Predisposición genética al cáncer infantil.

De la secuenciación masiva a la consulta clínica

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## Introducción por parte del doctorando y agradecimientos

La tesis "Predisposición genética al cáncer pediátrico. De la secuenciación masiva a la consulta clínica" es el resultado del trabajo realizado dentro de la beca de investigación concedida al doctorando por el Instituto de Investigación Sanitaria la Fe (ISSLaFe). En la XVIII edición de la Beca de Investigación para post-residentes, convocada en 2018, me concedieron ser uno de los beneficiarios del programa tras la defensa pública del proyecto "Predisposición genética al cáncer pediátrico. De la secuenciación masiva a la consulta clínica". En dicho contexto fue posible obtener la estabilidad suficiente como para iniciar el programa de doctorado de la Universidad de Valencia ese mismo año.  El trabajo de 3 años ha culminado con el depósito de la presente tesis realizada como compendio de 5 artículos y titulada de la misma manera que el proyecto en su día presentado al ISSLaFe.

El proyecto "Predisposición genética al cáncer pediátrico. De la secuenciación masiva a la consulta clínica" se propuso valorar desde un enfoque genético a todos los pacientes de nuevo diagnóstico en la Unidad de Oncología Pediátrica del Hospital La Fe. Los objetivos, materiales y métodos, los principales resultados de dicha valoración, así como los estudios genéticos realizados, quedan recogidos en la primera de las publicaciones incluidas en la tesis. Los dos trabajos siguientes, derivan del análisis minucioso de dos casos específicos, ambos incluidos en la serie principal de pacientes.

La ejecución del proyecto de investigación fue posible gracias a las aportaciones económicas realizadas por asociaciones de pacientes, las cuales han sido imprescindibles para la realización de los estudios genéticos comprometidos dentro del proyecto "Predisposición genética al cáncer pediátrico. De la secuenciación masiva a la consulta clínica". Así mismo, la puesta en marcha, desarrollo y culminación del trabajo ha tenido lugar gracias a la confianza depositada en el doctorando por parte de la jefa del servicio de Oncología Pediátrica y directora de la presente tesis, la Dra. Adela Cañete. Así mismo, el respaldo científico del Dr. Silvestre Oltra, del Dr. Jaime Font de Mora y de la Dra. Victoria Castel ha sido imprescindible para llevar a cabo las tareas comprometidas. El desarrollo del proyecto se ha hecho realidad gracias también al apoyo científico, personal y logístico de la Dra. Yania Yáñez, la Dra. Vanessa Segura, el Dr. Antonio Juan y de Désirée Ramal. Me gustaría muy especialmente agradecer también el apoyo de la Dra. María Tasso del Hospital General de Alicante por la confianza depositada, a Marián Lázaro por el análisis pareado de datos genéticos realizado junto al doctorando, así como a las compañeras de la consulta externa de oncología pediátrica, Maite y Reme por las facilidades puestas para informar a los pacientes y obtener sus muestras. Acabar también agradeciendo el apoyo de todos los

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## LISTA DE ABREVIATURAS:

NGS: secuenciación de nueva generación

TW: Tumor de Wilms

SBW: Síndrome de Beckwith-Wiedemann

gDNA: ADN genómico

LP: probablemente patogénica/o

P: patogénica/o

VUS: variante de significado incierto

SNV: variante de nucleótido único

CNV: variante en el número de copias

CNS tumors: Tumores del sistema nervioso central

RETI: Registro Español de Tumores Infantiles

WHO: Organización Mundial de la Salud (OMS)

WES: secuenciación de exoma completo

IVF: Fertilización in vitro

MRI: Resonancia magnética

MRD21: Retraso Mental Autosómico Dominante 21

LOH: pérdida de heterocigosidad

LOI: pérdida del *imprinting*

LF: Síndrome de Li-Fraumeni

# 1. Introducción

Los síndromes de predisposición al cáncer son un conjunto de entidades de base genética conocida, asociadas a un riesgo elevado, con respecto a la población general, de desarrollar distintos tumores sólidos o neoplasias hematológicas durante la época prenatal, infancia, adolescencia o edad adulta [1]. Uno número importante de dichas entidades se caracterizan por un debut de la enfermedad en la edad pediátrica. Si bien hay ciertos síndromes que pueden debutar tanto en la infancia como en edades posteriores, muchos se pueden estudiar como enfermedades propias del adulto o del niño. Los principales síndromes de predisposición hereditaria al cáncer que pueden o suelen debutar en la infancia, han sido bien categorizados [2-4].

La proporción de pacientes afectos de síndromes de predisposición genética al cáncer pediátrico se ha estudiado detalladamente durante las últimas décadas. En el año 1991, Narod et al [5] publicaron los resultados de un estudio retrospectivo basado en la revisión del registro de tumores infantiles británico atendiendo a los datos recogidos durante el periodo 1971-1983. Tras su análisis de más de 16.000 pacientes concluyeron que, al menos un 4.2% de los pacientes de la serie, presentarían un síndrome de predisposición genética al cáncer. Asumiendo la limitación de sus datos, concluyeron que el porcentaje de pacientes afectos por entidades genéticas de susceptibilidad al desarrollo de tumores infantiles podría ser mayor. En su trabajo se confirmó que el retinoblastoma sería el tumor con una mayor componente hereditario de los estudiados en la serie con hasta un 37.2% de casos asociados a una base genética [5].

Más recientemente y gracias al desarrollo y abaratamiento de las tecnologías de secuenciación de nueva generación (NGS) ha sido posible realizar, a nivel internacional, distintas aproximaciones dirigidas a estimar la proporción de niños afectos por enfermedades genéticas de predisposición en series largas de pacientes oncológicos.

Cabe destacar el trabajo del grupo de investigación del centro St. Jude Children's Research Hospital publicado en 2015 [6]. En dicho trabajo secuenciaron el genoma o exoma completo de 1120 niños con cáncer, si bien analizaron detenidamente la secuencia de 565 genes en cada uno de los pacientes. En este estudio reportan un 8.5% de niños portadores de variantes consideradas patogénicas o probablemente patogénicas de predisposición a la enfermedad oncológica padecida. En dicha serie, el gen donde se concentraron más variantes de significado patogénico fue *TP53*, seguido de *APC*, *BRCA2*, *NF1*, *PMS2*, *RB1* y *RUNX1*. Desde el año 2015, se han publicado otros trabajos en la misma línea de manera sucesiva, varios de ellos con series de más de 100

pacientes. El proyecto BASIC3 secuenció el exoma completo de 150 pacientes en línea germinal y reportó un 10% de alteraciones genéticas compatibles con el fenotipo del paciente [7]. La universidad de Columbia publicó los resultados de su proyecto PIPseq en 2016 [8]. Secuenciaron mediante exoma completo la línea germinal de 101 pacientes y detectaron alteraciones de predisposición en un 14% de los casos. La publicación en 2020 del grupo Australia Zero Childhood Cancer Program, trasladó los resultados de secuenciación de genoma completo en la línea germinal de 252 pacientes, detectando variantes patogénicas de predisposición al cáncer en el 16.2% de los casos de alto riesgo incluidos en el estudio [9].

El trabajo más reciente en el campo es el publicado por el grupo del Memorial Sloan Kettering Cancer Center el presente año 2021 [10]. En dicho estudio se secuencia de manera pareada la sangre y el tumor de 751 pacientes mediante un panel NGS de 468 genes. Mediante esta aproximación, encuentran alteraciones genéticas patogénicas y de predisposición a la enfermedad en un 13% de los pacientes.

Al margen del porcentaje exacto de pacientes pediátricos afectos por síndromes de predisposición al cáncer, es evidente que el número no es nada despreciable. En este contexto, la necesidad de diagnosticar estas entidades y establecer programas de seguimiento adaptados al riesgo, así como guías consensuadas de manejo a nivel terapéutico se ha puesto de manifiesto [11]. Gracias a la colaboración internacional, existen guías de seguimiento específicas para los principales síndromes de predisposición al cáncer. Son documentos en constante revisión y actualización, pero sin duda, constituyen un soporte imprescindible para el clínico en el manejo de los pacientes y una herramienta muy útil para favorecer una atención homogénea en los distintos territorios [12-26].

Por último, de cara a ser eficientes en el estudio genético de pacientes con cáncer pediátrico en busca de síndromes genéticos de predisposición al cáncer, se ha propuesto realizar el estudio genético únicamente en aquellos que cumplan algún criterio de sospecha. Durante los últimos años se han desarrollado herramientas de selección de pacientes para tal fin. La herramienta de Jongmans MC et al fue publicada en 2016 [27]; propone valorar genéticamente a aquellos pacientes que cumplan al menos un criterio de los recogidos en la herramienta. Dichos criterios se basan en los antecedentes familiares de cáncer, en el tipo tumoral padecido, la historia personal de varios tumores, la presencia o no de anomalías congénitas, así como la toxicidad experimentada a los tratamientos quimioterápicos.

## Childhood cancer, indication for referral to a clinical geneticist?

*If your patient fulfills one or more of the criteria mentioned below (one or more circles filled), he or she may benefit from referral to a clinical geneticist.*

**1.** Family history of the child with cancer

- O ≥ 2 malignancies at childhood age (≤ 18 years of age)
- O a first degree relative (parent or sibling) with cancer < 45 years of age
- O ≥ 2 second degree relatives with cancer < 45 years of age on the same side of the family
- O the parents of the child with cancer are related, i.e. consangious

**2.** A person with one of these tumors in childhood

- O Adrenocortical carcinoma
- O Atypical teratoid rhabdoid tumor
- O Cerebellar gangliocytoma
- O Choroid plexus carcinoma
- O Endolymphatic sac tumors
- O Hemangioblastoma
- O Hepatoblastoma
- O JMML
- O Low hypodiploid ALL
- O Malignant peripheral nerve sheath tumor
- O Medullary thyroid carcinoma
- O Medulloblastoma
- O Optic glioma
- O Ovarian sertoli-leydig cell tumor
- O Pleuropulmonary blastoma
- O Pituitary blastoma
- O Pineoblastoma
- O Retinoblastoma
- O Schwannoma
- O Subependymal giant cell tumor

**Or**    O   A cancer of adult age, i.e. colorectal cancer, ovarian cancer, basal cell carcinoma etc.

**3.** O **A child with two malignancies** one of those with onset < 18 years of age (unless the 2nd malignancy is consistent in time and/or tissue type with these expected from their treatment regimen).

**4.** O **A child with cancer and congenital anomalies or other specific symptoms**

| Sign | Think of |
|---|---|
| Congenital anomalies | Organs, bones, oral clefting, teeth, eyes, ears, brain, urogenital anomalies, etc. |
| Facial dysmorphisms | |
| Intellectual disability | |
| Aberrant growth | Length, head circumference, birth weight, asymmetric growth |
| Skin anomalies | Aberrant pigmentation i.e. > 2 café-au-lait spots, vascular skin changes, hypersensitivity for sunlight, multiple benign tumors of the skin |
| Hematological disorders | Pancytopenia, anemia, thrombocytopenia, neutropenia |
| Immune deficiency | |

**5.** O **A child with excessive treatment toxicity**

Tabla 1.-Jongmans MC et al. Recognition of genetic predisposition in pediatric cancer patients: An easy-to-use selection tool. Eur. J. Med. Genet. 2016, 59, 116–125.

Esta herramienta fue posteriormente revisada y actualizada por el grupo de trabajo de predisposición al cáncer de la Sociedad Alemana de Hematología y Oncología Pediátricas [2]. La

actualización se centra en la inclusión de entidades tumorales específicas no incluidas en la herramienta de Jongmans MC. La selección de enfermedades incorporadas en la guía fue realizada en base a una exhaustiva revisión de la bibliografía científica, recogiendo aquellas entidades con base genética conocida. Los pormenores en cuanto a genes implicados quedan adecuadamente recogidos en el manuscrito [2]. Su aplicación en la práctica clínica está siendo evaluada [28].

# Childhood cancer: Indication for genetic counseling?*

updated Jongmans criteria [Jongmans et al., 2016]

*if at least one criterion is fulfilled, your patient may benefit from genetic counseling*

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### 1. Family history (3 generation pedigree)
O ≥2 malignancies occurred in family members before age 18 years, including index patient
O Parent or sibling with current or history of cancer before age 45 years
O ≥2 first or second degree relatives in the same parental lineage with cancer before age 45 years
O The parents of the child with cancer are consanguineous

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### 2. One of the following Neoplasms was diagnosed:
O Adrenocortical carcinoma / adenoma
O ALL (low hypodiploid)
O ALL (ring chromosome 21)
O ALL (Robertsonian translocation 15;21)
O ALL relapse (*TP53* mutated)
O AML (Monosomy 7)
O Basal cell carcinoma
O Botryoid rhabdomyosarcoma of the urogenital tract (fusion-negative)
O Chondromesenchymal harmatoma
O Choroid plexus carcinoma / tumor
O Colorectal carcinoma
O Cystic nephroma
O Endolymphatic sack tumor
O Fetal rhabdomyoma
O Gastrointestinal stromal tumor
O Glioma of the optic pathway (with signs of NF1)
O Gonadoblastoma
O Hemangioblastoma
O Hepatoblastoma (*CTNNB1* wildtype)
O Hepatocellular carcinoma
O Infantile myofibromatosis
O Juvenile myelomonocytic leukemia
O Keratocystic odontogenic tumor
O Large cell calcifying Sertoli-cell-tumor
O Malignant peripheral nerve sheath tumor
O Medullary thyroid carcinoma
O Medulloblastoma (SHH activated)
O Medulloblastoma (WNT activated, *CTNNB1* wildtype)

O Medullary renal cell carcinoma
O Medulloepithelioma
O Melanoma
O Meningioma
O Myelodysplastic syndrome
O Myeloproliferative neoplasms (except CML)
O Myxoma
O Neuroendocrine tumor
O Paraganglioma / pheochromocytoma
O Parathyroid carcinoma / adenoma
O Pineoblastoma
O Pituitary adenoma / tumor
O Pituitary blastoma
O Pleuropulmonary blastoma
O Renal cell carcinoma
O Retinoblastoma
O Rhabdoid tumor
O Rhabdomyosarcoma with diffuse anaplasia
O Schwannoma
O Schwannomatosis
O Sertoli-Leydig cell tumor
O Sex cord stromal tumor with annular tubules
O Small cell carcin. of the ovary hypercalcemic type
O Squamous cell carcinoma
O Subependymal giant cell astrocytoma
O Thyroid carcinoma (non-medullary)
O Transient myeloproliferative disease
**O Other rare cancers or cancers that typically occur in adults, unusually early manifestation age**

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### 3. O Genetic tumor analysis reveals defect suggesting a germline predisposition

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### 4. O A patient with ≥2 malignancies (e.g. secondary, bilateral, multifocal, metachronous)

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### 5. O A child with cancer and congenital or other anomalies

| Sign | Think of |
|---|---|
| O Congenital anomalies | Abnormal organs, skeletal anomalies, oral clefting, abnormal teeth, urogenital anomalies, abnormal hearing or vision, etc. |
| O Facial dysmorphism | |
| O Mental impairment, developmental delay | Abnormal behavior, learning difficulties |
| O Abnormal growth | Height, head circumference, birth weight, hemihyperplasia, growth chart |
| O Skin anomalies | Abnormal pigmentation such as ≥2 café-au-lait spots, vascular lesions, hypersensitivity to sun, benign tumors, etc. |
| O Hematological abnormalities (not explained by current cancer) | Pancytopenia, anemia, thrombocytopenia, neutropenia, leukopenia, macrocytic erythrocytes |
| O Immune deficiency | Frequency of infections, lymphopenia |
| O Endocrine anomalies | Primary hyperparathyroidism, precocious puberty, gigantism/acromegaly, Cushing syndrome |

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### 6. O The patient suffers from excessive toxicity of cancer therapy

Tabla 2.-Ripperger T et al. Childhood cancer predisposition syndromes-A concise review and recommendations by the Cancer Predisposition Working Group of the Society for Pediatric Oncology and Hematology. Am. J. Med. Genet. A 2017, 173, 1017–1037.

Son por tanto más de cincuenta las entidades diagnósticas bien relacionadas con síndromes de predisposición al cáncer pediátrico. Si bien revisar cada una de ellas no es un objetivo de la presente tesis, sí se recoge a continuación una revisión resumida de la predisposición al Retinoblastoma, al tumor de Wilms y al meningioma.

## Retinoblastoma

El retinoblastoma es el tumor ocular más frecuente en la infancia. Representa aproximadamente el 3% de todos los tumores infantiles [29-30]. En el grupo de edad de menores de 5 años supone el 5% de los casos [29]. Atendiendo a la etiología, el retinoblastoma se puede dividir en dos categorías, hereditario y esporádico. Las formas hereditarias se caracterizan por la presencia de una mutación germinal en *RB1* (OMIM 180200) y la adquisición en la retina de un segundo evento exclusivamente somático que favorece el desarrollo tumoral siguiendo la hipótesis de Knudson [31]. Los casos esporádicos sin embargo no presentan variantes germinales, sino que se caracterizan por la adquisición de dos eventos somáticos en *RB1*, bien sea por variantes de nucleótido único, deleciones de parte o todo el gen y/o modificaciones epigenéticas.

Las formas hereditarias comprenden hasta un 40% del total. Ahora bien, el retinoblastoma hereditario aparece en la mayoría de los pacientes *de novo* (en hasta un 80%) y por tanto, sin historia familiar [32]. El síndrome se hereda con un carácter autosómico dominante y una alta penetrancia, la cual se ha estimado del 90-95% [32]. En las formas hereditarias, las mutaciones más frecuentes son variantes truncantes de tipo *nonsense* (37%) o *frameshift* (20%) comprendidas entre los exones 2 y 25 del gen *RB1* [33]. Los pacientes tienen mayor incidencia de formas bilaterales y multifocales, sin embargo, en hasta un 15% de los pacientes con presentación unilateral se detectan también mutaciones en línea germinal [31]. De hecho, se ha descrito que un 15% adicional de formas esporádicas serían pacientes con mutaciones constitucionales en mosaico, resultado de mutaciones postcigóticas durante el desarrollo embrionario [34]. En función de la distribución del mosaicismo, estos pacientes estarían también en riesgo o no de transmitir la variante patogénica a la descendencia.

Los pacientes con retinoblastoma hereditario padecen también susceptibilidad a desarrollar un tumor intracraneal de naturaleza típicamente neuro-ectodérmica. El pineoblastoma es el tumor más frecuente, pero otros tumores supra o paraselares han sido también descritos [35]. Por último, los casos hereditarios presentan un riesgo aumentado de desarrollar otros tumores primarios a lo largo de la vida, destacando el osteosarcoma y el melanoma [36]. Así mismo, estos

y otros tumores se presentan también de manera secundaria a los tratamientos administrados con una frecuencia alta respecto a la población general [37].

En base al riesgo de padecer retinoblastoma, así como otros tumores a lo largo de la vida, el último consenso internacional realizó las siguientes recomendaciones de seguimiento para los pacientes con retinoblastoma hereditario [23]:

**Table 1**

Hereditary RB surveillance protocol

| Age | Frequency |
| --- | --- |
| **Surveillance for intraocular RB**[a] | |
| Birth to 8 weeks | Nonsedated exams every 2 to 4 weeks |
| 8 weeks to 12 months | EUA monthly |
| 12 to 24 months | EUA every 2 months |
| 24 to 36 months | EUA every 3 months |
| 36 to 48 months | EUA every 4 months |
| 48 to 60 months | EUA every 6 months |
| 5 to 7 years[c] | Nonsedated exams every 6 months |
| **Surveillance for trilateral RB** | |
| Brain MRI at the time of RB diagnosis; some centers recommend a brain MRI every 6 months until 5 years old | |
| **Surveillance for second primary tumors** | |
| Education regarding second primary tumor risks and close attention to any new signs/symptoms | |
| Skin exam by the pediatrician during well child visits, to continue annually by the primary care physician or dermatologist for melanoma from age 18 | |
| Some consider WBMRI annually after age 8, but no consensus[b] | |

Abbreviation: WBMRI, whole-body MRI.

[a] On the basis of consensus recommendations of the American Association of Ophthalmic Oncologists and Pathologists.

[b] Or later, when the child is able to tolerate WBMRI without anesthesia.

[c] Some suggest continuing exams every 1 to 2 years after age 7.

Tabla 3.-Kamihara J, et al. Retinoblastoma and Neuroblastoma Predisposition and Surveillance. Clin. Cancer Res. 2017, 23, 98–106.

Por último, un reducido de grupo de pacientes con retinoblastoma hereditario presentan un fenotipo particular, caracterizado principalmente por orejas antevertidas, frente ancha y filtrum largo, así como un grado variable de discapacidad intelectual. Estos casos son parte de un síndrome de genes contiguos conocido como síndrome de deleción 13q-. El fenotipo de los pacientes está condicionado por los genes incluidos en la deleción [38], sin embargo, las regiones determinantes de las distintas manifestaciones clínicas siguen siendo un campo de continuo debate que requiere más estudio [39].

Todo paciente con retinoblastoma tiene indicación de estudio genético, de acuerdo a lo recogido por Ripperger T et al [2].

## Tumor de Wilms

El tumor de Wilms (TW) es un tumor de características embrionarias que se desarrolla en el riñón de niños típicamente menores de 5 años, disminuyendo la incidencia por encima de dicha edad [29-30]. Los tumores renales suponen aproximadamente el 5% del total, siendo el tumor de Wilms la entidad con mucho más frecuente de las que afectan al riñón, suponiendo hasta el 95% de los cánceres renales de debut en la edad pediátrica [29-30]. La supervivencia alcanza al 90% de los niños en los países occidentales. En un 5% de los pacientes la enfermedad se presenta de manera bilateral [29]. Un porcentaje de en torno al 10%-15% de pacientes con TW padecerían un síndrome de susceptibilidad a este tumor [29]. La presentación bilateral, la identificación de anomalías congénitas, la presencia de esclerosis renal mensagial y los antecedentes familiares de TW son altamente sugestivos de síndrome de predisposición. Sin embargo, en ausencia de al menos uno de estos datos, la probabilidad de padecer una entidad predisponente es muy baja [40]. La base molecular puede ser una variante genética constitucional o un cambio epigenético adquirido en las fases iniciales del desarrollo embrionario [40].

La predisposición al tumor de Wilms puede dividirse en dos grandes grupos. En primer lugar, **trastornos relacionados con el gen *WT1***. En segundo lugar, **entidades relacionadas con el locus 11p15.5.** Al margen de estos dos grandes grupos, hay un pequeño número de casos hereditarios que se relacionan con síndromes genéticos de otra índole que padecen también un riesgo aumentado de sufrir TW, pero sin ser éste el fenotipo principal [40].

Entre los trastornos relacionados con *WT1*, el fenotipo final va a depender del tipo de variante presente en el gen. En primer lugar, se han descrito algunas familias con TW en distintas generaciones y sin malformaciones genito-urinarias entre los miembros de la familia; la etiología

se ha podido demostrar y se relaciona con la presencia de mutaciones de *WT1* en heterocigosis que se heredan con carácter autosómico dominante. Nos referimos a este cuadro hereditario como **Predisposición a tumor de Wilms asociado a *WT1*** [41]. En este contexto, el riesgo de desarrollar un tumor de Wilms se estima del 30%. Por otra parte, en caso de producirse un síndrome de genes contiguos por deleción en la región 11p13, que incluya tanto a *WT1* como a *PAX6*, el fenotipo esperado sería un **síndrome de WAGR** (TW, aniridia, anomalías genitales y retraso en el desarrollo intelectual). La deleción de *PAX6* es responsable de la aniridia [25]. El riesgo de padecer tumor de Wilms en el síndrome de WAGR ronda el 50% [42]. Otra entidad destacable es el **síndrome de Denys-Drash**, producido, en la mayoría de los casos, como resultado de variantes de tipo *missense* en los exones 8 o 9 de *WT1*. El fenotipo consiste en fallo renal prematuro debido a una severa esclerosis mensangial, elevado riesgo de TW (cerca de un 75% de pacientes lo padecerían) y distintos grados de ambigüedad sexual o fenotipo genital próximo al femenino, tanto en pacientes mujeres como varones [40]. En pacientes con variantes patogénicas en el sitio de *splicing* del intrón 9 de *WT1* [43], el fenotipo es discretamente distinto al anterior y se conoce como **síndrome de Frasier.** En esta entidad los pacientes presentan de nuevo ambigüedad sexual o unos genitales externos semejantes a los femeninos, glomeruloesclerosis segmentaria y también riesgo de padecer gonadoblastoma. En este síndrome, la probabilidad de padecer nefroblastoma es bajo, pero el riesgo no se conoce con exactitud. El espectro clínico de pacientes con mutaciones germinales en *WT1*, se completaría con el cuadro **Anomalías genitourinarias sin fallo renal.** Ciertos individuos afectos de TW se caracterizan por la presencia de malformaciones genitourinarias, pero sin asociar fallo renal. Estos pacientes suelen presentar deleciones génicas completas de *WT1* o variantes truncantes de tipo *nonsense* o *frameshift.*

Las alteraciones en el locus 11p15 [44] se relacionan fundamentalmente con el síndrome de Beckwith-Wiedemann (SBW). Si bien el espectro clínico es variable, las manifestaciones principales del mismo se pueden resumir en: macrosomía, macroglosia, hemihiperplasia, hipoglucemia neonatal, onfalocele, visceromegalia (incluida la adrenocortical), anomalías renales y riesgo de padecer tumores embrionarios (tumor de Wilms, hepatoblastoma, neuroblastoma y rabdomiosarcoma fundamentalmente) [44]. La etiología molecular es en el 50% de los individuos, la pérdida de metilación en el centro de *imprinting* IC2 del cromosoma materno. En el 20%, disomía uniparental paterna en la región cromosómica 11p15. El 5% de los pacientes presenta una ganancia de metilación en el centro de *imprinting* 1 (IC1) situado también en 11p15. Variantes patogénicas en *CDKN1C* se encuentran así mismo implicadas entre las causas de un fenotipo tipo SBW.

En base a la información aquí recogida, se deduce que la inmensa de mayoría de pacientes con síndrome de predisposición al TW va a padecer malformaciones congénitas o antecedentes familiares como dato de sospecha para indicar el estudio genético en los pacientes. Pacientes con tumor de Wilms unilateral, sin malformaciones asociadas ni antecedentes familiares no tienen indicación de estudio genético.

Por último, y al margen de los dos grandes grupos aquí recogidos, son muchos otros los síndromes que asocian un riesgo aumentado de padecer TW con respecto a la población general [45]. Además, el conocimiento sigue avanzando y nuevos genes se han relacionado con la susceptibilidad a la enfermedad, como son *TRIM28, FBXW7, NYNRIN*, y *KDM3B* [46].

Un actualizado resumen de los síndromes de predisposición al tumor de Wilms, así como el consenso más reciente sobre las recomendaciones de seguimiento para la detección precoz del TW en pacientes con síndromes de predisposición, se recoge en la revisión del grupo Europeo de la SIOP (SIOP-Europe Host Genome Working Group) en colaboración con el Grupo de estudio de tumores renales de la SIOP [45].

Summary of cancer predisposition genes/syndromes with a reported risk of Wilms tumour (WT) development and surveillance recommendations.

| Syndrome/gene | | Estimated % of patients with this condition with WT | WT surveillance recommended? If yes: 3-monthly from birth until 7th birthday | Evidence* |
|---|---|---|---|---|
| *WT1* mutations | Exonic missense variants | ~50% | **Yes, renal US** | Strong |
| | Exonic truncating variants | ~80% | **Yes, renal US** | Strong |
| | Intron 9 variants | ~2% | No | Moderate |
| WAGR syndrome (11p13 deletion encompassing *WT1*) | | ~55% | **Yes, renal US** | Strong |
| Beckwith-Wiedemann syndrome/ spectrum (BWS/BWSp) | LOM IC2 | <1% | No | Moderate |
| | GOM IC1 | ~21% | **Yes, full abdominal US**[A] | Strong |
| | Paternal UPD 11p15 | ~8% | **Yes, full abdominal US**[A] | Strong |
| | *CDKN1C* mutation | ~1% | **Yes, full abdominal US**[A] | Moderate |
| | Classical BWS with negative tests | ~5% | **Yes, full abdominal US**[A] | Moderate |
| Lateralised overgrowth with ≥1 BWS feature | | Unknown | **Yes, full abdominal US**[A] | Moderate |
| Lateralised overgrowth without additional BWS features | | Unknown | No | Moderate |
| Perlman syndrome (*DIS3L2*) **(recessive)** | | ~64% | **Yes, renal US** | Strong |
| *PIK3CA*-related overgrowth (*PIK3CA*) **(somatic mosaic)** | | 1−5% | No | Moderate |
| Simpson-Golabi Behmel syndrome (*GPC3/GPC4*) | | ~3% | **Yes, full abdominal US**[A] | Moderate |
| *TRIM28* mutations | | >50% penetrance | **Yes, renal US** | Moderate |
| *REST* mutations | | >50% penetrance | **Yes, renal US** | Moderate |
| *CTR9* mutations | Truncating/splicing variants | Appears high | **Yes, renal US** | Moderate |
| | Missense variants | WT not reported | No | Moderate |
| *HACE1* mutations | | Unknown | No | Moderate |
| *KDM3B* mutations | | Appears low | No | Moderate |
| *FBXW7* mutations | | Unknown | No | Moderate |
| *NYNRIN* mutations **(recessive)** | | Unknown | No[B] | Moderate |
| Fanconi anaemia | FANC-D1 (*BRCA2*) **(recessive)** | ~20% | **Yes, renal US** | Strong |
| | FANC-N (*PALB2*) **(recessive)** | ~40% | **Yes, renal US** | Strong |
| | Other subtypes | WT not reported | No | Moderate |
| Mulibrey nanism (*TRIM37*) **(recessive)** | | ~6−8% | **Yes, renal US** | Moderate |
| Mosaic variegated aneuploidy (MVA) | *BUB1B* variants **(recessive)** | ~50% | **Yes, renal US** | Moderate |
| | *TRIP13* variants **(recessive)** | ~20% | **Yes, renal US** | Moderate |
| | *CEP57* variants **(recessive)** | WT not reported | **Yes, renal US** | Moderate |
| | MVA with unknown cause | WT not reported | **Yes, renal US** | Moderate |
| 9q22.3 microdeletion syndrome | | 10−20% | **Yes, renal US** | Moderate |
| 2p24.3 duplication (encompassing *MYCN*) | | Unknown | No | Moderate |
| Osteopathia striata with cranial sclerosis (*WTX*) **(X-linked)** | | Unknown, but appears >5% | **Yes, renal US** | Moderate |
| 2q37 deletion syndrome | Extending to 2q37.1 | 10−20% (3 cases) | **Yes, renal US** | Moderate |
| | More distal deletions | WT not reported | No | Moderate |
| Bloom syndrome (*BLM*) **(recessive)** | | ~3% | No | Moderate |
| DICER1 syndrome (*DICER1*) | | <2% | No[C,D] | Moderate |
| Li Fraumeni syndrome (*TP53*) | | Low | No[C] | Moderate |
| Neurofibromatosis type 1 (*NF1*) | | <1% | No[C] | Moderate |
| Hyperparathyroidism-jaw tumour syndrome (*CDC73*) | | <5% | No[C] | Moderate |
| Constitutional mismatch repair deficiency (*MSH2, MSH6, MLH1, PMS2*) **(recessive)** | | ~3% | No[C] | Moderate |
| Bohring-Opitz syndrome (*ASXL1*) | | ~7% | **Yes, renal US** | Moderate |
| Trisomy 13 | | <1% | No | Moderate |
| Trisomy 18 | | ~1% | No | Moderate |

Tabla 4.- Hol JA et al. Wilms tumour surveillance in at-risk children: Literature review and recommendations from the SIOP-Europe Host Genome Working Group and SIOP Renal Tumour Study Group. Eur J Cancer. 2021;153:51-63.

