ALTERACIONES CROMOSÓMICAS SEGMENTARIAS COMO MARCADOR PRONÓSTICO EN NEUROBLASTOMA Y SARCOMAS PEDIÁTRICOS

Tesis Doctoral:

Programa de Doctorado 3139 en Medicina

Doctorando

Antonio Juan Ribelles

Directora

Mª Adela Cañete Nieto

Solicitud de depósito

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de D/Dña. Antonio Juan Ribelles, estudiante del Programa de Doctorado **3139 Medicina** (RD99/2011) en Medicina de la Universitat de València, emiten informe favorable para la realización del depósito y la defensa de la tesis doctoral.

Fecha: Valencia, 4 de septiembre de 2020

Fdo.: Mª Adela Cañete Nieto

Directora

ESCUELA DOCTORAL

UNIVERSITAT DE VALÈNCIA

Introducción por parte del doctorando y agradecimientos

He dedicado los últimos 8 años de mi vida profesional a la asistencia clínica en Onco-hematología pediátrica. Los primeros años dentro del programa formativo de residencia en el Hospital U i P La Fe, posteriormente durante el primer año de especialista en el Hospital Vall d'Hebron de Barcelona y más adelante, de nuevo en el Hospital La Fe de Valencia. Aunque he tenido contacto con todos los campos de la especialidad incluyendo el trasplante de progenitores hematopoyético, he tenido especial dedicación a los tumores sólidos en la infancia, al desarrollo de nuevas terapias y los ensayos clínico precoces.

Durante estos pocos años, he sido testigo de muchos avances en términos de nuevos tratamientos y el perfeccionamiento del diagnostico anatomo-patológico y molecular de los tumores infantiles, destacando el neuroblastoma y los sarcomas. Los oncólogos pediátricos hemos pasado de conocer una enfermedad tan temible como un cáncer únicamente por sus características anatomo-patológicas (que a día de hoy siguen siendo el pilar fundamental sobre el que se basan los diagnósticos) al desarrollo gradual de múltiples técnicas complementarias que implican al campo de la biología molecular y que nos están permitiendo conocer cada vez con mayor detalle a nuestro "enemigo" contra el que nos enfrentamos cada día con nuestros pacientes. Algunos ejemplos de estas técnicas son la hibridación fluorescente in situ (FISH), las técnicas de reacción en cadena de la polimerasa con transcriptasa inversa (RT-PCR), las técnicas con array de SNP y CGH array y por último la secuenciación directa y la secuenciación masiva.

La introducción de estas técnicas nos ha permitido entender por qué un mismo tumor se comporta de forma diferente en un caso u otro y los motivos por los que algunos pacientes se curan de su enfermedad y otros recaen o son refractarios a las terapias habituales cuando parten de un mismo diagnóstico anatomo-patológico. Las técnicas han llegado a perfeccionarse tanto que en oncología durante los últimos años intentamos hacer aproximaciones de medicina personalizada intentando encontrar terapias diana que inhiben específicamente un tumor por poseer una mutación concreta. Algunas de estas terapias ya forman parte del tratamiento de primera línea de algunas neoplasias y de esta forma, se consigue la erradicación de la enfermedad incluso con menos efectos secundarios que los que observamos con los citostáticos clásicos.

Como la asistencia clínica, que en este campo siempre tiene un ritmo muy intenso, no me ha permitido poder profundizar en el conocimiento de la biología tumoural y las técnicas diagnósticas todo lo que me hubiera gustado, decidí invertir el tiempo de mi tesis doctoral a formarme y conocer un poco mejor estos métodos diagnósticos que hacen que pueda comprender mejor el comportamiento de los tumores a los que me enfrento en el día a día, siempre con la ayuda de los compañeros de laboratorio. Por este motivo y gracias al refuerzo positivo de Adela Cañete

(directora de la tesis) y la supervisión de Victoria Castel, he conseguido terminar los cuatro artículos de investigación que constituyen esta tesis por compendio de artículos.

La finalización de los cuatro trabajos que se presentan no hubiera podido ocurrir sin el apoyo de las personas que enumero a continuación: Empezando por compañeros y compañeras de trabajo diario, en especial Chema, Carol, Bárbara y Mara, el equipo de enfermería y auxiliares, el equipo de cirugía del Dr. Marco incluyendo a Javi y Jorge, las compañeras de radiología y medicina nuclear con especial mención a María Guasp, Cinta Sangüesa y Raquel Sánchez. A Gemma Llavador, patóloga infantil y al personal de laboratorio con especial mención a Jaime Font de Mora y Marta Llop. Para continuar, agradecer también enormemente al equipo de investigadores: Pablo, Sheila, Desi, Vanessa y Yania. Por último, por estar siempre cerca, agradecer los consejos a mi familia más cercana: Mi madre, mi padre, mi hermana y el respaldo de Pau.

Como resumen general, los primeros dos trabajos exploran las alteraciones cromosómicas segmentarias en neuroblastoma estudiadas por array de SNPs, en primer lugar, profundizando en la deleción del brazo largo del cromosoma 11 y sus connotaciones negativas. En segundo lugar, se exploran las agrupaciones de alteraciones cromosómicas que se presentan en este tumor con mayor frecuencia. Coincidiendo en el tiempo con el desarrollo de la secuenciación masiva y el programa de medicina personalizada que se formó en el Hospital La Fe he estudiado los resultados de 70 sarcomas por secuenciación con las recomendaciones de fármacos dirigidos que se han propuesto en el comité de medicina personalizada del centro. Esto ha permitido la implantación de las técnicas de secuenciación masiva al debut en todos los sarcomas pediátricos dentro de la cartera de servicios del centro al tratase de un centro de referencia nacional para sarcomas pediátricos. Por último, con una temática más diferente y enlazando con los comités de tumores moleculares, el cuarto artículo es el fruto de una encuesta realizada a nivel europeo sobre el funcionamiento de los comités de tumores en los diferentes países de la unión europea dentro del proyecto Expo-r-Net donde participé activamente para crear y formar parte de la red de referencia en cáncer infantil *PAEDCAN*.

Dado que los cuatro artículos que componen esta tesis están escritos en inglés, he decidido completar la tesis en el mismo idioma, aunque algunos de los apartados más relevantes como esta introducción, la hipótesis con objetivos principales y conclusiones figuran en castellano también. Espero haber aportado a la comunidad científica un pequeño grano de arena con estos trabajos y que puedan servir de base para nuevas investigaciones en un futuro.

El doctorando:
Antonio Juan Ribelles

ESCRITO SOBRE LAS REVISTAS QUE RECOGEN LOS TRABAJOS

La tesis realizada por el doctorando Antonio Juan Ribelles "ALTERACIONES CROMOSÓMICAS SEGMENTARIAS COMO MARCADOR PRONÓSTICO EN NEUROBLASTOMA Y SARCOMAS PEDIÁTRICOS" ha sido completada en forma de compendio de publicaciones siendo el autor el primer firmante en los cuatro trabajos. Tres de ellos ya han sido aceptados y publicados en revistas indexadas como exige la normativa de tesis por compendio de publicaciones de la Universitat de València. El doctorando ha sido el principal responsable de las 4 investigaciones, de la recolección de datos, análisis de resultados y además es el escritor en primera persona de los 4 artículos a los que se hace referencia. Además, los manuscritos no se han presentado para la defensa de ninguna otra tesis doctoral con anterioridad.

El **primer** artículo: "CLINICAL FEATURES OF NEUROBLASTOMA WITH 11Q DELETION: AN INCREASE IN RELAPSE PROBABILITIES IN LOCALIZED AND 4S STAGES" ha sido publicado en la revista *Scientific Reports*, perteneciente a *Nature Research*. Se trata de una revista abierta con un factor de impacto en los últimos 5 años de 4.576, de primer cuartil. Además, se trata de una de las revistas científicas más citadas del mundo con más de 350,000 citaciones en 2019.

El **segundo** artículo: "DISTRIBUTION OF SEGMENTAL CHROMOSOMAL ALTERATIONS IN NEUROBLASTOMA" y el **cuarto**: "SURVEY ON PAEDIATRIC TUMOUR BOARDS IN EUROPE: CURRENT SITUATION AND RESULTS FROM THE EXPO-R-NET PROJECT" se han publicado en *Clinical & Translational Oncology*, revista con amplia diseminación en la comunidad científica del campo de la oncología con un factor de impacto de 2.737.

El **tercer** artículo: "NEXT-GENERATION SEQUENCING IDENTIFIES POTENTIAL ACTIONABLE TARGETS IN PEDIATRIC SARCOMAS" está pendiente de aceptación en *International Journal of Oncology* con un factor de impacto de 3.889.

Fdo.: Directora de tesis

Mª Adela Cañete Nieto

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ABBREVIATION LIST

SCA: Segmental chromosomal alterations

NB: Neuroblastoma

NGS: Next-generation sequencing

CGH: Comparative genomic hybridization

MLPA: Multiplex ligation dependent probe amplification

PCR: Polymerase chain reaction

SNP: Single-nucleotide polymorphism

MNA: MYCN amplification

CNV: Copy number variations

MAF: Minor allele frequency

IDRF: Risk factors derived from imaging tests

INPC: International Neuroblastoma Pathology Classification

FISH: Fluorescence in situ hybridization

INRG: International neuroblastoma risk group

SIOPEN: Society of Paediatric Oncology European Neuroblastoma research network

NCA: Numerical chromosomal alterations

STS: Soft-tissue sarcoma

BS: Bone sarcoma

RMS: Rhabdomyosarcoma

EWS: Ewing Sarcoma

RT-PCR: Reverse transcription polymerase chain reaction

PMTB: Paediatric molecular tumour boards

EPSSG: European Paediatric Soft-Tissue Sarcoma Study Group

1. INTRODUCTION

1.1 SEGMENTAL CHROMOSOMAL ALTERATIONS

One of the most difficult, though rewarding, challenges in oncology is to determine how specific genetic alterations in certain tumours can affect prognosis and lead to targeted therapies for the individual patient. Current methods of gene-expression profiling have showed that tumour sub-types (previously thought to be homogenous from histological criteria alone), often have various underlying molecular signatures. Complex mutational events seem to promote a major impact on certain genes' expression that contribute to the induction and progression of a neoplasm and, therefore, on the aggressiveness of the tumour, response to therapy and final clinical outcome, including survival. The precise assessment of tumour-cell heterogeneity has therefore become a central focus of investigations in oncology. The ultimate objective of these efforts is to identify cancer sub-types that are driven by altered signalling pathways, whose genetic defects correlate perfectly with final prognosis and can offer attractive targets towards molecular intervention.

One of the goals of this doctoral thesis is to demonstrate how new genomic diagnostic techniques have contributed to the correct stratification of paediatric tumours by risk groups and how the existence of certain segmental chromosomal alterations (SCA) in neuroblastoma (NB) detected by means of molecular karyotyping and certain mutations observed by next-generation sequencing (NGS) in paediatric sarcomas are prognostic markers in these malignancies. Both of these techniques are described below.

1.1.1 MOLECULAR KARYOTYPING (SNP ARRAYS)

The most established method of diagnosing and differentiating tumour types is the detection of chromosomal aberrations by cytogenetic analysis. The emergence of molecular cytogenetic techniques, such as spectral karyotyping, fluorescence in situ hybridization and chromosome-based comparative genomic hybridization (CGH), led to a substantially improved resolution and genome coverage compared with conventional cytogenetics. Another highly sensitive and accurate method used in order to detect copy number changes is multiplex ligation dependent probe amplification (MLPA). This approach is based on polymerase chain reaction (PCR) amplification of ligated probes hybridised to target DNA sequences.

An attempt, in order to improve the detection of altered genomic regions combines classic CGH study with a microarray platform, generating the array CGH technique. This relies on competitive hybridization of fragmented and labelled tumour DNA together with also fragmented, but differentially labelled control DNA. The microarray platform enables a higher-resolution mapping of

genomic regions that contain copy-number variations, such as deletions and amplifications, and the interpretation of data from array CGH studies is usually simpler than data extracted from conventional CGH.

Another microarray-based technique: high-resolution single-nucleotide polymorphism (SNP) array analysis, offers even more advantages for a detailed study of cancer genome. SNP arrays enable high resolution detection of loss of heterozygosity, a common event in cancer formation, in addition to the identification of DNA copy-number aberrations at a resolution similar to that of array CGH. Therefore, SNP array technology can provide a global analysis of DNA copy-number alterations in human cancers and at the same time reveal important losses of heterozygosity, which would not be detected by CGH array or conventional cytogenetic analysis.

In NB, as mentioned before, MYCN amplification (MNA) and specific segmental chromosomal alterations are prognostic markers and the detection of these aberrations are carried out routinely in every new diagnosis. MLPA and SNP array analysis have both been used as part of NB diagnosis at the Oncology and Haematology unit at Hospital La Fe and H. Clínico of Valencia, and these are the methods on which both paper 1 and 2 of this doctoral thesis are based on.

1.1.2 NEXT GENERATION SEQUENCING

Molecular diagnosis of cancer was traditionally based on the detection of changes in a nucleotide or small deletions or insertions in one or more specific genes by means of Sanger sequencing. Because of the increasing number of genes involved in cancer, this technique is now expensive and difficult to use in daily practice. In addition, it requires a large amount of DNA, which is sometimes impossible to extract, and does not allow detection of changes in sub-clonal populations in DNA. The development of NGS sequencing has been a revolution in the approach to cancer diagnosis and has provided a more accessible and complete information on tumour molecular biology. Thanks to the development of these new technologies, together with the implementation of bioinformatic programs for data analysis, the identification of pathogenic alterations and new genes related to the development of a disease is now easier, faster and more cost-effective.

In addition to identifying the genetic alterations that cause a tumour, it is important to determine whether these are present only in the tumour (somatic mutation) or whether they are constitutive in the patient and are, therefore, found in the blood (germline mutation). Although only 5-10% of cancer is hereditary, there are several types of germline cancer produced by known mutations. In these cases, diagnosis and prognosis are different and require genetic counselling because the tumours can also affect other family members and be transmitted to their offspring. Recent studies

show that approximately half of germline involvement cases have no significant family history of cancer.

It is important to take into account the need to re-biopsy the tumour for molecular analysis in the event of a relapse, since the pattern of genetic alterations may change from the pattern identified at the time of diagnosis. In patients with NB and other tumours it has been described that the mutational load at relapse is higher than at the time of diagnosis.

In the last decade, the situation has changed from sequencing a maximum of 96 sequences of 800 nucleotides with first generation sequencers (Sanger method) to sequencing millions of DNA fragments with second generation equipment. This novel technology is based on the amplification of immobilised DNA on a solid surface and the parallel reading of millions of sequences. The simultaneous sequencing of this immobilised DNA enables a reduction in the sequencing time and the number of reagents required, greatly reducing the cost per sequenced nucleotide. The large amount of data generated in this process has been a great challenge for bioinformaticians that have developed specific analysis programs.

Massive sequencing allows the detection of point mutations, insertions, deletions, copy number variations (CNV) and translocations. The use of NGS in the detection of somatic variants in subpopulations of tumour cells present in a low proportion in the tumour sample is also remarkable. These sub-clonal mutations, undetectable by Sanger sequencing, are responsible in some cases for relapses or explain why some tumours are refractory to front line treatments.

In oncology, gene panel sequencing has commonly been generalised. These panels contain primers or probes for a previously well-known group of genes and allow targeted sequencing for a certain cancer. A large number of commercial panels exist, although they can also be personally designed. They allow the sequencing of known mutations (hot spots), complete genes and also detect translocations and copy number variations (CNV). This means that the design is optimised, allowing the sequencing of the genes of interest with a great coverage and reading depth. This makes the detection of very low frequency variants feasible, as well as fast and reliable.

The correct interpretation of the changes or genetic variants detected is key in precision medicine. The detection of these changes consists in identifying certain differences in an individual's DNA sequence when compared with a reference DNA. However, the determination of variants alone is insufficient, and interpretation by specialists who determine their clinical and molecular implications accurately is mandatory for an optimal therapeutic approach.

In order to interpret the variants correctly, there are several databases that classify pathogenic variants and other variants detected in healthy populations or polymorphisms. In the case of unknown variants, bioinformatic programs are used to provide predictions of “in silico”

pathogenicity. In order to detect pathogenic variants and exclude polymorphisms, different parameters are considered:

– Variant location: a variant can be exonic (can cause an amino acid change in the protein or a synonym change) or intronic (may or may not affect consensus splicing sites and may result in errors in protein coding).

– Minor allele frequency (MAF): frequency at which the least common allele appears in a given population. A high MAF is related to population polymorphisms.

– Coverage: number of times a nucleotide is sequenced. If the coverage of the variant is at a lower frequency than expected because of the tumour cellularity it can be a technique artifact or a cellular subclone with a different genomic profile. The artifacts are usually repeated in several samples of the same sequencing run and can be detected.

It is also important to establish the correct categorisation of the variant: benign, probably benign, probably pathogenic, pathogenic, or of uncertain clinical significance. A variant is considered benign when it has a MAF greater than 2% in the general population or when it is described within this clinical significance in specific databases (for example: ClinVar, http://www.ncbi.nlm.nih.gov/clinvar/ or COSMIC, http://cancer.sanger.ac.uk/cosmic,). An important limitation in paediatric oncology is that most variants are not described in adult databases, and therefore, the use of “in silico” predictors is mandatory, with specific programs that predict mutations’ pathogenicity based on different factors, such as allele frequency in variant databases (ExAC http://exac.broadinstitute.org/1000G, http://www.1000 genomes.org/,) protein conformation (SIFT http://sift.jcvi.org/y Polyphen2http://genetics.bwh.harvard.edu/pph2/) or splicing (HSF, http://www.umd.be/HSF/; NNSplice, http://www.fruitfly.org/seq_tools/splice.html; Netegene 2, http://www.cbs.dtu.dk/services/NetGene2/, and SpliceView, _COPY17~webgene/wwwspliceview_ex.html). If the variant is not described in these databases and several “in silico” predictors indicate that it is a benign change, it can be interpreted as a variant of uncertain or probably benign clinical significance. A variant is considered of uncertain clinical significance when it is not described in the databases but potentially could affect gene functionality. In these variants, all the predictors mentioned above are considered and a level of pathogenicity is established by integrating other data provided by clinicians, pathologists or bioinformaticians. On the other hand, a variant is considered as pathogenic when previous studies support its pathogenicity. Finally, it is considered as probably pathogenic when it is described in these terms

in previous literature or in the consulted databases or also, when it is not directly described but several predictors suggest that it is a pathogenic change.

In **paper 3** of this publication compendium, NGS technique is used in order to study a cohort of 70 paediatric patients with sarcomas.

1.2 NEUROBLASTOMA

Neuroblastoma (NB) is the most common extracranial solid tumour in children and accounts for 6-8% of all paediatric tumours. The average age at diagnosis is 2 years, and 90% are diagnosed before the age of 5. The most frequent location is the abdomen (especially on the adrenal gland), followed by mediastinum, pelvis, cervical region and other locations. It originates from neural crest cells, so it can secrete measurable catecholamines in urine. Symptoms are usually non-specific, and prognosis is markedly variable depending on clinical, pathological and biological factors. Treatment options are established according to the risk and stage of the disease.

The main characteristics of NB are:

- Ability to mature from undifferentiated forms (NB) to mature forms (ganglioneuroma).

- Capacity to regress spontaneously in low risk patients and favourable prognosis in patients under one year of age.

- Highly aggressive behaviour in high-risk forms (older patients, amplification of MYCN or tumours with segmental chromosomal imbalances).

Classification

There are numerous factors with prognostic value in NB, although some may not have an independent value and are associated with others of greater weight. The factors accepted by all cooperation groups are age, stage of disease and amplification of the MYCN oncogene. These factors can be divided into:

- Clinical factors: age at diagnosis and tumour stage according to INRGSS.

- Pathological factors: histological category and tumour differentiation degree.

- Biological factors: amplification of MYCN, presence of chromosomal segmental aberrations and ploidy.

Clinical factors

AGE: Infants younger than 18 months, whether they have a loco-regional tumour or disseminated disease, have a more favourable prognosis than patients older than 18 months. In contrast, patients older than 5 years with metastatic disease at diagnosis have a very poor prognosis.

CLINICAL STATUS: The first staging system developed for NB was the INSS in 1993 (Table 1), whose objective was to obtain a post-surgical staging system to allow a common meeting point between various working groups in each country. However, because of the degree of tumour

resection factor, this classification could not include cases of NB without surgical treatment and also depended on surgical skill and technique. Therefore, the INRG developed the INRGSS in 2009: a pre-surgical classification that considers the presence or not of risk factors derived from imaging tests (IDRF) and diagnostic metastasis (Table 2).

Stage	Description
1	Localised tumour confined to area of origin; complete gross macroscopic resection; identifiable ipsilateral and contralateral lymph nodes negative microscopically
2A	Localised tumour with incomplete gross macroscopic resection; identifiable ipsilateral and contralateral lymph nodes negative microscopically
2B	Unilateral tumour with complete or incomplete gross macroscopic resection with positive ipsilateral regional lymph nodes; contralateral lymph nodes negative microscopically
3	Tumour infiltrating across midline +/- regional lymph node involvement; unilateral tumour with contralateral lymph node involvement; midline tumour with bilateral lymph node involvement
4	Dissemination to distant lymph nodes, bone, bone marrow, liver, or other organs (except as defined in stage 4S
4S	Localised primary tumour with dissemination to skin, liver or bone marrow (<10% tumour cells and MIBG scan -ve in marrow). Limited to infants aged < 1 year.

Table 1. Brodeur, Garrett M, et al. International criteria for diagnosis, staging, and response to treatment in patients with neuroblastoma. Journal of Clinical Oncology 6 (12) 1874-1881

Stage	Description
L1	Localised tumour not involving vital structures, as defined by list of IDRFs*, and confined to one body compartment Includes spinal invasion that dos not fulfil the criteria for an IDRF
L2	Local-regional tumour with one or more IDRFs Includes ipsilateral tumour extending across 2 contiguous body compartments eg lower chest to upper abdomen
M	Distant metastatic disease (except stage MS) Includes distant lymph nodes and contralateral involvement of 2 body compartments eg right chest and left abdomen Ascites and pleural effusion, with or without identifiable malignant cells, not included
MS	Metastatic disease in children younger than 18 months (547 days), with metastases confined to skin, liver, and/or bone marrow Bone marrow involvement consists of < 10% of nucleated cells in culture smears or biopsy MIBG –ve in bone/ bone marrow Primary tumour may be with or without IDRFs ie L1 or L2

Table 2. The International Neuroblastoma Risk Group (INRG) staging system: an INRG Task Force report. Monclair T, Brodeur GM, Ambros PF et al. J Clin Oncol 2009;27:298-303.

Pathological factors

NB cells are small, blue and rounded, as for most paediatric tumours. The two distinguishing features are the tumour differentiation degree and the presence of Schwann or stromal cells. According to these criteria, neuroblastic tumours can be:

- NB: Poor stromal. Presence of at least 50% neuroblasts in the tumour mass, grouped in nests surrounded by fibromuscular partitions. Three types are distinguished: undifferentiated, poorly differentiated and in differentiation (at least 5% of the cells show differentiation towards ganglion cells).

- Ganglioneuroblastoma: Mostly glioneuromatous component, and to a lesser extent NB. Two subtypes are distinguished: nodular (stroma-rich and stroma-poor areas) and intermixed (stroma-rich).

- Ganglioneuroma: Predominant Schwanian stroma. Two subtypes are distinguished: maturing (neuroblasts in different stages) and mature (mature stromal cells and ganglioneuroma).

Depending on the age of the patient and the pathological characteristics of the tumour, the INPC (International Neuroblastoma Pathology Classification) distinguishes between favourable and unfavourable histology, adapted from the original classification described by Shimada et al.

Biological factors

MYCN: Considered the most relevant biological factor, the MYCN oncogene is located on chromosome 2p and is amplified in 20-25% of primary NB cases. MNA NBs have a very poor outcome, being associated with an advanced stage, rapid disease progression and low survival despite aggressive treatment. Given its high prognostic impact, MYCN status is a determining factor when selecting treatment. Therefore, its detection using fluorescence in situ hybridization (FISH) is used routinely.

PLOIDY: Alterations in DNA content or chromosome number (ploidy) are a frequent finding in NB. Its prognostic value has been observed in disseminated disease or stage 4, but not in low or intermediate risk tumours because they do not have amplified MYCN. In these cases, diploid tumours show more aggressive behaviour. On the other hand, hyperploids (55% of NB) have a more favourable prognosis. However, a risk analysis performed by INRG suggests that ploidy was only prognostic in NB in infants of a few months, in which the risk group can change.

STRUCTURAL CHROMOSOMAL ALTERATIONS (SCA): Segmental chromosomal imbalances and focal aberrations are very frequent in high-stage tumours and are a prognostic marker in particular subtypes of NB. Low stage tumours typically show whole-chromosome number alterations. More specifically, tumours with only numerical aberrations have a favourable prognosis, whilst any presence of segmental aberrations indicates a poor survival outcome. SCA are considered a bad prognostic factor in some groups of low and intermediate risk NB. However, as the majority of high-risk tumours show segmental aberrations, prognostic stratification based on the absence or presence of segmental aberrations is not considered in high-risk NB SIOPEN protocols. In some groups of the intermediate-risk protocol the presence of SCA is also not considered in risk stratification.

Deletion in the long arm of chromosome 11 is the SCA that has been associated with a worse prognosis in NB. Despite being second in terms of frequency after 17q gain, the prognosis in NB with 11q deletion is usually worse. The frequency of 11q alterations may vary between 20-45%, depending on the different series and study techniques used. These particular tumours have, in addition to segmental aberration in chromosome 11: a higher observed frequency of chromosomal breakage, a higher median age at diagnosis and poor prognosis (survival rates estimated of 35% at 8 years). There is a paucity of data comparing the outcome of 11q-deleted and MNA NB cohorts.

Pretreatment risk group	INRG stage	Age (months)	Histology	MYCN	11q aberration	Ploidy
Very low	L1/2		GN; GNBi			
	L1		GNBn; NB	NA		
	MS	<18		NA	No	
Low	L2	<18	GNBn; NB	NA	No	
	L2	≥18	GNBn; NB, diff	NA	No	
	M	<18	GNBn; NB, diff	NA	No	Hyperdiploid
Intermediate	L2		GNBn; NB	NA	Yes	
	M	<18		NA		Diploid
High	L1		GNBn; NB	A		
	L2	≥18	GNBn; NB	A		
	M	<18		A		
	M	≥18				
	MS	<18		NA	Yes	
	MS	<18		A		

Table 3. Adapted International neuroblastoma risk group (INRG) pretreatment classification.

The international NB risk group (INRG) proposed a staging system in 2009 that stratified NB in risk groups according to clinical and genetic factors, such as 11q aberration in some particular stages. Other factors taken into consideration were INRG stage (L1, L2, M, 4s), age, tumour differentiation grade and histologic pattern, ploidy and MYCN status (Table 3).

However, in some subgroups of the International Society of Paediatric Oncology European NB research network (SIOPEN) (mainly intermediate and high risk cases), the presence of 11q aberrations is not taken into account when classifying the risk group, which may give rise to concern among the treating physicians, given the possibility that these patients are receiving insufficient treatment. The presence of other SCA is also not considered in some groups.

Regarding other SCA, 17q gain, 2p gain and 11q deletion have been well described in previous literature and their impact on the final outcome has been defined for some of them. A few studies mention the possible role of +1q, -1p, -3p and -4p. Aside from these seven alterations, no other imbalances associated with outcome in NB have been established or studied in-depth. Very few studies have been undertaken aiming to identify aberrations that discriminate outcomes in high-risk patients and the roles of many other segmental imbalances still remain to be defined. Moreover,

there is scarce literature focusing on numerical chromosomal alterations (NCA) and how they may have an impact on prognosis.

To study in depth the characteristics and outcome of patients with 11q-deleted NB, a retrospective study has been conducted in a Spanish cohort comparing clinical features among 11q-del, MNA and other NB patients without any of these genetic abnormalities. This study is discussed in the first paper included in this doctoral thesis.

Secondly, in order to investigate associations of less frequent segmental imbalances and the influence of certain NCA, we conducted the next study and reviewed the previous literature. Our aim was to investigate and describe NB SCA distribution according to INRGS stage, MYCN status, mortality, relapse, age and particularly to study less frequent SCA in NB samples studied by molecular karyotyping using single nucleotide polymorphism (SNP) arrays. We placed special emphasis on associations between certain SCA that appear together recurrently in the same tumour. The role of some particular NCAs have also been analysed. The results of this investigation are reported in the second manuscript.

1.3 PAEDIATRIC SARCOMAS

Paediatric sarcomas account for over 20% of all paediatric solid malignant cancers and represent 13% of all paediatric malignancies. They also contribute substantially to cancer-related mortality and morbidity. With more than over 70 histological subtypes, sarcomas can arise from a primitive mesenchymal cell from almost every tissue in the human body and are classified into two main groups: soft tissue sarcomas (STS) and bone sarcomas (BS). In children, the highest incidence rates are reported amongst rhabdomyosarcoma (RMS), osteosarcoma and Ewing's sarcoma (EWS).

1.3.1 RHABDOMYOSARCOMA

Rhabdomyosarcoma is the most common form of soft tissue sarcoma in children and adolescents and accounts for 5% of all childhood malignancies. The peak incidence occurs at around the age of 5 and it is originated from primitive mesenchymal cells involved in the development of striated muscle. Tumours can be located anywhere in the body, even in those places where striated muscle is not usually found. The most frequent locations are the genitourinary region (24%), head and neck (26%), orbit (9%) and extremities (19%).

RMS is included in the group of undifferentiated, small, round and blue cell embryonal neoplasms. The key to the diagnosis of RMS is the presence of skeletal muscle differentiation. Diagnosis requires immunochemical techniques, such as staining for myogenin, MyoD, muscle-specific actin, myoglobin and desmin. Histological classification identifies a favourable group, comprising embryonal tumours and including botryoid and spindle cell variants, and another unfavourable histology group, comprising alveolar tumours.

Signs and symptoms of presentation are related to the location of the tumour, its volume and neighbouring structures. Tumours can present as an asymptomatic mass located in soft parts, as in the extremities. Orbital locations usually develop proptosis and parameningeal locations may appear, occurring in the nasal sinus or resulting in otic obstruction, mucopurulent discharge or cranial nerve palsies.

Embryonal and alveolar subtypes have different cytogenetic alterations. Most alveolar rhabdomyosarcomas have reciprocal t(2;13) or t(1;13) chromosomal translocations. t(2;13) appears in 55% of patients with an alveolar subtype and results in a chimeric fusion gene involving the *PAX3* gene with a member of the *FOXO1* family. t(1;13) involves *PAX7* and *FOXO1* genes and is present in 20% of alveolar RMS. Genes *PAX3* and *PAX7* encode the synthesis of proteins involved in the development of the nervous and musculoskeletal system. *FOXO1* encodes a transcription factor that regulates metabolism, cell proliferation and apoptosis. Chimeric fusion proteins resulting from these translocations inhibit myogenic differentiation and apoptosis and induce proliferation and

transformation, thus acting as oncogenic proteins. The translocation of these genes is usually studied in the laboratory by FISH or by reverse transcription polymerase chain reaction (RT-PCR).

In addition to these genetic abnormalities, there are other common alterations in RMS such as whole chromosome gains and losses and genome amplifications. These imbalances and losses occur equally in embryonal and alveolar tumours. In embryonal subtypes, the involvement of *P53* in Li-Fraumeni syndrome is well known. Other genes that play a role in embryonal RMS are *RAS, FGFR4* and *FBXW7*.

1.3.2 OSTEOSARCOMA

Osteosarcoma is the most common primary malignant tumour of the bone in childhood and is responsible of 4% of all childhood malignancies. The highest incidence occurs between the age of 10 and 14, and the most common location is the knee (distal femur and proximal tibia). Pain and swelling are the predominant initial symptoms.

Osteosarcoma cells have the ability to produce osteoid or immature bone. The critical factor in determining osteoid malignancy is the degree of anaplasia of the surrounding osteoblasts and how the osteoid matrix is produced directly by the sarcomatous cells. Virtually all osteosarcomas are high-grade and, pathologically, there are several variants; the most common is the osteoblastic variant, which is present in more than half of all cases. Other variants include chondroblastic, fibroblastic, telangiectatic and small cell osteosarcoma.

Since it was accepted that osteosarcoma is a systemic disease and that pulmonary micrometastasis were present at diagnosis, chemotherapy was introduced as part of the treatment. This increased event-free survival from 20% to 75% today in localised tumours. Around 15-20% of the patients present detectable metastases at diagnosis, mainly in the lungs. Prognosis in these patients is extremely poor.

Osteosarcoma is usually resistant to radiotherapy and this makes surgery extremely important in its treatment, with the aim of achieving a complete resection of all the areas where the disease is present. Chemotherapy is also used, mainly based on high-dose methotrexate, anthracyclines, alkylating agents and cisplatin. Other biological therapies also play a role. For example, mifamurtide is a specific ligand for the nucleotide-binding oligomerization domain-containing protein (NOD) receptor found on monocytes, dendritic cells and macrophages. It acts as a potent activator of these cells and has been demonstrated to improve overall survival from 70% to 78% in cases of localised osteosarcoma (Intergroup Trial INT-0133).

About one-third of the patients relapse, usually with metastasis in the lung, and survival in relapse is very poor. Surgical rescue is essential when it is possible to perform. Also, promising new drugs,

such as lenvatinib, are being developed and are currently used in combination with standard chemotherapy in relapsed cases in clinical trials.

A wide variety of both numerical and structural cytogenetic alterations have been found in osteosarcoma. There is a complex relationship between tumour development and progression and alterations in tumour suppressor genes. *TP53, RB1* and *CDKN2A* genes have been described to play a role in this neoplasm. It is known that the overexpression of P-glycoprotein confers a greater risk of relapse and, regarding this issue, the Spanish sarcoma research group (GEIS) has designed a study to determine the best treatment of localised osteosarcoma according to the presence or lack of expression of P-glycoprotein.

1.3.3 EWING SARCOMA

EWS is the second most common primary bone tumour in children and represents about 1.5% of all neoplasms under 15 years of age. It is a small, round cell tumour of neuroectodermal origin with evident neurogenic differentiation. The average age at presentation is 14 years and it affects long and flat bones as well as the axial skeleton. Pain is the most frequent symptom at diagnosis, generally in the tumoural area, but it can radiate and cause nerve compression. There may be swelling, functional impotence, fever, paresthesias and pathological fractures. Dissemination occurs by hematogenous spread and the most frequent locations of metastasis are the lungs (38%), bones (31%) and bone marrow (11%).

