proliferation marker Ki67 was observed in all tumors. LU-NB-1 and LU-NB-2 tumors had large, collapsed blood vessels, often observed in connection with a worse prognosis (347). Interestingly, there was no statistical difference between the

cured and not cured samples of the LU-NB-3 COJEC + surgery group regarding morphological differentiation and proliferation (**Manuscript Figure 1**).

Bulk whole genome copy number profiling and single cell DNA sequencing to unravel clonal dynamics

Samples from the parental organoids, control tumors, treated tumors and relapsed tumors were used. Our collaborators reconstructed one phylogenetic tree per PDX where they saw that each PDX had a high number of subclones and complex evolutionary branches (**Manuscript Figure 2**).

LU-NB-1 had a comparable genetic diversity in COJEC treated and control tumors, likely due to the whole-genome duplication found in the parental tumors' organoids. In short, no enrichment for specific resistant subclones was found in the treatment group. LU-NB-2 similarly revealed no evidence of a selective clonal sweep, but treatment specific copy number losses were found. LU-NB-3 presented with an additional gain in 17q+ and 1pq in both treatment and control, suggesting that the selection of clones was based on intrinsic evolutionary forces.

Overall, evidence showed that an increase of copy number burden aberrations correlated with time the tumor grew, suggesting that treatment did not cause any additional aberrations.

When our collaborators analyzed the single cell DNA sequencing data, they observed that LU-NB-1 again had a large genetic variety. They further observed that no subclone was detected in more than one tumor. On the other hand, for LU-NB-3, they were able to find two major subclones across all tumors. One of these subclones was found in both controls and treated tumors but enriched in the latter. Despite this, overall, there was no significant enrichment of specific subclones found that could explain the treatment resistance post COJEC treatment (**Manuscript Figure 3**).

Identification of transcriptomic signatures in COJEC treated tumors

Next, we performed RNA sequencing to investigate potential changes in the transcriptomics post-COJEC treatment. Unsupervised clustering resulted, as expected, in strong clustering by LU-NB PDX model. Within their respective PDX models, no specific pattern for LU-NB-1 and LU-NB-2 was observed. LU-NB-3 tumors that underwent COJEC + surgery, however, clustered together.

Analysis of the top 1000 variable genes for LU-NB-3 resulted in four distinct clusters. Cluster 1 was characterized by genes involved in early development, cluster 2 contained genes involved in the cell cycle, cluster 3 had a mixed signature and cluster 4 contained nervous system related genes. Interestingly, tumors that relapsed or regrew, had an upregulated expression in the early development cluster, while nervous system related genes were downregulated. Further, tumors that underwent surgery and were later cured differed from surgery samples that later

relapsed. Samples that later relapsed had a higher expression of the cell cycle cluster (cluster 2) and the mixed cluster (cluster 3), while the cured ones had a downregulation of cell cycle genes (cluster 2) and an upregulation of the nervous system cluster (cluster 4). This gene signature of the cured samples was then applied to patient-derived RNA sequencing data and correlated with a better prognosis. The signature of the samples that were not cured correlated with worse outcomes and had a higher expression of genes involved in DNA repair and cell cycle.

In summary, transcriptomic data revealed that tumors that were cured showed higher expression in nervous system developmental related genes, while treatment resistant NB had high expression of genes involved in the early embryonic development (**Manuscript Figure 4**).

Identification of specific NB phenotypes using transcription data

In the next part of the analysis, we compared gene clusters representing, relapsed tumors and cured tumors to normal developing human adrenal medulla. Relapsed tumors and immature Schwann cell precursors had an overlap in high expressed genes, while the cured samples resembled neuroblasts and chromaffin cells (**Manuscript Figure 4K**).

As mentioned in the introduction chapters, two distinct phenotypes have been established for NB: the adrenergic phenotype and the mesenchymal phenotype (35,36,348). Consistently, the cured tumors had a higher ADRN signature, and the relapsed tumors had a higher MES signature.

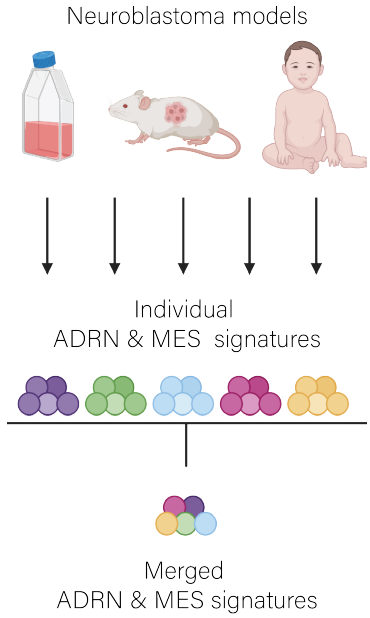

Figure 9 Process of establishing the merged ADRN and MES signature.
Individual signatures from various neuroblastoma models were merged to one list of genes.

In the next step, six previously published ADRN and MES signatures were merged with genes from our cluster 4 and 1 respectively (Figure 9). Unsurprisingly, the "merged ADRN" list was enriched in cured LU-NB-3 tumors and the "merged MES" signature enriched in the relapsed LU-NB-3 tumors. Interestingly, when applied to patient data, the "merged MES" and normal MES signature were not associated with a worse outcome, only low expression of the "merged ADRN" signature was.

The merged MES list was further split into two subcategories based on the groups of genes that were upregulated in different treatment groups. One part of the merged MES list was upregulated in LU-NB-3 surgery tumors that were later cured and characterized by genes involved in cell death, negative regulation of MAPK/ERK and cell differentiation. The other subcategory consisted of genes that were enriched in the relapsed and regrown population. This list was characterized by genes involved in cell proliferation, migration and early development (**Manuscript Figure 5**).

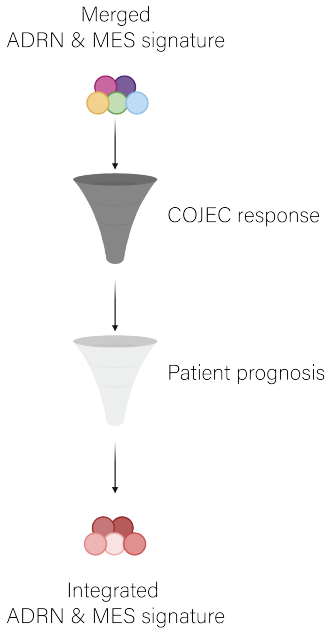

Figure 10 Curation of integrated ADRN and MES signature.
The previously established merged adrenergic and mesenchymal signature lists where filtered through the COEJC response of cured PDX tumors as well as through patietn samples to obtain an integrated list predictive of treatment outcome.

To further specify the merged ADRN and merged MES signature list, we decided to create a list that has improved predictive power in both patient and PDX models. For this, the ADRN gene signature of LU-NB-3 cured samples and the MES gene signature of relapsed samples were filtered through over 200 patient samples to obtain a new signature (Figure 10). This signature is referred to as "integrated signature" (**Manuscript Figure 6**) and contained genes representing various signature lists. Staining of LU-NB-3 with selected proteins off the integrated signature revealed the interesting finding that tumors were positive for both, ADRN and MES markers at surgery point, but only positive for MES at relapse.

We also investigated the change of *MYCN* expression in treated samples. For LU-NB-3, we observed lower expression in the surgery samples of the tumors that ended up being cured. This was confirmed by staining for MYCN where quantification showed a strong difference (**Manuscript Figure 7**).

