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Rastegar, Bahar

2026

Document Version:
Publisher's PDF, also known as Version of record

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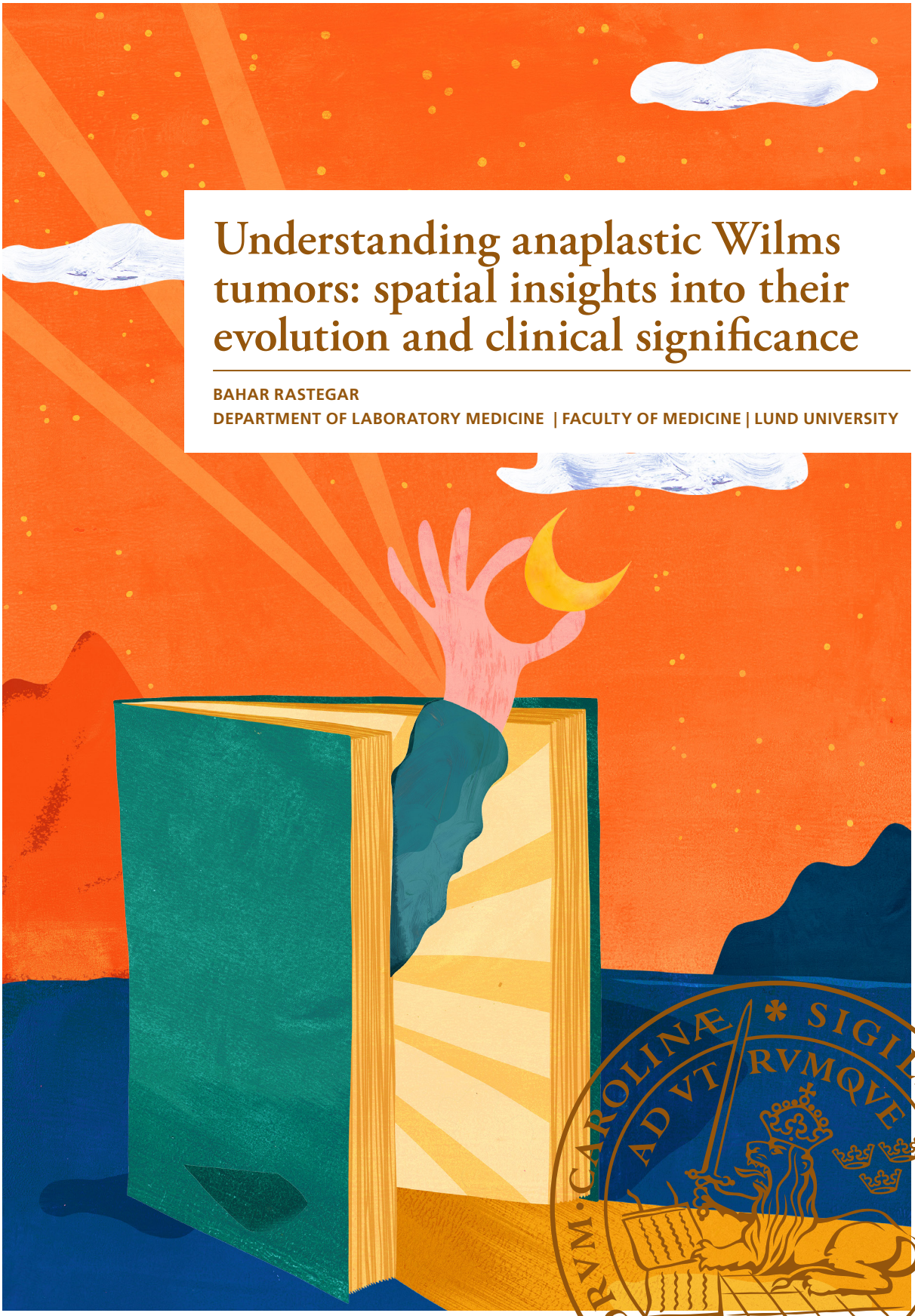

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DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Medicine at Lund University to be publicly defended on 27th of Febuary at 09.00 in Belfragesalen, BMC D15, Klinikgatan 32, Lund

Faculty opponent
Professor Sam Behjati MD, PhD

Department of Paediatric Oncology, University of Cambridge, England

Organization: LUND UNIVERSITY, Faculty of Medicine, Department of Laboratory Medicine

Document name: Doctoral Dissertation **Date of issue** Febuary 27th 2026

Author(s): Bahar Rastegar **Sponsoring organization:**

Title and subtitle: Understanding Anaplastic Wilms tumors: spatial insights into their evolution and clinical significance

Abstract:

Wilms tumor (WT) is one of the most common pediatric abdominal malignancies, with overall survival exceeding 90% in most cases. However, the subgroup of patients with advanced-stage WT and diffuse anaplasia (DA) faces a significantly poorer prognosis, largely due to chemotherapy resistance and the lack of effective targeted therapies. This thesis explores WT DA from multiple angles: tumor evolution, microenvironmental features, and molecular characteristics, to better understand its aggressive behavior and identify potential therapeutic targets.

Spatial mapping of subclonal architectures revealed that WT DA exhibits markedly complex phylogenies, characterized by high genetic diversity, saltatory and parallel evolution, and compartmentalized clonal patterns. *TP53* mutations were consistently associated with anaplastic regions and accompanied by increased copy number aberrations (CNAs) and regressive histological features.

Further, immunohistochemical analyses demonstrated that anaplastic cells have a high proliferative index, elevated levels of DNA double-strand breaks, and a gradual progression toward full-blown anaplasia, suggesting a mechanism of acquired tolerance to DNA damage that may underlie chemoresistance.

Finally, Glypican 3 (GPC3), a heparan sulfate proteoglycan, was identified as a promising therapeutic target. GPC3 expression was particularly strong and consistent in blastemal and anaplastic compartments of WT DA, and positively correlated with anaplastic features, proliferation, p53 expression, and apoptosis. These findings suggest that GPC3 may contribute to WT DA pathogenesis and merits further investigation in the context of targeted therapy.

In summary, WT DA is defined by complex tumor evolution, high genomic instability, and molecular traits that drive its resistance to treatment. These insights highlight the importance of anatomically informed tissue sampling for precision diagnostics and open avenues for novel therapeutic strategies, including GPC3-targeted approaches.

Key words: Wilms tumor, diffuse anaplasia, phylogeographies, clonal evolution, GPC3, single patient tissue microarrays

Classification system and/or index terms (if any) Supplementary bibliographical information

Language English **Number of pages**: 81

ISSN and key title: 1652-8220

ISBN: 978-91-8021-816-0

Recipient's notes Price Security classification

Signature Date 2025-12-15

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Lund 2026

ISBN 978-91-8021-816-0

ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University,
Lund, 2026

For all the patients and their families who are my driving force

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Preface

As a medical student, I often found it difficult to stay engaged. That was, until I stepped into the pediatric oncology ward. Something shifted. The complexity, humanity and strength in these children sparked a deep interest.

Initially, I questioned whether I had the emotional strength to work with children facing life-threatening illnesses. But I chose to view that emotional investment as a strength, not a limitation. That decision has shaped my path and brought me great fulfillment.

Working in pediatric oncology is a privilege. The courage and resilience of these children often surpass that of adults. As a pediatric oncologist, you become more than a clinician, you're a steady presence for families through years of treatment, and when needed, a guide in ensuring quality of life. It's one of the most meaningful aspects of our profession.

During my early clinical years, my colleague and friend Sofie Olsson Hau arranged a lunch meeting with Karin Jirström, the lunch that changed everything. I began working with pancreatic cancers, thinking I'd pursue general oncology. But after six months, I realized I couldn't let go of pediatric oncology.

When I shared this with Karin, she immediately reached out to David Gisselsson. In our meeting, I expressed my desire to focus on solid tumors with poor prognoses, hoping to make a meaningful contribution through research. Soon after, I connected with the pediatric oncology department, who offered me a future position. Our Ph.D. proposal was also awarded funding by the Swedish Childhood Cancer Foundation.

For a 26-year-old woman of Persian heritage, born in Malmö and educated in Poland, it felt surreal to see my dream within reach. In David's research group, I met my co-supervisor Linda (min stöttepelare), whose support has been invaluable throughout this journey.

The rest of the story unfolds in the pages that follow.

The front-page illustration, created by the talented Hanna Albrektson and modified by me is something I am truly honored to include in this thesis. It is more than an image it tells the story of my PhD journey. The open book represents the knowledge I have gained throughout these years, while the arm reaching for the stars reflects the moments when the path felt uncertain or intimidating. It symbolizes the curiosity that drove me forward and the determination to transform fear into growth.

Thesis at a glance

Paper I

- 1st author
- Published 2023
- *Clinical Cancer Research*

In summary:

Anaplastic Wilms tumors show distinct evolutionary patterns, with complex phylogenetic trees, high copy number aberrations, and progressive *TP53* mutations. These focal genetic changes drive secondary mutations, sustaining proliferation despite regressive features. Our findings highlight the need for comprehensive tumor sampling to fully understand tumor evolution.

Paper II

- 2nd author
- Published 2024
- *Modern Pathology*

In summary:

Chemoresistance in diffuse anaplastic Wilms tumors arises from a paradoxical mechanism where highly proliferative cells persist despite extensive genetic damage. This process is closely tied to *TP53* mutations and p53 expression, suggesting that anaplasia develops gradually through evolutionary adaptation. These insights highlight how tumor cells survive under genetic stress, reinforcing the complexity of anaplastic progression.

Paper III

- 1st author
- Unpublished manuscript

In summary:

The embryonal protein GPC3 is strongly and uniformly expressed in the blastemal component of Wilms tumors with diffuse anaplasia, suggesting a link to tumor aggressiveness. The low variation of GPC3 expression among blastemal tumor cells, indicates a potential therapeutic effect on the most proliferative tumor elements.

Patientberättelse: En resa genom barncancer

I arbetet med denna avhandling har jag haft förmånen att intervjua en tidigare patient med Wilms tumör och hennes mamma. Dottern är idag friskförklarad sedan sju år tillbaka. Deras berättelse ger en inblick i hur cancer påverkar både barnet och familjen. Nedan följer deras berättelse, återgiven med egna ord.

Dotterns berättelse

Det jag minns mest är hur jag åkte runt i sängarna, genom korridorerna, till strålningen. Jag minns också när jag skulle sövas, och det hände ofta. Det var mycket undersökningar, mycket väntan. Jag låg ofta i sängen och stirrade upp i taket. Ovissheten där var jobbig, men till slut blev det en vana.

Jag var aldrig rädd. Det var som att jag visste att jag skulle klara det. Vänner och kusiner som kom och hälsade på blev en viktig del av min återhämtning. Vi pratade, lekte, skrattade, inte om sjukdomen, utan om allt annat. Cancer är obekvämt, men nu i efterhand var det värt att gå igenom allt.

Nu när jag är frisk känns det bra, men det är en annan sorts känsla än för någon som aldrig haft cancer. Jag behöver fortfarande åka till sjukhuset, missa skolan och har mindre fritid än vänner som inte haft cancer. Men jag gillar att vara tillbaka på sjukhuset. Det känns tryggt. Jag känner mig säker här, med personalen, med lokalerna. Jag vill komma tillbaka och jobba här en dag.

Jag älskade krossade isbitar. Sköterskorna brukade krossa dem i små, små bitar och ge mig en sked. Det var en speciell smak, en smak av nostalgi. Jag äter det fortfarande hemma ibland. Alla hade sitt eget sätt att krossa dem, det var något fint i det.

Mammans berättelse

Diagnosen var fruktansvärd. Hon var ett så friskt barn, jag kunde inte förstå att hon hade cancer. Efter flera läkarbesök under tre veckor var det jag som till slut upptäckte tumören. Hon kräktes, gick ner i vikt. Jag var förtvivlad. Jag grät. Jag visste att något var fel.

En morgon låg hon i vår säng, solen lyste på hennes mage och då såg jag den, *Knölen*. Det var då jag förstod. Jag hade redan googlat på barn och cancer i magen. Wilms tumör dök upp. Det var som att jag visste.

Under sjukdomstiden var allt kritiskt. Vi hade en gård, djur, en liten lillasyster, men alla hjälptes åt. Farmor och mormor turades om att vara på Ronald McDonald med lillasyster. Det var svårt att träffa lillasyster då hon var frisk, och det kändes orättvist. Jag kände skuld för att det var jobbigt att se lillasyster. Men samtidigt, var det fint och en undanflykt från den verklighet vi hade. Dock kände jag ofta att jag

bara ville vara i ”bubblan” med storasyster på sjukhuset, där vi kände oss trygga och hade någon typ av kontroll.

Storasyster var bestämd. Ingen annan fick röra henne. Det var mest jag som fick göra allt, lyfta henne, flytta henne till röntgenbordet. Hon hade slangar överallt. Vi sov tillsammans i en 90 cm säng i fem månader. Hon ville att våra huvuden skulle ligga mot varandra. Den närheten, den betydde allt.

Jag kommer ihåg första gången vi fick åka hem på permission från sjukhuset, efter 5 månader. Det var snö ute. Vi åkte pulka. Allt kändes så overkligt.

När hon blev friskförklarad kändes det som att livet aldrig skulle bli sig likt igen. Man förstår vad som är viktigt. Jag minns när hon låg på operationsbordet i Göteborg - jag tänkte: ”*Jag kan bo i en koja i skogen, bara jag får med den här ungen levande härifrån.”*

Vi fick så mycket stöd under tiden på sjukhuset. Men efteråt, då fanns inget. Jag var utmattad, sökte hjälp och fick kontakt med en psykolog som arbetade med akuta krisreaktioner. Ingen hade verktygen jag behövde. Jag tog hand om det själv. Pratade med familjen. Hästarna hjälpte också, det var terapeutiskt. Jag hade dock önskat mer hjälp i efterhand. Vi fick mycket hjälp när vi var inneliggande, men jag var inte mottaglig då. Jag hade inte tid att tänka på mig själv.

Vi pratar inte mycket om sjukdomstiden. Bara när barnen vill. Jag vill gärna ventilera. Min syster, som är sjuksköterska, var ett stort stöd. Något vi fick tips om var att man skulle ta mycket bilder på tiden man var inneliggande för att prata om det i efterhand. Dem tittar vi på ibland. Men hon tycker det är jobbigt. Hon svarar: ”Nä, det tycker jag inte. Du tar konstiga bilder, och så ler hon.”

Hon minns inte så mycket. Det känns konstigt, att alla andra vet mer än hon som faktiskt var sjuk. Men hon var så stark. Jag minns när hon cyklade till strålbehandlingen med droppställningen. Hon är min hjälte.

När hon tog sina första steg igen efter operationen, ropade hon direkt: ”ANNA!” – till sin vuxna vän (barnkirurgen). Den relationen till vårdpersonalen har satt spår. Nu vill hon själv bli kirurg.

Reflektion

Det är just mötet med barnen och deras familjer som motiverar mig att arbeta kliniskt och att forska inom området. Deras berättelse är den mest betydelsefulla delen av min avhandling, eftersom den påminner om varför vi bedriver forskning: för att förbättra vården för dem som drabbas.

Denna berättelse ger en konkret inblick i hur ett barn och en förälder upplever tiden kring en cancerdiagnos och behandling. Barnets perspektiv visar hur trygghet kan byggas genom relationer, rutiner och närvaro, även i en komplex vårdmiljö.

Förälderns berättelse belyser den psykiska belastningen, men också den starka närheten och omsorgen som uppstår i krisen.

Berättelsen understryker vikten av kontinuitet i vården och hur relationen till vårdpersonal kan skapa trygghet och påverka barnets framtida syn på sjukvård. Den visar också att behovet av stöd kvarstår efter avslutad behandling, både för barnet och familjen.

Patient story: A journey through childhood cancer

As part of this thesis work, I had the privilege of interviewing a former Wilms tumor patient and her mother. The daughter has been in remission for seven years. Their story provides insight into how cancer affects both the child and the family. Below is their story, retold in their own words.

The daughter's story

What I remember most is being wheeled around in beds, through corridors, to radiation treatments. I also remember being put under anesthesia, which happened often. There were many examinations, lots of waiting. I often lay in bed staring at the ceiling. The uncertainty was hard, but eventually it became routine.

I was never afraid. It was as if I knew I would make it. Friends and cousins who came to visit were an important part of my recovery. We talked, played, laughed, we never talked about the illness, but about everything else. Cancer is uncomfortable, but looking back, it was worth going through it all.

Now that I'm healthy, it feels good, but it's a different feeling than for someone who has never had cancer. I still have to go to the hospital, miss school, and have less free time than friends who haven't been sick. But I like going back to the hospital. It feels safe. I feel secure there, with the staff and with the environment. One day, I want to come back and work there.

I loved crushed ice. The nurses used to crush it into tiny pieces and give me a spoonful. It had a special taste, a taste of nostalgia. I still eat it at home sometimes. Everyone had their own way of crushing it, and there was something beautiful about that.

The mother's story

The diagnosis was devastating. She was such a healthy child; I couldn't comprehend that she had cancer. After several visits to the doctor over three weeks, I was the one who finally discovered the tumor. She was vomiting, losing weight. I was desperate. I cried. I knew something was wrong.

One morning, she was lying in our bed, the sun shining on her stomach, and that's when I saw it, the lump. That's when I understood. I had already googled "children" and "cancer in the abdomen." Wilms tumor came up. It was as if I knew.

During treatment, everything was critical. We had a farm, animals, a little sister, everyone helped. Grandma and grandpa took turns staying at Ronald McDonald House with the little sister. It was hard to see her, because she was healthy, it felt unfair. I felt guilty for finding it hard to see her. But at the same time, it was nice and an escape from the reality we were living. Still, I often just wanted to stay in the

“bubble” with the big sister at the hospital, where we felt safe and had some sense of control.