Metastatic disease at the time of diagnosis is the factor that confers the worst prognosis. Other important variables are an initial tumour volume greater than 200 ml, axial location, and poor response to cytostatics. Chemotherapy with alkylating agents, anthracyclines, etoposide and vincristine is always used for treatment. It is important to perform a good local treatment with surgical resection and/or radiotherapy. Currently, the European trial EuroEwing2012 is evaluating the possible role of bisphosphonates in EWS treatment.

Regarding its molecular genetics, a chromosomal translocation has been identified involving chromosomes 11 and 22 affecting the EWS gene located in the long arm of chromosome 22 and the FLI-1 gene located in the long arm of chromosome 11. The genetic profile of EWS is dominated by this driving reciprocal chimeric translocation between EWSR1 and a variety of ETS partner transcription factors (FLI-1, ERG, ETV1, ETV4, FEV). These gene fusions act as an aberrant transcription factor that determines an epigenetic dysregulation that explains the biology of the tumours. Table 4 shows the molecular fusions that are identified in this tumour.

Translocation	Fusion	Frequency in ESFT
t(11;22)(q24;q12)	EWSR1–FLI1	~85%–90% of cases
t(21;22)(q22;q12)	EWSR1–ERG	~10% of cases
t(7;22)(p22;q12)	EWSR1–ETV1	Rare
t(17;22)(q12;q12)	EWSR1–ETV4	Rare
t(2;22)(q35;q12)	EWSR1–FEV	Rare
t(16;21)(p11;q22)	FUS–ERG	Rare
t(2;16)(q35;p11)	FUS–FEV	Rare

Table 4. Current TET–ETS fusion pairings identified in Ewing sarcoma. (ESFT: Ewing sarcoma family tumours). Precision medicine approaches for the management of Ewing sarcoma: current perspectives. Cictoria T Rizk, et al. Pharmacogenomics and Personalized Medicine 2019:12 9–14

1.3.4 SUMMARY SARCOMAS

Although each subtype has a different phenotype and molecular genetic profile, sarcomas are classified into two groups: a *genetically complex* group with a high mutational burden and complex karyotype and a *genetically simple* group, that contain a single and disease-specific translocation, amplification or mutation with a silent genomic background. Most paediatric sarcomas are included in the second group as they are mostly characterised by chromosomal translocations which lead to gene rearrangements acting as drivers that are critical for sarcomagenesis.

Paediatric RMS protocols currently classify this tumour according to molecular criteria, displacing the classical pathology classification, and they are classified as fusion positive (*PAX/FOXO1* translocation) and fusion negative RMS. The genetic profile of EWS is dominated by the driving reciprocal chimeric translocation between *EWSR1* and a variety of *ETS* partner transcription factors. These gene fusions act as an aberrant transcription factor that determines an epigenetic dysregulation that explains the tumour biology. On the other hand, osteosarcoma shows an extremely complex and unstable genome without a remarkable repetitive pattern. Sarcomas involving translocations can be detected by FISH and RT-PCR. Translocations are used by clinicians mostly as diagnostic markers. However, the resulting chimeric proteins of these translocations are not easily druggable and this hinders the development of inhibitors.

Both STS and BS display a highly aggressive behaviour. Significant advances have been made in improving outcomes in localised tumours since the addition of systemic chemotherapy and the refinement of local control decades ago, and approximately 2 out of 3 patients with localised stages survive. However, metastatic and relapsed sarcomas still have very poor survival rates. Scarce improvement has been observed in this area, despite an increasing insight in cancer biology.

At the same time, in recent years, the diagnosis of paediatric cancer, including sarcomas, has experienced an increase in its demands in terms of precision and quality criteria. New techniques have been introduced that complement pathological diagnosis, including immunochemistry, FISH, RT-PCR and NGS. In tertiary centres, these demands have been gradually assumed by clinicians, pathologists, geneticists and molecular biologists and precision medicine programs have been developed in order to expand our knowledge of tumour biology and defeat cancer with more precise information.

In the third paper we present the results on paediatric sarcomas from the Precision Medicine program for children and adolescents with solid tumours in relapse/progression carried out at a national reference centre for paediatric sarcomas. It is the first cohort published on paediatric sarcomas using NGS in Spain. This program has received samples from collaborative centres, providing a national perspective. Since September 2019, these studies are routinely carried out at diagnosis for every case of paediatric sarcoma.

1.4 PAEDIATRIC TUMOUR BOARDS

Multidisciplinary care is the hallmark of high-quality cancer management. This statement has been supported over the last decades where individual opinions have been displaced by collective and multidisciplinary decisions in approaching a patient with a complex oncologic disease. Although childhood cancer is a rare disease, it is the first cause of non-accidental death in childhood and sharing of expertise is paramount in this field. Several studies have shown that tumour boards lead to changes in diagnosis and staging of cancer patients, affect management decisions, and increase quality of care. Therefore, they should be an integral part of adult and childhood cancer patient care around the world.

A **paediatric tumour board** comprises a multidisciplinary team of experts including paediatric oncologists, radiologists, surgeons, radiotherapists, pathologists, and other disciplines as relevant for the respective cancer case to discuss patients' clinical cases where the diagnosis and treatment plan are complex. The patients' clinical management following a discussion in a tumour board takes into account the opinion of several experts participating in a particular meeting.

A **paediatric molecular tumour board** (PMBT) is a special type of tumour board where results extracted from NGS are discussed between member of several disciplines in order to deliver personalized treatment recommendations. When genomic and histopathological studies were completed, all members discuss the results and finally a report is transferred to the corresponding medical doctor. Once these recommendations are done, paediatric oncologists must find possible ways to access certain drugs, in the first place by means of clinical trials or, for example, as a compassionate use basis. A PMTB is usually composed by paediatric oncologists, geneticists, pharmacologists, pathologists, bioinformatics and molecular biology specialists. At Hospital U i P La Fe, the PMTB was created in November 2014 and in this forum, all the actionable targets and results in **manuscript 3** were discussed.

A **paediatric virtual tumour board** is a special type of multidisciplinary meeting based on videoconference systems that can involve experts from different centres. For practical reasons, these tumour board meetings need to be "virtual" using videoconference systems to allow regular meetings and discussions between experts in different countries. The virtual tumour board can, therefore, convene regularly and rapidly to discuss urgent cases and they avoid large gathering of physicians in the same physical room. During this year, because of the pandemic caused by COVID19 there has been an urgent need for the development of videoconferencing systems to implement virtual communication between health care professionals. However, there are enormous differences between European countries regarding facilities to develop paediatric virtual tumour boards. As a first step towards a European Paediatric Oncology Virtual Tumour Board Network, in the **fourth manuscript** we aimed to identify already existing paediatric tumour boards in Europe in

order to investigate the kind of technologies and logistics that are in place in the different countries and to explore current differences between European regions. This task was carried out within a working package of the ExPO-r-Net project (European Expert Paediatric Oncology Reference Network for Diagnostics and Treatment).

2. HYPOTHESIS AND OBJECTIVES

HYPOTHESIS

Paediatric tumour cytogenetic and sequencing studies generate a greater biological knowledge of these malignancies which enables a more adjusted risk stratification and the design of personalised therapeutic strategies.

OBJECTIVES

Main objective:

Describe the use of genomic studies in neuroblastoma and paediatric sarcomas from a translational point of view using cytogenetic and next-generation sequencing methods to obtain personalised diagnosis that can able better risk stratification and the incorporation of new therapeutic agents.

Specific objectives:

- Analyse the importance of the deletion of the long arm of chromosome 11 as a prognostic marker in neuroblastoma, especially in localised stages and 4s.

- Study the distribution of segmental chromosomal alterations according to already known risk factors in neuroblastoma.

- Identify possible associations of segmental chromosomal alterations in neuroblastoma and the role of certain numerical alterations.

- Describe the most frequent mutations detected by next-generation sequencing, their role in prognosis and the detection of possible therapeutic targets in a multi-centric cohort of paediatric sarcomas.

- Implement next-generation sequencing techniques for the study of paediatric sarcomas at first diagnosis in the Unit of Paediatric Oncology and at Hospital U i P La Fe.

- Identify paediatric oncology tumour boards in Europe and investigate the kind of technologies and logistics that are in place in different countries to explore current differences between regions.

3. MANUSCRIPT COMPENDIUM (METHODOLOGY AND RESULTS)

SUMMARY

MANUSCRIPT 1

CLINICAL FEATURES OF NEUROBLASTOMA WITH 11Q DELETION: AN INCREASE IN RELAPSE PROBABILITIES IN LOCALIZED AND 4S STAGES

Scientific Reports (Nature Research) (2019)

MANUSCRIPT 2

DISTRIBUTION OF SEGMENTAL CHROMOSOMAL ALTERATIONS IN NEUROBLASTOMA

Clinical & Translational Oncology (2020)

MANUSCRIPT 3

NEXT-GENERATION SEQUENCING IDENTIFIES POTENTIAL ACTIONABLE TARGETS IN PAEDIATRIC SARCOMAS

Under review in International Journal of Oncology (2020)

MANUSCRIPT 4

SURVEY ON PAEDIATRIC TUMOUR BOARDS IN EUROPE: CURRENT SITUATION AND RESULTS FROM THE EXPO-R-NET PROJECT

Clinical & Translational Oncology (2018)

MANUSCRIPT 1

CLINICAL FEATURES OF NEUROBLASTOMA WITH 11Q DELETION: AN INCREASE IN RELAPSE PROBABILITIES IN LOCALIZED AND 4S STAGES

Scientific Reports (Nature Research) (2019)

*2-year impact factor: 3.998
*5-year impact factor: 4.576
*Immediacy index: 0.527
*Eigenfactor® score: 1.23118
*Article influence score: 1.263
*Quartile: 1
* 7th most-cited journal in the world, with more than 350,000 citations in 2019

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MANUSCRIPT 1

CLINICAL FEATURES OF NEUROBLASTOMA WITH 11Q DELETION: AN INCREASE IN RELAPSE PROBABILITIES IN LOCALIZED AND 4S STAGES

INTRODUCTION

Neuroblastoma (NB) is a complex paediatric tumour and the most common extracranial solid malignancy in childhood. Clinical manifestations may range from aggressive growth despite intensive treatment, to cases in which spontaneous regression is reported. In the last decades, it has been observed that particular tumoural genomic changes correlate with its behaviour and outcome. It is well known that an *MYCN* amplification (MNA) is associated with aggressive tumours and a dismal prognosis. Other genomic features besides MNA, such as 11q deletion (11q-del) and 17q gain, represent segmental aberrations that take place in a remarkable number of cases.

Segmental chromosomal aberrations (SCA) are considered a bad or negative prognostic factor. The frequency of 11q alterations may vary between 20-45%, depending on the different series and study techniques used. These particular tumours have, in addition to segmental aberration in chromosome 11: a higher observed frequency of chromosomal breakage, a higher median age at diagnosis, and poor prognosis (survival rates estimated of 35% at 8 years). There is a poor amount of data comparing the outcome of 11q-deleted and MNA NB cohorts. The international NB risk group (INRG) proposed a staging system in 2009 that stratified NB in risk groups according to clinical and genetic factors, such as 11q aberration in some particular stages. Other factors taken into consideration were INRG stage (L1, L2, M, 4s), age, tumour differentiation grade and histologic pattern, ploidy and *MYCN* status.

However, in some subgroups of the International Society of Paediatric Oncology European neuroblastoma research network (SIOPEN) (mainly intermediate and high-risk cases), the presence of 11q aberrations is not taken into account when classifying the risk group, which may give rise to concern among the treating physicians, given the possibility that these patients are receiving insufficient treatment. To study in depth the characteristics and outcome of patients with 11q-deleted NB, a retrospective study has been conducted in a Spanish cohort comparing clinical features among 11q-del, MNA and other NB without any of these genetic abnormalities.

Methods

The aim in this study was to study and describe the 11q-del NB cases diagnosed throughout an 8-year period. This retrospective multi-centric study consists of 399 NB patients from 29 Spanish cooperating hospitals. Data collected from patients included age at diagnosis, primary tumour location, pathology, stage, MYCN status, 11q-del status, relapse, time until relapse, time from relapse to death, cause of death, follow up and current state of the patient. Staging and risk stratification were established according to INSS and INRGSS classifications. Tumour samples from all patients were referred to the Spanish reference centers for pathology and molecular biology NB studies. Samples were centrally reviewed and classified according to the International NB Pathology Classification (INPC)

Patients were registered in the file of the Spanish Society of Paediatric Hematology and Oncology (SEHOP) NB group database, and included for treatment mainly in SIOPEN trials according to period and clinical characteristics (INES, LINES, LNESG-I, EUNS and HRNBL1). In stage M patients, response to induction treatment chemotherapy was evaluated according to SIOPEN guidelines (High Risk NB Study 1.7 of SIOP-Europe)11. Informed consent for study participation, samples and data management were obtained in all cases from the patients 'parents, and all the patients were treated following SIOPEN-approved protocols.

Chromosome 11q-del was defined as a missing (deleted) copy of genetic material on the long arm (q) of chromosome 11. 11q23 was the most frequent region found to be deleted. Biological studies included MYCN status and 1p using FISH technique, and 11q status was initially studied by multiplex ligation-dependent probe amplification technique (MLPA), and from 2012 onwards by single nucleotide polymorphism (SNP) array (Affymetrix and Illumina) according to the European NB Quality Assessment (ENQUA) guidelines (Ambros IM et al.)

As recommended by the ENQUA guidelines, amplification of MYCN was defined as a higher than a 4-fold increase of MYCN signals in relation to the number of reference chromosome 2 signals. The increase or the additional copies (up to the 4-fold) were defined as MYCN gain.

The data used was summarized using mean, standard deviation (SD) and median (1st, 3rd Q) in the case of continuous variables, whereas absolute and relative frequencies were used for categorical variables. To assess the independent association of 11q-del with survival, Cox proportional hazard regression models were adjusted including MYCN status, stage and age at diagnosis. For event free survival (EFS), time to event was defined as the time from diagnosis until the time of first occurrence of relapse, progression or death. For OS, time to event was defined as time until death or until last contact if the patient was alive. Kaplan-Meier curves were compared with log-rank test. Age difference between the presence and absence of 11q-del was compared using Wilcoxon Rank

Sum test. 95% confidence Intervals of the effects were provided. Proportions were evaluated using Chi-square test. P-values below 0.05 were considered statistically significant. All analyses and graphs were performed using R software (version 3.5.0) with clickR (0.3.64) and survival (2.41-3) packages.

Results

A total of 399 children with NB were registered during this 8-year study period (2006 to 2013). Tumours without initial 11q determination were excluded from analysis (42 patients). The remaining 357 NB were tested for 11q-del. 60/357 patients were found to have this alteration (17%). The presence of 11q-del and MNA in the same tumour was almost mutually excluding (only 3 cases showed both abnormalities).

The vast majority of the cases had abdominal location (79%), followed by the thoracic area (10%). A smaller proportion of tumours was found in the neck and in the pelvis, and there was one retro-orbital case. Concerning tumour stage, 177 patients (50%) had a localized disease (stage 1, 2 or 3), 145 patients were diagnosed as stage 4 (40%) and 35 patients had stage 4s NB (10%). Median age at diagnosis was 1,37 years.

According to the INPC system (International NB Pathology Classification), 202 samples were classified as poorly differentiated NB (57%) leading this to be the most frequent diagnosis, followed by undifferentiated NB (14%). MNA was found in 63 patients (18% of the cases), whilst 38 tumours had MYCN gain (10%).

From the whole cohort, 105 patients died (30%) and 252 are alive with a median follow up of 5,7 years. The most important cause of death was tumour progression in 90% of the cases. Regarding the survivors, 222 patients remain free of disease (62%) and 30 patients are alive on treatment after relapse (8%). **Table 1** summarizes the clinical and biological characteristics of the patients.

Table 1. General description of the studied population.

Variable	n = 357
Age at diagnosis (years)	mean: 2.51 median: 1.37

Variable	n = 357
Location	
Abdominal	281 (79%)
Neck	8 (2%)
Neck-thoracic	6 (1.5%)
Pelvic	6 (1.5%)
Retro-orbital	1 (0.3%)
Thoracic	35 (10%)
Thoracic-abdominal	20 (6%)
11q-del	
No	297 (83%)
Yes	60 (17%)
MNA	
Amplified	63 (18%)
Gained	38 (10%)
Non amplified	256 (72%)
Stage	
1	85 (24%)
2	25 (7%)
3	67 (19%)
4	145 (40%)
4s	35 (10%)
Pathology	
GanglioNB	41 (11%)

Variable	n = 357
Non specified GanglioNB	6 (2%)
Differentiating NB	17 (5%)
Undifferentiated NB	52 (14%)
Poorly differentiated NB	202 (57%)
Anaplastic NB	2 (1%)
Non specified NB	37 (10%)
Current state	
Dead	105 (30%)
Alive with disease	30 (8%)
Alive without disease	222 (62%)
Cause of death	
Disease progression	94 (90%)
Other causes	11 (10%)

Initially, 11q status was considered as an unique factor and 11q-del population (n=60) was compared to the no-11q-del cases (n=297) including all age groups and stages. Overall survival (OS) was lower in 11q-del patients (60% at 5 years and 52% at 8 years) than in the cases lacking 11q-del (76% at 5 years and 72% at 8 years) p=0.014. Differences were noticeable when considering EFS. In 11q-del patients, EFS was 35% at 5 years and 32% at 8 years compared to the data in no-11q-del cases: 75% at 5 years and 73% at 8 years ($p<0.001$) with a median follow up of 5.7 years. **(Figure 1).**

Separating the data by stages (4, 4s or localized), it was observed that localized and 4s stages 11q-del patients had a poor EFS (approximately 45% and 40% respectively) **(Figure 2).** EFS was similar in 11q-del localized or 4s stages to no-11q-del stage 4 cases. This fact is very representative and further confirms that 11q-del status plays an important role in NB relapse. Statistically significant differences were observed when comparing 11q-del and non-11q-del EFS in localized NB and

stage 4s (p=0.001). Differences in stage 4 EFS were observed according to 11q-del status although this result was not statistically significant (p=0.32).

Figure 1. Event-free survival of 11q-del compared to no-11q-del neuroblastoma.

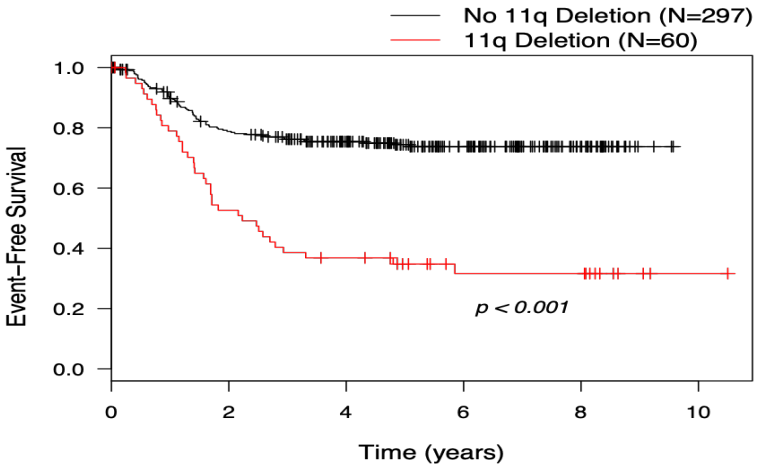
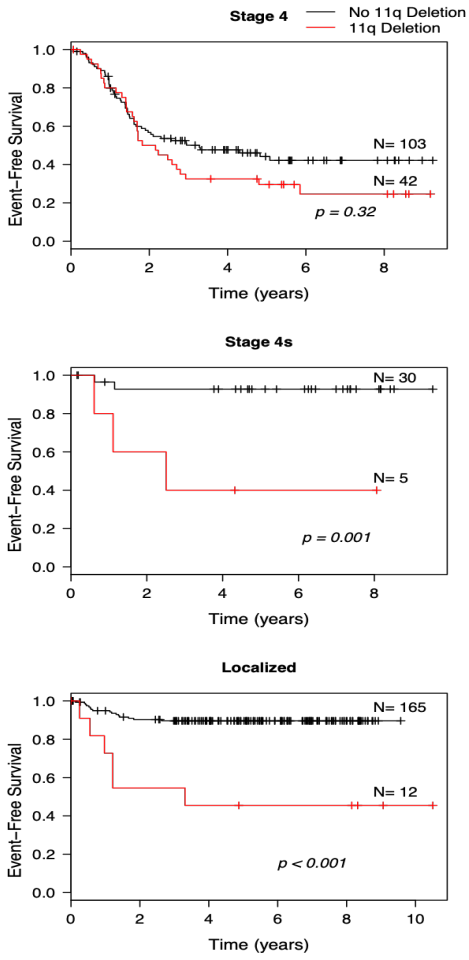
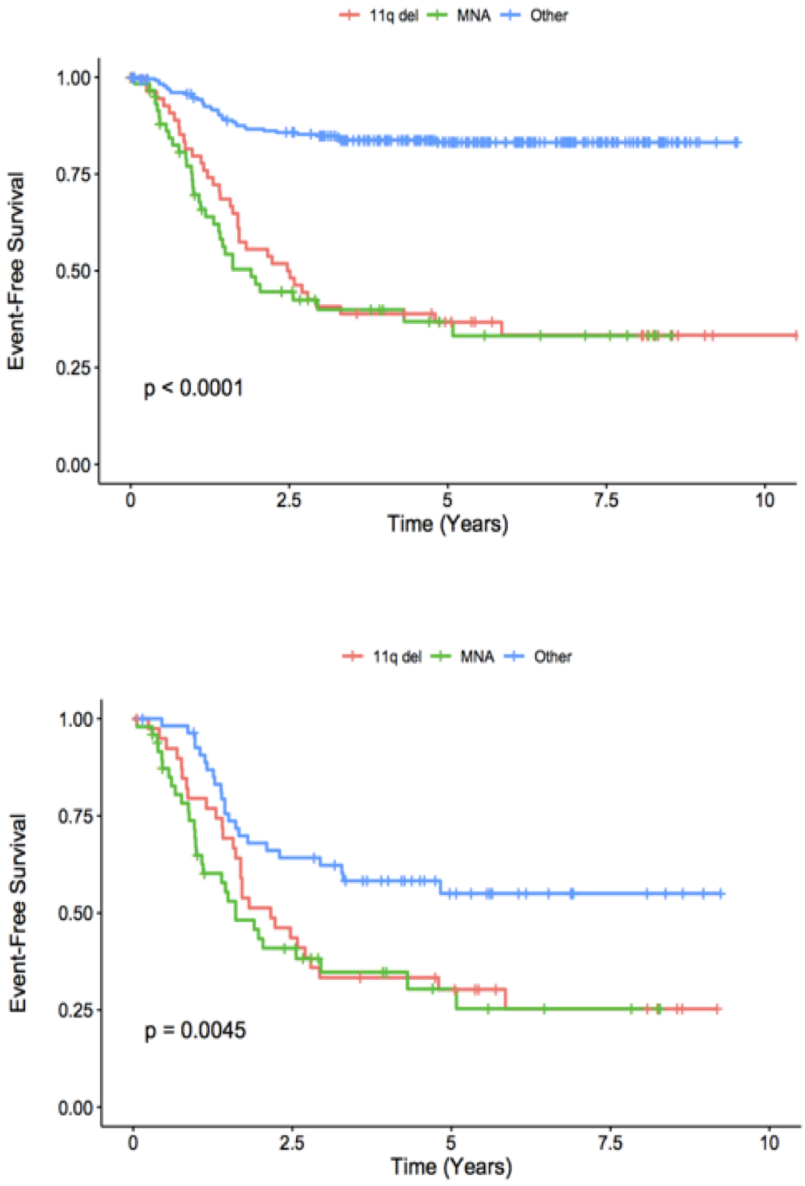


Figure 2. Event-free survival analysis by stages (stage 4, 4s and localized) comparing 11q-del vs no-11qdel NB cases.



Given the importance of MNA as an established prognostic factor associated with adverse outcome in NB, a comparison between EFS in MNA, 11q-del and the remaining NB cases without these alterations was made using Kaplan-Meier curves. Statistically significant results were found and no differences between MNA and 11q- del outcomes were observed (**Figure 3**; above: Entire cohort; below: Stage 4 NB).

Figure 3. Kaplan-Meier curve comparing EFS between 11q-del, MNA and the remaining cases of NB. (Above: Entire cohort. Below: Stage 4 NB).



In the study, three cohorts were compared: 57 patients with 11q-del, 60 patients with MNA, and other 237 cases without both alterations (other population). The only 3 cases containing both abnormalities were excluded. The general characteristics of these subgroups are summarized in **Table 2** and are the following:

AGE

Median age at diagnosis was found to be higher in the 11q cases (3.29 years at diagnosis) when compared with MNA cohort (2.02 years) and the remaining cases (0.92 years at diagnosis).

STAGE

When comparing tumour stage, 42 cases (73%) of 11q-del patients were classified as stage 4 at diagnosis. This figure is similar to the number of MNA stage 4 cases (80%) while only 55 cases out of 237 (23%) of the other NB cases were classified as stage 4.

MYCN GAIN

It was observed that out of sixty 11q-deleted NB, 20 had also MYCN gain (33.3%) whereas only 18 of the other 237 cases (without 11q-del) had MYCN gain (7.6%). These results are statistically significant ($p<0.001$) and show that despite 11q-del and MNA being almost mutually excluding, 11q-del is associated with MYCN gain. Most of the patients with both these aberrations (11q-del and MYCN gain) presented high-risk features, 16/20 were diagnosed as stage 4 and 15/20 were older than 18 months.

Table 2. Description of the three neuroblastoma subgroups: 11q-del, MNA and the remaining cases.

	11q del n = 57	**MNA n = 60**	**Other = 237**
Median age at diagnosis (years)	3.29	2.02	0.92
Stage			
1	3 (5%)	1 (2%)	80 (34%)
2	0 (0%)	0 (0%)	24 (10%)

	11q del n = 57	MNA n = 60	Other = 237
3	7 (13%)	9 (15%)	51 (22%)
4	42 (73%)	48 (80%)	55 (23%)
4s	5 (9%)	2 (3%)	27 (11%)
Number of relapses			
0	21 (37%)	26 (43%)	198 (84%)
1	26 (46%)	33 (55%)	31 (13%)
2 or more	10 (17%)	1 (2%)	8 (3%)
Death			
No	32 (56%)	18 (30%)	203 (85%)
Yes	25 (44%)	42 (70%)	34 (15%)

RELAPSE

A small number of tumour relapses were reported in the cohort without MNA or 11q alterations (relapses in 16% of the cases). On the other hand, more MNA or 11q-del NB recurred. 55% of the MNA tumours had one relapse and one case had two recurrences. Among the 11q-del cases, 36/57 patients relapsed (63%). 26 patients relapsed only once (46%) and ten cases with sequential relapses were detected (6 patients had two relapses, 2 patients had three relapses and 2 patients recurred four times) **(Table 2).**

In the relapse model (Cox regression), the population with higher relapse risk was stage 4s with 11q-del, with 7.581 HR (hazard ratio). MYCN homogeneous amplification was also associated with a higher relapse risk (HR 2.718). Localized tumours (stage 1,2,3) with 11q-del also have a higher risk (HR 3.312). Notably, 4s population and localized NB without 11q-del had the lowest HR (0.245 in both cases) showing how aberrations in 11q dramatically increase relapse rate in low risk NB. These results are statistically significant. **Table 3** summarizes relapse data in these subgroups of patients.

The clinical course of 11q-del NB patients was usually an insidious process with multiple relapses and longer courses with a poor final outcome, based on time until relapse, time from relapse to death and time from diagnosis to death. Patients with 11q-del relapsed later than the other cases.

Median time from diagnosis to relapse was 1.42 years, compared with 1.08 in the MNA cohort and 1.28 in the other NB relapsed patients. Relapsed 11q-del patients also had a longer median time from relapse to death (1.53 years) than MNA cases (0.49 years) and than the other NB cases (0.98 years). Finally, median time from diagnosis to death was longer in 11q-del patients (2.88 years) compared to MNA NBs (1.37 years) and the remaining patients (2.09 years).

Table 3. Relapse risk in neuroblastoma subgroups (Cox regression relapse model).

Relapse	HR	Lower 95%	Upper 95%	P-value
Stage 4s	0.245	0.056	1.065	0.061
Localized	0.245	0.133	0.449	<0.001
11q-del	1.847	1.087	3.138	0.023
MYCN gain	1	0.573	1.745	0.999
MYCN amplification	2.718	1.672	4.421	<0.001
Age < 18 months	0.405	0.252	0.653	<0.001
Stage 4s with 11q del	7.581	1.175	48.924	0.033
Localized with 11q del	3.312	1.128	9.727	0.029

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Statistical differences were observed in OS when comparing patients in the three groups. Mortality is higher in the MNA NB with 70% of deaths (42/60 compared with 44% in the 11q-del tumours (25/57) and 15% in the remaining patients (34/237). MNA patients had a HR of 3.673 ($p<0.001$). Therefore, by using Cox regression we conclude that MNA amplification was associated with a lower survival, a fact that has been widely identified **(Table 4).**

The most important differences in 11q-del NB were observed in final outcome in localized and stage 4s tumours. Localized stages with 11q-del showed higher risk of death (HR 3.119, p=0.082). Moreover, stage 4s patients with 11q-del presented a HR of 4.398, p=0.12). These results were not statistically significant, probably due to the low number of 11q-del localized and stage 4s cases. Even so, given the notoriousness of these values, they should be taken into account.

Table 4. Death risk in neuroblastoma subgroups (Cox regression survival model).

Death risk	HR	Lower 95%	Upper 95%	P-value
Stage 4s	0.391	0.112	1.363	0.14
Localized	0.185	0.093	0.37	<0.001
11q del	1.145	0.648	2.02	0.641
MYCN gain	1.251	0.689	2.273	0.462
MYCN amplification	3.673	2.243	6.015	<0.001
Age < 18 months	0.352	0.209	0.594	<0.001
Stage 4s with 11q del	4.398	0.681	28.409	0.12
Localized with 11q del	3.119	0.866	11.227	0.082

Discussion

During the last years many international efforts have been done trying to investigate why some particular genetic changes lead to more aggressive cases of NB. MNA is still the most relevant biological prognostic factor, especially in infants. However, in the last years, SCA including 11q alterations are also taken into account and affect risk stratification in some subgroups of NB. This is

one of the largest cohorts (n=357) that shows and further confirms worst outcomes in NB containing 11q-del. Patients have been studied homogeneously without previous selection and have a median follow up of 5.7 years. As it has been described, 11q-del NB is related to older age at diagnosis ($p<0.001$) and is also associated with more advanced stages of NB ($p<0.001$). Co-presence of 11q-del and MNA is extremely rare. Using Cox regression, we conclude that 11q-del as well as MNA is associated with a higher risk of relapse. The comparison between EFS in MNA, 11q-del and the remaining cases with Kaplan-Meier curves (Figure 3) further confirms the clinical value of 11q-del in NB, being equivalent to that of MNA.

In the cohort studied previously by Schleiermacher et al., it was observed that in 147 NB without MNA, a SCA profile was the strongest independent prognosis factor. In this cohort, 76% of the cases with SCA showed 11q-del. Caren et al., reported that median age at diagnosis was significantly higher in the 11q-del group compared to numeric chromosomal aberrations (NCA), MNA and 17q gain groups (42 months vs 3, 21 and 21 months, respectively). Prognosis was found to be poor in MNA and 11q-del groups (8 years OS 35%), but the median survival time after diagnosis was longer in 11q-del NB (40 vs 16 months). These observations are very similar to our findings as 11q-del NB patients in our study have shown higher median time to relapse, higher median time from relapse to death and higher median time from diagnosis to death.

Concerning age, similar conclusions have been reported by previous groups highlighting that 11q alteration is detected mostly in older patients. Cetinkaya C et al. reported a cohort of NB where median age at diagnosis was extremely different according to MNA or 11q-del. The results were 58.5 months in 11q-del NB vs 18 months in the MNA group. Analysis of the INRG database has also shown that in a cohort of younger patients (< 18 months) with stage 3 NB, the only independently associated factor with poor survival and EFS has been 11q-del status. These facts are completely concordant with our results. Adolescents with NB represent less than 5% of the cases and in most series, they are characterized by a high prevalence of SCA and a low incidence of MNA. The prevalence of 11q-del in this group is between 30 to 60%. ALK and ATRX mutations are more frequent in older patients too and ATRX mutated NB showed a higher number of SCA including 11q-del with a very poor outcome.

We also report that 11q-del is associated with MYCN gain. The proportion of MYCN gain in the 11q-del cohort is much higher than in other cases (33.3% versus 6.1%). Our data also confirms the association between 11q aberration and high-risk disease, specifically in the absence of MNA. MYCN-gain most likely occurs due to a larger gain of copies of the 2p chromosomal arm, rather than a focal gain, meaning that additional genes at 2p including ALK could contribute to NB pathogenesis and high-risk disease. Furthermore, recent findings have provided a potential link between this

inverse association between 11q aberration and MNA. More specifically, evidence has suggested that dysregulation of the microRNA let-7 plays a central role in the pathogenesis of NB and that either MNA or 11q loss are able to disrupt let-7, but the biological significance of this relationship is still waiting to be confirmed.

In the subset of patients with “good prognosis” (localized and 4s stages), 11q-del frequency is rather low. However, when these cases have 11q-del, prognosis has shown to be worse. Hence, some authors support that patients in low and intermediate risk groups with SCA such as 11q-del could benefit of intensified treatments. In the COG protocols, 11q-del is added to other factors (MYCN status and ploidy) in patients with localized tumours younger than 18 months. In SIOPEN studies, chromosomal segmentation aberrations are considered including 11q-del but these are not considered in all tumour stages. Currently, it is well recognized that 11q-del NB constitutes a distinct subgroup of aggressive malignancies, but with different features compared to MNA and therefore, the results show that some action points regarding treatment need to be further assessed in this field. A high frequency of chromosomal breakage, suggestive of a chromosomal instability, is one of the main features of 11q-del NB that has been previously reported. This shows that certain genes in 11q could be involved in the chromosomal instability phenotype, by haplo-insufficiency or inactivation of the second allele by mutation or epigenetic modification. Recurrent patterns of SCA or NCA suggest that NB is a cancer driven by copy number rather than by particular mutations. The fact that 11q is never lost on both chromosomes suggests that important genes are present on the remaining 11q copy, but that the second hit needed would be caused by another localized mutation or methylation event.