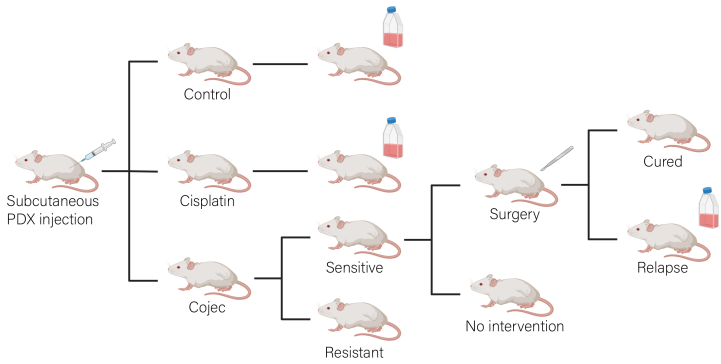

Figure 11 Schematic of *in vivo* COJEC treatment groups for tumor organoid sampling.
Mice were seperated into three different treatment groups, control, cisplatin treatment and COJEC treatment. Mice with tumors that responded to COJEC tretment were further seperated into surgery and no surgery groups. LU-NB-3R tumor organoids were derived from a mouse that underwent COJEC treatment, surgery and had a tumor that relapsed, indicated by the cell culture flask.

As a last step, we established tumor organoids from the treated PDXs (Figure 11). Using cisplatin and vincristine, we analyzed acquired treatment resistance after COJEC treatment. Comparison to the organoids derived from the control PDX tumors showed that there was no difference for LU-NB-1 and LU-NB-2. LU-NB-3 organoids derived from a relapsed tumor (LU-NB-3R) revealed an increase resistance to chemotherapeutics compared to organoids derived from a control tumor (Figure 12).

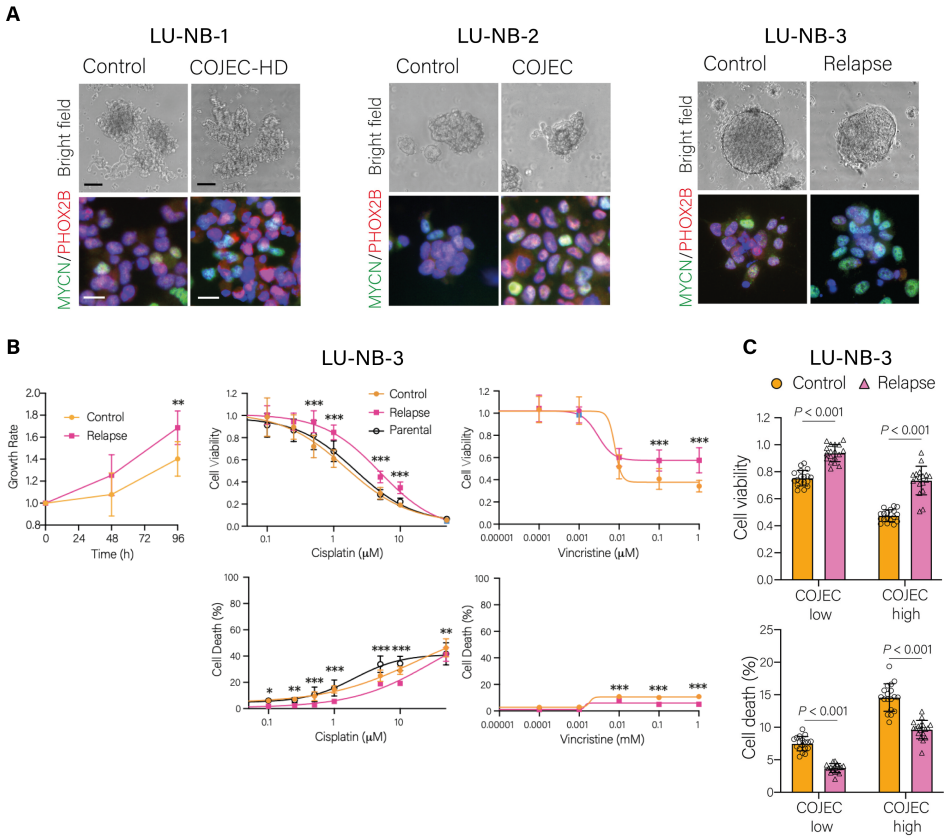

Figure 12 Characterization of tumor organoids after COJEC treatment.
Phenotypical changes in organoids derived from treated and control tumors of the three LU-NB models used in Paper I (A). Growth curves and cell viability and cell death curves of parental LU-NB-3, control treatment LU-NB-3 and relapsed LU-NB-3 derived organoids using cisplatin and vincristine (B). Comparison of cell viability and cell death after *in vitro* COJEC treatment using relapsed LU-NB-3 derived organoids and control treatment derived LU-NB-3 organoids (C) .

We further developed a protocol combining all five COJEC chemotherapeutics *in vitro* in two doses: COJEC High Dose (HD) and COJEC Low Dose (LD). Even after combining all COJEC drugs, LU-NB-3R organoids showed higher viability and reduced cell death compared to their control counterparts (Figure 12).

Using single cell DNA sequencing we were able to show that the organoids retained their genetic aberrations from *in vivo* to *in vitro*. We further identified that a clonal sweep occurred in the relapse which led to clone #8, which was defined by a few copy number alterations (CNAs), of unclear importance, taking over. Genetic aberrations from this clone were retained in the organoids and no novel changes were gained during transitioning between *in vivo* and *ex vivo*. RNA sequencing of the control organoids and the organoids derived from the relapse revealed that the

relapse had a downregulation in the previously identified cluster 4 (nervous system development related), in accordance with what was observed in the *in vivo* RNA sequencing data. Further, *MYCN* expression was increased in LU-NB-3R. Several ADRN genes were downregulated and several MES upregulated in the relapse. As a last step, LU-NB-3R organoids were reinjected subcutaneously into NSG mice and gave rise to new PDX tumors. These tumors were analyzed and showed morphological and transcriptional profiles like the parental tumor (**Manuscript Figure 8**).

In summary, we developed an *in vivo* and *in vitro* protocol to mimic the standard of care COJEC treatment for preclinical research and showed that transcriptional phenotypic cell states are associated with treatment resistance. My contributions to this paper were focused on the organoid establishment, drug treatment response analysis and growth analysis of said organoids.

Paper II: Targeted ferroptosis induction enhances chemotherapeutic efficacy in chemoresistant neuroblastoma

In Paper II, we explored ferroptosis as a potential vulnerability of neuroblastoma and investigated the interaction of selected ferroptosis inducing agents with COJEC.

Transcriptomic analysis of patient data and preclinical data

In the first part of the paper, we investigated the expression of genes involved in antioxidant pathways in patient RNA sequencing data. The three pathways we chose were: glutathione pathway, thioredoxin pathway and mevalonate pathway. In total we analyzed bulk RNA of three different patient cohorts, two single-nuclei transcriptome databases, spatial transcriptomic data of two patients with paired pre- and post-chemotherapy data, our NB PDX models and data from conventional NB cell lines. In short, we found supporting data that NB depends on the chosen pathways and that their activity correlated with outcome in patients and with chemotherapy resistance (**Manuscript Figure 1**).

In vitro analysis of ferroptosis inducing agents revealed four promising agents

Using our four PDX models, LU-NB-1, LU-NB-2, LU-NB-3 and the relapse model LU-NB-3R, we tested 16 different ferroptosis inducing agents (**Manuscript Table 1**). All models have *MYCN* amplification and varying responses to chemotherapeutic treatment. While all 16 drugs were effective in at least one model, we chose four treatments that were effective in all four models. This was determined by calculating the area under the curve for both viability and cell death (**Manuscript Supplementary Figure 2**). The drugs selected were auranofin, ML162, RSL3 and salinomycin. ML162 and RSL3 are both GPX4 inhibitors (349,350) (Figure 13). Auranofin is an FDA approved drug that was previously used to treat rheumatoid arthritis and is a gold-based small molecule. It is known to inhibit TrxR (351–353)

(Figure 14). Salinomycin is an antibacterial that has been shown to lead to lysosomal membrane disruption by sequestering iron in lysosomes (354). Ferroptosis was confirmed by staining tumor organoids for TRFR1 and 4HNE, and by analyzing lipid peroxidation (**Manuscript Figure 2**).