## Meningioma

Los meningiomas son tumores poco frecuentes en la edad pediátrica, a diferencia de lo que ocurre durante la vida adulta. Entre los niños, suponen menos del 2% del total de tumores cerebrales. Como ocurre en la inmensa mayoría de neoplasias, la biología tumoral es distinta con respecto a la observada en los pacientes adultos. En los niños, suelen ser tumores más agresivos (mayor grado tumoral, subtipo histológico más agresivo, mayores tasas de invasión cerebral) y tienen predilección por situarse en localizaciones atípicas. Además, en el contexto pediátrico, es más probable su asociación con síndromes genéticos de predisposición a tumores [47]. Por ello, Ripperger T et al, recogen el meningioma entre los diagnósticos que hacen recomendable una valoración genética en línea germinal. Entre las enfermedades genéticas que predisponen al desarrollo de meningiomas, destaca la **Neurofibromatosis tipo 2**. Entidad poco frecuente, con una incidencia poblacional de 1 de cada 25.000 recién nacidos [48] asociada a mutaciones germinales en el gen *NF2*, que se hereda con herencia autosómica dominante y una penetrancia del 100% [49]. Ahora bien, entre los síndromes potencialmente implicados se encuentran también la **Schwanomatosis asociada a *SMARCB1***, **Meningiomas de células claras asociado a *SMARCE1*** y **Susceptibilidad a meningioma asociada al gen *SUFU***. Así mismo, también aumenta el riesgo de meningiomas entre pacientes con síndrome de Cowden [50], síndrome de Werner [51] y síndrome de predisposición asociado a *BAP1*, el cual se encuentra fuertemente asociado al desarrollo de meningiomas de alto grado de características rabdoides [52]. Otros síndromes como neoplasia endocrina múltiple tipo 1 o el síndrome de Rubinstein-Taybi se han relacionado con el meningioma en base a casos aislados y por tanto, más evidencias son necesarias [52, 53].

Atendiendo al conocimiento disponible en torno a la incidencia de los síndromes de predisposición genética al cáncer pediátrico y la repercusión clínica de su detección y adecuada caracterización molecular, se puso en marcha el proyecto de investigación que dio lugar a la presente tesis.

## 2. Hipótesis y Objetivos

### Hipótesis

- Al menos un 10% de pacientes pediátricos con neoplasias sólidas y hematológicas padecen un síndrome genético de predisposición al cáncer identificable mediante técnicas convencionales y/o NGS.

### Objetivos

<u>Principal:</u>

- Establecer la prevalencia de síndromes de predisposición al cáncer en una cohorte de pacientes oncológicos diagnosticados en una unidad de oncología pediátrica.

<u>Específicos:</u>

- Ofrecer a todos los pacientes la posibilidad de ser estudiados genéticamente desde dicho enfoque.
- Realizar consejo genético en pacientes y familiares.
- Investigar nuevas asociaciones genotipo-fenotipo.

## 3. Metodología y Resultados (compendio de manuscritos)

**Manuscrito 1**

Germline Predisposition to Pediatric Cancer, from Next Generation Sequencing to Medical Care

*Cancers (MDPI) (2021)*

**Manuscrito 2**

Retinoblastoma and mosaic 13q deletion: a case report

*International Journal of Retina and Vitreous (BMC) (2021)*

**Manuscrito 3**

Germline Variant in CTCF links mental retardation to Wilms tumor predisposition

*En revisión European Journal of Human Genetics (Nature) (2021)*

**Manuscrito 4**

Li-Fraumeni: Will the detection in families increase the survival of its members?

*Anales de Pediatría (*Revista de la Asociación Española de Pediatría) (2019)

**Manuscrito 5**

Li–Fraumeni syndrome heterogeneity

*Clinical and Translational Oncology (Springer) (2020)*

MANUSCRITO 1

TÍTULO

Germline Predisposition to Pediatric Cancer, from Next Generation Sequencing to Medical Care

REVISTA

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## INTRODUCTION

Genetic predisposition plays an important role in cancer development. This fact is well-known in both adult and pediatric patient population [1, 2]. Environmental factors, involved in tumor onset during adult ages, are not so relevant in childhood [3]. However, the incidence and spectrum of mutations predisposing to cancer among children and adolescents are only partially understood [4, 5, 6]. Narod and colleagues claimed in 1991 that 10% of children with cancer had a genetic predisposition [7]. Several genes related to predisposition to different childhood malignancies have been described since then (myeloid leukemia [8] and lymphoblastic leukemia [9], neuroblastoma [10], medulloblastoma [11, 12, 13], osteosarcoma [14] and soft tissue and bone sarcomas [15, 16]). Meanwhile, knowledge on several disorders remains scarce, but current next generation sequencing technologies have expanded the frontiers of genetic predisposition research and, hence, the possibility of discovering new genotype-phenotype relationships [17].

Identifying cancer predisposition syndromes, defining them properly and establishing risk-adjusted surveillance programs are main goals of the scientific community [18, 19]. Recent literature provided follow-up guidelines for several cancer predisposition syndromes with broad consensus [20-34]. These recommendations open-up the work of healthcare professionals in case of detecting a genetic syndrome. Published guidelines are constantly being updated and are established as a useful framework for daily clinical practice.

The clinical feasibility of this genetic understanding is therefore clear and, the advantages can be summarized in the following points. This knowledge enables a personalized medical and/or surgical treatment for several patients (e.g.: Li-Fraumeni syndrome patients who have *TP53* mutations should not be exposed to ionizing radiation; surgical treatment should be conservative for hereditary retinoblastoma patients). It improves the selection of donors and the choice of the correct time for hematopoietic transplantation. This knowledge also allows to implement familial genetic counseling and transmit prognostic information to patients. It may accelerate the detection of associated non-tumor problems which may require early intervention (e.g.: patients with a *WT1* mutation, who may have insidious renal dysfunction). Moreover, unraveling a genetic condition that explains the phenotype may help face the psychological burden of such a diagnosis in some patients / parents. Finally, it could provide a better understanding of tumor development in specific cases [35].

The growing knowledge on pediatric predisposition cancer syndromes underlines the great necessity to transfer into clinical practice the vast genetic knowledge generated by genetic analysis. The present work aimed to assess the incidence of genetic alterations in a prospective

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cohort of pediatric patients by germline genetic analysis. We believe that at least 10% of our patients may suffer from a pediatric cancer predisposition syndrome. Therefore, we estimated that a relevant group of patients (at least 10%) could benefit from personalized follow-up recommendations or even personalized treatment. It was also expected to find at least 10% of families who could benefit from genetic counseling.

## METHODS

### Patient Study Cohort

All potential candidate patients were diagnosed in or referred to our center from other hospitals between March 2018 and March 2020. Those who relapsed in our institution during this period were also considered for inclusion. Patient eligibility was assessed between days one and sixty since the first hospital admission. The following inclusion criteria were required for study entry:

-Age between 0 and 18 years old.

-Final pathology diagnosis established.

-Germline origin blood sample availability.

-Patient clinical stability.

-Patient voluntary agreement to participate having understood the information related to the study.

-None exclusion criteria fulfilled.

Furthermore, the exclusion criteria that conditioned the withdrawal of the study:

-Rejection of the study by the patient and/or family.

-Unfavorable previous psychological evaluation.

-Diagnosis of a benign tumor without any known genetic basis for its development.

Patients who met the inclusion/exclusion criteria and agreed to enter the study were included. Patients who entered the project were clinically evaluated in a targeted way. A physical examination was performed and personal and family history were assessed, including a family tree. The information obtained was contrasted with Jongmans MC et al. criteria [36]. This tool allows the detection of patients who would benefit from personalized genetic counseling. In the event of any of the Jongmans MC et al. criteria being fulfilled and a genetic syndrome suspected, the patient was studied accordingly if a technique was available at the hospital (e.g.: *RB1* by PCR

and MLPA or *NF1/NF2* by a custom NGS panel). If no alterations were detected by these studies, the test was expanded by a custom NGS panel, *Onconano V2*. Patients who did not fulfil those three conditions were studied by the *Onconano V2* gene panel from the beginning. The workflow is shown in **Figure 1.**



**figure 1.** Workflow established for the study.

The custom *Onconano V2* panel was sequenced by the Institute of Genomic Medicine (Imegen – Health in Code Group). The technical report resulting from the genetic analyses was discussed by the Genetic Predisposition Committee of La Fe Hospital. A pediatric oncologist, a geneticist specialized in hereditary cancer and a molecular biologist were included in this committee. The final report prepared by the committee was delivered to the patient and family. Variants of uncertain significance were not communicated to the families. Pathogenic variants involved in the risk of cancer during childhood led to personalized recommendations for pediatric oncologists. Pathogenic or probably pathogenic variants with implications for cancer risk in adulthood guided family segregation studies and personalized follow-up of family members by the Genetic Counselling Unit. Likewise, pathogenic variants related to recessive diseases and their potential implications for the offspring were informed to the parents.

Patients and their families received pre- and post-testing genetic counseling. During the first visit, they were informed that the study consisted of sequencing a large NGS-based gene panel (390 genes) but that only a very low number of genes related to the pathology suffered by them or their children (score 1) are included in it. They were notified that exclusively the analysis of these

genes, included as score 1, could get cause-effect conclusions for their individual cases. Moreover, they were advised that all the remaining data obtained from the analysis would be used for research purposes by the research team within the framework of the project. They were warned that some doubtful information may emerge from the study and that we could propose to continue studying different issues in the patient and / or family for research purposes. Nevertheless, in no case this latest information would allow us to obtain evidence for the specific patient. We also commit not to harm or increase the number of patient and family medical visits when doing these complementary tests. Thereupon, patients and / or parents signed the informed consent being aware of all this. Therefore, when identifying variants that were of interest from a research point of view, the families received the pertinent information during the post-testing visit. Accordingly, complementary studies (such as family segregation analysis) were carried out within this theoretical framework.

## NGS Panel, Sequencing and Analysis Features

The *Onconano V2* custom panel was developed in collaboration with Agilent and designed to detect mutations (point mutations, including single-nucleotide variants and small indels) and CNVs (deletions or duplications) in 390 genes related to pediatric cancer (supplementary data 1). The main established genes related to genetic predisposition to pediatric cancer were also covered. Genomic DNA (gDNA) from blood or other tissue was extracted using the commercial extraction kits RecoverAll™ and the QIAamp DNA Investigator Kit (Qiagen). Concentration was measured by fluorometric quantification using a Qubit fluorometer with the Qubit dsDNA BR Assay kit (Thermo Fisher Scientific) and Qubit dsDNA HS Assay kit (Invitrogen). DNA Integrity Number (DIN) was determined using the DNA ScreenTape assay (Agilent Technologies). The cut-off DIN value was 3. Library preparation followed the manufacturer's recommendations. Libraries were then loaded onto the NextSeq 550 system (Illumina) for massive library sequencing in "Stand-alone" mode with 2 x 150 paired-end reads following the manufacturer's instructions. For bioinformatics analysis, the alignment to the reference sequence-Genome Reference Consortium Human Build 37 (GRCh37), annotation and variant calling followed a custom pipeline through the DataGenomics platform by Imegen. For the CNV analysis, in-house scripts by Imegen were used to obtain a fractional coverage based on a correlation between the number of normalized reads of a region in respect to the number of DNA copies for that region. A minimum inter-sample variability was guaranteed by homogenizing experimental conditions between different samples and genomic regions. CNV calls were classified by DataGenomics based on their credibility, using a scoring algorithm that takes into account parameters such as log2 ratio, event size, proximity

and type of contiguous events. CNV plots provided by the platform were manually reviewed to discard possible artifacts and validated by digital PCR or MLPA.

The panel genes were classified into 3 scores, depending on their involvement at the hereditary cancer level in order to facilitate further manual analysis. Genes involved in predisposition to the patient's tumor were studied as score 1. Genes involved in predisposition to other tumors as score 2 and other genes related to pediatric cancer at the somatic level and included in the panel were included as score 3. The analysis was performed with the DataGenomics software. Filters were applied to remove from the analysis variants with an MAF(minor allele frequency) >0.02 and variants in non-coding regions (flanking splicing sites up to +/- 10 nucleotides were excluded from filters). Changes described as polymorphic according to gnomAD browser data were also removed from the analysis. The study of the variants was carried out with the help of the Varsome, COSMIC, professional HGMD and Pcan.stjude.org websites, as well as those available for specific genes. Information obtained from *in silico* predictions was also considered.

The variants were classified as benign, likely benign, VUS (variant of uncertain significance), likely pathogenic (LP) and pathogenic (P) following ACMG recommendations [37]. In addition, some VUS, were considered to be potentially involved in genetic predisposition to the disease. However, for many of them, evidence was scarce in this clinical context. Despite a comprehensive *in-silico* analysis, a review of the available literature and a discussion of the variants in expert committee, no strong conclusions could be drawn. For these variants of uncertain significance, potentially involved in predisposition to the patient's cancer but without enough evidence to be considered probably pathogenic, an internal nomenclature was established. Variants of potential pathogenic significance (VOPPS) was the term used for these variants.

## RESULTS

### Patient Cohort and Genetic Variants Identified

Overall, 223 patients were assessed for inclusion during the specified period. Finally, 170 patients fully met the inclusion criteria and agreed to participate in the study. The parents signed the informed consent in all cases, but the patients were also informed according to their ages and, patients older than 12 years signed specific documents.

The male-female distribution was 60%-40% and the mean age was 7.2 years (0-18). The most common diagnosis was leukemia (45 cases; 26.5%), followed by CNS tumors (26 cases; 15.3%),

lymphomas (20; 11.8%), neuroblastoma and peripheral nervous system tumors (19; 11.2%), bone tumors (14; 8.2%), soft-part sarcomas (12; 7.1%), renal tumors (9; 5.3%), retinoblastoma (8; 4.7%), liver tumors (4; 2.3%), germ-cell tumors (3; 1.8%), melanoma and other skin tumors (1; 0.5%) and other tumor types (9; 5.3%) (**figure 2**).



**Figure 2.** Distribution of patients (%) by tumor type.

These percentages were compared to those collected in the Spanish Registry of Pediatric Tumors (RETI) [38]. Statistically significant differences were not found when comparing the incidence rates for these tumor types between the RETI series (age group 0-19 years; 1980-2017) and our cohort. Following the workflow established, a total of 153 patients were studied with *Onconano V2*, and the remaining 17 cases exclusively by conventional techniques or other NGS panels (supplementary data 2).

A pathogenic variant predisposing to the patient's tumor was detected in 16 cases (16/170; 9.4%). Regarding the genes involved in predisposition, the most frequently altered was the *RB1* gene (6/16; 37.5%), followed by *NF1* (3/16; 18.8%); other mutated genes were *DICER1, NF2, SUFU, TP53, XPC* and *SOS1*. Moreover, a patient diagnosed with trisomy 21 was included in the cohort. In addition, likely pathogenic mutations that may be involved in predisposition to the patient's tumor were identified in ten other cases (10/170; 5.9%). These 26 pathogenic and likely pathogenic variants detected are summarized in **table 1 and figure 3.**

**Table 1.** Pathogenic or likely pathogenic variants considered to be involved (pathogenic) or maybe involved (likely pathogenic) in patient's disease.

| Patient number | Diagnosis | Gene variant / genomic alteration | Categorization |
|---|---|---|---|
| 10 | Retinoblastoma (unilateral) | 13q12.13-q21.2 deletion | Pathogenic |
| 11 | Retinoblastoma (unilateral) | RB1 c.844G>T (p.E282*) | Pathogenic |
| 12 | Pilocytic astrocytoma | NF1 C.910C>T (p.R304*) | Pathogenic |
| 15 | Ewing sarcoma | CHEK2 c.254C>G (p.P85R) | Likely pathogenic |
| 31 | Neuroblastoma | SOS1 c.1300G>A (p.G434R) | Pathogenic |
| 36 | Pilocytic astrocytoma | MRE11 c.659+1G>A | Likely pathogenic |
| 39 | Neuroblastoma | PALB2 c.2747A>T (p.E916V) | Likely pathogenic |
| 44 | B-ALL | Trisomy 21 | Pathogenic |
| 51 | Retinoblastoma (bilateral) | RB1 c.2104 C>T (p.Q702*) | Pathogenic |
| 59 | Neuroblastoma | BRCA1 c.68_69del (p.E23Vfs*17) | Likely pathogenic |
| 64 | Retinoblastoma (unilateral) | 13q12q21 deletion | Pathogenic |
| 65 | Plexiform neurofibroma | NF1 c.4084C>T (p.R1362*) | Pathogenic |
| 66 | Retinoblastoma (bilateral) | RB1 c.224G>A (p.W75*) | Pathogenic |
| 89 | B-ALL | ATM c.1402_1403del (p.K468Efs*18) | Likely pathogenic |
| 103 | Cutaneous angiosarcoma | XPC c.1643_1644delTG (p.V548Afs*25) (homozygous) | Pathogenic |
| 105 | Wilms tumor | CTCF c.353T>A (p.I118K) | Likely pathogenic |
| 108 | Embryonal rhabdomyosarcoma | TP53 c.559G>A (p.G187S) | Pathogenic |
| 110 | B-ALL | CDKN2A deletion | Likely pathogenic |
| 113 | Medulloblastoma SHH | SUFU c.71dup (p.A25Gfs*23) | Pathogenic |
| 116 | B-ALL | JAK3 c.1465C>T (p.Q489*) and JAK3 c.1442-2A>G | Likely pathogenic |
| 118 | B-ALL | CHEK2 c.497A>G (p.N166S) | Likely pathogenic |
| 120 | Burkitt lymphoma | CHEK2 c.470T>C (p.I157T) | Likely pathogenic |
| 127 | Schwannoma CNS (NF1 phenotype) | NF1 c.2251+1 G>A | Pathogenic |
| 150 | Vestibular schwannoma (bilateral) | NF2 c.115-2A>G | Pathogenic |
| 156 | Pineoblastoma | DICER1 c.2026C>T (p.R676*) | Pathogenic |
| 169 | Retinoblastoma (bilateral) | RB1 c.2548_2552delCAGA-T (Q850Gfs*3) | Pathogenic |

**Figure 3.** Genes and tumor types whereby pathogenic or likely pathogenic variants were identified.

Other pathogenic/likely pathogenic variants were not considered to be involved in predisposition to patient tumors because they were related to recessive diseases, but without any evidence to associate them with the patient's cancer. However, their involvement cannot be ruled out in certain cases: *ERCC3* (patient 159; Ewing sarcoma), *XPC* (patient 151; ependymoma), *FANCM* (patient 149; neuroblastoma), *PIK3CG* (patient 111; lymphoma), *RECQL4* (patient 89; leukemia), *NBN* (patient 78; atypical teratoid rhabdoid tumor), *FANCL* (patient 42; rhabdomyosarcoma) and *CEP57* (patient 18; astrocytoma) (details on the variants can be found in **table 2A** and supplementary data 3). Besides that, some VUS might be attributed potential pathogenicity. Hence, they might be involved in predisposition to the tumor suffered by the patients, but the lack of evidence leads to classifying them as VUS according to the ACMG criteria. These variants were classified as VOPPS. Variants of these characteristics were detected in the genes *ING4* (patient 153; carcinoid tumor), *NF1* (patient 144; neuroblastoma), *FANCD2* (patient 143; lymphoma), *IGF1R* (patient 139; Wilms tumor), *ALK* (patient 119; leukemia), *FAT1* (patient 81; HGG), *CHEK2* (patient 78; teratoid/rhabdoid tumor), *RET* (patient 72; leukemia) and *SH2B3* (patient 48; leukemia) (more in **table 2B;** supplementary data 3). Variants of uncertain significance or likely benign not previously reported in databases or with a higher incidence than expected in cancer patients are also collected in supplementary data 3.

**Table 2. 2A.-** Pathogenic or likely pathogenic variants considered as not involved in the tumor etiology by the Pediatric Cancer Predisposition Committee. **2B.-** Variants of uncertain significance to which potential pathogenicity was attributed by the committee (Variants of potential pathogenic significance - VOPPS).

| Table 2A P/LP variants not predisposing to patient's tumor | | |
|---|---|---|
| Patient number | Diagnosis | Gene variant |
| 18 | Pilocytic astrocytoma | *CEP57* c.241C>T (p.R81*) |
| 42 | Alveolar rhabdomyosarcoma | *FANCL* c.40del (p. L14Cfs*27) |
| 42 | Alveolar rhabdomyosarcoma | *XPC* c.1643_1644del (p. V548Afs*25) |
| 78 | Atypical teratoid rhabdoid tumor | *NBN* c.1648_1651del (p. K550Gfs*8) |
| 89 | B-ALL | *RECQL4* c.2336_2357del (p.D779Cfs*57) |
| 111 | Lymphoblastic lymphoma | *PIK3CG* c.2340dup (p. E781Rfs*4) |
| 149 | Neuroblastoma | *FANCM* c.2161-1G>A |
| 151 | Ependymoma | *XPC* c.1643_1644del (p. V548Afs*25) |
| 159 | Ewing sarcoma | *ERCC3* c.583C>T (p. R195T*) |
| Table 2B VOPPS variants | | |
| Patient number | Diagnosis | Gene variant |
| 48 | B-ALL | *SH2B3* c.622G>C (p.E208Q) |
| 72 | B-ALL | *RET* c.2331C>A (p.N777K) |
| 78 | Atypical teratoid rhabdoid tumor | *CHEK2* c.342G>T (p.W114C) |
| 81 | High grade glioma | *FAT1* c.10990del (p.Q3664Sfs*10) |
| 119 | B-ALL | *ALK* c.3467G>A (p.C1156Y) |
| 139 | Wilms tumor | *IGF1R* c.3367A>G (p.M1123V) |
| 143 | Lymphoblastic lymphoma | *FANCD2* c.2204G>A (p.R735Q) |
| 144 | Neuroblastoma | *NF1* c.2998C>A (p.R1000S) |
| 153 | Carcinoid tumor | *ING4* c.109+1G>C |

Overall and considering all P/LP variants identified, related or not to genetic predisposition to patient's tumor, 35 out of 170 patients/families (20.6%) carried at least one of these variants. Families received this information and adequate genetic counseling.

### Jongmans MC et al 2016 Tool Evaluation

A total of 50 patients (29%) met the indication for referral to a clinical geneticist according to the Jongmans MC et al. criteria during the targeted assessment carried out after inclusion. Among them, pathogenic predisposing mutations were detected in 15 cases (15/50; 30%). It can be seen from this that 94% of the total of pathogenic variants predisposing to pediatric cancer detected in the study (15/16) were found in patients who met the Jongmans MC et al. criteria. In addition, five out of ten variants (50%) classified as likely pathogenic were detected among patients who met the Jongmans MC et al. criteria. Therefore, pathogenic and likely pathogenic mutations were identified in 40% of the patients chosen by the tool (20/50). Considering as predisposition variants

only the 16 pathogenic mutations, the Jongmans MC et al. tool was found to have a sensitivity of 94% and a specificity of 77% in our cohort. Taking into consideration both pathogenic and likely pathogenic variants probably involved in predisposition, the sensitivity would be 77% and specificity 79%. For the 120 patients who did not meet the indication for referral to a clinical geneticist based on the Jongmans MC et al criteria, pathogenic predisposition mutations were detected only in one case (0.8%). Out of the 120 patients, five carried likely pathogenic variants according to the ACMG criteria (4.2%).

The phenotype-genotype correlations of those patients carrying likely pathogenic variants are described below:

*CTCF Variant c.1337-T > A and Wilms Predisposition*

Patient number 105 corresponds to a 2-year-old child diagnosed with bilateral Wilms tumor. The phenotype was intellectual development at the limit of normality, bilateral cryptorchidism, patent foramen ovale, minor facial dysmorphism, such as a prominent forehead, leafy and arched eyebrows, long filtrum and thin upper lip. Therefore, the evaluation using the Jongmans MC et al tool was positive; however, it did not suggest any diagnosis. The NGS study identified the likely pathogenic *CTCF* variant c.1337-T>A (p.I446K) (NM_006565.4), with an allelic frequency of 50%. This variant was confirmed in homozygosity both in tumor DNA and RNA. The family segregation study confirmed that the variant occured de novo in the patient. The detection of this variant in the clinical context of the patient, having adequately ruled out other entities predisposing to Wilms tumor, led us to the diagnosis of mental retardation, autosomal dominant 21 [39]. After multidisciplinary assessment, we considered that this variant might predispose to Wilms tumor in the context of MRD21; this tumor has not been reported in other MRD21 patients to date.

*BRCA1 c.68_69del Variant and Neuroblastoma Susceptibility*

Patient number 59 was diagnosed with poorly differentiated mediastinal neuroblastoma at the age of 6 months (*NMYC* not amplified; without segmental chromosomal alterations in SNP Array). Parents were consanguineous, but data suggestive of a familial predisposition syndrome were not detected. The evaluation using the Jongmans MC tool was positive (consanguinity). NGS study was carried out since there was no suspicion of a specific entity. The *BRCA1* variant c.68_69del e.E23Vfs*17 (NM_007294.3) was detected in heterozygosity. The relationship between *BRCA1* mutations and predisposition to neuroblastoma is based on casual findings in specific cases, such as our patient. The *BRCA1*-Neuroblastoma risk ratio is still under study; therefore, the

implications of this variant in tumor development are currently undetermined. The parents refused the family segregation study and no additional family information was provided.

*CHEK2 c.497A > G Variant and B-cell ALL Risk*

Patient number 118 suffered from B-cell acute lymphoblastic leukemia when he was one year old, without other remarkable personal clinical data. Her mother had breast cancer at age 41 and a non-informative *BRCA1* and *BRCA2* study result. Colon cancer in her grandfather on of the mother´s side at age 73 stands out in the family history, as well as Hodgkin's lymphoma at age 45 in one of the mother's three siblings. There are no cases of cancer reported on the father's side. The NGS study identified heterozygous *CHEK2* c.497A>G (p.N166S) NM_007194.3. Segregation study confirmed the maternal origin of the variant and the remaining members of the family are under study. Based on the evidence available for *CHEK2* mutations in breast cancer, this variant might be involved in the mother's breast cancer [40]. However, evidence supporting the relationship between *CHEK2* variants and the risk of leukemia is still limited [41].

*CDKN2A Deletion and Leukemia*

Patient number 110 was diagnosed with common B-cell ALL at the age of 3 years. Her mother was diagnosed with metastatic melanoma and died of the disease at a young age. Following the established workflow, the *Onconano V2* panel was sequenced. A mono-allelic *CDKN2A* deletion was detected and it was confirmed by MLPA. There was a high probability that the *CDKN2A* deletion was inherited from the mother, but it could not be confirmed. While the relationship between melanoma and *CDKN2A* is well known, information on the involvement of the *CDKN2A* gene in leukemia predisposition is scarce. However, a possible association of some *CDKN2A* polymorphisms (rs3731249 and rs3731217) with ALL risk in pediatric age has been proposed [42, 43, 44]. In this context, we concluded that the detected deletion might have facilitated the tumor development in the patient, although currently available evidence is insufficient.

*JAK3 Mutations and Familial Leukemia*

Patient number 116 is a 5-year-old girl with a diagnosis of common B-ALL (Acute lymphoblastic leukemia). Her mother also had ALL at the age of 5 years. The mother survived and is now 36 years old. The remaining information available on the maternal side was not contributory. Given that the patient met the Jongmans MC et al. criteria, the patient's sample was sequenced. Two

CIS heterozygous and likely pathogenic variants were identified in the *JAK3* gene. The detected variants were *JAK3* c.1465C>T (p.Q489*) and *JAK3* c.1442-2A>G. Family segregation study of both variants was completed. The mother carried both variants, while the father did not. The *JAK3* c.1442-2A>G variant, located closer to the N-terminal end than the other variant, is thought to be a null variant leading to loss of protein function. Loss-of-function mutations in homozygosity or compound heterozygosity are associated with severe combined immunodeficiency, whose inheritance is autosomal recessive. However, heterozygous loss-of-function mutations have not been associated with leukemia predisposition to date. Given the peculiarity of the family history, we considered the variant(s) to be likely pathogenic. Whether the variant(s) is involved in predisposition to leukemia suffered by the mother and daughter is completely unknown.

Other data of interest related to these and other specific cases carrying LP variants are shown in supplementary data 4 **and figure 4**. Some of the complementary studies carried out in specific patients or families in response to the detection of some variants are contained in supplementary data 4.



**Figure 4.** Family tree of patient number 15. Despite not fulfilling the Jongmans MC criteria nor the revised criteria by Ripperger, the patient's family history of cancer was still suggestive for genetic cancer predisposition and the genetic counseling was advised based on this information. The adolescent was diagnosed with extraosseous Ewing sarcoma. A *CHEK2* variant was detected by the NGS *Onconano V2* panel. The variant was described in the general population (gnomAD reports three total heterozygotes). However, it was described six times in ClinVar and three times in cancer patients (uncertain clinical significance (ID 233261)). It was not found in other databases. In addition, it is a variant studied functionally on one occasion. It was reported in the literature in a patient with hereditary breast cancer, with functional *in vitro* study that demonstrated a 50% reduction in kinase activity (PMID: 22114986), although the location

of the variant is outside of a functional domain. The variant was found to be of maternal origin and family history of melanoma was identified in the grandfather and great-uncle in this branch of the family. In addition, the grandfather had had a second tumor at an older age. Based on the ACGM criteria and family information, the variant was classified as likely pathogenic.

## DISCUSSION

This study presents the results of *Onconano V2* NGS panel sequencing of germline samples from a large cohort of pediatric oncology patients. On the basis of our results, it can be concluded that up to 9.4% patients have a genetic predisposition syndrome which explains the cancer they suffer. Meanwhile, considering that an additional 5.9% of the patients carry likely pathogenic variants, a few of which might be involved in susceptibility to the disease, this figure might be higher. The results obtained are consistent with previously published data [4, 5, 6]. Recent results from the MSK-IMPACT cohort also point the same way [45]. New predisposition genes have been described in the last two years [12] and these genes were not included in the panel; therefore, the figure presented might be considered conservative.

Due to the high number of patients to be assessed from a germline point of view, selection tools to enable concise assessment may improve the decision-making in this field. We evaluated the usefulness of the Jongmans MC et al tool for this purpose in our cohort. The tool showed high sensitivity for the detection of patients with currently well-categorized predisposition syndromes in our cohort (94%). A Li-Fraumeni patient was the only case of well-established and undetected genetic syndrome by the tool. He was a 5-year-old patient diagnosed with anaplastic embryonal rhabdomyosarcoma. No other data of interest were found in the medical records. The presence of a family history of cancer was ruled out. A variant considered pathogenic was detected in the *TP53* gene and the diagnosis of Li-Fraumeni syndrome was made. Family studies could not be expanded because of the early death of the patient and loss of contact with parents. The association between soft-tissue sarcoma and Li-Fraumeni syndrome is high [46]. The benefit of studying *TP53* at least in patients with anaplastic rhabdomyosarcoma is reinforced by current evidence [47]. Ripperger T et al. modified the Jongmans MC criteria [48] and their updated tool would have detected this case; this tool had achieved a sensitivity of 100% in the detection of pathogenic variants within the analyzed cohort in this study. The Ripperger T et al. revision also added rare entities specific to cancer predisposing syndromes in order to improve the sensitivity of the selection tool (e.g., Botryoid rhabdomyosarcoma of the urogenital tract). The inclusion of patients with acute myeloid leukemia, based on the 2016 WHO recommendations, was also

considered [49]. Currently, this would be the most appropriate tool for patient selection in order to recommend a genetic study.

Our results highlight the challenge of interpreting genetic variants in the context of predisposition to pediatric cancer. The cases carrying LP variants and above presented are only an example of frequent difficulties found throughout the series. Variants in genes *CHEK2, MRE11, PALB2 and ATM* reported for patients 15, 36, 39, 89, 120 present similar challenges. These clinical cases require constant re-evaluation based on the evidence available at any given time. The same is true for rare VOUS in the general population, especially those to which we attribute potential pathogenicity and designated VOPPS (variants located in *ING4, IGF1R, NF1, FANCD2, ALK, CHEK2, FAT1* and *SH2B3* genes). This subsequent work should be considered from the beginning in order to quantify properly the resources that will be required in the long term. Despite the limitations found for variant interpretation, this work has allowed to detect genetic variants that might be related to new genotype-phenotype associations for different pathologies. These data could be investigated in larger patient cohorts by international collaborative groups.

This kind of clinical approximation, with so many personal and family implications, demands a comprehensive assessment of the advantages and disadvantages detected. The NGS technology used has allowed us to reliably test for SNVs and CNVs in 390 gene regions in a single test. Despite the lower cost and accessibility of this technology at present, a cost/effectiveness assessment should be carried out. Based on the results obtained, the detection of the main pediatric cancer predisposition syndromes could be possible through a considerably smaller and less expensive gene panel than *Onconano V2.* A pre-test approach based on a tool such as that of Jongmans MC et al or Ripperger T et al can achieve an adequate selection of most of the patients that should be studied. In fact, one of the main conclusions raised from this work is that, outside of Ripperger T et al criteria and, therefore, out of the syndromes included in their review, no genetic alteration with evidence of being responsible for the disease suffered by the patient has been identified. Accordingly, sequencing broad panels such as ours or WES would make sense only and exclusively in the field of research or when facing extremely particular clinical cases. Therefore, for daily clinical practice and in order to detect cancer predisposition syndromes, the analysis of genes not related to the entities collected by Ripperger T et al gives rise to more doubts than certainties. In consequence, their testing would not be recommended outside the research field.