So far, the mechanism by which hemizygous deletion in 11q leads to high risk features is unknown, and therapies targeting this alteration have not been totally developed. Some of the genes located in this chromosomal area have been studied and seem to have important relation with the adverse prognosis that the ablation produces. Particularly, TSLC1 (CADM1; cell adhesion molecule 1) located in 11q23.3 has an important role as tumour suppressor gene in NB and has also been related in oncogenesis. Recently, while looking for new possibilities and alternative therapies in patients with relapsed 11q-del tumours, ATM hemizygosis (11q22.3) in the presence of functional TP53 (17p13.1) has shown in vitro and in vivo response to PARP inhibitors.

The gene ATM is within this chromosomic locus and has the role of repairing DNA damage by homologous recombination. Efficient repair of damaged DNA strands helps maintain the stability of the cell's genetic information. H2AFX (H2A Histone Family Member X) also located in 11q23.3 is a member of the nucleosome structure and thereby plays an important role in transcription regulation, chromosomal stability, DNA repair and replication. In fact, ATM protein phosphorylates H2AFX

during response to double-strand breaks (DSB). Therefore, the loss of H2AFX also suggests a potential utility of PARP inhibitors and could be related in the described responses.

Poly ADP-ribose polymerase (PARP) is a protein that signals DNA damage and facilitates DNA repair. PARP catalyzes the addition of ADP-ribose to DNA, histones, topoisomerases and helicases and has a critical function in cellular replication, transcription, differentiation, gene regulation, protein degradation and spindle maintenance. Inhibition of PARP results in persistent single strand DNA breaks leading to stalled replication forks and double strand DNA breaks. PARP inhibition produces DNA damage that leads to cell cycle arrest and apoptosis. PARP inhibitors are being evaluated in cancers with defective DNA repair mechanisms alone or in combination with cytotoxic therapy or radiation. The addition of PARPi to second line chemotherapy in 11q-del NB patients could be an attractive combination for these patients that is currently under exploration.

In the basis of these facts and other similar hypotheses of previous revisions, we think that within the international groups, new frontline strategies are required to be developed in order to improve the outcome of NB patients with 11q-del.

MANUSCRIPT 2

DISTRIBUTION OF SEGMENTAL CHROMOSOMAL ALTERATIONS IN NEUROBLASTOMA

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MANUSCRIPT 2

DISTRIBUTION OF SEGMENTAL CHROMOSOMAL ALTERATIONS IN NEUROBLASTOMA

INTRODUCTION

Neuroblastoma (NB) is a complex, heterogeneous tumour and the most common extracranial solid malignancy in childhood. It originates in the sympathetic nervous system and is responsible of approximately 15% of childhood cancer deaths. Clinical manifestations vary from aggressive growth despite intensive treatment to some cases of spontaneous regression. Age and stage are prognostic factors and particular genomic changes in the tumour correlate with its behavior and outcome. *MYCN* amplification (MNA) is associated with aggressive tumours and a poor prognosis in all the tumour stages.

Segmental chromosomal imbalances and focal aberrations are very frequent in high-stage tumours and are a prognostic marker in particular subtypes of NB. Low stage tumours typically show whole-chromosome number alterations. More specifically, tumours with only numerical aberrations have a favorable prognosis, whilst any presence of segmental aberrations indicates poor survival. Segmental chromosomal alterations (SCA) are considered a bad prognostic factor in some groups of low and intermediate risk NB. However, as the majority of high-risk tumours show segmental aberrations, prognostic stratification based on the absence or presence of segmental aberrations is not considered in high-risk NB SIOPEN protocols. In some groups of the intermediate-risk protocol the presence of SCA is also not considered in risk stratification.

Specific SCA, such as 17q gain, 2p gain and 11q deletion are well described in previous literature and their impact in final outcome has been well-defined in most of them. A few studies mention the possible role of 1q gain, 1p deletion, 3p deletion and 4p deletion. Aside of these seven alterations no other imbalances have been established and in-depth associated with outcome in NB. Very few studies have been undertaken aiming to identify aberrations that discriminate outcome in high-risk patients and the role of many other segmental imbalances is still awaiting to be defined. Moreover, there is scarce literature focusing on numerical chromosomal alterations (NCA) and how they may have an impact in prognosis.

In order to investigate the role of less frequent segmental imbalances and certain NCA, we reviewed previous literature and conducted the following study. Our aim was to describe the distribution of SCA in NB according to INRGSS stage, *MYCN* status, age, mortality and relapse. Special emphasis

was made on associations between particular SCA which recurrently appeared together in the same tumours. The role of some particular NCA has also been analyzed.

MATERIAL AND METHODS

This retrospective multi-centric study has been conducted with 155 NB tumour samples collected in 29 cooperating Spanish hospitals between 2013 and 2018. Data collected from patients included age at diagnosis, primary tumour location, stage, *MYCN* status, 11q status, treatment received, relapses, follow up and results from SNP arrays including NCA and SCA profiling.

Staging and risk stratification were established according to INSS and INRGSS classifications. Tumour samples from all patients were referred to Hospital U i P La Fe for pathology and molecular biology studies. Samples were centrally reviewed and classified according to the International NB Pathology Committee (INPC) system. Biological studies included *MYCN* status studied by FISH and 11q status studied by SNP arrays (Affymetrix) according to the European NB Quality Assessment (ENQUA) guidelines. Patients were registered in the Spanish Society of Paediatric Hematology and Oncology (SEHOP) NB group database file and included for treatment mainly in SIOPEN trials (LINES, LNESG-II, EUNS and HRNBL1). As recommended by the ENQUA guidelines, amplification of *MYCN* was defined as a more than a 4-fold increase of *MYCN* signals in relation to the number of chromosomes 2. Cases with 2-4-fold increase in *MYCN* signal were defined as *MYCN* gain.

The 155 NB samples were studied by molecular karyotyping using SNP arrays Affymetrix CytoScan HD to determine SCA and NCA profiles. Quality and quantity of DNA was determined by spectrophotometry (Nanodrop 2000). In order to process the arrays, DNA samples chosen met optimum quality standards such as a relation A260/A280 (ratio DNA/RNA) between 1.8-2, a relation DNA/alcohols above 1.8, a sufficient DNA concentration and a proper DNA integrity. DNA quantity and integrity was assessed on an Agilent 2200 TapeStation using a DNA ScreenTape.

The database was compiled with patient data that met inclusion criteria, such as the availability of molecular karyotype reports that showed some type of numerical or segmental chromosomal alteration. Those patients whose samples were not suitable for genomic studies or with no available reports were not considered. NB without SCA and NCA were also excluded from analysis (n=8). Loss of heterozygosity (LOHs) detected were also not considered in the analysis.

Data were summarized using mean (standard deviation) and median (1st and 3rd quartiles) for numerical variables and absolute frequency (relative frequency) for categorical variables. To find an association between the presence of NCA and SCA with the variables age, *MYCN* status and INRGSS stage, two logistic regression models were adjusted. To find clinical (age, INRGSS stage)

or biological (*MYCN* status, SCA, NCA) factors associated with relapse and mortality, two cox models penalized by elastic net were adjusted. The penalty factor (lambda) was selected by taking the median of 200 replicates of the value that minimized the cross-validation error in each replicate. Frequency bar charts were used to show the summary of data, conditional plots to show the relationship between whole-chromosome 19 gain and the studied variables and box plots to illustrate the comparison between groups of quantitative variables. Survival data were illustrated using Kaplan-Meier graphs. A p-value less than 0.05 was considered significant.

All study actions were done under the appropriate ethics code and informed consents for samples and data management were obtained in all cases from the patients' parents or guardians. The study was performed in accordance with the Declaration of Helsinki.

RESULTS

General description

A total of 155 patients with NB were studied between 2013 and 2018 **(Table 1)**. NB without SCA and NCA were excluded from analysis (n=8). Median and mean age at diagnosis were found to be 14.7 and 32.6 months respectively (median range 0.06-364.3, interquartile range 32.5). According to the INRGSS classification, 26 of the cases (18%) were considered as L1, 43 cases (29%) as L2, 51 cases (35%) were stage M at diagnosis and 27 (18%) were classified as stage Ms. MNA was observed in 24 NB samples (16%), 12 tumours had *MYCN* gain (8%) whereas in the remaining 111 cases, *MYCN* status was non-altered (76%). During follow-up, 28 patients died (19%) and 59 patients had at least one relapse (40%) with a median follow up of 32 months. Segmental and numerical chromosomal rearrangements studied with SNP arrays showed 45 tumours lacking SCA and only containing NCA (31%) and other 102 NB that contained SCA (69%), despite NCA status.

Table 1. General description of the studied population (n=147) according to INRGSS stage, presence of SCA, MYCN status, mortality and relapses.

VARIABLE	n = 147
Median age at diagnosis (months)	14.7
Stage (INRGSS)	
L1	26 (18%)
L2	43 (29%)
M	51 (35%)
Ms	27 (18%)
SCA / NCA	
SCA	102 (69%)
NCA only	45 (31%)
MYCN	
Non-amplified	111 (76%)
Gain	12 (8%)
Amplified	24 (16%)
Death	
No	119 (81%)
Yes	28 (19%)
Relapse	
No	88 (60%)
Yes	59 (40%)

The most frequent SCA detected was +17q observed in 34% of the tumours (n=50), followed by +2p (n=38) and -11q (n=37). Other frequent imbalances found were -1p (n=29), +1q (n=21), +11q (n=18), -3p (n=17), +1p (n=16), -4p (n=15) and +11p (n=15). +2q, +6q and +7q were found in 13 tumours. Frequency of SCA is shown in **Figure 1**. Overall survival (OS) was significantly lower (p=0.028) in tumours with SCA **(Figure 2).**

Figure 1. Frequency bar chart showing distribution of SCA determinations obtained by molecular karyotyping.

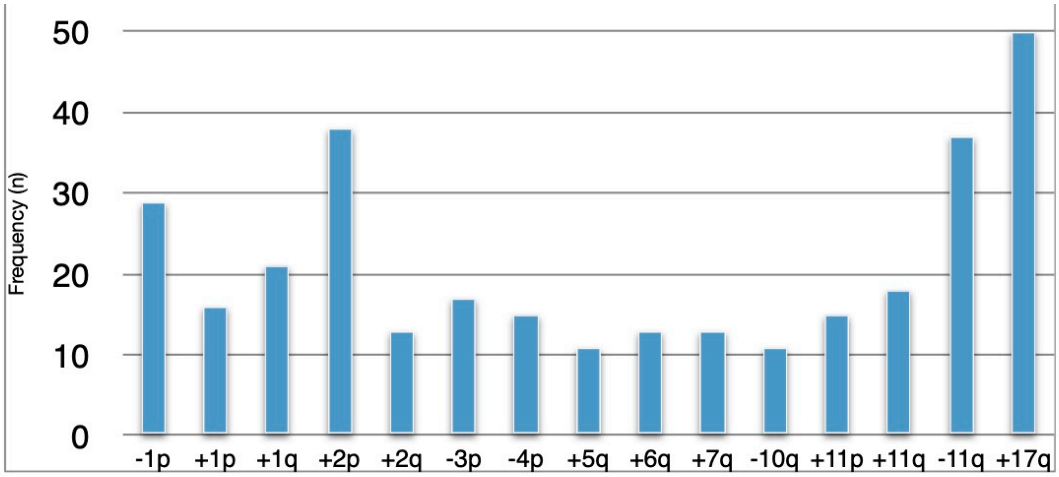
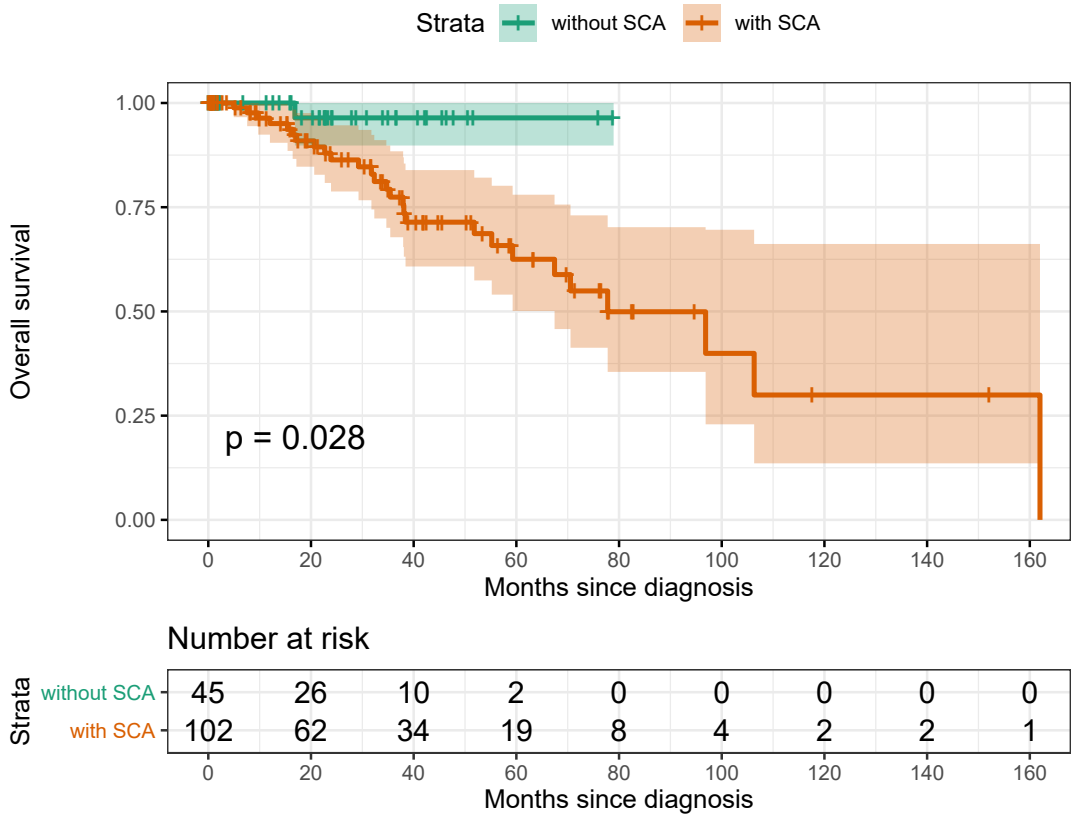


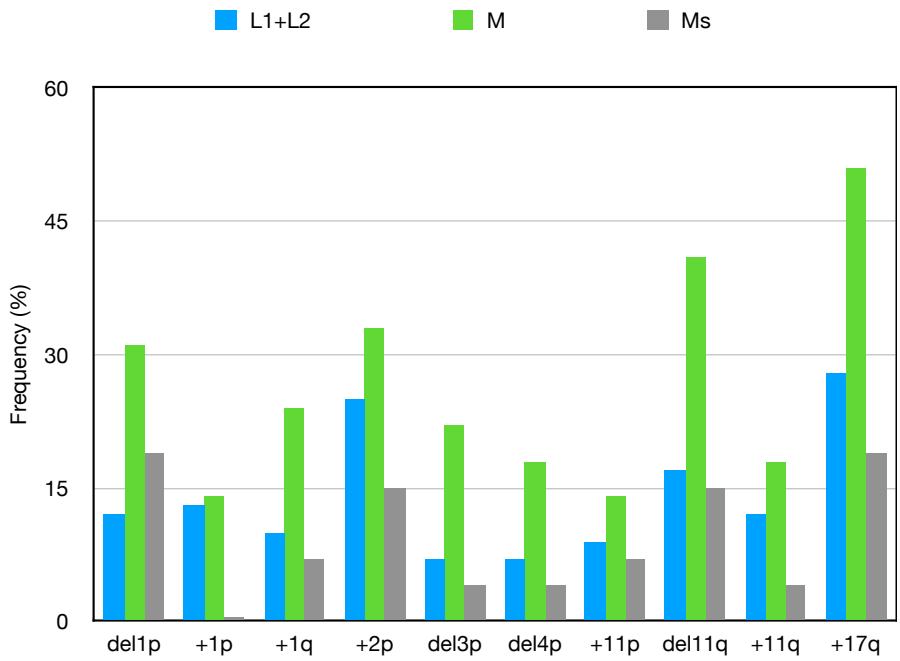
Figure 2. Kaplan-Meier graph comparing overall survival stratified by copy number profile: SCA (n=102) and non-SCA (only NCA) neuroblastoma (n=45).



SCA distribution according to INRGSS stage

The tumour stage that presented less SCA was stage Ms (41%), followed by L1 (62%), L2 (65%) and finally stage M in which 47/51 tumours contained at least one SCA (92%). Only 4 NB classified as stage M did not show SCA, with 3 of them being alive. Gain in 17q was the most common alteration found in every stage (51% in stage M, 28% in localized and 19% in Ms). SCA distribution according to INRGSS stage can be observed in **Figure 3.**

Figure 3. Frequency bar chart (%) showing SCA distribution according to INRGSS stage (L1 + L2, M and Ms stage)



SCA distribution according to *MYCN* status

NB with MNA contained SCA in 96% of the tumours whereas NB without MNA only had SCA in 65% of the cases. In the tumours showing MYCN gain, 58% presented an SCA. The most frequent alteration in non-MNA tumours was 11q deletion whereas +17q was the most common SCA detected in MNA NB. In *MYCN* gained subgroup both +17q and +2p were identified as the most common SCA (33% in both).

SCA distribution according to age

In this study, the presence of SCA was significantly associated with older patients. 93% of patients older than 18 months at diagnosis (62/67 patients) have at least one SCA. Specific SCA that were

significantly associated with older age were +1q and -3p. The most frequent SCA found in patients younger than 18 months was +17q.

SCA distribution according to mortality

A total amount of 28 patients died during the follow-up period because of disease progression and 27 of them (96%) had at least one SCA. Only 1 SCA-free patient died during the follow up period. 98% of the patients without SCA survived whereas 74% of the patients with SCA survived. The most frequent SCA in the mortality subgroup (surpassing +17q) was -11q while +17q was the most frequent alteration in the patients that survived. Other frequently observed SCA in the cases that died were -3p and +11p, whilst the most frequent SCA found in survivors following +17q were +1q and -10p. A longer follow-up period is required to achieve further conclusions, especially in patients with 11q deleted NB that usually relapse later compared to patients with MNA NB.

SCA distribution according to relapse

Relapsed NB presented SCA in 85% of the cases. NB with SCA relapsed in 49% of the cases whereas only 20% of the patients relapsed in the SCA-free group (9/45). The SCA most frequently detected in the relapsed patients was -11q, surpassing +17q. On the other hand, in the non-relapsed patients' group, the most common SCA observed were +17q and -1p.

SCA combinations

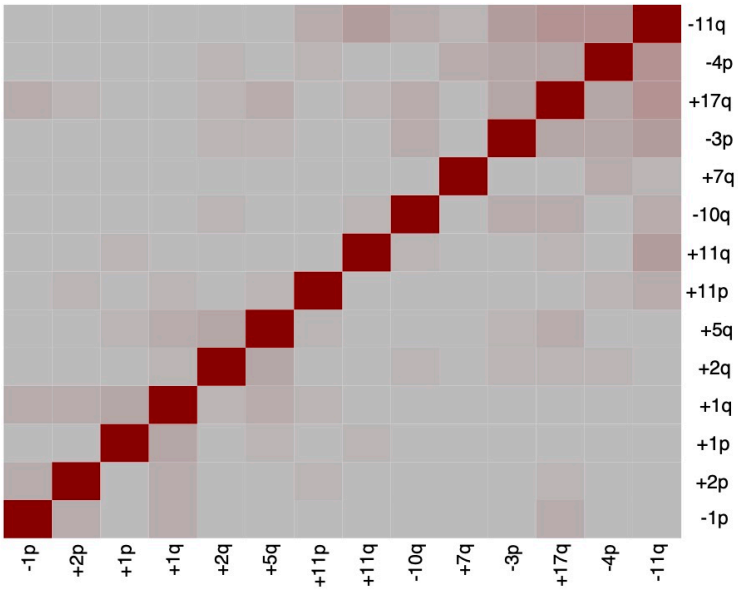
To the date, the most recurrent SCA reported have been +17q, +2p, -11q, +1q, -1p, -3p and -4p. Other specific alterations have not been widely reported, except from isolated reports such as 6q loss, described by Depuydt et al in a subgroup of "ultra-high-risk" NB, or 7p gain described by Pezzolo et al as a protective factor.

In this study, according with previous literature, the most recurrent alterations described were +17q (50 cases) that was detected in 49% of NB with SCA, followed by +2p (38 cases) and -11q in the third place (37 NB). Other recurrent alterations found were -1p (n=29), +1q (n=21), -3p (n=17) and -4p (n=15). However, other SCA less described in previous literature have also been found in a significant proportion such as +1p, +2q, +5q, +6q, +7q, -10q, +11p and +11q (Figure 1).

The most common alteration associated with a higher number of other segmental rearrangements was -11q followed by -4p. To study possible associations and recurrent patterns of combinations in NB, several SCA including the most frequent ones were studied separately. Cluster graph **(Figure**

4) shows associations between the most recurrent SCA. +17q have significant associations with +5q and -10q. +2p was correlated together with +11p whilst +1q appeared with +1p, +5q and +11p alterations. 11q deletion was the aberration mostly related to other chromosomal imbalances (+7q, -10q, +11p and +11q) and -4p appeared more frequently together with +2q and +7q. In addition, -3p correlated with +7q and -10q whilst 1p deletion wasn't associated with other particular SCA and was demonstrated as the less frequent SCA associated with other segmental imbalances.

Figure 4. Cluster color intensity graph showing associations between the most recurrent SCA (association probability between SCA in the same tumour is higher as the red shading intensity increases).



NCA distribution

In this study, 45/147 NB presented NCA without SCA (31%). Survival in this group was high (98%) and most of these NB were classified as localized or Ms tumours. Whole chromosome gains and losses were found more frequently than SCA although sometimes both NCA and SCA were detected in the same tumour. In this cohort, tumours with SCA and NCA behaved as tumours with only SCA. The most frequent NCA observed has been gain in chromosome 7 (86/147) followed by +18 (67/147), +17 (66/147), +12 (63/147) and +13 (62/147). OS in tumours with or without these numeric aberrations has been studied and no differences have been detected in survival regarding these alterations.

However, a small subgroup with whole gain in chromosome 19 (n= 22) that included NB of all tumour stages was analyzed and patients with this alteration showed an outstanding favorable outcome. Gain in chromosome 19 in this cohort was significantly associated with other low risk features in NB such as the absence of SCA (OR 0.178) and lower INRGSS tumour stage (OR 0.558) **(figures 5 and 6)**. The Kaplan-Meier graph **(figure 7)** illustrates OS comparison between the cases with whole-chromosome 19 gain (n=22) and the cases with no numeric alterations in chromosome 19 (n=102), observing remarkable differences between these two groups (p=0.031). Cases with whole loss of chromosome 19 (n=23) have been excluded in this comparison. These differences were not observed when analyzing other NCA.

Figure 5. Conditional plot showing association between whole-gain in chromosome 19 and SCA presence (n19gain=22).

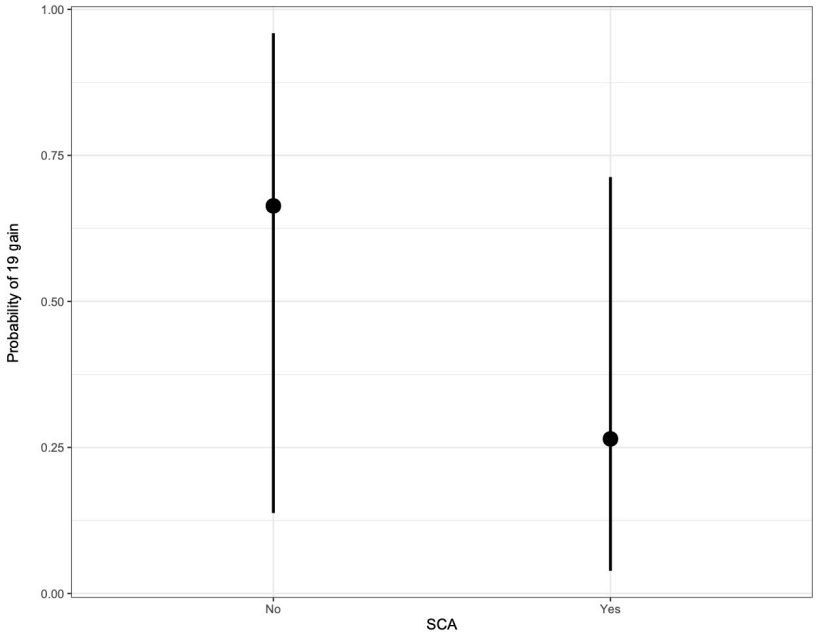


Figure 6. Conditional plot showing association between whole-gain in chromosome 19 and INRGSS stage (n19gain=22).

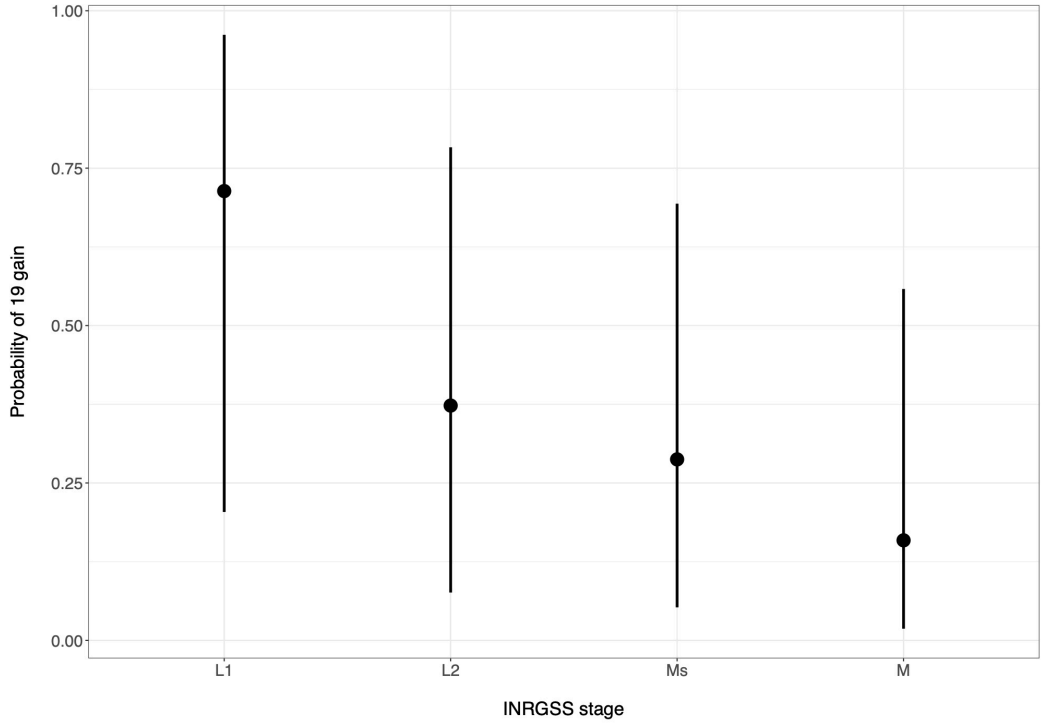
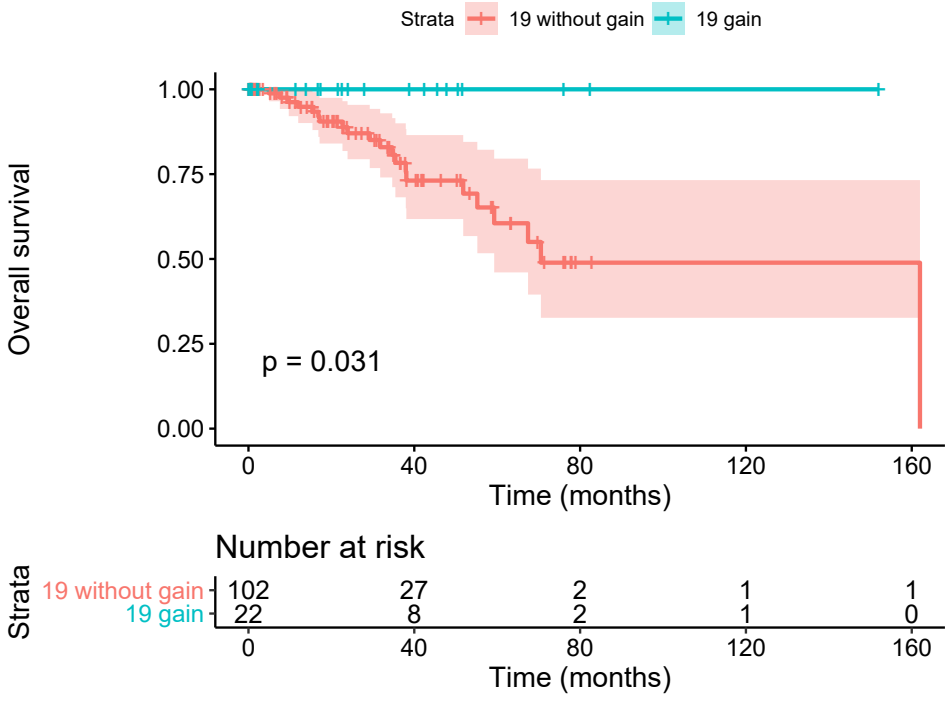


Figure 7. Kaplan-Meier graph comparing overall survival in 22 neuroblastoma with whole-gain in chromosome 19 (n19=1) and 102 tumours without numeric alterations in chromosome 19 (n19=0).



DISCUSSION

Clinical heterogeneity in NB is a well-recognized feature of this cancer and as more insight is reported in this tumours' biology, this fact becomes gradually more understandable. Somatic chromosomal copy number alterations have shown to be associated with prognosis, especially in low and intermediate risk cases and this information is used in SIOPEN protocols to stratify risk. While aggressive NB phenotypes usually contain SCA (translocations, amplifications or deletions), favorable cases are mostly SCA-free and frequently, complete gains or losses in whole chromosomes are observed. In terms of outcome, it has been highlighted that, tumours containing both SCA and NCA behave as cases that present SCA solely, thus proving the relevance of SCA above NCA. Gain of 17q, loss in 1p and loss in 11q are the most frequent and studied segmental imbalances up until now and this is further confirmed in the present study. Excluding these, few studies have improved our insight in other SCA and how these combine together.

Gain of 17q is the most frequent SCA observed in sporadic NB and has previously been associated with a higher age, 1p deletion and MNA. In this cohort, 17q gain appears in 34% of the tumours and in 49% of the NB with SCA. Approximately 50% of the NB samples with 17q gain contained -1p and -11q. Prognosis value of 17q gain as an unique factor is still unknown and has been controversial. Lastowska M et al. and Bown N et al. related 17q gain to worst outcomes whilst in the cohort reported by Spitz R et al. no association between 17q gain and an inferior prognosis was found. A poor outcome prevails when 17q gain is combined to the other mentioned SCA.

Genetic alterations in the long arm of chromosome 11 are more diverse. Allelic loss is the alteration mostly observed but unbalanced translocations (in 11q21 and 11q22 regions), deletions in 11q23 and inversion in 11q21-q23 regions have also been described. Loss in 11q is inversely related to amplification of *MYCN*. In this study co-existence of both abnormalities was detected in only 4% of the cases. Moreover, deletion in 11q is associated with very bad outcome and higher chances of relapse. The existence of this alteration in low and intermediate risk tumours as well as in Ms tumours configures very aggressive NB phenotypes.

The frequency of 11q alterations may vary between 20-45%, (25% in this study) and it is associated with a higher median age at diagnosis. As further confirmed in this cohort, 11q deletion is the alteration most frequently associated with other concomitant SCA and a higher number of chromosomal breakage points. Furthermore,11q deletion was the most frequent SCA (above +17q) in the relapse and mortality subgroups. In our study, the number of tumours with 11q deletion was higher in relapsing patients compared to those who did not, as well as in those who died. This has

been described in previous studies comparing the poor prognosis of 11q deletion, similar to those of MNA NB.

The incidence of 2p gain in this cohort was relatively high (25%) and appeared in tumours with advanced INRGSS stage and tumours with *MYCN* gain. 2p gain has been related before with advanced disease stages at diagnosis and decreased average 5-year OS and EFS. Oncogene *MYCN* is localized in chromosome 2p. Although amplification of *MYCN* is the most robust genetic factor that is correlated with poor outcome, very little is known about the role of *MYCN* gain and other 2p gains. A remarkable fact is that *MYCN* gain has been related in previous studies to 11q deletion. Most of the patients with both of these aberrations (11q-del and *MYCN* gain) presented high-risk features in this study. *ALK* gene is also located on the short arm of chromosome 2 (2p23) and encodes a protein called anaplastic lymphoma kinase, which is a receptor tyrosine kinases (RTK). Changes in *ALK* lead to an abnormal version of the protein that is constitutively active, which induces abnormal proliferation of immature nerve cells and leads to NB. Previous reports suggested that *ALK* copy number multiplication, if occurring concurrently with *MYCN*, significantly reduces patients' survival especially for the intermediate and high-risk group and that 2p gain tumours encompassing the *ALK* locus also associate worse outcome in these patients with NB.

Deletion in 1p has been the fourth more frequent SCA found in this study. Several tumour suppressor genes have been identified in this particular region although there is not enough evidence supporting their role in NB etiology. Loss of chromosome 1p has been previously described by Caron et al as a strong prognostic factor in patients with NB, independently of age and stage that reliably identifies patients at high-risk in stages I, II, and 4s, which are otherwise clinically favorable. In this study, deletion in 1p was found to be the most frequent SCA after +17q, -11q and +2p (29 cases harboring -1p) but surprisingly, it is the alteration less related to other chromosomal breakage.

Spitz et al, reported 26 cases with 3p changes (18%), most of them deletions, in a cohort of 144 NB. They associated this alteration with stage 4: 20 out of 59 (34%) versus 6 out of 85 (7%) that were in stages 1-3 and 4s, p=0.007. Median age in the group with 3p changes was higher ($p < 0.001$) tan in those with no 3p aberrations. Aberrations in chromosome 3p and 11q were found to be highly associated with each other ($p < 0.001$), as further confirmed in the present study. Moreover, patients with 3p and 11q aberrations with localized or stage 4s tumours showed a clearly worse outcome compared to those without any alterations ($p= 0.002$ and $p=0.0027$ respectively). It has been suggested that the association of 3p deletion with 11q deletion may constitute a new high-risk subgroup within non-MNA tumours, both localized, 4s and disseminated. In this study, 17 cases with 3p deletion were identified, and 76% of them had 17q gain (13/17 cases).