Validation of the four chosen ferroptosis inducing drugs in vivo

Auranofin and RSL3 were well tolerated by mice. ML162 was not toxic to mice but was poorly absorbed *in vivo* and had to be discarded from further experiments. Salinomycin was not well tolerated and was substituted with a derivate named ironomycin (354). Ironomycin was well tolerated by mice and was shown to be effective *in vitro* in all four PDX models. *In vivo* tests using LU-NB-1, however, showed no significant change in tumor growth for ironomycin (**Manuscript Supplementary Figure 3**).

Treatment of NB PDX models using RSL3 showed successful reduction in tumor size

RSL3 was tested via two different routes: intra-tumoral and subcutaneously adjacent to the tumor. RSL3 showed effect in reduced tumor growth and extended survival of the mice, with clear changes in the cancer cell morphology. One mouse had to be euthanized early due to metastatic spread of the tumor, even after the primary tumor had shrunk. Interestingly, staining of the RSL3 treated tumors showed an increase in cells positive for the ADRN marker TH and a decrease of the embryonic marker SOX9 chosen as a representing MES marker (**Manuscript Figure 3**). Intra-tumor injections of RSL3 had an even bigger impact on the tumors which displayed extracellular matrix remodeling and a strong reduction of cancer cells. RNA sequencing on these samples was not possible, as RSL3 treated samples could not be reliably mapped to the human genome (**Manuscript Supplementary Figure 3**).

Due to the promising results of RSL3 in LU-NB-1, we decided to further test it using our more chemo sensitive model LU-NB-3 and its relapse LU-NB-3R which we established in Paper I. Both models responded to RSL3 treatment and for LU-NB-3, 4/8 mice were cured at the end of experiment. Some of the control tumors experienced a size reduction as well, most likely due to damage from intra-tumoral injections. Controls of LU-NB-3R did not show any impact by choice of injection route and 5/7 tumors treated with RSL3 had a reduction in size. At the end of the experiment, one mouse remained cured (**Manuscript Figure 3**).

Combination of COJEC and RSL3 – an unsuccessful story

Due to its promising results *in vivo* and *in vitro*, we investigated the potential of combining RSL3 with the standard of care COJEC treatment. Synergy analysis was performed using two of our *in vitro* models, LU-NB-1 and LU-NB-3R. COJEC treatment consisted of COJEC LD and COJEC HD, as established in Paper I. The results showed an inhibitory effect when combining RSL3 with COJEC (**Manuscript Figure 4**).

As NB is known to have a high expression of the drug pump MRP1, we suspected it was the leading cause for the antagonism. Furthermore, we observed that COJEC treatment led to increased expression of the MRP1 gene *ABCC1I* in our models (**Manuscript Supplementary Figure 4).** In addition, MRP1 relies on GSH for the out of cell transport of vincristine and etoposide (355). As RSL3 can lead to an increase of GSH in cells, we suspected that this increased the efflux of COJEC drugs and reduced the effectiveness.

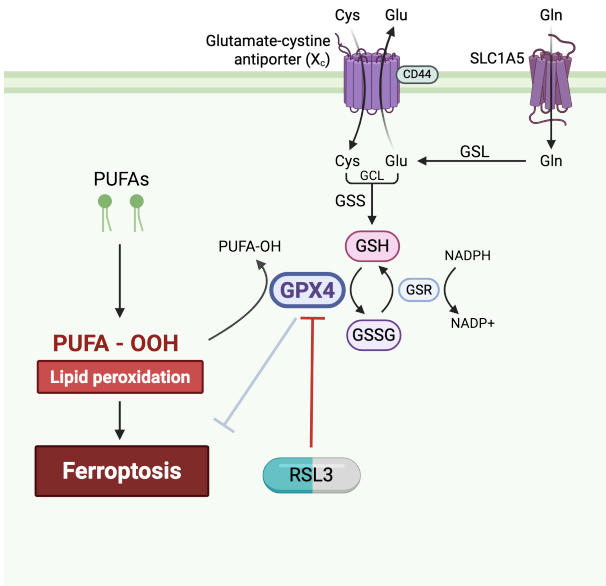

Figure 13 RSL3 inhibition on the glutathione pathway.
RSL3 inhibits GPX4, which thereby stops the rescue of the cell by reducing PUFA-OOH to PUFA-OH. This leads to accumulation of lipid peroxidation which then triggers ferroptosis.

Therefore, we decided to co-treat our tumor organoids with buthionine sulfoxamine (BSO) which depletes cellular GSH. However, while GSH levels were reduced, the viability of cells did not increase when combining COJEC, RSL3 and BSO (**Manuscript Supplementary Figure 4**). As BSO could not rescue the inhibitory effect, we decided to perform synergy analysis of the individual COJEC drugs with RSL3. The inhibitory effect was shown to be driven by etoposide and carboplatin. While cisplatin and vincristine had an additive and even synergistic effects, etoposide specifically showed a strong inhibitory profile. Literature research suggested that etoposide can lead to increase in GPX4 and could therefore inhibit the effect of RSL3 (356). As COJEC treatment significantly increases *GPX4* expression in our PDX models and patient data revealed similar increased

expression in post-treatment data, we decided to perform a partial knockout experiment using siRNA. Knockdown of *GPX4* in combination with RSL3 and COJEC treatment rescued the antagonism. Combination of siRNAs with RSL3 further completely abolished the effect of RSL3, while COJEC and siRNA treatment did not alter COJEC efficacy (**Manuscript Figure 4**). This confirmed our suspicion that etoposide is the culprit of the antagonism found.

Interestingly, we further found evidence that COJEC treatment increased the expression of known ferroptosis inhibitors *FSP1* and *HSPB1*, indicating that COJEC treatment overall could increase cancer cells resistance to ferroptosis.

Auranofin, as a single agent and in combination with COJEC, has promise as a NB treatment agent

Auranofin during single drug treatment showed reduced tumor growth and changes in the morphology of the cancer cells when administered with higher frequency (**Manuscript Figure 3**). Combination of auranofin with COJEC resulted in an additive effect *in vitro* and even increased survival of mice *in vivo* (**Manuscript Figure 5 and Figure 6**).

Transcriptomic analysis of *in vivo* material revealed a downregulation of metastasis related pathways and tumor malignancy associated pathways. Staining of tumor tissue revealed increase in ferroptosis associated markers such as 4HNE and TRFR1. Increase in iron deposits was identified in the combination samples. Oxidative stress was assessed by 8OHdG staining which was increased in the auranofin tumors and the combination tumors. Interestingly, a shift from MES to ADRN expression was observed in both the auranofin and combination groups by staining for SOX9 and TH, indicating that this shift is mainly lead by auranofin and not hindered by COJEC (**Manuscript Figure 6**).