Big sister was determined. No one else was allowed to touch her. Mostly, I did everything, lifting her, moving her to the X-ray table. She had tubes everywhere. We slept together in a 90 cm bed for five months. She wanted our foreheads to touch. That closeness meant everything.

I remember the first time we were allowed to go home on leave from the hospital, after five months. There was snow outside. We went sledding. Everything felt surreal.

When she was declared healthy, it felt like life would never be the same again. You realize what really matters. I remember when she was lying on the operating table in Gothenburg, I thought: “I can live in a hut in the woods, as long as I get this child out of here alive.”

We received so much support during the hospital stay. But afterward, there was nothing. I was exhausted, sought help, and eventually got in touch with a psychologist who worked with acute crisis reactions. No one had the tools I needed. I handled it myself. Talked to family. The horses helped too, it was therapeutic. But I wish there had been more help afterward. We got a lot of help while we were admitted, but I wasn’t receptive then. I didn’t have time to think about myself.

We don’t talk much about the illness now, only when the children want to. I would like to talk about it more. My sister, who is a nurse, was a great support. One tip we got was to take lots of pictures during the hospital stay to talk about it later. We look at them sometimes. But she finds it hard. She says: “No, I don’t think its hard. You take weird pictures,” and then she smiles.

She doesn’t remember much. It feels strange that everyone else knows more than the one who was actually sick. But she was so strong. I remember when she cycled to radiation with the IV pole. She is my hero.

When she took her first steps again after surgery, she immediately shouted: “ANNA!”- to her adult friend (the pediatric surgeon). That relationship with the healthcare staff left a mark. Now she wants to become a surgeon herself.

Reflection

It is the encounter with children and their families that motivates me to work clinically and to conduct research in this field. Their story is the most meaningful part of my thesis because it reminds us why we do research: to improve care for those who are affected.

This story provides a concrete insight into how a child and a parent experience the time surrounding a cancer diagnosis and treatment. The child’s perspective shows how security can be built through relationships, routines, and presence, even in a

complex care environment. The parent's story highlights the psychological burden, but also the deep closeness and care that arise in a crisis.

The story underscores the importance of continuity in care and how relationships with healthcare professionals can create a sense of safety and influence the child's future view of healthcare. It also shows that the need for support persists after treatment ends, for both the child and the family.

Populärvetenskaplig sammanfattning

Wilms tumör, även kallad nefroblastom är den vanligaste njurtumören hos barn. Den drabbar flickor och pojkar lika ofta. Majoriteten av de drabbade barnen är under 6 år. När den tyske kirurgen Carl Max Wilhelm Wilms först beskrev tumören 1899 var prognosen dyster. Inledningsvis bestod behandlingen enbart av kirurgiskt avlägsnande av den drabbade njuren, vilket resulterade i låg överlevnad. Ett genombrott kom på 1950-talet när läkare började kombinera kirurgi med strålbehandling av området kring tumören. Detta ökade överlevnaden till omkring 50%. 1970-talet markerade början på en ny era inom behandlingen av Wilms tumör. Upptäckten att cytostatika var särskilt effektivt mot denna tumörform revolutionerade behandlingsmetoderna. Wilms tumör blev den första barncancerformen där cytostatika visade tydliga resultat. Den moderna behandlingen av Wilms tumör är en framgångssaga inom barncancervården. Genom att kombinera olika behandlingsmetoder, en kombination av kirurgi, strålbehandling och cellgifter har överlevnaden ökat dramatiskt. Nio av tio barn som drabbas av Wilms tumör blir idag friska. Denna förbättring i överlevnad är ett lysande exempel på hur medicinsk forskning och utveckling av behandlingsmetoder kan förändra livet för många unga patienter och deras familjer.

Wilms tumör ger sällan några uppenbara symptom, man söker vård när en förälder upptäcker en svullnad i magen. Detta är den vanligaste anledningen till att tumören är så pass stor vid diagnostillfället. Inte heller andra symptom som kan förekomma vid Wilms tumör är särskilt uppenbara: feber, trötthetskänsla, blod i urinen och förstoppning. Det vanligaste tillvägagångssättet för diagnos är genom ultraljudsundersökning, men ibland kan man behöva genomgå en skiktröntgen. Behandling av tumören innebär alltid cellgiftsbehandling till en början för att minska storleken på tumören och göra operationen mindre komplicerad. Efter operationen undersöks tumören under mikroskop för att bestämma tumörtyp och hur länge och vilken typ av cellgiftsbehandling patienten ska genomgå. Ett fåtal patienter behöver även få strålbehandling mot den njuren där tumören suttit. Många barn blir friskförklarade efter behandling, men ett fåtal patienter som har tumörtypen diffus anaplasi har en mycket sämre prognos och har inte samma överlevnadsstatistik. Det är dessa tumörer som jag har fokuserat på inom min forskning. Genom att analysera biologisk heterogenitet och mikromiljö i tumörerna ville vi utröna mer om hur anaplastisk Wilms tumör uppkommer och utforska om det finns nya, alternativa terapier för att öka överlevnad och minska biverkningar av nuvarande behandling.

I den första studien kartlade vi de genetiska förändringar som leder fram till anaplasi i Wilms tumör. Vi använde en unik samling av hela tumörsnitt för att undersöka hur cancercellerna förändras över tid och rum inom tumören. Genom att analysera genetiska förändringar, särskilt mutationer i *TP53*-genen och förändringar i antalet genkopior, fick vi en djupare förståelse för hur anaplasi utvecklas. Vi studerade

också hur tumörcellerna förändrar sitt utseende och sin funktion under denna process. Vår forskning gav en detaljerad bild av hur Wilms tumörer utvecklas till mer aggressiva former. Detta kan hjälpa oss att bättre förstå varför vissa tumörer är svårare att behandla och kan leda till nya sätt att angripa dessa utmanande cancerformer i framtiden. Vi upptäckte att anaplastiska tumörer har en mer komplex genetisk struktur än andra Wilms tumörer. De uppvisar större mångfald, oregelbundenhet och avvikelser i sitt "släktträd" av cancerceller. Dessutom har de fler kopietalsförändringar i sitt DNA. Våra fynd tyder på att de mutationer i *TP53*-genen som ofta ses vid anaplasi uppkommer i ett litet område och sedan utlöser en kedjereaktion av ytterligare genetiska förändringar. Detta sker samtidigt som tumören fortsätter att växa snabbt. Studien understryker vikten av att ta prover från olika delar av tumören för att få en fullständig bild av dess genetiska sammansättning. Denna kunskap kan i framtiden leda till bättre behandlingsstrategier för barn med denna aggressiva form av Wilms tumör.

I vår andra studie fokuserade vi återigen på anaplastiska celler. Vår hypotes var att dessa celler kan fortsätta växa och sprida sig även under cellgiftsbehandling. Vi trodde att detta berodde på att de har en unik förmåga att överleva trots omfattande skador på sitt DNA och stora förändringar i antalet genkopior. Genom att testa denna idé hoppades vi få en bättre förståelse för varför vissa Wilms tumörer är resistenta mot behandling. Denna kunskap kan i framtiden leda till nya, mer effektiva behandlingsmetoder för barn med denna svåra form av cancer. Med denna studie upptäckte vi att i områden där cancercellerna ser särskilt onormala ut (det vi kallar hög-gradig pleomorfism), fortsätter cellerna att dela sig snabbt. Detta sker trots att cellerna har omfattande skador på sitt DNA och många förändringar i antalet genkopior. Denna ovanliga förmåga verkar hänga ihop med höga nivåer av ett protein som kallas p53, samt mutationer i genen som styr produktionen av detta protein (den redan nämnda *TP53*-genen). Normalt sett skulle celler med så mycket genetisk skada sluta dela sig eller dö. Men dessa aggressiva cancerceller tycks ha hittat ett sätt att överleva och fortsätta växa trots skadorna. Det verkade därmed inte sannolikt att nya varianter av cellgifter och strålning, vars mekanism är att allvarligt skada DNA, kunde hjälpa oss att bota fler barn med anaplastisk Wilms tumör.

Med vår tredje studie ville vi därför utforska vägar till nya behandlingar för anaplastisk Wilms tumör. I den studien undersökte vi ett protein vid namn Glypican 3 (GPC3) som förekommer i höga nivåer i vissa former av barncancer enligt andra forskare. Vi fann att i de diffust anaplastiska Wilms tumörerna, förekommer GPC3 i anmärkningsvärt höga nivåer. Vi upptäckte samband mellan GPC3 och centrala cancerprocesser: proteinet korrelerar tydligt med snabb celldelning, förändringar i det viktiga p53-proteinet och till och med ökad celldöd. Dessa resultat öppnar spännande möjligheter. GPC3 skulle kunna utvecklas till ett mål för framtidens immunterapi eller fungera som en prognostisk markör som hjälper oss att förutse hur aggressiv en specifik tumör är. För barn med Wilms tumör kan detta innebära mer skräddarsydda och effektiva behandlingar.

List of papers

The thesis is based on the following papers:

I. Rastegar B, Andersson N, Karlsson J, Chattopadhyay S, Valind A, Durand G, Jansson C, Romerius P, Jirström K, Holmquist Mengelbier L, Gisselsson D. Resolving the pathogenesis of treatment-resistant anaplastic Wilms tumors through spatial mapping of cancer cell evolution. *Clin Cancer Res. 2023 Jul 14;29(14):2668-2677*

II. K. Uno, B. Rastegar, C. Jansson, G. Durand, A. Valind, S. Chattopadhyay, A. Bertolotti, S. Cicerie, F. Spreafico, P. Collini, D. Perotti, L. Holmquist Mengelbier, D. Gisselsson. A gradual transition toward anaplasia in Wilms tumor through tolerance to genetic damage. *Modern Pathology 2024 Vol. 37 Issue 1*

III. Rastegar B, Uno K, Chattopadhyay S Jansson C, Bertolotti A, Ciceri S, Perotti D, Spreafico F, Collini P, Holmquist Mengelbier L, Gisselsson D. Strong glypican 3 expression in blastemal components of Wilms tumor with diffuse anaplasia. *Manuscript unpublished.*

Abbreviations

WT	Wilms tumor
DA	Diffuse anaplasia
WT DA	Wilms tumor with diffuse anaplasia
CNA	Copy number aberrations
GPC3	Glypican-3
CCS	Childhood cancer survivor
TMA	Tissue microarray
WAGR	Wilms tumor aniridia genitourinary anomalies and mental retardation
VMA	Vanillylmadelic acid
HVA	Homovanillic acid
VA	Vincristine and Actinomycin D
AVD	Actinomycin D, Vincristine and Doxorubicin
WT1	Wilms tumor protein 1
VEGF	Vascular endothelial growth factor
WMS	Whole mount section
IR	Intermediate-risk
BT	Blastemal-type
FFPE	Formalin-fixed, Paraffin-embedded
H&E	Hematoxylin and Eosin
TMA	Tissue microarray
FF	Fresh frozen
LOH	Loss of heterozygosity
SNP	Single nucleotide polymorphism
BAF	B-Allele frequency
TAPS	Tumor aberration prediction suite
TCF	Tumor cell fraction
MCF	Mutated cell fraction
MP	Maximum parsimony
ML	Maximum likelihood
MMP	Modified maximum parsimony
PSR	Phylogenetic species richness
TDS	Targeted deep sequencing
TCT	Tumor cell twinning
COEX	Clonal coexistance
VAR	Subclonal variance
SWE	Clonal sweeps
IHC	Immunohistochemistry
dsDNA	Double-stranded DNA

Introduction

Overview of pediatric oncology

Although remarkable progress has been made in the management of childhood cancers over the past five decades, these diseases continue to represent a significant global public health challenge. Enhanced diagnostic methods, refined risk stratification, and advances in treatment modalities have significantly improved outcomes for children with cancer [1]. Today, thanks to advances in modern therapies and comprehensive supportive care, approximately 80% of affected children can be successfully treated, when having access to the right resources [2]. So far, only around 10% of the world's children reside in high-income countries, where access to advanced medical care is more readily available [3].

Over the past five decades, pediatric oncology has experienced profound transformation, largely driven by the development and continual modification of chemotherapy protocols through the collaborative efforts of research groups in North America and Europe. These collaborations have enabled the creation of more effective treatment regimens, optimizing the use of existing agents and resulting in substantial improvements in survival across nearly all pediatric cancer types. Progress in stem cell transplantation has provided a critical therapeutic option for children whose cancers are resistant to initial treatment. Advances in molecular diagnostics, minimal residual disease detection, and genetic profiling have enabled more personalized and effective treatments. Improved imaging technologies have enhanced the detection of metastases, while surgical and radiation techniques have become more precise. The emergence of targeted therapies and immunotherapies has further expanded treatment options for specific patient groups. Pediatric cancer mortality has significantly decreased [4, 5]. Pediatric tumors present unique challenges due to their rarity, biological heterogeneity, distinct pathogenetic mechanisms compared to adult cancers, a higher incidence of hereditary predisposition, and the imperative to balance optimal survival with minimizing long-term treatment-related sequelae [4].

Key challenges

While survival rates for many pediatric cancers have improved significantly, these gains have come with a growing awareness of the long-term consequences of treatment. These include cardiomyopathy from anthracyclines and chest radiotherapy, as well as secondary malignancies linked to radiation, alkylating agents, and topoisomerase inhibitors. Among childhood cancer survivors, over 70% develop at least one chronic health condition within 30 years of diagnosis, with 42% experiencing severe or life-threatening complications [6]. Survivors face complex late effects including second malignancies, cardiovascular disease, and endocrine disorders that significantly impact quality of life [7]. Recent evidence indicates that approximately 40–50% of survivors will develop at least one endocrine disorder during their lifetime. Radiation to endocrine organs such as the hypothalamus, pituitary, thyroid, and gonads is the strongest predictor of later dysfunction. These effects are both dose- and time-dependent, with higher doses and longer intervals after treatment increasing risk. Endocrine disorders may emerge decades post-therapy, emphasizing the need for lifelong follow-up. [8, 9]. Cardiovascular late effects in childhood cancer survivors commonly include cardiomyopathy or heart failure, valvular disease, pericardial disorders, and coronary artery disease. Among those treated with anthracyclines, the prevalence of cardiomyopathy ranges from about 16% for symptomatic cases to nearly 50% when assessed by echocardiographic evidence of impaired cardiac function [10, 11].

By age 50, childhood cancer survivors experience twice as many severe chronic health conditions as their healthy peers [12], and their mortality rate is ten times higher than that of the general population [13].

The growing use of targeted therapies has also revealed unique toxicities in the developing child [14-17]. Immunotherapy is not without risks. Adverse effects such as aplastic anemia, hypothyroidism, and hypophysitis have been reported with monoclonal antibody therapy and may have lifelong consequences in children [18]. However, compared to conventional chemotherapy, immunotherapy offers the advantage of high cell specificity, potentially reducing long-term toxicity [19]. Today, pediatric oncology drug development focuses both on improving outcomes for patients with high-risk tumors and on integrating novel agents to reduce long-term treatment-related morbidity [20]. Pediatric cancers affect approximately 16 out of every 100,000 children aged 1 to 18 years [21], a frequency that meets the EU and US criteria for classification as rare or orphan diseases [22]. Drug development in pediatric oncology faces several obstacles, including smaller patient populations compared to adults and the typically low mutational burden of childhood cancers [20].

As D'Angio noted as early as 1975, [23] "cure is not enough." The significant improvement in survival rates has resulted in a growing population of childhood cancer survivors (CCSs) reaching adulthood [24]. However, decades of research have

demonstrated that, compared to the general population, survivors of childhood cancer face an increased risk of early mortality, the development of secondary malignancies, and a wide spectrum of late clinical and psychosocial effects related to both the disease itself and its treatment [6, 12, 25-27]. Growing knowledge of late treatment-related toxicities has led to the development of risk stratification systems for each cancer type, based on clinical, biological, or genetic factors. This approach allows for tailored treatment intensity, aiming to minimize toxicity without compromising cure rates. Notably, these strategies have contributed to a reduction in late mortality among more recently treated CCSs [28] and have influenced risk models for certain chronic conditions [29]. Lifelong follow-up is now considered essential for all CCSs, enabling prevention, early detection, and management of late effects, as well as timely interventions to mitigate or improve adverse outcomes [30]. With the increasing number of survivors, a major challenge in their care is ensuring effective long-term follow-up and integrating evidence-based clinical practice guidelines into routine clinical practice. This isn't just a challenge for healthcare providers, but also a challenge for infrastructure that should be able to provide CCS adequate care [31].

Unique aspects of childhood cancer

The development of cancer in adults is attributed to the progressive build-up of mutations throughout an individual's life. Advances in recent decades have established genetic predisposition as a key factor in some childhood cancers, with epigenetics emerging as an important contributor [32]. Compared to adult cancers, childhood cancers typically exhibit a lower somatic mutation burden [33] and is rather strongly influenced by germline mutations in cancer predisposition genes [34, 35]. Several well-characterized cancer predisposition syndromes have been identified in children, including Li–Fraumeni syndrome, neurofibromatosis, Fanconi anemia, DICER1 syndrome, multiple endocrine neoplasia, retinoblastoma predisposition, rhabdoid tumor predisposition syndrome, APC-associated polyposis, BRCA-related syndromes, and constitutional mismatch repair deficiency, among others [33, 36, 37]. The genes involved in these syndromes are primarily involved in DNA repair, cell cycle regulation, and apoptosis, and are linked to a broad range of cancer types [38].

Wilms tumor

WT exhibits a striking similarity to the early stages of kidney formation, both in its structural appearance and gene expression patterns. This parallel reflects the tumor's origins in the developmental processes of nephrogenesis [33]. Most commonly, these tumors exhibit a triphasic structure, interweaving three distinct cell

populations: undifferentiated blastemal cells, maturing epithelial components and developing stromal elements. This three-part composition is the hallmark of classic WT. However, the cellular makeup can vary. Some tumors deviate from this pattern, presenting as biphasic variants with only two of these cellular elements. Even rarer are monophasic tumors, composed of a single cell type. These variations in cellular architecture highlight the spectrum of differentiation within WTs, reflecting the complexity of disrupted renal development [39, 40].