On the other hand, pre- and post-psychological test assessment plays a key role in proper long-term management. This has been proved as something basic in different areas of genetics [50]

and is especially important in this field, with so many consequences in the personal and family sphere. Moreover, the turnaround time required for suitable clinical implementation is a huge challenge in this context. Workflow based on pre-test clinical and psychological evaluation, patient/family information in a context of high emotional stress, germline sample collection, sequencing and analysis, committee discussion and reporting require a constant evaluation of deadlines. Undoubtedly, if the turnaround time is too long, some of the potential clinical benefit may be lost. However, the optimal time mainly depends on the specific case of each tumor type, or even of each patient. This work also highlights the importance of an expert committee, and not only in the field of research since the challenges derived from studying relatively well-known genes and syndromes remain remarkable. This multidisciplinary approach has already been shown to be very useful in other fields of personalized medicine [51], but it slowly emerges as a key component in this research area. Nonetheless, expert knowledge of each of the analyzed genes is an arduous task for any human group. Therefore, consultation with other national and international experts is presented as a useful tool in this field. The implementation of networks focusing on pediatric cancer predisposition syndromes will be essential in the following years.

In summary, it should be noted that in nearly 20% of the patients were identified genetic data that could have personal and family implications. In a few of them (9.4%), a genetic syndrome was diagnosed; thereby, the information was clinically useful for the patient. However, uncertainty was transferred to families in several cases when analyzing genes previously unrelated to pediatric cancer predisposition syndromes. In fact, outside of the genes and syndromes included in the Ripperger T et al criteria, not a single cancer predisposition syndrome was identified in this study. For this purpose, it seems preferable in clinical practice to sequence a highly selected gene panel rather than a large one or the whole exome after evaluating and choosing the patients with a selection tool such as that of Jongmans MC et al or Ripperger T et al.

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MANUSCRITO 2

TÍTULO

Retinoblastoma and mosaic 13q deletion: a case report

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INTRODUCTION

Retinoblastoma is a rare tumor that occurs in young children's retina. About 40% of patients diagnosed with retinoblastoma have a predisposing genetic condition [1]. Most of them carry heterozygous truncating *RB1* mutations in the germline. Some patients present isolated deletions of one of the two *RB1* alleles, and at-risk patients are exceptionally 13q-syndrome cases [2]. Because of the fact that 98% of retinoblastoma cases begin after a double *RB1* hit, according to

Knudson's hypothesis [3], all these children are at a major risk of being affected. 13q deletion syndrome was first described by Allderdice et al, after studying two pediatric patients in 1969 [4]. The first patient affected by the syndrome including retinoblastoma was reported in 1983 [5]. Several cases have been communicated during the past 50 years and the syndromic phenotype has been characterized. Intellectual disability, facial anomalies, several malformations and retinoblastoma risk stand out as the most prominent signs amongst other previously described abnormalities. However, the tumor would not be able to progress easily in 13q- syndrome even if a second *RB1* hit were present. It has been hypothesized that some genes deleted together with *RB1* would be necessary for retinoblastoma development. Available data suggest that 13q deletions larger than 1 Mb—and particularly those including *MED4* and *SUCLA2*—are associated with unilateral forms or without retinoblastoma development [6]. Improvements in cytogenetic analysis has enabled better molecular characterization of 13q- syndrome cases and more accurate genotype–phenotype correlations. Depending on the deleted chromosomal bands, three clinical groups may be established [7]:

Group 1: 13q12.2–13q32. Mild intellectual disability, growth delay, limb malformations, and retinoblastoma risk (when the *RB1* gene is deleted [chromosomal position 13q14.2].

Group 2: 13q32. Severe brain malformations and developmental delay.

Group 3: 13q33–13q34. Minor congenital malformations but severe intellectual impairment. Some patients with 13q-syndrome are affected by a mosaic disease and a few cases have been described [8–11]. Bestetti et al. reported a patient with mosaic 13q deletion syndrome including *RB1* but no retinoblastoma [8].

## METHODS AND RESULTS

### Case presentation

A 6-month-old girl conceived by in vitro fertilization (IVF) (own oocytes and anonymous donor sperm) was admitted to the hospital because of leukocoria and strabismus. Past medical history and physical examination were unremarkable except for clinodactyly of the right fifth finger. Indirect ophthalmoscopic examination and examination under anesthesia was performed by ophthalmologists. Orbital ultrasound and magnetic resonance imaging (MRI) scans showed a 14×13×11 mm left intraocular mass located in the lower-external retinal side. Retinal detachment

was also detected. Other tumoral lesions were ruled out by an ophthalmologist and MRI in both retina and brain. Diagnosis of Retinoblastoma was made and based on International Classification for Intraocular Retinoblastoma, a grade E was established. The patient received intra-arterial melphalan but due to a local vasospasm in her left leg, the treatment was discontinued. Afterwards, four courses of conventional chemotherapy were administered (vincristine, carboplatin and etoposide). A partial response was achieved, but, despite chemotherapy, the disease progressed few weeks later and the affected eye was enucleated. On the basis of global recommendations, the *RB1* gene was studied in germline DNA from peripheral blood lymphocytes. Exon–intron boundaries of *RB1* were amplified by conventional PCR and then sequenced by the Sanger method; no mutations were detected. A Multiplex Ligation-dependent Probe Amplification (MLPA) assay was used to test for *RB1*-gene deletions and duplications (SALSA P047-C1). The detected values were relatively low but within the normal range (Fig. 1A) and a complete *RB1* deletion in mosaicism was suspected. A genomic SNP array (AfymetrixCytoScan 750 array) was performed and a 13q deletion of 35.7 Mb from 13q12.13 to 13q21.2 (arr[hg19]13q12.13q21.2(26,555,387–62,280,955)×1–2) detected in around 40% of cells (Fig. 1B) was confirmed.

Fig. 1 *RB1* deletion in the context of mosaic 13q deletion. A Multiplex Ligation-dependent Probe Amplifcation (MLPA) assay looking for *RB1* gene deletions or duplications (SALSA P047-C1) in germline DNA from peripheral blood lymphocytes. The detected values were low but not consistent with a heterozygous deletion. The suspicion was a complete *RB1* deletion in mosaicism. B) Genomic SNP array (AfymetrixCytoScan 750 array) reports a 13q deletion in mosaicism. It is a deletion of 35.7 Mb from 13q12.13 to 13q21.2 (arr[hg19] 13q12.13q21.2(26,555,387–62,280,955)×1–2) observed in about 40% of all determinations.

This result was further confirmed by cytogenetic karyotype analysis of cultivated lymphocytes previously stimulated with phytohemagglutinin. Fifty metaphases were analyzed and, two cell clones were detected. A majority cell line (44 cells) presented 46 chromosomes whose identification with G bands (resolution level of 400–500 bands) did not show numerical or structural alterations (46, XX). A minor cell line (6 cells) with 46 chromosomes showed the presence of an interstitial deletion in the long arm of chromosome 13 (Fig. 2).

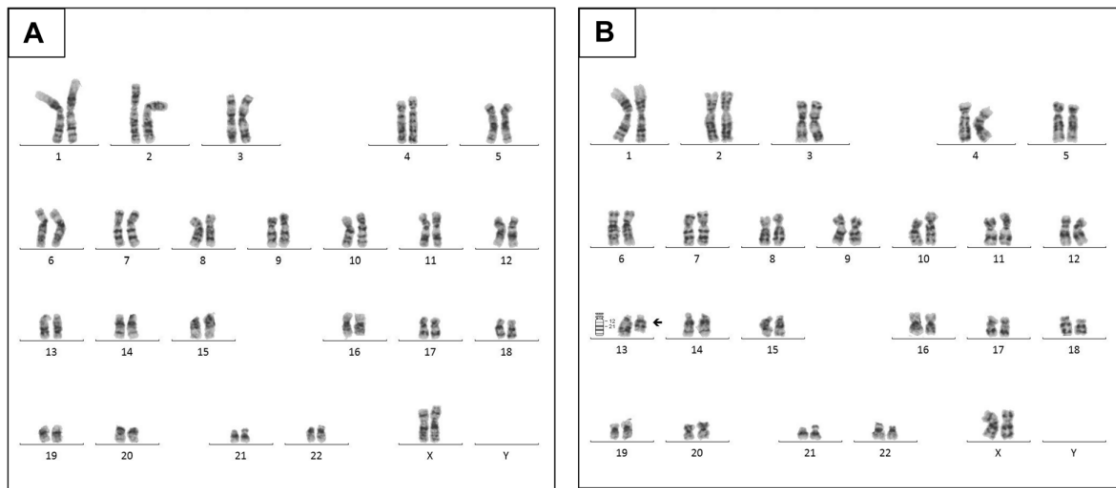Fig. 2 Cytogenetic karyotype from cultivated lymphocytes previously stimulated with phytohemagglutinin. Karyotype 46,XX,del(13)(q12q21) [6]/46,XX[44]. A) A majority cell line (44 cells): 46 chromosomes whose identification with G bands (resolution level of 400–500 bands) does not show numerical or structural alterations (46, XX). B) A minor cell line (6 cells): 46 chromosomes but shows the presence of an interstitial deletion in the long arm of chromosome 13.

A *RB1*-specifc FISH probe (LSI13) performed from swab oral mucosa cells evidenced 13q deletion in around 40% of the cells. We performed an Afymetrix Oncoscan array for both her tumor-free paraffin-embedded retina and fixed retinoblastoma sample. The healthy retina carried the 13q deletion in mosaicism but in about 50% of the studied cells. However, all retinoblastoma sample cells carried the deletion in heterozygosity (Fig. 3). Neither LOH (Loss of Heterozygosity) nor chromothripsis were detected in 13q bands. Furthermore, 6p12.3pter gain (3 total copies) and 6q25.3qter loss (1 total copy) were reported exclusively in the tumor sample. Looking for second hit mutations in *RB1*, we applied a custom designed NGS panel (Onconano V2) that included the *RB1, BCOR* and *CREBPP* genes (among other 400 commonly mutated genes in pediatric cancer). The study detected only one pathogenic single-nucleotide variant, *RB1* c.958C>T (p.Arg320Ter) (NM_000321.2 chromosomal position 13–48,941,648-C-T; allele frequency of 25%). Copy number variations in 6p, 6q and 13q were again observed.

Fig. 3 Afymetrix Oncoscan array performed from tumor-free paraffin-embedded retina and also from fixed retinoblastoma sample. The results have been analyzed with the Chromosome Analysis Suite software, applying the following filters in the analysis: at least 500 altered markers at 500 kb for CNV and at least 1 marker altered in 20 mb for LOH. The genome version was Hg19. Oncoscan by Afymetrix does not allow calculating the mosaicism percentage; therefore, the figures obtained are an approximation. A) Afymetrix Oncoscan array from tumor-free paraffin-embedded healthy retina. It carries the 13q deletion in mosaicism but in about 50% of studied cells. B) Afymetrix Oncoscan array from fixed retinoblastoma sample. 13q deletion is detected with a frequency consistent with heterozygosity in all tumor cells. Neither LOH nor chromothripsis in 13q bands were detected. 6p12.3pter gain and 6q25.3qter loss were detected as well.

After molecular diagnosis and completing the treatment, the patient was placed on surveillance. The right eye has been free of disease and the child is 42 months old now. She does not present growth retardation at the moment (weight and height in the 50th percentile; cranial perimeter in the 90th). Neither cardiac, eye nor other malformations have been detected and neurological development has been normal (Fig. 4). Informed consent for genetic studies and for taking and sharing pictures was obtained from both parents.

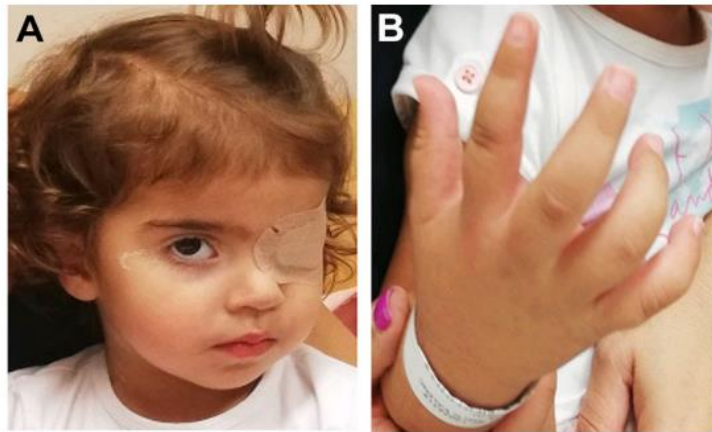Fig. 4 A) Patient's face. Left eye enucleated and waiting for prosthetics at the time of taking the photo. The patient's face has no noteworthy malformations. The wavy hair is not striking, the length of the forehead does not seem pathological at the age of 2. Other facial features are considered normal. B) Right hand Clinodactyly of the right fifth finger. She has no other limb malformations.

## DISCUSSION

We described the case of a child with 13q-mosaicism affected by retinoblastoma. The unilateral presentation agrees with previous data available for 13q deletions larger than 1 Mb including *MED4* and *SUCLA2* [6]. As in this case, retinoblastoma with both genes deleted is associated with less tumor aggressiveness compared with tumors whose genes are conserved [6]. Retinoblastoma seems to be caused by a double hit in *RB1* approaching 98% cases (by mutation, deletion, promoter methylation or intra-genic chromothripsis) [12, 13]. Few retinoblastoma cases would start because of *MYCN* amplification [13]. 13q deletion syndrome patients would not be an exception. In fact, we confirmed a second *RB1* hit (*RB1* p.Arg320Ter) in the tumor. However, double *RB1* hit only gives rise to retinoma; therefore, subsequent epigenetic or genetic changes would give an advantage for tumor progression. The sequence of events capable of causing a malignant phenotype is only partially known. Epigenetic deregulation secondary to homozygous *RB1* loss drives an increase in KIF14 and E2F3 levels [14] and could lead to the expression of the SYK oncogene as well. Moreover, cellular control mediated by p53 is inactivated as a result of high expression of MDM2 and MDM4 in retinoblastoma [14]. In addition, cytogenetic analysis has shown recurrent CNVs (copy number variation) among retinoblastoma tumors, which are mainly chromosomal gains at 1q, 2p, 6p, 13q and 19q and losses at 13q, 16q and 17p [15]. These recurrent aberrations allow to establish as a possible hypothesis that genes located at these loci could be related to retinoblastoma progression [15], yet no conclusive data are available about this at the moment. We looked for CNVs in the tumor and discovered a chromosomal gain in 6p12.3pter, which is one of the most frequently reported CNVs in retinoblastoma [15]. However,

we also detected a less common deletion of 6q25.3qter. The deletion of this region has already been described among non-13q-deletion syndrome patients, although rarely [16]. Sixty OMIM genes are located in this region, and several of them are associated with different cancers, but none with retinoblastoma. A terminal 6q deletion may be present in ovarian cancer and neuroblastoma [17] and seems to be related to bad prognosis in neuroblastoma [17]. The fact that this deletion could play a role in retinoblastoma development in the context of 13q-syndrome is unknown. Furthermore, NGS approaches have detected a low rate of mutations in retinoblastoma. Several studies support retinoblastoma as one of the less mutated human tumors. Only *BCOR* (mutated in 13% of tumors) and *CREBPP* mutations occur frequently in retinoblastoma [18]. Therefore, retinoblastoma presents a stable genome with few genetic events described and epigenetic deregulation appears to have a notable role [19]. Studies based on RNA-sequencing could continue to shed light on the genes and signaling pathways involved in retinoblastoma development [20]. In regards to common mutated genes in retinoblastoma, we determined *BCOR* and *CREBPP* status without detecting pathogenic variants. We did not find other variants considered pathogenic or likely pathogenic in 400 genes commonly mutated in pediatric cancer beyond *RB1*. The patient carries the deletion 13q12.13–13q21.2 and, therefore, fts in Group 1 of the clinical classification for 13q-syndrome [7]. Patients with band 13q14 deleted typically present with mild facial anomalies such as high forehead, short nose, small upper lip, curly hair and down-turned corners of the mouth [6]. Our patient does not show these facial features. Furthermore, deletion of *NUFIP1*, located in 13q14.12, and *PCDH8*, in 13q21.1, may be crucial for developmental delay [6]. Both of them are deleted in our patient, but the degree of mosaicism in her central nervous system is unknown. In fact, she is neurologically normal. Moreover, other common abnormalities in Group 1 are micrognathia and microcephaly but these are related to loss in the 13q21.33q31.1 and 13q21.32q21.33 regions, respectively [6]. Our patient's deletion finishes at 13q21.2; therefore, she does not present either micrognathia or microcephaly, because those regions are not affected. About 75% of patients with large deletions present short height, but this is not the case of our patient (50th percentile). Genes involved in short height have not been clearly defined. The *BRCA2* gene, located in 13q13.1, may be lost in some 13q-patients. Heterozygous mutations in this gene predispose to breast and ovarian cancer syndrome in adulthood [21] and a complete deletion of this gene might predispose to these tumors as well. However, the occurrence of these two tumors has not been reported in 13q-syndrome to date. Our patient loses *BRCA2*; therefore, she may benefit from risk-adapted surveillance strategies for breast/ovarian cancer. After confirming retinoblastoma diagnosis in a child, genetic study of *RB1* in the germline is mandatory. Any phenotypic manifestation, including

minor peculiarities (clinodactyly of the fifth finger in our case) should raise suspicion of 13q-syndrome, and it should be studied, given the fact that mosaic forms exist.

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MANUSCRITO 3

TÍTULO:

**Germline Variant in *CTCF* links mental retardation to Wilms tumor predisposition**

REVISTA:

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INTRODUCTION

Several molecular alterations are recurrently detected in Wilms tumors (WT), but 11p aberrations have a prominent role. *WT1* and several genes placed in 11p15.5 locus are commonly dysfunctional in Nephroblastoma[1–2]. *IGF2* is located at this locus and, it is commonly overexpressed in sporadic WT[2]. The Imprinting Control Center (ICR) is a 2.4 Kb in length region positioned between *IGF2* and *H19* gene (H19-ICR) which controls the expression of both genes. Physiologically, maternal *IGF2* allele is silenced through a refined imprinting regulation carried out on this region. ICR DNA is unmethylated on the maternal allele and methylated on the paternally derived allele. *IGF2* can be expressed when ICR is methylated and therefore, it occurs only in the paternal allele. Conversely, *H19* is only expressed from the unmethylated maternal

allele. This accurate regulation requires the presence of wild type CTCF protein[3]. This pattern of regulation on 11p15.5 locus is usually disrupted in WT. The somatic biallelic expression of *IGF2* in WT can be induced as a result of two independent mechanisms: 1) Duplication of the paternal allele by LOH (loss of heterozygosity) and paternal uniparental disomy or 2) Increased ICR methylation on maternal allele (Loss of imprinting; LOI)[4].

A broad spectrum of constitutional genetic mutations, genomic aberrations and epigenetic deregulation are known to predispose to WT development. The Knudson's two-hit hypothesis fits correctly for different genetic syndromes which include WT in their phenotype[1]. Among them highlight those associated with *WT1* mutations or deletions[5] and Beckwith-Wiedemann syndrome (BWS).

Constitutional *CTCF* mutations, including missense mutations within zinc-finger domains, have been detected among patients with Mental Retardation Autosomal Dominant 21 (MRD21; OMIM #615502)[6]. These patients usually exhibit a short stature, microcephaly, intellectual disability with a broad clinical spectrum, minor facial dysmorphism and cardiac anomalies[7–9]. Complete *CTCF* loss of function in germline is lethal in mice during embryonal development and probably in humans as well[10]. Heterozygous germline variants in *CTCF* gene have not been previously related to WT risk.

METHODS

NGS-based gene panel was Pediatric-OncoPanelDx *(*by Imegen). DNA was isolated from blood with RecoverAll kit (Invitrogen) and from paraffin-embedded tumor-selected section with QIAamp DNA Investigator Kit (QIAGEN). DNA quantification and integrity were assessed with Qubit and TapeStation. Libraries were prepared with Agilent SureSelect customized panel (254 genes, 0.8 Mb, Agilent XT-HS). Sequencing by Illumina NextSeq at 2x150bp; Depth>1000X. Bioinformatic analysis was performed with BWA-MEM aligner, caller variant VarDict and annotation by Ensembl Variant Effect Predictor. Quality metrics performed through Picard Pipeline (Broad Institute).

RNA was isolated from the paraffined tumor using RNeasy Mini Kit (Qiagen) and the retrotranscription with Taqman reverse transcription reagents (Applied Biosystems) and primers 5´TGTGCGATTACGCCAGTGTAGA3´ ; 5´GGCTCCTCCTCATCCTCATTGT3´.

DNA isolated from paraffin tumor was analyzed by molecular karyotyping with SNPa (Oncoscan, Affymetrix). SNPa results were analyzed with Chromosome Analysis Suite software (Affymetrix, ChAS; version 3.1; GRCh37 (hg19)). SNPa data quality was assessed with the internal array quality control parameter "Median of the Absolute Values of all Pairwise Differences". SNPa data were plotted and interpreted as previously described[11].

## RESULTS

### Patient data:

Unrelated white Caucasian parents conceived spontaneously a male. The father previously suffered a seminoma with lung metastasis. Mother's pregnancy was normal without any detectable ultrasound alterations. The child was born with an age-appropriate weight, height and cranial perimeter. Bilateral cryptorchidism was observed and, a bilateral renal ectasia (grade II) confirmed the first days of life. During neonatal age he was admitted in hospital due to pathological hypoglycemia. A heart murmur was auscultated and, an interatrial communication type oval fossa diagnosed. He also presented a mild ascending aortic ectasia. During the next months he grew normally with psychomotor development at the lower limit of normal range. He also presented a constitutionally minor facial dysmorphia consisting on: a prominent forehead, bushy and arched eyebrows, long philtrum and thin upper lip (Figure 1A).



Figure 1. (A) Phenotypical manifestations of MRD21 consisting on prominent forehead, bushy and arched eyebrows, long philtrum and thin upper lip.

At 26 months old, magnetic resonance imaging revealed a solid right kidney nodule in the lower pole (4x4.1cm) and an additional focal lesion in the left kidney (1.9cm maximum diameter), suggesting a bilateral WT. Chemotherapy treatment was given according to Umbrella SIOP-RTSG-2016 protocol[12] followed by a nephron sparing surgery. The right kidney lesion displayed

neoplastic proliferation with blastemal and epithelial components observed in similar amounts (Figure 1B). No signs of anaplasia were observed and, no tumor infiltration was present in the resected margin. Nuclear WT1 expression was observed by immunohistochemistry, reinforcing WT diagnosis (Figure 1B). The left kidney lesion corresponded to a nephrogenic neoplastic nodule with nephroblastomatous-like characteristics, consisting on blastema component with considerable mitotic activity without signs of anaplasia.
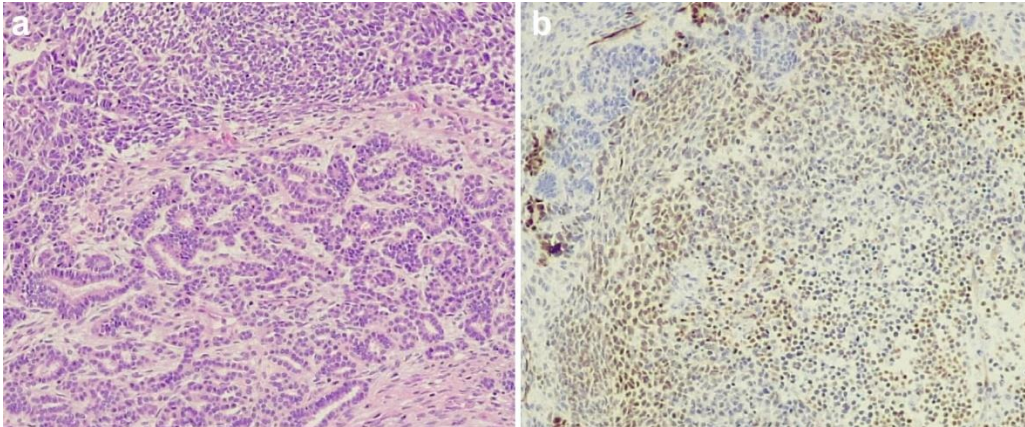


Figure 1B. (B; a) Hematoxylin eosin 324 staining at 20× magnification revealing mesenchymal, epithelial and blastomatous 325 components in the tumor. (B; b) Positive immunostaining for WT1 showing a major nuclear localization.

No pathogenic single nucleotide exclusively somatic variants were detected by NGS. The likely pathogenic variant *CTCF* c.1337 T>A (p.I446K) (exon 7, NM_006565.4) was detected heterozygous in the germline (VAF 51%) and homozygous in the tumor (VAF 97%; 659x). The homozygous detection of the *CTCF* variant was further confirmed in expressed tumor RNA (Figure 2D). The variant was confirmed to be *de novo* in the patient after a family segregation study (Figure 2A-C). We next used NGS results to compare CNVs within *CTCF* exons between tumor and blood DNA (green and red lines respectively, Figure 2E). Despite the noisiness of the technique, no significant variations were detected (yellow line, Figure 2E) suggesting that there were no internal deletions, gains or exome alterations aside of the single nucleotide variant found.

We performed a pan-genomic analysis of the tumor in order to confirm *CTCF* status as well as to identify other genetic alterations. Notably, SNPa data didn't reveal neither numerical nor segmental chromosomal alterations in the tumor (Figure 2F). The only alteration was an LOH covering the whole *q* arm of chromosome 16, including *CTCF* gene.
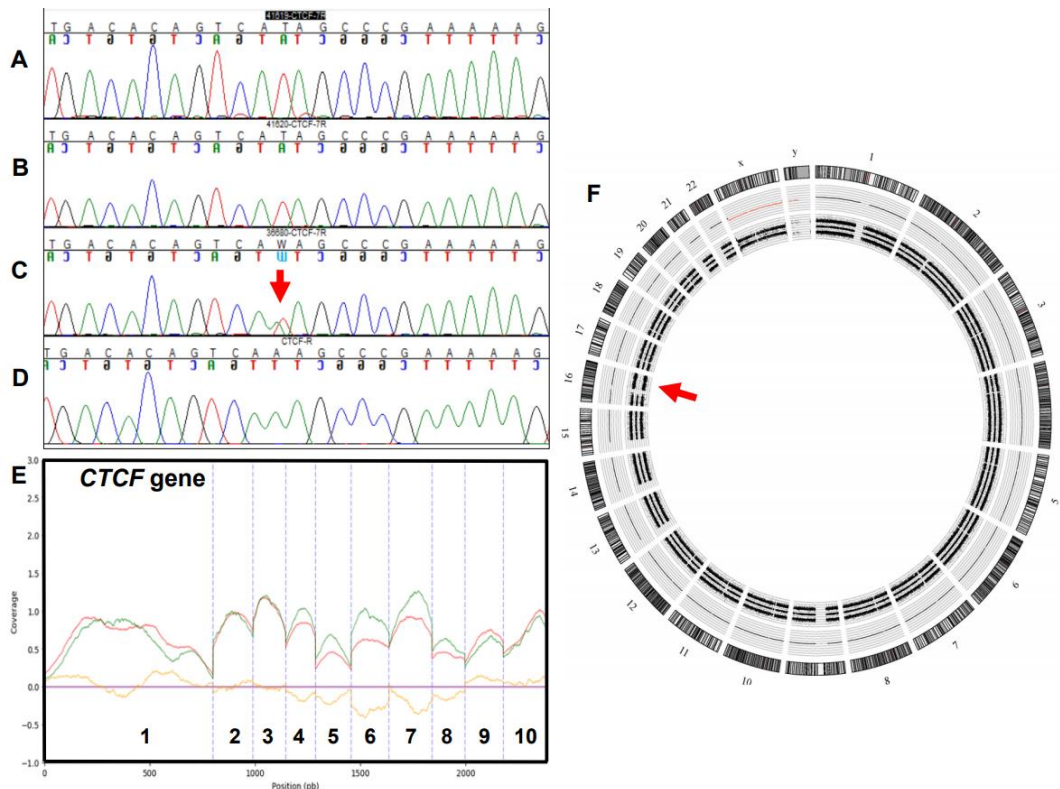
Figure 2. Sequencing alignment of *CTCF* variant c.1337 T>A (p.I446K) in (A) father's germinal DNA, (B) mother´s germinal DNA, (C) patient's germinal DNA and (D) patient's tumor cDNA. (E) Copy number variations (CNV) along CTCF exons (indicated in the bottom of each segmented area) derived from NGS sequencing data from germline DNA (green line) and tumor DNA (red line). Differences between germline and tumor CNV defined by the yellow line reveal no internal CNV alterations in CTCF's coding sequence in the tumor. (F) Molecular karyotype in circus-plot of the reported Wilms tumor. Allele peaks (inner plots) and weighted Log2ratio (middle plots) information were obtained from Affymetrix software ChAS. The only alteration detected was an LOH in the whole 16q arm (where CTCF gene is located).

In accordance with the first international expert consensus statement in BWS[12], the BWSp diagnosis was assessed in our patient, who would obtain a final score of three points. DNA methylation testing for both 11p15.5 imprinting control centers *H19/IGF2:IG* DMR (IC1) and *KCNQ1OT1:TSS* DMR (IC2) was indicated; the result was negative. *CDKN1C* loss of function mutations were ruled out as well. Considering these molecular results, the patient´s clinical features were consistent with MRD21.

## DISCUSSION

MRD21 was firstly reported by Gregor *et al*. in 2013[6] and afterwards characterized in the largest series[9]. To our knowledge, this is the first diagnosed MRD21 patient that suffers from WT.

Considering the rarity of bilateral WT among children, the oddity of MRD21, and the data here reported, we hypothesize *CTCF* variant c.1337 T>A (p.I446K) as a link between MRD21 and WT predisposition. In fact, *CTCF* mutations within zinc-finger domains have been described among WT patients[12]. One of two variants reported by Filippova *et al.* among WT samples is located within zinc-finger domain 7 (R448Q). In the presence of this variant, they describe CTCF failure to bind the *Igf2/H19* sites[13]. The variant detected with a 100% allelic frequency in the tumor of our patient, located as well within zinc-finger 7, might be responsible of analogous effects. This result suggests that during tumor evolution 16q-wild-type arm was replaced by a copy of the 16q arm containing the *CTCF* variant, thus resulting in an LOH.

LOH at 16q occurs in nearly 20% of WT patients and is an independent prognostic factor among low histological stage tumors[14]. 16q LOH was reported to be associated with loss of imprinting (LOI) at 11p15 and *CTCF* reduced expression in a group of patients[15]. Whereas *CTCF* gene is located at 16q22 and is a basic element in normal imprinting at 11p15, its haploinsufficiency might be responsible for 11p LOI and therefore, it may explain a driver mechanism in some WT[15]; however, more data are needed. Moreover, hypermethylation of a CTCF binding site downstream of the *WT1* gene promoter would disturb the normal transcriptional regulation of *WT1* and it might be considered oncogenic in WT[16]. Although the mechanisms are only partially known, *CTCF* is currently thought to play a role in regulating *WT1* gene expression in WT. However, these data are not sufficient to demonstrate a driver role for these alterations. In fact, Cresswell GD *et al.*[17], supported that 16q is a heterogeneous event in WT that is unlikely to be a driver.

Mild forms of BWS have been described without fitting classic criteria[18] but, the phenotype in our patient is clearly more compatible with MRD21 than with BWSp. However, the patient was excluded from BWSp diagnosis, based on current recommendations[19]. In this case, the presence of the variant in germline would justify the development of MRD21.

Thus, the results here reported only suggest an implication in tumor development of 16q LOH carrying a *CTCF* likely pathogenic variant and, a possible predisposition to WT development in patients with MRD21.

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MANUSCRITO 4

TÍTULO:

Li-Fraumeni: Will the detection in families increase the survival of its members?

REVISTA:

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INTRODUCTION

li-Fraumeni syndrome is a rare cancer predisposition syndrome with an autosomal dominant pattern of inheritance and of variable phenotypic expression associated with germline mutations in gene *TP53*. This disease predisposes to the development of a wide variety of malignant tumours. The most frequent tumours are soft-tissue sarcomas (rhabdomyosarcoma and others), osteosarcoma, breast cancer in premenopausal women, hypodiploid leukaemia, brain tumours (choroid plexus carcinoma, glioblastoma and medulloblastoma) and adrenocortical carcinoma. These tumours may develop at any age, including in children. The prevalence of this syndrome is not well known, as it is without a doubt underdiagnosed. Since follow-up of families has not proven to improve long-term survival, to date no programme has been established for detection of affected individuals. Circumstances are changing in regard to this predisposition syndrome. According to a study published by Villani et al. in 20111 and updated in 2016, it is possible to carry out a follow-up that could potentially increase long-term survival.