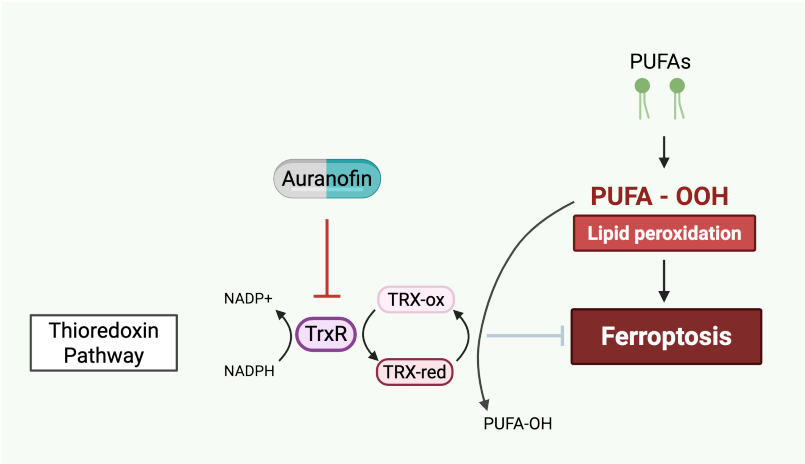

Figure 14 Auranofin inhibition of TrxR.
TrxR normally reduzes the oxidized state of TRX to the redox state, which can then reduce PUFA-OOH to PUFA-OH. By inhibiting TrxR using auranofin, this process is stopped, leading to lipid peroxidation which triggers ferroptosis.

Further *in vitro* assessment of the mechanism confirmed that auranofin inhibits TrxR. RNA sequencing for two of our LU-NB PDX models revealed upregulation in autophagy related pathways in auranofin and combination samples. Expression of NCOA4, a known ferritinophagy mediator which binds ferritin and transfers it to lysosomes, was highly increased in auranofin and combination samples. In fact, other important genes in ferritinophagy and genes related to iron overload response were consistently upregulated (**Manuscript Figure 5**). As ferritinophagy is a specific subtype of autophagy, we assessed whether ferritinophagy is a result of TrxR inhibition or an off-target effect. Ee tested two additional TrxR inhibiting agents: D9, another gold-based molecule, and curcumin, a polyphenol. We performed live-cell staining using these three agents as well as salinomycin as ferritinophagy is its known mechanism. Consistently, the agents showed strong lysosome accumulation and a decrease in calcein, which is known to reduce signal when encountering free intracellular iron, compared to the control.

As mentioned in the section about RSL3, COJEC treatment increased expression of ferroptosis inhibitors. RNA sequencing analysis of auranofin and combination treated samples showed a reduction in expression of *HSPB1*. Further, the previously mentioned increase of the MRP1 drug pump after COJEC treatment was decreased (**Manuscript Figure 5 and Supplementary Figure S6**).

Interestingly, auranofin and combination treatment samples had a reduced expression of *CD44* (**Manuscript Figure 5 and Supplementary Figure S6**). *CD44* is a stem cell marker that has been shown to enable an iron-dependent epigenetic plasticity and has influence over the stabilization of glutathione synthesis (357,358).

As CD44 in NB has been shown to promote aggressive disease and is a marker in the MES phenotype (123,359), we used the in Paper I established integrated ADRN and MES signature, signatures derived from Patel et al. and signatures derived from Van Groningen (35,119). The Van Groningen signature revealed inconsistent results, but the other two signature lists showed a clear reduction in the MES phenotype in auranofin, and combination treated samples (**Manuscript Supplementary Figure S6**). This suggests that there is an auranofin mediated reduction in MES associated genes.

In summary, we tested a total of 17 ferroptosis inducing drugs *in vitro* and confirmed efficacy *in vivo* for three of the selected agents. While RSL3 is a promising agent to target ferroptosis in NB, it has an inhibitory effect when combined with the standard of care treatment COJEC. Auranofin, although having a lower effect as a single drug on NB, resulted in an additive effect with COJEC and countered COJEC-mediated resistance changes. My experimental contributions to this paper consisted of the *in vitro* drug testing of various single agents and confirmation of lipid peroxidation of the selected agents for further testing. Additionally, I performed synergy testing of RSL3 and COJEC, including assessment of single agents' combination with RSL3. I further investigated the ferritinophagy effect of auranofin and the other TrxR inhibitory agents. Lastly, I aided with stainings and scoring of tumor tissues of *in vivo* experiments using auranofin.

Paper III: Evaluation of TRPA1 as a Therapeutic Target in *MYCN*-Amplified Neuroblastoma

In Paper III we investigated the potential of targeting TRPA1 as a novel way to treat neuroblastoma.

Expression of TRPA1 *across patient data and preclinical models*

TRPA1 expression was assessed both on protein and RNA level. For the protein levels, tumor micro arrays (TMAs) with samples of 63 patients were used. TRPA1 was found in all samples, with most tumors expressing it in more than 50% of the tumor cells. *TRPA1* expression on RNA level was investigated using two publicly available single-cell datasets (119,348). Subpopulations of NB cells showed expression, both in the differentiated and undifferentiated category, as well as stroma cells (**Manuscript Figure 1)**. We further investigated the expression in large publicly available data sets (SEQC) which showed that low *TRPA1* expression in patients with tumors that are non-*MYCN* amplified or in cohorts with mixed *MYCN* expression had worse prognosis. There was no clear association between survival and *TRPA1* expression in *MYCN* amplified tumors, so we decided to focus on *MYCN* amplified neuroblastoma in the following investigations.

We decided to investigate the dependance of various NB cell lines on TRPA1 using Depmap. Depmap is a publicly available website containing datasets of siRNA or

CRISPR knockdowns on cell lines to investigate their dependance on specific genes (360). Knockout of *TRPA1* using their modeling revealed a dependance, suggesting TRPA1 as a potential treatment option. Using RNA sequencing data from Paper I, we found that *TRPA1* expression was further increased in relapsed samples after COJEC treatment, compared to control tumors. We therefore decided to confirm protein expression in our models using immunofluorescent staining and western blots. Immunofluorescence showed a clear expression across all LU-NB tumor organoids models and quantifications of the western blots revealed that our more chemosensitive model LU-NB-3 had the lowest protein expression **(Manuscript Figure 2)**.

LU-NB PDX derived tumor organoids are sensitive to TRPA1 inhibition

Next, we investigated the effect of TRPA1 inhibition *in vitro* using three commercially available inhibitors, AP-18, A967079 and Bay 390. Interestingly, viability of all four tumor organoid models was reduced, but cell death only increased in high doses. We therefore concluded that inhibition of TRPA1 resulted in a change of proliferation. Furthermore, morphological changes were observed in LU-NB-3 indicating differentiation, which was further supported by immunofluorescent staining of treated organoids using TH and SOX9, markers often included in ADRN and MES phenotype signatures. We observed an increase in TH staining in A967079 LU-NB-3 samples, although the overall response between the models showed mixed results (**Manuscript Figure 3 and Supplementary Figure 3**).

Inhibition of TRPA1 alone is not successful in reducing tumor size

After promising *in vitro* results, we moved on to investigate the potential of A967079 and Bay 390 *in vivo* using LU-NB-3R due to its higher expression of *TRPA1* than the parental LU-NB-3. Although treatment was tolerated well by mice, there was no impact observed in tumor growth. RNA sequencing however revealed that A967079 treatment upregulated pathways involved in the neuron development and Bay 390 treatment resulted in upregulation of cell cycle related genes (**Manuscript Figure 4)**.

Pre-treatment using TRPA1 inhibition enhances chemotherapy

As a last step, we investigated the synergistic effect of TRPA1 inhibition with COJEC. While the common combination system of adding treatments at the same time resulted in an inhibitory effect, pretreatment of LU-NB tumor organoids showed an additive and even synergistic effect in both A967079 and Bay 390. This suggests that pre-treatment with TRPA1 inhibitors could be used to achieve a higher effect of COJEC or even allow the reduction of dose used (**Manuscript Figure 5)**.