Epidemiology

WT represents the most prevalent renal malignancy in the pediatric population. Epidemiological studies have demonstrated notable geographic and ethnic variation in its incidence [41, 42]. For instance, the annual incidence rate of WT in East Asia is significantly lower compared to North America and Europe, with reported figures of approximately 4.3 cases per million children versus 8–9 cases per million, respectively. Within the United States, children of African/American descent exhibit the highest incidence rate (9.7 per million), whereas those of Asian/Pacific Islander descent show the lowest (3.7 per million) [41].

Accurate estimation of the global incidence of WT remains challenging, particularly in resource-limited settings. This difficulty is largely attributable to the absence of comprehensive, population-based childhood cancer registries and the variable quality of available data, which may be incomplete due to underreporting of cases or limited diagnostic capacity [2, 43, 44]. Furthermore, in regions with constrained healthcare resources, approximately 50% of children present with metastatic disease at the time of diagnosis, reflecting delays in detection and access to healthcare [45]. The mean age at diagnosis is 36 months, with most cases occurring between 1 and 4 years of age. Globally, WT tend to present earlier in boys, peaking around 1 year, compared to a peak between 1 and 3 years in girls [41].

Etiology and risk factors

Around 17% of WTs develop as part of a malformation syndrome [46]. Of these, about 10% are linked to known genetic predisposition [47].

Syndromic predisposition to WT includes:

- Wilms tumor-aniridia syndrome (WAGR) (11p13; *WT1* gene deletion)
- Denys-Drash syndrome (defect in the *WT1*gene)
- Beckwith-Wiedemann syndrome (11p15.5; WT2 region deletion)
- Frasier syndrome (defect in the *WT1* gene)

- Li-Fraumeni syndrome (pathogenic variation in the *TP53* gene)
- Simpson-Golabi-Behmel syndrome (mutation in the *GPC3* gene)
- Fanconi anemia (biallelic pathogenic variants in *BRCA2* or *PALB2*)
- Mosaic variegated aneuploidy (pathogenic variations of biallelic *BUB1B* or *TRIP13*)
- Perlman syndrome (biallelic pathogenic variants in *DIS3L2*) [48]

Clinical presentation

The clinical presentation of WT is typically an asymptomatic mass in the abdomen which is found by a family member or a health professional. Most are diagnosed before 5 years of age [49-51]. Only 20% of the patients present with:

- Hematuria
- Abdominal pain
- Hypertension
- Fever
- Weight loss
- Urinary tract infection
- Constipation

Although uncommon, metastatic WTs may present with symptoms such as dyspnea due to pulmonary involvement, abdominal pain from liver metastases, or vascular complications including tumor thrombus in the renal vein or vena cava, and varicocele [52].

Clinical assessment

The clinical evaluation includes documentation of the patient's age, gender, body weight, length, and the presence of any dysmorphic features. Functional status is assessed using the Lansky or Karnofsky Performance Scales, depending on the patient's age. One should note blood pressure and body temperature, as well as any clinical signs of infection. In addition, the presence of an abdominal mass is evaluated through palpation, noting its location and size if detectable.

Initial laboratory work-up includes both blood and urine analyses. Blood investigation includes a full blood cell count, renal function markers such as creatinine and blood urea nitrogen, lactate dehydrogenase, liver enzymes, and serum

calcium levels. Coagulation parameters are assessed, given that approximately 5% of patients with WT may develop acquired von Willebrand's disease. Blood typing, along with baseline viral screening are done.

Urine analysis includes measurement of catecholamine metabolites vanillylmandelic acid (VMA) and homovanillic acid (HVA) to exclude neuroblastoma.

Initial imaging includes abdominal ultrasound as the first-line modality for evaluating suspected abdominal masses, assessing tumor size, location, and vascular involvement. Abdominal MRI is used as the preferred complementary imaging due to its superior soft tissue contrast and lack of ionizing radiation. Chest X-ray is performed at diagnosis as a baseline, while chest CT is used to detect pulmonary metastases. Additional imaging, such as MIBG scintigraphy, brain MRI, or echocardiography, is performed when clinically indicated. A short schematic description in the figure below (Figure 1) [53].

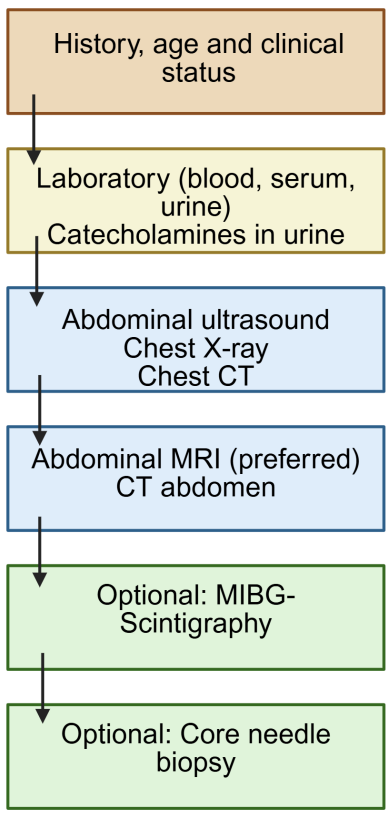


Figure 1. Schematic overview of clinical assessment in Wilms tumor.
Including key steps such as patient history, physical examination, laboratory investigations, and imaging modalities [53].

Histopathological assessment

Adequate tumor sampling is essential to ensure accurate pathological assessment [54]. WTs are stratified into three histological risk groups: low, intermediate, and high, based on post-chemotherapy histology [54, 55]. Classification relies on evaluating the proportion of viable tumor components and chemotherapy-induced changes. The initial step is to determine the presence of DA. If absent, the next step is to quantify both macroscopically and histologically the proportion of chemotherapy-induced changes (necrosis, fibrosis, foamy macrophages) and viable tumor tissue [54].

Differentiating between non-anaplastic and anaplastic WTs is crucial in both the international SIOP and the North American COG treatment protocols. The diagnostic criteria for anaplasia are consistent across both approaches, as anaplasia is unaffected by preoperative therapy [56]. Diagnostic features of anaplasia include abnormal, enlarged multipolar mitoses and markedly enlarged, hyperchromatic nuclei, typically three times the size of adjacent nuclei of the same cell type (Figure 2) [56, 57].

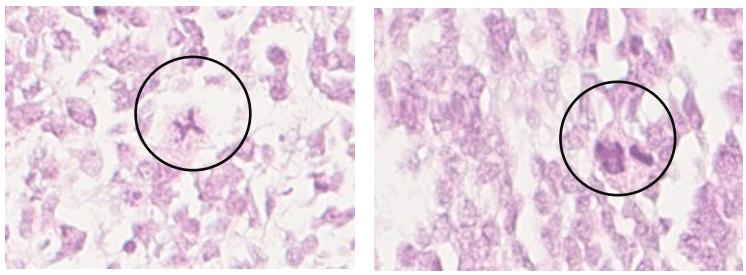


Figure 2. Diagnostic features of anaplastic Wilms tumor.
A) WT DA, encircled area shows mitotic figure. B) WT DA with hyperchromatic, enlarged cell nuclei.

Anaplasia is classified as either focal or diffuse. DA is defined by non-circumscribed anaplastic areas or the presence of anaplasia outside the kidney, such as in vessels, the renal sinus, extracapsular sites, or metastases. Additionally, focal anaplasia accompanied by widespread nuclear atypia ('nuclear unrest') or identified in limited samples (e.g., biopsies) is also considered diffuse [56]. Focal anaplasia is defined as one or more well-demarcated areas of anaplasia confined to the primary tumor, without evidence of anaplasia or significant nuclear atypia in extrarenal or extratumoral sites. Multiple foci are acceptable if each is small enough to fit within a single microscopic section and all other criteria for FA are met [56]. According to the UMBRELLA 2016 protocol, a diagnosis of FA is limited to a maximum of two foci, each no larger than 15 mm in diameter [57], and without evidence of anaplasia or significant nuclear atypia in extrarenal or extratumoral sites. If these criteria are

met, the tumor is classified as intermediate-risk and managed similarly to other intermediate-risk, non-anaplastic WTs [54].

Table 1 – Classifying Wilms tumor after preoperative chemotherapy

Tumor type	Criteria	Histological risk group
Completely necrotic	No viable tumor	Low
Regressive	>=67% chemotherapy induced changes, regardless of the specific viable WT component.	Intermediate
Epithelial (Figure 3A)	At least 33% viable tissue, of which >=67% is epithelial and <10% is blastemal.	Intermediate
Stromal (Figure 3B)	At least 33% viable tissue, of which >=67% is stromal and <10% is blastemal.	Intermediate
Mixed	At least 33% with no single component exceeding 67%, except when epithelial and/or stromal elements coexist with more than 10% blastemal tissue.	Intermediate
Focal anaplasia	One or two foci of anaplasia, with each focus measuring no more than 15mm in diameter.	Intermediate
Blastemal (Figure 3C)	At least 33% viable tissue, of which >=67% is blastemal and the remaining portion consisting of any other component.	High
Diffuse anaplasia	Presence of mitotic figures, hyperchromasia and/or enlarged nuclei.	High

Table inspired by Vujanic et al. 2022 [54]

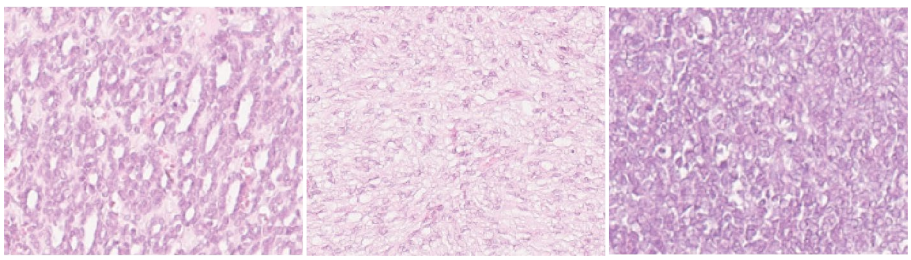


Figure 3. Triphasic histology in Wilms tumor.
A) WT with stromal predominance. B) WT with epithelial predominance. C) WT with blastemal predominance.

Treatment

Tumor staging of WT - an important parameter for risk stratification and treatment:

- Stage I – Confined to the kidney.
- Stage II - Locally infiltrative but completely removed.
- Stage III - Tumors exhibit residual disease within the abdomen.
- Stage IV - Distant metastasis.
- Stage V – Bilateral WT [54].

Initial classification into local stage, stage IV, or stage V is essential, as it guides the choice of preoperative treatment. Following surgery, the final staging, based on tumor characteristics, lymph node involvement, and histological subtype determines the postoperative therapeutic approach.

***Localized Wilms tumors (Stage I–III)*:** In infants under six months of age, primary surgery is generally recommended following a multidisciplinary evaluation of the risk of tumor rupture versus the benefits of preoperative chemotherapy. For all other patients with localized WT, preoperative treatment with vincristine and actinomycin D (VA) is advised. In cases where the diagnosis of WT is uncertain, fine needle aspiration or core needle (Tru-cut) biopsy should be considered

Pre-operative chemotherapy, includes 2 drugs which are given during 4 weeks; VA. Both are given intravenously (Figure 4).

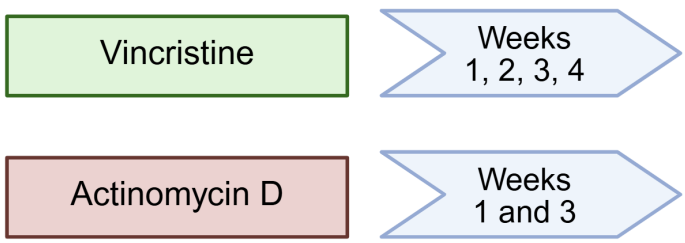


Figure 4. Preoperative Treatment Strategies in Wilms Tumor.
Schematic overview of treatment approaches before surgery.

Tumor reassessment by imaging should be conducted at week 4. Surgical intervention is ideally scheduled for weeks 5 to 6. If surgery is postponed, although this is not recommended, an additional dose of vincristine should be administered.

Postoperative risk stratification in WT is based on histological subtype, local tumor stage, and preoperative tumor volume. In the current SIOP protocol, tumor volume has been introduced as an additional prognostic factor for a specific subgroup. Patients with intermediate-risk histology (excluding stromal and epithelial

subtypes), local stage II or III, and a residual tumor volume >500 ml after preoperative chemotherapy are assigned to intensified treatment with Actinomycin D, Vincristine and Doxorubicin (AVD). This approach is supported by data from the SIOP 2001 study, which showed that large tumor volume in this subgroup was associated with poorer outcomes when treated with VA alone. In contrast, for patients with stage I intermediate-risk tumors or any stage with stromal or epithelial histology, large residual volume did not significantly impact prognosis.

Stage I, Low Risk Histology: No further treatment

For stage I WT with low-risk histology, no postoperative treatment is required. Final treatment decisions should await central pathology review. If delayed, one additional dose of vincristine may be considered.

Stage I, Intermediate Risk Histology

Vincristine is given for 4 weeks and Actinomycin D is given week 2.

Stage I, High Risk Histology

This regimen also applies to patients with stage II or III disease, FA, mixed or regressive histology, and residual tumor volume >500 ml following preoperative chemotherapy. The total duration of postoperative chemotherapy is 27 weeks (Figure 5).

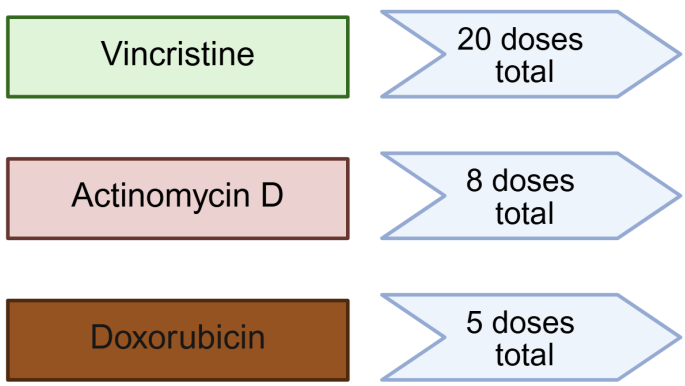


Figure 5. Schematic representation of postoperative treatment strategies for Wilms tumor, Stage I, high risk histology.

Stage II/III Low and Intermediate Risk Histology

This treatment is indicated for all patients with local stage II and III disease (Figure 6). In cases with focal anaplasia, mixed or regressive histology, and residual tumor volume >500 ml after preoperative chemotherapy, doxorubicin is added to AV (resulting in AVD, as used for high-risk stage I). The total duration of postoperative

chemotherapy is 27 weeks. Another important note is that stage III tumors always need local radiotherapy.

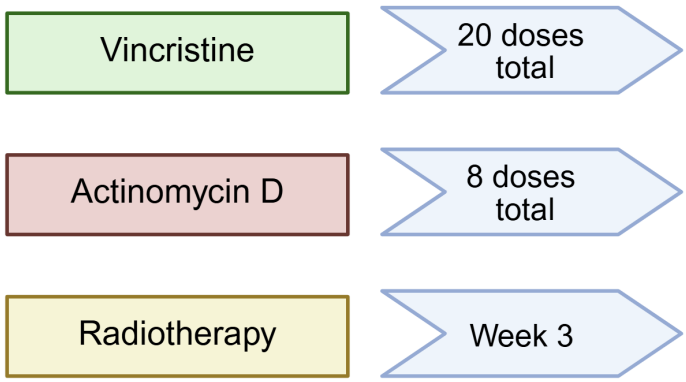


Figure 6. Schematic representation of postoperative treatment strategies for Wilms tumor, Stage II/III, low and intermediate risk histology.

Stage II/III High Risk Histology: High Risk Regimen

Postoperative treatment lasts 34 weeks and consists of two alternating chemotherapy regimens, each combining two drugs and given every 21 days. Treatment begins once the patient has recovered from surgery, typically within 21 days after completing preoperative chemotherapy. Local radiotherapy is required for all stage III tumors and for stage II tumors with DA (Figure 7). It is not indicated for stage II blastemal-type tumors.

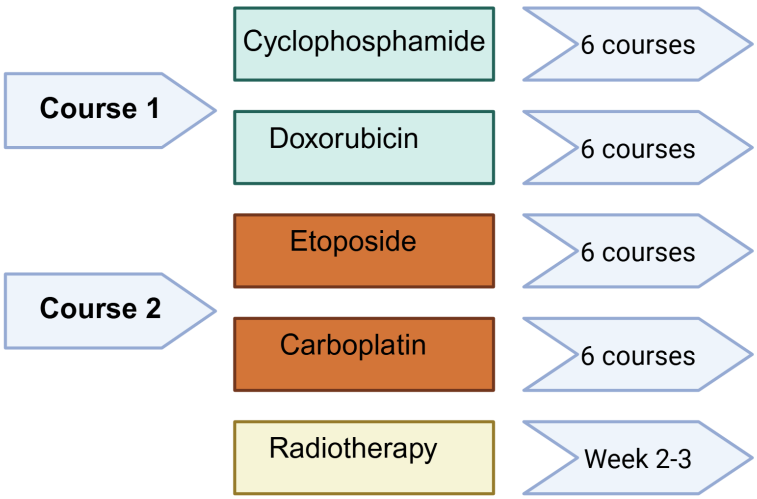


Figure 7. Schematic representation of postoperative treatment strategies for Wilms tumor, Stage II/III, high risk histology.

Metastatic Wilms Tumor (Stage IV): Patients with stage IV WT receive risk-adapted treatment based on the size and response of metastatic lesions, particularly in the lungs.