Gain of 1q was associated in this study with +1p, +5q and +11p. The high presence of this alteration in other series in NB raised the possibility that genes involved in cancer development could be located in this region. By analyzing this phenomenon in other paediatric tumours such as retinoblastoma, a potential oncogene known as *KIF14* was identified. Its role in NB has yet to be defined.

In this study, we demonstrate that 4p deletion (n=15) is the SCA most associated with other chromosome imbalances after 11q deletion as observed in Figure 4. 4p deletion predominates in the population older than 18 months (73%) and is also associated with relapse (12/15 cases relapsed). The paired-like homeobox 2B gene (*PHOX2B*) is located in chromosome 4p13. This gene plays an essential role in autonomic nervous system differentiation and has been identified as a sensitive and specific biomarker for minimal residual disease detection in NB. Mutations in *PHOX2B* have been found in individuals with neural crest disease involvement. A low frequency of heterozygote germline alterations in *PHOX2B* have also been identified in sporadic NB patients and in patients with familial NB. *C. Krona*, et al, investigated the role of *PHOX2B* and data from this case suggest a model in which progressive growth and additional genetic changes occurred from the first tumour after activation of the second *PHOX2B* allele by chromosome 4p deletion and 17q gain. Our results further confirm the association between 4p deletion and 17q gain.

One of the most frequently observed alterations was 11p gain (10.2%). In previous studies, this has been associated with 11q deletion via translocation or unbalanced deletion. This statement can account for the high presence of this aberration in the relapse group of our cohort, an event to which 11q deletion is associated.

Depuydt et al, identified an extremely poor outcome in patients with distal 6q losses. In their study 5,9% of the patients harbored 6q deletion and 10-year overall survival was only 3.4%. They also observed that only 25% of the tumours with 6q loss also had MNA and that 6q loss contained more breakpoints than tumours without this abnormality. In our cohort only 5/147 cases presented 6q deletion, all of them being classified as stage M and mortality detected in this subgroup was 40%.

The majority of previous research has focused on the existence of SCA and how their presence is linked to a worse outcome. Very few studies refer to the possible influence of particular NCA in NB prognosis. Apparently when SCA and NCA appear together in NB, the negative influence of SCA is predominant and therefore the possible influence of some NCA cannot be evaluated. However, when only NCA are present, Parodi S, et al. recently described that loss of whole chromosome X can predict prognosis of NB patients with only numerical genomic profile. In this study that included DNA copy-number data of 174 NB with NCA genomic profile the association between poor EFS and whole

chromosome X alteration was reported. In our study 19/147 NB had whole-chromosome X loss and most of these tumours were associated with the presence of SCA (13/19) and mortality was 32%.

We report a significant favorable outcome in patients with whole numeric gain in chromosome 19 (p=0.028). Although this has been detected in a small group of 22 cases, this hasn't been observed when analyzing other numerical alterations. Whole gain in chromosome 19 in this study is associated with lower INRGSS stage and the absence of SCA. It is known that this chromosome has the highest gene density of all human chromosomes, more than double the genome-wide average. The large clustered gene families, corresponding high guanine-cytosine content, CpG islands and density of repetitive DNA indicate a chromosome rich in biological and evolutionary significance. Mutations in tumour suppressor genes and other alterations in chromosome 19 have been related to multiple types of human cancer such as leukemia, lymphoma and other solid tumours as gliomas and lung cancer. Nakamura et al described frequent loss of heterozygosity on chromosome 19 in secondary glioblastomas and how tumour suppressor genes located on chromosome 19q13.3 have a role in progression of low-grade astrocytomas to secondary high-grade glioblastoma. Gain in chromosome 19 has also been the most common abnormality detected by comparative genomic hybridization by Alvarez S. et al in acute megakaryocytic leukemia, a rare subtype of acute myeloid leukemia that is most commonly associated with Down syndrome in children. In NB, loss of heterozygosity in 19q has also been related before with locally aggressive tumours but, so far, whole numeric changes in this chromosome have not been related to outcome in NB. A larger cohort is probably required to confirm the impact of this alteration in prognosis and the appropriateness of considering this abnormality as a biological marker.

In conclusion, we report that SCA are not randomly distributed and are concentrated on recurrent chromosomes. The most frequently affected chromosomes are recurrent in specific prognostic groups according to NB well established stratifying factors (age, INRGSS stage and *MYCN* status). Presence of SCA is associated with older age and MNA. Specific segmental brakes are associated and appear together with other SCA, specially 11q deletion and 4p deletion. Whole gain in chromosome 19 is related with the absence of SCA and a lower INRGSS stage. A small subset of patients with a whole numeric gain in chromosome 19 and a better outcome has been identified.

MANUSCRIPT 3

NEXT-GENERATION SEQUENCING IDENTIFIES POTENTIAL ACTIONABLE TARGETS IN PAEDIATRIC SARCOMAS

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MANUSCRIPT 3

NEXT-GENERATION SEQUENCING IDENTIFIES POTENTIAL ACTIONABLE TARGETS IN PAEDIATRIC SARCOMAS

INTRODUCTION

Paediatric sarcomas account for over 20% of all paediatric solid malignant cancers and represent 13% of all paediatric malignancies. They also contribute substantially to cancer-related mortality and morbidity. With more than over 70 histologic subtypes, sarcomas can arise from a primitive mesenchymal cell from almost every tissue in the human body and are classified into two main groups: soft tissue sarcomas (STS) and bone sarcomas (BS). The highest incidence rates in children are reported amongst rhabdomyosarcoma (RMS), osteosarcoma and Ewing's sarcoma (EWS). Although each subtype has a different phenotype and genetic profile, they are classified into two molecular groups: a genetically complex group with a high mutational burden and com-plex karyotype, and a genetically simple group containing a single and disease-specific translocation, amplification or mutation with a silent genomic background. Most paediatric sarcomas are included in the second group as they are mostly characterized by chromosomal translocations that result in hybrid genes acting as drivers that are critical for sarcomagenesis.

Paediatric RMS protocols, currently classify this tumour based on the presence of PAX/FOXO1 translocation and distinguish between fusion positive or fusion negative RMS. The genetic profile of EWS is dominated by the driving reciprocal chimeric translocation between EWSR1 and a variety of ETS partner transcription factors. These gene fusions act as oncogenic transcription fac-tors that trigger transcriptomic and epigenetic dysregulations that explain tumours' biology. In contrast to EWS and RMS, osteosarcoma shows an extremely complex and unstable genome but without a remarkable repetitive pattern. In most clinical settings, sarcomas involving translocations are detected by fluorescence in situ hybridization (FISH) and reverse transcriptase polymerase chain reaction (RT-PCR). Translocations are used by clinicians mostly as diagnostic markers. However, the resulting chimeric proteins of these translocations are not easily druggable and hinder the development of inhibitors. Table 1 shows the most frequent fusion transcripts in paediatric sarcomas.

Both STS and BS display a highly aggressive behavior. During the last decades, addition of systemic chemotherapy has improved outcome of localized tumours resulting in the survival of 2 out of three patients. However, metastasic and relapsed sarcomas still have very poor survival rates. Despite the knowledge gained in cancer biology, aetiology and in the implementation of novel di-

agnostic techniques and omics, scarce improvement has been observed in advanced stage STS and BS.

Table 1. Main fusion transcripts in paediatric sarcomas: Chromosomal translocation, gene tran-scripts and expected frequences. RMS: rhabdomyosarcoma; STS: soft-tissue sarcoma.

DIAGNOSIS	TRANSLOCATION	FUSION	FREQUENCY
Alveolar RMS	t(2;13)(q35;q14) t(1;13)(p36;q14)	PAX3 / FOXO1 PAX7 / FOXO1	60% 20%
Ewing sarcoma	t(11;22)(q24;q12) t(21;22)(q22;q12) t(7;22)(p22;q12) t(2;22)(q35;q12) t(16;21)(p11;q22)	EWSR1 / FLI1 EWSR1 / ERG EWSR1 / ETV1 EWSR1 / FEV FUS / ERG	85% 10% <1% <1% <1%
Desmoplastic small round cell tumor	t(11;22)(p13;q12.2)	EWRS1 / WT1	>95 %
Infantile fibrosarcoma	t(12;15)(p13.2;q25.3)	ETV6 / NTRK3	70 %
Synovial sarcoma	t(X;18)(p11.2;q11.2-11.23) t(X;18)(p11.2;q11.2-11.23) t(X;18)(p11.2;q11.2-11.23)	SS18 / SSX1 SS18 / SSX2 SS18 / SSX4	64% 35% 1%
Clear cell STS	t(12;22)(q13;q12)	EWSR1 / ATF1	>90%

During the last years, precision and quality criteria for the diagnosis of paediatric cancers including sarcomas, has experienced an increased demand. New techniques have been introduced that complement pathological diagnosis including immunochemistry, FISH, RT-PCR and next-generation sequencing (NGS). These demands have been gradually assumed by clinicians, pathologists, geneticists and molecular biologists in tertiary reference hospitals. In addition, pre-cision medicine programs have been developed in order to expand our knowledge of tumour biol-ogy and defeat cancer with more precise pharmacological targets. We present the results in paediatric sarcomas from the Precision Medicine program for children and adolescents with solid tumours in relapse/progression carried out at a national reference centre for paediatric sarcomas. This program has received samples from collaborative centres, providing a national perspective. Since September 2019, these studies are routinely carried out at diagnosis in every paediatric sarcoma.

MATERIALS AND METHODS

Study subjects

A total of 70 sarcoma samples from paediatric patients treated at a reference institution for pediatric sarcomas or at other Spanish center from February 2015 to March 2020 were included. Thirty patients were analyzed at diagnosis and forty patients were studied at relapse or refractory disease. Re-biopsy at relapse was highly encouraged. Initially, only high-risk tumours were exceptionally considered and studied before first line treatment and since September 2019 every pediatric sarcoma was studied at diagnosis by NGS.

The program was approved by the Ethics committee of the center. Parents signed the informed consent and were informed about the possibility of finding germline mutations and accepting or refusing to be informed. Consent was also required when performing NGS studies at diagnosis. Every procedure was performed according to the Declaration of Helsinki.

Study samples

Fresh tumour samples were requested. Paraffined-embedded tumours and/or pretreatment biopsies were only used if fresh samples were unavailable. Peripheral blood samples were simultaneously collected in 45 cases. All tumour samples were reviewed by a board-certified pathologist to confirm histology and estimate tumour cell content. Immunochemistry techniques (p-AKT, PDL1, p-EGFR, c-KIT, PTEN, Her2neu, p53) and FISH (NTRK1 / 3, ALK, BRAF) were also performed. Only samples with >30% tumour cell content were considered for further genomic testing. The selected tumour material and peripheral blood samples were sent to a biobank for DNA extraction and subsequently to the laboratory for sequencing analysis. In some cases, based on previous literature and according to the sequencing results obtained for each tumour type, studies were completed with SNP array analysis.

DNA extraction

DNA extraction from tumour and blood samples was carried out using the QIAamp® DNA Investigator kit (QIAGEN® ref.56504) or QIAamp® DNA Mini Kit (QIAGEN® ref. 51, 304, respectively, following manufacturer instructions. The concentration and absorbance ratios were measured with NanoDrop 2000®13.

RNA extraction and cDNA generation

RNA was extracted with the RecoverAll™ Total Nucleic Acid Isolation Kit following manufacturer's protocol. Total RNA was quantified with the with the Qubit™ RNA HS Assay Kit (ThermoFIsher Scientific), and cDNA was obtained with the SuperScript™ IV VILO™ Master Mix.

Sequencing studies, data interpretation and variant calling

Commercial and customized NGS panels that included the consensus gene list and that pro-duced an average coverage of 1000X and homogeneity with a minimum of 85% were used for the analysis of relapse or refractory patients: Ion Ampliseq Cancer Hotspot Panel v2 (Thermo Fisher Scientific), Human Comprehensive® Cancer Panel (Qiagen©), Paediatric-OncoPanelDx® (Imegen) and Onconano Gene Panel (Paediatric Oncology Group-IIsLaFe). For analysis of newly diagnosed samples, the Oncomine Childhood Research Assay® was used (Ref: A36485).

Gene panels included at least the following: ABL1, AKT1, ALK, BRAF, CDKN2A, CSF1R, CTNNB1, EGFR, ERBB2, ERBB4, EZH2, FGFR1, FGFR2, FGFR3, FLT3, GNA11, GNAQ, HRAS, IDH1, IDH2, JAK2, JAK3, KIT, KRAS, MET, MPL, NRAS, PDGRFA, PIK3CA, PTEN, PTPN11, RB1, RET, SMARCB1, SMO, and TP53.

For NGS data analysis, variant calling was based on the genome version GRCh37 (hg19). Genetic variants detected in both, blood and paired tumour samples were classified as germline variants, whereas variants detected exclusively in tumours were categorized as somatic variants.

Variant annotation was carried out applying an algorithm of filters in order to discard non clinically relevant variants: those with an allelic frequency < 5%, changes in non-coding regions (excluding those variants in exon splicing sites +/− 10 nucleotides), synonymous variants (excluding those coding variants nearby splicing sites +/− 4 positions), variants with high frequency in the general population (MAF > 0.01), polymorphic changes (SNPs) without clinical relevance found in healthy population or described as benign by several sources or our genomic database. The remaining variants were classified according to international recommendations as pathogenic, likely pathogenic, benign, likely benign or of uncertain significance based on literature or disease databases (COSMIC, ClinVar, HGMD, CiVIC or St Jude PeCan).

Pathogenic or likely pathogenic variants were reviewed and approved by the paediatric molecular tumour board (PMBT) committee, and further confirmed using direct Sanger sequencing. Actionable variant was referred as a genomic change that suggests an alteration with biological activity that could be targeted with a specific therapy already used in vivo. Targeted therapies were preferentially recommended to be administered within clinical trials but also as compassionate use basis if trials weren't available. Median time between biopsy/surgery and molecular tumour board recommendation was 5 weeks.

Paediatric molecular tumour board discussion

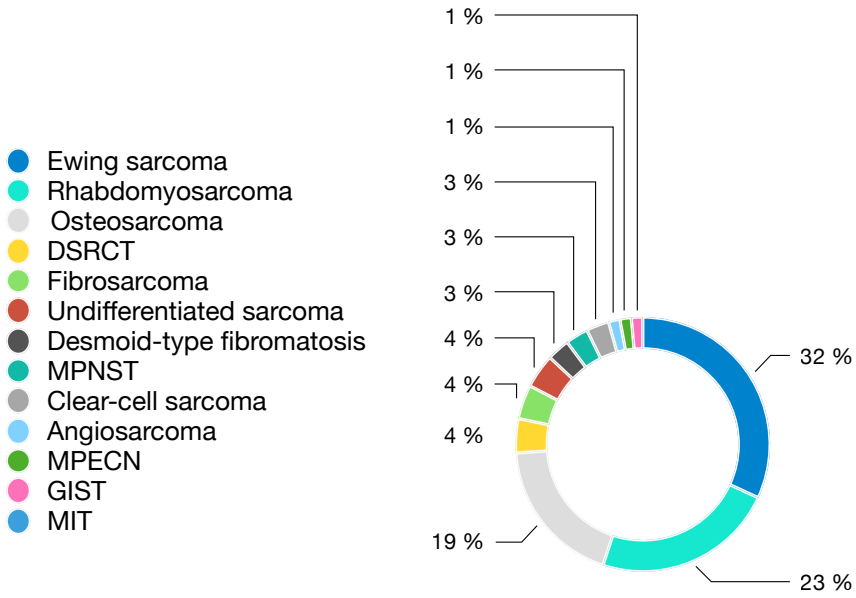
The PMTB was created in November 2014 and composed by paediatric oncologists, pharmacologists, geneticists, pathologists, molecular biologists and bioinformatics. The PMBT established the consensus gene panel for the NGS analysis. After the completion of pathological and genomic studies, results were discussed in periodical meetings in the PMTB and a final report was transferred to the corresponding physician. The workflow was based on previous pilot studies.

RESULTS

Clinical characteristics

A total of 70 paediatric and adolescent patients with STS and BS from 10 Spanish cooperating sites were included in a 5-year period from February 2015 to March 2020. Patients' median age at study entry was 11.5 years with a range of 1-18 years. Forty-one per cent of the patients were female and 59% were male. Distribution of tumour type is shown in Figure 1.

Figure 1. Distribution of sarcoma type amongst reported cases. DSRCT: desmoplastic small round cell tumour; MPNST: malignant peripheral nerve sheath tumour; MPECN: malignant perivascular epitheloid cell neoplasm; GIST: gastrointestinal stromal tumour; IMT: myofibroblastic inflammatory tumour.



The most frequent tumours were EWS (n=22), RMS (n=16) and osteosarcoma (n=13). Thirty patients were studied at diagnosis (43%), 22 patients at first relapse (31%), 15 patients at second or successive recur-rences (21%) and 3 patients when found to be refractory to first line treatment (4%). Somatic variants described as pathogenic or likely pathogenic using international system classifications15 were reported. The main characteristics of the selected patients are detailed in Table 2.

Table 2. Clinical characteristics of the studied population (n=70). STS: soft-tissue sarcoma.

VARIABLE	N (n=70)
DIAGNOSIS	
Ewing Sarcoma	22
Rhabdomyosarcoma	16
Osteosarcoma	13
Desmoplastic small round cell tumour	3
Infantile fibrosarcoma	3
Undifferentiated sarcoma	3
Desmoid-type fibromatosis	2
Malignant nerve sheath tumour	2
Clear cell STS	2
Angiosarcoma	1
Malignant Perivascular Epithelioid Cell Neoplasm	1
Gastrointestinal stromal tumour	1
Myofibroblastic inflammatory tumour	1
AGE	
0-4	6
5-8	16
9-12	20
13-18	28
SEX	
Female	29
Male	41
CLINICAL STATUS	
Diagnosis	30
First relapse	22
Second or next relapses	15
Refractory	3

NGS results

Twenty-two out of 70 patients (31%) had at least one pathogenic or probably pathogenic alteration identified by NGS as with a mean of 1,4 mutations per patient. Most of the cases had one unique mutation. A total of 30 different pathogenic or likely pathogenic mutations in 18 different genes were detected amongst the 22 patients. Mutations were detected in relapsed or refractory sarcomas (57%) and also at first diagnosis (43%).

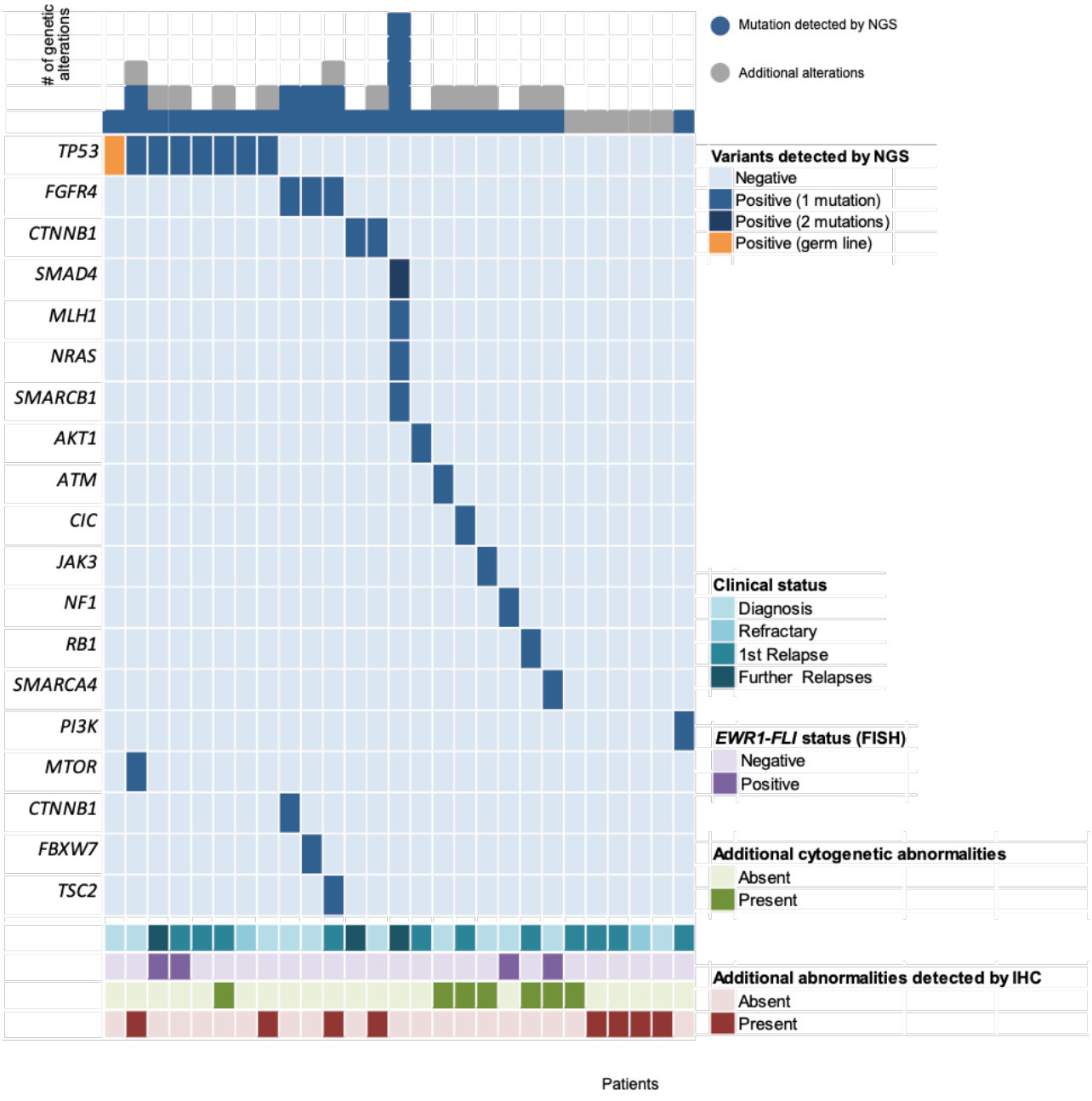
Diagnostic sarcoma fusion genes detected by FISH or RT-PCR were only used for diagnosis but were not considered for precision medicine recommendations as no targeted treatments are available for these alterations to date. Overall, TP53 was the most frequently affected gene (27%) and preferentially identified in EWS, RMS and angiosarcoma. Three embryonal rhabdomyosarcomas harbored alterations in FGFR4 whilst the two aggressive fibromatosis and an embryonal RMS had CTNNB1 mutations. Identified gene and variant alterations are shown in Table 3. Including information obtained by complementary techniques (immunochemistry and FISH) up to 27 patients had an identified alteration (39% of the cases). A summary of the molecular alterations spotted in these 27 patients is shown in Figure 2.

Table 3. Tumour sequencing results: Mutated gene, variant, sarcoma subtype and clinical status. DSRCT: desmoplastic small round cell tumour; RMS: rhabdomyosarcoma; EWS: Ewing sarcoma; DTF: desmoid-type fibromatosis; MPNST: malignant peripheral nerve sheath tumour.

GENE	VARIANT	TUMOUR TYPE	CLINICAL STATUS
TP53	c.1040C>T(p.A347V)	DSRCT	Relapse
	c.559G>C(p.G187R)	Embryonal RMS	Diagnosis
	c.906_907delCCinsTT (p.R303*)	Angiosarcoma	Diagnosis
	c.742C>T (p.R248W)	EWS	Diagnosis
	c.404G>T (p.C135F)	EWS	Relapse
	c.817C>T (p.R272C)	Alveolar RMS	Refractory
	c.448A>G (p.Y163C)	EWS	Relapse
	c.614A>G(p.Y205C)	EWS	Relapse

FGFR4	c.1648G>A(p.V550M)	Embryonal RMS	Relapse
	c.1648 G>C (p.V550L)	Embryonal RMS	Diagnosis
	c.1648 G>C(p.V550L)	Embryonal RMS	Diagnosis
CTNNB1	c.134C>T(p.S45F)	DTF	Diagnosis
	c.134C>T(p.S45F)	DTF	Relapse
	c.133_134delTCinsCT (p.S45L)	Embryonal RMS	Diagnosis
SMAD4	c.302G>A(p.W101*)	Alveolar RMS	Relapse
	c-370G>A(p.D124N)	Alveolar RMS	Relapse
ATM	c.7032G>A(p.W2344*)	MPNST	Diagnosis (secondary tumour)
NRAS	c.176C>T (p.A59V)	Alveolar RMS	Relapse
CIC	c5939_5943del (p.G1980Vfs*78)	Clear cell renal sarcoma	Relapse
FBXW7	c.1394G>A (p.R465H)	Embryonal RMS	Diagnosis
RB1	c. 361 C>T (p.Q121*)	Osteosarcoma	Relapse
AKT1	c.138C>A(p.D46E)	MPNST	Relapse
JAK3	c.2164G>A(p.V722I)	EWS	Diagnosis
PI3K	c.1624G>A(p.E542K)	Embryonal RMS	Relapse
SMARCB1	c.1135>A (p.A379T)	Alveolar RMS	Relapse
MLH1	c.1138G>A(p.A380T)	Alveolar RMS	Relapse
MTOR	c.6644C>T(p.S2215F)	Angiosarcoma	Diagnosis
TSC2	c.5158C>T(p.R1720W)	Embryonal RMS	Relapse
SMARCA4	c.1135>A (p.A379T)	DSRCT	Diagnosis
NF1	c.2087G>A (p.W696*)	EWS	Diagnosis

Figure 2. Molecular alterations observed in the 27 patients (each column represents one patient). IHC: immunochemistry.



Clinical translation

After discussion of the biological results in the PMTB, 18 actionable variants (26%) were identified and formal recommendations were submitted to the respective physicians. RMS was the tumour in which more actionable variants were observed (39%), particularly embryonal histology (28%). Two osteosarcoma patients presented actionable alterations. Despite the number of EWS cases

included in the study (22), only one patient had an actionable variant. This result points out the difficulties to implement a precision medicine strategy in EWS tumours.

Six patients out of the whole cohort received targeted treatment (9%), observing clinical benefit in five of them (78%):

A thirteen-year-old female with a radio-induced abdominal malignant nerve sheath tumour, 10 years after neuroblastoma treatment was found to have a mutation in ATM. After radical surgery and standard chemotherapy she underwent disease progression. Targeted treatment with PARP inhibitor olaparib and temozolamide was administered, resulting in disease stabilization during one month with a clear disease control. She received treatment during two months before a subsequent progression.

A twelve-year-old female with malignant perivascular epithelioid cell kidney tumour with lung metastasis was found to have positive p-AKT with immunochemistry and targeted treatment with sirolimus and sorafenib was initiated after observing no response to classic sarcoma chemotherapy. A slight response was observed in tumour size and the disease was stabilized according to RECIST 1.1 criteria for a 5-month period. This achievement had not been possible with the previous schedules administered. On the third place, a nine-year old female with first local and metastasic osteosarcoma relapse with positive mTOR immunochemistry was treated with an oral mTOR inhibitor during a two-month period after failure of standard treatments. Unfortunately, progression was observed after the third month.

Another twelve-year-old male affected by a mediastinal myofibroblastic inflammatory tumour with ALK translocation detected by FISH. Disease progression was observed after standard chemotherapy (IVA regime) and surgery. Targeted treatment with ALK inhibitor ceritinib was started and a very good partial response was observed. Finally, a thirteen-year-old female with a stomach GIST with positive c-KIT diagnosis (CD-117) by immunohistochemistry is currently receiving imatinib after radical surgery and has achieved complete response.

Future treatment options were available in 12 patients (17%) that are at the moment in complete response or receiving other standard treatments. Altogether, implementing NGS with complementary diagnostic techniques such as immunohistochemistry and FISH in a precision medicine approach for targeted treatment of sarcomas, a disease control rate of 23% would potentially be achieved. The summary of the recommendations and clinical responses are shown in Table 4.

Table 4. Clinical translation: Actionable variants detected, molecular tumour board recommendations, targeted treatments administered and results observed. EWS: Ewing sarcoma; IQ: immunochemistry; RMS: rhabdomyosarcoma; DTF: desmoid-type fibromatosis; MPNST: malignant peripheral nerve sheath tumour; LOH: loss of heterozygosity; CNV: copy number variation; MIT: myofibroblastic inflammatory tumour; GIST: gastrointestinal stromal tumour; MPECN: malignant perivascular epithelioid cell neoplasm.

TUMOUR HISTOLOGY	ACTIONABLE VARIANT	OTHER BIOLOGIC INFORMATION	COMMITTEE RECOMENDATION	TREATMENT ADMINISTERED	MAXIMUM RESPONSE
EWS	*TP53* c.742C>T (p.R248W)	*PDL-1* + (IQ 5%)	*PRIMA-1* / *PD-L1* inhibitors	No (future option)	-
Embryonal RMS	*FGFR4* c.1648 G>C (p.V550L) *FBXW7* c.1394G>A (p.R465H)	-	Ponatinib / Erdafitinib	No (future option)	-
Embryonal RMS	*TP53* c.559G>C (p.G187R) in germline	*P-AKT* + (IQ 50%)	*mTOR* inhibitor. Li-Fraumeni follow-up	No (future option)	-
Embryonal RMS	*TSC2* c.5158C>T (p.R1720W) *FGFR4* c.1648G>A (p.V550M)	*mTOR* + (IQ 100% cytoplasm)	*mTOR* inhibitor	No (future option)	-
Embryonal RMS	*PI3K* c.1624G>A (p.E542K)	-	*mTOR* inhibitor	No (future option)	-
Embryonal RMS	*FGFR4* c.1648 G>C (p.V550L) *CTNNB1* c.133_134delTCinsCT (p.S45L)	-	Ponatinib / Erdafitinib	No (future option)	-

Alveolar RMS	*SMAD4* c.302G>A (p.W101*) *SMAD4* c-370G>A (p.D124N) NRAS c.176C>T (p.A59V)	-	Palbociclib + Venetoclax	Yes (<1 month)	PD
Alveolar RMS	*TP53* c.817C>T (p.R273C)	-	*PRIMA-1*	No (future option)	-
Angiosarcoma	*TP53 c.906_907delCCinsTT (p.R303*)* *MTOR* c.6644C>T (p.S2215F)	*P-AKT* + (IQ 60% membrane and cytoplasm) Patient with Xerodermapigmentosum	*mTOR* inhibitor	No (future option)	-
DTF	*CTNNB1* c.134C>T (p.S45F)	IQ betacatenin +	Beta-catenin inhibitor	No (future option)	-
DTF	*CTNNB1* c.134C>T (p.S45F)	IQ betacatenin +	Beta-catenin inhibitor	No (future option)	-
MPNST	*ATM* c.7032G>A (p.W2344*)	11q deletion	*PARP* inhibitor	Yes (2 months)	SD
Osteosarcoma	*RB1* c. 361 C>T (p.Q121*)	Gain chromosomes: +14,+20,+21, Segmental imbalances: 2p, 17q. LOH 3, 16. *PD-L1* + (IQ 20%)	*PD-L1* inhibitors	No (medical decision)	-
Osteosarcoma	-	*mTOR* + (IQ 60% cytoplasm)	*mTOR* inhibitor	Yes (2 months)	SD
Undifferentiated sarcoma	-	CNV: Deletion in genes *ARID1A, MTOR, NRAS, SDHB*	Tazemetostat / Vorinostat	No (future option)	-
MIT	-	FISH: *ALK* +	Ceritinib	Yes (3 months)	PR
GIST	-	IQ: C-KIT+ (CD117)	Imatinib	Yes (20 months)	CR
MPECN	-	*P-AKT* + (IQ 100%)	Sirolimus + Sorafenib	Yes (5 months)	SD

DISCUSSION

Genetic variation is one of the main characteristics of paediatric sarcomas. This is mostly explained because despite being originated from a mesenchymal cell, they constitute different histologic entities with different genomic landscapes that explain their unequal behaviours. Beside pathology, chromosomal segmental aberrations, changes in ploidy and specific gene alterations are routinely used in order to guide intensity of treatment in paediatric oncology protocols.

It is worth noting important differences spotted when comparing adult with paediatric NGS studies in sarcomas. Epidemiologically, sarcomas represent less than 1% of all solid malignant cancers in adult population while they represent 20% of all paediatric solid malignant cancers. Therefore, the first main difference lies in the fact that the magnitude of the problem is proportionally much higher in paediatric population. Furthermore, adult type cancers such as epithelial neoplasms arise after accumulation of multiple sequential mutations directly linked to environmental exposures, and arise within differentiated adult tissues. Mesenchymal tumours, such as sarcomas appear both in adult and paediatric population. However, specific histologic subtypes and clinical progression are age-dependent, suggesting differential pathogenetics and underlying molecular mechanisms for tumour initiation and clinical behavior in the different age subgroups.

In this study, we found that the overall mutational load in our cohort was relatively low when compared to adult studies. This might be explained by the fact that adult sarcomas are mostly driven by mutagenic exposure from environmental factors, whereas most of paediatric cancers contain a relatively small number of mutations and frequently display unique gene rearrangements. Although this restricts the targeted treatment to available drugs, it also makes them attractive candidates for drug discovery.

In order to improve outcome, international efforts amongst cooperative groups have been carried out developing genomic precision medicine programs. These programs aim to bring NGS approaches into the clinical practice and require the identification of patients that might benefit from targeted therapies. Once these targets are identified, in paediatric population it is important to communicate these results, as well as possible toxicities observed by compassionate use basis as dosing is more complex when compared to adult population. Hence, the importance of promoting paediatric phase I clinical trials in order to titrate infant dosing.

In this study, we conclude that the most frequent somatic mutation observed in paediatric sarcomas occurs in TP53 (27% of the pathogenic mutations detected by NGS). This information correlates with adult sarcoma cohorts such as the study presented by Groisberg et al. Xiaosheng et al.

compared overall survival (OS) time between TP53-mutated and TP53-wildtype cancers in 20 adult cancer types. They reported that patients with TP53 mutations had lower survival compared with those without TP53 mutations in colon, lung and pancreas adenocarcinoma, acute myeloid leukemia and other epithelial cancers. In paediatric oncology, the clinical significance of somatic TP53 mutations remains unrecognized and no routine testing or therapy intensification is considered. Recent studies suggest that mutation in TP53 in localized EWS is not a reliable prognostic marker. In order to target TP53, small molecules that reactivate mutant p53 by restoring wild-type conformation have been identified by various approaches. APR-246 alone is currently being tested in prostate or ovarian cancers or in combination with azacitidine in myeloid malignancies in adult phase I-II trials. No studies are currently recruiting paediatric population.