In summary, we investigated the expression of TRPA1 on both protein and RNA level in patient data, PDX data, tumor organoids and cell lines. We further assessed inhibitors and their effect on NB tumor organoids *in vitro,* either as single agents or in combination with COJEC. *In vivo* results with currently available inhibitors showed no reduction in tumor growth but revealed changes in RNA expression depending on which inhibitor was used. My contributions included the finding of TRPA1 overexpression in relapsed samples, which started the project. I oversaw and participated in experiments, analysis, writing and revision process, which gave me the opportunity to lead the project from the start to publishing.

Paper IV: A humanized *in vivo* model of neuroblastoma bone metastasis

In this paper we aimed to establish an *in vivo* model to mimic bone marrow metastasis of NB based on our previous work (328).

Mimicking the metastatic process via different administration routes

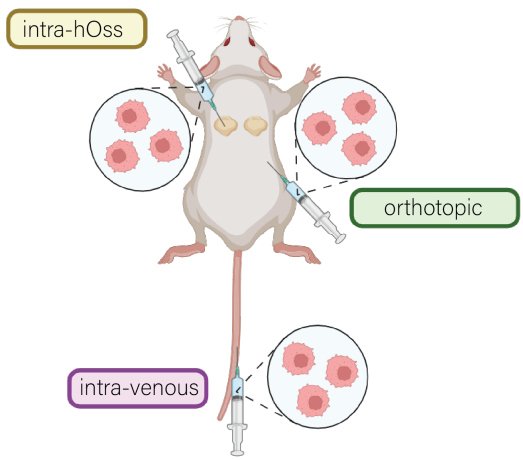

Figure 15 hOss injection methods used.
Three different injection methods to mimic the metastatic spread were used. Orthotopic injections represented the migration from primary tissue to hOss. Intra-venous injections were used to recapitulate NB cells in the circlation and intra-hOss injections to represent already established metastasis.

hOss were established as described in the method section by differentiating MSOD-B to chondrogenic tissue that was then transplanted into NSG mice. To mimic the different steps of metastasis in this project, we used three injection methods (Figure

15). The previously proved successful intra-hOss injections were used to mimic metastasis that already established in the bone marrow niche, intra-venous injections were used to introduce tumor cells into the circulating blood flow and orthotopic injections were used for the primary disease giving rise to metastasis. During all experiments, we used *in vivo* live imaging relying on the bioluminescent signal of our transduced LU-NB PDX cells.

PDX NB cells migrate to hOss after intra-venous injection

As mentioned, we used intra-venous injections to mimic the blood-born metastatic spread of NB cells. Mice who received intra-hOss injections were used as an engraftment control for the LU-NB PDX cells. IVIS *in vivo* imaging of intra-venous injected mice revealed engraftment in 6/7 mice for LU-NB-1 after 14 weeks. For LU-NB-2, engraftment was slower, as we only observed one mouse with engraftment in hOss after 20 weeks. Immunohistochemistry (IHC) staining for NB marker PHOX2B was used to confirm metastasis in hOss and organs. While metastasis was observed in liver and hOss, we could not observe any in the lungs.

NB PDX cells can travel from primary tumors to hOss, but not in large numbers

Next, we injected mice bearing hOss with LU-NB-1 cells orthotopically to give rise to a primary tumor. We then observed growth using the IVIS until tumor burden was too large and mice had to be sacrificed. During the observation period we encountered the challenge that the signal from the primary tumor was incredibly strong and overtaking. Registering smaller signals from metastasis was not possible, so we tried *ex vivo* imaging of hOss and liver for one mouse. We were able to observe signal in one hOss which was later confirmed by IHC. As we confirmed the metastasis later via IHC, no advantage of *ex vivo* imaging was observed.

Chemotherapeutic treatment of NB PDX cells in hOss is successful in most cases

As a last experiment, we used direct intra-hOss injection of the slower growing LU-NB-2 to represent already engrafted metastasis. In addition, we applied the previously in Paper I established COJEC treatment. As treatment control, we further injected additional mice subcutaneously with the same batch of LU-NB-2 cells. Treatment of subcutaneous mice showed expected tumor reduction.

Intra-hOss injected mice were separated into control and COJEC treatment groups. Treatment lasted for five weeks unless a pre-established bioluminescent threshold was reached. This threshold was determined by data from previously performed intra-hOss injection experiments. The signal in the COJEC treated mice reduced rapidly, and IHC for PHOX2B revealed that four out of five mice had no NB cells left in hOss. One mouse, however, presented MRD which was not detectable during IVIS imaging, mimicking the scenario we often observe in the clinic.

In summary, we used three different administration routes to mimic the metastatic process in our humanized *in vivo* model. We further applied treatment to hOss with

NB PDX cells engrafted, which resulted in complete response in all mice. Lastly, we were able to observe hidden MRD in one of the treated mice, replicating a common issue observed in the clinic. My contributions to this project included planning experiments, differentiation of MSOD-B cells to hOss, *in vivo* imaging and surgeries during experiments, staining of the harvested hOss and organs as well as writing of the manuscript and analysis of data.

Discussion

What did we learn about chemoresistance in NB?

One major focus of this thesis was to better understand the chemoresistance of neuroblastoma in primary, relapsed and metastatic disease, in hope to improve the outcome of patients. In the following sections, the main findings will be summarized and discussed.

What did we learn about chemoresistance in neuroblastoma?

In **Paper I**, we mainly observed that changes in the tumors followed the Darwinian selection process. However, the selection pressure could not be directly linked to treatments as it was detected in both control and treated tumors. This suggested that it was a time-dependent Darwinian selection instead of a treatment induced one. Additionally, we did not find any CNAs or microdeletions that could directly explain the treatment resistance of NB. However, two of the genes with microdeletions, *MACROD2* and *LSAMP*, are of potential interest. *MACROD2* deletions have been found to impair PARP1 activity and thus disrupt proper DNA repair mechanisms in other cancers (361). *LSAMP* has been further identified as a NB tumor suppressant (362). Overall, the data suggests that relapse and resistance mechanisms in neuroblastoma are mainly transcriptionally driven instead of genetically.

This is further supported by the fact that MES cells were found in relapses of treatment sensitive cells and already present before treatment in the chemoresistant models. Some cells however were found with both ADRN and MES markers, as also shown by the work of Yuan et al. (120). This phenotype dependent resistance suggests a Lamarckian induction rather than a Darwinian selection. While no long-term observation of the tumor was performed, the stability of the MES phenotype even after withdrawing treatment supports the suggestion that the MES state is stable, as needed for the Lamarckian induction.

Our finding of chemoresistance being driven by MES-like NB cells goes accordance with other literature. For example, van Groningen et al. and Thirant et al. showed that MES-like cells were associated with therapy resistance to chemotherapy

(35,123). In addition, it has been shown that MES-like cells can evade other targeted therapies, such as ALK inhibition and GD-2 targeted immunotherapy (134,363). Taken together, development of therapies targeting specifically MES-like cells or therapies inducing a shift from the resistant MES-like to the treatment sensitive ADRN state should be investigated to overcome NB treatment resistance. Especially since our obtained data suggests that COJEC might select for MES cells, which contribute to relapse and resistance, a combination with a therapeutic agent that induces a shift to ADRN state, or even target both states, could successfully overcome this problem.