Preoperative chemotherapy is initiated with AV or AVD depending on lung nodule size:

- Nodules ≥3 mm are considered metastatic and treated with preoperative AVD.
- Nodules 1–2 mm are not classified as metastases and receive AV, similar to localized disease.

Reassessment before surgery is essential to evaluate response and guide postoperative therapy.

Postoperative chemotherapy is tailored:

- Patients with complete response of lung metastases may avoid pulmonary radiotherapy.
- Those with residual nodules ≥3 mm typically receive intensified chemotherapy and lung irradiation.
- Radiotherapy is indicated for persistent metastatic lesions and may include pulmonary, hepatic, or other sites depending on disease spread.

Total treatment duration and intensity vary based on histology, metastatic burden, and response to initial therapy [53]

Molecular and genetic background

Embryologic background and key genetic drivers of Wilms tumor

Findings over the past decade have reinforced the long-standing view that WT originates from embryonic rather than fully developed renal tissue [58]. WTs often exhibit a distinctive histology, comprising cells resembling undifferentiated metanephric mesenchyme (blastema) alongside more differentiated elements such as stroma and tubular epithelial cells, both normally derived from metanephric mesenchyme which indicates that these tumors result from disrupted kidney development [59]. Several genes that are recurrently mutated in WTs play essential roles also in normal renal development [60-62].

WT was among the first tumor types examined by Knudson during the formulation of his ‘two-hit’ hypothesis of tumor suppressor gene inactivation [63]. Much of what we know about the link between WT and kidney development stems from research

on the Wilms tumor protein 1 (WT1) tumor suppressor gene located on chromosome band 11p13 [64-67]. WTs harbor inactivating mutations in the *WT1* gene sporadically, while some exhibit loss of heterozygosity (LOH) and lastly there is a few percent that show homozygous deletions at this site. The WAGR syndrome has played a key role in elucidating the molecular genetics of WT. This syndrome is characterized by cytogenetically detectable constitutional deletions affecting chromosome band 11p13. Such deletions lead to hemizygosity of genes within this region and represent the first genetic alteration or 'hit' in tumor initiation. Subsequent studies have demonstrated that the second genetic event, which is not constitutional but somatic and triggers tumor growth, involves this same chromosomal locus [68].

WT1 regulates cell growth, differentiation, and apoptosis and is expressed in the kidney, gonads, spleen, and mesothelium. WT1 plays a pivotal role in renal and gonadal embryogenesis, and its constitutional disruption leads to genitourinary developmental abnormalities [69]. The *CTNNB1* gene encodes β-catenin, a transcriptional co-activator that drives Wnt signaling by promoting target gene transcription. This pathway is essential for normal renal development, while activating *CTNNB1* mutations disrupts the mesenchymal-to-epithelial transition, a critical step in nephrogenesis. Such alterations contribute to WT formation by maintaining cells in an undifferentiated state [58]. WT2, located at 11p15, comprises two imprinted domains: IGF2/H19 and KIP2/LIT1. Genomic imprinting is an epigenetic mechanism in which gene expression depends on parental origin. IGF2 encodes a growth factor essential for renal development, whereas H19 produces non-coding RNAs that may function as tumor suppressors. It has been shown that this region is affected by LOH and loss of imprinting in WT patients [70]. *AMER1* deletions or mutations typically occur later in WT evolution. This gene is a component of the β-catenin destruction complex and plays an important role in kidney development. Mutations in *SIX1* and *SIX2*, two genes critical for renal development, have also been identified in WTs. These alterations occur more frequently in blastemal-predominant tumors than in necrotic or regressive types [58].

Two genes that are involved in high-risk WT are *MYCN* and *TP53*. *MYCN* copy number gain or mutation is associated with poor outcome [71]. *TP53* mutations are strongly associated with tumor progression in WT. *TP53* mutations are particularly linked to anaplastic histology, where the majority of cases show *TP53* disruption [72]. *TP53* mutations represent late events in WT evolution and therefore tend to occur regionally. Notably, nearly all anaplastic areas harbor *TP53* mutations or LOH, although these alterations are not exclusive to anaplastic tumors.

LOH at chromosomal regions 1p and 16q is recognized as a significant marker of unfavorable prognosis in WT [58]. These loci are thought to harbor genes involved in tumor suppression, and their loss correlates with more aggressive disease behavior. Importantly, clinical studies have demonstrated that the negative

prognostic impact of LOH at these sites can be counteracted by intensifying treatment protocols, thereby improving patient outcomes [58, 73]. Initial studies of gene expression in WT suggested a possible role of the 1q chromosomal region in tumor recurrence [74]. Although the clinical relevance of 1q gain as a prognostic biomarker is increasingly evident, the underlying biological mechanism driving its impact on disease outcome remains unclear. To date, no single gene within 1q has been conclusively identified as the primary determinant of poor prognosis. Instead, it is likely that multiple genes within this large chromosomal region, influenced by increased copy number, collectively contribute to the observed phenotype [58].

Tumor cell heterogeneity and phylogenetics

Cancer evolution is traditionally viewed as a gradual process driven by the sequential accumulation of genetic and epigenetic alterations. However, emerging evidence suggests that tumor development can follow diverse evolutionary trajectories. Two concepts that illustrate the alternative patterns are: parallel evolution and saltatory evolution. Parallel evolution describes the independent acquisition of similar traits in closely related lineages under comparable selective pressures. Unlike convergent evolution, it occurs among species sharing a recent common ancestor, making their descendants even more alike. In cancer, parallel evolution is reflected by identical genomic alterations such as whole-chromosome changes arising independently in different regions of the same tumor [75]. Saltatory evolution in cancer refers to sudden bursts of genomic change rather than gradual accumulation. Such leaps often result from catastrophic events like chromosomal instability or chromothripsis, enabling rapid tumor progression and challenging the traditional Darwinian view of gradual evolution [76].

Genomic instability in cancer refers to an increased rate of somatic mutations and copy number changes. These events generate cell-to-cell variability, contributing to tumor cell heterogeneity [77]. Tumor cell heterogeneity provides the molecular diversity upon which selective pressures act, promoting tumor progression and often leading to resistance against cancer therapies. As a result, many novel treatments fail to achieve lasting efficacy [78].

Tumor cell heterogeneity can be observed across both spatial and temporal dimensions, appearing in various regions of the tumor and evolving over time. Spatial heterogeneity is typically categorized into intratumoral heterogeneity, which refers to variation within a single tumor mass, and intertumoral heterogeneity, which encompasses differences between separate tumor sites in the same patient or across different patients (Figure 8). Tumor heterogeneity is a feature of most malignancies, but rare in benign tumors [79-84].

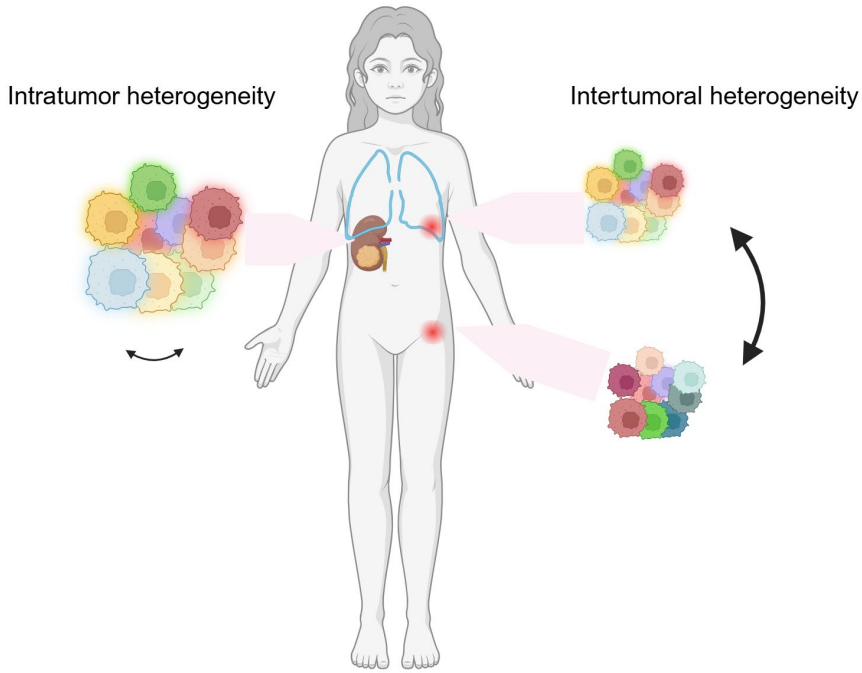


Figure 8. Tumor heterogeneity.
Illustration of both intratumoral and intertumoral heterogeneity. Inspired by Alexandra Petersson.

In evolutionary biology, ancestral relationships between species are commonly illustrated using branching diagrams known as phylogenetic trees (Figure 9). A phylogenetic tree is a graphical representation of evolutionary relationships among biological entities. It is widely employed in evolutionary biology to investigate genetic connections between species, estimate the timing of their divergence at genetic, phenotypic, or temporal levels, and to identify both similarities and differences among them [85].

Phylogenetic trees help pinpoint when species diverged and allow for comparisons of their similarities and differences. In a similar way, detailed analysis of the cancer genome enables the reconstruction of evolutionary relationships between cancer cell clones across different tumor regions [80, 86]. The evolutionary trajectory of a tumor can be reconstructed by estimating the most likely sequence of genomic events. This is achieved by analyzing the relative abundance of each alteration across different samples, following the deconvolution of identified subclonal populations [87].

Phylogenetic diagrams can offer valuable insights into the behavior of specific subclones, including their potential to metastasize, resist treatment, and contribute to disease recurrence [79, 80, 82, 88-90]. The stem of a phylogenetic tree represents

alterations that are common to all cells across all sampled regions. These are typically referred to as truncal, clonal, or stem alterations. Changes confined to branches of trees correspond to mutations found in only a subpopulation of cancer cells, for example localized to just one or a few sites, or subclonal alterations in one or all sites [85].

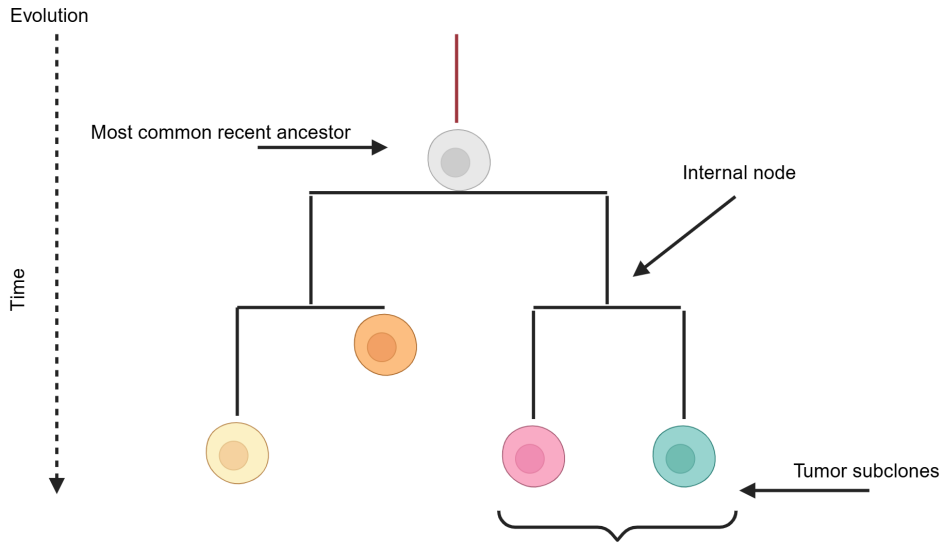


Figure 9. A phylogenetic tree illustrating the ancestral relationships among tumor subclones.

Significance of anaplasia for Wilms tumor

Anaplasia was first identified in 1978 as a histological feature associated with poor prognosis in WT. As previously described, it is characterized by markedly enlarged, hyperchromatic nuclei and the presence of atypical multipolar mitotic figures [91]. Anaplasia in WT is classified into two subtypes: Focal anaplasia, defined as involvement of less than 10% of the tumor, and DA, where 10% or more of the tumor exhibits anaplastic features [56]. Patients with focal anaplasia have considerably better outcomes [56, 92], with WT DA being more aggressive and more resistant to conventional chemotherapy [93]. Although both focal anaplasia and WT DA share the histologic hallmark of anaplasia, they differ significantly in nearly all other clinical and biological characteristics [93]. WT DA patients are often older when diagnosed [94, 95]. WT DA are associated with a significantly higher tumor stage at diagnosis compared to non-anaplastic WTs, with a greater proportion of cases presenting with regional lymph node involvement or distant metastases [92, 94, 96-98]. Anaplastic cells in WT exhibit a combination of increased mutation

rates, reduced cell death and accelerated proliferation. This biological profile could enable them to explore a broader range of adaptive environments compared to non-anaplastic cells. Such adaptability is evident in their ability to survive under selective pressures like chemotherapy, as well as in metastatic niches such as lymph nodes and lungs. Based on these observations, Vujanic, et.al [93] propose an expanded definition of tumor aggressiveness in WT, which encompasses not only rapid growth and invasive behavior leading to high-stage disease, but also intrinsic resistance to chemotherapy [93].

Tumor microenvironment in Wilms tumor

Immune microenvironment

The immune system plays a complex role in cancer development. While immunosurveillance can eliminate tumor cells, immune cells also exert selective pressure, driving immunoediting and immune escape, which may promote tumor progression. Unlike adult tumors, which are typically rich in T cells, pediatric tumors are characterized by an immune infiltrate dominated by innate immune cells, primarily myeloid cells and macrophages [99]. This difference may be attributed to the lower mutational burden observed in pediatric tumors [100]. Immunotherapy for pediatric renal tumors faces challenges similar to those in other solid tumors, including an immunosuppressive tumor microenvironment, pronounced intra- and intertumoral heterogeneity, and variability in tumor biology related to histology, molecular features, and stage [101]. Checkpoint inhibitor immunotherapy often shows limited efficacy in pediatric solid tumors, largely due to their low immunogenicity, likely reflecting their origin from embryonal cells and transcriptional dysregulation, rather than the gradual mutation accumulation characteristic of adult cancers [102].

In adult oncology, research on the tumor immune microenvironment has shown considerable promise in identifying novel prognostic markers [103] and therapeutic strategies, offering the added benefit of reducing the toxicity associated with conventional chemotherapy-based treatments [19]. Most immunotherapies for solid childhood cancers have failed to demonstrate a significant antitumor effect. While checkpoint inhibitors such as anti–PD-L1 have achieved greater outcomes compared to conventional chemotherapy in adult cancers [104], temporary results from the largest pediatric trial, KEYNOTE-051, reported minimal to no antitumor activity of pembrolizumab, an anti–PD-1 monoclonal antibody [105]. The only notable exception is anti-GD2 monoclonal antibody therapy, which has shown efficacy in neuroblastoma [106].

Studies on WT microenvironment are limited due to its rarity, but available data consistently show that WTs are immunologically cold tumors [99]. They exhibit

low CD8+ T-cell infiltration and are dominated by immunosuppressive cells such as T-regs and M2-like macrophages. This correlates with a cytokine milieu rich in IL-10, TGF-β, and indoleamine 2,3-dioxygenase, which suppress cytotoxic T-cell responses (Figure 10). The low mutational burden and absence of neoantigens further contribute to immune evasion [99]. These features make WT resistant to checkpoint inhibitor therapy, highlighting the need for strategies that convert cold tumors into hot ones, such as pro-inflammatory stimuli, oncolytic viruses, bispecific antibodies, or immunogenic cell death induced by chemotherapy or radiotherapy [88, 107, 108].

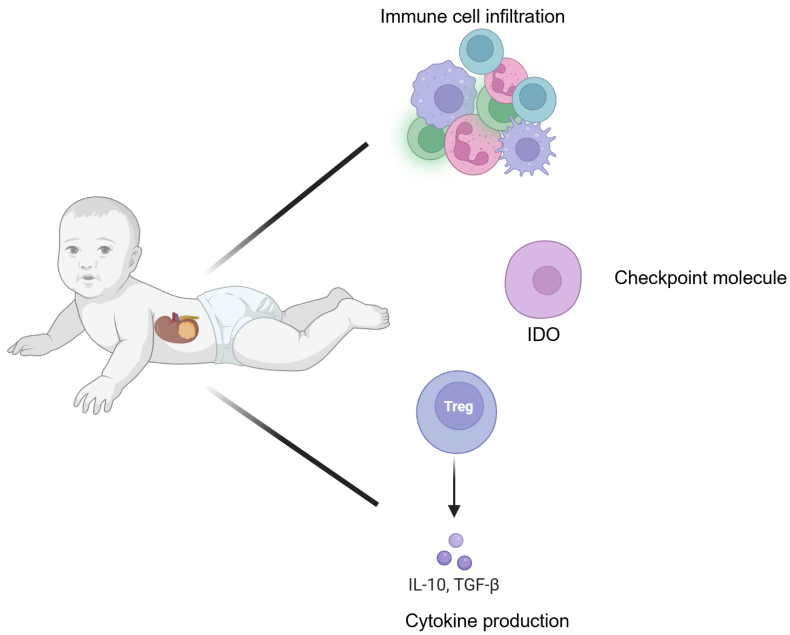


Figure 10. Tumor microenvironment in Wilms tumor.
This image illustrates an infant with Wilms tumor and highlights key components of its tumor microenvironment (TME) that contribute to immune evasion and tumor progression. The cluster of colorful cells represents various immune cells infiltrating the tumor site. Idoleamine 2,3-dioxygenase (IDO) is shown as a checkpoint molecule. Lastly, regulatory T cells produce cytokines, such as IL-10 and TGF-β [19].