Mutations in Fibroblast Growth Factor Receptor 4 (FGFR4) have also been described in paediatric sarcomas, most outstandingly in RMS. Higher FGFR4 expression in RMS has been associated with advanced-stage cancer and poor survival. FGFR4 pathogenic mutations appear in 33% of the embryonal RMS studied in our cohort and all of them received a targeted recommendation therapy. FGFR4 codifies for a cell surface tyrosine kinase (TK) receptor that is involved in normal myogenesis and muscle regeneration. It has been reported that human embryonal RMS cells have increased FGFR4 mRNA expression compared to normal human myoblasts, and FGFR4 pathway blockade decreases proliferation. In fact, over-expression and mutational activation of FGFR4 has been reported in RMS, promoting tumour progression. FGFR4 signaling is also a com-mon mechanism of oncogenesis in fusion positive RMS (usually alveolar subtype).

Alterations in FGFR4 are clinically relevant because they are actionable targets in patients with RMS. New generation of multi-kinase inhibitors are under current development such as ponatinib (AP-24534), an orally administered TK inhibitor that was initially developed as an inhibitor for BCL-ABL. Ponatinib recently received FDA approval for the treatment of adult patients with Philadelphia chromosome positive acute lymphoblastic leukemia and chronic myeloid leukemia resistant to other TK inhibitors. Inhibition profile of ponatinib includes other TK such as c-KIT, PDGFR, FLT3, SRC and FGFR. Moreover, inhibition of FGFR family members with ponatinib has been demonstrated in preclinical models with bladder cancer, endometrial cancer, breast, lung and colon cancer. Samuel Q. Li et al tested a panel of RMS cell lines over-expressing FGFR4, all of them exhibiting sensitivity to five different TK inhibitors including ponatinib, cediranib, nintedanib, dovitinib and danusertib. They observed that ponatinib resulted to be the most powerful FGFR4 inhibitor, inhibiting both, mutated and wild-type FGFR4 cell growth. It also inhibited tumour development expressing FGFR4 in vivo. Currently, ponatinib is being tested in clinical trials including paediatric patients (NCT03934372). Erdafitinib is also being tested in a phase II trial for tumours with FGFR mutations. (NCT03210714).

The CTNNB1 gene provides instructions to form the protein beta-catenin. The relationship between the Wnt/beta-catetin signaling pathway and desmoid-type fibromatosis (DTF) has been widely studied and it has been reported that the vast majority of DTF tumours (up to 85%) harbor a mutation in exon 3 of the CTNNB1 gene (beta-catetenin). These mutations lead to an abnormally stable beta-catenin protein that is more resistant to proteolytic degradation and accumulates within the cells. Excess of beta-catenin promotes an uncontrolled proliferation of cells, allowing the formation of DTF.

Therapeutic options targeting Wnt/betacatenin signaling pathway are limited and have not been tested in paediatric population. Accumulation of beta-catenin in the nucleus triggers transcription of Wnt-specific genes responsible for the control of cell fate decisions. The development of drugs targeting mutated or altered beta-catenin signaling, or its interaction with CBP, TCF, GSK3β or APC (which are essential to complete its function) has been difficult due to the toxicity of the new compounds. Several of them are currently in Phase 1 clinical trials, such as the PRI-724 molecule (NCT01302405, NCT02413853, NCT01764477, and NCT01606579) that prevents the interaction of beta-catenin with CBP. Despite these and other approaches, there are no clinical trials available for paediatric patients with Wnt/beta-catenin inhibitors. All DTF studied in our cohort har-bored mutations in CTNNB1.

In the study, a patient with malignant nerve sheath tumour and ATM mutation was treated with PARP inhibitors in combination with olaparib. The ataxia telangiectasia gene (ATM), localized in 11q22-q23, plays an important role in maintaining genomic integrity. It regulates the double-strand DNA breaks repair and activates different checkpoints in the cell cycle. ATM is associated with some types of leukemia and lymphoma and it has also been described in neuroblastoma with 11q deletion. Poly ADP-ribose polymerase (PARP) is a protein that signals DNA damage and contributes towards DNA repair. PARP catalyzes the addition of ADP-ribose to DNA, helicases, topoisomerases and histones. It also has a critical role in transcription, cellular replication, gene regulation, differentiation, spindle maintenance and protein degradation. PARP inhibition produces persistent single strand DNA breaks leading to double strand DNA breaks and finally produces DNA damage leading to apoptosis and cell cycle arrest. Preclinical studies show that ATM mutated neuroblastoma cells also succumb to apoptosis when treated with PARP inhibitors and neuroblastomas with 11q deletion are extremely sensitive to conventional chemotherapy combined with PARP inhibitors. The patient in the study managed a short period of stable disease but progressed rapidly afterwards. Other mutations considered as uncertainly significant in ATM have been detected but no recommendations were issued because no previous clinical evidence was found. Currently, early phase trials with PARP inhibitors are recruiting paediatric patients with diverse malignancies.

Recent studies in RMS have revealed recurrent mutations in the RAS pathway, particularly affecting NRAS. Dolghik et al demonstrated that PIK3CA played a critical role in the activation of the PI3K/AKT/mTOR pathway in NRAS mutant RMS. They noted that NRAS-mutated RMS cells particularly relied on PIK3CA to prevent cell death upon NRAS silencing or MEK inhibition. Their data showed that specific PIK3CA knockdown was sufficient to cooperatively trigger cell death together with pharmacological MEK inhibition. In addition, pharmacological inhibitors of MEK or NRAS knockdown synergize with the PIK3CA specific inhibitor BYL719 to trigger cell death in NRAS-mutated RMS cells. All this data supports the rationale for the combination of MEK and PIK3CA specific inhibitors in NRAS mutated RMS. This recommendation is a future option for one of the patients studied in our cohort.

In this study, a patient diagnosed with c-KIT positive (CD-117) GIST was treated with imatinib and so far, has maintained complete response after surgery. Another patient with ALK+ myofibroblastic inflammatory tumour received treatment with ceritinib obtaining a partial response. Both of these rare sarcomas have a classical alteration that has been widely reported before.

In conclusion, we have observed that the incorporation of NGS results into paediatric sarcoma clinical practice is feasible and allows personalized treatments with acceptable disease control rates in the relapse setting. However, some of the recommendations are still issued taking into account other biologic information such as pathology, immunochemistry or FISH techniques and therefore, the real paper of NGS on its own in paediatric sarcomas could be smaller. At the moment, as the integration NGS as a routine diagnostic technique has been limited this is difficult to estimate, although the situation is changing and sequencing studies are gradually becoming wide-spread. Further investigations are required to confirm this hypothesis.

In this study, up to 23% of the patients would obtain clinical benefit by implementing this precision medicine approach complementing routine diagnostic techniques. Although the understanding of paediatric sarcomas' biology has improved in a relatively short period of time, outcomes in high-risk tumours remain poor and regarding new therapeutic strategies, very few advances have been highlighted. This emphasizes that strong, international efforts are still required in order to improve implementation of new diagnostic techniques, impulse paediatric drug development and access to clinical trials in childhood. Finally, we would like to stress the importance of treating childhood, adolescent and young adult sarcomas and other types of cancers in specialized units, with all the available expertise and distinct requirements involving this particular population.

MANUSCRIPT 4

SURVEY ON PAEDIATRIC TUMOUR BOARDS IN EUROPE: CURRENT SITUATION AND RESULTS FROM THE EXPO-R-NET PROJECT

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MANUSCRIPT 4

SURVEY ON PAEDIATRIC TUMOUR BOARDS IN EUROPE: CURRENT SITUATION AND RESULTS FROM THE EXPO-R-NET PROJECT

Introduction

Multidisciplinary care is the hallmark of high-quality cancer management. This statement has been supported over the last decades where individual opinions have been displaced by collective and multidisciplinary decisions in approaching a patient with a complex oncologic disease. Although childhood cancer is a rare disease, it is the first cause of non-accidental death in childhood and sharing of expertise is paramount in this field. Several studies have shown that tumour boards lead to changes in diagnosis and staging of cancer patients, affect management decisions, and increase quality of care. Therefore, they should be an integral part of adult and childhood cancer patient care around the world.

A paediatric tumour board comprises a multidisciplinary team of experts including paediatric oncologists, radiologists, surgeons, radiotherapists, pathologists and other disciplines as relevant for the respective cancer case to discuss patients' clinical cases where the diagnosis and treatment plan are complex. The patients' clinical management following a discussion in a tumour board takes into account the opinion of several experts participating in a particular meeting.

ExPO-r-Net (European Expert Paediatric Oncology Reference Network for Diagnostics and Treatment) is a project supported by the European Commission aiming to prepare for a Paediatric Oncology European Reference Network. This network will facilitate the cross-border healthcare for children and young people with cancer to access expertise and specialist healthcare in a EU Member State other than the Member State of affiliation. European Reference Networks (ERNs) are a feature of the EU Directive on Cross-Border Healthcare aiming to unite the best specialists from across Europe to tackle complex or rare medical conditions that require highly specialized healthcare and a concentration of knowledge and resources.

One of the missions in the ExPO-r-Net project is the creation of a virtual tumour board network across Europe. A virtual tumour board is a special type of multidisciplinary meeting based on videoconference systems that can involve experts from different centres. For practical reasons these

tumour board meetings need to be “virtual” using videoconference systems to allow regular meetings and discussions between experts in different countries. The virtual tumour board can, therefore, convene regularly and rapidly to discuss urgent cases.

As a first step towards a European Paediatric Oncology Virtual Tumour Board Network, ExPO-r-Net aimed to identify European already existing tumour boards of paediatric oncology units to investigate the kind of technologies and logistics that are in place in the different countries and to explore current differences between European regions.

Materials and Methods

A standard questionnaire with twenty questions regarding several features of multidisciplinary practice in paediatric oncology was designed by Hospital Universitari i Politècnic La Fe (València). Data collected included information from the centres, number of patients treated per year, infrastructure for meetings, organization/logistics and clinical decision-making. The majority of the questions were multiple choice but some open questions, which allowed free text explanations, were also included. The questionnaire went through an internal review first and, finally, an external review was done by the University of Birmingham (UK), as part of the quality assessment work package in the ExPO-r-Net project.

After finalisation, the survey was distributed to the national leaders of the European National Paediatric Haematology and Oncology Societies (NaPHOS) by email. Contacts were provided by the European Society for paediatric oncology (SIOPE). Participants were sent an introductory email asking for information about the paediatric oncology treatment situation in their country (number of centres and number of patients treated per centre). If participation was accepted, a second email with an invitation link to complete an electronic survey, set up via a freely available web-survey tool, was forwarded. The survey was disseminated by the NaPHOS in each country to all the centres with a paediatric oncology unit. Surveys were completed online between July 2015 and March 2016. A simplified version of this questionnaire is presented in this paper (Figure 1).

Figure 1. Simplified version of the european questionnaire

1. Number of new cancer patients seen at your institution per year?
A. < 15 cases/year. B. 15-25 cases/year. C. 25-50 cases/year. D. 50-75 cases/year. E. > 75 cases/year

2. Do you have a Paediatric Multidisciplinary Tumour Board? A. Yes. B. No

3. How often do you meet? A. Weekly. B. Biweekly. C. Monthly. D. Other

4. What is the usual length of your Paediatric Tumour Board Meeting? A. Up to 1 h B. 60–120 min C. 120–180 min D. 120-180 min. E. More than 3 hours

5. Indicate the number of cases discussed by your Paediatric Tumour Board Meeting in a single meeting: A. 1–2 cases B. 3–4 cases C. 5–6 cases D. 7–9 cases E. 10–12 cases F. > 12 cases

6. The Paediatric Tumour Board Meeting room has: A. A specific room for this purpose B. Equipment for projecting and viewing radiology images/specimen biopsies C. Equipment connected to PACS (Picture Archiving and Communications System) D. Access to retrospective images/reports during the meeting

7. In the case of specialist not belonging to your institution, how do you manage it? A. They move to our institution for the meeting B. Teleconference C. Videoconference D. They do not participate

8. Does your Paediatric Tumour Board Meeting room have videoconferencing facilities? A. Yes, H. 323 Video Conference System B. Yes, web-based videoconference system (Skype, Adobe Connect, etc.) C. No

9. Are patients informed that a Paediatric Tumour Board will review their treatment/care? A. Yes. B. No

10. If Paediatric tumour board recommendations are not followed, is the patient informed? A. Yes. B. No.

11. Do you have any standard operational procedures? A. Yes. B. No.

12. Do you usually make a report with the final recommendations? A. No. B. Yes, in paper format. C. Yes, in electronic database.

13. Paediatric Tumour Board recommendations are: A. Mandatory B. Optional

14. Is there electronic data exchange with other hospitals? A. Yes. B. No.

15. Do you participate in any Virtual Paediatric Tumour Board? A. Yes B. No.

Results were collected and analyzed at Hospital La Fe. Questionnaires from 30 countries were received and a descriptive analysis was performed from of the information provided. For comparative analysis between geographical regions, respondents were grouped into four European geographical regions: Northern, Central, Southern and Eastern, following the methodology of the EUROCARE-5

population-based study. Statistical significance among regions was assessed by multinomial logistic regression and $p<0.05$ was considered as statistically significant.

Results

1. The countries

The following 30 European countries were included in the study: Austria, Belgium, Bosnia-Herzegovina, Bulgaria, Croatia, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Lithuania, Macedonia, Netherlands, Norway, Poland, Portugal, Romania, Serbia, Slovak Republic, Slovenia, Spain, Sweden, Switzerland, and United Kingdom.

2. Total responses

A total of 23 NaPHOS responded (77%) and distributed the questionnaire to the respective centres in their country specialised in childhood cancer care. This corresponds to a total of 212 institutions; 121/212 (57%) responded.

3. Existence of multidisciplinary tumour boards and number of patients

The first question of the survey addressed the existence of a tumour board in the institution; 110/121 centres (91% of the respondents) confirmed regular multidisciplinary tumour board meetings while in 11 centres (9%) they were not part of routine practice. All of the latter were smaller centres treating less than 25 new cases per year.

The survey also reflected that 62% of centres had an annual accrual rate of more than 50 patients, 23% accrued between 25 and 50 patients and only 15% accrued less in varying numbers.

4. Meeting organization

The frequency of the interdisciplinary meetings was also studied. Weekly boards were held in 43% of the centres, biweekly meetings in 23%, and monthly ones in 21% and other frequencies in the remaining 13% of the centres. The duration of tumour board meetings was reported to be less than 60 minutes by 42% of centres, 60 to 120 minutes in 45% and 120 to 180 minutes in 9% whilst 4% reported duration of more than 180 minutes.

The number of cases discussed during each meeting is another important parameter: more than 12 patients are addressed per tumour board in 9% of the centres, 7-11 cases in 26%, 3-6 cases in 48% and 1-2 cases in 17%.

5. Infrastructure for meetings

In 44% of the institutions, standard operational procedures are in place whilst they do not exist in 56%. Reports summarizing discussions held and recommendations are available in paper format for 45% of the institutions, electronic format for 43% and are unavailable for 12%.

We also addressed the availability of a specific room to hold tumour board meetings: 66% of the centres had a specific room for this purpose whilst 34% didn't. A projector to visualize images for all the staff present was available in 80% of sites. Of those 68% had access to PACS (Picture Archiving and Communication System). Finally, 77% of centres had direct access to the clinical records from the same room.

6. Clinical decision making

The decision-making processes at sites have been addressed and found that discussions were particularly fruitful if all specialists involved in the patients' care were present. However, in 55% of the centres discussions happen without all involved specialists being present while in 45% of them a particular patient was not discussed unless all disciplines were represented.

In the vast majority of cases (93%), patients were informed that their case was going to be discussed in a multidisciplinary forum whilst this was not the case in 7% of centres. While 53% of the institutions made recommendations or action points addressed in the tumour board mandatory, there were regarded as only optional actions by 47% of the centres.

7. Information exchange

The survey also addressed the participation of members from different institutions. Physical movement of physicians occurred in 54%. Videoconferencing as a mean to share common discussion was reported by 20% of the centres whilst 5% are using teleconferencing. Finally, in 19% of centres specialists from different institutions never participate in these meetings.

On a national, basis second consultations are requested by 62% of the centres whilst internationally 64% were reported. It must be mentioned here that some of the reporting institutions located in smaller countries are the only ones for paediatric oncology. Therefore, there is no possibility of a national consultation.

8. Virtual / ICT logistics

Evaluation of the current state of videoconferencing and IT facilities at sites was one of the main themes to be addressed for the purpose of a baseline. Centres were asked if videoconferencing facilities are available according to the H.323 standard for audio and video multimedia teleconferencing, web-based videoconference systems (Skype, Adobe Connect, Webex, etc) or if no facilities were available. As reported in this survey of the date of this report, 52% of the centres didn't have any kind of access to videoconferencing, 28% had access to web videoconferencing systems but only 20% had H.323-based videoconferencing systems.

Electronic data exchange was done in 57% of the institutions. In 77% of them this happens prior to the tumour board so that cases may be well prepared. Of the 43% of centres currently not exchanging electronic data, 33% indicated that this would actually be an urgent necessity.

Finally, we specifically assessed the number of centres that already participate in virtual tumour boards since this is a major issue related to the set-up of European cross-border, virtual tumour board network. Surprisingly, only 14% of the centres participated already in virtual meetings at the time of evaluation. The vast majority of centres in Europe (86%) don't usually carry out this type of meetings.

9. Comparative analysis between regions

To investigate the differences in the functioning of tumour boards between geographical regions, comparative analysis was performed taking into consideration 108 responses from 21 countries. Countries with a rate of response below 40% were excluded and the answers from the centres without multidisciplinary boards were also excluded from analysis.

The respondents were grouped into four geographical regions in Europe: Northern, Central, Southern and Eastern.8 Statistical significance among regions was assessed by multinomial logistic regression and $p<0.05$ was considered indicating statistical significance. Global results of the percentages of availability of certain issues related to multidisciplinary boards are provided in Table 1.

Table 1. Percentages of tumour board facilities available in the different European geographical regions

	Northern (n=16)	Central (n=23)	Southern (n=55)	Eastern (n=14)
Existence of SOPs	67%	71%	29%	36%
Specific room for tumour board	81%	96%	60%	36%
Projector available	94%	91%	74%	43%
Videconferencing facilities	69%	87%	33%	21%
Virtual tumour boards	44%	30%	4%	0%

A comparison of the results obtained from the answers in Northern and Central Europe with Southern and Eastern European countries has been done. Statistically significant differences in several

aspects regarding the functioning and the availability of facilities for tumour boards had been observed.

Firstly, there were significant differences in the existence of standard operational procedures (SOPs) with Eastern and Southern countries having less SOPs when compared with Northern and Central countries (p=0.002). Hence, in Northern and Central European countries more standardization to support the functioning of the meetings was in place.

Differences have also been observed regarding the availability of a specific room to carry out the tumour board meetings and a projector to view the images of the patients' discussed. Significant differences (p=0.001) in both cases have been observed.

Regarding videoconferencing facilities, respondents were asked if videoconferencing systems in their institution were H.323 based, web based or if no technological facilities were available. The majority of the centres in Southern and Eastern European countries answered that there were no videoconferencing facilities available to allow communication with other national or international centres. On the other hand, more than 50% of the institutions in North and Central Europe had either H.323 or web-based videoconferencing tools to support communications with other centres (Figure 2). Finally, virtual tumour boards were in place in 44% of the Northern European centres, in 30% of the central European ones, whilst only 4% of the Southern and none of the Eastern European responding centres had such facilities available. Differences in videoconferencing facilities and virtual tumour boards are shown graphically in Figure 3.

Figure 2. Percentage of videoconferencing tools available in european geographical regions

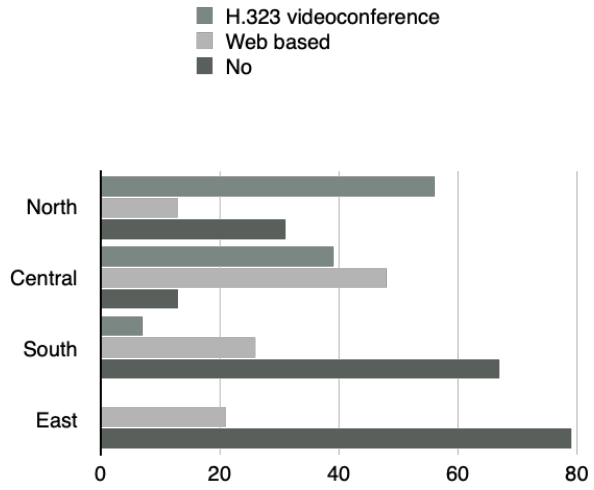
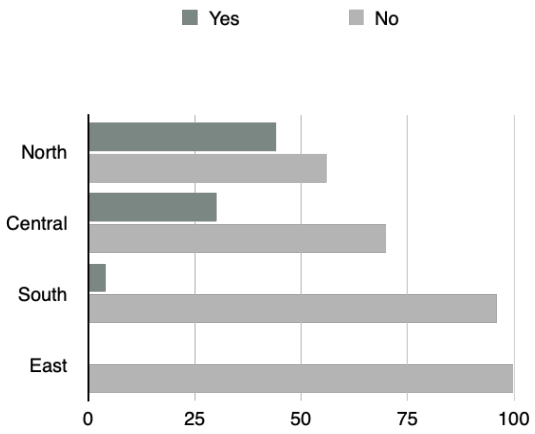


Figure 3. Existence of virtual tumour boards in european geographical regions (percentage)



Discussion

Multidisciplinary tumour board meetings are conducted worldwide to manage well with the help of multidisciplinary teams the treatment of cancer patients. They deliver a higher standard of care based on simultaneous involvement of different specialists for specific purposes related to the establishment of the right diagnosis and the planning of respective treatment components. Several national and international guidelines emphasize the importance of multidisciplinary team management of children and adolescents with cancer and need to be reinforced as standard of care for paediatric patients with cancer.

Most of the paediatric oncologists involved in this survey confirmed that tumour boards are an essential tool for the management of complex cases. In contrast to adult cancer tumour boards addressing rather patients with a single adult cancer entity in one forum, most paediatric tumour boards cover a wide scope of paediatric cancer entities. All paediatric cancers are rare by definition and hence children benefit from treatment plans discussed and agreed upon in multidisciplinary teams. Once annual accrual rates are below 100 patients, the experience of a single site remains limited for specific very rare entities. Hence, the whole paediatric oncology community may benefit from the introduction of a well-established virtual tumour board system to better share knowledge and expertise particularly in complex cases and very rare cancer entities.

In the vast majority of surveyed countries across Europe tumour boards take place to ensure appropriate decisions in the best interest of the respective child. Although tumour boards apparently already existed in more than 90% of the surveyed centres, the organization and facilities available were quite different in respective countries. Therefore, it is necessary in Europe to establish harmonised processes when aiming to create an international virtual tumour board network. In addition, potential legal responsibilities of physicians participating in virtual tumour boards need to be addressed. However, the responsibility ultimately needs to stay with the respective treating physician and hence inside the respective health care provider responsible for the patient's treatment.

Most importantly this survey showed important, statistically significant, differences related to many tumour board aspects among European geographical regions. Such differences were particularly evident with regard to availability of equipment for videoconferencing and communication processes. This lack of equity between different countries may explain in part why as of today paediatric patients suffering of the same cancer entity have different chances of survival depending on the country where they receive treatments. To overcome such current inequalities across Europe, the ExPO-r-Net project and the creation of the European Reference Network (ERN) for Paediatric Cancer (PaedCan) both are aiming for better access to expertise advice and facilitation of communications between EU member states. Whenever possible the information and expertise should travel rather than the patients and only those in need for specific interventions requiring a high level of expertise will be directed to centres able to provide this very special care.

This is the first European survey within the Paediatric Oncology community involving such a wide range of countries and different health systems. A previous survey was done at a national level in Spain showing the picture of a single country. However, this survey reflects the current practice of paediatric oncologists working in multidisciplinary teams in order to take important treatment decisions together all aiming to achieve the best possible outcome for a particular patient in Europe. The whole community may benefit from improved and harmonised organisation of virtual tumour boards to assure best care of paediatric cancer patients. There is a real need for well-functioning, interoperable ICT solutions across Europe and respective national health systems.

The ExPO-r-Net project has contributed to identify the needs across Europe and has developed tumour board solutions for a few very rare tumour entities. The future for the established ERN PaedCan network lies within the tools building on this experience, and provided now by the European

commission to achieve the next level of sharing and caring within the network and in particular, regarding identified cross boarder health care patient management.

4. FINAL CONCLUSIONS

In the studies that form this doctoral thesis, the importance of molecular techniques for the diagnosis and risk stratification of paediatric tumours such as molecular karyotyping and next-generation sequencing has been proven once again. Segmental chromosomal alterations in neuroblastoma and more specifically, within the chromosome, point mutations, insertions, deletions, translocations and CNV detected by next-generation sequencing in sarcomas, distinguish between more or less aggressive tumour subtypes, with a different response to chemotherapy and different survival rates. At the moment, there are very few cytogenetic and molecular factors with enough evidence to be considered in first-line therapy stratification but some of the conclusions drawn by previous clinical trials already include specific molecular information of the tumours (11q deletion, MYCN amplification, FOXO1 gene fusion, etc) and this has enabled us to intensify the treatment load in the cases with a worst biological prognosis.

As more data becomes available, taking into account the results of these complementary techniques, it is very likely that new risk groups will be incorporated. In fact, some of the future protocols of European cooperative groups such as protocol FaR-RMS for rhabdomyosarcoma within the EpSSG and future SIOPEN protocols for high-risk and low and intermediate risk neuroblastoma consider biological factors for an initial risk group stratification.

Below, I detail the main and other more specific conclusions drawn from the publication of the four articles.

Main conclusions

- 11q deletion in NB confers a poor prognosis in all tumour stages and particularly in localized and Ms tumours. Its negative connotations in prognostic terms are comparable to amplification of MYCN.

- Segmental chromosomal alterations in NB are not randomly distributed, but are concentrated in recurrent chromosomes and appear in characteristic patterns according to previously established prognostic factors (age, INRGSS stage and MYCN status).

- The incorporation of massive sequencing studies together with pathological techniques in paediatric sarcomas is plausible, can benefit up to 23% of patients and allows personalized treatments to be found, especially in the relapse setting.

Specific conclusions

- 11q deletion in NB is related to older age at diagnosis, more advanced stages and it is associated with a higher risk of relapse.

- The co-existence of 11q deletion and MNA is extremely rare.

- There is a relationship between 11q deletion and MYCN gain whose origin is unknown.

- The presence of SCA in NB is associated with older age at diagnosis and with MNA.

- The SCA that is most frequently associated with the presence of other segmental alterations is 11q deletion followed by 4p deletion.

- Numerical gain on chromosome 19 is associated with the absence of SCA, less advanced INRGSS stage and better prognosis.

- TP53 is the most frequent mutation detected by next-generation sequencing in paediatric sarcomas, followed by FGFR4 and CTNNB1.

- The genomic profile of paediatric sarcomas and other types of paediatric cancer differs considerably from the genomic profiles of neoplasms in adults.

- Paediatric tumour boards are an essential tool for the management of complex cases and exist in 91% of European centres.

- There is a lack of equity and important differences in some paediatric tumour board aspects amongst European geographical regions, especially regarding availability of videoconferencing equipment and communication processes.

Possible future research possibilities

As future research possibilities I would suggest a detailed exploration of the biological relationship between 11q deletion and MYCN gain. It is very striking that there is an inverse relationship between MYCN amplification and that 11q deletion is associated with MYCN gain. Possible comparative trials could also be proposed by intensifying the therapy of patients with 11q deletion where this biological finding does not increase the therapeutic burden.

Another possible, less investigated issue is prognostic involvement of certain numerical chromosomal alterations in NB. Since segmental chromosomal alterations have such a strong influence in the prognosis of NB, few studies have focused on analyzing separately the possible influence of gains and losses of complete chromosomes in NB. This could be of real interest and there are recent publications on this topic in addition to the second publication that constitutes this thesis.

Regarding next-generation sequencing in sarcomas we have presented a single cohort of 70 sarcomas that includes up to 13 different histologies. Therefore, I think that many research lines remain to be explored, including a detailed study in each tumour subtype with larger paediatric cohorts. As the use of these techniques becomes more widespread, we will be able to draw more focused conclusions for each tumour type and this may encourage research into new lines of personalized treatment in paediatric cancer.

Finally, as for paediatric tumour boards, one of the deficits revealed by the COVID19 pandemic in 2020 has been the precariousness of the health systems in terms of new technologies, information technology and videoconferencing systems. The complex cases that are treated in paediatric oncology units shouldn't be managed without multidisciplinary board discussions and there is an immediate and real emergency in the implementation of appropriate videoconferencing and communication facilities in health-care centers with all the research lines that can go along with this audiovisual development within the health systems.

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6. RESUMEN DE LA TESIS EN CASTELLANO

1. INTRODUCCIÓN

NEUROBLASTOMA

Tumor descrito por primera vez en por el patólogo alemán Virchow en 1864, el neuroblastoma (NB) es el tumor sólido extracraneal más frecuente entre la población pediátrica, y forma parte de los tumores derivados del sistema nervioso simpático (en cadena simpática o ganglios simpáticos). Otros tumores también derivados de la cresta neural son el ganglioneuroblastoma y el ganglioneuroma. Las características del neuroblastoma son las siguientes:

- Capacidad de madurar de formas indiferenciadas (neuroblastomas) a formas maduras (ganglioneuroma).
- Capacidad de regresar espontáneamente.
- Buen pronóstico en pacientes menores de un año.
- Comportamiento altamente agresivo en formas de alto riesgo (con metástasis, o presencia de amplificación del oncogén MYCN).

Diagnóstico

El diagnóstico del NB se basa en tres pilares fundamentales: la clínica, las pruebas de imagen y en especial la histopatología, ya que otorga el diagnóstico definitivo y posibilita su estratificación pronóstica, la cual es esencial para decidir el esquema de tratamiento adecuado. Ante la sospecha de tumoración torácica o abdominal, se realizan pruebas de imagen que muestran la tumoración: radiografía de tórax o ecografía abdominal. El estudio del tumor primario y de sus posibles metástasis debe completarse con TC o RM, siendo la

RM la prueba de elección para tumores con afectación medular y por la evitación de radiación ionizante.

Si existe sospecha de NB, la prueba a realizar es una gammagrafía ósea con I123 MIBG (meta-yodo benzil-guanidina). La MIBG es captada de forma selectiva, utilizándose una gamma-cámara que emite una imagen corporal total permitiendo la identificación de posibles núcleos metastásicos. A pesar de que, recientemente, la tomografía por emisión de positrones (PET) con F18-FDG haya ganado protagonismo entre algunos grupos, la I123 MIBG continúa siendo de elección al ser más específica. No obstante, El PET puede ser útil en casos MIBG negativos, o en pacientes con afectación de partes blandas. Por otro lado, se están empezando a considerar nuevas técnicas de imagen nuclear como SPECT (Single Photon Emission Computerized Tomography) o PET/TC, para obtener una mejoría en la resolución espacial y permitir una medición cuantitativa de la afectación.

En cuanto al estudio inicial del NB, debe hacerse un aspirado bilateral de médula ósea (en ambas crestas ilíacas) junto con biopsia bilateral de médula ósea. La afectación de una sola muestra ya diagnostica NB metastásico, mientras que para descartar afectación metastásica de médula ósea se precisa que las cuatro muestras obtenidas sean negativas (los requisitos y métodos técnicos son revisados por la iniciativa INRG, o International Neuroblastoma Risk Group). Sin embargo, la obtención de material tumoral en ocasiones puede ser complicada, debido a la localización del tumor. Por ello, el INSS (International Neuroblastoma Staging System) establece los siguientes criterios al diagnóstico:

- Diagnóstico anatomopatológico de NB en una muestra de tejido tumoral, con o sin inmunohistoquímica o elevación de catecolaminas.

- Infiltración de médula ósea en el aspirado o en la biopsia por células tumorales junto con aumento de catecolaminas urinarias.

La importancia de la obtención de material tumoral también reside en el diagnóstico molecular, pues posibilita su caracterización biológica: tanto el oncogén MYCN como el

perfil de alteraciones cromosómicas, así como el gen ALK, todos ellos con impacto pronóstico y en la evolución terapéutica.

Adicionalmente, el estudio inicial incluye marcadores en sangre y en orina recogida en 24 horas. Los marcadores en sangre incluyen LDH, enolasa específica neuronal y ferritina, mientras que los marcadores en orina evalúan la excreción de catecolaminas como VMA (ácido vanilmandélico), HVA (ácido homovanílico) y dopamina, frecuentemente elevados en el NB. Si bien la elevación de estos marcadores no es específica, puede servirnos de apoyo y orientación al diagnóstico en la clasificación pronóstica.

Clasificación

Hay numerosos factores con valor pronóstico en el NB, aunque algunos pueden no tener un valor independiente y se presentan asociados con otros de mayor peso. Los factores admitidos por todos los grupos de cooperación son la edad, el estadio de la enfermedad y la amplificación del oncogén MYCN. Los factores pueden dividirse en:

a) Factores clínicos (edad al diagnóstico y estadio tumoral según el INRGSS)

b) Factores histopatológicos (categoría histológica y grado de diferenciación tumoral)

c) Factores biológicos (amplificación del oncogén MYCN, presencia o no de aberraciones genéticas en el brazo largo del cromosoma 11 y ploidía).

a) Factores clínicos

EDAD AL DIAGNÓSTICO: Los lactantes menores de 18 meses, tanto si presentan un tumor loco-regional o enfermedad diseminada, tienen un pronóstico más favorable que los pacientes mayores de 18 meses. En cambio, los pacientes mayores de 5 años con

enfermedad metastásica al diagnóstico tienen un mal pronóstico, aunque su enfermedad sigue un curso más lento en comparación a niños más pequeños (de entre 1 año y medio y 5 años).