In **Paper II**, we investigated the effect of the FDA approved drug auranofin on neuroblastoma as monotherapy and in combination. Interestingly, we were able to observe a higher population of NB cells with ADRN phenotype after treatment with auranofin. This phenomenon remained even when treated in combination with COJEC. Based on the data obtained in this study, it is not clear if auranofin induces a switch in phenotype or targets MES-like cells. However, the combination showed that COJEC can be combined with an agent that reduces selection for the relapses inducing MES-like cells.

In **Paper III**, we identified that TRPA1 was higher expressed in relapsed NB cells than in other NB cells. Due to its function as a calcium channel and previously reported role of calcium in differentiation (234), TRPA1 became a target of interest for us. Together with the information we obtained from **Paper I** that COJEC selects for mesenchymal states, a hypothesis was formed that *TRPA1* expression could be increased in MES cells. However, inhibition of TRPA1 lead to varying results in the different NB models, with mixed changes in ADRN/MES related protein expression and mixed response by inhibitor used. While we could not directly confirm TRPA1s involvement in the phenotypical state of NB, we obtained indication that it can influence the cell state both due to visual differentiation and changes in protein expression. Further, pre-treatment of NB tumor organoids increased efficacy of COJEC, going in accordance with other work that showed that TRPA1 inhibition led to increased sensitivity of breast cancer cells to chemotherapy (238).

What about chemoresistance in metastatic neuroblastoma?

With our growing knowledge of NB cells chemoresistance, the question remains on how the phenotypic signatures are expressed in metastasis. Research on patient samples revealed that disseminated neuroblastoma cells exhibited transcriptional and phenotypical differences from the primary tumor. Disseminated tumor cells retain their plasticity, but often present in the immature MES-like state and are known to remodel the metastatic niche (268,364). Taken into context with our findings in **Paper I**, these MES-like cells are resistant to chemotherapy and induce

relapses. Interestingly, analysis of patient samples also provided evidence that aspirates where NB was present included also a higher number of MSCs. These MSCs differed functionally from normal MSCs, as their self-renewal capacity is reduced (269). Due the limited number of patients and small size of bone marrow aspirates, pre-clinical research relies on *in vivo* models to further unravel BM metastases and the included chemoresistance.

However, our options to study the influence of the bone marrow niche on resistance mechanisms are limited, as most models rely on human cancer to murine TME interaction. In **Paper IV**, we therefore aimed to develop an *in vivo* model using a humanized bone marrow niche. In this study we aimed to validate the ability of NB PDX cells to engraft via different injection methods. Additionally, we applied chemotherapeutic pressure to NB cells that were already engrafted in hOss. While we could not assess the phenotype of cells that engrafted and even resisted chemotherapy at this point, future work focusing on this, using spatial omics approaches, could provide answers desperately needed.

While our current hOss model gives us novel opportunities to study metastasis in NB in a more humanized context, the model could be further improved and optimized. MSOD-Bs were derived from an adult donor. To further refine this model, it would be of interest to establish an immortalized MSC line from a pediatric donor, which would represent the adipose composition in the BM niche more accurately. In addition, working toward an even more humanized BM niche by including human derived hematopoietic stem cells after sublethal radiation of mice would open opportunities to test immunotherapeutic treatment options. As research showed that the immune composition in the BM niche is altered in NB metastasis, this would further allow us to study how to re-sensitize the area to treatment and further improve our understanding of NB BM disease.

Future perspectives in the context of chemoresistance in NB

Based on the findings in Paper I, it would be of interest to compare the RNA expression of specific clones at different stages during therapy. Results could provide information on whether treatment affects cells with a specific phenotypic state or if there is a switch in phenotype to resist treatment. Using barcoded cells, DNA and RNA sequencing data can be connected, and clones can be tracked. Additionally, using agents such as auranofin that have been observed to reduce MES-like enriched cells, one could unravel whether this is done via inducing phenotypic switching or targeting MES cells.

While these suggestions would provide us with very important data, current knowledge about the MES and ADRN phenotypes could already provide relevant information in the clinic. Routine phenotyping of biopsies could indicate treatment response of tumors and aid clinicians in designing more personalized therapies. For

this, a unified ADRN and MES signature would need to be established. While our integrated signature showed promise by correlating with patient outcome, higher sampling of patient material could help refine these signatures.

Furthermore, with the usage of the established *in vitro* and *in vivo* protocols, screening for therapies that can target both the MES and ADRN phenotypes without diminishing the effect of COJEC will improve our selection for potential novel therapeutic options.

Future perspectives with the focus on metastatic modelling

In **Paper IV**, we used orthotopic injection of NB cells to mimic the process of primary disease to metastatic disease. While the current focus lied heavily on confirmation of metastatic spread to hOss, it could be of interest to investigate the effect the primary tumor had on hOss without metastatic engraftment. As previously mentioned, NB has been shown to use EV to prime the metastatic site for engraftment. Using already obtained material, more insight into this process and the changes MSCs, as well as other cell types, undergo could be investigated.

The current standard of establishing patient derived models relies on orthotopic transplantation of tumor material to the adrenal gland (112,298,324). Trials of using the current hOss method and injecting them with BM aspirated NB cells could be a way of providing an "orthotopic transplantation method" for BM metastasis. While current hOss might not have the optimal niche representation, they could still provide a platform to increase cell population before establishment *in vitro*.

As briefly touched upon before, the currently used MSOD-B cells could be improved by deriving MSCs from pediatric donors. This could be taken further by deriving MSCs of bone marrow aspirates of patients, differentiating them to hOss which then get injected with NB harvested from the same aspirate. This could provide a model to study and trial therapies to optimize the treatment protocol for individual patients, taking into account the altered states of MSCs reported in the literature (269).

Lastly, with the development of novel *in vitro* bioreactor systems, the possibility to mimic metastatic processes outside of animal models is slowly becoming reality. The EU-funded project B2B focusses on developing a metastasis *in vitro* system to mimic the spread of breast cancer cells from primary tumor to hOss (336). Implementing similar systems in the field of NB could shorten the time needed to establish the model and allow for fine-tuning of aspects such as treatment and sampling prior to testing *in vivo*.

Ferroptosis – The new miracle target in neuroblastoma?

Results from **Paper II** show that targeting ferroptosis is a promising approach, individually and in combination with COJEC, to target high-risk *MYCN* amplified neuroblastoma. Depending on the agent used, the combined approach is even more effective than COJEC alone. However, not all mechanisms of action are effective in all patients and some agents with the same mechanism of action can negatively interact with chemotherapy.

Selection of mechanism of action is important in NB

As presented in the data, we investigated three pathways involved in ferroptosis in NB patient data. We were able to observe that high expression in these pathways correlated with prognosis. During *in vitro* studies, we used a total of 17 ferroptosis inducing agents targeting mechanisms across these pathways. Although only four drugs were chosen for further testing, other agents should be considered in future studies as well. For example, Artesunate could be investigated, as it is an approved medication. While it was not as effective in all four NB models as the other agents, it could provide a stronger effect when combined with COJEC or turn out to be more effective when tested on a larger panel of neuroblastoma tumors.

With our growing understanding of NB, a more personalized therapy that targets specific phenotypes or metabolic processes becomes a close reality. In connection to ferroptosis, selection of agents should be based on mechanism of action that matches the pathways upregulated in the patient. Additionally, a focus on interaction of chemotherapy with ferroptosis inducing agent is important.

Is synergy the most important factor when using combinations?