Inflammatory and hypoxia-driven pathways in Wilms tumor

In normal kidney tissue, vascular endothelial growth factor (VEGF) expression is localized to proximal and distal convoluted tubules. In WT, VEGF was consistently detected within the stromal component across all analyzed specimens, with lower levels observed in blastemal areas and minimal expression in epithelial regions [109]. This distribution pattern closely parallels that of COX-2 and HIF-1,

suggesting a coordinated role in angiogenesis and hypoxia response. HIF-1 is upregulated under hypoxic conditions driving VEGF expression and adaptation to low oxygen. COX-2 expression correlates with tumor progression and poor prognosis. Consistent with this, most inflammatory proteins similar to immune cells were also predominantly localized within the tumor stroma, underscoring the central role of this compartment in shaping both vascular and immune aspects of the microenvironment [109]. The presence of HIF-1α suggests hypoxia-driven pathways are involved. More broadly, hypoxia is a well-recognized feature of rapidly growing solid tumors, including pediatric cancers, where it promotes angiogenesis and immune suppression through HIF-1 activation and VEGF upregulation. These findings highlight COX-2, VEGF, and HIF-1 as potential therapeutic targets in WT [109]. Although VEGF inhibitors initially showed promise, their clinical benefit proved limited, as they triggered compensatory upregulation of other pro-angiogenic pathways and were associated with toxicity and additional adverse effects [110]. It has also been disappointing in adult cancer treatment due to therapy resistance and the drugs have limited response [110, 111].

Glypican-3 in Wilms tumor

GPC3 has emerged as an important factor in WT biology, with studies showing that GPC3 is markedly overexpressed in both primary and metastatic WTs compared to normal kidney tissue and other renal tumor types [112]. GPC3 is a membrane-bound heparan sulfate proteoglycan that plays a role in regulating cell growth and differentiation. Mutations in the GPC3 gene are commonly observed in children with Simpson-Golabi-Behmel syndrome, a condition associated with an increased risk of developing pediatric malignancies, including WT and hepatoblastoma [112]. GPC3 is physiologically expressed during embryonic development in the placenta, fetal liver, lung, and kidney, but is either absent or present at very low levels in most adult tissues. Similar to other glypicans, the GPC3 core protein and its heparan sulfate side chains interact with multiple regulatory molecules involved in key developmental pathways, including Wnt, Hedgehog, and fibroblast growth factor signaling. Notably, GPC3 binds Wnt ligands and promotes activation of the canonical Wnt/β-catenin signaling cascade (Figure 11), which is essential for normal kidney and liver development. Dysregulation of Wnt/β-catenin signaling is a frequent feature of pediatric embryonal tumors, underscoring the relevance of GPC3 in tumorigenesis [113].

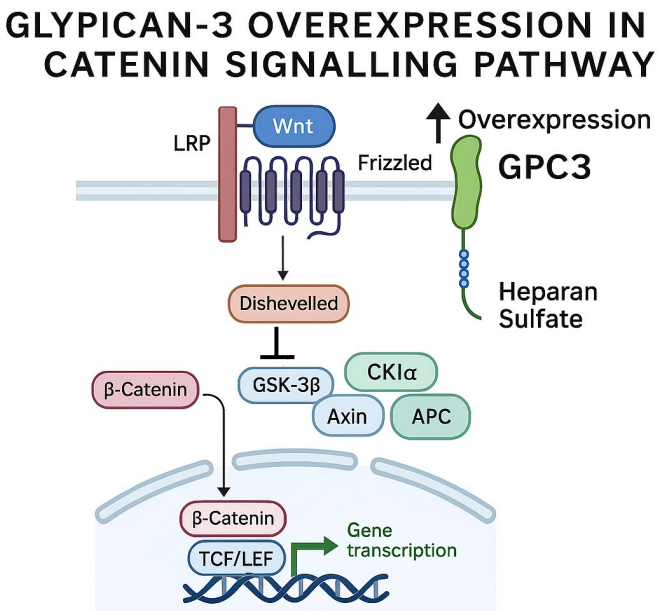


Figure 11. GPC3 overexpression in the Wnt/β-catenin signaling pathway.
GPC3, a membrane-bound heparan sulfate proteoglycan, facilitates Wnt ligand binding to the LRP and Frizzled receptors, enhancing pathway activation. This interaction strengthens Dishevelled activity, which inhibits the β-catenin destruction complex (GSK-3β, CKIα, AXIN and APC), allowing β-catenin to accumulate in the cytoplasm. Stabilized β-catenin translocates to the nucleus, where it associates with TCF/LEF transcription factors to drive gene expression, some which are involved in cell proliferation and survival [114], [115].

Aims of the thesis

General aim:

To increase the understanding of the pathogenesis of anaplasia in WT and its underlying genetic evolution with the ultimate aim to find new ways to target these aggressive tumors.

Specific aims:

Paper I *– Resolving the pathogenesis of anaplastic Wilms tumors through spatial mapping of cancer cell evolution*

1. Identify key molecular and evolutionary events underlying the development of WT DA.
2. Map cancer cell evolution across anatomical compartments by analyzing subclonal architecture using high-resolution copy-number profiling and *TP53* mutation analysis.
3. Characterize spatial constraints on tumor evolution through phylogenetic reconstruction and whole-mount section (WMS) analysis.
4. Assess the relationship between genetic complexity and morphologic features, including CNA burden and regressive changes.
5. Evaluate the role of multi-regional sampling in high-risk WTs.

Paper II *- A gradual transition toward anaplasia in Wilms tumor through tolerance to genetic damage*

1. Investigate mechanisms underlying chemoresistance in WT DA focusing on cellular tolerance to DNA damage and CNAs.
2. Assess associations between anaplasia severity and biological markers, including *TP53* status, proliferation index, DNA double-strand (dsDNA) breaks, and CNA burden.

3. Identify pre-anaplastic cell populations that may give clues to mechanisms promoting anaplasia.
4. Clarify the role of *TP53* alterations and genomic instability in driving anaplasia and treatment resistance.

Paper III *- Strong glypican 3 expression in blastemal components of Wilms tumors with diffuse anaplasia*

1. Evaluate GPC3 expression patterns in different histologic WT compartments (stromal, epithelial, blastemal, and anaplastic), with a focus on DA.
2. Compare GPC3 expression between WT DAs and non-anaplastic WTs using single patient tissue microarrays.
3. Assess correlations between GPC3 expression and key biological features, including anaplasia severity, proliferation, p53 expression, apoptosis, marker for endothelial- and T-cells (CD31, CD3).
4. Determine the potential of GPC3 as a therapeutic target in high-risk WTs, considering intratumoral variability of protein expression.
5. Explore the relationship between GPC3 expression and tumor aggressiveness, highlighting its role in WT DA pathogenesis.

Methodological considerations

Methods in Paper I-III

Patient cohort

The study cohort in Paper I consists of 20 patients with WT. All preoperatively treated according to SIOP guidelines at Skåne University Hospital in Lund, between the years 1992-2020. The distribution of cases after chemotherapy, categorized by the SIOP histologic risk classification, was: 10 intermediate-risk (IR) tumors, 5 blastemal-type (BT) tumors and lastly 5 WT DA.

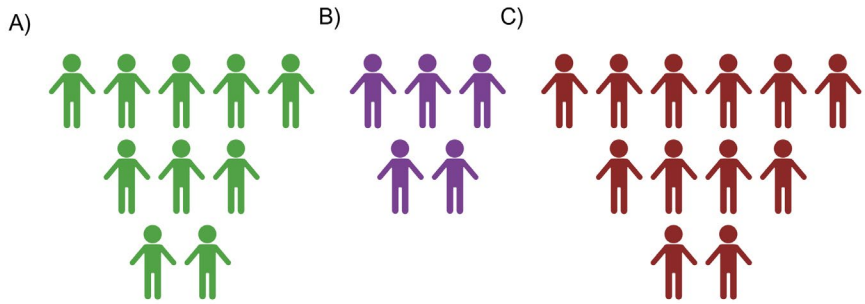


Figure 12. Patient cohort.
Retrospective cohort from from Lund and Milan. A) 10 Lund patients with intermediate-risk tumors. B) 5 Lund patients with blastemal-type tumors. C) Lastly, the only tumor-group with both cohorts included: 12 patients with diffuse anaplasia.

In addition, seven DA patients were included from Fondazione IRCCS Istituto Nazionale dei Tumori, Milan (Figure 12). At Skåne University Hospital, all available BT and DA cases from a 20-year period were included, while IR WTs from the same timeframe were selected based on sufficient tumor cell content after chemotherapy. Clinical diagnoses were verified by two pathologists (primary and SIOP reference). Tumor samples obtained during diagnostic procedures were included based on the presence of formalin-fixed paraffin-embedded (FFPE) blocks with viable tumor tissue and documented localization within the primary tumor. For Swedish WT cases, WMSs were preferred, but conventional paraffin blocks were

used when WMSs were unavailable. To achieve extensive anatomical representation, some WMSs were complemented with conventional block samples [116, 117]. In paper I we only included the Swedish cohort, whilst in paper II-III, both cohorts were included.

Single-patient tissue microarrays

To efficiently analyze large numbers of tissue samples from multiple patients or different tumor regions within a single patient, TMAs are commonly used. The TMA technique has become essential for oncological biomarker research and has significantly advanced our understanding of tumor biology [118]. Although constructing TMAs is labor-intensive, they provide an efficient and cost-effective approach for assessing protein expression and are widely applied in cancer biomarker research [118].

TMAs are created by extracting cylindrical cores from archival FFPE tissue blocks and embedding them into a recipient paraffin block to form a tissue matrix (Figure 13). Thin sections from this block can then be mounted on glass slides for analyses such as protein expression by immunohistochemistry or nucleic acid evaluation using fluorescence in situ hybridization [119]. Compared to whole tissue sections, TMAs may provide less precise information on intratumoral heterogeneity. This limitation can be reduced by sampling multiple cores from different donor blocks and creating single patient TMAs. Single patient TMAs were used in paper II and III.

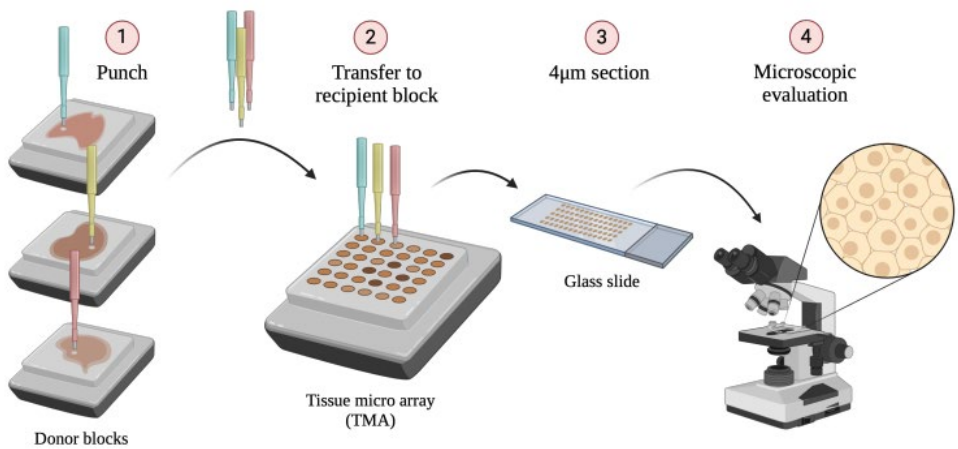


Figure 13. Tissue microarray workflow.
Schematic representation of TMA construction followed by subsequent microscopic evaluation. Reproduced from Petersson A, Pancreatic cancer evolution across space and time: From diagnosis to terminal disease [doctoral thesis], Lund University, 2024, with permission.

Multiregional sampling and DNA preparation

To assess spatial tumor heterogeneity, hematoxylin and eosin (H&E)-stained slides were overlaid with a 1 × 1 cm grid. Regions with necrosis or debris were excluded. Corresponding areas were identified on FFPE blocks, and two 1 mm cores were taken from each grid square using the Tissue-Tek Quick-Ray system. The cores were pooled per square to form one sample. DNA was extracted using the AllPrep DNA/RNA FFPE kit (Qiagen) following standard protocols. The number of samples per tumor varied depending on size and cellularity [116]. Two 2-mm cores were sampled adjacent to the regions used for DNA extraction to construct two parallel tissue microarrays (TMA-A and TMA-B). This method was used in paper I-II whereas TMA cores were utilized in paper II-III.

Tumor tissue analyses in this thesis were performed on FFPE specimens. FFPE is the gold standard for clinical diagnostics and pathology, as it preserves tissue architecture and allows long-term storage at room temperature [120]. By sectioning thin slices from FFPE blocks, various microscopic analyses can be performed, including diagnostic evaluation of cellular and tissue morphology, protein expression, and other biomarkers. A limitation of FFPE preservation is nucleic acid degradation, particularly RNA [121]. Both formalin fixation and the chemicals used for paraffin removal prior to DNA extraction can contribute to DNA fragmentation and introduce sequence artifacts. In genomics, fresh frozen (FF) tissue is often preferred for sequencing, as it typically yields higher-quality DNA with less degradation compared to FFPE specimens. Although FF tissue offers superior DNA quality, it requires ultra-low temperature storage. In contrast, FFPE specimens can be stored at room temperature and remain widely used in clinical practice and generally provide adequate quality for sequencing and diagnostics. Their availability through biobanks enables large, robust cohort studies, which often outweigh the limitations in DNA integrity [121].

SNP array

Large-scale CNA and LOH were analyzed using the OncoScan CNV assay, a SNP (single nucleotide polymorphism) microarray platform specifically optimized for FFPE tissue samples [122]. For each SNP locus analyzed with the OncoScan assay, two key metrics are generated for downstream analysis: the $\log_2$ ratio ($\log_2$R) and the B-allele frequency (BAF) [123, 124]. The $\log_2$R reflects copy number by comparing the combined signal intensity for each SNP to a reference sample. A value of 0 indicates a normal copy number (typically diploid), while positive and negative values correspond to gains and losses, respectively. In contrast, BAF represents the proportion of the non-reference allele at a given SNP and is calculated as the intensity of the B-allele divided by the total allele intensity. Heterozygous SNPs in diploid regions have a BAF of 0.5, whereas homozygous SNPs show values

of 0 or 1. Deviations from these expected values occur in the presence of CNAs, enabling inference of complex allelic configurations. Our cutoff for scoring a positive CNA was ≥5 Mbp in size, encompassing at least 20 SNPs, and represented by more than one data point in the tumor aberration prediction suite (TAPS) (see below, Figure 15). When analyzing bulk tumor DNA, both $\log_2$R and BAF are influenced by normal cell admixture. Importantly, deviations from expected values assuming 100% tumor purity can be used to estimate the tumor cell fraction (TCF) for each sample [124].

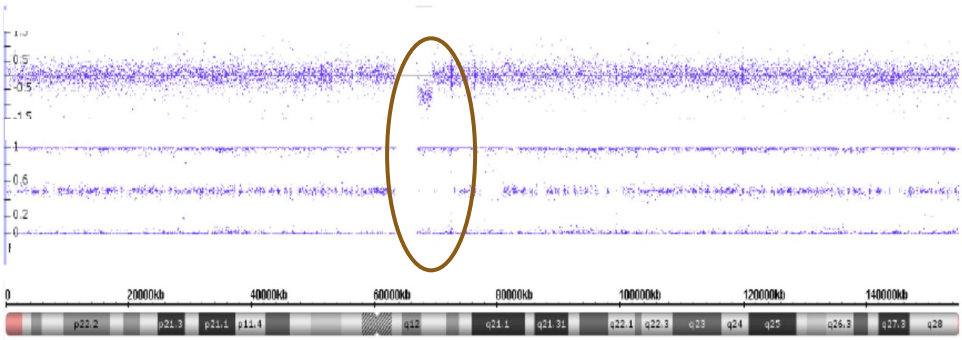


Figure 14. SNP array analysis of chromosome X demonstrating a chromosomal loss on the q-arm.

SNP array analysis was performed by using three main tools: Chromosome Analysis Suite for initial data processing, and Nexus Copy Number together with TAPS for calculation of allelic configuration, clonality and ploidy level [125]. These methods were used in papers I-II.

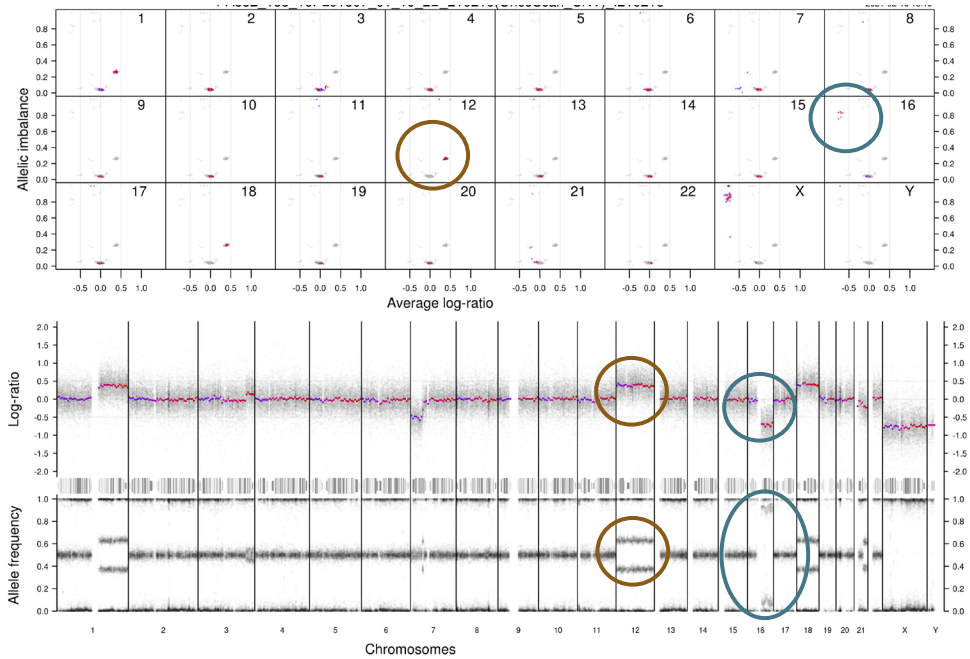
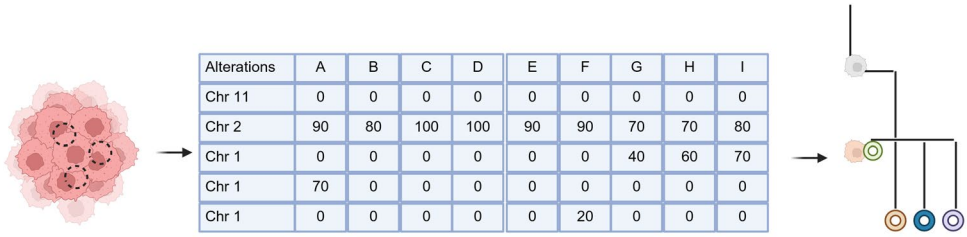


Figure 15. TAPS plot illustrating genome-wide copy number and allelic imbalance.
The top panel shows allelic imbalance across chromosomes 1–22 and sex chromosomes (X and Y), plotted as a function of average log-ratio. Each dot represents a genomic segment, with deviations from the center indicating allelic imbalance. The bottom panel displays log-ratio values (middle track) and allele frequency (lower track) across all chromosomes. Vertical lines separate individual chromosomes. Red and blue lines in the log-ratio track represent smoothed estimates of copy number changes. This visualization integrates copy number and allelic imbalance to identify regions of chromosomal gain, loss, and potential uniparental disomy.