METHODS

We present the case of a previously healthy boy aged 2 years that visited the emergency services of his local hospital due to a convulsive seizure (complex partial seizure). The evaluation started with imaging tests and, due to the suspicion of a space-occupying lesion, he was transferred to the referral hospital. The imaging tests revealed a malignant tumour with disseminated meningeal involvement throughout the neuraxis, and a primary lesion inside the brain (tumour in the left posterior clinoid region with diffuse meningeal dissemination) (Fig. 1).
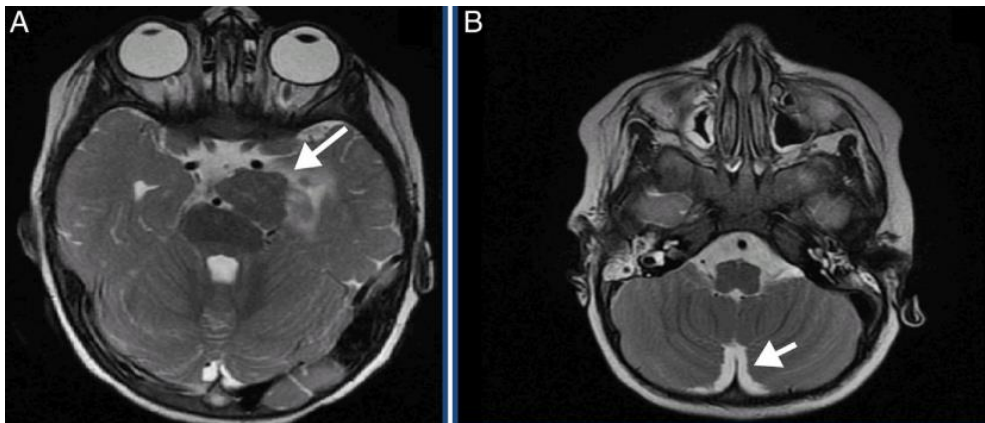


Figure 1 (A) Main lesion. Suspected primary tumour. Solid tumour measuring 30 mm (CC) × 27 mm (AP) × 20 mm (TR) in the left side of the pontine cistern. (B) Disseminated meningeal involvement. Thick irregular enhancement extending through the lateral sulcus and perisylvian cortex, interpeduncular, quadrigeminal and suprasellar cisterns, pineal recess and, ventral medulla oblongata.

The histological diagnosis based on the gross and microscopic examination of the biopsy sample obtained by craniotomy was malignant meningioma (high cellularity with cells with a clear cytoplasm and nuclei squeezed to the periphery, other cells with eosinophilic cytoplasm and nuclei pushed to the side, others with large hyperchromatic and pleomorphic nuclei). The cells were arranged in sheets alternating with compressed vessels, with foci of eosinophilic basement membrane material mixed between the tumour cells. Absence of whorls or psammoma bodies. Absence of chondromyxoid stroma. Immunohistochemistry staining: strong positive staining with of CK AE1-AE 3 and INI 1 antibodies. Focal expression of EMA, vimentin, synaptophysin and very focally PLAP. Staining negative for CD 117, OCT3/4 and alpha-fetoprotein. Negative for CD45, CD68, GFAP and desmin. The intense expression of cytokeratins and focal positive staining for EMA and vimentin supported the diagnosis of malignant meningioma and ruled out choroid plexus carcinoma. The patient was treated with chemotherapy in adherence with the protocol established by the SEHOP for children aged less than 3 years, as surgery and irradiation were not indicated due to the extent of dissemination and the young age of the patient. The patient

received the prescribed treatment and complications during follow-up were managed as they arose (secondary hydrocephalus with a ventriculoperitoneal shunt, convulsive seizures that were difficult to control, refractory vomiting, resting tremor, changes in behaviour). Despite treatment, the disease progressed, and the patient died 6 months after onset. A family history was taken at the time of diagnosis, revealing multiple cases of cancer in the paternal side (see the genetic family tree, Fig. 2). The family was referred to the cancer genetic counselling unit. The diagnosis of Li-Fraumeni syndrome was confirmed by genetic testing (c.430C > T p.Q144* missense mutation in exon 5 of the *TP53* of the patient). This testing had not been performed before in this family, despite the highly indicative family tree.

A subsequent evaluation of the family confirmed that the father and other relatives in his side of the family carried the same mutation.
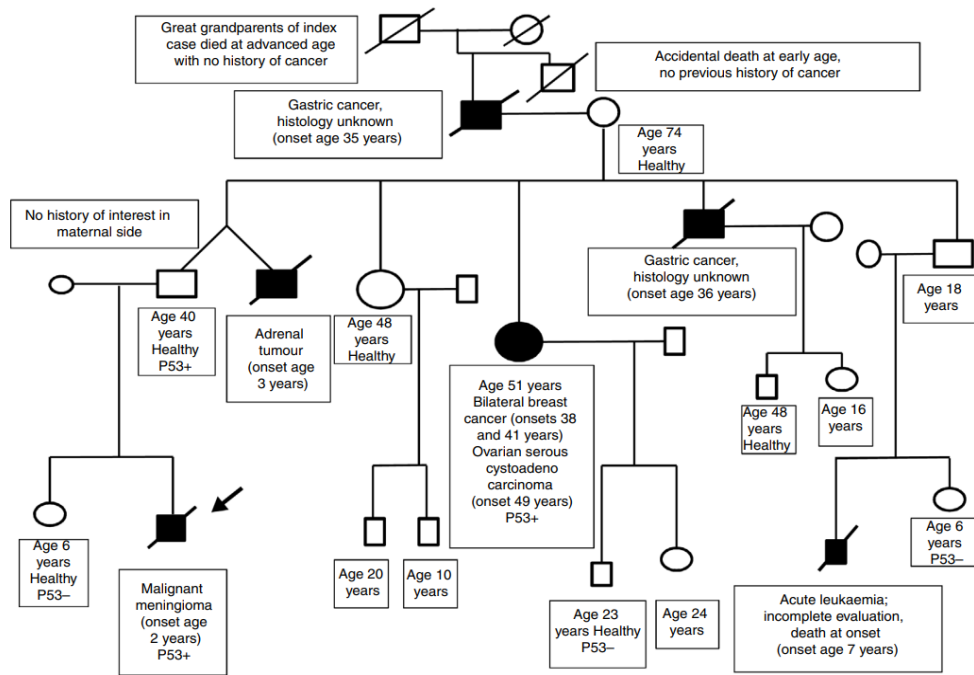


Figure 2 Family tree. The index case is in the bottom left of the image.

### DISCUSSION

According to the article published by Villani et al., it is possible to increase overall and long-term survival in carriers of *TP53* germline mutations. This article led the American Association for Cancer Research to set up a meeting of international experts on Li Fraumeni syndrome to evaluate and publish the current knowledge on the disease. Furthermore, the Li-Fraumeni-

Syndrome-Cancer Predisposition-Syndrome Registry 01 research protocol was launched with their cooperation with the aim of developing a worldwide register of patients with Li-Fraumeni and other cancer predisposition syndromes. Earlier detection in our patient could have made it possible to modify the course of the disease. Furthermore, other members of the family could have benefitted from adequate followup. As paediatricians, it is important that we remain aware of this syndrome when conducting the initial evaluation of a healthy infant in this context. In cases of families with a history of cancer in multiple individuals, the family should be referred to a specialised genetic counselling service. The family evaluation would start with an affected adult with the aim of benefitting healthy newborns that could potentially be carriers of the mutation.

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MANUSCRITO 5

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REVIEW

## Li–Fraumeni syndrome

Li–Fraumeni syndrome (LFS) is a rare predisposing cancer disease transmitted by autosomal dominant inheritance. The variable clinical expressions of this syndrome are an extreme challenge for individualized surveillance [1]. This particular syndrome was described for the first time by Li and Fraumeni in 1969 [2]. Li–Fraumeni disorder predisposes to malignant tumors development. These tumors can appear throughout the life of the patient. Cancer types observed in LFS patients include: soft tissue sarcomas [3, 4], osteosarcoma [5, 6], breast cancer [7, 8], brain tumors, leukemia [9, 10] and adrenocortical carcinoma [11] (#151623 OMIM). However, aggressiveness and the number of tumors vary to a great extending among different patients. Cumulative incidence for development of at least one tumor at 30 years old is estimated to be 50%, while it is near 100% at 70 years old [12]. Cancer risk at early ages is higher in women due to breast cancer risk. Cumulative incidence in women at 70 years old is 54% for breast cancer, 15% for soft tissue sarcomas, 6% for brain tumors, and 5% for osteosarcoma. For male patients, however, the reported figures are 22%, 19%, and 11% for soft tissue sarcomas, brain tumors, and osteosarcoma, respectively. Fifty percent of patients with a malignant tumor developed a second tumor over the next 10 years [12]. Several patients with many malignant primary tumors have been described in the literature [13]. Approximately 70% of families affected by classical tumors carry germinal mutations in TP53. However, 40% of patients with Li–Fraumeni-like (LFL) phenotype (families with other malignant tumors, different from classical tumors) carry *TP53* deleterious mutations. *TP53* mutations, associated to LFS or LFL, are mainly located in the DNA binding domain. Only few cases harbor *TP53* mutations outside this hotspot location [14, 15]. Pathogenic *TP53* variants do not explain all phenotypic manifestations. Mutations within the cell cycle checkpoint gene *CHEK2* have also been reported in some LFS or LFL families without detectable *TP53* mutations [16–20]. However, there are still relatively few reports of such mutations. Despite the fact that *CHEK2* is no longer considered as a major determinant of LFS, a number of studies support the hypothesis that *CHEK2* gene may act as a factor contributing to individual tumor development in families with LFL tumors. In addition to *CHEK2*, mutations in *POT1* (protection of telomeres 1) have also been associated with the risk of developing several tumor types and have been detected in LFL families [21, 22]. *POT1* encodes a nuclear protein that is essential for telomere maintenance. A higher telomeric fragility has been demonstrated in patients affected by pathogenic *POT1* variants [16]. There are still a significant number of LFS/LFL families for whom no underlying genetic determinant has been identified. For this reason, many

authors have studied the influence of *BAX* [23], *CDKN1A/p21* (cell cycle arrest mediator) [24], *PTEN* (associated to PTEN hamartoma syndrome) [25], *PRDM* and *GAS8* [26] in LFS families without detectable *TP53* mutations. However, none of them has been identified as determinant of LFS. Advances in next-generation sequencing (NGS) technologies, have allowed the identification of TP53 pathogenic variants in patients with malignant tumors and without clinical suspicion of LFS. Therefore, tumor development predisposition in those cases seems to be related to these particular variants [27–29]. Consequently, new bioinformatics tools (not clinical data alone) have been suggested to detect suitable patients for genetic studies [30]. So far, LFS and LFL cases have been commonly classified based on clinical descriptions. New strategies, focused not only on clinical data, but also on molecular alterations, would be more suitable for LFS and LFL classification. Following this idea, nomenclature should also be adapted, and therefore, "*TP53* Cancer Predisposition Syndrome" and "*CHEK2* Cancer Predisposition Syndrome" could be new nomenclatures. All patients with one or more malignant tumors that are clearly related to either of the pathogenic variants (*TP53* or *CHEK2*), might be affected by one of these two proposed entities ("TP53 Cancer Predisposition Syndrome" or "CHEK2 Cancer Predisposition Syndrome"), respectively. Sub-classifications could be also possible, but the molecular basis (germline *TP53* or *CHEK2* pathogenic variant) should be the start point to correctly classifying patients in syndromic entities (based on present knowledge). Li–Fraumeni syndrome might be an exclusion diagnosis, when *TP53* or *CHEK2* alterations were not found and, the family or personal story suggests the LF or LFL syndrome.

## Li–Fraumeni syndrome dependent on pathogenic variants in *TP53*

Tumors more frequently associated to *TP53* germline mutations are: soft tissue sarcomas, osteosarcoma, breast cancer, brain tumors, leukemia and adrenocortical carcinoma. However, many other different tumor types have also been described: phyllodes tumor, choroid plexus tumors, and melanoma. Additionally, more infrequent tumor types included: lung, digestive tract, thyroid tumors, ovary, colon, lymphoma, and childhood malignant meningioma [31–44]. Up to now, causes of phenotypic differences among families affected by different predisposing mutations to LFS are poorly understood. Furthermore, the potential causes of phenotypic differences among members of the same family are not known. Factors influencing those phenotypic differences will be reviewed below.

### *TP53* gene

*TP53* encodes a tumor suppressor protein which in response to oncogenic mutations or DNA damage triggers a transcriptional program to regulate DNA repair mechanisms, cell cycle progression and apoptosis [45, 46]. *TP53* is essential for regulating cell division and preventing tumor formation [47–50]. It also plays a key role in aging [51, 52], cellular metabolism [53, 54], regulation of homeostasis [55] and immune function [46, 56, 57]. Tetramer formation of p53 is essential for its tumor suppressor function. This oligomerization is modulated by the protein concentration of p53, post-translational modifications, and/or interactions with its binding proteins [58]. The active protein conformation induces cell cycle arrest, senescence, and apoptosis through transcriptional regulation of some target genes or non-transcriptional pathways [59]. It is accepted that p53-dependent transcriptional activation occurs by binding to a consensus DNA sequence called the p53 response element in target genes promoters. Fischer M et al. meta-analysis concluded that p53 is not a direct repressor of transcription, but solely activates its target genes [60]. Therefore, p53 acts mainly as conductor conditioning the transcription of several genes: *p21, MDM2, GADD45, BAX, XPC, XPE and 14-3-3σ* [61]. This well-scored transcriptional program performs many of the described *TP53*/p53 tumor suppressor functions. Somatic mutations in this central gene are frequently observed in human cancers [62–64] and the knowledge about *TP53*/p53 in tumors has been useful to understand the phenotypic differences in patients with Li–Fraumeni syndrome.

## Mutated *TP53* gene

*TP53* is mutated in more than 50% of human cancers and disrupted in practically the rest of them [65]. Approximately 80% of *TP53* mutations are single point mutations (the majority of *TP53* well accepted alterations are missense mutations). Moreover, the gene has hotspot mutations [66], in fact, its central domain (nucleotides 102-292) alone accounts for 90% of the changes [66]. Tumor suppressor gene inactivation does not follow the Knudson model for *TP53* (this model implies the inactivation sequence of the two alleles). The p53 protein is especially inactivated by "dominance negative" effect of pathogenic missense variants. The mutated p53 monomers bind and inactivate wildtype p53 monomers. Beside the loss of function (common to all pathogenic *TP53* variants) and the dominant-negative effect on the wild-type p53 activity of pathogenic missense variants, the mutant p53 could also acquire new oncogenic functions, the so-called "gain-of-functions". [67]. Therefore, some missense *TP53* mutations (R282, R175, Y220, R248 and R273) might not only alter the protein function by disrupting the DNA-binding capacity [31], but also can favor a greater oncogenic activity [68]. As a consequence and speaking about Li–Fraumeni patients, a more aggressive phenotype associated to some pathogenic missense

variants (gain-of-function variants) has been observed in large patient cohorts [69, 70]. In this regard, Amadou et al. in a review of 1730 patients found an earlier age of tumor onset in patients with missense mutations (21.3 years), compared to those with all types of loss of function mutations (28.5 years) or genomic rearrangements (35.8 years). Notably, most of children with LFS in this study carried missense mutations [71]. Tumors with missense *TP53* mutations occur earlier in life and are frequently associated to specific histological subtypes [72]. Ognjanovic et al. described that globally, pathogenic missense mutations in exons encoding the DNA binding domain, were more frequently observed in patients with rhabdomyosarcoma and osteosarcoma while loss of function mutations were more frequent in patients with leiomyosarcoma [72]. In addition, not only the type of variant, but also, the location of the variant may cause certain types of tumors to be more frequent than others [73]. Olivier et al. described that brain tumors were associated with missense *TP53* mutations located in the DNA-binding loop that contact the minor groove of DNA, whereas adrenal gland carcinomas were associated with missense mutations located in the loops opposing the protein–DNA contact surface [73]. The greatest compilation of information regarding the genotype–phenotype relationship is found in the IARC (International Agency for Research on Cancer) TP53 Database. The type and location of *TP53* variants may condition a different biological activity of the protein and facilitate the development of certain tumor types. However, despite having the same genetic alteration in *TP53*, there are significant differences among families, which cannot be explained by the type of mutation.

## Polymorphic variants of *TP53*

The presence of certain polymorphisms within *TP53* sequence may determine LFS clinical presentation since these polymorphisms may modify the oncogenic activity of the p53 protein. A novel p.Gly360Val *TP53* variant (in a linker region near the tetramerization domain) is known to be responsible for a phenomenon called enhanced transactivation: transcriptional activation of *TP53* target genes conditions the up-regulation of several p53 response elements and, as a result, the final function of p53 in the cell is modified [74]. The effects of this variant in cancer phenotype among families and members of the same family remain unknown. Otherwise, it was postulated that *TP53* PIN3 polymorphic variant (hg19 chr17: 7579690; a 16 bp duplication in intron 3) may contribute to the phenotypic diversity of germline *TP53* mutations associated with LFS/LFL patients. [75]. Indeed, Marcel et al. reported that the heterozygous *TP53* PIN3 variant supposed a difference of 19.0 years in the mean age at the first diagnosis in *TP53* mutation carriers. The polymorphic variant delayed the appearance of the first tumor [75]. Sagne et al. also observed that cancer tended to occur approximately 15 years later in mutation carriers who also carried

the polymorphic variant *TP53* PIN3 [76]. Another example is the p.Pro72Arg allele of *TP53*; Bougeard et al. described that the mean age of tumor onset in Arg allele carriers (21.8 years) was significantly different from the mean age of tumor onset from those with Pro/Pro (34.4 years) [77]. Marcel et al. reported anticipation of 8.3 years when Arg allele was present [75] (Fig. 1). These polymorphic variants could explain the diversity of tumor patterns among members of the same family.



*TP53* gene is located in 17p13.1 and it is organized in 11 well-defined exons. Codifying protein regions are referred over every numbered exon. Polymorphic *TP53* variants that could explain Li–Fraumeni heterogeneity are p.Gly360Val, p. Pro72Arg and *TP53* PIN3

## Polymorphic variants in MDM2 gene

Murine double minute 2 (*MDM2*) plays an important role in *TP53* regulation. *MDM2* encodes an E3 ubiquitin-protein ligase that mediates ubiquitination of p53, leading to its degradation by the proteasome. This gene is itself transcriptionally regulated by p53. Therefore, the encoded protein can promote tumor formation by targeting p53, if it does not function well. In fact, overexpression or amplification of this locus is detected in a variety of different cancers (Fig. 2). It has been proposed that certain polymorphic variants of *MDM2* can condition its function and, therefore, could explain clinical differences among families or members of the same family with LFS. The most outstanding example is *MDM2* SNP309 (hg19 chr12: 69202580; T–>G variation), which has been described as a modifier of tumor phenotype. This particular polymorphism increases the expression of *MDM2* and, as a consequence an attenuation of the p53 pathway is detected [77–80]. Bougeard et al. reported an accelerated phenotype among *MDM2* SNP309 G allele carriers. The mean age of tumor onset in *MDM2* SNP309 G allele carriers (19.6 years) was significantly different from that observed in patients homozygous for the T allele (29.9 years, $p < 0.05$). Their

data also supported an amplified effect on the age of tumor onset by the *TP53* p. Pro72Arg allele [77]. Ruijs et al. published that among the *TP53* germline mutation carriers, a significant difference was seen in the mean age of tumor onset for the SNP309 G allele group, that is, 29.7 years as compared to the SNP309 homozygous T group 45.5 years (P=0.005) [78]. In the same way, Macedo et al. studied the median age at first diagnosis among Li–Fraumeni patients carrying *TP53* R337H mutation. The median age at first diagnosis was earlier in *MDM2* SNP309 GG carriers when compared to other genotypes for both tumors analyzed in their study (adrenocortical carcinoma and breast cancer); however, they did not demonstrate a statistically significant difference [79]. Renaux-Petel et al. published results concordant with these and also reported other interesting data about *MDM2* 285G and 309G polymorphism interactions. They reported that the *MDM2* 285-309 G–G is a higher risk haplotype in patients with germline *TP53* mutations and, therefore, suggesting that the *MDM2* 309G variation is deleterious when its effect is not neutralized by the 285C variation [80]. Unfortunately, not enough information is available in concrete populations to translate to Li–Fraumeni patients polymorphic data with prognosis implications. Nowadays, physicians could not personalize surveillance programs based on polymorphic data. Nevertheless, we consider mandatory to study *TP53* PIN3, *TP53* p. Pro72Arg and *MDM2* SNP309 for all Li–Fraumeni patients. The study of at least these three *TP53* polymorphisms (mainly MDM2) is the only way to assess their impact on individual and familial diversity of tumor patterns. To do so, national and international contributions integrating all this information joined to *TP53* mutation type and clinical data is the way to follow.



DNA damage drives p53 activation. Protein p53 develops transcription factor functions that condition cell cycle stop and apoptosis activation and also stimulates *MDM2* transcription. Murine double minute 2 (MDM2 protein) plays an important role in p53/TP53 regulation. *MDM2* encodes an E3 ubiquitin-protein ligase that mediates ubiquitination of p53, leading to its degradation by the proteasome

## microRNA regulation pattern

It is known that certain microRNAs are members of *TP53* transcriptional program. It has been proposed that miR-605 (regulator of loop p53-MDM2) could affect the tumor phenotype in LFS [81]. When cellular stress is present, p53 escapes the p53:Mdm2 negative feedback to accumulate rapidly and to induce cell cycle arrest and apoptosis. Xiao et al. demonstrated that miR-605 is transcriptionally activated by p53 and post-transcriptionally represses Mdm2. The activation of p53 upregulates miR-605 expression, via interacting with the promoter region of the gene [81]. Based on the knowledge about p53-miR-605-MDM2 interactions, polymorphic variants in miR-605 gene and their role in Li–Fraumeni phenotype were studied. Indeed, the variant G allele of miR-605 (Hg 19 chr10: 53059406) was proposed by Id Said B and Malkin D as modifier of the LFS phenotype. They described a 10-year acceleration in the mean age of LFS tumor onset when miR-605 (Hg 19 chr10: 53059406) is present, supporting their hypothesis [82].

Moreover, miR-34A is a key component of the p53 regulatory network. It was shown that p53 regulates the expression of miR-34A, representing an important mechanism of p53 signaling. Members of the miR-34 family were proposed as the most prevalent p53-induced miRNAs and are frequently silenced in variety of tumor entities, suggesting that they are important tumor suppressors [83]. Accordingly, miR-34A is inactivated by hypermethylation across many histologic types of primary tumors from patients with LFS. Malkin D group described that loss of function *TP53* mutations were significantly associated with hypermethylation at the locus encoding miR-34A ($P<0.001$) in germline, and this observation was validated in an independent patient cohort (P $P<0.001$). At tumoral level, miR-34A hypermethylation was associated with decreased overall survival in a cohort of 29 patients with choroid plexus carcinomas ($P<0.05$).

In conclusion, the systematic study of polymorphic variants in *TP53* and *MDM2* genes could enrich Li–Fraumeni knowledge, as commented above. In the same way, the polymorphism miR-605 (Hg 19 chr10: 53059406 G allele) and the methylation pattern at the locus encoding miR-34A should be mandatory when a Li–Fraumeni patient carrying a *TP53* mutation is diagnosed. We will be able to enhance our comprehension of this entity sharing this information internationally.

## Copy number variations

Copy number variations (CNV) among Li–Fraumeni patients carrying *TP53* mutations are understudied. *TP53* dysfunction causes an increased number of copy number variations due to tumor instability [85–87]. Shlien et al. published that LFS *TP53* mutation carriers present an increased CNV both in tumors and germline [88]. They studied a cohort of 53 individuals from Li–Fraumeni families, 33 were *TP53* mutation carriers and 20 harbored wild-type *TP53* (controls). Controls displayed a median of 2 CNVs per genome in germline. However, the *TP53* mutation carriers displayed a significant increase in CNVs (a mean of 12.19 CNVs) (p=0.01). They also suggested a dose–response relationship between CNV frequency and severity of the LFS phenotype. Interestingly, they showed even greater number of CNVs among those *TP53* carriers affected by cancer, than those which have not developed cancer yet. Moreover, they found two genes involved in recurrent duplications among LFS families: *MLLT4* and *ADAM12.* They proposed that CNV frequency, or another high-resolution measure of instability, may help to define the nature and severity of the germline *TP53* mutations found in LFS families [88]. This hypothesis was tested by Arifn et al. in a family with clinical data of anticipation. They analyzed CNV exceeding 10 kb in size. They concluded that CNV composition did not show significant variation among family members, despite their differences in *TP53* mutation carriage and in cancer status [89]. Furthermore, Silva et al. did not find any difference in the total number of germline CNV present in LFS patients versus controls. However, they noted a highly significant increase (>fivefold) in the rare CNVs (estimated based both on DGV and db Var) in TP53 DNA-binding domain mutation carriers as compared both to controls and to p.R337H carriers. They proposed that different microarray technologies used by Shlien et al. could be the origin of their hopeful results [90]. Total number of germline CNVs cannot be used to stratify risk assessment for Li–Fraumeni patients based on present knowledge. Nevertheless, deletions or duplications in concrete genome regions could explain some phenotypic differences among families or members of the same family. Larger cohort and homogeneous populations of Li–Fraumeni patients sharing *TP53* mutation should be studied in this way to obtain conclusive results.

## Telomeric length variations

The influence of telomere length in final phenotypic differences has been studied among the carriers of germline mutations in *TP53*. Human telomeres are nucleoprotein complexes at chromosome ends, consisting of TTAGGG repeats and associated telomere-binding proteins. In germ cells, telomeres range from 10 to 15 kb in length. Telomeres protect chromosomes from nuclease degradation and chromosome rearrangements and serve as mitotic clocks that monitor the number of cell divisions. A possible link between p53, telomeres, tumor initiation, and

anticipation in LFS, has been suggested [91–93]. Based on this hypothesis, Trkova et al. published that the telomere length in peripheral blood cells was shorter among *TP53* mutation carriers than in general population. They did not find progressive telomere shortening among Li–Fraumeni generations. However, they observed a trend (not statistically significant) of earlier onset of cancer in individuals with shorter telomeres and vice versa [94]. Tabori et al. published that telomere length was significantly shorter in affected than in non-affected *TP53* mutation carriers. They concluded that telomere length could explain earlier age of onset of tumors in successive generations of the same family with identical *TP53/MDM2*-SNP309 genotypes [95]. Not enough information is available in this way to reach to conclusions and to take clinical decisions. More in-depth studies are needed.

## Oxidative stress cell level

So far, there is just one published study in the literature that compares levels of oxidative stress between *TP53* carriers and controls. Macedo et al. reported an increase in cellular oxidative stress among patients with the p53R *TP53* variant (p.Arg337His). Specifically, an increase in erythrocyte GPx activity and carbonyl levels in plasma (indicator of protein oxidative damage) in mutation carriers compared to noncarriers. In addition, a significant increase in malondialdehyde levels (indicative of increased lipid peroxidation) has been demonstrated in *TP53* p.Arg337His mutation carriers. Thus, the cellular oxidative damage level could also partially explain the different phenotype among LFS families and members of the same LFS family. To the best of our knowledge, this phenomenon has not been studied in large patient cohorts [96]

## Epigenetic regulation of TP53 expression

 The *TP53* promoter is highly regulated. Different mechanisms participate in a delicate control. A direct binding of several transcription factors in *TP53* promoter is well described. Saldaña-Meyer et al. reviewed the *TP53* epigenetic regulation extensively [97]. *TP53* human promoter has several conserved transcription factor binding motifs. Different transcription factors bind TP53 promoter and upregulate its expression. They are Myc/Max, USF, AP-1, ETS2, NFκB, RREB-1, ETS2, YY, NF, HOXA5, p53/p73, pituitary homeobox 1 (hPitx1) and ISGF3 (formed by Stat1, Stat2 and IRF-9). Moreover, kinase C δ (PKCδ) although does not bind the *TP53* promoter, promotes *TP53* transactivation. Nevertheless, Pax and BCL6 transcription factors inhibit the TP53 promoter. ETS1 also binds on the human TP53 promoter, but its effects are not well described [97]. A particular transcription factor is E2F1 which binds *TP53* promoter and has a direct role in the induction of

mutant p53 [97]. Furthermore, *TP53* human promoter has a CTCF binding site downstream of a CpG island. CTCF influences transcriptional regulation of *TP53*. In fact, when knocking-down *CTCF*, the human *TP53* gene loses its expression supporting its relevant contribution to *TP53* expression regulation [97, 98]. Otherwise, the *TP53* gene promoter regulation by DNA methylation remains controversial. Present knowledge points to the lack of methylation over the *TP53* core promoter. Therefore, other mechanisms might be involved (methylation of genomic regions different from promoters) [97]. Finally, microRNAs can negatively regulate *TP53* gene expression and if deregulated can promote cancer. The best known examples are: miRNA-125a and miRNA-125b which represses p53 post-transcriptionally. MicroRNA-504, microRNA-25, miRNA-30d and LincRNA-p21 interfere as well with p53 functions [97]. The anti-sense RNA Wrap53 is necessary for the proper transcription of *TP53* [97]. *TP53* is regulated by multiple transcription factors and microRNAs, which are epigenetically regulated. Thus, a certain pattern of epigenetic regulation of all these regulatory genes could condition a wild-type and mutated p53 cellular level, variable from one individual to another, which might explain phenotypic differences among members of the same Li–Fraumeni family. No studies were developed either studying plasma levels of these regulatory elements or methylation pattern of their codifying genes among Li–Fraumeni patients. It could be a way to explore in the future

## Epigenetic regulation of genes regulated by TP53

Genetic and epigenetic alterations may be involved in the phenotypic variability of LFS. p53 regulates several pathways, including the thymine DNA glycosylase (TDG) pathway, which regulates the DNA methylation of several genes. Fortes et al. compared the DNA methylation pattern of genes related to the TDG pathway among germline *TP53* mutations carriers, patients with wild-type *TP53*, and healthy individuals. Finally, no significant differences were found. However, increased TDG expression was detected in patients with p.R337H *TP53* mutation affected by adrenocortical carcinoma. Further studies in larger patient cohorts are necessary to evaluate the clinical impact of epigenetic alterations on genes potentially involved in LFS variability [99]

## Other elements to consider

The presence of mutations in certain RecQ DNA helicases (like *BLM* (Bloom syndrome (BS) protein) and *WRN* (Werner syndrome protein)) would affect *TP53* function. The Harris CC group suggests that p53 mediates the cooperation of p53 and BLM to induce apoptosis. Therefore,

certain variants in these genes might affect, at least partially, the function of *TP53* [100]. The elements that might condition the tumor phenotype in LFS are detailed in Table 1.

**Table 1** Elements that may condition phenotypic differences, among patients carrying the same *TP53* variant

|  | Regulatory element | References |
|---|---|---|
| **Genetics** | | |
| Polymorphic variants in *TP53* gene | *TP53* p.G360V | Id Said et al. [74] |
|  | *TP53* PIN3 | Marcel et al. [75] |
|  | *TP53* p.Pro72Arg | Bougeard et al. [77] |
| Polymorphic variants in *MDM2* gene | *MDM2* SNP309 G allele | Bougeard et al. [77] |
| Polymorphic variants in microRNAs | microRNA 605 (rs2043556 GG) variant | Id Said et al. [82] |
| **Genomics** | | |
| Copy number variations (CNVs) | Presence of rare CNVs | Silva et al. [90] |
| Telomeric length | Telomeric length shortening | Tabori et al. [95] |
| **Epigenomics** | | |
| *TP53* transcriptional and post-transcriptional regulation | Diversity among individuals in regulation pattern | Saldaña-Meyer et al. [97] |
| microRNA-34 | miR-34A methylation pattern | Samuel et al. [84] |
| **Metabolomics** | | |
| Oxidative stress cell level | Protein oxidative damage level | Macedo et al. [96] |
|  | Lipid oxidative damage level | |

## Environmental components

Phenotypic differences are detected among Li–Fraumeni patients from different geographical origins. Environment could affect tumor development among *TP53* carriers, therefore, life style, diet and environmental exposures joined to all above said, probably condition the final phenotype. An environmental component may be responsible for the differences observed among families from different origins that share *TP53* mutation [89]. None large cohorts studying its influence has been published. Moreover, founding mutations are very common in certain regions and exceptional in others, and this makes comparative studies difficult.