As described in the introduction of this thesis, synergy defines a combination of therapeutics that yield a treatment response that is greater than the sum of the individual treatments. While in an ideal world all combination therapies should work like that, the current synergy testing mainly applies to pre-clinical results. In practice however, it has been shown that most therapeutic combinations in the clinic have an additive or even sub-additive effect and humans very rarely experience treatments that control the tumors more than additively (365). Synergistic effects in pre-clinical research are often limited to a handful of models, while clinical data represents several models. Hwangbo et al. further stated that if translated to the clinic, additive results would not exhibit an improved effect on progression-free-

survival compared to monotherapy, since this would only consider a small number of patients. On a large population of patients however, additive effects show a clear improvement when treated with combinations compared to monotherapies, as more tumor models with varying sensitivities are included (365). Based on this, it is arguable if achieving synergy should be the goal when testing combinations in preclinical research. One should consider instead using synergy testing more as a tool to find combinations that do not inhibit each other by focusing on different mechanisms of action.

In **Paper II**, we showed that RSL3, while being effective as a single agent, had an inhibitory interaction with COJEC. During consecutive tests, we were able to pinpoint this interaction to etoposide. Due to this counteracting interaction and the not yet addressed but limited bioavailability of RSL3, we discontinued further experiments. However, one could argue that excluding etoposide from COJEC or by scheduling the RSL3 treatment to occur during treatment cycles without etoposide, preferably with the additive and synergistic components of COJEC, could still be an interesting option. Of course, this would depend heavily on the bioavailability, half-time and other factors of both drugs, which at this point cannot be evaluated.

In **Paper III**, we observed similar issues that TRPA1 inhibition resulted in inhibitory effects when combined with COJEC. When adapting the treatment schedule to COJEC being added after the TRPA1 inhibition, we were able to prevent this effect. In the context of clinical use, studies have shown that *TRPA1* is significantly enriched in patients with chronic pain and has been connected to chemotherapy induced neuropathy (366,367). Marcotti et al. showed that the modulation of TRPA1 could prevent chemotherapy induced peripheral neuropathy in mice (368). By identifying that treatment at the same time causes an inhibitory effect, pre-treating with a TRPA1 inhibitor could still be of interest as a preventative measure for chemotherapy induced side-effects while potentially boosting the treatment.

Auranofin, which was used in **Paper II** as well, showed an additive effect when used with COJEC and improved survival during *in vivo* experiments. While this makes it an optimal candidate for further investigation in the clinic, additional questions remain. Auranofin has been approved and used in other diseases (353), but the translation of the dosage used in the *in vivo* experiment to the dosage that would be needed in the clinic to achieve similar effects, needs to be researched.

With these arguments, synergy should mainly be used to identify combinations and treatment strategies that are not inhibitory, instead of focusing on only finding the "optimal" combination. Additionally, this allows the investigation of mechanistic pathways that cause the inhibition which is information that could be used to still implement promising treatments in the clinic, without reducing the individual treatments effect.

TRPA1 – a potential to be revisited in the future

In **Paper III**, we investigated the potential of inhibiting TRPA1 as a form of neuroblastoma treatment. However, *in vivo* monotherapy showed no impact on tumor growth *in vivo*. While it would have been of interest to trial pre-treatment with TRPA1 and subsequent treatment with COJEC *in vivo*, the choice of currently available inhibitors was a deciding factor against it. During our experiments, we observed that the effects of the two inhibitors used resulted in varying outcomes on RNA level and in protein expression. For example, analysis of bulk RNA sequencing of *in vivo* treated tumors revealed that A967079 resulted in an upregulation of genes involved in neuron development. Bay 390 on the other hand seemed to have influenced genes related to the cell cycle. While the data in this study was promising, the fact of varying results from the inhibition of the same target suggests that it would be of more interest to investigate efficacy of an already approved drug, rather than investigating inhibitors limited to preclinical research. To date, there is however no inhibitor for TRPA1 approved for clinical use. Results of a proof-of-concept study using a novel TRPA1 antagonist, LY3526318, were published with the focus on chronic pain. However, no significant difference in pain was observed between treated group and placebo group. While previous tests with healthy participants did not reveal any side-effects, this study showed a safety concern due to potentially drug-induced liver toxicity (369). Similarly, clinical tests using the TRPA1 inhibitor ISC 17536 resulted in no significant difference between treatment groups (370).

While it would be of clinical interest to investigate the potential of using TRPA1 inhibition to prevent or alleviate chemotherapy-induced neuropathic pain while increasing chemotherapy efficacy, the lack of clinically approved and active inhibitors suggest that his investigation should be revisited at a later point.

Limitations

General limitations for all projects

Limitations regarding all papers in this thesis included the usage of immunocompromised mice. Previous research has shown that immune cells are capable of interacting with NB cells, specifically with the MES-like phenotypes (371), which we were not able to recapitulate due to the reduced immune system. The strains used in the projects were NSG and/or nude mice. NSG mice lack mature B and T cells as well as NK cells (372). Nude mice are genetically modified to prevent thymus development, therefore lacking mature T-cells. This strain however still has functioning B and NK cells (373). In the context of ferroptosis, the immune

system has been shown to play an important role in ferroptosis and should be considered in future studies (374). The NB PDXs used were of small sample size and all *MYCN* amplified. Conclusions made from this cannot be generalized to all neuroblastomas due to their difference in genetics and biology. With the addition of non-*MYCN* amplified PDXs, we would have been able to better represent the entire patient population. This was not done due to the lack of appropriate models during the studies performed. However, with the use of TMAs and scRNA/bulk RNA sequencing datasets which included patients without *MYCN* amplification, we were able to expand the material and investigate the potential to apply similar treatments to these patient groups. In addition, relying on sequencing data of patients goes in accordance with the 3Rs of animal research, allowing us to reduce the number of animals needed for our research.

What are the limitations of the *in vivo* COJEC protocol?

In Paper I, we established a novel *in vivo* protocol containing all five chemotherapeutic drugs used in the clinic. In preclinical research, it is common to use one, maybe two chemotherapeutic drugs. This does not fully recapitulate the treatment pressure tumor cells undergo and skew results in combination treatments. Using all five chemotherapeutics in a cyclic manner, we were able to provide a standardized protocol to use as a basis for combination studies or studies on the effect of COJEC on the tumor cells.

However, the dosage the mice received is not perfectly accurate and translatable from the human doses, as the murine drug metabolism differs from the human metabolism. Further, the chemotherapeutic agents are given in a shorter time frame of 40 days and with variation in their combinations compared to the exact schedule of the clinic. In addition to this, our *in vivo* protocol uses one-time administration of therapeutics, while patients receive the same drug sometimes over days. In patients, administration is done intra-venously, while mice received the treatment intra-peritoneal. Regarding the tumor location, PDX models with subcutaneous tumors were used for simplicity of measuring tumor sizes and for easier access during surgeries. This does not recapitulate the correct TME that tumor cells have in patients, where primary tumors are most often found at the adrenal gland. While orthotopic injections could have been performed, alternative forms of measuring tumor sizes, using for example ultrasound or the live imaging system IVIS, would have been needed. While being able to measure tumor growth with IVIS, it is not as accurate and surgeries for total resection are complicated.

Most of these mentioned limitations are of technical nature and while our protocol is not a perfect recapitulation, it improved our understanding of treatment impact on neuroblastoma cells in the *in vivo* setting. Compared to the traditional use of one chemotherapeutic, it advances the current modelling platform and provides new possibilities for *in vivo* drug screening.

What are the limitations of the *in vitro* COJEC protocol?

Besides the development of the *in vivo* version of COJEC, we also used an *in vitro* version of COJEC treatment to assess the chemoresistance of relapsed and treated PDX derived organoids.