Subclonal deconvolution and phylogenetic tree reconstruction

Subclonal deconvolution was performed using the DEVOLUTION algorithm (v1.1) based on measured mutation cell fractions (MCFs). For each patient, a segment file listing all genetic alterations, including chromosomal position, type (gain, loss, or CNNI), and MCF was provided as input. Clonal deconvolution involves using data on genetic alterations and their frequencies from individual tumor samples to determine which subclones are present in each region. This information is then applied to reconstruct the evolutionary relationships between subclones through phylogenetic analysis (Figure 16). Clones were defined as cell populations with unique genetic profiles present in ≥90% of tumor cells, and subclones as those present in <90%. Phylogenetic trees were then reconstructed using maximum parsimony (MP), maximum likelihood (ML), and a modified maximum parsimony (MMP) approach. Using the event matrix as input, two phylogenetic trees were constructed based on the detected subclones, applying both MP and ML approaches.

The MP method seeks the simplest tree, minimizing the number of genetic changes required to explain the data, whereas the ML method identifies the tree that is statistically most likely given the observed alterations. Similar to MP, the MMP method aims to construct the simplest possible phylogenetic tree but additionally seeks to minimize back mutations and can incorporate information about alterations that are more likely to occur in parallel. In cases of conflicting data, the MMP method produced phylogenies that were more biologically plausible and ensured consistency with the pigeonhole principle across all samples [87, 116]. This method was used in paper I.

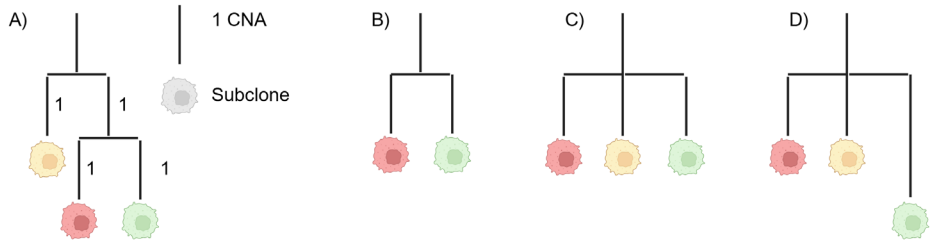


Alterations	A	B	C	D	E	F	G	H	I
Chr 11	0	0	0	0	0	0	0	0	0
Chr 2	90	80	100	100	90	90	70	70	80
Chr 1	0	0	0	0	0	0	40	60	70
Chr 1	70	0	0	0	0	0	0	0	0
Chr 1	0	0	0	0	0	20	0	0	0

Figure 16. Workflow from tumor sampling to phylogenetic analysis.
(1) Multiregional sampling of tumor tissue. (2) Construction of a matrix summarizing all samples and the proportion of cells carrying each detected alteration. (3) Computational clonal deconvolution to identify distinct subclones, followed by phylogenetic reconstruction illustrating the evolutionary relationships among subclones.

Calculating the evolutionary complexity

To compare phylogenetic characteristics between clinical subgroups, we calculated three metrics for each phylogenetic tree (Figure 17): phylogenetic species richness (PSR), divergence, and irregularity. PSR represents the total sum of phylogenetic distances between all detected subclones. Divergence reflects the average relatedness within the tree, calculated as PSR divided by the number of pairwise comparisons. Irregularity measures deviation from a star-like phylogeny and was computed as the variance in distances from the stem to all subclones [126]. This method was used in paper I.



E)

Tree	PSR	Divergence	Irregularity
B	2	1	0
C	6	2	0
D	8	2,66	0,33

Figure 17. Tree complexity.
Illustration of methods for evaluating phylogenetic tree complexity by calculating PSR, divergence, and irregularity. A) A phylogenetic tree. B-D) Three different tree configurations evaluated for PSR, divergence and irregularity. E) Summary table of all calculations. PSR increases with a greater number of branches or longer branch lengths. Divergence rises as branch lengths become longer. Irregularity equals zero in perfectly symmetrical trees but increases as the structure deviates from a star-like topology.

TP53 targeted sequencing

For TP53 analysis we used targeted sequencing (TDS), which concentrates on selected genes or specific genomic regions. DNA samples with high-quality OncoScan SNP array profiles and sufficient material were selected for deep *TP53* sequencing [116]. TDS focuses on a predefined subset of coding and non-coding regions. This approach is cost-effective and enables high sequencing depth, which helps mitigate challenges associated with FFPE samples [127]. The targeted sequencing was done with the Agilent SureSelectXT capture kit. Libraries were prepared and sequenced on an Illumina NovaSeq 6000 using 150 base pair paired-end reads by Eurofins Genomics. Data processing included standard Illumina base-calling and demultiplexing. Sequence reads were aligned to the GRCh37 human reference genome using BWA MEM, with sorting and duplicate removal handled by Samtools [128]. Somatic small variants were identified using Mutect2 in multisample mode, allowing joint analysis of all samples from the same patient against a panel of normal controls included in the study. Raw variant calls were filtered with the GATK FilterMutectCalls tool, using default settings except for increased allowance in regions with multiple nearby events to avoid excluding subclonal mutations [129]. Only variants present in at least 5% of reads and with a minimum coverage of 50 in at least one sample were considered further, which helped exclude likely noise from low-quality DNA. Ultimately, remaining mutations were manually reviewed using the Integrative Genomics Viewer to ensure accuracy [130].

Histopathological evaluation

***Anaplasia scoring*:** Each TMA core was assessed for three key features of anaplasia: hyperchromasia, multipolar mitoses, and enlarged nuclei. Hyperchromasia was defined as intensely stained chromatin in cells slightly larger than adjacent cells, excluding mitotic figures. Multipolar mitoses were characterized by abnormal mitotic figures with multiple spindle poles; other abnormalities such as chromosome lagging, acentric fragments, or anaphase bridging were not included. Enlarged nuclei were identified when one or more nuclei were at least three times the size of neighboring nuclei [91]. Each feature was assigned one point, resulting in a possible score of 0–3 per core. Areas scoring 2 or 3 were classified as anaplastic because the small size of TMA cores compared to standard histology slides makes it unlikely to observe all criteria within a single core, even if sampled from an anaplastic region [117].

Nuclear unrest: Nuclear unrest was defined as the presence of tumor cells with enlarged, hyperchromatic nuclei that do not meet the criteria for anaplasia [131]. The evaluation was based on previously published criteria, where nuclear unrest was graded from 1 to 3 according to the following features [97]:

Grade 1: Small, rounded blastemal cells with minimal disorder and nuclear diameters similar to those of red blood cells.

Grade 3: Marked irregular disarray with nuclei larger than grade 1 but slightly smaller than those observed in anaplastic cells.

Grade 2: Intermediate morphology between grades 1 and 3.

For comparative purposes, we additionally defined grade 0 as areas lacking blastemal histology or cellular atypia, and grade 4 as areas corresponding to an anaplasia score of 2 or 3 in our tiered system.

Immunohistological evaluation

***Protein expression assessment*:** Immunohistochemistry was performed on FFPE TMA sections to detect p53, Ki67, and γH2AX proteins. After deparaffinization, antigen retrieval, and blocking, sections were incubated with primary antibodies followed by HRP-conjugated secondary detection and visualization using DAB. Slides were counterstained, scanned at 40× magnification, and evaluated digitally. For p53 expression, the proportion of p53-positive areas was recorded, considering only high-intensity nuclear staining as positive. In blastemal regions, the percentage of p53-positive cells was assessed primarily when staining differed markedly from adjacent stromal or epithelial areas. Proliferation index by Ki67 expression was evaluated as the percentage of positive tumor cells within each area (0–100%), irrespective of histological subtype. DNA double strand breaks was assessed by γH2AX expression based on two distinct nuclear staining patterns: strong diffuse

staining (entire nucleus stained, representing apoptosis) and dot staining (≥10 visible nuclear dots, representing dsDNA breaks). For each TMA core, the number of cells exhibiting these patterns was counted in three randomly selected high-power fields, and the average was calculated. Necrotic areas were excluded from analysis. To specifically evaluate Ki67 and γH2AX expression in anaplastic cells, only cells with enlarged nuclei were considered [117].

Tumor vascularity

Analysis of tumor vascularity by CD31 detection was performed by dividing each TMA core into three 250 µm squares (Figure 18). Overall histology was first assessed for the entire core. Within each square, CD31-positive vessels were counted and histology recorded, and the mean value across the three squares was calculated.

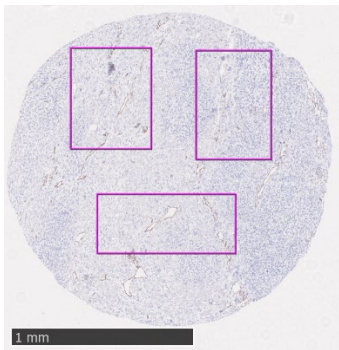


Figure 18. CD31-positive vessels.
Assessment of vascularity in a TMA core. Three selected regions are evaluated for CD31-positive vessels, identified by endothelial cells exhibiting brown staining.

Immune cells detection

For T-cell detection we analyzed CD3, and its positive expression was defined as staining in either the cytoplasm or the cell membrane. Each TMA core was analyzed by selecting three regions, each measuring 0.0650 µm². The histological characteristics of the entire core and the corresponding regions were documented. Each region was further subdivided into four quadrants to facilitate counting of total cells and CD3-positive cells. The median percentage of CD3-positive cells was then calculated.

Lastly, for GPC3 detection in paper III, the entire TMA core was evaluated, and staining intensity was categorized into four grades: none, weak, moderate, and strong (Manuscript figure 1) [112]. Intensity grading was determined manually

through consensus among multiple observers. In addition, the percentage of GPC3-positive staining within each core was recorded. Histological components exhibiting GPC3 positivity were noted. In cases of heterogeneous staining within a single core, the final grade was assigned based on the region showing the highest staining intensity.

Statistical analysis

In paper I comparisons of phylogenetic tree parameters were performed using the Mann–Whitney U test with continuity correction, and P values were adjusted for multiple comparisons using the Bonferroni method. Correlations of CNAs, mitotic count, and anaplasia were assessed using Pearson correlation. To analyze associations between CNA burden, anaplasia grade, mitotic rate, and regressive histology, a negative binomial hierarchical model was applied. This model included a case-specific random intercept to account for within-case correlation, specified in R as:

CNA ~ Anaplasia + Mitoses + Regression + (1|Case).

Separate models were fitted for anaplastic and non-anaplastic tumors.

In paper II non-normally distributed data were analyzed using nonparametric tests. Differences between two groups were assessed with the Mann–Whitney U test, and comparisons among multiple groups with the Kruskal–Wallis test. A two-way Kruskal–Wallis analysis was applied to account for potential confounders. *TP53* status (wild type, LOH, mutation) was compared across groups using chi-square tests with Bonferroni correction. Statistical significance was set at $P < 0.05$. Analyses were performed in SPSS Statistics v27 (IBM).

In paper III we used spearman correlation to evaluate associations between GPC3 expression and parameters such as Ki67 index, p53 expression, γH2AX staining, CD31, and CD3 expression. As the data were non-normally distributed, non-parametric tests were applied. Group differences were assessed using the Kruskal–Wallis test, followed by Dunn's post-hoc test with Bonferroni correction for multiple comparisons. This approach enabled comparison of medians across groups and identification of specific pairwise differences. All analyses were performed in R Studio (version 4.4.2). For comparisons of GPC3 grading and tumor subtypes pairwise Kruskal–Wallis tests were used.

Ethical considerations

These retrospective studies were independent of clinical management and did not affect treatment decisions. All analyses were performed on archival tumor specimens collected during routine diagnostics or surgery, causing no additional harm. Although the material dates from 1992-2020 within the Swedish cohort, it remains a valuable resource for research. Sampling was carefully planned to meet study objectives while preserving tissue for future use. TMAs were employed as an efficient method to analyze multiple tumor regions simultaneously while maintaining the integrity of remaining material. All data were pseudonymized to ensure de-identification and protect patient confidentiality.

For paper I the study was approved by the Swedish Ethical Review Authority (reference no. 2023-01550-01) and for paper II and III the studies were approved by the Swedish Ethical Review Authority (reference no. 2023-01550-01) and the ethical committee of the Fondazione IRCCS Istituto Nazionale dei Tumori in Italy (reference no. INT 222/20). Written informed consent for histologic and genomic analyses was obtained from all the patients' parents. The studies comply with the Helsinki declaration.

Results

The detailed results are reported in the original publications and are only briefly summarized here.

Paper I

Resolving the Pathogenesis of Anaplastic Wilms Tumors through Spatial Mapping of Cancer Cell Evolution

Summary of key findings

Compared to WT without DA, those with DA exhibited markedly greater intratumoral heterogeneity, characterized by a higher number of genetically distinct subclones and more complex phylogenetic architectures. WT DA demonstrated elevated phylogenetic species richness, divergence, and irregularity. All regions displaying classical anaplastic morphology harbored *TP53* alterations. *TP53* mutations were commonly followed by saltatory evolutionary patterns and parallel loss of the remaining wild-type allele across multiple regions. Increasing morphologic features of anaplasia correlated with higher CNA burden and regressive changes. Compartments delineated by fibrous septa or areas of necrosis/regression frequently (73%) coincided with the emergence of novel clonal CNAs, although complete clonal sweeps within these compartments were uncommon.

Complex patterns of combined branching and linear evolution

Whole-genome CNA profiling was performed on multiple regions per tumor (median: 9) across 20 WTs: 10 IR, five BT, and five with DA. All cases received preoperative actinomycin D and vincristine; one metastatic case also received doxorubicin. In total, 169 regions were analyzed, identifying 144 genetically distinct populations classified as clonal (≥90% of cells) or subclonal (<90%). After subclonal deconvolution, phylogenetic trees were reconstructed for 16 tumors using CNA profiles combined with *TP53* sequencing data. One case had no allelic imbalance and three cases had only a single clonal population. The reconstructed phylogenetic trees revealed highly diverse structural patterns; there were tumors with linear and branching patterns, but most tumors displayed a mix of linear and

branching evolutionary patterns (Figure 19 A-C) (Paper I, figure 3D-R) across the tumor landscape, underscoring that intratumoral CNA heterogeneity is a defining feature of WTs.

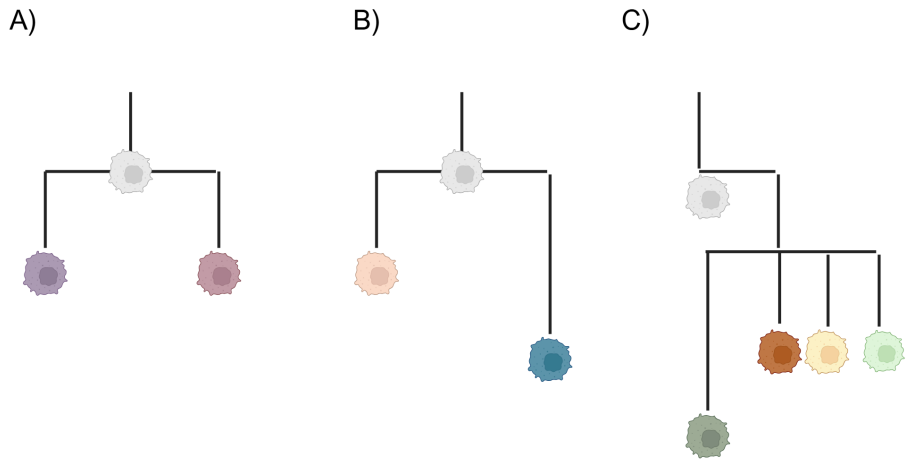


Figure 19. Reconstructed phylogenetic trees.
A) Linear pattern. B) Branching pattern. C) Mix of linear and branching.

TP53 mutation and allelic loss through convergent evolution

While IR and BT tumors showed considerable variability in phylogenetic tree architecture, all DA cases consistently displayed multigenerational trees combining both branching and linear evolutionary patterns. In 4 of 5 diffuse anaplasia cases, one or more *TP53* mutations occurred together with allelic imbalances resulting in loss of the remaining wild-type allele. All tumors with classical DA morphology exhibited *TP53* inactivation, accompanied by evidence of parallel evolution across anatomically distinct anaplastic regions. In three cases, this parallel evolution was preceded by an earlier TP53 disruption, either homozygous or hemizygous.