ESTADIO CLÍNICO: El primer sistema de estadiaje desarrollado para el NB fue el INSS en 1993, cuyo objetivo era obtener un sistema de estadiaje post-quirúrgico para permitir un punto de encuentro común entre los diversos grupos de trabajo de cada país. No obstante, al contar con el factor de grado de resección tumoral, esta clasificación no puede incluir casos de NB sin tratamiento quirúrgico y además dependía de la destreza y técnica quirúrgicas. Por ello, el INRG desarrolló el INRGSS en 2009: una clasificación pre-quirúrgica que contempla la presencia o no de factores de riesgo derivados de pruebas de imagen (IDRF) y de metástasis al diagnóstico. Los nuevos grupos de estadiaje obtenidos fueron:

b) Factores histopatológicos

En cuanto a la anatomía patológica del NB, sus células son pequeñas, azules y redondeadas, y los dos rasgos distintivos son el grado de diferenciación del tumor y la presencia de células de Schwann o estroma. Atendiendo a estos criterios, los tumores neuroblásticos pueden ser:

- Neuroblastoma. Pobre en estroma. Presencia de al menos 50% de neuroblastos en la masa tumoral, agrupados en nidos rodeados de tabiques fibromusculares. Se distinguen tres tipos: indiferenciado, pobremente diferenciado, y en diferenciación (al menos 5% de las células muestran diferenciación hacia células ganglionares)

- Ganglioneuroblastoma. Componente mayormente glioneuromatoso, y en menor medida de neuroblastoma. Se distinguen dos subtipos: nodular (zonas compuestas ricas en estroma y pobres en estroma) y entremezclado (rico en estroma).

- Ganglioneuroma. Estroma schwaniano predominante. Se distinguen dos subtipos: en maduración (neuroblastos en diferentes estadios) y maduro (células de estroma maduro y ganglionares).

En función de la edad del paciente y de las características anatomopatológicas del tumor, la INPC (International Neuroblastoma Pathology Classification) distingue entre histología favorable e histología desfavorable, adaptado a partir de la clasificación original de Shimada et al:

c) Factores biológicos

ONCOGÉN MYCN: Considerado el factor biológico más relevante, el oncogén MYCN o N-MYC se localiza en el cromosoma 2p y se encuentra amplificado en un 20-25% de los casos primarios de NB. La amplificación es una característica que del tumor que aparece al diagnóstico y que no se modifica con el curso de la enfermedad. Es un factor de mal pronóstico, ya que se relaciona con un estadiaje más avanzado, una rápida progresión de enfermedad, baja supervivencia pese a tratamientos agresivos y su presencia condiciona el tratamiento como casos de alto riesgo. Dada su alta implicación pronóstica, el estatus de MYCN es determinante a la hora de escoger entre distintos esquemas de tratamiento. Por lo tanto, su detección mediante la técnica de elección FISH (hibridación in situ por fluorescencia) se utiliza de forma rutinaria en caso de diagnóstico de NB.

PLOIDÍA: Las alteraciones en el contenido de ADN o en el número de cromosomas (ploidía) son un hallazgo frecuente en el NB. Su valor pronóstico se ha observado en enfermedad diseminada o estadio 4, pero en tumores de riesgo bajo o intermedio por no tener MYCN amplificado. En estos casos, los tumores diploides muestran un comportamiento más agresivo. Por otro lado, los hiperploides (55% de los NB) tienen un pronóstico más favorable. No obstante, un análisis de riesgo realizado por el INRG refleja que la ploidía

sólo era pronóstica en NB en lactantes de pocos meses, en los que el grupo de riesgo sí puede cambiar.

ALTERACIONES CROMOSÓMICAS ESTRUCTURALES: Los NB agresivos muestran alteraciones cromosómicas segmentarias (SCA) más frecuentemente (traslocaciones desequilibradas, amplificaciones o delecciones), mientras que los NB con pronóstico favorable suelen presentar alteraciones cromosómicas numéricas (NCA) como ganancias o pérdidas completas. Si estas alteraciones coexisten, pronósticamente predominan las SCA (peor pronóstico y tendencia a recaída en NB sin amplificación MYCN). Recientemente se incorpora en los sistemas de clasificación pronóstica la ganancia 11q, asociada a inestabilidad cromosómica y a menor supervivencia. Otras pérdidas cromosómicas incluyen 1p, 4p, 9p, 14q… entre otras, y las ganancias descritas son MYCN y 17q. Por otro lado, recientemente se han descrito mutaciones en el gen ALK, asociadas a NB hereditario y esporádico. El ALK es un oncogén situado en el cromosoma 2, cuya función es aún desconocida. Sin embargo, sí se ha observado su efecto como iniciador del NB y como potenciador de la agresividad del MYCN. Inhibidores del ALK como crizotinib se han utilizado recientemente en ensayos clínicos, mostrando beneficios en resultados preliminares.

SARCOMAS PEDIÁTRICOS

Los sarcomas pediátricos representan más del 20% de todos los cánceres malignos sólidos pediátricos y representan el 13% de todas las malignidades pediátricas. También contribuyen sustancialmente a la mortalidad y la morbilidad relacionadas con el cáncer. Con más de 70 subtipos histológicos, los sarcomas pueden surgir de una célula mesenquimal primitiva de casi todos los tejidos del cuerpo humano y se clasifican en dos grupos principales: sarcomas de tejidos blandos (STB) y sarcomas óseos (BS). En los niños, las mayores tasas de incidencia se registran en el rabdomiosarcoma (RMS), el osteosarcoma y el sarcoma de Ewing (EWS).

Aunque cada subtipo tiene un fenotipo y un perfil genético molecular diferentes, los sarcomas se clasifican en dos grupos: un grupo “genéticamente complejo” con una elevada carga de mutaciones y cariotipo complejo y un grupo “genéticamente simple”, que contienen una translocación, amplificación o mutación única y específica de la enfermedad con un trasfondo genómico silente. La mayoría de los sarcomas pediátricos se incluyen en el segundo grupo, ya que se caracterizan principalmente por translocaciones cromosómicas que dan lugar a reordenamientos genéticos que actúan como impulsores de la sarcomagénesis.

Los protocolos de RMS pediátrico clasifican actualmente este tumor según criterios moleculares, desplazando la clasificación clásica de la patología, y se clasifican como RMS de fusión positiva (translocación PAX/FOXO1) y RMS de fusión negativa. El perfil genético del RMS está dominado por la translocación quimérica recíproca impulsora entre el RMS1 y una variedad de factores de transcripción asociados al RMS. Estas fusiones de genes actúan como un factor de transcripción aberrante que determina una disregulación epigenética que explica la biología del tumor. Por otro lado, el osteosarcoma muestra un genoma extremadamente complejo e inestable sin un patrón repetitivo evidente. Los sarcomas que implican translocaciones pueden ser detectados por FISH y RT-PCR. Las translocaciones son utilizadas por los médicos principalmente como marcadores de diagnóstico. Sin embargo, las proteínas quiméricas resultantes de estas translocaciones no son fácilmente subsidiarias de inhibición y esto dificulta el desarrollo de fármacos inhibidores.

Tanto los STS como los BS muestran un comportamiento altamente agresivo. Se han logrado avances significativos en la mejora de los resultados de los tumores localizados desde que se añadió la quimioterapia sistémica y el perfeccionamiento del control local hace décadas, y aproximadamente 2 de cada 3 pacientes con estadios localizados sobreviven. Sin embargo, los sarcomas metastásicos y los sarcomas recidivantes siguen teniendo tasas de supervivencia muy bajas. Se han observado escasas mejoras en este ámbito, a pesar de que cada vez se tiene más conocimiento de la biología del cáncer.

Al mismo tiempo, en los últimos años, el diagnóstico del cáncer pediátrico, incluidos los sarcomas, ha experimentado un aumento de sus exigencias en términos de precisión y criterios de calidad. Se han introducido nuevas técnicas que complementan el diagnóstico patológico, entre ellas la inmunohistoquímica, FISH, RT-PCR y NGS. En los centros terciarios, estas demandas han sido asumidas gradualmente por clínicos, patólogos, genetistas y biólogos moleculares y se han desarrollado programas de medicina de precisión para ampliar nuestros conocimientos sobre la biología de los tumores y vencer el cáncer con una información más precisa.

En el tercer trabajo presentamos los resultados sobre los sarcomas pediátricos del programa de medicina de precisión para niños y adolescentes con tumores sólidos en recaída/progresión realizado en un centro de referencia nacional para sarcomas pediátricos. Es la primera cohorte publicada sobre sarcomas pediátricos utilizando NGS en España. Este programa ha recibido muestras de centros colaboradores, lo que proporciona una perspectiva nacional. Desde septiembre de 2019, estos estudios se realizan de forma rutinaria en el momento del diagnóstico para cada caso de sarcoma pediátrico.

COMITÉS DE TUMORES PEDIÁTRICOS

El cuidado multidisciplinar es el sello distintivo del manejo de alta calidad del cáncer. Esta afirmación ha sido apoyada en las últimas décadas, en las que las opiniones individuales han sido desplazadas por decisiones colectivas y multidisciplinarias al tratar a un paciente con una enfermedad oncológica compleja. Aunque el cáncer infantil es una enfermedad rara, es la primera causa de muerte no accidental en la infancia y compartir la experiencia es primordial en este campo como en todos los campos que tratan enfermedades infrecuentes en medicina. Varios estudios han demostrado que los debates en comités de tumores pueden suponer cambios en el diagnóstico y el estadiaje de los pacientes con cáncer, afectan a las decisiones de tratamiento y aumentan la calidad de la atención recibida. Por lo tanto, deben formar parte de la atención integral de los pacientes pediátricos con cáncer.

Un comité de tumores pediátrico está formado por un equipo multidisciplinar de expertos que incluye oncólogos pediátricos, radiólogos, cirujanos, radioterapeutas, patólogos y otras disciplinas relevantes para tratar enfermedades oncológicas, con el fin de debatir los casos clínicos de los pacientes en los que el diagnóstico y el plan de tratamiento son complejos. El tratamiento clínico de los pacientes tras un debate en un comité de tumores tiene en cuenta la opinión de varios expertos que participan en una reunión determinada.

Un **comité de tumores molecular pediátrico** (PMBT) es un tipo especial de comité en el que los resultados extraídos de las pruebas de secuenciación masiva se debaten entre miembros de varias disciplinas con el fin de ofrecer recomendaciones de tratamiento personalizadas. Una vez finalizados los estudios genómicos e histopatológicos, todos los miembros debaten los resultados y finalmente se transfiere un informe al médico correspondiente. Una vez hechas estas recomendaciones, los oncólogos pediátricos deben encontrar posibles formas de acceder a determinados fármacos, idealmente mediante la inclusión en ensayos clínicos o, por ejemplo, mediante uso compasivo. Un PMBT suele estar compuesta por oncólogos pediátricos, genetistas, farmacólogos, patólogos, especialistas en bioinformática y biología molecular. En el Hospital U i P La Fe se creó el PMTB en noviembre de 2014 y en este foro se discutieron todos los objetivos y resultados accionables del artículo 3 de esta tesis.

Un **comité de tumores virtual pediátrico** es un tipo especial de reunión multidisciplinar basada en sistemas de videoconferencia en el que pueden participar expertos de diferentes centros. Por razones prácticas, estas reuniones deben ser "virtuales" y utilizar sistemas de videoconferencia para permitir la celebración de reuniones y debates periódicos entre expertos de diferentes países. Por consiguiente, los comités de tumores virtuales pueden reunirse con regularidad y rapidez para evaluar casos urgentes y evitan las grandes aglomeraciones de personal sanitario físicamente en la misma sala. Durante este año, debido a la pandemia causada por el COVID19, ha habido una necesidad urgente de desarrollar sistemas de videoconferencia para poner en práctica la comunicación virtual entre los profesionales de la salud. Sin embargo, existen enormes diferencias entre los países europeos en cuanto a las instalaciones para desarrollar comités de tumores virtuales pediátricos. Como primer paso hacia una Red Europea de Comités de Tumores virtuales

en Oncología Pediátrica, en el cuarto artículo nos propusimos identificar los comités de tumores pediátricos ya existentes en Europa para investigar el tipo de tecnologías y logística que existen en los diferentes países y explorar las diferencias actuales entre las regiones europeas. Esta tarea se llevó a cabo dentro de un paquete de trabajo del proyecto ExPO-r-Net (Red de Referencia de Expertos Europeos en Oncología Pediátrica para el Diagnóstico y el Tratamiento).

2. HIPÓTESIS Y OBJETIVOS

HIPÓTESIS

El estudio citogenético y por secuencación de los tumores pediátricos aplicado a la clínica genera un mayor conocimiento biológico de estas enfermedades y mejora la estratificación de riesgo del paciente, permitiendo diseñar estrategias terapéuticas individualizadas.

OBJECTIVOS

Objetivo principal:

Profundizar en el estudio genómico del neuroblastoma y de los sarcomas pediátricos desde el punto de vista traslacional mediante el empleo de métodos citogenéticos y de secuenciación masiva para obtener un diagnóstico personalizado que permita categorizar el riesgo con mayor precisión y la incorporación de nuevos agentes terapéuticos.

Objetivos específicos:

- Analizar la importancia de la deleción del brazo largo del cromosoma 11 como marcador pronóstico en neuroblastoma, sobretodo en estadios localizados y 4s.

- Estudiar la distribución de las alteraciones cromosómicas segmentarias según factores de riesgo ya conocidos en neuroblastoma.

- Identificar posibles asociaciones de alteraciones cromosómicas segmentarias en neuroblastoma y el papel de ciertas alteraciones numéricas.

- Describir las mutaciones más frecuentes detectadas por secuenciación masiva, su implicación en el pronóstico y la detección de posibles dianas terapéuticas en una cohorte multicéntrica de sarcomas pediátricos.

- Implementar las técnicas de secuenciación masiva para el estudio de los sarcomas pediátricos al debut de la enfermedad en la Unidad de Onco-hematología Pediátrica del Hospital U i P La Fe.

- Identificar comités de tumores pediátricos en Europa e investigar el tipo de tecnología y logística que existen en los diferentes países para explorar las diferencias actuales entre regiones.

3. METODOLOGÍA Y RESULTADOS

ARTÍCULO 1

CLINICAL FEATURES OF NEUROBLASTOMA WITH 11Q DELETION: AN INCREASE IN RELAPSE PROBABILITIES IN LOCALIZED AND 4S STAGES

Scientific Reports (Nature Research) (2019)

ARTÍCULO 2

DISTRIBUTION OF SEGMENTAL CHROMOSOMAL ALTERATIONS IN NEUROBLASTOMA

Clinical & Translational Oncology (2020)

ARTÍCULO 3

NEXT-GENERATION SEQUENCING IDENTIFIES POTENTIAL ACTIONABLE TARGETS IN PAEDIATRIC SARCOMAS

En revision en International Journal of Oncology (2020)

ARTÍCULO 4

SURVEY ON PAEDIATRIC TUMOUR BOARDS IN EUROPE: CURRENT SITUATION AND RESULTS FROM THE EXPO-R-NET PROJECT

Clinical & Translational Oncology (2018)

4. RESUMEN DE RESULTADOS Y CONCLUSIONES FINALES

En los estudios que componen esta tesis por artículos se ha comprobado una vez más el peso y la importancia que han adquirido durante los últimos años las técnicas moleculares como las técnicas citogenéticas de cariotipo molecular y la secuenciación masiva para el diagnóstico y estratificación del riesgo de los tumores pediátricos extra-cerebrales por excelencia. Las alteraciones cromosómicas segmentarias en el neuroblastoma y a nivel más específico dentro del cromosoma, las mutaciones puntuales, inserciones, deleciones, translocaciones y CNV detectadas por secuenciación masiva en los sarcomas, disciernen entre subtipos de tumores más o menos agresivos, con una respuesta mayor o menor a la quimioterapia, una mayor o menor tendencia de reaparecer y por tanto con una mayor o menor supervivencia. Por el momento existen pocos factores citogenéticos y moleculares con suficiente evidencia para ser considerados en la estratificación de los tumores en primera línea pero algunas de las conclusiones a las que se ha llegado por medio de ensayos clínicos que incluyen variables dependientes en la categorización molecular de los tumores (deleción de 11q, amplificación de MYCN, fusión del gen FOXO1, etc) nos han permitido intensificar la carga de tratamiento en los casos con peor pronóstico y a medida que se van conociendo más datos es muy probable que se incorporen nuevos grupos de riesgo atendiendo a los resultados de estas técnicas complementarias. De hecho, por estos motivos en algunos de los futuros protocolos de grupos cooperativos europeos como el protocolo para primera línea del rabdomiosarcoma FaR-RMS dentro del EpSSG y los futuros protocolos para el neuroblastoma tanto de alto riesgo como de riesgo bajo e intermedio de SIOPEN consideran algunos factores biológicos para una estratificación de riesgo inicial.

A continuación, detallo las conclusiones principales y otras más específicas extraídas del trabajo de los cuatro artículos.

Resultados principales

- La deleción de 11q en NB confiere mal pronóstico a la enfermedad en todos los estadios, destacando su importancia en los tumores localizados y Ms. Sus connotaciones negativas en términos pronósticos son comparables a la amplificación de MYCN.

- Las alteraciones cromosómicas segmentarias en NB no se distribuyen de manera aleatoria, sino que se concentran en unos cromosomas recurrentes y aparecen agrupadas en patrones característicos según factores pronósticos establecidos previamente (edad, estadio INRGSS y status de MYCN).

- La incorporación de los estudios de secuenciación masiva junto con las técnicas anatomo-patológicas en los sarcomas pediátricos es plausible, puede beneficiar hasta un 23% de los pacientes y permite hallar tratamientos personalizados sobretodo en el contexto de las recaídas.

Conclusiones específicas

- La presencia de la deleción 11q en NB se relaciona con mayor edad al diagnóstico, estadios más avanzados y se asocia a un mayor riesgo de recidiva.

- La co-presencia de deleción de 11q y MNA es extremadamente infrecuente.

- Existe una relación entre la deleción de 11q y la ganancia de MYCN cuyo origen es desconocido.

- La presencia de SCA en NB se asocia con una mayor edad al diagnóstico y con la presencia de MYCN amplificado.

- La SCA que se asocia con mayor frecuencia a la presencia de otras alteraciones segmentarias es la deleción de 11q seguida por la deleción en 4p.

- La ganancia numérica en el cromosoma 19 se asocia a ausencia de SCA, estadio INRGSS menos avanzado y mejor pronóstico.

- TP53 es la mutación más frecuente detectada por secuenciación masiva en sarcomas pediátricos, seguida de FGFR4 y CTNNB1.

- El perfil genómico de los sarcomas pediátricos y otros tipos de cáncer pediátrico difiere considerablemente de los perfiles genómicos de las neoplasias en adultos.

- Los comités de tumores pediátricos son un instrumento esencial para el manejo de los casos complejos y existen en el 91% de los centros europeos.

- Hay una falta de equidad y diferencias importantes en algunos aspectos de los comités de tumores pediátricos entre las regiones geográficas europeas, especialmente en lo que respecta a la disponibilidad de equipos de videoconferencia y los sistemas de comunicación.

Posibles líneas de investigación futuras

Como posibles líneas de investigación futura destacaría la exploración en detalle de la relación biológica entre la deleción del 11q y la ganancia de MYCN. Resulta muy llamativo que haya una relación inversa entre la amplificación de MYCN y que se asocie la deleción de 11q con la ganancia de MYCN. También se podrían proponer posibles ensayos comparativos intensificando la terapia de los pacientes con deleción del 11q en los que este hallazgo biológico no supone un incremente de la carga terapéutica.

Otra posible línea de investigación menos trabajada es la posible implicación pronóstica de determinadas alteraciones cromosómicas numéricas en NB. Al tener tanto peso las alteraciones cromosómicas segmentarias en el pronóstico del NB escasos trabajos se han centrado en analizar separadamente la posible influencia de ganancias y pérdidas de cromosomas completos en el NB. Esto podría tener un interés real y existen publicaciones recientes al respecto además de la segunda publicación que constituye esta tesis.

En cuanto a la secuenciación masiva en sarcomas hemos presentado una cohorte única de 70 sarcomas que incluye hasta 13 neoplasias diferentes. Por lo tanto, pienso que quedan muchas líneas de investigación por explotar, incluyendo el estudio detallado en cada subtipo tumoral con cohortes de mayor volumen. A medida que se vaya extendiendo el uso de estas técnicas podremos extraer conclusiones más focalizadas a cada tipo tumoral y posiblemente esto fomente la investigación en nuevas líneas de tratamiento personalizadas para el tratamiento del cáncer pediátrico.

Finalmente, respecto a los comités de tumores, uno de los déficits que ha manifestado la pandemia COVID19 en el año 2020 ha sido la precarización de los sistemas sanitarios en lo que respecta a las nuevas tecnologías, la informática y los sistemas de videoconferencia. Los casos complejos que se tratan en unidades de oncología pediátrica no pueden prescindir de comités multidisciplinares y existe una urgencia inmediata y real en la implementación en los centros sanitarios de medios virtuales de comunicación adecuados y esto conlleva todas las líneas de investigación que pueden acompañar a este desarrollo audiovisual en el ámbito de los centros sanitarios.

7. DOCUMENTARY APPENDIX: ORIGINAL MANUSCRIPT COMPENDIUM

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Clinical Features of Neuroblastoma with 11q Deletion: An Increase in Relapse Probabilities in Localized and 4S Stages

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Neuroblastoma (NB) is a heterogeneous tumor with an extremely diverse prognosis according to clinical and genetic factors, such as the presence of an 11q deletion (11q-del). A multicentric study using data from a national neuroblastic tumor database was conducted. This study compared the most important features of NB patients: presence of 11q-del, presence of *MYCN* amplification (MNA) and remaining cases. A total of 357 patients were followed throughout an 8-year period. 11q-del was found in sixty cases (17%). 11q-del tumors were diagnosed at an older age (median 3.29 years). Overall survival (OS) was lower in 11q-del patients (60% at 5 years), compared to all other cases (76% at 5 years) $p = 0.014$. Event free survival (EFS) was 35% after 5 years, which is a low number when compared with the remaining cases: 75% after 5 years ($p < 0.001$). Localized tumors with 11q-del have a higher risk of relapse (HR = 3.312) such as 4 s 11q-del patients (HR 7.581). 11q-del in NB is a dismal prognostic factor. Its presence predicts a bad outcome and increases relapse probability, specially in localized stages and 4 s stages. The presence of 11q aberration should be taken into consideration when stratifying neuroblastoma risk groups.

Neuroblastoma (NB) is a complex pediatric tumor and the most common extracranial solid malignancy in childhood[1]. Clinical manifestations may range from aggressive growth despite intensive treatment, to cases in which spontaneous regression is reported. In the last decades, it has been observed that particular tumoural genomic changes correlate with its behaviour and outcome. It is well known that an *MYCN* amplification (MNA) is associated with aggressive tumors and a dismal prognosis. Other genomic features besides MNA, such as 11q deletion (11q-del) and 17q gain, represent segmental aberrations that take place in a remarkable number of cases.

Segmental chromosomal aberrations (SCA) are considered a bad or negative prognostic factor. The frequency of 11q alterations may vary between 20–45%[1], depending on the different series and study techniques used. These particular tumors have, in addition to segmental aberration in chromosome 11: a higher observed frequency of chromosomal breakage, a higher median age at diagnosis, and poor prognosis[2,3] (survival rates estimated of 35% at 8 years). There is a poor amount of data comparing the outcome of 11q-deleted and MNA NB cohorts.

The international NB risk group (INRG) proposed a staging system in 2009 that stratified NB in risk groups according to clinical and genetic factors, such as 11q aberration in some particular stages[4]. Other factors taken into consideration were INRG stage (L1, L2, M, 4 s), age, tumor differentiation grade and histologic pattern, ploidy and *MYCN* status.

However, in some subgroups of the International Society of Pediatric Oncology European neuroblastoma research network (SIOPEN) (mainly intermediate and high risk cases), the presence of 11q aberrations is not taken into account when classifying the risk group, which may give rise to concern among the treating physicians, given the possibility that these patients are receiving insufficient treatment. To study in depth the characteristics

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and outcome of patients with 11q-deleted NB, a retrospective study has been conducted in a Spanish cohort comparing clinical features among 11q-del, MNA and other NB without any of these genetic abnormalities.

Methods

Our aim is to study and describe the 11q-del NB cases diagnosed throughout an 8-year period. This retrospective multi-centric study consists of 399 NB patients from 29 Spanish cooperating hospitals. Data collected from patients included age at diagnosis, primary tumor location, pathology, stage, *MYCN* status, 11q-del status, relapse, time until relapse, time from relapse to death, cause of death, follow up and current state of the patient. Staging and risk stratification were established according to INSS and INRGSS classifications[5–7]. Tumor samples from all patients were referred to the Spanish reference center for pathology and molecular biology NB studies. Samples were centrally reviewed and classified according to the International NB Pathology Classification (INPC)[8–10].

Patients were registered in the file of the Spanish Society of Pediatric Hematology and Oncology (SEHOP) NB group database, and included for treatment mainly in SIOPEN trials according to period and clinical characteristics (INES, LINES, LNESG-I, EUNS and HRNBL1). In stage M patients, response to induction treatment chemotherapy was evaluated according to SIOPEN guidelines (High Risk NB Study 1.7 of SIOP-Europe)[11]. Informed consent for study participation, samples and data management were obtained in all cases from the patients' parents, and all the patients were treated following SIOPEN-approved protocols.

Chromosome 11q-del was defined as a missing (deleted) copy of genetic material on the long arm (q) of chromosome 11. *11q23* was the most frequent region found to be deleted. Biological studies included *MYCN* status and *1p* using FISH technique, and *11q* status was initially studied by multiplex ligation-dependent probe amplification technique (MLPA), and from 2012 onwards by single nucleotide polymorphism (SNP) array (Affymetrix and Illumina) according to the European Neuroblastoma Quality Assessment (ENQUA) guidelines (Ambros IM *et al.*)[12–14].

As recommended by the ENQUA guidelines, amplification of *MYCN* was defined as a higher than a 4-fold increase of *MYCN* signals in relation to the number of reference chromosome 2 signals. The increase or the additional copies (up to the 4-fold) were defined as *MYCN* gain[15,16].

The data used was summarized using mean, standard deviation (SD) and median (1^{st}, 3^{rd} Q) in the case of continuous variables, whereas absolute and relative frequencies were used for categorical variables. To assess the independent association of 11q-del with survival, Cox proportional hazard regression models were adjusted including *MYCN* status, stage and age at diagnosis. For event free survival (EFS), time to event was defined as the time from diagnosis until the time of first occurrence of relapse, progression or death. For OS, time to event was defined as time until death or until last contact if the patient was alive. Kaplan-Meier curves were compared with log-rank test. Age difference between the presence and absence of 11q-del was compared using Wilcoxon Rank Sum test. 95% confidence Intervals of the effects were provided. Proportions were evaluated using Chi-square test. P-values below 0.05 were considered statistically significant. All analyses and graphs were performed using R software (version 3.5.0) with clickR (0.3.64) and survival (2.41–3) packages.

Ethics approval and consent to participate. All study actions have been done under the appropriate ethics code and consent was obtained from all patients. The study was performed in accordance with the Declaration of Helsinki.

Results

A total of 399 children with NB were registered during this 8-year study period (2006 to 2013). Tumors without initial 11q determination were excluded from analysis (42 patients). The remaining 357 NB were tested for 11q-del. 60/357 patients were found to have this alteration (17%). The presence of 11q-del and MNA in the same tumor was almost mutually excluding (only 3 cases showed both abnormalities).

The vast majority of the cases had abdominal location (79%), followed by the thoracic area (10%). A smaller proportion of tumors was found in the neck and in the pelvis, and there was one retro-orbital case. Concerning tumor stage, 177 patients (50%) had a localized disease (stage 1, 2 or 3), 145 patients were diagnosed as stage 4 (40%) and 35 patients had stage 4 s NB (10%). Median age at diagnosis was 1,37 years.

According to the INPC system[4] (International NB Pathology Classification), 202 samples were classified as poorly differentiated NB (57%) leading this to be the most frequent diagnosis, followed by undifferentiated NB (14%). MNA was found in 63 patients (18% of the cases), whilst 38 tumors had *MYCN* gain (10%).

From the whole cohort, 105 patients died (30%) and 252 are alive with a median follow up of 5,7 years. The most important cause of death was tumor progression in 90% of the cases. Regarding the survivors, 222 patients remain free of disease (62%) and 30 patients are alive on treatment after relapse (8%). Table 1 summarizes the clinical and biological characteristics of the patients.

Initially, 11q status was considered as a unique factor and 11q-del population ($n = 60$) was compared to the no-11q-del cases ($n = 297$) including all age groups and stages. Overall survival (OS) was lower in 11q-del patients (60% at 5 years and 52% at 8 years) than in the cases lacking 11q-del (76% at 5 years and 72% at 8 years) $p = 0.014$. Differences were noticeable when considering EFS. In 11q-del patients, EFS was 35% at 5 years and 32% at 8 years compared to the data in no-11q-del cases: 75% at 5 years and 73% at 8 years ($p < 0.001$) with a median follow up of 5.7 years. (Fig. 1).

Separating the data by stages (4, 4 s or localized), it was observed that localized and 4 s stages 11q-del patients had a poor EFS (approximately 45% and 40% respectively) (Fig. 2). EFS was similar in 11q-del localized or 4 s stages to no-11q-del stage 4 cases. This fact is very representative and further confirms that 11q-del status plays an important role in NB relapse. Statistically significant differences were observed when comparing 11q-del and non-11q-del EFS in localized NB and stage 4 s ($p = 0.001$). Differences in stage 4 EFS were observed according to 11q-del status although this result was not statistically significant ($p = 0.32$).

Variable	n = 357
Age at diagnosis (years)	mean: 2.51
	median: 1.37
Location	
Abdominal	281 (79%)
Neck	8 (2%)
Neck-thoracic	6 (1.5%)
Pelvic	6 (1.5%)
Retro-orbital	1 (0.3%)
Thoracic	35 (10%)
Thoracic-abdominal	20 (6%)
11q-del	
No	297 (83%)
Yes	60 (17%)
MNA	
Amplified	63 (18%)
Gained	38 (10%)
Non amplified	256 (72%)
Stage	
1	85 (24%)
2	25 (7%)
3	67 (19%)
4	145 (40%)
4 s	35 (10%)
Pathology	
GanglioNB	41 (11%)
Non specified GanglioNB	6 (2%)
Differentiating NB	17 (5%)
Undifferentiated NB	52 (14%)
Poorly differentiated NB	202 (57%)
Anaplastic NB	2 (1%)
Non specified NB	37 (10%)
Current state	
Dead	105 (30%)
Alive with disease	30 (8%)
Alive without disease	222 (62%)
Cause of death	
Disease progression	94 (90%)
Other causes	11 (10%)

Table 1. General description of the studied population.

Given the importance of MNA as an established prognostic factor associated to adverse outcome in neuroblastoma, a comparison between EFS in MNA, 11q-del and the remaining NB cases without these alterations was made using Kaplan-Meier curves. Statistically significant results were found and no differences between MNA and 11q-del outcomes were observed (Fig. 3; above: Entire cohort; below: Stage 4 NB).

In the study, three cohorts were compared: 57 patients with 11q-del, 60 patients with MNA, and other 237 cases without both alterations (other population). The only 3 cases containing both abnormalities were excluded. The general characteristics of these subgroups are summarized in Table 2 and are the following.

Age. Median age at diagnosis was found to be higher in the 11q cases (3.29 years at diagnosis) when compared with MNA cohort (2.02 years) and the remaining cases (0.92 years at diagnosis).

Stage. When comparing tumor stage, 42 cases (73%) of 11q-del patients were classified as stage 4 at diagnosis. This figure is similar to the number of MNA stage 4 cases (80%) while only 55 cases out of 237 (23%) of the other NB cases were classified as stage 4.

***MYCN* gain.** It was observed that out of sixty 11q-deleted NB, 20 had also *MYCN* gain (33.3%) whereas only 18 of the other 237 cases (without 11q-del) had *MYCN* gain (7.6%). These results are statistically significant ($p < 0.001$) and show that despite 11q-del and MNA being almost mutually excluding, 11q-del is associated with *MYCN* gain. Most of the patients with both these aberrations (11q-del and *MYCN* gain) presented high-risk features, 16/20 were diagnosed as stage 4 and 15/20 were older than 18 months.

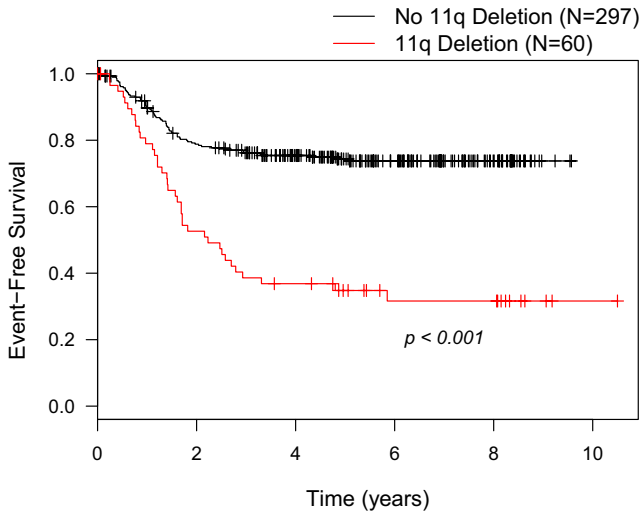

Figure 1. Event-free survival of 11q-del compared to no-11q-del neuroblastoma.

Relapse. A small number of tumor relapses were reported in the cohort without MNA or 11q alterations (relapses in 16% of the cases). On the other hand, more MNA or 11q-del NB recurred. 55% of the MNA tumors had one relapse and one case had two recurrences. Among the 11q-del cases, 36/57 patients relapsed (63%). 26 patients relapsed only once (46%) and ten cases with sequential relapses were detected (6 patients had two relapses, 2 patients had three relapses and 2 patients recurred four times) (Table 2).

In the relapse model (Cox regression), the population with higher relapse risk was stage 4 s with 11q-del, with 7.581 HR (hazard ratio). *MYCN* homogeneous amplification was also associated with a higher relapse risk (HR 2.718). Localized tumors (stage 1, 2, 3) with 11q-del also have a higher risk (HR 3.312). Notably, 4 s population and localized NB without 11q-del had the lowest HR (0.245 in both cases) showing how aberrations in 11q dramatically increase relapse rate in low risk NB. These results are statistically significant. Table 3 summarizes relapse data in these subgroups of patients.