Like in the *in vivo* version, there are limitations to this protocol. On a more technical side, the *in vitro* protocol is even more simplified than the *in vivo* version. Instead of administration in cycles, the chemotherapeutics are administered at the same time. This is mainly due to: 1) method to measure the viability used in this study and 2) technical limitation of the culturing method used. For our study, cells were seeded in 96 well plates, with the intend of future use in drug combination tests. As the process of changing media in 96 well plates, with free floating tumor organoids, is challenging, the approach of combining and applying COJEC at the same time was used. Developments of so-called bioreactors which supply continued flow to the tumor organoids could enable researchers to administer drugs in a cyclic manner. However, these bioreactors are not standard use yet. Regarding viability testing, alternative methods such as fixing organoids and staining for proliferation markers could have been used. However, this approach takes a long time and is not suitable for high-throughput testing.

What are the limitations of the metastatic model?

Several limitations of the hOss model used in **Paper IV** have been touched upon already in the previous chapters. As a summary, while hOss are an improved humanized niche compared to the femur of a mouse, it is not a perfectly accurate representation due to the limited numbers of human derived cell types. In addition, the injection methods used in the studies do not always recapitulate the full metastatic process.

During this study, we used three different injection methods to mimic the metastatic spread. Each of these methods comes with their own advantages and limitations. Starting with the orthotopic injections, we quickly encountered the issue that the primary tumors bioluminescent signal overtook, making it difficult to track the metastatic spread to the hOss. However, it represents the metastatic spread the most accurately, as the experiment starts at primary tumor. Intra-venous injections were used to mimic the next step in metastasis, the circulation. In contrast to the “natural” process, cells that were injected did not have to undergo changes from their primary tumor state to migrate to the vascular system. While the number of tumors cells found in the patients circularly system can vary between the individual patients and cancers (375), the ratio of injected cells to blood volume in this study is likely higher than observed in patients. As a last step, we used intra-hOss injections to mimic metastasis that was already engrafted in the BM niche. Cells injected in this manner did not undergo the changes and cellular stress connected to migration to the circular

system and the migration to the surrounding tissue. In addition, the number of cells injected into the hOss exceeds the number of cells that would normally enter the BM niche at the same time, ensuring that at least a small population survived and engrafted.

One of the mayor components lacking in the hOss model are hematopoietic stem cells and the resulting human derived immune system. A recent publication showed that NB metastasis had increased protein expression of PD-L1 compared to the non-metastasis groups. In addition, their research showed an enrichment in a specific set of tumor associated macrophages in metastatic samples which interacted with a subpopulation metastatic NB cells, potentially aiding in their progression (376). This suggests that the future addition of a human derived immune system would be of great interest to improve the model capacity of hOss.

As already mentioned in some other chapters of this thesis, the MSOD-B cells used in **Paper IV** were originally derived from an adult donor, which results in hOss with higher adipocyte content than expected for a pediatric patient. Obtaining and deriving MSCs from a pediatric donor could improve our model by more accurately representing the adipose cell number. Especially with the focus on personalized medicine, finding ways to use primary pediatric MSCs to not just successfully but routinely establish hOss could provide options for testing specific therapies or investigating resistance mechanisms for each patient.

Future Perspectives

As already mentioned in the sections above, there are future perspectives to consider for the individual projects.

For **Paper I**, it would be of high interest to investigate the RNA expression of specific NB clones before, during and after therapy using barcodes. This could provide more insight into the switching between phenotypes under treatment pressure of specific cells. In addition, using DNA sequencing, results could be compared to data available from patient studies previously published (113,114) to investigate clonal evolution. Further, using the established treatment protocols both *in vitro* and *in vivo*, they could be used to further assess potential combinations before clinical trials, as has for example already been done in **Paper II** and **Paper III**.

For **Paper II**, auranofin showed additive effect in combination with COJEC. However, future investigations into dose translation from mouse experiments to humans should be considered. As for RSL3, while the combination had an inhibitory effect, experiments investigating the timing of treatments could assess if RSL3 still could be of interest in the clinic. However, currently available forms of RSL3 cannot be used systemically due to pharmacokinetics. Hao et al. showed an interesting approach using RSL3-loaded liposomes which showed an impact in treating glioblastoma (377). Work like this suggests that ongoing developments could allow a revisit of the use of RSL3 in neuroblastoma with an adapted treatment schedule.

For **Paper III**, as discussed previously, the currently available inhibitors are an impactful limitation. With a clinically approved TRPA1 inhibitor, it would be of interest to re-investigate TRPA1 and a potential treatment target, especially with its role in peripheral neuropathy in mind.

For **Paper IV**, future perspectives for the hOss model could include the investigation of reported EV priming the premetastatic niche, assessment of ADRN/MES state of the MRD observed after treatment and their use as an orthotopic injection site of patient derived metastasis. A focus on development of patient derived MSC lines for further improvement of the model together with HSC transplantation could also be a focus point.

Conclusions

In summary, the following important points were made in each paper:

For **Paper I** we used PDXs of high-risk, *MYCN*-amplified NB and developed an *in vivo* protocol to mimic a clinically relevant treatment protocol using the five-chemotherapy regiment (COJEC) from the clinic. Using genomic and transcriptomic analysis on the treated and control tumors, we were able to show that tumors with intrinsic resistance had high genetic heterogeneity and displayed a more MES-like, immature phenotype. Tumors that responded to treatment well had an ADRN phenotype. By merging existing signatures and filtering them through treatment response and patient data, we were able to establish an integrated signature for both phenotypical states. These integrated signatures correlated with patient outcome. When moving the treated PDX tumors to an *in vitro* tumor organoid culture, we observed that relapsed samples retained their resistance, ADRN and MES signatures, and tumorigenicity. In conclusion, we showed that transcriptional states instead of genetic mutations seem to be the drivers of chemotherapy resistance in NB and inducing NB cells to express ADRN signatures could be beneficial for treatment success.

For **Paper II** we explored the potential of exploiting NB addiction to iron and cysteine to trigger Ferroptosis. Two promising therapeutic agents were identified, RSL3 and auranofin. While RSL3 showed remarkable effect *in vivo*, the bioavailability issue and its inhibitory effect when combined with the first line of treatment, COJEC, reduced its potential in clinical use. While the exclusion of etoposide from the protocol would prevent the inhibitory effect, a refined way of delivering RSL3 to tumor cells is needed. Auranofin, while less effective as a singular drug, had an additive effect when combined with COJEC. Transcriptional analysis post-treatment revealed an increase in the treatment sensitive ADRN phenotype, suggesting either an induced shift in NB cells or a targeting of MES-like cells. Together with the fact that auranofin is already FDA approved, it makes it an ideal therapy option for clinical testing.

In **Paper III** we investigated the potential use of TRPA1 inhibition in neuroblastoma, as it has previously been shown that calcium is relevant in differentiation and chemoresistance. While *in vitro* results showed promising effect by reducing proliferation and inducing morphological differentiation, *in vivo* treatment did not affect tumor growth. Interestingly, pre-treatment with TRPA1

inhibitors lead to an additive and even synergistic effect with COJEC. As TRPA1 has been shown to be involved in the development of peripheral neuropathy, inhibition of TRPA1 before COJEC could be of interest to prevent neuropathy and increase the effect of the chemotherapies. Further development of refined TRPA1 inhibitors would be needed to investigate this option.

In **Paper IV** we focused on the development of a novel humanized mouse model to mimic NB bone metastasis. Using three different injection methods, we could simulate the metastatic process from primary tumor to established metastasis in the BM niche. In addition, we were able to show that chemotherapeutic treatment of NB engrafted in the humanized BM niche reduced their numbers but could result in minimal residual disease, representing an important issue observed in the clinic. Future use of this model could provide insights into the metastatic process of NB, aid in testing novel therapeutic options targeting BM disease and improve our understanding of the mechanism behind resistant, minimal residual disease.

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