## Anticipation?

A decrease in the age at cancer onset and an increase in more LFS-specific cancers in successive generations have been suggested [101, 102]. The genetic mechanisms proposed to explain this heterogeneity include accumulation of copy number variations (CNVs) with successive generations, and progressive telomere shortening [88]. Arifn et al. studied a dataset of 269 pedigrees of *TP53* germline mutation carriers. Although, they reported a decrease in age at first cancer onset in multigeneration pedigrees, their observations did not ft with a classical model of anticipation. Nevertheless, only pedigrees with three or four generations showed a delayed age

at first cancer onset in the older generations of *TP53* mutation carriers. Then, they suggested that the founder patient of such pedigrees may carry, in addition to germline *TP53* mutation, rare independent genetic modifiers that attenuate the risk of early cancer. These genetic variants might allow cancer-free survival until post-reproduction age of founders. Based on these observations, they proposed the term "genetic regression" instead of anticipation [103]. To understand this phenomenon, they looked for CNVs larger than 10 kb and for telomere length shortening among kindred affected by LFS-specific cancer, but did not discover significant differences. Moreover, they did not find neither more frequent *MDM2*-SNP309 G allele nor *TP53* PIN3 among affected children compared with their previous generations [103]. Otherwise, this group used whole-genome sequencing (WGS) analysis among family members and identified interesting rare single-nucleotide variants (SNVs). A curious example was a father (non-carrier *TP53*) who transmitted a rare SNV to two out of four *TP53* mutation carrier children. Children with *TP53* mutation and the rare SNV developed an early cancer but not the two *TP53* mutation carrier children who not carried that rare SNV. Such rare SNV may be considered as candidate-modifier genes that may modulate age at cancer onset. Deeper studies looking for these variants could be important [103]. In fact, Franceschi et al. reported recently an affected child who inherited the *TP53* mutation from his affected mother (breast cancer in adulthood age) and received from their non-affected father 25 predicted deleterious variants including a nonsense mutation in *ERCC3*. They proposed that those inherited mutations are possible candidate modifiers linked to *TP53* [104]. Undiscovered genetic variants could determine also Li–Fraumeni heterogeneity among members of the same family

## Conclusions

It is very difficult to elucidate the genotype–phenotype relationship in LFS. Based on the evidence described in the present review, not only would the genotype condition phenotypic peculiarities, but also the epigenome seems to play a key role, although to date, studies in this field are scarce. The current knowledge of LFS makes it difficult to state individual recommendations adapted to the risk at all levels of clinical care (genetic counseling in assisted reproductive treatments, pediatric or medical oncology). Therefore, it is urgent to increase the understanding of this devastating entity. The systematic and coordinated study of all the elements involved in LFS is the only way to move forward.

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## 4. Conclusiones

<u>Principales conclusiones:</u>

-De acuerdo a los resultados derivados de estudiar una serie de 170 pacientes pediátricos con cáncer en nuestro centro, se establece que un 9.4% padece un síndrome genético de predisposición hereditaria al cáncer.

-Un 5.9% adicional de pacientes incluidos en nuestra serie, son portadores de variantes probablemente patogénicas que pueden estar implicadas en la predisposición genética a la enfermedad padecida, pero no hay evidencia suficiente para confirmar una relación causa-efecto.

-Al estudiar la línea germinal de pacientes mediante un panel NGS de gran tamaño (390 genes relacionados con cáncer pediátrico), se detectan variantes patogénicas o probablemente patogénicas que requieren asesoramiento genético personal y/o familiar en hasta un 20% de pacientes.

-Las herramientas de selección de pacientes oncológicos que se deben estudiar desde un punto de vista genético, como la de Jongmans MC o esta modificada (Ripperger et al.) presentan una elevada sensibilidad para detectar a los pacientes con síndromes de predisposición genética al cáncer (Jongmans MC, sensibilidad del 94%; Ripperger T et al, sensibilidad del 100%).

<u>Conclusiones específicas:</u>

-La valoración de los pacientes oncológicos desde un punto de vista germinal en la práctica clínica, debería realizarse considerando los criterios de Ripperger T et al, para a continuación estudiar los genes asociados a la enfermedad sospechada en la línea germinal.

-La realización de grandes paneles de genes (como fue nuestro caso) o genomas/exomas, tendría sentido en la actualidad únicamente en un contexto de investigación.

-Variantes germinales en *CTCF* se asocian a la entidad Retraso mental autosómico dominante 21, la cual condiciona distintos grados de discapacidad intelectual y podrían a su vez predisponer a tumor de Wilms.

-Se reporta el primer paciente con síndrome 13q- en mosaico afecto de retinoblastoma. Las características genéticas derivadas del análisis de su tumor son concordantes con lo publicado hasta la fecha en Retinoblastoma.

-El meningioma maligno podría ser parte del fenotipo Li-Fraumeni.

-La heterogeneidad del síndrome Li-Fraumeni es parcialmente conocida, la variante específica detectada en *TP53* condiciona la clínica de los pacientes.

-La variabilidad fenotípica intrafamiliar no se puede explicar en base a la variante de *TP53* padecida; otros cambios genéticos y/o epigenéticos podrían condicionar la expresión clínica. Factores ambientales pueden estar también implicados.

<u>Posibles investigaciones futuras:</u>

-Evaluación de las consecuencias psicológicas de la información facilitada en una consulta de consejo genético en predisposición hereditaria al cáncer pediátrico.

-Realización de estudios de coste-eficiencia en el campo de la predisposición genética al cáncer pediátrico (WES/WGS *vs* paneles grandes de genes *vs* paneles dirigidos).

-Estudiar en series grandes de pacientes con tumor de Wilms y fenotipo sindrómico la prevalencia de variantes germinales en *CTCF.*

-Profundizar en las posibles implicaciones de las variantes germinales de *JAK3, CDKN2A* y *CHEK2* en la predisposición a leucemia en la infancia.

-Estudiar en series amplias de pacientes con síndrome de cáncer de mama y ovario hereditario el antecedente de neuroblastoma con el objetivo de valorar la posible asociación entre variantes de *BRCA1* y predisposición a neuroblastoma.

-Integrar el análisis genético, cromosómico, epigenético y transcriptómico en el campo de la heterogeneidad del síndrome de Li-Fraumeni en base al conocimiento disponible, en busca de asociaciones genotipo-fenotipo en familias concretas.

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# 6. Resumen de la tesis

## Introducción:

La predisposición genética al cáncer pediátrico es una realidad bien caracterizada en la actualidad. Son decenas los síndromes genéticos adecuadamente categorizados que predisponen a uno o más tumores durante la infancia y/o vida adulta. Fruto del consenso internacional, existen herramientas que facilitan la detección de estos pacientes, así como guías clínicas de seguimiento y adaptación del manejo terapéutico. Las repercusiones personales y familiares que implica su detección exigen un asesoramiento genético exquisito. La adecuada recopilación de la información clínica, una detallada información pre-test, así como una inteligible información post-test son elementos clave en el consejo genético a realizar en el campo del cáncer hereditario.

Atendiendo exclusivamente a la población pediátrica con cáncer, los datos disponibles apuntan a que aproximadamente un 10% de pacientes serían portadores de variantes genéticas de predisposición a su enfermedad. A medida que avanza el conocimiento y las tecnologías de secuenciación de genes, se descubren nuevas entidades de etiología genética asociadas al riesgo de padecer cáncer. Los trabajos más recientes sugieren un porcentaje de en torno al 15% de niños con síndromes de predisposición genética al cáncer pediátrico en cohortes no seleccionadas por tipo tumoral de pacientes oncológicos.

En base al conocimiento disponible, el retinoblastoma sería el tumor con mayor componente hereditario, pero se relaciona únicamente con un gen, *RB1*. Existen dos grandes formas de predisposición a retinoblastoma. En primer lugar, pacientes portadores de variantes patogénicas en *RB1* (variantes de nucleótido único o variantes en el número de copias), sin malformaciones asociadas, siendo este grupo de pacientes la mayoría. Se diferencian principalmente de las formas no hereditarias por la presentación bilateral o multifocal de la enfermedad en un porcentaje de pacientes significativamente mayor. Un segundo grupo de pacientes con susceptibilidad a padecer retinoblastoma, son niños con cuadros malformativos asociados al síndrome de deleción 13q. En este último grupo, el fenotipo de los pacientes parece estar condicionado por la región delecionada y por tanto, por los genes en ella ubicados. Se han descrito pacientes afectos de la enfermedad en mosaico, en los cuales, la clínica depende de los territorios corporales afectos.

Otro tumor con un componente hereditario nada despreciable es el tumor de Wilms. La predisposición al mismo se puede dividir en dos grandes grupos. Alteraciones en el gen *WT1* o en el locus 11p15.5. En función del evento génico producido en *WT1,* el fenotipo puede ser uno de

los siguientes: Predisposición a tumor de Wilms asociado a *WT1,* síndrome de WAGR, síndrome de Denys-Drash, síndrome de Frasier, anomalías genitourinarias sin fallo renal. Por otro lado, las alteraciones en el locus 11p15 se relacionan fundamentalmente con el síndrome de Beckwith-Wiedemann. La sospecha de uno de estos síndromes se basa principalmente en las malformaciones genitourinarias de los pacientes y el fallo renal en el primer grupo (si bien no todos presentan este fenotipo) y la macrosomía, hemihiperplasia, hipoglucemia neonatal, onfalocele, visceromegalia (incluida la adrenocortical), así como las anomalías renales en el segundo. Si bien son muchos los matices fenotípicos entre entidades y dentro de cada enfermedad, se puede concluir que pacientes con tumor de Wilms unilateral, sin malformaciones asociadas ni antecedentes familiares muy probablemente no padecerán un síndrome de predisposición.

Por último, una entidad de desarrollo intracraneal poco frecuente en la infancia es el meningioma. En la edad pediátrica, su aparición se asocia fuertemente con la presencia de una neurofibromatosis tipo 2 y más raramente aparece en el contexto de una Schwanomatosis asociada a *SMARCB1,* asociado a *SMARCE1 (*meningioma de células claras), así como en el contexto de susceptibilidad a meningioma asociada al gen *SUFU*.

## Hipótesis y objetivos:

### Hipótesis

Al menos un 10% de pacientes pediátricos con neoplasias sólidas y hematológicas padecen un síndrome genético de predisposición al cáncer identificable mediante técnicas convencionales y/o *Next Generation Sequencing* (NGS).

### Objetivos

Principal:

Establecer la prevalencia de síndromes de predisposición al cáncer en una cohorte de pacientes oncológicos diagnosticados en una unidad de oncología pediátrica.

Específicos:

- Ofrecer a todos los pacientes la posibilidad de ser estudiados genéticamente desde dicho enfoque.
- Realizar consejo genético en pacientes y familiares.
- Investigar nuevas asociaciones genotipo-fenotipo.

## Metodología y resultados:

### Manuscrito 1

Germline Predisposition to Pediatric Cancer, from Next Generation Sequencing to Medical Care

*Cancers (MDPI) (2021)*

En el primero de los manuscritos incluidos en la presente tesis, se recoge la valoración de la susceptibilidad genética al cáncer en 170 pacientes oncológicos pediátricos. A todos los pacientes diagnosticados en nuestro centro durante un periodo de dos años (Marzo 2018-Marzo 2020), se les presentó el proyecto y se les ofreció entrar en el estudio. Únicamente los pacientes que cumplieron los criterios de inclusión entraron en el estudio. Los criterios fueron: Edad entre 0 y 18 años; diagnóstico patológico final establecido; disponibilidad de muestra de sangre de origen germinal; estabilidad clínica del paciente; aceptación del paciente a participar habiendo entendido la información relacionada con el estudio; no padecer un tumor benigno sin base genética conocida para su desarrollo.

Los pacientes y sus familias recibieron asesoramiento genético antes y después de la prueba. Durante la primera visita se les informó que el estudio consistía en secuenciar un panel de genes amplio (390 genes) basado en tecnología NGS, pero también de que únicamente incluye un número muy bajo de genes relacionados con la patología que padecen ellos o sus hijos. Se les notificó que exclusivamente el análisis de estos genes previamente relacionados con su enfermedad, podría obtener conclusiones de causa-efecto para sus casos individuales. Además, se les informó que todos los datos restantes obtenidos del análisis serían utilizados con fines de investigación por el equipo de investigación en el marco del proyecto. Se les advirtió que del estudio puede surgir alguna información dudosa y que podríamos proponer continuar estudiando diferentes aspectos en el paciente y / o familia con fines de investigación. Sin embargo, en ningún caso esta última información nos permitiría obtener evidencia para el paciente concreto. También nos comprometimos a no dañar ni aumentar el número de visitas médicas de pacientes y familiares al realizar estas pruebas complementarias. Acto seguido, los pacientes y / o padres firmaron el consentimiento informado siendo conscientes de todo ello.

Los pacientes fueron incluidos en la serie de manera prospectiva, sin excluir ninguna neoplasia maligna. Los pacientes fueron explorados físicamente, se recogió la historia familiar y fueron valorados mediante la herramienta de Jongmans MC et al. En caso de existir la sospecha de un

síndrome específico y la posibilidad de estudiar la entidad sospechada mediante un test incluido en la cartera de servicios del servicio de genética, el paciente se estudió por esta vía. Sin embargo, en caso de no disponer del estudio genético de interés o de no tener una sospecha de un síndrome concreto, el paciente se estudió mediante el panel custom *Onconano V2.*

El panel *Onconano V2* fue secuenciado por el Instituto de Medicina Genómica (Imegen - Health in Code Group). Las variantes se clasificaron como benignas, probablemente benignas, VUS (variante de significado incierto), probablemente patogénica (LP) y patogénica (P) siguiendo las recomendaciones de la ACMG. Además, se consideró que algunas VUS estaban potencialmente implicadas en la predisposición genética a la enfermedad. Para estas variantes de significado incierto, potencialmente involucradas en la predisposición al cáncer del paciente pero sin evidencia suficiente para ser consideradas probablemente patógenas, se estableció una nomenclatura interna. Variantes de potencial importancia patogénica (VOPPS) fue el término utilizado para estas variantes.

El informe técnico resultante de los análisis genéticos fue discutido por el Comité de Predisposición Genética del Hospital La Fe. En este comité se incluyó a un oncólogo pediatra, un genetista especializado en cáncer hereditario y un biólogo molecular. El informe final elaborado por el comité fue entregado al paciente y su familia. Las variantes de significado incierto no se comunicaron a las familias. Las variantes patogénicas implicadas en el riesgo de cáncer durante la infancia llevaron a recomendaciones personalizadas para los oncólogos. Variantes patogénicas o probablemente patogénicas con implicaciones de riesgo de cáncer en la edad adulta, condujeron a la realización de estudios de segregación familiar y seguimiento personalizado de los familiares en la Unidad de Consejo Genético del Hospital La Fe. Así mismo, se informó a los padres sobre las variantes patogénicas relacionadas con enfermedades recesivas y sus potenciales implicaciones para la descendencia.

Se evaluó la inclusión de 223 pacientes durante el período especificado. Finalmente, 170 pacientes cumplieron los criterios de inclusión y aceptaron participar en el estudio. Los padres firmaron el consentimiento informado en todos los casos, pero también se informó a los pacientes según sus edades y los pacientes mayores de 12 años firmaron documentos específicos.

La distribución hombre-mujer fue del 60%-40% y la edad media fue de 7,2 años (0-18). El diagnóstico más frecuente fue leucemia (45 casos; 26,5%), seguido de tumores del SNC (26 casos; 15,3%), linfomas (20; 11,8%), neuroblastoma y tumores del sistema nervioso periférico (19; 11,2%), tumores óseos ( 14; 8,2%), sarcomas de partes blandas (12; 7,1%), tumores renales (9;

5,3%), retinoblastoma (8; 4,7%), tumores hepáticos (4; 2,3%), tumores de células germinales (3; 1,8%), melanoma y otros tumores cutáneos (1; 0,5%) y otros tipos de tumores (9; 5,3%)

Estos porcentajes se compararon con los recogidos en el Registro Español de Tumores Pediátricos (RETI). No se encontraron diferencias estadísticamente significativas al comparar las tasas de incidencia de estos tipos de tumores entre la serie RETI (grupo de edad 0-19 años; 1980-2017) y nuestra cohorte. Siguiendo el flujo de trabajo establecido, se estudiaron un total de 153 pacientes con Onconano V2, y los 17 casos restantes exclusivamente mediante técnicas convencionales u otros paneles NGS.

Se detectó una variante patogénica de predisposición al tumor del paciente en 16 casos (16/170; 9,4%). En cuanto a los genes implicados en la predisposición, el más frecuentemente alterado fue el gen *RB1* (6/16; 37,5%), seguido del gen *NF1* (3/16; 18,8%); otros genes mutados fueron *DICER1, NF2, SUFU, TP53, XPC y SOS1*. Además, se incluyó en la cohorte a un paciente diagnosticado de trisomía 21. Además, en otros diez casos se identificaron mutaciones probablemente patogénicas que podrían estar implicadas en la predisposición al tumor del paciente (10/170; 5,9%).

Además, a algunos VUS se les podría atribuir una potencial patogenicidad. Por tanto, podrían estar implicadas en la predisposición al tumor que padecen los pacientes, pero la falta de evidencia lleva a clasificarlas como VUS según los criterios de la ACMG. Estas variantes se clasificaron como VOPPS. Se detectaron variantes de estas características en los genes *ING4, NF1, FANCD2, IGF1R, ALK, FAT1, CHEK2, RET y SH2B3.*

Un total de 50 pacientes (29%) cumplieron al menos uno de los criterios de Jongmans MC et al. durante la evaluación realizada después de la inclusión. Entre ellos, se detectaron mutaciones patogénicas predisponentes en 15 casos (15/50; 30%). De esto se desprende que el 94% del total de variantes patogénicas predisponentes al cáncer pediátrico detectadas en el estudio (15/16) se encontraron en pacientes que cumplieron los criterios de Jongmans MC et al. Además, cinco de las diez variantes (50%) clasificadas como probablemente patogénicas se detectaron entre los pacientes que cumplieron con los criterios de Jongmans MC et al. Por lo tanto, se identificaron mutaciones patogénicas y probablemente patogénicas en el 40% de los pacientes elegidos por la herramienta (20/50). Considerando como variantes de predisposición solo las 16 mutaciones patogénicas, la herramienta de Jongmans MC et al. obtuvo una sensibilidad del 94% y una especificidad del 77% en nuestra cohorte. En los 120 pacientes que no cumplieron la indicación de derivación a un genetista clínico según los criterios de Jongmans MC et al, se detectaron mutaciones de predisposición patogénica solo en un caso (0,8%). De los 120 pacientes, cinco portaban variantes probablemente patogénicas según los criterios de la ACMG (4,2%).

## Manuscrito 2

Retinoblastoma and mosaic 13q deletion: a case report

*International Journal of Retina and Vitreous (BMC) (2021)*

El síndrome de deleción 13q se conoce desde el año 1969. Desde entonces, se han publicado distintas series, donde se recoge el espectro clínico de la enfermedad. En aquellos casos que involucran al gen *RB1* (13q14.2), el paciente tiene riesgo de padecer retinoblastoma. Durante los últimos años, se han reportado pacientes con esta entidad en mosaico y una clínica compatible con la conocida para el síndrome 13q- pero condicionada por los territorios corporales portadores de la misma.

El segundo trabajo de la presente tesis recoge los datos clínicos de una paciente afecta de retinoblastoma y portadora de una deleción de 13q en mosaico, que sí afectaría a la retina en su caso, como se pudo demostrar, facilitando el desarrollo de un retinoblastoma tras la aparición de un segundo hit en *RB1*. Este pudo a su vez ser evidenciado en el retinoblastoma padecido, junto a otras anomalías citogenéticas características del retinoblastoma. Se trata del primer paciente con un síndrome de deleción 13q- en mosaico afecto de retinoblastoma recogido en la literatura.

La paciente debutó con 6 meses e ingresó en el hospital por leucocoria y estrabismo. La historia clínica y el examen físico no fueron llamativos excepto por la clinodactilia del quinto dedo derecho. El examen oftalmoscópico indirecto y el examen bajo anestesia fueron realizados por oftalmólogos. La ecografía orbitaria y la resonancia magnética (RM) mostraron una masa intraocular izquierda de 14 × 13 × 11 mm ubicada en el lado inferior externo de la retina. También se detectó desprendimiento de retina. El oftalmólogo descartó otras lesiones tumorales y la resonancia magnética tanto en retina como en cerebro descartó otros tumores. Se realizó el diagnóstico de retinoblastoma y, en base a la Clasificación Internacional de Retinoblastoma Intraocular, se estableció un grado E. La paciente recibió melfalán intraarterial pero debido a un vasoespasmo local en su pierna izquierda, se suspendió el tratamiento. Posteriormente, se administraron cuatro ciclos de quimioterapia convencional (vincristina, carboplatino y etopósido). Se logró una respuesta parcial, pero, a pesar de la quimioterapia, la enfermedad progresó pocas semanas después y se enucleó el ojo afectado. Sobre la base de las

recomendaciones internacionales, se estudió el gen *RB1* en el ADN de la línea germinal de linfocitos de sangre periférica; no se detectaron mutaciones. Se utilizó un ensayo MLPA para evaluar posibles deleciones y duplicaciones del gen *RB1.* Los valores detectados fueron relativamente bajos pero dentro del rango normal. Se sospechó una deleción completa de *RB1* en mosaico. Se realizó un SNP genómico (AfymetrixCytoScan 750) y se detectó una deleción 13q de 35,7 Mb de 13q12.13 a 13q21.2 (ar [hg19] 13q12.13q21.2 (26,555,387–62,280,955) × 1–2) en alrededor del 40% de células analizadas.

Una sonda FISH específica de *RB1* (LSI13), a partir de células de mucosa oral, mostró una deleción de 13q en alrededor del 40% de las células. Realizamos un array de SNPs (Afymetrix Oncoscan) tanto en su retina sana, libre de infiltración tumoral, así como en la muestra de retinoblastoma. La retina sana fue portadora de la deleción 13q en mosaico, pero en cerca del 50% de las células estudiadas. Sin embargo, todas las células de la muestra de retinoblastoma fueron portadoras de la deleción en heterocigosis. No se detectaron LOH (pérdida de heterocigosidad) ni cromotripsis en las bandas 13q. Además, la ganancia de 6p12.3pter (3 copias en total) y la pérdida de 6q25.3qter (1 copia en total) se detectaron exclusivamente en la muestra de tumor. Buscando mutaciones de nucleótido único, realizamos un panel NGS (Onconano V2) en el tumor. El estudio detectó sólo una variante patogénica *(RB1* c.958C> T (p.Arg320Ter) (NM_000321.2 posición cromosómica 13-48,941,648-C-T; frecuencia alélica del 25%).

## Manuscrito 3

Germline Variant in CTCF links mental retardation to Wilms tumor predisposition

*En revisión European Journal of Human Genetics (Nature) (2021)*

El tercer manuscrito, recoge de manera elocuente la posible asociación entre una variante germinal en el gen *CTCF* y la predisposición a tumor de Wilms. La asociación entre variantes germinales en *CTCF* y discapacidad intelectual ha sido descrita recientemente y condicionaría un síndrome llamado Retraso mental autosómico dominante 21. Este síndrome puede venir asociado a malformaciones menores a nivel cardíaco, genitourinario y otras. El caso estudiado en este manuscrito se trata de un paciente afecto de tumor de Wilms bilateral, malformaciones genitourinarias y capacidad intelectual en el límite bajo de la normalidad. Se detectó mediante un panel NGS una variante probablemente patogénica en *CTCF* tanto en línea germinal, donde se identificó en heterocigosis, como en el tumor analizado, donde se demostró en homocigosis. Pudo confirmarse una LOH en la región cromosómica 16q, donde se encuentra ubicado dicho

gen. La variante apareció *de novo* en el paciente, el cual es el segundo hijo de un varón tratado con quimioterapia los meses previos a la concepción del paciente.

## Manuscrito 4

Li-Fraumeni: Will the detection in families increase the survival of its members?

*Anales de Pediatría (*Revista de la Asociación Española de Pediatría) (2019)


El manuscrito número 4 recoge la historia clínica de un niño diagnosticado de meningioma maligno, entidad excepcional en la edad pediátrica. La recopilación de la historia familiar permitió sospechar un síndrome de Li-Fraumeni, que se confirmó en el paciente mediante estudio dirigido del gen *TP53* por PCR convencional. El caso índice, fue el hijo de un varón sano que se confirmó así mismo portador de la misma variante, la cual se detectó también en distintos miembros de la familia diagnosticados de tumores del espectro clínico Li-Fraumeni. En base a lo publicado en la literatura, sería el primer paciente con Li-Fraumeni diagnosticado de un meningioma maligno en la edad pediátrica.

Presentamos el caso de un niño de 2 años previamente sano que acudió a los servicios de urgencias de su hospital por una crisis convulsiva (crisis parcial compleja). La evaluación se inició con pruebas de imagen y, ante la sospecha de lesión ocupante de espacio, fue trasladado al hospital de referencia. Las pruebas de imagen revelaron un tumor maligno con afectación meníngea diseminada a lo largo del neuro-eje y una lesión primaria en el interior del cerebro.

El diagnóstico histológico basado en el examen macroscópico y microscópico de la muestra de biopsia obtenida por craneotomía fue de meningioma. El paciente fue tratado con quimioterapia de acuerdo con el protocolo establecido por la SEHOP para menores de 3 años, ya que la cirugía y la irradiación no estaban indicadas por la extensión de la enfermedad y la corta edad del paciente. A pesar del tratamiento, la enfermedad progresó y el paciente falleció 6 meses después del debut. Se recogió la historia familiar en el momento del diagnóstico, revelando múltiples casos de cáncer en el lado paterno. La familia fue remitida a la unidad de asesoramiento genético. El diagnóstico de síndrome de Li-Fraumeni se confirmó mediante pruebas genéticas (c.430C> T p.Q144 * en el exón 5 del *TP53* del paciente). Esta prueba no se había realizado antes en esta familia, a pesar del árbol genealógico altamente sugestivo.

Una evaluación posterior de la familia confirmó que el padre y otros parientes de su rama familiar portaban la misma mutación.

**Manuscrito 5**

Li–Fraumeni syndrome heterogeneity

*Clinical and Translational Oncology (Springer) (2020)*

El manuscrito número 5 es un artículo de revisión del síndrome Li-Fraumeni, centrado en los aspectos moleculares con condicionan la heterogeneidad tumoral del síndrome. Además de la enorme variabilidad clínica entre familias, al observar familias concretas, resulta llamativa la enorme heterogeneidad clínica. Si bien gran parte de las diferencias entre familias se pueden explicar en base a la variante específica padecida, dicho razonamiento no es útil al analizar familias concretas. Son muchos los aspectos moleculares que podrían estar condicionando la expresión clínica, los principales, quedan recogidos en esta exhaustiva revisión y pueden resumirse en los siguientes puntos.

De manera adicional a la variante patogénica detectada en la familia en el gen *TP53*, hay una serie de variantes polimórficas en el propio gen que podrían estar influyendo en la variable expresión clínica evidenciada entre familiares. Estas serían las variantes hasta la fecha implicadas: *TP53* p.G360V; *TP53* PIN3; *TP53* p.Pro72Arg. De manera complementaria, a nivel del gen *MDM2*, el cual codifica para la proteína MDM2, implicada en la regulación de p53, se ha publicado la relación entre el polimorfismo *MDM2* SNP309 G y  la clínica final del síndrome de Li-Fraumeni. Adicionalmente, a nivel de la secuencia del ADN, se ha establecido así mismo una relación estadísticamente significativa entre el polimorfismo microRNA 605 (rs2043556 GG) y el fenotipo familiar. La heterogeneidad del cuadro clínico, parece no relacionarse exclusivamente con la variante patogénica de *TP53* o ciertos polimorfismos presentes a lo largo de la secuencia del ADN, sino también, a nivel genómico, el grado de acortamiento de los telómeros o la presencia de ciertas variantes en el número de copias poco frecuentes, podrían estar también participando de la heterogeneidad clínica. La epigenómica podría estar también implicada y en dicha línea se ha podido relacionar el patrón de metilación en el miR-34A y las manifestaciones clínicas del síndrome.

En base a estas y a nuevas evidencias, se antoja imprescindible la integración de todo el conocimiento disponible en el estudio clínico de familias, de cara obtener una adecuada

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comprensión del cuadro clínico. Todo ello, con el objetivo de poder trasladar una información pronóstica, unas recomendaciones de seguimiento y un manejo terapéutico adaptado al riesgo individual de cada miembro de la familia.

## Conclusiones finales

<u>Principales conclusiones:</u>

-En la serie de 170 pacientes pediátricos con cáncer estudiados en nuestro centro, un 9.4% padece un síndrome genético de predisposición hereditaria al cáncer.

-Un 5.9% adicional de pacientes incluidos en nuestra serie, son portadores de variantes probablemente patogénicas que pueden estar implicadas en la predisposición genética a la enfermedad padecida, pero no hay evidencia suficiente para confirmar una relación causa-efecto.

-Al estudiar la línea germinal de pacientes mediante un panel NGS de gran tamaño (390 genes relacionados con cáncer pediátrico), se detectan variantes patogénicas o probablemente patogénicas que requieren asesoramiento genético personal y/o familiar en hasta un 20% de pacientes.

-Las herramientas de selección de pacientes oncológicos que se deben estudiar desde un punto de vista genético, como la de Jongmans MC o esta modificada (Ripperger et al.) presentan una elevada sensibilidad para detectar a los pacientes con síndromes de predisposición genética al cáncer (Jongmans MC, sensibilidad del 94%; Ripperger T et al sensibilidad del 100%).

<u>Conclusiones específicas:</u>

-La valoración de los pacientes oncológicos desde un punto de vista germinal en la práctica clínica, debería realizarse considerando los criterios de Ripperger T et al, para a continuación estudiar los genes asociados a la enfermedad sospechada en la línea germinal.

-La realización de grandes paneles de genes (como fue nuestro caso) o genomas/exomas, tendría sentido en la actualidad únicamente en un contexto de investigación.

-Variantes germinales en *CTCF* se asocian a la entidad Retraso mental autosómico dominante 21, la cual condiciona distintos grados de discapacidad intelectual y podrían a su vez predisponer a tumor de Wilms.

-Se reporta el primer paciente con síndrome 13q- en mosaico afecto de retinoblastoma. Las características genéticas derivadas del análisis de su tumor son concordantes con lo publicado hasta la fecha en Retinoblastoma.

-El meningioma maligno podría ser parte del fenotipo Li-Fraumeni.

-La heterogeneidad del síndrome Li-Fraumeni es parcialmente conocida, el tipo de variante condiciona la clínica de los pacientes.

-La variabilidad fenotípica intrafamiliar no se puede explicar en base a la variante de *TP53* padecida; otros cambios genéticos y/o epigenéticos podrían condicionar la expresión clínica. Factores ambientales pueden estar también implicados.


Posibles investigaciones futuras:

-Evaluación de las consecuencias psicológicas de la información facilitada en una consulta de consejo genético e n predisposición hereditaria al cáncer pediátrico.

-Realización de Estudios de coste-eficiencia en el campo de la predisposición genética al cáncer pediátrico (WES/WGS *vs* paneles grandes de genes *vs* paneles dirigidos).

-Estudiar en series grandes de pacientes con tumor de Wilms y fenotipo sindrómico la prevalencia de variantes germinales en *CTCF.*

-Profundizar en las posibles implicaciones de las variantes germinales de *JAK3, CDKN2A* y *CHEK2* en la predisposición a leucemia en la infancia.

-Estudiar en series amplias de pacientes con síndrome de cáncer de mama y ovario hereditario el antecedente de neuroblastoma en la infancia con el objetivo de valorar la posible asociación entre variantes de *BRCA1* y predisposición a neuroblastoma.

-Integrar el análisis genético, cromosómico, epigenético y transcriptómico en el campo de la heterogeneidad del síndrome de Li-Fraumeni en base al conocimiento disponible, en busca de asociaciones genotipo-fenotipo en familias concretas.

# 7. Apéndice (1): Otras publicaciones relacionadas del doctorando

-Gargallo P, Yáñez Y, Juan A, Segura V, Balaguer J, Torres B, Oltra S, Castel V, Cañete A.  Review: Ewing Sarcoma predisposition. Pathol Oncol Res. 2020; 26(4):2057-2066.

-Gargallo P et al. Precision Medicine in relapsed or refractory pediatric solid tumors: A collaborative Spanish initiative. Translational medicine communications. 2019.

-Gargallo P, Oltra JS, Yáñez Y, Segura V, Balaguer J, Cañete A. Retinoblastoma: Towards an Earlier Diagnosis. Arch Soc Esp Oftalmol. 2018;93(9):439-443.

8. Apéndice (2): Compendio de manuscritos originales

Artículo 1.