The clinical course of 11q-del NB patients was usually an insidious process with multiple relapses and longer courses with a poor final outcome, based on time until relapse, time from relapse to death and time from diagnosis to death. Patients with 11q-del relapsed later than the other cases. Median time from diagnosis to relapse was 1.42 years, compared with 1.08 in the MNA cohort and 1.28 in the other NB relapsed patients. Relapsed 11q-del patients also had a longer median time from relapse to death (1.53 years) than MNA cases (0.49 years) and than the other NB cases (0.98 years). Finally, median time from diagnosis to death was longer in 11q-del patients (2.88 years) compared to MNA NBs (1.37 years) and the remaining patients (2.09 years).

Survival/event free survival. Statistical differences were observed in OS when comparing patients in the three groups. Mortality is higher in the MNA NB with 70% of deaths (42/60 compared with 44% in the 11q-del tumors (25/57) and 15% in the remaining patients (34/237). MNA patients had a HR of 3.673 ($p < 0.001$). Therefore, by using Cox regression we conclude that MNA amplification was associated with a lower survival, a fact that has been widely identified (Table 4).

The most important differences in 11q-del NB were observed in final outcome in localized and stage 4 s tumors. Localized stages with 11q-del showed higher risk of death (HR 3.119, $p = 0.082$). Moreover, stage 4 s patients with 11q-del presented a HR of 4.398, $p = 0.12$). These results were not statistically significant, probably due to the low number of 11q-del localized and stage 4 s cases. Even so, given the notoriousness of these values, they should be taken into account.

Discussion

During the last years many international efforts have been done trying to investigate why some particular genetic changes lead to more aggressive cases of NB. MNA is still the most relevant biological prognostic factor, specially in infants[17]. However, in the last years, SCA including *11q* alterations are also taken into account and affect risk stratification in some subgroups of NB. This is one of the largest cohorts ($n = 357$) that shows and further confirms worst outcomes in NB containing 11q-del. Patients have been studied homogeneously without previous selection and have a median follow up of 5.7 years. As it has been described, 11q-del NB is related to older age at diagnosis ($p < 0.001$) and is also associated with more advanced stages of NB ($p < 0.001$). Co-presence of 11q-del and MNA is extremely rare. Using Cox regression, we conclude that 11q-del as well as MNA is associated with a higher risk of relapse. The comparison between EFS in MNA, 11q-del and the remaining cases with Kaplan-Meier curves (Fig. 3) further confirms the clinical value of 11q-del in NB, being equivalent to that of MNA.

In the cohort studied previously by Schleiermacher *et al.*, it was observed that in 147 NB without MNA, a SCA profile was the strongest independent prognosis factor. In this cohort, 76% of the cases with SCA showed 11q-del[4]. Caren *et al.*, reported that median age at diagnosis was significantly higher in the 11q-del group

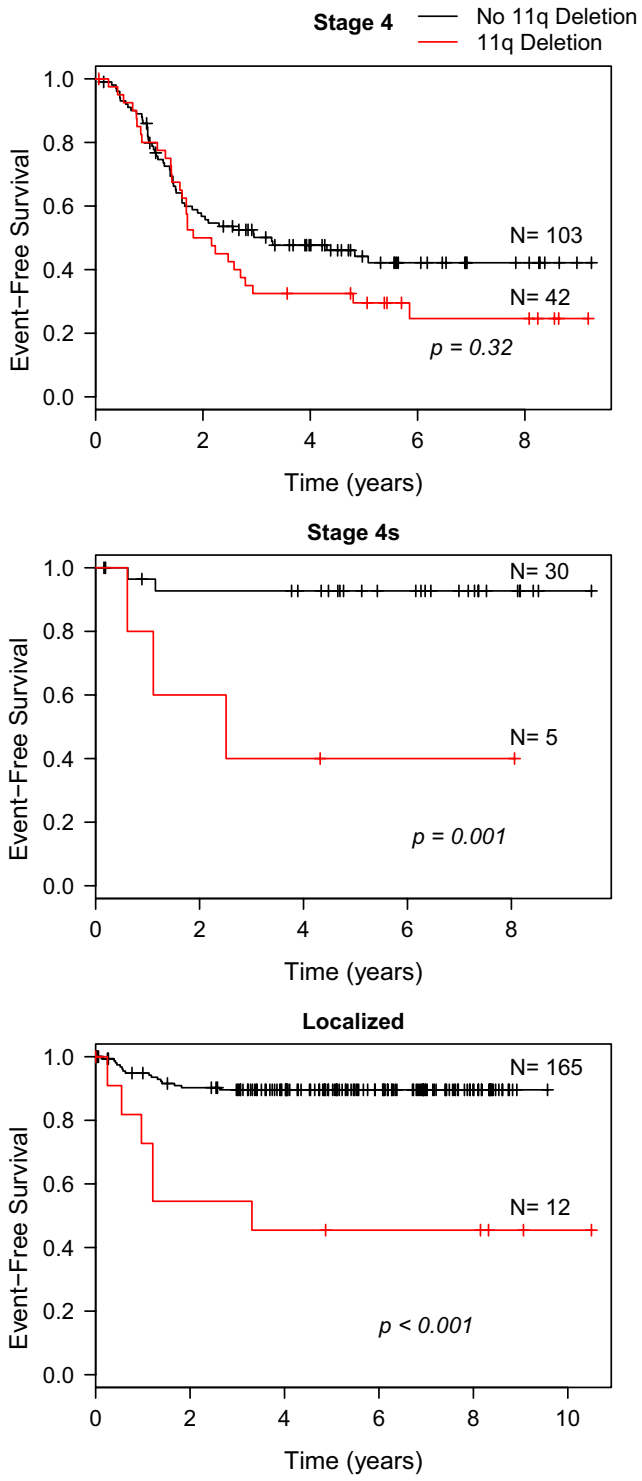

Figure 2. Event-free survival analysis by stages (stage 4, 4 s and localized) comparing 11q-del vs no-11q del NB cases.

compared to numeric chromosomal aberrations (NCA), MNA and 17q gain groups (42 months vs 3, 21 and 21 months, respectively). Prognosis was found to be poor in MNA and 11q-del groups (8 years OS 35%), but the median survival time after diagnosis was longer in 11q-del NB (40 vs 16 months)[1,3]. These observations are very similar to our findings as 11q-del NB patients in our study have shown higher median time to relapse, higher median time from relapse to death and higher median time from diagnosis to death.

Concerning age, similar conclusions have been reported by previous groups highlighting that 11q alteration is detected mostly in older patients. Cetinkaya C *et al.* reported a cohort of NB where median age at diagnosis was extremely different according to MNA or 11q-del. The results were 58.5 months in 11q-del NB vs 18 months

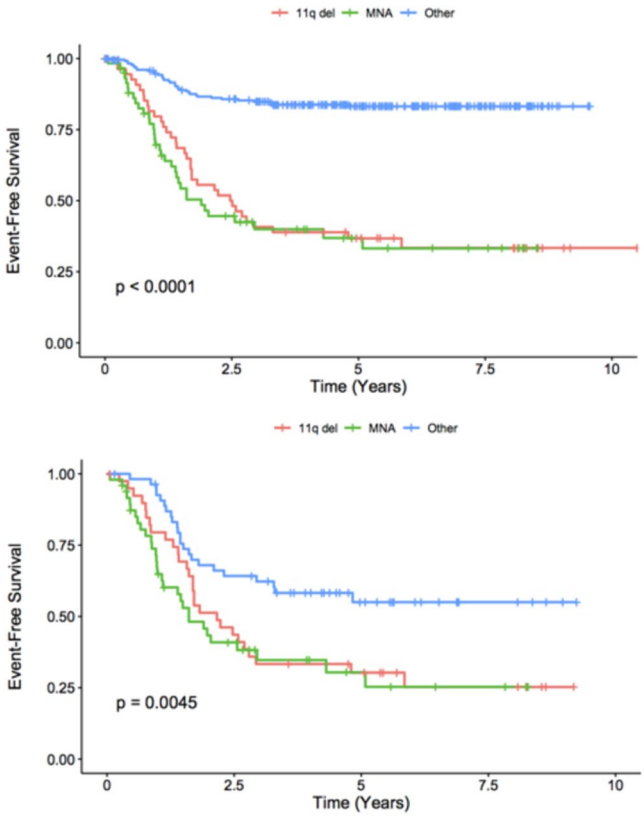

Figure 3. Kaplan-Meier curve comparing EFS between 11q-del, MNA and the remaining cases of NB. (Above: Entire cohort. Below: Stage 4 NB).

	11q del n = 57	MNA n = 60	Other = 237
Median age at diagnosis (years)	3.29	2.02	0.92
Stage			
1	3 (5%)	1 (2%)	80 (34%)
2	0 (0%)	0 (0%)	24 (10%)
3	7 (13%)	9 (15%)	51 (22%)
4	42 (73%)	48 (80%)	55 (23%)
4 s	5 (9%)	2 (3%)	27 (11%)
Number of relapses			
0	21 (37%)	26 (43%)	198 (84%)
1	26 (46%)	33 (55%)	31 (13%)
2 or more	10 (17%)	1 (2%)	8 (3%)
Death			
No	32 (56%)	18 (30%)	203 (85%)
Yes	25 (44%)	42 (70%)	34 (15%)

Table 2. Description of the three neuroblastoma subgroups: 11q-del, MNA and the remaining cases.

in the MNA group[1,18]. Analysis of the INRG database has also shown that in a cohort of younger patients (<18 months) with stage 3 NB, the only independently associated factor with poor survival and EFS has been 11q-del status. These facts are completely concordant with our results[1,19]. Adolescents with neuroblastoma represent less than 5% of the cases and in most series they are characterized by a high prevalence of SCA and a low incidence of MNA. The prevalence of 11q-del in this group is between 30 to 60%[1]. ALK and ATRX mutations are more frequent in older patients too and ATRX mutated NB showed a higher number of SCA including 11q-del[20,21] with a very poor outcome.

We also report that 11q-del is associated to *MYCN* gain. The proportion of *MYCN* gain in the 11q-del cohort is much higher than in other cases (33.3% versus 6.1%). Our data also confirms the association between 11q aberration and high-risk disease, specifically in the absence of MNA. *MYCN*-gain most likely occurs due to a larger

Relapse	HR	Lower 95%	Upper 95%	P-value
Stage 4 s	0.245	0.056	1.065	0.061
Localized	0.245	0.133	0.449	<0.001
11q-del	1.847	1.087	3.138	0.023
MYCN gain	1	0.573	1.745	0.999
MYCN amplification	2.718	1.672	4.421	<0.001
Age < 18 months	0.405	0.252	0.653	<0.001
Stage 4 s with 11q del	7.581	1.175	48.924	0.033
Localized with 11q del	3.312	1.128	9.727	0.029

Table 3. Relapse risk in neuroblastoma subgroups (Cox regression relapse model).

Death risk	HR	Lower 95%	Upper 95%	P-value
Stage 4 s	0.391	0.112	1.363	0.14
Localized	0.185	0.093	0.37	<0.001
11q del	1.145	0.648	2.02	0.641
MYCN gain	1.251	0.689	2.273	0.462
MYCN amplification	3.673	2.243	6.015	<0.001
Age < 18 months	0.352	0.209	0.594	<0.001
Stage 4 s with 11q del	4.398	0.681	28.409	0.12
Localized with 11q del	3.119	0.866	11.227	0.082

Table 4. Death risk in neuroblastoma subgroups (Cox regression survival model).

gain of copies of the 2p chromosomal arm, rather than a focal gain, meaning that additional genes at 2p including ALK could contribute to NB pathogenesis and high risk disease. Furthermore, recent findings have provided a potential link between this inverse association between 11q aberration and MNA. More specifically, evidence has suggested that dysregulation of the microRNA *let-7* plays a central role in the pathogenesis of neuroblastoma and that either MNA or 11q loss are able to disrupt *let-7*, but the biological significance of this relationship is still waiting to be confirmed[22].

In the subset of patients with "good prognosis" (localized and 4 s stages), 11q-del frequency is rather low. However, when these cases have 11q-del, prognosis has shown to be worse[23]. Hence, some authors support that patients in low and intermediate risk groups with SCA such as 11q-del could benefit of intensified treatments[24]. In the COG protocols, 11q-del is added to other factors (*MYCN* status and ploidy) in patients with localized tumors younger than 18 months. In SIOPEN studies, chromosomal segmentation aberrations are considered including 11q-del but these are not considered in all tumor stages. Currently, it is well recognized that 11q-del NB constitutes a distinct subgroup of aggressive malignancies, but with different features compared to MNA and therefore, the results show that some action points regarding treatment need to be further assessed in this field. A high frequency of chromosomal breakage, suggestive of a chromosomal instability, is one of the main features of 11q-del NB that has been previously reported. This shows that certain genes in 11q could be involved in the chromosomal instability phenotype[3], by haplo-insufficiency or inactivation of the second allele by mutation or epigenetic modification[1]. Recurrent patterns of SCA or NCA suggest that NB is a cancer driven by copy number rather than by particular mutations. The fact that 11q is never lost on both chromosomes suggests that important genes are present on the remaining 11q copy, but that the second hit needed would be caused by another localized mutation or methylation event[1].

So far, the mechanism by which hemizygous deletion in 11q leads to high risk features is unknown, and therapies targeting this alteration have not been totally developed. Some of the genes located in this chromosomal area have been studied and seem to have important relation with the adverse prognosis that the ablation produces. Particularly, TSLC1 (CADM1; cell adhesion molecule 1) located in 11q23.3 has an important role as tumor suppressor gene in NB and has also been related in oncogenesis[25]. Recently, while looking for new possibilities and alternative therapies in patients with relapsed 11q-del tumors, ATM hemizygosis (11q22.3) in the presence of functional TP53 (17p13.1) has shown *in vitro* and *in vivo* response to PARP inhibitors[26].

The gene ATM is within this chromosomic locus and has the role of repairing DNA damage by homologous recombination. Efficient repair of damaged DNA strands helps maintain the stability of the cell's genetic information[27]. H2AFX (H2A Histone Family Member X) also located in 11q23.3 is a member of the nucleosome structure and thereby plays an important role in transcription regulation, chromosomal stability, DNA repair and replication. In fact, ATM protein phosphorylates H2AFX during response to double-strand breaks (DSB). Therefore the loss of H2AFX also suggests a potential utility of PARP inhibitors and could be related in the described responses[26,28].

Poly ADP-ribose polymerase (PARP) is a protein that signals DNA damage and facilitates DNA repair. PARP catalyzes the addition of ADP-ribose to DNA, histones, topoisomerases and helicases and has a critical function in cellular replication, transcription, differentiation, gene regulation, protein degradation and spindle maintenance.

Inhibition of PARP results in persistent single strand DNA breaks leading to stalled replication forks and double strand DNA breaks. PARP inhibition produces DNA damage that leads to cell cycle arrest and apoptosis. PARP inhibitors are being evaluated in cancers with defective DNA repair mechanisms alone or in combination with cytotoxic therapy or radiation[29,30]. The addition of PARPi to second line chemotherapy in 11q-del neuroblastoma patients could be an attractive combination for these patients that is currently under exploration.

In the basis of these facts and other similar hypotheses of previous revisions, we think that within the international groups, new frontline strategies are required to be developed in order to improve the outcome of neuroblastoma patients with 11q-del.

Data Availability

Data and materials are available in the national neuroblastic tumor data base in University Hospital La Fe.

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Author Contributions

Antonio Juan Ribelles and Adela Cañete: Main authors. Wrote manuscript text. Sandra Barberá and Bárbara Juan: Data collection and english translation. Yania Yáñez, Pablo Gargallo and Victoria Castel: Wrote parts of the manuscript. Vanessa Segura, Rosa Noguera, Marta Piqueras and Jaime Font de Mora: Molecular testing. Sample management. Victoria Fornés-Ferrer: Biostatistical analysis.

Additional Information

Competing Interests: The authors declare no competing interests.

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Distribution of segmental chromosomal alterations in neuroblastoma

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RESEARCH ARTICLE

Distribution of segmental chromosomal alterations in neuroblastoma

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Abstract

Background Neuroblastoma (NB) is a heterogeneous tumor with extremely diverse prognosis according to clinical and genetic factors such as specific combinations of chromosomal imbalances.

Methods Molecular karyotyping data from a national neuroblastic tumor database of 155 NB samples were analyzed and related to clinical data.

Results Segmental chromosomal alterations (SCA) were detected in 102 NB, whereas 45 only displayed numerical alterations. Incidence of SCA was higher in stage M (92%) and MYCN amplified (MNA) NB (96%). Presence of SCA was associated with older age, especially 1q gain and 3p deletion. 96% of the deaths were observed in the SCA group and 85% of the relapsed NB contained SCA. The alteration most commonly associated with a higher number of other segmental rearrangements was 11q deletion, followed by 4p deletion. Whole-chromosome 19 gain was associated with lower stages, absence of SCA and better outcome.

Conclusions SCA are not randomly distributed and are concentrated on recurrent chromosomes. The most frequently affected chromosomes identify prognostic factors in specific risk groups. SCA are associated with older age and MNA. We have identified a small subset of patients with better outcome that share whole-chromosome 19 numeric gain, suggesting its use as a prognostic biomarker in NB.

Keywords Segmental chromosomal alterations · 11q deletion · Whole gain chromosome 19 · *MYCN* amplification · Molecular karyotyping

Introduction

Neuroblastoma (NB) is a complex, heterogeneous tumor and the most common extracranial solid malignancy in childhood [1]. It originates in the sympathetic nervous system and is responsible for approximately 15% of childhood cancer deaths [2]. Clinical manifestations vary from aggressive growth despite intensive treatment to some cases of spontaneous regression. Age and stage are prognostic factors and particular genomic changes in the tumor correlate with its behavior and outcome. *MYCN* amplification (MNA) is associated with aggressive tumors and a poor prognosis in all the tumor stages.

Segmental chromosomal imbalances and focal aberrations are very frequent in high-stage tumors and are a prognostic marker in particular subtypes of NB [3]. Low-stage tumors typically show whole-chromosome number alterations. More specifically, tumors with only numerical aberrations have a favorable prognosis, while any presence of segmental aberrations indicates poor survival [4]. Segmental chromosomal alterations (SCA) are considered a bad prognostic factor in some groups of low- and intermediate-risk NB [5]. However, as the majority of high-risk tumors show segmental aberrations, prognostic stratification based on the absence or presence of segmental aberrations is not

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considered in high-risk NB SIOPEN protocols. In some groups of the intermediate-risk protocol, the presence of SCA is also not considered in risk stratification [5].

Specific SCA, such as 17q gain, 2p gain and 11q deletion, are well described in previous literature and their impact in final outcome has been well defined in most of them [6]. A few studies mention the possible role of 1q gain, 1p deletion, 3p deletion and 4p deletion [7–10]. Aside of these seven alterations, no other imbalances have been established and in-depth associated with outcome in NB. Very few studies have been undertaken aiming to identify aberrations that discriminate outcome in high-risk patients and the role of many other segmental imbalances is still awaiting to be defined. Moreover, there is scarce literature focusing on numerical chromosomal alterations (NCA) and how they may have an impact on prognosis.

To investigate the role of less frequent segmental imbalances and certain NCA, we reviewed previous literature and conducted the following study. Our aim was to describe the distribution of SCA in NB according to INRGSS stage, *MYCN* status, age, mortality and relapse. Special emphasis was made on associations between particular SCA which recurrently appeared together in the same tumors. The role of some particular NCA has also been analyzed.

Materials and methods

This retrospective multi-centric study was conducted with 155 NB tumor samples collected from 29 cooperating Spanish hospitals between 2013 and 2018. Data collected from patients included age at diagnosis, primary tumor location, stage, *MYCN* status, 11q status, treatment received, relapses, follow-up and results from SNP arrays including NCA and SCA profiling.

Staging and risk stratification were established according to INSS and INRGSS classifications [11–13]. Tumor samples from all patients were referred to Hospital U i P La Fe for pathology and molecular biology studies. Samples were centrally reviewed and classified according to the International NB Pathology Committee (INPC) system [14–16]. Biological studies included *MYCN* status studied by FISH and 11q status studied by SNP arrays (Affymetrix) according to the European NB Quality Assessment (ENQUA) guidelines [17–19]. Patients were registered in the Spanish Society of Pediatric Hematology and Oncology (SEHOP) NB group database file and included for treatment mainly in SIOPEN trials (LINES, LNESG-II, EUNS and HRNBL1 [20]). As recommended by the ENQUA guidelines, amplification of *MYCN* was defined as a more than a fourfold increase of *MYCN* signals in relation to the number of chromosomes 2. Cases with two- to fourfold increase in *MYCN* signal were defined as *MYCN* gain [21, 22].

The 155 NB samples were studied by molecular karyotyping using SNP arrays Affymetrix CytoScan HD to determine the SCA and NCA profiles. The quality and quantity of DNA were determined by spectrophotometry (Nanodrop 2000). To process the arrays, DNA samples chosen met optimum quality standards such as a relation A260/A280 (ratio DNA/RNA) between 1.8–2, a relation DNA/alcohols above 1.8, a sufficient DNA concentration and a proper DNA integrity. DNA quantity and integrity were assessed on an Agilent 2200 TapeStation using a DNA ScreenTape.

The database was compiled with patient data that met inclusion criteria, such as the availability of molecular karyotype reports that showed some type of numerical or segmental chromosomal alteration. Those patients whose samples were not suitable for genomic studies or with no available reports were not considered. NB without SCA and NCA were also excluded from analysis ($n = 8$). Loss of heterozygosity (LOHs) detected were not considered in the analysis.

Data were summarized using mean (standard deviation) and median (1st and 3rd quartiles) for numerical variables and absolute frequency (relative frequency) for categorical variables. To find an association between the presence of NCA and SCA with the variables age, *MYCN* status and INRGSS stage, two logistic regression models were adjusted. To find clinical (age, INRGSS stage) or biological (*MYCN* status, SCA, NCA) factors associated with relapse and mortality, two Cox models penalized by elastic net were adjusted. The penalty factor (lambda) was selected by taking the median of 200 replicates of the value that minimized the cross-validation error in each replicate. Frequency bar charts were used to show the summary of data, conditional plots to show the relationship between whole-chromosome 19 gain and the studied variables and box plots to illustrate the comparison between groups of quantitative variables. Survival data were illustrated using Kaplan–Meier graphs. A p value less than 0.05 was considered significant.

All study actions were done under the appropriate ethics code and informed consents for samples and data management were obtained in all cases from the patients' parents or guardians. The study was performed in accordance with the Declaration of Helsinki.

Results

General description

A total of 155 patients with NB were studied between 2013 and 2018 (Table 1). NB without SCA and NCA were excluded from analysis ($n = 8$). Median and mean age at diagnosis were found to be 14.7 and 32.6 months, respectively (median range 0.06–364.3, interquartile range 32.5).

Table 1 General description of the studied population ($n=147$) according to INRGSS stage, presence of SCA, MYCN status, mortality and relapses

Variable	$N=147$
Median age at diagnosis (months)	14.7
Stage (INRGSS)	
L1	26 (18%)
L2	43 (29%)
M	51 (35%)
Ms	27 (18%)
SCA/NCA	
SCA	102 (69%)
NCA only	45 (31%)
MYCN	
Non-amplified	111 (76%)
Gain	12 (8%)
Amplified	24 (16%)
Death	
No	119 (81%)
Yes	28 (19%)
Relapse	
No	88 (60%)
Yes	59 (40%)

According to the INRGSS classification, 26 of the cases (18%) were considered as L1, 43 cases (29%) as L2, 51 cases (35%) were stage M at diagnosis and 27 (18%) were classified as stage Ms. MNA was observed in 24 NB samples (16%) and 12 tumors had *MYCN* gain (8%), whereas in the remaining 111 cases, *MYCN* status was non-altered (76%). During follow-up, 28 patients died (19%) and 59 patients had at least one relapse (40%) with a median follow up of 32 months. Segmental and numerical chromosomal rearrangements studied with SNP arrays showed 45 tumors lacking SCA and only containing NCA (31%) and other 102 NB that contained SCA (69%), despite NCA status.

The most frequent SCA detected was +17q observed in 34% of the tumors ($n=50$), followed by +2p ($n=38$) and −11q ($n=37$). Other frequent imbalances found were −1p ($n=29$), +1q ($n=21$), +11q ($n=18$), −3p ($n=17$), +1p ($n=16$), −4p ($n=15$) and +11p ($n=15$). +2q, +6q and +7q were found in 13 tumors. Frequency of SCA is shown in Fig. 1. Overall survival (OS) was significantly lower ($p=0.028$) in tumors with SCA (Fig. 2).

SCA distribution according to INRGSS stage

The tumor stage that presented less SCA was stage Ms (41%), followed by L1 (62%), L2 (65%) and finally stage M in which 47/51 tumors contained at least one SCA (92%). Only four NB classified as stage M did not show SCA, with three of them being alive. Gain in 17q was the most common alteration found in every stage (51% in stage M, 28% in localized and 19% in Ms). SCA distribution according to INRGSS stage can be observed in Fig. 3.

SCA distribution according to *MYCN* status

NB with MNA contained SCA in 96% of the tumors, whereas NB without MNA only had SCA in 65% of the cases. In the tumors showing MYCN gain, 58% presented an SCA. The most frequent alteration in non-MNA tumors was 11q deletion, whereas +17q was the most common SCA detected in MNA NB. In *MYCN* gained subgroup, both +17q and +2p were identified as the most common SCA (33% in both).

SCA distribution according to age

In this study, the presence of SCA was significantly associated with older patients. 93% of patients older than 18 months at diagnosis (62/67 patients) have at least one SCA. Specific SCA that were significantly associated with older age were +1q and −3p. The most frequent SCA found in patients younger than 18 months was +17q.

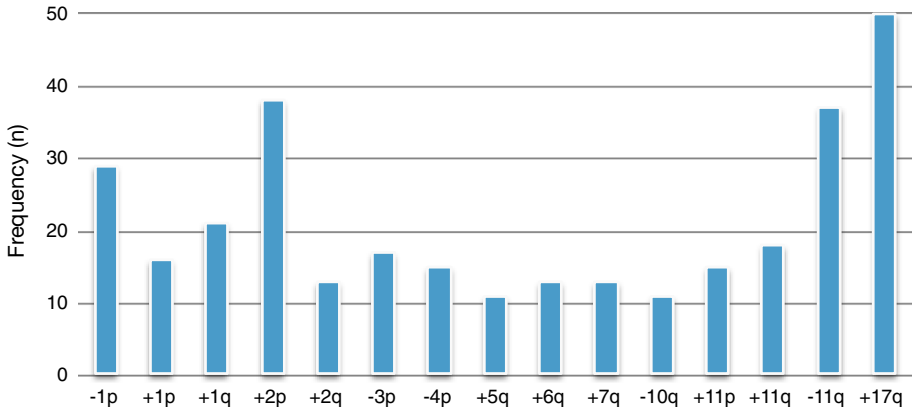

Fig. 1 Frequency bar chart showing distribution of SCA determinations obtained by molecular karyotyping

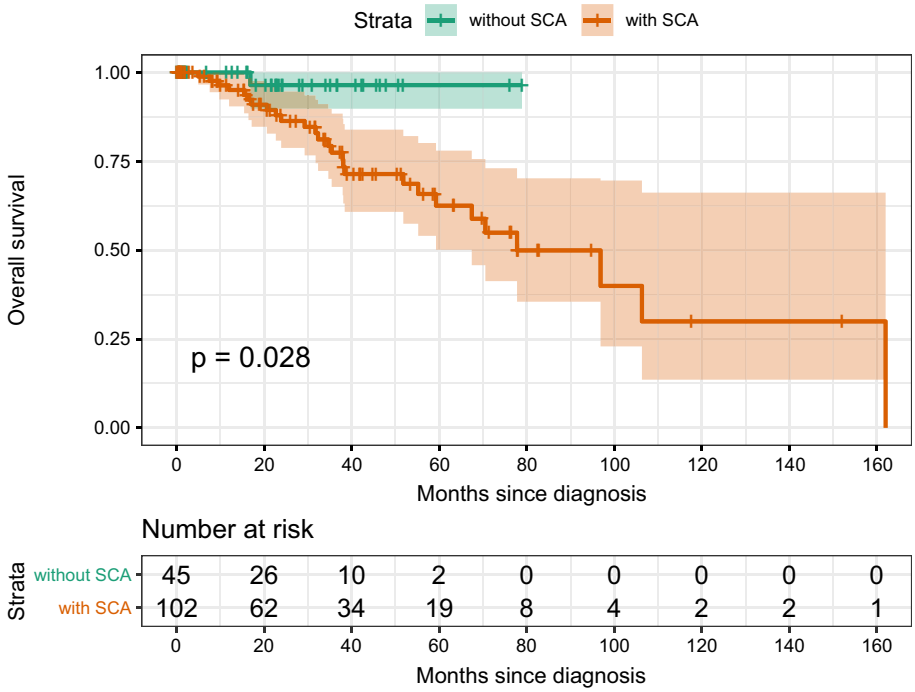

Fig. 2 Kaplan–Meier graph comparing overall survival stratified by copy number profile: SCA ($n = 102$) and non-SCA (only NCA) neuroblastoma ($n = 45$)

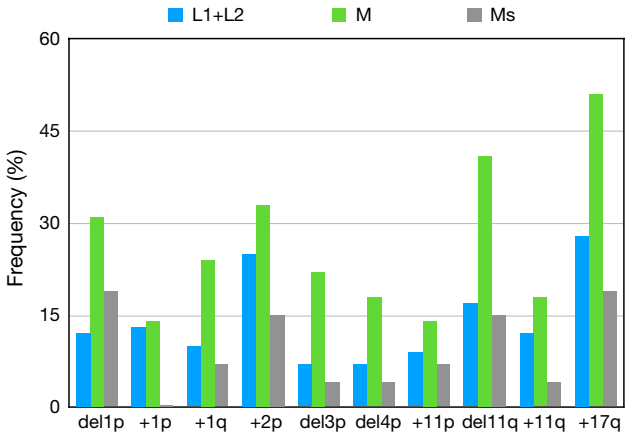

Fig. 3 Frequency bar chart (%) showing SCA distribution according to INRGSS stage (L1 + L2, M and Ms stage)

SCA distribution according to mortality

A total amount of 28 patients died during the follow-up period because of disease progression and 27 of them (96%) had at least one SCA. Only one SCA-free patient died during the follow up period. 98% of the patients without SCA survived, whereas 74% of the patients with SCA survived. The most frequent SCA in the mortality subgroup (surpassing + 17q) was − 11q, while + 17q was the most frequent alteration in the patients that survived. Other frequently observed SCA in the cases that died were − 3p and + 11p, while the most frequent SCA found in survivors following + 17q were + 1q and − 10p. A longer follow-up period is required to achieve further conclusions, especially in patients with 11q deleted NB that usually relapse later compared to patients with MNA NB.

SCA distribution according to relapse

Relapsed NB presented SCA in 85% of the cases. NB with SCA relapsed in 49% of the cases, whereas only 20% of the patients relapsed in the SCA-free group (9/45). The SCA most frequently detected in the relapsed patients was − 11q, surpassing + 17q. On the other hand, in the non-relapsed patients group, the most common SCA observed were + 17q and − 1p.

SCA combinations

To date, the most recurrent SCA reported have been + 17q, + 2p, − 11q, + 1q, − 1p, − 3p and − 4p. Other specific alterations have not been widely reported, except from isolated reports such as 6q loss, described by Depuydt et al. [23] in a subgroup of "ultrahigh-risk" NB, or 7p gain described by Pezzolo et al. [7] as a protective factor.

In this study, according to previous literature, the most recurrent alterations described were + 17q (50 cases) that was detected in 49% of NB with SCA, followed by + 2p (38 cases) and − 11q in the third place (37 NB). Other recurrent alterations found were − 1p ($n = 29$), + 1q ($n = 21$), − 3p ($n = 17$) and − 4p ($n = 15$). However, other SCA less described in previous literature have also been found in a

significant proportion such as + 1p, + 2q, + 5q, + 6q, + 7q, − 10q, + 11p and + 11q (Fig. 1).

The most common alteration associated with a higher number of other segmental rearrangements was − 11q, followed by − 4p. To study possible associations and recurrent patterns of combinations in NB, several SCA including the most frequent ones were studied separately. Cluster graph (Fig. 4) shows associations between the most recurrent SCA. + 17q had significant associations with + 5q and − 10q. + 2p was correlated together with + 11p, while + 1q appeared with + 1p, + 5q and + 11p alterations. 11q deletion was the aberration mostly related to other chromosomal imbalances (+ 7q, − 10q, + 11p and + 11q) and − 4p appeared more frequently together with + 2q and + 7q. In addition, − 3p correlated with + 7q and − 10q, while 1p deletion was not associated with other particular SCA and was demonstrated as the less frequent SCA associated with other segmental imbalances.

NCA distribution

In this study, 45/147 NB presented with NCA without SCA (31%). Survival in this group was high (98%) and most of these NB were classified as localized or Ms tumors. Whole chromosome gains and losses were found more frequently than SCA, although sometimes both NCA and SCA were detected in the same tumor. In this cohort, tumors with SCA and NCA behaved as tumors with only SCA. The most frequent NCA observed has been gain in chromosome 7 (86/147), followed by + 18 (67/147), + 17 (66/147), + 12 (63/147) and + 13 (62/147). OS in tumors with or without these numeric aberrations has been studied and no differences have been detected in survival regarding these alterations.

However, a small subgroup with whole gain in chromosome 19 ($n = 22$) that included NB of all tumor stages was analyzed and patients with this alteration showed an outstanding favorable outcome. Gain in chromosome 19 in this cohort was significantly associated with other low-risk features in NB such as the absence of SCA (OR 0.178) and lower INRGSS tumor stage (OR 0.558) (Figs. 5 and 6). The Kaplan–Meier graph (Fig. 7) illustrates OS comparison between the cases with whole-chromosome 19 gain ($n = 22$) and the cases with no numeric alterations in chromosome 19 ($n = 102$), observing remarkable differences between these two groups ($p = 0.031$). Cases with whole loss of chromosome 19 ($n = 23$) have been excluded in this comparison. These differences were not observed when analyzing other NCA.