High complexity of tumor phylogenies in diffuse anaplasia

WT DA exhibited significantly greater phylogenetic complexity compared to IR and BT tumors. They also harbored a higher overall CNA burden and a markedly increased number of genetically distinct subclonal populations (median = 13) relative to IR tumors (median =3), with BT tumors being in between (median =7). In summary, these results demonstrate that the increased evolutionary complexity in DA manifests through multiple features, including a greater number of subpopulations (clones and subclones), elevated PSR and divergence, and higher asymmetry within phylogenetic trees (Paper I, figure 4 A-E).

Clonal evolution within and across anatomic compartments

We aimed to compare clonal evolution occurring within compartments to events associated with the formation of new compartments, reflected as genetic variation between them. To achieve this, we analyzed CNA maps across WMSs (phylogeographies) to assess shifts in the subclonal landscape within compartments versus those observed across compartments (Figure 20A-B).

Shifts in the subclonal landscape were categorized into four evolutionary trajectories [132] tumor cell twinning (TCT), representing identical and homogeneous clonal composition; clonal coexistence (COEX); subclonal variation (VAR); and clonal sweeps (SWE), defined as the emergence of a new clone occupying ≥90% of tumor cells, with or without daughter clones [132]. Within compartments, the most common trajectory was TCT (Figure 20A), indicating largely homogeneous copy-number profiles. Variation within compartments was limited to shifting VAR (Figure 20A) or the presence of the same subclones, i.e. COEX. SWE was rare within compartments (2% of comparisons). In contrast, 76% of intercompartmental comparisons showed SWE (Figure 20B), while TCT and occasional VAR were observed, but COEX was absent. These findings suggest that new compartments typically arise from existing ones through processes involving a single subclone rather than multiple coexisting clones.

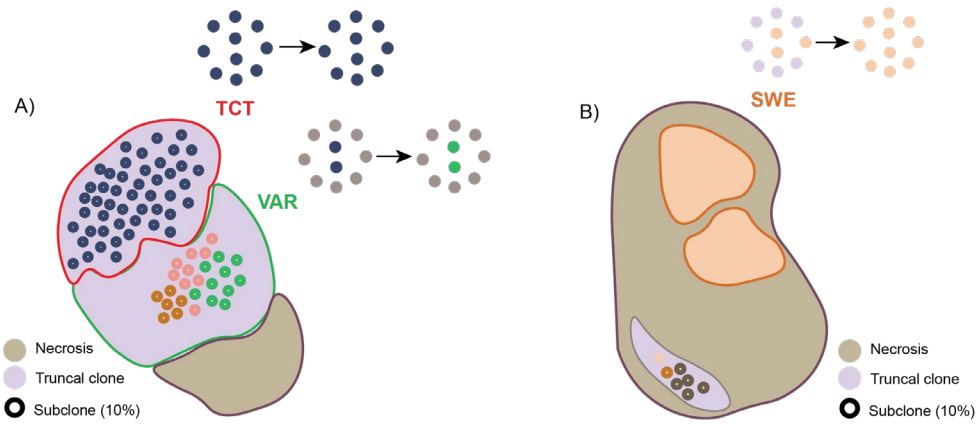


Figure 20. Assesing evolutionary trajectories within two different phylogeographies.
A) Phylogeography with TCT within the same compartment and VAR within the same compartment. B) SWE across compartments.

Paper II

A Gradual Transition Toward Anaplasia in Wilms Tumor through Tolerance to Genetic Damage

Summary of key findings

Proliferation index and DNA damage (γH2AX dot expression) increased with higher anaplasia scores. Nearly all regions with full-scale anaplasia (95.6%) showed *TP53* mutations or LOH, accompanied by CNAs. Interestingly, areas with wild-type *TP53* but LOH of the gene and only one anaplastic feature (score 1) also exhibited significantly higher proliferation, DNA damage, and CNAs compared to non-anaplastic regions (score 0), suggesting these may represent pre-anaplastic populations under selective pressure for *TP53* mutations. Overall, these findings indicate that chemoresistance in WT DA may partly reflect a high proliferative capacity despite an elevated baseline of genomic instability in anaplastic cells. Our findings support a gradual evolution of anaplasia in WT, appearing progressively and beginning with *TP53* LOH, followed by *TP53* mutation, ultimately leading to full scale anaplasia [117]. Thus, chemoresistance in these patients may partly stem from the high proliferative capacity of anaplastic cells combined with their increased tolerance to CNAs and dsDNA breaks [117].

Anaplastic cells maintain a proliferative state despite a high burden of double-stranded DNA breaks and high p53 protein levels

Anaplasia was scored on single-patient TMAs. All H&E-stained TMA cores (27 WTs; 769 analyzed cores/tumor areas) were evaluated for anaplastic features and assigned an anaplasia score ranging from 0 to 3. Anaplastic cells (scores 2–3) exhibited strong nuclear p53 accumulation (Paper II, figure 2B) together with Ki67 positivity (Paper II, figure 2G-H) and strong and dot γH2AX staining (apoptosis and the latter dsDNA breaks), indicating ongoing dsDNA damage (Paper II, figure 2E-F). Importantly, these three markers were co-expressed within the same cells, demonstrating that despite p53 accumulation and extensive DNA damage, the cells rarely underwent apoptosis and continued to proliferate. This staining pattern was consistent across histologic subtypes (IR, BT, DA) and nuclear unrest grades, supporting the conclusion that WT DA are highly proliferative even under genotoxic stress.

Distribution of TP53 mutations and loss of heterozygosity

To identify *TP53* mutations, we performed targeted deep sequencing on 122 tumor DNA samples (from 20 WTs). No *TP53* mutations were found in IR WTs. All pathogenic *TP53* mutations occurred in DA cases (17/122 samples, 14%), except for one blastemal case with a 3′UTR variant in a non-anaplastic area (score 1) without p53 accumulation. One DA case (DA-4) lacked *TP53* mutations despite multiple anaplastic regions. Overall, 95.6% of areas with anaplastic histology

(scores 2-3) had *TP53* mutations or LOH (Paper II figure 3B). Alterations were significantly more frequent in score 1 areas than score 0. Almost all IR WT samples (98.6%) were TP53 wild type, whereas BT WTs showed LOH in 28% and mutations in 2.5%. In DA tumors, two-thirds of areas harboured *TP53* alterations. In summary, pathogenic *TP53* mutations were restricted to DA tumors, and their frequency increased with anaplasia severity.

TP53 mutations confer continued proliferation and genetic instability

We next compared protein expression patterns across areas with different *TP53* status (wild type, LOH, mutation). As expected, p53 expression was significantly higher in regions with *TP53* mutations compared with wild-type (Paper II, figure 3C). Proliferation (Ki67) was also increased in areas with *TP53* LOH or mutations compared to wild type (Paper II figure 3D).

Apoptosis, indicated by strong diffuse γH2AX staining, was most extensive in *TP53*-mutated regions (Paper II, figure 3E). In contrast, γH2AX dot staining reflecting dsDNA breaks without apoptosis rose progressively with increasing genomic instability following the hierarchy: combined *TP53* mutation and LOH > mutation alone > LOH alone > wild type (Paper II, figure 3F). Overall, both *TP53* LOH and mutation areas exhibited enhanced proliferation and DNA damage in comparison to wild-type areas.

The number of copy number aberrations correlates to anaplasia but depends on TP53 status

We analyzed CNA burden in relation to histology, anaplasia score, and *TP53* status. IR cases had significantly fewer CNAs than BT or DA cases, with no difference between BT and DA. Areas with anaplastic histology (scores 2–3) showed markedly higher CNA levels than areas with scores 0–1; among non-anaplastic regions, score 1 areas exceeded score 0. CNA burden was greatest in regions with *TP53* LOH, followed by *TP53* mutations, and lowest in wild-type areas. Differences in CNA levels across anaplasia scores largely reflected varying frequencies of *TP53* alterations.

Paper III

Strong Glypican 3 Expression in Blastemal Components of Wilms Tumors with Diffuse Anaplasia

Summary of key findings

GPC3 expression was markedly higher in blastemal compartments of WT DAs compared to IR and BT WTs. GPC3 correlated positively with anaplastic features, proliferation, p53 expression, and apoptosis. Its strong association with anaplasia,

consistent blastemal expression, and low intratumoral variation suggest a potential role in WT DA pathogenesis and warrant further evaluation as a therapeutic target.

GPC3 expression overview

GPC3 expression was assessed by immunohistochemistry (IHC) in single-patient TMAs of IR-, BT-, and DA-WTs using a semi-quantitative scoring system based on staining intensity and percentage of positive cells. Identification of histological compartments (blastema, epithelium, stroma, and DA) was aided by adjacent H&E-stained sections. Strong staining was observed in all subgroups but was mostly focal in IR WTs (≤10% of core area in over half of cases; average 13%). In contrast, BT and WT DAs showed a broader distribution of strong staining (1–90% of core area) with the average of 30%, indicating more extensive expression in these tumor types.

In depth analysis of GPC3 expression in blastemal elements

Most TMA cores from BT WTs showed strong GPC3 expression. We therefore focused on blastemal areas positive for GPC3 across all WT subgroups (IR, BT, DA). GPC3 expression was significantly higher in blastemal areas of WT DAs compared to IR and BT ($p<0.001$) (Paper III, figure 2A), with no significant difference between IR and BT. Whole-core analysis confirmed stronger staining in WT DA. Blastemal GPC3 positivity was common across subtypes: 90% of IR cores, 88% of BT cores, and 86% of DA cores contained GPC3-positive blastema, indicating widespread expression in blastemal compartments.

GPC3 expression positively correlates with anaplasia and tumor aggressiveness

GPC3 expression in TMA cores correlated positively with anaplasia ($\rho=0.35$), proliferation (Ki67; $\rho=0.50$), p53 accumulation ($\rho=0.30$), and apoptosis (γH2AX; $\rho=0.50$) (all $p<0.001$). Conversely, GPC3 expression showed negative correlations with CD31 ($\rho=-0.25$) and CD3 ($\rho=-0.30$), markers of vascularity and T-cell presence, respectively. Group-wise comparisons confirmed significant differences in GPC3 expression across anaplasia grades (0–3) and between low, intermediate, and high levels of Ki67, p53, and γH2AX (Paper III, figure 4A-D).

Discussion

In my three papers, I have focused on anaplastic WT and looked into their evolution and its clinical significance, hoping to gain insights that could lead to more precise diagnostics and effective treatments for children with this tumor type.

In **paper I** we mapped clonal architecture in 20 WTs (IR, BT, and DA) using copy number profiling and *TP53* mutation analysis, followed by phylogenetic reconstruction. DA tumors showed greater tree complexity, higher CNA burden, and more subpopulations than IR and BT WTs. In WT DA *TP53* alterations, mutations and allelic loss were late, subclonal events, often initiating anaplasia and followed by branching and saltatory evolution. Parallel *TP53* changes across compartments suggest multifocal progression. Macroscopic compartments reflected clonal sweeps, highlighting the need for cross-compartment sampling in WT analysis. In **paper II**, we explored the emergence of anaplasia in WTs and its link to chemoresistance. DA (5–10% of cases) occurs independently of treatment protocol [94, 95] but strongly correlates with poor response and recurrence [5, 95]. Anaplastic regions showed high proliferation despite extensive DNA damage and CNA burden, suggesting gradual progression toward anaplasia. *TP53* LOH appeared early and *TP53* mutations later, increasing with anaplasia score and driving genomic instability. The early CNA instability likely imposes selective pressure for *TP53* inactivation, enabling continued proliferation and resistance. Our findings support a stepwise model where *TP53* alterations precede CNA accumulation, culminating in fully anaplastic morphology. In **paper III** we demonstrated a strong association between GPC3 expression and tumor aggressiveness, with the highest levels observed in the blastemal components of WT DA. GPC3 expression correlated with increased cell turnover, apoptosis, and p53 accumulation. These findings suggest that GPC3 may play an important role in the pathogenesis or progression of WT DA and merits further investigation as a potential therapeutic target. Notably, the relatively low intratumoral variability in blastemal GPC3 expression indicates that most tumor cells could be accessible to therapy targeted against this protein. We observed that GPC3-rich tumors display a paradoxical profile of both high proliferative activity and increased apoptosis. This dynamic interplay between rapid cell division and programmed cell death may underlie the complex biological behavior of WT DA, including the saltatory evolution previously described as a hallmark of WT DA [116]. Interestingly, while earlier studies have reported GPC3 expression to be independent of the cell cycle [112], our findings suggest otherwise.

Limitations and strengths of the studies

The different studies have several limitations that should be acknowledged. The sample size was relatively small, which may restrict the generalizability of the findings. To strengthen the validity of our findings and increase statistical power, broader international collaboration is essential to include a larger cohort of patients.

Patients with focal anaplasia could not be included due to insufficient material. Although focal and diffuse anaplasia differ primarily in the risk group, extent and distribution of anaplastic areas, additional studies are needed to confirm whether our observations also apply to focal anaplasia. Another potential source of bias is that the vast majority of analyzed samples were obtained after preoperative chemotherapy, making it impossible to assess the direct impact of chemotherapy on anaplastic cells. Furthermore, the use of FFPE tissue introduced technical limitations, as the DNA is often fragmented and of low quality. Finally, some tumors exhibited extensive regressive changes, reducing the amount of viable tumor tissue available for analysis and potentially limiting the depth of genetic characterization. Also, further research with larger patient populations is needed to fully understand the implications of GPC3 expression in WT and its potential as a therapeutic target.

Although the number of patients included in this study was limited, a major strength lies in the extensive sampling strategy. Multiple regions were analyzed from each tumor, utilizing single-patient TMAs specifically constructed for this purpose. This approach enabled a detailed spatial assessment of tumor heterogeneity and minimized the risk of overlooking subclonal variation.

Another strength is the integration of complementary methodologies including copy number profiling, targeted *TP53* analysis, subclonal deconvolution and phylogenetic reconstruction. Papers II and III extend this approach by offering an in-depth characterization of the WT microenvironment, encompassing proliferative activity, apoptosis, vascularity, protein expression, anaplasia scoring, dsDNA breaks, immune infiltration and the potential application of GPC3 as a target for therapeutic intervention.

Clinical context and evolutionary patterns

Patients with WT DA are generally older than those with non-anaplastic tumors [133]. This may reflect the timing of *TP53* mutations as late events in tumor evolution, as described in paper I and paper II. The late emergence of *TP53* mutations and anaplasia indicates that aggressive WTs ‘grow bad’ over time, rather than originating as inherently aggressive [117]. Our findings indicate that TP53 alterations often arise through parallel evolution leading to multifocal anaplasia,

which subsequently triggers saltatory evolution and extensive accumulation of CNAs [116] which could explain the complex tumor heterogeneity within WTs. It also emphasizes that WT DA is not simply a histologic variant but a distinct evolutionary state with amplified branching, species richness, divergence, and irregularity at the phylogenetic level. When TP53 function is lost, the cell's ability to maintain genomic integrity collapses, enabling chromothripsis, chromosomal instability or many CNAs in a single step [134].

In paper I we found that in 4 out of 5 WT DA cases, *TP53* mutations were present, and all four exhibited classical anaplastic morphology. Whilst in one of the cases with anaplastic morphology, there was no *TP53* mutation. This raised the question if factors within the tumor microenvironment might contribute to WT DA pathogenesis and resistance to treatment? - A question that ultimately led to the focus of my final study.

TP53 alterations as a biomarker – timing matters

Several studies have explored the potential of *TP53* alterations as prognostic biomarkers and their utility in guiding treatment strategies, including the use of liquid biopsies. However, our findings in Papers I and II indicate that *TP53* alterations whether sequence mutations or allelic loss are consistently late events in WT evolution. This temporal pattern raises important questions regarding the clinical applicability of *TP53* as an early predictive marker. If *TP53* disruption occurs predominantly at advanced stages of tumor progression, its value for early risk stratification or treatment planning may be limited. Instead, *TP53* status might serve more effectively as an indicator of aggressive disease or impending anaplastic transformation rather than as a primary determinant for initial therapeutic decisions. *TP53* LOH appears early, as noted in paper II, could that be clinically useful as a predictive marker for anaplasia? Early recognition of such pre-anaplastic changes might help adapt surveillance.

Links between *TP53* alterations and GPC3 expression

By combining information from paper I with paper III, we observed that the WT DA case lacking *TP53* mutations also showed no GPC3 expression. Histological assessment of the corresponding TMA cores revealed a predominance of stromal and blastemal components (Figure 21), rather than a purely stromal pattern possibly explaining its classification as DA given the blastemal contribution. Similarly, in additional two Italian DA cases, we noted the same histological features and the absence of GPC3 expression. This raises the question of whether the absence of

TP53 mutations and GPC3 expression in these DA cases is related to their specific histological composition. However, targeted *TP53* sequencing was not performed for the mentioned Italian cases, but I do wonder, do they also lack *TP53* mutations?

Notably, two of the abovementioned cases experienced disease progression or relapse, suggesting that absence of GPC3 expression and potentially *TP53* alterations does not preclude aggressive clinical behavior. Our findings emphasize that other molecular or microenvironmental factors likely contribute to the WT DA phenotype and its poor outcome. This raises the question of whether there is a distinct subgroup of WT DA with a mesenchymal growth pattern, not dependent on p53 pathway disruption and GPC3 overexpression? There were no reports of a DICER1 syndrome association in these cases, not was their morphology dominated by high-level pleomorphism, which makes it unlikely that the cases in question were misdiagnosed anaplastic sarcomas of the kidney.

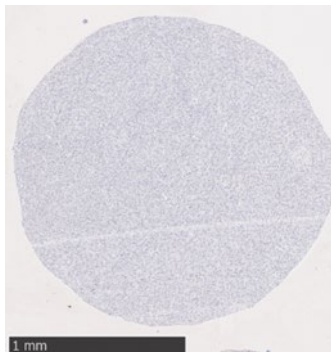


Figure 21. Wilms tumor with diffuse anaplasia.
Tissue microarray core illustrating stromal and blastemal components of Wilms tumor.