*Article*

# Germline Predisposition to Pediatric Cancer, from Next Generation Sequencing to Medical Care

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**Simple Summary:** The idea that motivated the design of the project is to offer a genetic germline analysis to all pediatric patients diagnosed in our pediatric oncology unit. The main objective is to determine the incidence of predisposing genetic variants when studying a cohort of pediatric cancer patients using an NGS gene panel. The custom panel employed is designed to detect variants in a large number of genes involved in pediatric cancer in order to be able to identify new genotype–phenotype relationships. The data obtained are valuable for estimating the incidence of predisposing genetic alterations, due to the large number of pediatric patients included in the study. Furthermore, the novel results collected in the main document, which suggest the involvement of new genes in the predisposition to different oncological diseases, are worthwhile.

**Abstract:** Knowledge about genetic predisposition to pediatric cancer is constantly expanding. The categorization and clinical management of the best-known syndromes has been refined over the years. Meanwhile, new genes for pediatric cancer susceptibility are discovered every year. Our current work shares the results of genetically studying the germline of 170 pediatric patients diagnosed with cancer. Patients were prospectively recruited and studied using a custom panel, *OncoNano V2*. The well-categorized predisposing syndromes incidence was 9.4%. Likely pathogenic variants for predisposition to the patient's tumor were identified in an additional 5.9% of cases. Additionally, a high number of pathogenic variants associated with recessive diseases was detected, which required family genetic counseling as well. The clinical utility of the Jongmans MC tool was evaluated, showing a high sensitivity for detecting the best-known predisposing syndromes. Our study confirms that the Jongmans MC tool is appropriate for a rapid assessment of patients; however, the updated version of Ripperger T criteria would be more accurate. Meaningfully, based on our findings, up to 9.4% of patients would present genetic alterations predisposing to cancer. Notably, up to 20% of all patients carry germline pathogenic or likely pathogenic variants in genes related to cancer and, thereby, they also require expert genetic counseling. The most important consideration is that the detection rate of genetic causality outside Jongmans MC et al. criteria was very low.

## 1. Introduction

Genetic predisposition plays an important role in cancer development. This fact is well-known in both the adult and pediatric patient population [1,2]. Environmental factors, involved in tumor onset during adult ages, are not so relevant in childhood [3]. However, the incidence and spectrum of mutations predisposing to cancer among children and adolescents are only partially understood [4–6]. Narod and colleagues claimed, in 1991, that 10% of children with cancer had a genetic predisposition [7]. Several genes related to predisposition to different childhood malignancies have been described since then (myeloid leukemia [8] and lymphoblastic leukemia [9], neuroblastoma [10], medulloblastoma [11–13], osteosarcoma [14] and soft tissue and bone sarcomas [15,16]). Meanwhile, knowledge on several disorders remains scarce, but current next generation sequencing technologies have expanded the frontiers of genetic predisposition research and, hence, the possibility of discovering new genotype–phenotype relationships [17].

Identifying cancer predisposition syndromes, defining them properly and establishing risk-adjusted surveillance programs are main goals of the scientific community [18,19]. Recent literature provided follow-up guidelines for several cancer predisposition syndromes with a broad consensus [20–34]. These recommendations open up the work of healthcare professionals in the case of detecting a genetic syndrome. Published guidelines are constantly being updated and are established as a useful framework for daily clinical practice.

The clinical feasibility of this genetic understanding is, therefore, clear and the advantages can be summarized in the following points: This knowledge enables a personalized medical and/or surgical treatment for several patients (e.g., Li–Fraumeni syndrome patients who have *TP53* mutations should not be exposed to ionizing radiation; surgical treatment should be conservative for hereditary retinoblastoma patients). It improves the selection of donors and the choice of the correct time for hematopoietic transplantation. This knowledge also allows to implement familial genetic counseling and transmit prognostic information to patients. It may accelerate the detection of associated non-tumor problems which may require early intervention (e.g., patients with a *WT1* mutation, who may have insidious renal dysfunction). Moreover, unraveling a genetic condition that explains the phenotype may help face the psychological burden of such a diagnosis in some patients/parents. Finally, it could provide a better understanding of tumor development in specific cases [35].

The growing knowledge on pediatric predisposition cancer syndromes underlines the great necessity to transfer into clinical practice the vast genetic knowledge generated by a genetic analysis. The present work aims to assess the incidence of genetic alterations in a prospective cohort of pediatric patients by a germline genetic analysis. We believe that at least 10% of our patients may suffer from a pediatric cancer predisposition syndrome. Therefore, we estimate that a relevant group of patients (at least 10%) could benefit from personalized follow-up recommendations or even personalized treatment. It is also expected to find at least 10% of families who could benefit from genetic counseling.

## 2. Materials and Methods

### 2.1. Patient Study Cohort

All potential candidate patients were diagnosed in or referred to our center from other hospitals between March 2018 and March 2020. Those who relapsed in our institution during this period were also considered for inclusion. Patient eligibility was assessed between days one and sixty since the first hospital admission. The following inclusion criteria were required for study entry:

- Age between 0 and 18 years old.
- Final pathology diagnosis established.
- Germline origin blood sample availability.
- Patient clinical stability.
- Patient voluntary agreement to participate having understood the information related to the study.
- No exclusion criteria fulfilled.

  Furthermore, the exclusion criteria that conditioned the withdrawal of the study:
- Rejection of the study by the patient and/or family.
- Unfavorable previous psychological evaluation.
- Diagnosis of a benign tumor without any known genetic basis for its development.

Patients who met the inclusion/exclusion criteria and agreed to enter the study were included. Patients who entered the project were clinically evaluated in a targeted way. A physical examination was performed and personal and family history were assessed, including a family tree. The information obtained was contrasted with Jongmans MC et al. criteria [36]. This tool allows the detection of patients who would benefit from personalized genetic counseling. In the event of any Jongmans MC et al. criteria being fulfilled and a genetic syndrome suspected, the patient was studied accordingly if a technique was available at the hospital (e.g., *RB1* by PCR and MLPA or *NF1/NF2* by a custom NGS panel). If no alterations were detected by these studies, the test was expanded by a custom NGS panel, *OncoNano V2*. Patients who did not fulfil those three conditions were studied by the *OncoNano V2* gene panel from the beginning. The workflow is shown in Figure 1.
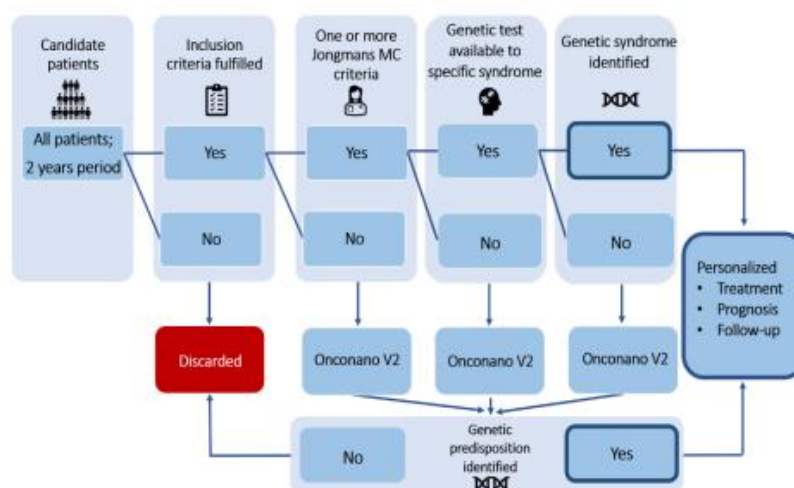


**Figure 1.** Workflow established for the study.

The custom *OncoNano V2* panel was sequenced by the Institute of Genomic Medicine (Imegen-Health in Code Group). The technical report resulting from the genetic analyses was discussed by the Genetic Predisposition Committee of La Fe Hospital. A pediatric oncologist, a geneticist specialized in hereditary cancer and a molecular biologist were included in this committee. The final report prepared by the committee was delivered to the patient and family. Variants of uncertain significance were not communicated to the families. Pathogenic variants involved in the risk of cancer during childhood led to personalized recommendations for pediatric oncologists. Pathogenic or probably pathogenic variants with implications for cancer risk in adulthood guided family segregation studies and personalized follow-up of family members by the Genetic Counselling Unit. Likewise,

pathogenic variants related to recessive diseases and their potential implications for the offspring were informed to the parents.

Patients and their families received pre- and post-testing genetic counseling. During the first visit, they were informed that the study consisted of sequencing a large NGS-based gene panel (390 genes), but that only a very low number of genes related to the pathology suffered by them or their children (score 1) was included in it. They were notified that exclusively the analysis of these genes, included as score 1, could obtain cause–effect conclusions for their individual cases. Moreover, they were advised that all the remaining data obtained from the analysis would be used for research purposes by the research team within the framework of the project. They were warned that some doubtful information may emerge from the study and that we could propose to continue studying different issues in the patient and/or family for research purposes. Nevertheless, in no case would this latest information allow us to obtain evidence for the specific patient. We were also committed not to harm or increase the number of patient and family medical visits when conducting these complementary tests. Thereupon, patients and/or parents signed the informed consent being aware of all this. Therefore, when identifying variants that were of interest from a research point of view, the families received the pertinent information during the post-testing visit. Accordingly, complementary studies (such as family segregation analysis) were carried out within this theoretical framework.

### 2.2. NGS Panel, Sequencing and Analysis Features

The *OncoNano V2* custom panel was developed in collaboration with Agilent and designed to detect mutations (point mutations, including single-nucleotide variants and small indels) and CNVs (deletions or duplications) in 390 genes related to pediatric cancer (File S1). The main established genes related to genetic predisposition to pediatric cancer were also covered. Genomic DNA (gDNA) from blood or other tissue was extracted using the commercial extraction kits RecoverAll™ and the QIAamp DNA Investigator Kit (QIAGEN, Hilden, Germany). Concentration was measured by fluorometric quantification using a Qubit fluorometer with the Qubit dsDNA BR Assay kit (Thermo Fisher Scientific, Waltham, MA, USA) and Qubit dsDNA HS Assay kit (Invitrogen, Waltham, MA, USA). DNA Integrity Number (DIN) was determined using the DNA ScreenTape assay (Agilent Technologies, Santa Clara, CA, USA). The cut-off DIN value was 3. Library preparation followed the manufacturer's recommendations. Libraries were then loaded onto the NextSeq 550 system (Illumina, San Diego, CA, USA) for massive library sequencing in "Stand-alone" mode with $2 \times 150$ paired-end reads following the manufacturer's instructions. For bioinformatics analysis, the alignment to the reference sequence Genome Reference Consortium Human Build 37 (GRCh37), annotation and variant calling followed a custom pipeline through the DataGenomics platform by Imegen. For the CNV analysis, in-house scripts by Imegen were used to obtain a fractional coverage based on a correlation between the number of normalized reads of a region in respect to the number of DNA copies for that region. A minimum inter-sample variability was guaranteed by homogenizing experimental conditions between different samples and genomic regions. CNV calls were classified by DataGenomics based on their credibility, using a scoring algorithm that took into account parameters such as log2 ratio, event size, proximity and type of contiguous events. CNV plots provided by the platform were manually reviewed to discard possible artifacts and validated by digital PCR or MLPA.

The panel genes were classified into 3 scores, depending on their involvement at the hereditary cancer level in order to facilitate further manual analysis. Genes involved in predisposition to the patient's tumor were studied as score 1. Genes involved in predisposition to other tumors as score 2 and other genes related to pediatric cancer at the somatic level and included in the panel were included as score 3. The analysis was performed with the DataGenomics software. Filters were applied to remove from the analysis variants with an MAF (minor allele frequencies) > 0.02 and variants in non-coding regions (flanking splicing sites up to $\pm 10$ nucleotides were excluded from filters). Changes described as polymorphic

according to gnomAD browser data were also removed from the analysis. The study of the variants was carried out with the help of the VarSome, COSMIC, professional HGMD and Pcan.stjude.org websites, as well as those available for specific genes. Information obtained from in silico predictions was also considered.

The variants were classified as benign, likely benign, VUS (variant of uncertain significance), likely pathogenic (LP) and pathogenic (P) following ACMG recommendations [37]. In addition, some VUSs were considered to be potentially involved in genetic predisposition to the disease. However, for many of them, evidence was scarce in this clinical context. Despite a comprehensive in silico analysis, a review of the available literature and a discussion of the variants in expert committee, no strong conclusions could be drawn. For these variants of uncertain significance, potentially involved in predisposition to the patient's cancer but without enough evidence to be considered probably pathogenic, an internal nomenclature was established. Variants of potential pathogenic significance (VOPPS) was the term used for these variants.

## 3. Results

### 3.1. Patient Cohort and Genetic Variants Identified

Overall, 223 patients were assessed for inclusion during the specified period. Finally, 170 patients fully met the inclusion criteria and agreed to participate in the study. The parents signed the informed consent in all cases, but the patients were also informed according to their ages and patients older than 12 years signed specific documents.

The male–female distribution was 60–40% and the mean age was 7.2 years (0–18). The most common diagnosis was leukemia (45 cases; 26.5%), followed by CNS tumors (26 cases; 15.3%), lymphomas (20; 11.8%), neuroblastoma and peripheral nervous system tumors (19; 11.2%), bone tumors (14; 8.2%), soft-part sarcomas (12; 7.1%), renal tumors (9; 5.3%), retinoblastoma (8; 4.7%), liver tumors (4; 2.3%), germ-cell tumors (3; 1.8%), melanoma and other skin tumors (1; 0.5%) and other tumor types (9; 5.3%) (Figure 2).

### TUMOR TYPE



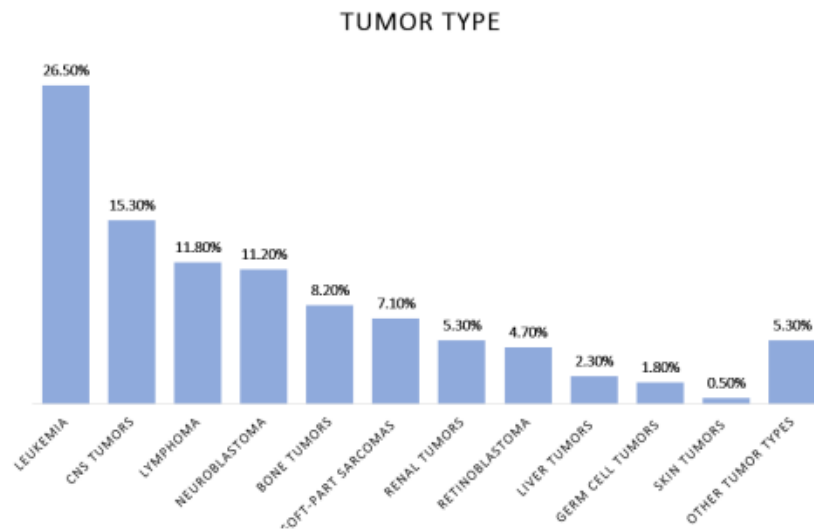**Figure 2.** Distribution of patients (%) by tumor type.

These percentages were compared to those collected in the Spanish Registry of Pediatric Tumors (RETI) [38]. Statistically significant differences were not found when comparing the incidence rates for these tumor types between the RETI series (age group 0–19 years; 1980–2017) and our cohort. Following the workflow established, a total of
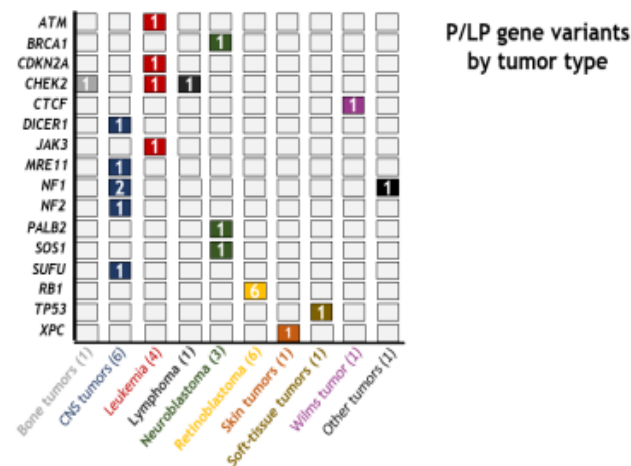
153 patients was studied with *OncoNano V2*, and the remaining 17 cases exclusively by conventional techniques or other NGS panels (File S2).

A pathogenic variant predisposing to the patient's tumor was detected in 16 cases (16/170; 9.4%). Regarding the genes involved in predisposition, the most frequently altered was the *RB1* gene (6/16; 37.5%), followed by *NF1* (3/16; 18.8%); other mutated genes were *DICER1, NF2, SUFU, TP53, XPC* and *SOS1*. Moreover, a patient diagnosed with trisomy 21 was included in the cohort. In addition, likely pathogenic mutations that could be involved in predisposition to the patient's tumor were identified in ten other cases (10/170; 5.9%). These 26 pathogenic and likely pathogenic variants detected are summarized in Table 1 and Figure 3.

**Table 1.** Pathogenic or likely pathogenic variants considered to be involved (pathogenic) or maybe involved (likely pathogenic) in patient's disease.

| Patient Number | Diagnosis | Gene Variant/Genomic Alteration | Categorization |
|---|---|---|---|
| 10 | Retinoblastoma (unilateral) | 13q12.13-q21.2 deletion | Pathogenic |
| 11 | Retinoblastoma (unilateral) | *RB1* c.844G>T (p.E282*) | Pathogenic |
| 12 | Pilocytic astrocytoma | *NF1* C.910C>T (p.R304*) | Pathogenic |
| 15 | Ewing sarcoma | *CHEK2* c.254C>G (p.P85R) | Likely pathogenic |
| 31 | Neuroblastoma | *SOS1* c.1300G>A (p.G434R) | Pathogenic |
| 36 | Pilocytic astrocytoma | *MRE11* c.659+1G>A | Likely pathogenic |
| 39 | Neuroblastoma | *PALB2* c.2747A>T (p.E916V) | Likely pathogenic |
| 44 | B-ALL | Trisomy 21 | Pathogenic |
| 51 | Retinoblastoma (bilateral) | *RB1* c.2104 C>T (p.Q702*) | Pathogenic |
| 59 | Neuroblastoma | *BRCA1* c.68_69del (p.E23Vfs*17) | Likely pathogenic |
| 64 | Retinoblastoma (unilateral) | 13q12q21 deletion | Pathogenic |
| 65 | Plexiform neurofibroma | *NF1* c.4084C>T (p.R1362*) | Pathogenic |
| 66 | Retinoblastoma (bilateral) | *RB1* c.224G>A (p.W75*) | Pathogenic |
| 89 | B-ALL | *ATM* c.1402_1403del (p.K468Efs*18) | Likely pathogenic |
| 103 | Cutaneous angiosarcoma | *XPC* c.1643_1644delTG (p.V548Afs*25) (homozygous) | Pathogenic |
| 105 | Wilms tumor | *CTCF* c.353T>A (p.I118K) | Likely pathogenic |
| 108 | Embryonal rhabdomyosarcoma | *TP53* c.559G>A (p.G187S) | Pathogenic |
| 110 | B-ALL | *CDKN2A* deletion | Likely pathogenic |
| 113 | Medulloblastoma SHH | *SUFU* c.71dup (p.A25Gfs*23) | Pathogenic |
| 116 | B-ALL | *JAK3* c.1465C>T (p.Q489*) and *JAK3* c.1442-2A>G | Likely pathogenic |
| 118 | B-ALL | *CHEK2* c.497A>G (p.N166S) | Likely pathogenic |
| 120 | Burkitt lymphoma | *CHEK2* c.470T>C (p.I157T) | Likely pathogenic |
| 127 | Schwannoma CNS (NF1 phenotype) ** | *NF1* c.2251+1 G>A | Pathogenic |
| 150 | Vestibular schwannoma (bilateral) | *NF2* c.115-2A>G | Pathogenic |
| 156 | Pineoblastoma | *DICER1* c.2026C>T (p.R676*) | Pathogenic |
| 169 | Retinoblastoma (bilateral) | *RB1* c.2548_2552delCAGA-T (Q850Gfs*3) | Pathogenic |

** Schwannoma is not an NF1 feature, but the patient fulfilled an NF1 diagnosis according to the NIH criteria with >6 café-au-lait spots (CAL), axillary freckling and a proven neurofibroma.

**Figure 3.** Genes and tumor types whereby pathogenic or likely pathogenic variants were identified.

Other pathogenic/likely pathogenic variants were not considered to be involved in predisposition to patient tumors because they were related to recessive diseases, but without any evidence to associate them with the patient's cancer. However, their involvement cannot be ruled out in certain cases: *ERCC3* (patient 159; Ewing sarcoma), *XPC* (patient 151; ependymoma), *FANCM* (patient 149; neuroblastoma), *PIK3CG* (patient 111; lymphoma), *RECQL4* (patient 89; leukemia), *NBN* (patient 78; atypical teratoid rhabdoid tumor), *FANCL* (patient 42; rhabdomyosarcoma) and *CEP57* (patient 18; astrocytoma) (details on the variants can be found in Table 2 and File S3). Besides that, some VUSs might be attributed to a potential pathogenicity. Hence, they might be involved in predisposition to the tumor suffered by the patients, but the lack of evidence leads to classifying them as VUSs according to the ACMG criteria. These variants were classified as VOPPS. Variants of these characteristics were detected in the genes *ING4* (patient 153; carcinoid tumor), *NF1* (patient 144; neuroblastoma), *FANCD2* (patient 143; lymphoma), *IGF1R* (patient 139; Wilms tumor), *ALK* (patient 119; leukemia), *FAT1* (patient 81; HGG), *CHEK2* (patient 78; teratoid/rhabdoid tumor), *RET* (patient 72; leukemia) and *SH2B3* (patient 48; leukemia) (more in Table 2; File S3). Variants of uncertain significance or likely benign not previously reported in databases or with a higher incidence than expected in cancer patients were also collected in File S3.

Overall, and considering all P/LP variants identified, related or not to genetic predisposition to patient's tumor, 35 out of 170 patients/families (20.6%) carried at least one of these variants. Families received this information and adequate genetic counseling.

### 3.2. Jongmans MC et al., 2016, Tool Evaluation

A total of 50 patients (29%) met the indication for referral to a clinical geneticist according to the Jongmans MC et al. criteria during the targeted assessment carried out after inclusion. Among them, pathogenic predisposing mutations were detected in 15 cases (15/50; 30%). It can be seen from this that 94% of the total of pathogenic variants predisposing to pediatric cancer detected in the study (15/16) was found in patients who met the Jongmans MC et al. criteria. In addition, five out of ten variants (50%), classified as likely pathogenic, were detected among patients who met the Jongmans MC et al. criteria. Therefore, pathogenic and likely pathogenic mutations were identified in 40% of the patients chosen by the tool (20/50). Considering predisposition variants as only the 16 pathogenic mutations, the Jongmans MC et al. tool was found to have a sensitivity of 94% and a specificity of 77% in our cohort. Taking into consideration both pathogenic and likely pathogenic variants probably involved in predisposition, the sensitivity would be

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77% and specificity 79%. For the 120 patients who did not meet the indication for referral to a clinical geneticist based on the Jongmans MC et al. criteria, pathogenic predisposition mutations were detected only in one case (0.8%). Out of the 120 patients, five carried likely pathogenic variants according to the ACMG criteria (4.2%).

**Table 2.** Pathogenic or likely pathogenic variants considered as not involved in the tumor etiology by the Pediatric Cancer Predisposition Committee. Variants of uncertain significance to which potential pathogenicity was attributed by the committee (variants of potential pathogenic significance—VOPPS).

| P/LP Variants Not Predisposing to Patient's Tumor | | |
|---|---|---|
| **Patient Number** | **Diagnosis** | **Gene Variant** |
| 18 | Pilocytic astrocytoma | *CEP57* c.241C>T (p.R81*) |
| 42 | Alveolar rhabdomyosarcoma | *FANCL* c.40del (p. L14Cfs*27) |
| 42 | Alveolar rhabdomyosarcoma | *XPC* c.1643_1644del (p. V548Afs*25) |
| 78 | Atypical teratoid rhabdoid tumor | *NBN* c.1648_1651del (p. K550Gfs*8) |
| 89 | B-ALL | *RECQL4* c.2336_2357del (p.D779Cfs*57) |
| 111 | Lymphoblastic lymphoma | *PIK3CG* c.2340dup (p. E781Rfs*4) |
| 149 | Neuroblastoma | *FANCM* c.2161-1G>A |
| 151 | Ependymoma | *XPC* c.1643_1644del (p. V548Afs*25) |
| 159 | Ewing sarcoma | *ERCC3* c.583C>T (p. R195T*) |
| **VOPPS Variants** | | |
| **Patient Number** | **Diagnosis** | **Gene Variant** |
| 48 | B-ALL | *SH2B3* c.622G>C (p.E208Q) |
| 72 | B-ALL | *RET* c.2331C>A (p.N777K) |
| 78 | Atypical teratoid rhabdoid tumor | *CHEK2* c.342G>T (p.W114C) |
| 81 | High grade glioma | *FAT1* c.10990del (p.Q3664Sfs*10) |
| 119 | B-ALL | *ALK* c.3467G>A (p.C1156Y) |
| 139 | Wilms tumor | *IGF1R* c.3367A>G (p.M1123V) |
| 143 | Lymphoblastic lymphoma | *FANCD2* c.2204G>A (p.R735Q) |
| 144 | Neuroblastoma | *NF1* c.2998C>A (p.R1000S) |
| 153 | Carcinoid tumor | *ING4* c.109+1G>C |

The phenotype–genotype correlations of those patients carrying likely pathogenic variants are described below:

### 3.3. CTCF Variant c.1337-T>A and Wilms Predisposition

Patient number 105 corresponds to a 2-year-old child diagnosed with bilateral Wilms tumor. The phenotype was intellectual development at the limit of normality, bilateral cryptorchidism, patent foramen ovale, minor facial dysmorphism, such as a prominent forehead, leafy and arched eyebrows, a long filtrum and thin upper lip. Therefore, the evaluation using the Jongmans MC et al. tool was positive; however, it did not suggest any diagnosis. The NGS study identified the likely pathogenic *CTCF* variant c.1337-T>A (p.I446K) (NM_006565.4), with an allelic frequency of 50%. This variant was confirmed in homozygosity both in tumor DNA and RNA. The family segregation study confirmed that the variant occurred de novo in the patient. The detection of this variant in the clinical context of the patient, having adequately ruled out other entities predisposing to Wilms tumor, led us to the diagnosis of mental retardation, autosomal dominant 21 [39]. After a multidisciplinary assessment, we considered that this variant might predispose to Wilms

tumor in the context of MRD21; this tumor has not been reported in other MRD21 patients to date.

### 3.4. BRCA1 c.68_69del Variant and Neuroblastoma Susceptibility

Patient number 59 was diagnosed with poorly differentiated mediastinal neuroblastoma at the age of 6 months (*NMYC* not amplified; without segmental chromosomal alterations in SNP Array). Parents were consanguineous, but data suggestive of a familial predisposition syndrome were not detected. The evaluation using the Jongmans MC tool was positive (consanguinity). An NGS study was carried out since there was no suspicion of a specific entity. The *BRCA1* variant c.68_69del e.E23Vfs*17 (NM_007294.3) was detected in heterozygosity. The relationship between *BRCA1* mutations and predisposition to neuroblastoma is based on casual findings in specific cases, such as our patient (10). The *BRCA1*–Neuroblastoma risk ratio is still under study; therefore, the implications of this variant in tumor development are currently undetermined. The parents refused the family segregation study and no additional family information was provided.

### 3.5. CHEK2 c.497A>G Variant and B-Cell ALL Risk

Patient number 118 suffered from B-cell acute lymphoblastic leukemia when he was one year old, without other remarkable personal clinical data. Her mother had breast cancer at age 41 and a non-informative *BRCA1* and *BRCA2* study result. Colon cancer in her grandfather on the mother's side at age 73 stood out in the family history, as well as Hodgkin's lymphoma at age 45 in one of the mother's three siblings. There are no cases of cancer reported on the father's side. The NGS study identified heterozygous *CHEK2* c.497A>G (p.N166S) NM_007194.3. A segregation study confirmed the maternal origin of the variant and the remaining members of the family are under study. Based on the evidence available for *CHEK2* mutations in breast cancer, this variant might be involved in the mother's breast cancer [40]. However, evidence supporting the relationship between *CHEK2* variants and the risk of leukemia is still limited [41].
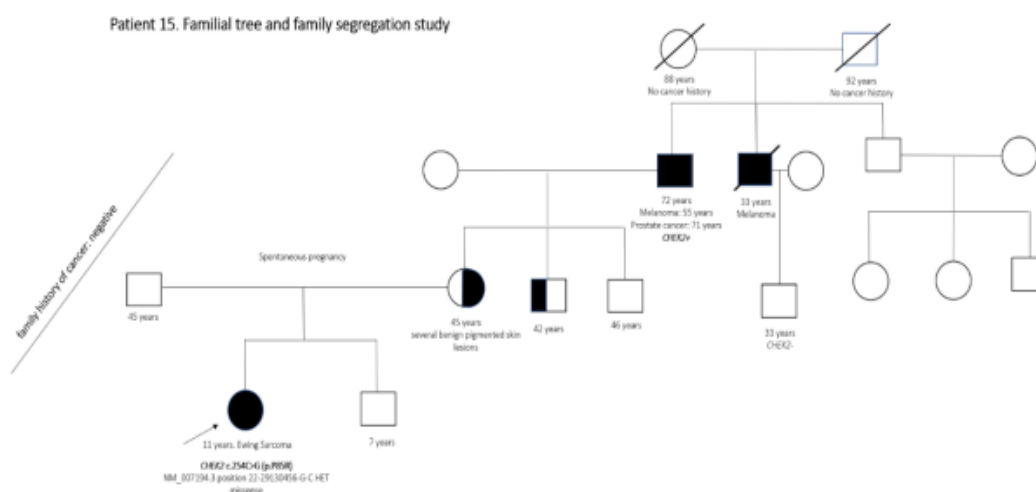
### 3.6. CDKN2A Deletion and Leukemia

Patient number 110 was diagnosed with common B-cell ALL at the age of 3 years. Her mother was diagnosed with metastatic melanoma and died of the disease at a young age. Following the established workflow, the *OncoNano V2* panel was sequenced. A mono-allelic *CDKN2A* deletion was detected and it was confirmed by MLPA. There was a high probability that the *CDKN2A* deletion was inherited from the mother, but it could not be confirmed. While the relationship between melanoma and *CDKN2A* is well known, information on the involvement of the *CDKN2A* gene in leukemia predisposition is scarce. However, a possible association of some *CDKN2A* polymorphisms (rs3731249 and rs3731217) with ALL risk in pediatric age has been proposed [42–44]. In this context, we concluded that the detected deletion might have facilitated the tumor development in the patient, although currently available evidence is insufficient.

### 3.7. JAK3 Mutations and Familial Leukemia

Patient number 116 was a 5-year-old girl with a diagnosis of common B-ALL (acute lymphoblastic leukemia). Her mother also had ALL at the age of 5 years. The mother survived and was now 36 years old. The remaining information available on the maternal side was not contributory. Given that the patient met the Jongmans MC et al. criteria, the patient's sample was sequenced. Two CIS heterozygous and likely pathogenic variants were identified in the *JAK3* gene. The detected variants were *JAK3* c.1465C>T (p.Q489*) and *JAK3* c.1442-2A>G. A family segregation study of both variants was completed. The mother carried both variants, while the father did not. The *JAK3* c.1442-2A>G variant, located closer to the N-terminal end than the other variant, was thought to be a null variant leading to the loss of protein function. Loss-of-function mutations in homozygosity or compound heterozygosity are associated with severe combined immunodeficiency, whose inheritance

is autosomal recessive. However, heterozygous loss-of-function mutations have not been associated with leukemia predisposition to date. Given the peculiarity of the family history, we considered the variant(s) to be likely pathogenic. Whether the variant(s) was involved in predisposition to leukemia suffered by the mother and daughter is completely unknown.

Other data of interest related to these and other specific cases carrying LP variants are shown in File S4 and Figure 4. Some of the complementary studies carried out in specific patients or families in response to the detection of some variants are contained in File S4.



Patient 15. Familial tree and family segregation study

**Figure 4.** Family tree of patient number 15. Despite not fulfilling the Jongmans MC criteria nor the revised criteria by Ripperger, the patient's family history of cancer was still suggestive for genetic cancer predisposition and the genetic counseling was advised based on this information. The adolescent was diagnosed with extraosseous Ewing sarcoma. A *CHEK2* variant was detected by the NGS *OncoNano V2* panel. The variant was described in the general population (gnomAD reports three total heterozygotes). However, it was described six times in ClinVar and three times in cancer patients (uncertain clinical significance (ID 233261)). It was not found in other databases. In addition, it is a variant studied functionally on one occasion. It was reported in the literature in a patient with hereditary breast cancer, with functional in vitro study that demonstrated a 50% reduction in kinase activity [45], although the location of the variant was outside of a functional domain. The variant was found to be of maternal origin and family history of melanoma was identified in the grandfather and great-uncle in this branch of the family. In addition, the grandfather had had a second tumor at an older age. Based on the ACGM criteria and family information, the variant was classified as likely pathogenic.