Discussion

Clinical heterogeneity in NB is a well-recognized feature of this cancer and as more insight is reported in this tumors' biology, this fact becomes gradually more understandable. Somatic chromosomal copy number alterations have shown to be associated with prognosis, especially in low- and

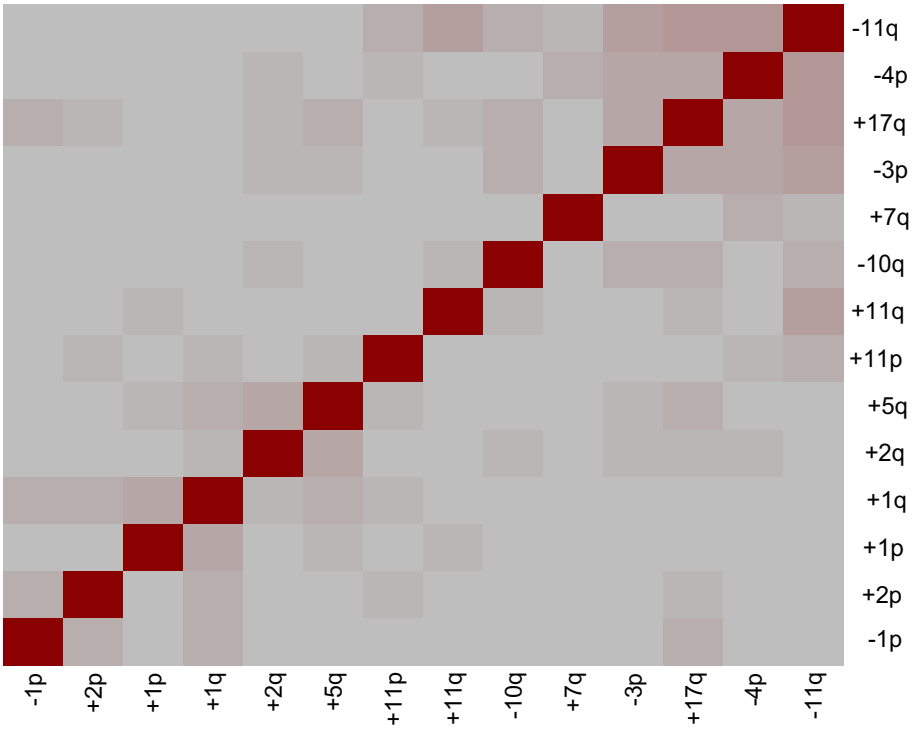

Fig. 4 Cluster color intensity graph showing associations between the most recurrent SCA (association probability between SCA in the same tumor is higher as the red shading intensity increases)

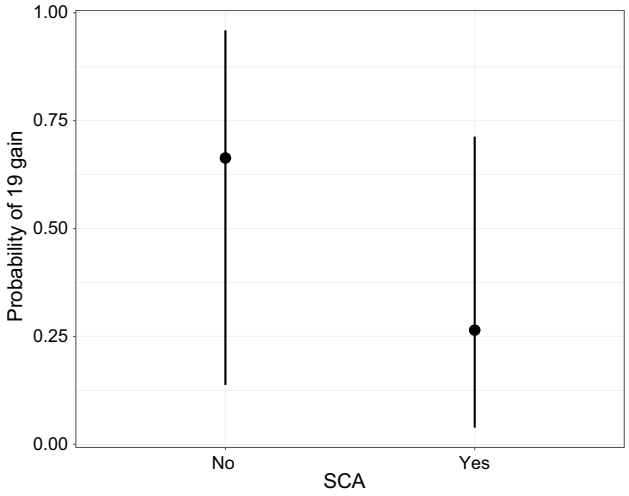

Fig. 5 Conditional plot showing association between whole gain in chromosome 19 and SCA presence (n19gain = 22)

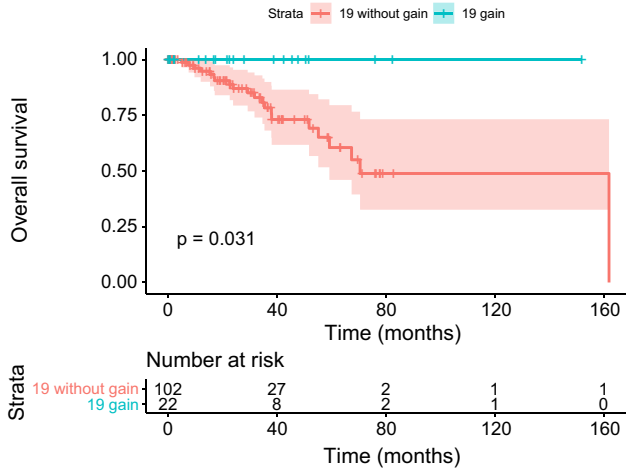

Fig. 7 Kaplan–Meier graph comparing overall survival in 22 neuroblastoma with whole gain in chromosome 19 (n19 = 1) and 102 tumors without numeric alterations in chromosome 19 (n19 = 0)

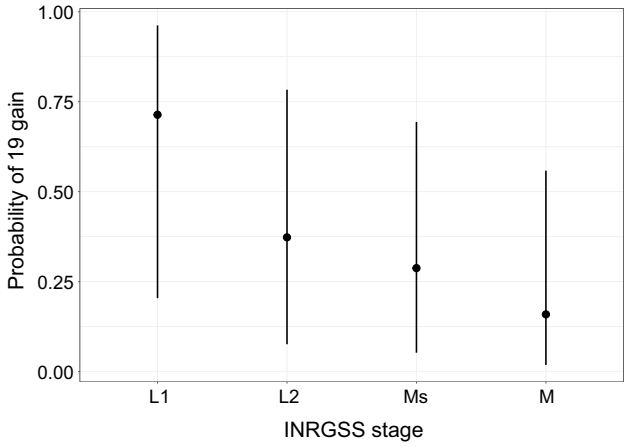

Fig. 6 Conditional plot showing association between whole gain in chromosome 19 and INRGSS stage (n19gain = 22)

intermediate-risk cases and this information is used in SIOPEN protocols [5, 20] to stratify risk. While aggressive NB phenotypes usually contain SCA (translocations, amplifications or deletions), favorable cases are mostly SCA free and frequently complete gains or losses in whole chromosomes are observed. In terms of outcome, it has been highlighted that tumors containing both SCA and NCA behave as cases that present SCA solely, thus proving the relevance of SCA above NCA. Gain of 17q, loss in 1p and loss in 11q are the most frequent [3] and studied segmental imbalances up until now and this is further confirmed in the present study. Excluding these, few studies have improved our insight into other SCA and how these combine together.

Gain of 17q is the most frequent SCA observed in sporadic NB and has previously been associated with a higher age, 1p deletion and MNA. In this cohort, 17q gain appears in 34% of the tumors and in 49% of the NB with SCA. Approximately, 50% of the NB samples with 17q gain contained − 1p and − 11q. Prognosis value of 17q gain as a unique factor is still unknown and has been controversial. Lastowska et al. [24] and Bown et al. [25] related 17q gain to worst outcomes, while in the cohort reported by Spitz et al. [26] no association between 17q gain and an inferior prognosis was found. A poor outcome prevails when 17q gain is combined to the other mentioned SCA [27].

Genetic alterations in the long arm of chromosome 11 are more diverse. Allelic loss is the alteration mostly observed, but unbalanced translocations (in 11q21 and 11q22 regions), deletions in 11q23 and inversion in 11q21–q23 regions have also been described [28]. Loss in 11q is inversely related to amplification of *MYCN* [8]. In this study, co-existence of both abnormalities was detected in only 4% of the cases. Moreover, deletion in 11q is associated with very bad outcome and higher chances of relapse [27, 28]. The existence of this alteration in low- and intermediate-risk tumors as well as in Ms tumors configures very aggressive NB phenotypes [29, 30].

The frequency of 11q alterations may vary between 20 and 45% [30], (25% in this study) and it is associated with a higher median age at diagnosis [30]. As further confirmed in this cohort, 11q deletion is the alteration most frequently associated with other concomitant SCA and a higher number of chromosomal breakage points. Furthermore, 11q deletion was the most frequent SCA (above + 17q) in the relapse and mortality subgroups. In our study, the number of tumors with 11q deletion was higher in relapsing patients compared to those who did not, as well as in those who died. This has been described in previous studies comparing the poor

prognosis of 11q deletion, similar to those of MNA NB [30, 31].

The incidence of 2p gain in this cohort was relatively high (25%) and appeared in tumors with advanced INRGSS stage and tumors with *MYCN* gain [32]. 2p gain has been related before with advanced disease stages at diagnosis and decreased average 5-year OS and EFS [32]. Oncogene *MYCN* is localized in chromosome 2p. Although amplification of *MYCN* is the most robust genetic factor that is correlated with poor outcome, very little is known about the role of *MYCN* gain and other 2p gains [6]. A remarkable fact is that *MYCN* gain has been related in previous studies to 11q deletion [30]. Most of the patients with both of these aberrations (11q-del and *MYCN* gain) presented high-risk features in this study. *ALK* gene is also located on the short arm of chromosome 2 (2p23) and encodes a protein called anaplastic lymphoma kinase, which is a receptor tyrosine kinases (RTK). Changes in *ALK* lead to an abnormal version of the protein that is constitutively active, which induces abnormal proliferation of immature nerve cells and leads to NB. Previous reports suggested that *ALK* copy number multiplication, if occurring concurrently with *MYCN*, significantly reduces patients' survival especially for the intermediate- and high-risk group and that 2p gain tumors encompassing the *ALK* locus also associate with worse outcome in these patients with NB [6].

Deletion in 1p has been the fourth more frequent SCA found in this study. Several tumor suppressor genes have been identified in this particular región, although there is not enough evidence supporting their role in NB etiology [9]. Loss of chromosome 1p has been previously described by Caron et al. [9] as a strong prognostic factor in patients with NB, independently of age and stage that reliably identifies patients at high risk in stages I, II, and 4 s, which are otherwise clinically favorable. In this study, deletion in 1p was found to be the most frequent SCA after + 17q, − 11q and + 2p (29 cases harboring − 1p), but surprisingly it is the alteration less related to other chromosomal breakage.

Spitz et al. [10] reported 26 cases with 3p changes (18%), most of them deletions, in a cohort of 144 NB. They associated this alteration with stage 4: 20 out of 59 (34%) versus 6 out of 85 (7%) that were in stages 1–3 and 4 s, $p = 0.007$. Median age in the group with 3p changes was higher ($p < 0.001$) than in those with no 3p aberrations. Aberrations in chromosomes 3p and 11q were found to be highly associated with each other ($p < 0.001$), as further confirmed in the present study. Moreover, patients with 3p and 11q aberrations with localized or stage 4 s tumors showed a clearly worse outcome compared to those without any alterations ($p = 0.002$ and $p = 0.0027$ respectively) [10]. It has been suggested that the association of 3p deletion with 11q deletion may constitute a new high-risk subgroup within non-MNA tumors, both localized, 4 s and disseminated. In this study, 17 cases with 3p deletion were identified, and 76% of them had 17q gain (13/17 cases).

Gain of 1q was associated in this study with + 1p, + 5q and + 11p. The high presence of this alteration in other series in NB raised the possibility that genes involved in cancer development could be located in this region. By analyzing this phenomenon in other pediatric tumors such as retinoblastoma, a potential oncogene known as *KIF14* was identified. Its role in NB has yet to be defined [33].

In this study, we demonstrate that 4p deletion ($n = 15$) is the SCA most associated with other chromosome imbalances after 11q deletion, as observed in Fig. 4. 4p deletion predominates in the population older than 18 months (73%) and is also associated with relapse (12/15 cases relapsed). The paired-like homeobox 2B gene (*PHOX2B*) is located in chromosome 4p13. This gene plays an essential role in autonomic nervous system differentiation and has been identified as a sensitive and specific biomarker for minimal residual disease detection in NB. Mutations in *PHOX2B* have been found in individuals with neural crest disease involvement [34]. A low frequency of heterozygote germline alterations in *PHOX2B* has also been identified in sporadic NB patients and in patients with familial NB. Krona et al. [34] investigated the role of *PHOX2B* and data from this case suggest a model in which progressive growth and additional genetic changes occurred from the first tumor after activation of the second *PHOX2B* allele by chromosome 4p deletion and 17q gain [34]. Our results further confirm the association between 4p deletion and 17q gain.

One of the most frequently observed alterations was 11p gain (10.2%). In previous studies, this has been associated with 11q deletion via translocation or unbalanced deletion [35]. This statement can account for the high presence of this aberration in the relapse group of our cohort, an event to which 11q deletion is associated.

Depuydt et al. [23], identified an extremely poor outcome in patients with distal 6q losses. In their study 5, 9% of the patients harbored 6q deletion and 10-year overall survival was only 3.4%. They also observed that only 25% of the tumors with 6q loss also had MNA and that 6q loss contained more breakpoints than tumors without this abnormality [23]. In our cohort only 5/147 cases presented 6q deletion, all of them being classified as stage M and mortality detected in this subgroup was 40%.

The majority of previous research has focused on the existence of SCA and how their presence is linked to a worse outcome. Very few studies refer to the possible influence of particular NCA in NB prognosis. Apparently when SCA and NCA appear together in NB, the negative influence of SCA is predominant and therefore the possible influence of some NCA cannot be evaluated. However, when only NCA are present, Parodi et al. [36] recently described that loss of whole chromosome X can predict prognosis of NB

patients with only numerical genomic profile. In this study that included DNA copy-number data of 174 NB with NCA genomic profile the association between poor EFS and whole chromosome X alteration was reported. In our study, 19/147 NB had whole-chromosome X loss and most of these tumors were associated with the presence of SCA (13/19) and mortality was 32%.

We report a significant favorable outcome in patients with whole numeric gain in chromosome 19 ($p = 0.028$). Although this has been detected in a small group of 22 cases, this has not been observed when analyzing other numerical alterations. Whole gain in chromosome 19 in this study is associated with lower INRGSS stage and the absence of SCA. It is known that this chromosome has the highest gene density of all human chromosomes, more than double the genome-wide average. The large clustered gene families, corresponding high guanine-cytosine content, CpG islands and density of repetitive DNA indicate a chromosome rich in biological and evolutionary significance [37]. Mutations in tumor suppressor genes and other alterations in chromosome 19 have been related to multiple types of human cancer such as leukemia, lymphoma and other solid tumors as gliomas and lung cancer. Nakamura et al. [38] described frequent loss of heterozygosity on chromosome 19 in secondary glioblastomas and how tumor suppressor genes located on chromosome 19q13.3 have a role in progression of low-grade astrocytomas to secondary high-grade glioblastoma. Gain in chromosome 19 has also been the most common abnormality detected by comparative genomic hybridization by Alvarez et al. [39] in acute megakaryocytic leukemia, a rare subtype of acute myeloid leukemia that is most commonly associated with Down syndrome in children. In NB, loss of heterozygosity in 19q has also been related before with locally aggressive tumors [40], but, so far, whole numeric changes in this chromosome have not been related to outcome in NB. A larger cohort is probably required to confirm the impact of this alteration in prognosis and the appropriateness of considering this abnormality as a biological marker.

In conclusion, we report that SCA are not randomly distributed and are concentrated on recurrent chromosomes. The most frequently affected chromosomes are recurrent in specific prognostic groups according to NB well-established stratifying factors (age, INRGSS stage and *MYCN* status [41]). Presence of SCA is associated with older age and MNA. Specific segmental brakes are associated and appear together with other SCA, specially 11q deletion and 4p deletion. Whole gain in chromosome 19 is related to the absence of SCA and a lower INRGSS stage. A small subset of patients with a whole numeric gain in chromosome 19 and a better outcome have been identified.

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Compliance with ethical standards

Conflict of interest The authors declare no competing financial interests and no other non-financial competing interests.

Ethical approval (Research involving human participants and/or animals) The study was approved by the Ethics and Investigation Committee Hospital U i P La Fe (CEIm). The study was performed according to the Declaration of Helsinki.

Informed consent All study actions were performed under the appropriate ethics code, and consent was obtained from all patients within the clinical trial recruited for treatment.

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RESEARCH ARTICLE

Survey on paediatric tumour boards in Europe: current situation and results from the ExPo-r-Net project

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Abstract

Background Under the ExPO-r-NeT project (European Expert Paediatric Oncology Reference Network for Diagnostics and Treatment), we aimed to identify paediatric oncology tumour boards in Europe to investigate the kind of technologies and logistics that are in place in different countries and to explore current differences between regions.

Methods A 20-question survey regarding several features of tumor boards was designed. Data collected included infrastructure, organization, and clinical decision-making information from the centres. The survey was distributed to the National Paediatric Haematology and Oncology Societies that forwarded the survey to the sites. For comparative analysis, respondents were grouped into four geographical regions.

Results The questionnaire was distributed amongst 30 countries. Response was obtained from 23 (77%) that altogether have 212 paediatric oncology treating centres. A total of 121 institutions answered (57%). Ninety-one percent of the centres hold multidisciplinary boards; however, international second consultations are performed in 36% and only 15% participate on virtual tumor boards. Videoconferencing facilities and standard operational procedures (SOPs) are available in 49 and 43% of the centres, respectively. There were statistically significant differences between European regions concerning meeting infrastructure and organization/logistics: specific room, projecting equipment, access to medical records, videoconferencing facilities, and existence of SOPs.

Conclusion Paediatric tumor boards are a common feature in Europe. To reduce inequalities and have equal access to healthcare, a virtual network is needed. Important differences on the functioning and access to technology between regions in Europe have been observed and need to be addressed.

Keywords Paediatric cancer · Multidisciplinary care · Virtual tumour boards · European survey · European reference networks

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Introduction

Multidisciplinary care is the hallmark of high-quality cancer management [1]. This statement has been supported over the last decades where individual opinions have been displaced by collective and multidisciplinary decisions in approaching a patient with a complex oncologic disease. Although childhood cancer is a rare disease, it is the first cause of non-accidental death in childhood and sharing of expertise is paramount in this field. Several studies have shown that tumour boards lead to changes in diagnosis and staging of cancer patients, affect management decisions, and increase quality of care [2, 3]. Therefore, they should be an integral part of adult and childhood cancer patient care around the world [4–7].

A paediatric tumour board comprises a multidisciplinary team of experts including paediatric oncologists, radiologists, surgeons, radiotherapists, pathologists, and other disciplines as relevant for the respective cancer case to discuss patients' clinical cases where the diagnosis and treatment plan are complex. The patients' clinical management following a discussion in a tumour board takes into account the opinion of several experts participating in a particular meeting.

ExPO-r-Net (European Expert Paediatric Oncology Reference Network for Diagnostics and Treatment) is a project supported by the European Commission aiming to prepare for a Paediatric Oncology European Reference Network. This network will facilitate the cross-border healthcare for children and young people with cancer to access expertise and specialist healthcare in an EU Member State other than the Member State of affiliation. European Reference Networks (ERNs) are a feature of the EU Directive on Cross-Border Healthcare aiming to unite the best specialists from across Europe to tackle complex or rare medical conditions that require highly specialised healthcare and a concentration of knowledge and resources.

One of the missions in the ExPO-r-Net project is the creation of a virtual tumour board network across Europe. A virtual tumour board is a special type of multidisciplinary meeting based on videoconference systems that can involve experts from different centres. For practical reasons, these tumour board meetings need to be "virtual" using videoconference systems to allow regular meetings and discussions between experts in different countries. The virtual tumor board can, therefore, convene regularly and rapidly to discuss urgent cases.

As a first step towards a European Paediatric Oncology Virtual Tumour Board Network, ExPO-r-Net aimed to identify European already existing tumour boards of paediatric oncology units to investigate the kind of technologies and logistics that are in place in the different countries and to explore current differences between European regions.

Materials and methods

A standard questionnaire with 20 questions regarding several features of multidisciplinary practice in paediatric oncology was designed by Hospital Universitari i Politècnic La Fe (València). Data collected included information from the centres, number of patients treated per year, infrastructure for meetings, organization/logistics, and clinical decision-making. The majority of the questions were multiple choice but some open questions, which allowed free text explanations, were also included. The questionnaire went through an internal review first and, finally, an external review was done by the University of Birmingham (UK), as part of the quality assessment work package in the ExPO-r-Net project.

After finalisation, the survey was distributed to the national leaders of the European National Paediatric Haematology and Oncology Societies (NaPHOS) by email. Contacts were provided by the European Society for paediatric oncology (SIOPE). Participants were sent an introductory email asking for information about the paediatric oncology treatment situation in their country (number of centres and number of patients treated per centre). If participation was accepted, a second email with an invitation link to complete an electronic survey, set up via a freely available Web-survey tool, was forwarded. The survey was disseminated by the NaPHOS in each country to all the centres with a paediatric oncology unit. Surveys were completed online between July 2015 and March 2016. A simplified version of this questionnaire is presented in this paper (Fig. 1).

Results were collected and analysed at Hospital La Fe. Questionnaires from 30 countries were received and a descriptive analysis was performed from of the information provided. For comparative analysis between geographical regions, respondents were grouped into four European geographical regions: Northern, Central, Southern, and Eastern, following the methodology of the EUROCARE-5 population-based study [8]. Statistical significance among regions was assessed by multinomial logistic regression and $p < 0.05$ was considered as statistically significant.

Results

The countries

The following 30 European countries were included in the study: Austria, Belgium, Bosnia-Herzegovina, Bulgaria, Croatia, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia,

1. Number of new cancer patients seen at your institution per year?
A. < 15 cases/year. B. 15-25 cases/year. C. 25-50 cases/year. D. 50-75 cases/year. E. > 75 cases/year

2. Do you have a Paediatric Multidisciplinary Tumour Board? A. Yes. B. No

3. How often do you meet? A. Weekly. B. Biweekly. C. Monthly. D. Other

4. What is the usual length of your Paediatric Tumour Board Meeting? A. Up to 1 h B. 60–120 min C. 120–180 min D. 120-180 min. E. More than 3 hours

5. Indicate the number of cases discussed by your Paediatric Tumour Board Meeting in a single meeting: A. 1–2 cases B. 3–4 cases C. 5–6 cases D. 7–9 cases E. 10–12 cases F. > 12 cases

6. The Paediatric Tumour Board Meeting room has: A. A specific room for this purpose B. Equipment for projecting and viewing radiology images/specimen biopsies C. Equipment connected to PACS (Picture Archiving and Communications System) D. Access to retrospective images/reports during the meeting

7. In the case of specialist not belonging to your institution, how do you manage it? A. They move to our institution for the meeting B. Teleconference C. Videoconference D. They do not participate

8. Does your Paediatric Tumour Board Meeting room have videoconferencing facilities? A. Yes, H. 323 Video Conference System B. Yes, web-based videoconference system (Skype, Adobe Connect, etc.) C. No

9. Are patients informed that a Paediatric Tumour Board will review their treatment/care? A. Yes. B. No

10. If Paediatric tumour board recommendations are not followed, is the patient informed? A. Yes. B. No.

11. Do you have any standard operational procedures? A. Yes. B. No.

12. Do you usually make a report with the final recommendations? A. No. B. Yes, in paper format. C. Yes, in electronic database.

13. Paediatric Tumour Board recommendations are: A. Mandatory B. Optional

14. Is there electronic data exchange with other hospitals? A. Yes. B. No.

15. Do you participate in any Virtual Paediatric Tumour Board? A. Yes B. No.

Fig. 1 Simplified version of the European questionnaire

Lithuania, Macedonia, Netherlands, Norway, Poland, Portugal, Romania, Serbia, Slovak Republic, Slovenia, Spain, Sweden, Switzerland, and United Kingdom.

Total responses

A total of 23 NaPHOS responded (77%) and distributed the questionnaire to the respective centres in their country specialised in childhood cancer care. This corresponds to a total of 212 institutions; 121/212 (57%) responded.

Existence of multidisciplinary tumour boards and number of patients

The first question of the survey addressed the existence of a tumour board in the institution; 110/121 centres (91% of the respondents) confirmed regular multidisciplinary tumour board meetings, while in 11 centres (9%), they were not part of routine practice. All the latter were smaller centres treating less than 25 new cases per year.

The survey also reflected that 62% of centres had an annual accrual rate of more than 50 patients, 23% accrued

between 25 and 50 patients, and only 15% accrued less in varying numbers.

Meeting organization

The frequency of the interdisciplinary meetings was also studied. Weekly boards were held in 43% of the centres, biweekly meetings in 23%, and monthly ones in 21% and other frequencies in the remaining 13% of the centres. The duration of tumour board meetings was reported to be less than 60 min by 42% of centres, 60–120 min in 45%, and 120–180 min in 9%, whilst 4% reported duration of more than 180 min.

The number of cases discussed during each meeting is another important parameter: more than 12 patients are addressed per tumour board in 9% of the centres, 7–11 cases in 26%, 3–6 cases in 48%, and 1–2 cases in 17%.

Infrastructure for meetings

In 44% of the institutions, standard operational procedures are in place, whilst they do not exist in 56%. Reports summarizing discussions held and recommendations are available in paper format for 45% of the institutions, electronic format for 43%, and are unavailable for 12%.

We also addressed the availability of a specific room to hold tumour board meetings: 66% of the centres had a specific room for this purpose, whilst 34% did not. A projector to visualize images for all the staff present was available in 80% of sites. Of those 68% had access to PACS (Picture Archiving and Communication System). Finally, 77% of centres had direct access to the clinical records from the same room.

Clinical decision-making

The decision-making processes at sites have been addressed and found that discussions were particularly fruitful if all specialists involved in the patients' care were present. However, in 55% of the centres, discussions happen without all involved specialists being present, while in 45% of them, a particular patient was not discussed unless all disciplines were represented.

In the vast majority of cases (93%), patients were informed that their case was going to be discussed in a multidisciplinary forum, whilst this was not the case in 7% of centres. While 53% of the institutions made recommendations or action points addressed in the tumour board mandatory, there were regarded as only optional actions by 47% of the centres.

Information exchange

The survey also addressed the participation of members from different institutions. Physical movement of physicians occurred in 54%. Videoconferencing as a mean to share common discussion was reported by 20% of the centres, whilst 5% are using teleconferencing. Finally, in 19% of centres, specialists from different institutions never participate in these meetings.

On a national basis, second consultations are requested by 62% of the centres, whilst internationally, 64% were reported. It must be mentioned here that some of the reporting institutions located in smaller countries are the only ones for paediatric oncology. Therefore, there is no possibility of a national consultation.

Virtual/ICT logistics

Evaluation of the current state of videoconferencing and IT facilities at sites was one of the main themes to be addressed for the purpose of a baseline. Centres were asked if videoconferencing facilities are available according to the H.323 standard for audio and video multimedia teleconferencing, Web-based videoconference systems (Skype, Adobe Connect, Webex, etc.), or if no facilities were available. As reported in this survey of the date of this report, 52% of the centres did not have any kind of access to videoconferencing, 28% had access to Web videoconferencing systems, but only 20% had H.323-based videoconferencing systems.

Electronic data exchange was done in 57% of the institutions. In 77% of them, this happens prior to the tumour board, so that cases may be well prepared. Of the 43% of centres currently not exchanging electronic data, 33% indicated that this would actually be an urgent necessity.

Finally, we specifically assessed the number of centres that already participate in virtual tumour boards, since this is a major issue related to the setup of European cross-border, virtual tumour board network. Surprisingly, only 14% of the centres participated already in virtual meetings at the time of evaluation. The vast majority of centres in Europe (86%) do not usually carry out this type of meetings.

Comparative analysis between regions

To investigate the differences in the functioning of tumour boards between geographical regions, comparative analysis was performed taking into consideration 108 responses from 21 countries. Countries with a rate of response below 40%

Table 1 Percentages of tumour board facilities available in the different European geographical regions

	Northern (n = 16) (%)	Central (n = 23) (%)	Southern (n = 55) (%)	Eastern (n = 14) (%)
Existence of SOPs	67	71	29	36
Specific room for tumour board	81	96	60	36
Projector available	94	91	74	43
Videoconferencing facilities	69	87	33	21
Virtual tumour boards	44	30	4	0

were excluded and the answers from the centres without multidisciplinary boards were also excluded from analysis.

The respondents were grouped into four geographical regions in Europe: Northern, Central, Southern, and Eastern [8]. Statistical significance among regions was assessed by multinomial logistic regression and $p < 0.05$ was considered indicating statistical significance. Global results of the percentages of availability of certain issues related to multidisciplinary boards are provided in Table 1.

A comparison of the results obtained from the answers in Northern and Central Europe with Southern and Eastern European countries has been done. Statistically significant differences in several aspects regarding the functioning and the availability of facilities for tumour boards had been observed.

First, there were significant differences in the existence of standard operational procedures (SOPs) with Eastern and Southern countries having less SOPs when compared with Northern and Central countries ($p = 0.002$). Hence, in Northern and Central European countries, more standardization to support the functioning of the meetings was in place.

Differences have also been observed regarding the availability of a specific room to carry out the tumour board meetings and a projector to view the images of the patients' discussed. Significant differences ($p = 0.001$) in both cases have been observed.

Regarding videoconferencing facilities, respondents were asked if videoconferencing systems in their institution were H.323-based, Web-based, or if no technological facilities were available. The majority of the centres in Southern and Eastern European countries answered that there were no videoconferencing facilities available to allow communication with other national or international centres. On the other hand, more than 50% of the institutions in North and Central Europe had either H.323 or Web-based videoconferencing tools to support communications with other centres (Fig. 2). Finally, virtual tumour boards were in place in 44% of the Northern European centres, in 30% of the central European ones, whilst only 4% of the Southern and none of the Eastern European responding centres had such facilities available. Differences in videoconferencing facilities and virtual tumour boards are shown graphically in Fig. 2.

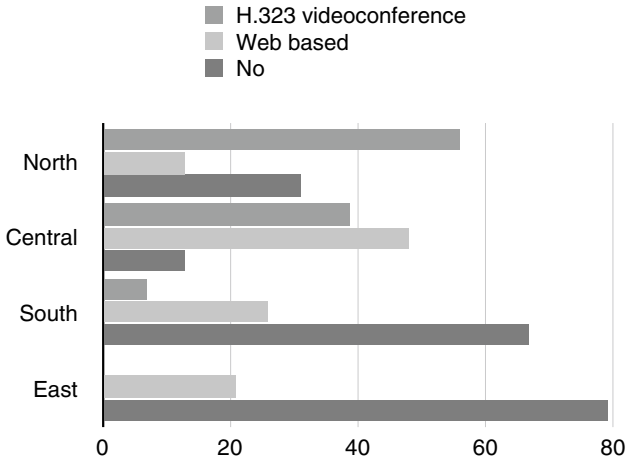

Fig. 2 Percentage of videoconferencing tools available in European geographical regions

Discussion

Multidisciplinary tumour board meetings are conducted worldwide to manage well with the help of multidisciplinary teams the treatment of cancer patients. They deliver a higher standard of care based on simultaneous involvement of different specialists for specific purposes related to the establishment of the right diagnosis and the planning of respective treatment components [9]. Several national and international guidelines emphasize the importance of multidisciplinary team management of children and adolescents with cancer [10–12] and need to be reinforced as standard of care for paediatric patients with cancer (Fig. 3).

Most of the paediatric oncologists involved in this survey confirmed that tumour boards are an essential tool for the management of complex cases. In contrast to adult cancer tumour boards addressing rather patients with a single adult cancer entity in one forum, most paediatric tumour boards cover a wide scope of paediatric cancer entities. All paediatric cancers are rare by definition and hence children benefit from treatment plans discussed and agreed upon in multidisciplinary teams. Once annual accrual rates are below 100 patients, the experience of a single site remains limited

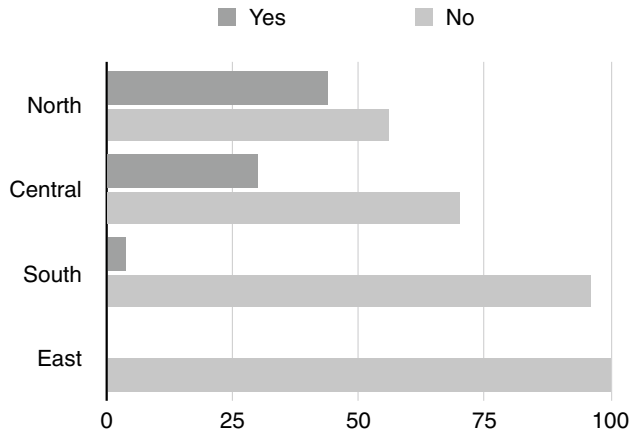

Fig. 3 Existence of virtual tumor boards in European geographical regions (percentage)

for specific very rare entities. Hence, the whole paediatric oncology community may benefit from the introduction of a well-established virtual tumour board system to better share knowledge and expertise particularly in complex cases and very rare cancer entities.

In the vast majority of surveyed countries across Europe, tumour boards take place to ensure appropriate decisions in the best interest of the respective child. Although tumour boards apparently already existed in more than 90% of the surveyed centres, the organization and facilities available were quite different in respective countries. Therefore, it is necessary in Europe to establish harmonised processes when aiming to create an international virtual tumour board network. In addition, potential legal responsibilities of physicians participating in virtual tumour boards need to be addressed [13–16]. However, the responsibility ultimately needs to stay with the respective treating physician and hence inside the respective health care provider responsible for the patient's treatment.

Most importantly, this survey showed important, statistically significant, differences related to many tumour board aspects among European geographical regions. Such differences were particularly evident with regard to availability of equipment for videoconferencing and communication processes. This lack of equity between different countries may explain in part why as of today paediatric patients suffering of the same cancer entity have different chances of survival depending on the country where they receive treatments. To overcome such current inequalities across Europe, the ExPO-r-Net project and the creation of the European Reference Network (ERN) for Paediatric Cancer (PaedCan) both are aiming for better access to expertise advice, and facilitation of communications between EU member states. Whenever possible, the information and expertise should travel rather than the patients and only those in need for specific interventions requiring a high level of expertise will be directed to centres able to provide this very special care.

This is the first European survey within the Paediatric Oncology community involving such a wide range of countries and different health systems. A previous survey was done at a national level in Spain showing the picture of a single country [17]. However, this survey reflects the current practice of paediatric oncologists working in multidisciplinary teams to take important treatment decisions together all aiming to achieve the best possible outcome for a particular patient in Europe. The whole community may benefit from improved and harmonised organisation of virtual tumour boards to assure best care of paediatric cancer patients. There is a real need for well-functioning, interoperable ICT solutions across Europe, and respective national health systems.

The ExPO-r-Net project has contributed to identify the needs across Europe and has developed tumour board solutions for a few very rare tumour entities. The future for the established ERN PaedCan network lies within the tools building on this experience, and provided now by the European commission to achieve the next level of sharing and caring within the network and in particular, regarding identified cross boarder health care patient management.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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