Although both *TP53* alterations and GPC3 overexpression are associated with aggressive features in WT DA, they act through distinct biological mechanisms. p53 functions as a tumor suppressor, and its loss via mutation or LOH removes critical genomic checkpoints, allowing cells with DNA damage to continue proliferating. This leads to the accumulation of dsDNA breaks and CNAs, which are strongly linked to chemoresistance and the emergence of full-scale anaplasia. In contrast, GPC3 is an oncofetal protein that promotes growth signaling through pathways such as Wnt/β-catenin and IGF2. High GPC3 expression correlates with a paradoxical combination of increased proliferation and apoptosis, potentially driving rapid clonal shifts and the saltatory evolution characteristic of WT DA. Thus, while both factors contribute to tumor aggressiveness, *TP53* loss primarily facilitates genomic instability, whereas GPC3 amplifies proliferative signaling. Their convergence may create a highly dynamic and treatment-resistant tumor phenotype.

Microenvironmental features and tumor behaviour

Interestingly, despite their aggressive phenotype, GPC3-high tumors exhibit reduced vascularity, as indicated by low CD31 expression. This suggests a microenvironment where tumor growth outpaces vascular development, resulting in heterogeneous perfusion, nutrient deprivation, and hypoxia [135]. Such conditions may influence both treatment response and disease progression [136]. Furthermore, we observed a negative correlation between GPC3 expression and the T-cell marker CD3, supporting the notion of an immunologically "cold" microenvironment typical of aggressive WT subtypes [137].

Novel therapeutic perspectives

Doxorubicin is commonly used in high-risk WTs. However, in Paper II we demonstrate that the DA subtype maintains a high proliferative rate despite extensive dsDNA breaks. Since doxorubicin exerts its cytotoxic effect primarily through DNA intercalation and subsequent functional arrest [138], this observation raises the possibility that WT-DA's ability to proliferate under genotoxic stress contributes to poor response to DNA-damaging agents such as doxorubicin.

The strong link between GPC3 overexpression and WT DA opens potential clinical opportunities. Drawing parallels with hepatocellular carcinoma, where GPC3-based serum assays have proven useful for both initial diagnosis and post-treatment monitoring [112], we propose that similar strategies could be explored for WT. However, this approach requires validation in larger patient cohorts. For high-risk WT patients, current cytotoxic regimens are nearing the limits of tolerable dosing [139], underscoring the need for novel therapeutic strategies such as targeted approaches. The predominant overexpression of GPC3 in DA and BT WTs suggests that GPC3 could serve as a promising target for tailored therapy, potentially reducing the adverse effects associated with conventional high-intensity chemotherapy.

Future perspectives

Paper I *– Tumor Heterogeneity and Evolution*

- The extensive intratumoral heterogeneity and complex phylogenetic architecture observed in WT DA underscore the need for **multi-region sampling**. Future studies should explore **liquid biopsy approaches** to capture this diversity non-invasively and monitor clonal evolution during treatment.
- Understanding the timing and distribution of *TP53* alterations could inform **risk stratification** and **adaptive therapy models**, where treatment intensity is tailored to evolutionary trajectories rather than static histology. This approach could allow treatment to be tailored based on the timing of *TP53* mutations. If these mutations typically occur late, therapy could be de-escalated to avoid overtreatment and reduce toxicity. Conversely, if they arise early, upfront treatment could be intensified to address the higher-risk trajectory.

Paper II *– Chemoresistance and Proliferation Under Genotoxic Stress*

- The ability of anaplastic cells to proliferate despite DNA damage suggests that **DNA-damaging agents alone may be insufficient** for WT DA. The issue is further complicated by the general lack in WT DA of driver mutations that are targetable by today's precision drugs.
- Other treatment options, from entirely new angles, seem to be necessary to improve outcomes for patients with WT DA.

Paper III *– GPC3 as a Biomarker and Therapeutic Target*

- The strong and relatively uniform expression of GPC3 in blastemal and anaplastic components positions it as a promising candidate for **targeted therapy** (e.g., monoclonal antibodies, vaccines, or antibody-drug conjugates).
- Future research should validate **GPC3-based serum assays** for early detection and treatment monitoring, drawing on experience from hepatocellular carcinoma.
- The observed immunologically "cold" microenvironment suggests that **combination strategies** involving GPC3-targeted therapy and **immune modulation** could be explored to improve outcomes.

Conclusion

Based on the findings presented in this thesis, the following conclusions were drawn:

- Extensive intratumoral heterogeneity was observed in WTs.
- WT DA exhibited greater phylogenetic complexity, higher CNA burden, and more subclonal diversity compared to intermediate-risk and blastemal-type tumors.
- *TP53* mutations and allelic loss were late, subclonal events, confined to branches rather than the trunk of tumor phylogenies, supporting a focal origin of anaplasia. *TP53* alterations correlated strongly with anaplasia severity.
- Macroscopic compartments reflect clonal sweeps, indicating that monoclonal spread followed by clonal expansion is the predominant mechanism of compartment formation. These findings underscore the need for cross-compartmental sampling to capture the full genetic landscape of WTs.
- WT DA exhibit a high proliferative capacity despite carrying extensive dsDNA breaks and CNAs, indicating that genomic instability does not impede cell division in these regions.
- Anaplasia appears to emerge gradually through an intermediate state characterized by *TP53* loss of heterozygosity.
- GPC3 overexpression is strongly associated with aggressive features of WTs, with the highest levels observed in the blastemal components of WT DA.
- GPC3 expression correlates with high proliferative activity, increased apoptosis, and p53 accumulation, suggesting a role in WT DA pathogenesis and progression.
- The relatively low intratumoral variability of GPC3 in blastemal regions indicates that most proliferative tumor cells could be accessible to GPC3-targeted therapy.
- Despite their aggressive nature, GPC3-rich tumors show reduced vascularity and an immunologically “cold” microenvironment, characterized by low CD31 and CD3 expression.

Acknowledgements

I am deeply grateful to everyone who supported me throughout this work. While it is impossible to name everyone without missing someone, I would like to express my sincere thanks to those who made this journey unforgettable:

I would especially like to thank my main supervisor, **David Gisselsson Nord**. I still remember my first day at BMC 13 when you said: "Sit in on a meeting with me and Subhayan, then you'll know how meetings look in this group." I sat there, understood nothing, blushed, and replied: "Yes, okay, I understand how it should be." I could never have imagined that years later, we would be discussing research and planning next steps together. Your enthusiasm is contagious and has made this journey both enjoyable and inspiring. You have given me an education I could not have received anywhere else. Thank you for believing in me and pushing me forward when needed, knowing that I work best under pressure. I am forever grateful for the years I have been your PhD student and look forward to many more years of collaboration.

My co-supervisor, **Linda Holmquist Mengelbier**, who truly has been my *stöttepelare*, as I wrote in my preface. There are not enough words to thank you for your guidance, for translating when I did not understand David, for always answering my texts and calls, comforting me, and helping me find my way through this journey. Thank you for always laughing with me and holding my hand through my PhD. You have been by my side every step of the way. I could not have done this without you, nor would I have wanted to.

Karin Jirström, my co-supervsior, who initially started as my main supervisor but, knowing my passion for pediatric oncology, guided me toward David. Without you, I would never have been so fortunate to connect with the research world. I still remember you calling colleagues in pediatric oncology and saying: *"You have to hire her."* I am deeply grateful for everything you taught me and for paving the way for this journey. Who would have thought that a lunch at CRC would lead me here? You were the beginning of it all, and I will never forget that. Thank you for being the start of everything.

Patrik Romerius, my co-supervisor and clinical supervisor. You have been an invaluable sounding board on how to balance clinical work with PhD life. Living a "double life" is not easy, but you have guided me through it, helped me de-stress

when needed, and always believed in me. Thank you for all your wisdom and always making me laugh and not take it all so seriously.

Jacek Toporski, my co-supervisor, thank you for your support and for meeting me back in 2019 when I was determined to pursue pediatric oncology. That conversation not only opened the door to this path but also revealed our shared appreciation for both Poland and Iran.

My group - **Jenny Karlsson**, in you and Linda I have found a special bond for which I am forever grateful. Thank you for all the coffee breaks (and chat breaks), the podcast and music tips (even if my Christmas playlist makes you laugh), and for being the aesthetic Adobe Illustrator Queen who taught me everything about choosing the "right" colors. Thank you for making SNP analysis so much more fun!

Subhayan Chattopadhyay - thank you for catching me when you see on my face that I'm struggling with bioinformatics in the BMC kitchen and explaining it in a way I can actually understand. And all other disasters I come running to you for, HERO!

Natalie Andersson, thank you for your brilliant mind and your trees - you make everything look so effortless and it's truly inspiring!

Anders Valind, thank you for your help and bioinformatics-brain throughout my time as a PhD student – so happy to continue working with you at pediatric oncology department!

Kaname Uno, it has been such a joy getting to know you during this journey. I've learned so much about Japan, airplanes, and p53 during the times we shared a room. **Caroline Jansson** and **Geoffroy Durand** - thank you for all the time and effort you have put into the lab work that made it possible for me to write this thesis! I would like to thank all members of our group, present and previous members!

Ioannis Orfanos, you have been both a mentor and a friend at the clinic. I have learned so much from you, and every single presentation during my PhD has benefited from your honest feedback, for which I am extremely grateful. Your comments, from 'Remove this slide, lost interest' to 'You had me there, but now I'm bored,' have helped me more than you know. Thank you for always being there when I needed help and guidance (and yes, this probably covers all the bets I lost against you!). Joking aside, thank you for everything.

To former and current fellow PhD students at clinical genetics, THANK you for all the fun lunches, exchanging ideas and a special thanks to all my roommates, I've had a few throughout the years.

Sofie Olsson Hau – my idol. Thank you for guiding me through life and for finding me. **Sebastian Lundgren**, my toastmaster, life would have been so boring without you in it. **Hanna Thorsson**, my roommate for a short but cozy time – thank you for hyping me up, sharing snacks, always checking in on me and helping me during my

last-minute questions. Never forget how great you are! **Louise Ahlgren,** thank you for all the laughs and hangouts in your and Hannas room. **Saskia and Josephine**, thank you for being the best roommates during this stressful time, making me laugh and rooting for me, so happy for the move to D14, I got to know you. **Karim**, who would I have had all the middle east talks with and comparing all the good foods if it wasn't for you. **Fanny**, this autumn and winter wouldn't have been the same without you. **Alexandra Svensson, Sara Wahlin, and Christina Siesing** – thank you for all the fun and great moments we've shared! I would like to thank all members both previous and current members of Karin Jirströms group.

Clinical Genetics – I am proud to be part of this division. I would like to thank all my colleagues here, I have spent so many years with you all, feels weird to say bye, I prefer – see you later <3

The atmosphere at the department makes it a pleasure to come to work, and I am grateful for how welcoming everyone has been despite my irregular presence over the years. It is a privilege and an honor to be surrounded by such an inspiring environment, where exceptional research is carried out. Thank you for always making me laugh during lunches and listening to all my silly TV shows I recommend to you guys (especially **Calle** and **Tina**). **Anette,** what would we all do without all your help?

To all **co-authors** in the publications included in this thesis - thank you for your support, input, and collaboration while working on the papers.

Mina barnläkarkollegor och vänner, tack för att ni får jobbet så roligt. Tänk att man kan ha så fina vänner på jobbet! Tack för all pepp under den här tiden och för att ni har erbjudit hjälp och byten av jourer under höst/vinter!

Felicia och Annika - vi har känt varandra sedan vi var sex år gamla, och nu har vi fyra barn som leker tillsammans och som kommer att ha varandra genom livet, precis som vi har haft varandra. Att få gå igenom alla livets kapitel med er är en ynnest. Jag vet att jag ibland försvinner under en sten när jag är stressad, men som alltid genom livet finns vi där för varandra. Tack för att ni finns och för att ni är mina systrar genom livet.

Mona och Ebba - jag vet inte om jag hade vågat välja barnonkologi om inte du, Mona, hade uppmuntrat mig. Jag har alltid varit rädd att bli för känslomässigt involverad och osäker på om jag skulle klara det. Men då sa du: ”Det finns inget som får dig att lysa upp så mycket som när vi är på barnonkologiavdelningen. Det är en styrka att du är som du är och blir så involverad.” Att studera utomlands gav mig DIG - min persiska syster som jag gjort galna resor med, pluggat tills vi blivit gråhåriga och som jag alltid kommer att skratta genom livet med. Ebba, i Gdansk träffade jag även dig. Vad vore livet utan alla dina råd och alla stunder vi haft ihop på diverse trappor i Gdansk med en kaffekopp, där vi stöttat varandra? Du har varit en så stor del av att hjälpa mig genom denna process - allt

från att komma ner till min halvtid, lyssna på alla telefonsamtal och alltid tro på mig.

Esther, min lilla älskling, Golam. Du har ändrat allt för mig. Att få ha dig som dotter är den största lyxen någonsin! Du är rolig, den härligaste människan jag vet, du utmanar mig och framförallt har du lärt mig om vad som är viktigt här i livet.

Lillasyster som har varit och är i min mage - du har gjort denna resa extra tuff (tips: skriv inte en avhandling i första trimestern!). Men som med allt annat har du gett mig perspektiv. Under en tuff jobbperiod har jag haft dig och storasyster att tänka på, och då känns allt mycket lättare och livet så mycket finare. Förhoppningsvis kommer du i maj, och åh vad du är efterlängtad!

Axel, min älskling (Stålmannen – han jobbar med stål och älskar det, denna korta mening kommer göra honom mallig). Tänk att jag i alla år innan jag träffade dig tänkt: ”äh, antingen skaffar jag ett gäng katter och blir ensamstående mamma eller så får jag hitta en partner jag tolererar?” Inte visste jag att jag kunde ha sån tur att jag får spendera livet med den absolut bästa och finaste människan jag vet. Du är den stabila grunden jag står på, för vi alla vet att jag kan vara en ”strukturerad röra” som har allt i ”huvudet”, höftar och springer genom livet utan tålamod för allt ska hända NU och så står du där med mig och med dig vet jag att allt i livet blir bra, för vi har ju varandra.

Mamma och pappa, ni är utan tvekan de bästa föräldrarna och vännerna jag har. Jag tror inte att jag hade lyckats med så många saker i livet om det inte vore för er. Ni har alltid, alltid trott på mig, vilket har gjort att jag sällan ser problem, utan försöker ta mig igenom det mesta med inställningen att allt kommer lösa sig. Och det mindsetet har jag fått från er. För om man kan fly från krig, starta ett liv i Svalöv och sedan flytta till Malmö, älska ägg och sill men ändå vara de mest stolta perserna jag vet då klarar man allt. Ni är mina förebilder. Tack för att ni även är världens bästa morföräldrar.

مامان و بابا – بدون شک شما بهترین والدین و بهترین دوستانی هستید که می‌توانستم داشته باشم .فکر نمی‌کنم بدون شما در زندگی به این همه موفقیت دست پیدا می‌کردم .شما همیشه، همیشه به من ایمان – داشتید و همین باعث شد که من به ندرت مشکلات را ببینم و همیشه فکر کنم همه چیز درست می‌شود و این طرز فکر را از شما گرفته‌ام .چون اگر کسی بتواند از جنگ فرار کند، در سوئد زندگی جدیدی بسازد، بعد به مالمو نقل مکان کند، عاشق تخم‌مرغ و شاماهی شود و در عین حال افتخارآمیزترین ایرانی‌هایی باشد که می‌شناسم، پس من هم از پس همه چیز برمی‌آیم .شما الگوی من هستید .ممنونم که بهترین پدربزرگ و مادربزرگ دنیا هم هستید

Min lillebror **Reza** du är den smartaste och snällaste personen jag känner. Din kompis googlade på mig för många år sedan och såg att jag var anmäld som doktorand. Jag minns fortfarande hur imponerad du var, vilket gör mig mallig eftersom du är den jag ser upp till. Du är så cool att jag inte ens kan förklara vad du jobbar med. När jag satt med Statistik 2 minns jag att jag sa: ”Men jag förstår ju

absolut ingenting, vem kan detta?!" Då berättade du att du hade studerat detta på universitetet - DÅ fick du inte vara ifred. Tack för att du drog mig igenom den kursen.

När jag först fick reda på att jag väntade lillasyster var jag helt säker på att jag hade en lillebror i magen, för jag hoppades att Esther skulle få ha en "Reza" genom livet. Jag vet att jag fick den bästa lillebror i världen. Som dessutom lät mig sno alla pengar du sparade när du var barn, bara för att du tyckte synd om mig som aldrig lyckades spara (har fortfarande hål i fickan). **Benedikte**, tack för att du gifter dig med min bror och blir en del av vår familj.

Sahar och Maryam – för att ni alltid finns där för mig och är mina bästa kusiner.

Till mina älskade svärföräldrar **Susanna och Gunnar**, tänk vilken tur att jag har er i mitt liv. Tack för allt ni gör för mig och Esther. **Elin och Daniel** – är det nu vi firar med en bender?

Jag skulle kunna fortsätta i evigheter, men det ska jag inte. Jag är så himla glad och tacksam för ALLA som har hjälpt, stöttat, peppat och hållit min hand under denna resa. Jag trodde aldrig att jag skulle klara något sånt här – utan er hade jag aldrig varit här.

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Printed by Media-Tryck, Lund 2026
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About the author

I am nearing completion of my pediatric residency and will pursue subspecialization in pediatric oncology. I have a particular interest in solid tumors and in developing therapeutic strategies that combine efficacy with reduced toxicity, aiming to achieve better prognoses and preserve long-term quality of life for affected children.

LUND UNIVERSITY

FACULTY OF MEDICINE

Division of clinical genetics
Department of Laboratory Medicine

Lund University, Faculty of Medicine
Doctoral Dissertation Series 2026:18
ISBN 978-91-8021-816-0
ISSN 1652-8220