## 4. Discussion

This study presented the results of an *OncoNano V2* NGS panel sequencing of germline samples from a large cohort of pediatric oncology patients. On the basis of our results, it can be concluded that up to 9.4% patients had a genetic predisposition syndrome which explained the cancer they suffered. Meanwhile, considering that an additional 5.9% of the patients carried likely pathogenic variants, a few of which might be involved in susceptibility to the disease, this figure might be higher. The results obtained were consistent with previously published data [4–6]. Recent results from the MSK-IMPACT cohort also pointed the same way [46]. New predisposition genes have been described in the last two years [12] and these genes were not included in the panel; therefore, the figure presented might be considered conservative.

Due to the high number of patients to be assessed from a germline point of view, selection tools to enable a concise assessment may improve the decision making in this field. We evaluated the usefulness of the Jongmans MC et al. tool for this purpose in our cohort. The tool showed a high sensitivity for the detection of patients with currently well-categorized predisposition syndromes in our cohort (94%). A Li–Fraumeni patient

was the only case of a well-established and undetected genetic syndrome by the tool. He was a 5-year-old patient diagnosed with anaplastic embryonal rhabdomyosarcoma. No other data of interest were found in the medical records. The presence of a family history of cancer was ruled out. A variant considered pathogenic was detected in the *TP53* gene and the diagnosis of Li–Fraumeni syndrome was determined. Family studies could not be expanded because of the early death of the patient and loss of contact with parents. The association between soft-tissue sarcoma and Li–Fraumeni syndrome is high [47]. The benefit of studying *TP53* at least in patients with anaplastic rhabdomyosarcoma is reinforced by current evidence [48]. Ripperger T et al. modified the Jongmans MC criteria [18] and their updated tool would have detected this case; this tool had achieved a sensitivity of 100% in the detection of pathogenic variants within the analyzed cohort in this study. The Ripperger T et al. revision also added rare entities specific to cancer predisposing syndromes in order to improve the sensitivity of the selection tool (e.g., Botryoid rhabdomyosarcoma of the urogenital tract). The inclusion of patients with acute myeloid leukemia, based on the 2016 WHO recommendations, was also considered [49]. Currently, this would be the most appropriate tool for patient selection in order to recommend a genetic study.

Our results highlighted the challenge of interpreting genetic variants in the context of predisposition to pediatric cancer. The cases carrying LP variants and above presented were only an example of frequent difficulties found throughout the series. Variants in genes *CHEK2, MRE11, PALB2 and ATM* reported for patients 15, 36, 39, 89 and 120 presented similar challenges. These clinical cases required constant re-evaluation based on the evidence available at any given time. The same was true for rare VOUS in the general population, especially those to which we attributed potential pathogenicity and designated VOPPS (variants located in *ING4, IGF1R, NF1, FANCD2, ALK, CHEK2, FAT1* and *SH2B3* genes). This subsequent work should be considered from the beginning in order to properly quantify the resources that will be required in the long term. Despite the limitations found for variant interpretation, this work has allowed to detect genetic variants that might be related to new genotype–phenotype associations for different pathologies. These data could be investigated in larger patient cohorts by international collaborative groups.

This kind of clinical approximation, with so many personal and family implications, demands a comprehensive assessment of the advantages and disadvantages detected. The NGS technology used allowed us to reliably test for SNVs and CNVs in 390 gene regions in a single test. Despite the lower cost and accessibility of this technology at present, a cost/effectiveness assessment should be carried out. Based on the results obtained, the detection of the main pediatric cancer predisposition syndromes could be possible through a considerably smaller and less expensive gene panel than *OncoNano V2*. A pretest approach based on a tool such as that of Jongmans MC et al. or Ripperger T et al. could achieve an adequate selection of most of the patients that should be studied. In fact, one of the main conclusions raised from this work was that, outside of Ripperger T et al. criteria and, therefore, out of the syndromes included in their review, no genetic alteration with evidence of being responsible for the disease suffered by the patient was identified. Accordingly, sequencing broad panels such as ours or WES would make sense only and exclusively in the field of research or when facing extremely particular clinical cases. Therefore, for daily clinical practice and in order to detect cancer predisposition syndromes, the analysis of genes not related to the entities collected by Ripperger T et al. gave rise to more doubts than certainties. In consequence, their testing would not be recommended outside the research field.

On the other hand, pre- and post-psychological test assessments play a key role in proper long-term management. This has been proved as something basic in different areas of genetics [50] and is especially important in this field, with so many consequences in the personal and family sphere. Moreover, the turnaround time required for suitable clinical implementation is a huge challenge in this context. Workflow based on a pre-test clinical and psychological evaluation, patient/family information in a context of high emotional stress, germline sample collection, sequencing and analysis, committee discussion and

reporting require a constant evaluation of deadlines. Undoubtedly, if the turnaround time is too long, some of the potential clinical benefit may be lost. However, the optimal time mainly depends on the specific case of each tumor type, or even of each patient. This work also highlighted the importance of an expert committee, and not only in the field of research, since the challenges derived from studying relatively well-known genes and syndromes remain remarkable. This multidisciplinary approach has already been shown to be very useful in other fields of personalized medicine [51], but it slowly emerges as a key component in this research area. Nonetheless, expert knowledge of each of the analyzed genes is an arduous task for any human group. Therefore, consultation with other national and international experts is presented as a useful tool in this field. The implementation of networks focusing on pediatric cancer predisposition syndromes will be essential in the following years.

## 5. Conclusions

In summary, it should be noted that, in nearly 20% of the patients, genetic data were identified that could have personal and family implications. In a few of them (9.4%), a genetic syndrome was diagnosed; thereby, the information was clinically useful for the patient. However, uncertainty was transferred to families in several cases when analyzing genes previously unrelated to pediatric cancer predisposition syndromes. In fact, outside of the genes and syndromes included in the Ripperger T et al. criteria, not a single cancer predisposition syndrome was identified in this study. For this purpose, it seems preferable in clinical practice to sequence a highly selected gene panel rather than a large one or the whole exome after evaluating and choosing the patients with a selection tool such as that of Jongmans MC et al. or Ripperger T et al.

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Artículo 2.

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**CASE REPORT**

**Open Access**

# Retinoblastoma and mosaic 13q deletion: a case report

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## Abstract

**Background:** Patients with 13q-syndrome are at risk of retinoblastoma when the *RB1* gene, located in the chromosomal band 13q14.2, is deleted. This syndrome is frequently associated with congenital malformations and developmental delay, although these signs could be mild. Mosaic 13q-deletion patients have been previously reported in the literature; their phenotype is variable, and they may not be recognized.

**Case presentation:** Retinoblastoma diagnosed in a child with 13q-mosaicism confirmed in blood, oral mucosa, healthy retina and retinoblastoma. A second *RB1* hit is present exclusively in the retinoblastoma sample (*RB1* c.958C>T p.Arg320Ter). Other detected molecular events in retinoblastoma are 6p12.3pter gain and 6q25.3qter loss. Clinical examination is unremarkable except for clinodactyly of the right fifth finger.

**Discussion and conclusions:** We describe a case of mosaic 13q deletion syndrome affected by retinoblastoma. Molecular data obtained from the tumor analysis are similar to previous data available about this malignancy. High clinical suspicion is essential for an adequate diagnosis of mosaic cases.

**Keywords:** Retinoblastoma, 13q-syndrome, Mosaicism, Cytogenetics, Molecular genetics

## Background

Retinoblastoma is a rare tumor that occurs in young children's retina. About 40% of patients diagnosed with retinoblastoma have a predisposing genetic condition [1]. Most of them carry heterozygous truncating *RB1* mutations in the germline. Some patients present isolated deletions of one of the two *RB1* alleles, and at-risk patients are exceptionally 13q-syndrome cases [2]. Because of the fact that 98% of retinoblastoma cases begin after a double *RB1* hit, according to Knudson's hypothesis [3], all these children are at a major risk of being affected.

13q deletion syndrome was first described by Allderdice et al. after studying two pediatric patients in 1969 [4]. The first patient affected by the syndrome including

retinoblastoma was reported in 1983 [5]. Several cases have been communicated during the past 50 years and the syndromic phenotype has been characterized. Intellectual disability, facial anomalies, several malformations and retinoblastoma risk stand out as the most prominent signs amongst other previously described abnormalities. However, the tumor would not be able to progress easily in 13q-syndrome even if a second *RB1* hit were present. It has been hypothesized that some genes deleted together with *RB1* would be necessary for retinoblastoma development. Available data suggest that 13q deletions larger than 1 Mb—and particularly those including *MED4* and *SUCLA2*—are associated with unilateral forms or without retinoblastoma development [6].

Improvements in cytogenetic analysis has enabled better molecular characterization of 13q-syndrome cases and more accurate genotype–phenotype correlations. Depending on the deleted chromosomal bands, three clinical groups may be established [7]:

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- Group 1: 13q12.2–13q32. Mild intellectual disability, growth delay, limb malformations, and retinoblastoma risk (when the *RB1* gene is deleted [chromosomal position 13q14.2].
- Group 2: 13q32. Severe brain malformations and developmental delay.

- Group 3: 13q33–13q34. Minor congenital malformations but severe intellectual impairment.

Some patients with 13q-syndrome are affected by a mosaic disease and a few cases have been described [8–11]. Bestetti et al. reported a patient with mosaic 13q



**Fig. 1** *RB1* deletion in the context of mosaic 13q deletion. **A** Multiplex Ligation-dependent Probe Amplification (MLPA) assay looking for *RB1* gene deletions or duplications (SALSA P047-C1) in germline DNA from peripheral blood lymphocytes. The detected values were low but not consistent with a heterozygous deletion. The suspicion was a complete *RB1* deletion in mosaicism. **B** Genomic SNP array (AffymetrixCytoScan 750 array) reports a 13q deletion in mosaicism. It is a deletion of 35.7 Mb from 13q12.13 to 13q21.2 (arr[hg19] 13q12.13q21.2(26,555,387–62,280,955) × 1–2) observed in about 40% of all determinations

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**Fig. 2** Cytogenetic karyotype from cultivated lymphocytes previously stimulated with phytohemagglutinin. Karyotype 46,XX,del(13)(q12q21) [6]/46,XX[44]. **A** A majority cell line (44 cells): 46 chromosomes whose identification with G bands (resolution level of 400–500 bands) does not show numerical or structural alterations (46, XX). **B** A minor cell line (6 cells): 46 chromosomes but shows the presence of an interstitial deletion in the long arm of chromosome 13

deletion syndrome including *RB1* but no retinoblastoma [8].
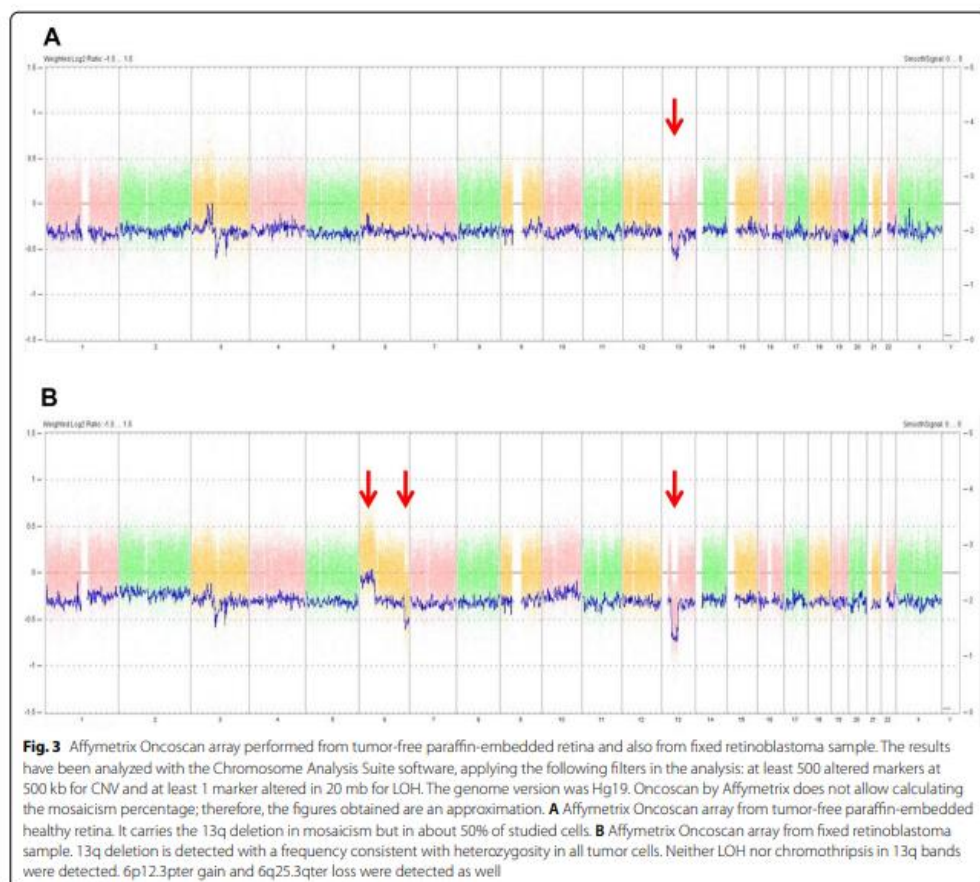
## Case presentation

A 6-month-old girl conceived by in vitro fertilization (IVF) (own oocytes and anonymous donor sperm) was admitted to the hospital because of leukocoria and strabismus. Past medical history and physical examination were unremarkable except for clinodactyly of the right fifth finger. Indirect ophthalmoscopic examination and examination under anesthesia was performed by ophthalmologists. Orbital ultrasound and magnetic resonance imaging (MRI) scans showed a $14 \times 13 \times 11$ mm left intraocular mass located in the lower-external retinal side. Retinal detachment was also detected. Other tumoral lesions were ruled out by an ophthalmologist and MRI in both retina and brain. Diagnosis of Retinoblastoma was made and, based on *International Classification for Intraocular Retinoblastoma*, a grade E was established. The patient received intra-arterial melphalan but due to a local vasospasm in her left leg, the treatment was discontinued. Afterwards, four courses of conventional chemotherapy were administered (vincristine, carboplatin and etoposide). A partial response was achieved, but, despite chemotherapy, the disease progressed few weeks later and the affected eye was enucleated.

On the basis of global recommendations, the *RB1* gene was studied in germline DNA from peripheral blood lymphocytes. Exon–intron boundaries of *RB1* were amplified by conventional PCR and then sequenced by the Sanger method; no mutations were detected. A Multiplex Ligation-dependent Probe Amplification (MLPA) assay was used to test for *RB1*-gene deletions and duplications (SALSA P047-C1). The detected values were relatively low but within the normal range (Fig. 1A) and a complete *RB1* deletion in mosaicism was suspected. A genomic SNP array (AffymetrixCytoScan 750 array) was performed and a 13q deletion of 35.7 Mb from 13q12.13 to 13q21.2 (arr[hg19] 13q12 .13q21.2(26,555,387–62,280,955) × 1–2) detected in around 40% of cells (Fig. 1B) was confirmed. This result was further confirmed by cytogenetic karyotype analysis of cultivated lymphocytes previously stimulated with phytohemagglutinin. Fifty metaphases were analyzed and two cell clones were detected. A majority cell line (44 cells) presented 46 chromosomes whose identification with G bands (resolution level of 400–500 bands) did not show numerical or structural alterations (46, XX). A minor cell line (6 cells) with 46 chromosomes showed the presence of an interstitial deletion in the long arm of chromosome 13 (Fig. 2). A *RB1*-specific FISH probe (LSI13) performed from swab oral mucosa cells evidenced 13q deletion in around 40% of the cells.

We performed an Affymetrix Oncoscan array for both her tumor-free paraffin-embedded retina and fixed retinoblastoma sample. The healthy retina carried the 13q

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**Fig. 3** Affymetrix Oncoscan array performed from tumor-free paraffin-embedded retina and also from fixed retinoblastoma sample. The results have been analyzed with the Chromosome Analysis Suite software, applying the following filters in the analysis: at least 500 altered markers at 500 kb for CNV and at least 1 marker altered in 20 mb for LOH. The genome version was Hg19. Oncoscan by Affymetrix does not allow calculating the mosaicism percentage; therefore, the figures obtained are an approximation. **A** Affymetrix Oncoscan array from tumor-free paraffin-embedded healthy retina. It carries the 13q deletion in mosaicism but in about 50% of studied cells. **B** Affymetrix Oncoscan array from fixed retinoblastoma sample. 13q deletion is detected with a frequency consistent with heterozygosity in all tumor cells. Neither LOH nor chromothripsis in 13q bands were detected. 6p12.3pter gain and 6q25.3qter loss were detected as well
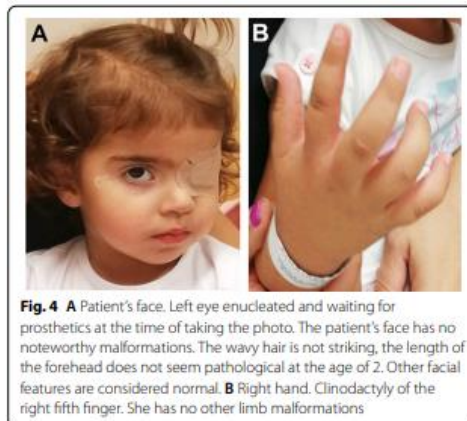
deletion in mosaicism but in about 50% of the studied cells. However, all retinoblastoma sample cells carried the deletion in heterozygosity (Fig. 3). Neither LOH (Loss of Heterozygosity) nor chromothripsis were detected in 13q bands. Furthermore, 6p12.3pter gain (3 total copies) and 6q25.3qter loss (1 total copy) were reported exclusively in the tumor sample.

Looking for second hit mutations in *RB1,* we applied a custom designed NGS panel (*Onconano V2*) that included the *RB1, BCOR* and *CREBPP* genes (among other 400 commonly mutated genes in pediatric cancer). The study detected only one pathogenic single-nucleotide variant, *RB1* c.958C>T (p.Arg320Ter) (NM_000321.2 chromosomal position 13–48,941,648-C-T; allele

frequency of 25%). Copy number variations in 6p, 6q and 13q were again observed.

After molecular diagnosis and completing the treatment, the patient was placed on surveillance. The right eye has been free of disease and the child is 42 months old now. She does not present growth retardation at the moment (weight and height in the 50th percentile; cranial perimeter in the 90th). Neither cardiac, eye nor other malformations have been detected and neurological development has been normal (Fig. 4).

Informed consent for genetic studies and for taking and sharing pictures was obtained from both parents.

**Fig. 4 A** Patient's face. Left eye enucleated and waiting for prosthetics at the time of taking the photo. The patient's face has no noteworthy malformations. The wavy hair is not striking, the length of the forehead does not seem pathological at the age of 2. Other facial features are considered normal. **B** Right hand. Clinodactyly of the right fifth finger. She has no other limb malformations

## Discussion and conclusions

We described the case of a child with 13q-mosaicism affected by retinoblastoma. The unilateral presentation agrees with previous data available for 13q deletions larger than 1 Mb including *MED4* and *SUCLA2* [6]. As in this case, retinoblastoma with both genes deleted is associated with less tumor aggressiveness compared with tumors whose genes are conserved [6].

Retinoblastoma seems to be caused by a double hit in *RB1* approaching 98% cases (by mutation, deletion, promoter methylation or intra-genic chromothripsis) [12, 13]. Few retinoblastoma cases would start because of *MYCN* amplification [13]. 13q deletion syndrome patients would not be an exception. In fact, we confirmed a second *RB1* hit (*RB1* p.Arg320Ter) in the tumor.

However, double *RB1* hit only gives rise to retinoma; therefore, subsequent epigenetic or genetic changes would give an advantage for tumor progression. The sequence of events capable of causing a malignant phenotype is only partially known. Epigenetic deregulation secondary to homozygous *RB1* loss drives an increase in KIF14 and E2F3 levels [14] and could lead to the expression of the *SYK* oncogene as well. Moreover, cellular control mediated by p53 is inactivated as a result of high expression of *MDM2* and *MDM4* in retinoblastoma [14].

In addition, cytogenetic analysis has shown recurrent CNVs (copy number variation) among retinoblastoma tumors, which are mainly chromosomal gains at 1q, 2p, 6p, 13q and 19q and losses at 13q, 16q and 17p [15]. These recurrent aberrations allow to establish as a possible hypothesis that genes located at these loci could be related to retinoblastoma progression [15], yet no conclusive data are available about this at the moment. We looked for

CNVs in the tumor and discovered a chromosomal gain in 6p12.3pter, which is one of the most frequently reported CNVs in retinoblastoma [15]. However, we also detected a less common deletion of 6q25.3qter. The deletion of this region has already been described among non-13q-deletion syndrome patients, although rarely [16]. Sixty OMIM genes are located in this region, and several of them are associated with different cancers, but none with retinoblastoma. A terminal 6q deletion may be present in ovarian cancer and neuroblastoma [17] and seems to be related to bad prognosis in neuroblastoma [17]. The fact that this deletion could play a role in retinoblastoma development in the context of 13q-syndrome is unknown.

Furthermore, NGS approaches have detected a low rate of mutations in retinoblastoma. Several studies support retinoblastoma as one of the less mutated human tumors. Only *BCOR* (mutated in 13% of tumors) and *CREBPP* mutations occur frequently in retinoblastoma [18]. Therefore, retinoblastoma presents a stable genome with few genetic events described and epigenetic deregulation appears to have a notable role [19]. Studies based on RNA-sequencing could continue to shed light on the genes and signaling pathways involved in retinoblastoma development [20]. In regards to common mutated genes in retinoblastoma, we determined *BCOR* and *CREBPP* status without detecting pathogenic variants. We did not find other variants considered pathogenic or likely pathogenic in 400 genes commonly mutated in pediatric cancer beyond *RB1*.

The patient carries the deletion 13q12.13–13q21.2 and, therefore, fits in Group 1 of the clinical classification for 13q-syndrome [7]. Patients with band 13q14 deleted typically present with mild facial anomalies such as high forehead, short nose, small upper lip, curly hair and down-turned corners of the mouth [6]. Our patient does not show these facial features. Furthermore, deletion of *NUFIP1*, located in 13q14.12, and *PCDH8*, in 13q21.1, may be crucial for developmental delay [6]. Both of them are deleted in our patient, but the degree of mosaicism in her central nervous system is unknown. In fact, she is neurologically normal. Moreover, other common abnormalities in Group 1 are micrognathia and microcephaly but these are related to loss in the 13q21.33q31.1 and 13q21.32q21.33 regions, respectively [6]. Our patient's deletion finishes at 13q21.2; therefore, she does not present either micrognathia or microcephaly, because those regions are not affected. About 75% of patients with large deletions present short height, but this is not the case of our patient (50th percentile). Genes involved in short height have not been clearly defined.

The *BRCA2* gene, located in 13q13.1, may be lost in some 13q-patients. Heterozygous mutations in this

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gene predispose to breast and ovarian cancer syndrome in adulthood [21] and a complete deletion of this gene might predispose to these tumors as well. However, the occurrence of these two tumors has not been reported in 13q-syndrome to date. Our patient loses *BRCA2*; therefore, she may benefit from risk-adapted surveillance strategies for breast/ovarian cancer.

After confirming retinoblastoma diagnosis in a child, genetic study of *RB1* in the germline is mandatory. Any phenotypic manifestation, including minor peculiarities (clinodactyly of the fifth finger in our case) should raise suspicion of 13q-syndrome, and it should be studied, given the fact that mosaic forms exist.

### Abbreviations
MRI: Magnetic resonance imaging; LOH: Loss of heterozygosity; CNV: Copy number variation; NGS: Next generation sequencing.

### Availability of data and materials
Data generated or analyzed during this study are included in this published article.

## Declarations

### Ethics approval and consent to participate:
The study was accepted by CEIm la Fe (Ethics Commitee la Fe) on March 13, 2018. Project reference number: 2017/0546. Project title: genetic predisposition to childhood cancer. From NGS to clinical consultation.

### Consent for publication
Parents consented to participate in the study and they allowed us to publish pictures of their child. Documents are available for consultation.

### Competing interests
The authors declare that they have no competing interests.

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## Artículo 3.

Gargallo P, Oltra S, Tasso M, Balaguer J, Yáñez Y, Dolz S, Calabria I, Martínez F, Segura V, Juan-Ribelles A, Llavador M, Castel V, Cañete A, Font de Mora J. Germline variant in Ctcf links mental retardation to Wilms tumor predisposition. Eur J Hum Genet. 2022 Nov;30(11):1288-1291. doi: 10.1038/s41431-022-01105-x. Epub 2022 Apr 22. PMID: 35459888; PMCID: PMC9626537.

Artículo 4.

## Li-Fraumeni: ¿la detección de familias aumentaría la supervivencia entre sus miembros?

## Li-Fraumeni: Will the detection in families increase the survival of its members?

*Sr. Editor:*

El síndrome de Li-Fraumeni es una rara entidad de predisposición genética al cáncer, de herencia autosómica dominante y expresividad variable, relacionada con alteraciones germinales en el gen TP53. Dicha entidad predispone al desarrollo de una amplia variedad de tumores malignos. Los tumores más característicos son los sarcomas de tejido blando (rabdomiosarcoma y otros), osteosarcoma, cáncer de mama en mujeres premenopáusicas, leucemia hipodiploide, tumores cerebrales (carcinoma de plexos coroideos, glioblastoma y meduloblastoma) y carcinoma adrenocortical. Dichos tumores pueden presentarse a cualquier edad, incluida la pediátrica. La prevalencia no es bien conocida pues es sin duda una entidad infradiagnosticada. Dado que el seguimiento de familias no había demostrado aumentar la supervivencia a largo plazo, no existe hasta la fecha un programa de detección de pacientes.

La situación con respecto a dicho síndrome de predisposición está cambiando. De acuerdo con el trabajo publicado por Villani et al. en 2011[1], y actualizado en 2016[2], es posible ofrecer un seguimiento que potencialmente permita aumentar la supervivencia a largo plazo.

Se presenta el caso de un varón de 2 años que consulta de forma urgente en el hospital de zona por crisis convulsiva (crisis parcial compleja), estando previamente asintomático. Se inicia estudio con prueba de imagen y, ante la sospecha de lesión ocupante de espacio cerebral, fue remitido al centro de referencia. En las pruebas de imagen realizadas, se detectó afectación tumoral maligna y diseminada por las cubiertas meníngeas de todo el neuroeje, y una lesión principal a nivel intracerebral (tumor retroclinoideo izquierdo con diseminación meníngea difusa) (fig. 1).

El diagnóstico anatomopatológico de la biopsia realizada por craniectomía fue de meningioma maligno (alta densidad celular constituida por células de citoplasma claro con núcleos lateralizados, otras células citoplasma eosinófilo también con núcleos lateralizados, otras núcleos grandes hipercromáticos y pleomórficos). Las células se disponen en sábana alternando con vasos congestivos y focalmente con material eosinófilo tipo membrana basal entre la celularidad tumoral. No remolinos ni cuerpos de psamoma. No estroma condromixoide. Las células expresan por inmunohistoquímica intensamente CK AE1-AE 3 e INI 1. Focalmente expresan EMA, vimentina, sinaptofisina y muy focalmente PLAP. Negatividad para CD 117, OCT3/4 y alfafetoproteína. Negatividad para CD45, CD68, GFAP y desmina. La expresión intensa para citoqueratinas con positividad focal para EMA y vimentina apoya el diagnóstico de meningioma maligno y no apoya diagnóstico de carcinoma de plexos coroideos.

Se administró quimioterapia de acuerdo con protocolo SEHOP para para menores 3 años, pues la cirugía y la radioterapia no estaban indicadas por lo diseminado de la enfermedad y la corta edad del niño. Se manejaron las complicaciones que surgieron a lo largo de su seguimiento (hidrocefalia secundaria con válvula de derivación ventrículo-peritoneal, crisis convulsivas de difícil control, vómitos incoercibles, temblor de reposo, alteraciones del comportamiento) y se administró el tratamiento previsto. A pesar de ello, la enfermedad acabó progresando y el paciente falleció 6 meses después del inicio.

Al diagnóstico, se valoró la historia familiar, detectando múltiples casos de cáncer en la familia paterna (se adjunta árbol genealógico familiar [fig. 2]). Se remitió a la unidad de consejo genético en cáncer. El diagnóstico de Li-Fraumeni se confirmó genéticamente (mutación c.430C>T p.Q144* exón 5 (sin sentido) del gen TP53 del paciente)[3]. Dicho diagnóstico, no se había realizado con anterioridad en una familia con un árbol genealógico altamente indicativo. Posteriormente se ha podido estudiar a la familia y confirmar que el padre es portador de la misma variante, así como otros miembros de la rama paterna.

De acuerdo con el artículo publicado por Villani et al., es posible aumentar la supervivencia a largo plazo y de
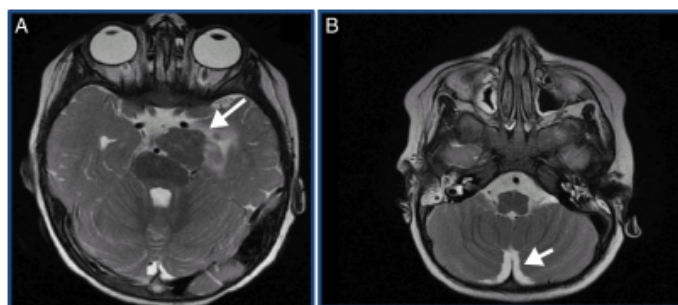


**Figura 1**   A) Lesión principal. Sospecha de tumor primario. Tumoración sólida, de 30 mm (CC) × 27 mm (AP) × 20 mm (T), en la vertiente izquierda de la cisterna prepontina. B) Diseminación tumoral meníngea. Realce leptomeníngeo seudonular grosero que se extiende por valles y cisuras de Silvio, cisternas interpeduncular, cuadrigeminal, prequiasmática, receso pineal y receso anterior superficie anterior del bulbo.
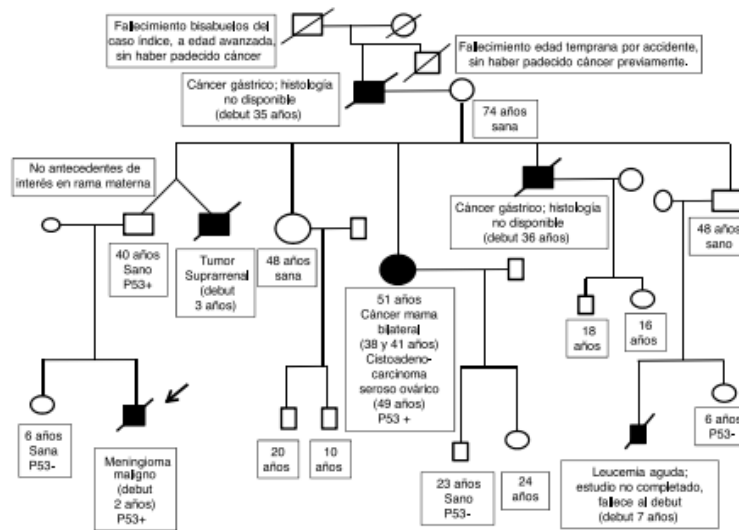
**Figura 2** Árbol familiar. Véase el caso índice en el margen inferior izquierdo de la imagen.

forma global de los portadores de alteraciones germinales en TP53. Esta publicación ha servido de fundamento para la comunicación del trabajo coordinado por la American Association for Cancer Research en torno al síndrome de Li-Fraumeni[4] y que cuenta con colaboración internacional de expertos en la materia. Además, con dicho apoyo, se ha puesto en marcha el ensayo académico Li-Fraumeni-Syndrome-Cancer-Predisposition-Syndrome Registry 01, dirigido a elaborar un registro mundial de pacientes con síndrome de Li-Fraumeni, entre otros síndromes de predisposición.

La detección previa de nuestro paciente podría haber cambiado la historia natural de la enfermedad. Además, otros miembros de la familia se podrían haber beneficiado de un seguimiento apropiado[5].

En este contexto, es importante que los pediatras tengamos presente dicho diagnóstico, al realizar la anamnesis inicial de un recién nacido sano. En caso de familias con múltiples antecedentes oncológicos, debe derivarse la familia a una unidad de consejo genético especializada[6].

Dicho estudio familiar, comenzaría con un adulto afectado para acabar beneficiando a un recién nacido sano, potencialmente portador.

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# Artículo 5.

**REVIEW ARTICLE**

# Li–Fraumeni syndrome heterogeneity

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## Abstract

Clinical variability is commonly seen in Li–Fraumeni syndrome. Phenotypic heterogeneity is present among different families affected by the same pathogenic variant in *TP53* gene and among members of the same family. However, causes of this huge clinical spectrum have not been studied in depth. *TP53* type mutation, polymorphic variants in *TP53* gene or in *TP53*-related genes, copy number variations in particular regions, and/or epigenetic deregulation of *TP53* expression might be responsible for clinical heterogeneity. In this review, recent advances in the understanding of genetic and epigenetic aspects influencing Li–Fraumeni phenotype are discussed.

**Keywords** Li–Fraumeni syndrome · Genotype · Phenotype · Epigenome · Pediatrics

## Abbreviations

LFS    Li–Fraumeni syndrome
LFL    Li–Fraumeni-like
NGS    Next-generation sequencing
CNV    Copy number variations
WGS    Whole-genome sequencing

## Li–Fraumeni syndrome

Li–Fraumeni syndrome (LFS) is a rare predisposing cancer disease transmitted by autosomal dominant inheritance. The variable clinical expressions of this syndrome are an extreme challenge for individualized surveillance [1]. This particular syndrome was described for the first time by Li and Fraumeni in 1969 [2]. Li–Fraumeni disorder predisposes to malignant tumors development. These tumors can appear throughout the life of the patient. Cancer types observed in LFS patients include: soft tissue sarcomas [3, 4], osteosarcoma [5, 6], breast cancer [7, 8], brain tumors, leukemia [9, 10] and adrenocortical carcinoma [11] (#151623 OMIM).

However, aggressiveness and the number of tumors vary to a great extending among different patients.

Cumulative incidence for development of at least one tumor at 30 years old is estimated to be 50%, while it is near 100% at 70 years old [12]. Cancer risk at early ages is higher in women due to breast cancer risk. Cumulative incidence in women at 70 years old is 54% for breast cancer, 15% for soft tissue sarcomas, 6% for brain tumors, and 5% for osteosarcoma. For male patients, however, the reported figures are 22%, 19%, and 11% for soft tissue sarcomas, brain tumors, and osteosarcoma, respectively. Fifty percent of patients with a malignant tumor developed a second tumor over the next 10 years [12]. Several patients with many malignant primary tumors have been described in the literature [13].

Approximately 70% of families affected by classical tumors carry germinal mutations in *TP53*. However, 40% of patients with Li–Fraumeni-like (LFL) phenotype (families with other malignant tumors, different from classical tumors) carry *TP53* deleterious mutations. *TP53* mutations, associated to LFS or LFL, are mainly located in the DNA-binding domain. Only few cases harbor *TP53* mutations outside this hotspot location [14, 15].

Pathogenic *TP53* variants do not explain all phenotypic manifestations. Mutations within the cell cycle checkpoint gene *CHEK2* have also been reported in some LFS or LFL families without detectable *TP53* mutations [16–20]. However, there are still relatively few reports of such mutations. Despite the fact that *CHEK2* is no longer considered as a major determinant of LFS, a number of studies support the hypothesis that *CHEK2* gene may act as a factor contributing

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to individual tumor development in families with LFL tumors. In addition to *CHEK2*, mutations in *POT1* (protection of telomeres 1) have also been associated with the risk of developing several tumor types and have been detected in LFL families [21, 22]. *POT1* encodes a nuclear protein that is essential for telomere maintenance. A higher telomeric fragility has been demonstrated in patients affected by pathogenic *POT1* variants [16].

There are still a significant number of LFS/LFL families for whom no underlying genetic determinant has been identified. For this reason, many authors have studied the influence of *BAX* [23], *CDKN1A/p21* (cell cycle arrest mediator) [24], *PTEN* (associated to *PTEN* hamartoma syndrome) [25], *PRDM* and *GAS8* [26] in LFS families without detectable *TP53* mutations. However, none of them has been identified as determinant of LFS.

Advances in next-generation sequencing (NGS) technologies, have allowed the identification of *TP53* pathogenic variants in patients with malignant tumors and without clinical suspicion of LFS. Therefore, tumor development predisposition in those cases seems to be related to these particular variants [27–29]. Consequently, new bioinformatics tools (not clinical data alone) have been suggested to detect suitable patients for genetic studies [30].

So far, LFS and LFL cases have been commonly classified based on clinical descriptions. New strategies, focused not only on clinical data, but also on molecular alterations, would be more suitable for LFS and LFL classification. Following this idea, nomenclature should also be adapted, and therefore, "*TP53* Cancer Predisposition Syndrome" and "*CHEK2* Cancer Predisposition Syndrome" could be new nomenclatures. All patients with one or more malignant tumors that are clearly related to either of the pathogenic variants (*TP53* or *CHEK2*), might be affected by one of these two proposed entities ("*TP53* Cancer Predisposition Syndrome" or "*CHEK2* Cancer Predisposition Syndrome"), respectively. Sub-classifications could be also possible, but the molecular basis (germline *TP53* or *CHEK2* pathogenic variant) should be the start point to correctly classifying patients in syndromic entities (based on present knowledge). Li–Fraumeni syndrome might be an exclusion diagnosis, when *TP53* or *CHEK2* alterations were not founded and the family or personal story suggests the LF or LFL syndrome.

## Li–Fraumeni syndrome dependent on pathogenic variants in *TP53*

Tumors more frequently associated to *TP53* germline mutations are: soft tissue sarcomas, osteosarcoma, breast cancer, brain tumors, leukemia and adrenocortical carcinoma. However, many other different tumor types have also been described: phyllodes tumor, choroid plexus tumors, and melanoma. Additionally, more infrequent tumor types included: lung, digestive tract, thyroid tumors, ovary, colon, lymphoma, and childhood malignant meningioma [31–44].

Up to now, causes of phenotypic differences among families affected by different predisposing mutations to LFS are poorly understood. Furthermore, the potential causes of phenotypic differences among members of the same family are not known. Factors influencing those phenotypic differences will be reviewed below.

### TP53 gene

*TP53* encodes a tumor suppressor protein which in response to oncogenic mutations or DNA damage triggers a transcriptional program to regulate DNA repair mechanisms, cell cycle progression and apoptosis [45, 46]. TP53 is essential for regulating cell division and preventing tumor formation [47–50]. It also plays a key role in aging [51, 52], cellular metabolism [53, 54], regulation of homeostasis [55] and immune function [46, 56, 57].

Tetramer formation of p53 is essential for its tumor suppressor function. This oligomerization is modulated by the protein concentration of p53, post-translational modifications, and/or interactions with its binding proteins [58]. The active protein conformation induces cell cycle arrest, senescence, and apoptosis through transcriptional regulation of some target genes or non-transcriptional pathways [59]. It is accepted that p53-dependent transcriptional activation occurs by binding to a consensus DNA sequence called the p53 response element in target genes promoters. Fischer M et al. meta-analysis concluded that p53 is not a direct repressor of transcription, but solely activates its target genes [60]. Therefore, p53 acts mainly as conductor conditioning the transcription of several genes: *p21, MDM2, GADD45, BAX, XPC, XPE* and *14-3-3σ* [61]. This well-scored transcriptional program performs many of the described TP53/p53 tumor suppressor functions.

Somatic mutations in this central gene are frequently observed in human cancers [62–64] and the knowledge about *TP53*/p53 in tumors has been useful to understand the phenotypic differences in patients with Li–Fraumeni syndrome.

### Mutated TP53 gene

TP53 is mutated in more than 50% of human cancers, and disrupted in practically the rest of them [65]. Approximately 80% of *TP53* mutations are single point mutations (the majority of *TP53* well accepted alterations are missense mutations). Moreover, the gene has hotspot mutations [66], in fact, its central domain (nucleotides 102-292) alone accounts for 90% of the changes [66]. Tumor suppressor gene inactivation does not follow the Knudson model for

*TP53* (this model implies the inactivation sequence of the two alleles). The p53 protein is especially inactivated by "dominance negative" effect of pathogenic missense variants. The mutated p53 monomers bind and inactivate wild-type p53 monomers. Beside the loss of function (common to all pathogenic *TP53* variants) and the dominant-negative effect on the wild-type p53 activity of pathogenic missense variants, the mutant p53 could also acquire new oncogenic functions, the so-called "gain-of-functions". [67]. Therefore, some missense *TP53* mutations (R282, R175, Y220, R248 and R273) might not only alter the protein function by disrupting the DNA-binding capacity [31], but also can favor a greater oncogenic activity [68]. As a consequence, and speaking about Li–Fraumeni patients, a more aggressive phenotype associated to some pathogenic missense variants (gain-of-function variants) has been observed in large patient cohorts [69, 70]. In this regard, Amadou et al. in a review of 1730 patients found an earlier age of tumor onset in patients with missense mutations (21.3 years), compared to those with all types of loss of function mutations (28.5 years) or genomic rearrangements (35.8 years). Notably, most of children with LFS in this study carried missense mutations [71].

Tumors with missense *TP53* mutations occur earlier in life and are frequently associated to specific histological subtypes [72]. Ognjanovic et al. described that globally, pathogenic missense mutations in exons encoding the DNA-binding domain, were more frequently observed in patients with rhabdomyosarcoma and osteosarcoma while loss of function mutations were more frequent in patients with leiomyosarcoma [72]. In addition, not only the type of variant, but also, the location of the variant may cause certain types of tumors to be more frequent than others [73]. Olivier et al. described that brain tumors were associated with missense *TP53* mutations located in the DNA-binding loop that contact the minor groove of DNA, whereas adrenal gland carcinomas were associated with missense mutations located in the loops opposing the protein–DNA contact surface [73]. The greatest compilation of information regarding the genotype–phenotype relationship is found in the IARC (International Agency for Research on Cancer) *TP53* Database.

The type and location of *TP53* variants may condition a different biological activity of the protein, and facilitate the development of certain tumor types. However, despite having the same genetic alteration in *TP53*, there are significant differences among families, which cannot be explained by the type of mutation.
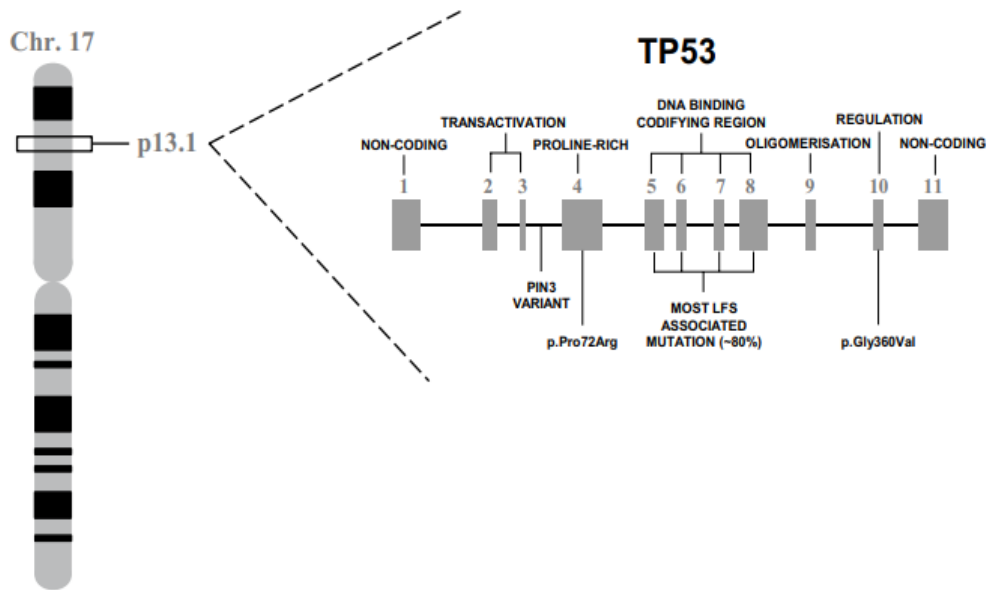
## Polymorphic variants of TP53

The presence of certain polymorphisms within *TP53* sequence may determine LFS clinical presentation since these polymorphisms may modify the oncogenic activity of the p53 protein. A novel p.Gly360Val *TP53* variant (in a

linker region near the tetramerization domain) is known to be responsible for a phenomenon called *enhanced transactivation*: transcriptional activation of *TP53* target genes conditions the up-regulation of several p53 response elements and, as a result, the final function of p53 in the cell is modified [74]. The effects of this variant in cancer phenotype among families and members of the same family remain unknown. Otherwise, it was postulated that *TP53* PIN3 polymorphic variant (hg19 chr17: 7579690; a 16 bp duplication in intron 3) may contribute to the phenotypic diversity of germline *TP53* mutations associated with LFS/LFL patients. [75]. Indeed, Marcel et al. reported that the heterozygous *TP53* PIN3 variant supposed a difference of 19.0 years in the mean age at the first diagnosis in *TP53* mutation carriers. The polymorphic variant delayed the appearance of the first tumor [75]. Sagne et al. also observed that cancer tended to occur approximately 15 years later in mutation carriers who also carried the polymorphic variant *TP53* PIN3 [76]. Another example is the p.Pro72Arg allele of *TP53*; Bougeard et al. described that the mean age of tumor onset in Arg allele carriers (21.8 years) was significantly different from the mean age of tumor onset from those with Pro/Pro (34.4 years) [77]. Marcel et al. reported anticipation of 8.3 years when Arg allele was present [75] (Fig. 1). These polymorphic variants could explain the diversity of tumor patterns among members of the same family.

## Polymorphic variants in MDM2 gene

Murine double minute 2 (*MDM2*) plays an important role in *TP53* regulation. *MDM2* encodes an E3 ubiquitin-protein ligase that mediates ubiquitination of p53/TP53, leading to its degradation by the proteasome. This gene is itself transcriptionally regulated by p53. Therefore, the encoded protein can promote tumor formation by targeting p53, if it does not function well. In fact, overexpression or amplification of this locus is detected in a variety of different cancers (Fig. 2). It has been proposed that certain polymorphic variants of *MDM2* can condition its function and, therefore, could explain clinical differences among families or members of the same family with LFS. The most outstanding example is *MDM2* SNP309 (hg19 chr12: 69202580; T−>G variation), which has been described as a modifier of tumor phenotype. This particular polymorphism increases the expression of *MDM2* and, as a consequence an attenuation of the p53 pathway is detected [77–80]. Bougeard et al. reported an accelerated phenotype among *MDM2* SNP309 G allele carriers. The mean age of tumor onset in *MDM2* SNP309 G allele carriers (19.6 years) was significantly different from that observed in patients homozygous for the T allele (29.9 years, $p < 0.05$). Their data also supported an amplified effect on the age of tumor onset by the TP53 p. Pro72Arg allele [77]. Ruijs et al. published that among

**Fig. 1** *TP53* gene is located in 17p13.1 and it is organized in 11 well-defined exons. Codifying protein regions are referred over every numbered exon. Polymorphic *TP53* variants that could explain Li–Frau-meni heterogeneity are p.Gly360Val, p. Pro72Arg and TP53 PIN3 polymorphic variant
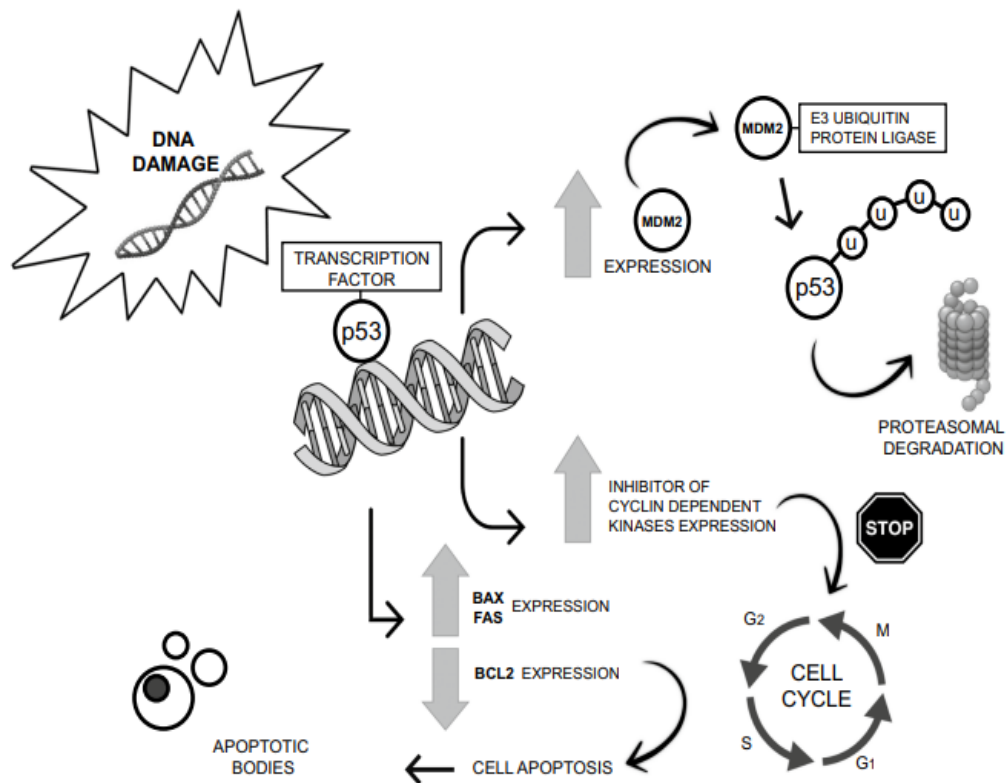
the *TP53* germline mutation carriers, a significant difference was seen in the mean age of tumor onset for the SNP309 G allele group, that is, 29.7 years as compared to the SNP309 homozygous T group 45.5 years ($P = 0.005$) [78]. In the same way, Macedo et al. studied the median age at first diagnosis among Li–Fraumeni patients carrying *TP53* R337H mutation. The median age at first diagnosis was earlier in *MDM2* SNP309 GG carriers when compared to other genotypes for both tumors analyzed in their study (adrenocortical carcinoma and breast cancer); however, they did not demonstrate a statistically significant difference [79]. Renaux-Petel et al. published results concordant with these, and also reported other interesting data about *MDM2* 285G and 309G polymorphism interactions. They reported that the *MDM2* 285-309 G–G is a higher risk haplotype in patients with germline TP53 mutations and, therefore, suggesting that the *MDM2* 309G variation is deleterious when its effect is not neutralized by the 285C variation [80].

Unfortunately, not enough information is available in concrete populations to translate to Li–Fraumeni patients polymorphic data with prognosis implications. Nowadays, physicians could not personalize surveillance programs based on polymorphic data. Nevertheless, we consider mandatory to study *TP53* PIN3, *TP53* p. Pro72Arg and *MDM2* SNP309 for all Li–Fraumeni patients. The study of at least these three TP53 polymorphisms (mainly *MDM2*) is the only way to

assess their impact on individual and familial diversity of tumor patterns. To do so, national and international contributions integrating all this information joined to *TP53* mutation type and clinical data is the way to follow.

### microRNA regulation pattern

It is known that certain microRNAs are members of *TP53* transcriptional program. It has been proposed that miR-605 (regulator of loop p53-MDM2) could affect the tumor phenotype in LFS [81]. When cellular stress is present, p53 escapes the p53:Mdm2 negative feedback to accumulate rapidly and to induce cell cycle arrest and apoptosis. Xiao et al. demonstrated that miR-605 is transcriptionally activated by p53 and post-transcriptionally represses Mdm2. The activation of p53 upregulates miR-605 expression, via interacting with the promoter region of the gene [81]. Based on the knowledge about p53-miR-605-MDM2 interactions, polymorphic variants in *miR-605* gene and their role in Li–Fraumeni phenotype were studied. Indeed, the variant G allele of *miR-605* (Hg 19 chr10: 53059406) was proposed by Id Said B and Malkin D as modifier of the LFS phenotype. They described a 10-year acceleration in the mean age of LFS tumor onset when miR-605 (Hg 19 chr10: 53059406) is present, supporting their hypothesis [82].

**Fig. 2** DNA damage drives p53 activation. Protein p53 develops transcription factor functions that condition cell cycle stop and apoptosis activation and also stimulates *MDM2* transcription. Murine double minute 2 (MDM2 protein) plays an important role in p53/*TP53* regulation. *MDM2* encodes an E3 ubiquitin-protein ligase that mediates ubiquitination of p53, leading to its degradation by the proteasome

Moreover, miR-34A is a key component of the p53 regulatory network. It was shown that p53 regulates the expression of miR-34A, representing an important mechanism of p53 signaling. Members of the miR-34 family were proposed as the most prevalent p53-induced miRNAs and are frequently silenced in variety of tumor entities, suggesting that they are important tumor suppressors [83]. Accordingly, miR-34A is inactivated by hypermethylation across many histologic types of primary tumors from patients with LFS. Malkin D group, described that loss of function *TP53* mutations were significantly associated with hypermethylation at the locus encoding miR-34A ($P < 0.001$) in germline, and this observation was validated in an independent patient cohort ($P < 0.001$) [84]. At tumoral level, miR-34A hypermethylation was associated with decreased overall survival in a cohort of 29 patients with choroid plexus carcinomas ($P < 0.05$) [84].

In conclusion, the systematic study of polymorphic variants in *TP53* and *MDM2* genes could enrich Li–Fraumeni knowledge, as commented above. In the same way, the polymorphism *miR-605* (Hg 19 chr10: 53059406 G allele) and the methylation pattern at the locus encoding miR-34A should be mandatory when a Li–Fraumeni patient carrying a *TP53* mutation is diagnosed. We will be able to enhance our comprehension of this entity sharing this information internationally.

## Copy number variations

Copy number variations (CNV) among Li–Fraumeni patients carrying *TP53* mutations are understudied. *TP53* dysfunction causes an increased number of copy number variations due to tumor instability [85–87]. Shlien et al. published that LFS *TP53* mutation carriers present an increased CNV both in tumors and germline [88]. They studied a cohort of 53 individuals from Li–Fraumeni families, 33 were *TP53* mutation carriers and 20 harbored wild-type *TP53* (controls). Controls displayed a median of 2 CNVs per

genome in germline. However, the *TP53* mutation carriers displayed a significant increase in CNVs (a mean of 12.19 CNVs) ($p = 0.01$). They also suggested a dose–response relationship between CNV frequency and severity of the LFS phenotype. Interestingly, they showed even greater number of CNVs among those TP53 carriers affected by cancer, than those which have not developed cancer yet. Moreover, they found two genes involved in recurrent duplications among LFS families: *MLLT4* and *ADAM12*. They proposed that CNV frequency, or another high-resolution measure of instability, may help to define the nature and severity of the germline *TP53* mutations found in LFS families [88]. This hypothesis was tested by Ariffin et al. in a family with clinical data of anticipation. They analyzed CNV exceeding 10 kb in size. They concluded that CNV composition did not show significant variation among family members, despite their differences in *TP53* mutation carriage and in cancer status [89]. Furthermore, Silva et al. did not find any difference in the total number of germline CNV present in LFS patients versus controls. However, they noted a highly significant increase (> fivefold) in the rare CNVs (estimated based both on DGV and db Var) in *TP53* DNA-binding domain mutation carriers as compared both to controls and to p.R337H carriers. They proposed that different microarray technologies used by Shlien et al. could be the origin of their hopeful results [90].

Total number of germline CNVs cannot be used to stratify risk assessment for Li–Fraumeni patients based on present knowledge. Nevertheless, deletions or duplications in concrete genome regions could explain some phenotypic differences among families or members of the same family. Larger cohort and homogeneous populations of Li–Fraumeni patients sharing *TP53* mutation should be studied in this way to obtain conclusive results.

## Telomeric length variations

The influence of telomere length in final phenotypic differences has been studied among the carriers of germline mutations in *TP53*. Human telomeres are nucleoprotein complexes at chromosome ends, consisting of TTAGGG repeats and associated telomere-binding proteins. In germ cells, telomeres range from 10 to 15 kb in length. Telomeres protect chromosomes from nuclease degradation and chromosome rearrangements and serve as mitotic clocks that monitor the number of cell divisions. A possible link between p53, telomeres, tumor initiation, and anticipation in LFS, has been suggested [91–93]. Based on this hypothesis, Trkova et al. published that the telomere length in peripheral blood cells was shorter among *TP53* mutation carriers than in general population. They did not find progressive telomere shortening among Li–Fraumeni generations. However, they observed a trend (not statistically significant) of

earlier onset of cancer in individuals with shorter telomeres and vice versa [94]. Tabori et al. published that telomere length was significantly shorter in affected than in non-affected *TP53* mutation carriers. They concluded that telomere length could explain earlier age of onset of tumors in successive generations of the same family with identical TP53/MDM2-SNP309 genotypes [95]. Not enough information is available in this way to reach to conclusions and to take clinical decisions. More in-depth studies are needed.

## Oxidative stress cell level

So far, there is just one published study in the literature that compares levels of oxidative stress between *TP53* carriers and controls. Macedo et al. reported an increase in cellular oxidative stress among patients with the p53R *TP53* variant (p.Arg337His). Specifically, an increase in erythrocyte GPx activity and carbonyl levels in plasma (indicator of protein oxidative damage) in mutation carriers compared to non-carriers. In addition, a significant increase in malondialdehyde levels (indicative of increased lipid peroxidation) has been demonstrated in *TP53* p.Arg337His mutation carriers. Thus, the cellular oxidative damage level could also partially explain the different phenotype among LFS families and members of the same LFS family. To the best of our knowledge, this phenomenon has not been studied in large patient cohorts [96].

## Epigenetic regulation of TP53 expression

The *TP53* promoter is highly regulated. Different mechanisms participate in a delicate control. A direct binding of several transcription factors in *TP53* promoter is well described. Saldaña-Meyer et al. reviewed the *TP53* epigenetic regulation extensively [97]. *TP53* human promoter has several conserved transcription factor binding motifs.

Different transcription factors bind *TP53* promoter and upregulate its expression. They are Myc/Max, USF, AP-1, ETS2, NFκB, RREB-1, ETS2, YY, NF, HOXA5, p53/p73, pituitary homeobox 1 (hPitx1) and ISGF3 (formed by Stat1, Stat2 and IRF-9). Moreover, kinase C δ (PKCδ) although does not bind the *TP53* promoter, promotes *TP53* transactivation. Nevertheless, Pax and BCL6 transcription factors inhibit the *TP53* promoter. ETS1 also binds on the human *TP53* promoter, but its effects are not well described [97]. A particular transcription factor is E2F1 which binds *TP53* promoter and has a direct role in the induction of mutant p53 [97].

Furthermore, *TP53* human promoter has a CTCF binding site downstream of a CpG island. CTCF influences transcriptional regulation of TP53. In fact, when knocking-down CTCF, the human *TP53* gene loses its expression supporting

its relevant contribution to *TP53* expression regulation [97, 98].

Otherwise, the *TP53* gene promoter regulation by DNA methylation remains controversial. Present knowledge points to the lack of methylation over the *TP53* core promoter. Therefore, other mechanisms might be involved (methylation of genomic regions different from promoters) [97].

Finally, microRNAs can negatively regulate *TP53* gene expression and if deregulated can promote cancer. The best known examples are: miRNA-125a and miRNA-125b which represses p53 post-transcriptionally. MicroRNA-504, micro-RNA-25, miRNA-30d and LincRNA-p21 interfere as well with p53 functions [97]. The anti-sense RNA Wrap53 is necessary for the proper transcription of *TP53* [97].

*TP53* is regulated by multiple transcription factors and microRNAs, which are epigenetically regulated. Thus, a certain pattern of epigenetic regulation of all these regulatory genes could condition a wild-type and mutated p53 cellular level, variable from one individual to another, which might explain phenotypic differences among members of the same Li–Fraumeni family. No studies were developed either studying plasma levels of these regulatory elements or methylation pattern of their codifying genes among Li–Fraumeni patients. It could be a way to explore in the future.

## Epigenetic regulation of genes regulated by TP53

Genetic and epigenetic alterations may be involved in the phenotypic variability of LFS. p53 regulates several pathways, including the thymine DNA glycosylase (TDG) pathway, which regulates the DNA methylation of several genes. Fortes et al. compared the DNA methylation pattern of genes related to the TDG pathway among germline

*TP53* mutations carriers, patients with wild-type *TP53*, and healthy individuals. Finally, no significant differences were found. However, increased TDG expression was detected in patients with p.R337H *TP53* mutation affected by adrenocortical carcinoma. Further studies in larger patient cohorts are necessary to evaluate the clinical impact of epigenetic alterations on genes potentially involved in LFS variability [99].

## Other elements to consider

The presence of mutations in certain RecQ DNA helicases (like BLM (Bloom syndrome (BS) protein) and WRN (Werner syndrome protein)) would affect *TP53* function. The Harris CC group suggests that p53 mediates the cooperation of p53 and BLM to induce apoptosis. Therefore, certain variants in these genes might affect, at least partially, the function of *TP53* [100].

The elements that might condition the tumor phenotype in LFS are detailed in Table 1.

## Environmental components

Phenotypic differences are detected among Li–Fraumeni patients from different geographical origins. Environment could affect tumor development among *TP53* carriers, therefore, life style, diet and environmental exposures joined to all above said, probably condition the final phenotype. An environmental component may be responsible for the differences observed among families from different origins that share *TP53* mutation [89]. None large cohort studying its influence has been published. Moreover,

**Table 1** Elements that may condition phenotypic differences, among patients carrying the same *TP53* variant

|  | Regulatory element | References |
| --- | --- | --- |
| Genetics |  |  |
| Polymorphic variants in *TP53* gene | *TP53* p.G360V | Id Said et al. [74] |
|  | *TP53* PIN3 | Marcel et al. [75] |
|  | *TP53* p.Pro72Arg | Bougeard et al. [77] |
| Polymorphic variants in *MDM2* gene | *MDM2* SNP309 G allele | Bougeard et al. [77] |
| Polymorphic variants in microRNAs | microRNA 605 (rs2043556 GG) variant | Id Said et al. [82] |
| Genomics |  |  |
| Copy number variations (CNVs) | Presence of rare CNVs | Silva et al. [90] |
| Telomeric length | Telomeric length shortening | Tabori et al. [95] |
| Epigenomics |  |  |
| *TP53* transcriptional and post-transcriptional regulation | Diversity among individuals in regulation pattern | Saldaña-Meyer et al. [97] |
| microRNA-34 | miR-34A methylation pattern | Samuel et al. [84] |
| Metabolomics |  |  |
| Oxidative stress cell level | Protein oxidative damage level | Macedo et al. [96] |
|  | Lipid oxidative damage level |  |

founding mutations are very common in certain regions and exceptional in others, and this makes comparative studies difficult.

## Anticipation?

A decrease in the age at cancer onset and an increase in more LFS-specific cancers in successive generations have been suggested [101, 102]. The genetic mechanisms proposed to explain this heterogeneity include accumulation of copy number variations (CNVs) with successive generations, and progressive telomere shortening [88]. Ariffin et al. studied a dataset of 269 pedigrees of *TP53* germline mutation carriers. Although, they reported a decrease in age at first cancer onset in multigeneration pedigrees, their observations did not fit with a classical model of anticipation. Nevertheless, only pedigrees with three or four generations showed a delayed age at first cancer onset in the older generations of *TP53* mutation carriers. Then, they suggested that the founder patient of such pedigrees may carry, in addition to germline *TP53* mutation, rare independent genetic modifiers that attenuate the risk of early cancer. These genetic variants might allow cancer-free survival until postreproduction age of founders. Based on these observations, they proposed the term "genetic regression" instead of anticipation [103]. To understand this phenomenon, they looked for CNVs larger than 10 kb and for telomere length shortening among kindred affected by LFS-specific cancer, but did not discover significant differences. Moreover, they did not find neither more frequent *MDM2*-SNP309 G allele nor *TP53* PIN3 among affected children compared with their previous generations [103]. Otherwise, this group used whole-genome sequencing (WGS) analysis among family members and identified interesting rare single-nucleotide variants (SNVs). A curious example was a father (non-carrier TP53) who transmitted a rare SNV to two out of four *TP53* mutation carrier children. Children with *TP53* mutation and the rare SNV developed an early cancer but not the two *TP53* mutation carrier children who not carried that rare SNV. Such rare SNV may be considered as candidate-modifier genes that may modulate age at cancer onset. Deeper studies looking for these variants could be important [103]. In fact, Franceschi et al. reported recently an affected child who inherited the *TP53* mutation from his affected mother (breast cancer in adulthood age) and received from their non-affected father 25 predicted deleterious variants including a nonsense mutation in *ERCC3*. They proposed that those inherited mutations are possible candidate modifiers linked to *TP53* [104]. Undiscovered genetic variants could determine also Li–Fraumeni heterogeneity among members of the same family.

## Conclusions

It is very difficult to elucidate the genotype–phenotype relationship in LFS. Based on the evidence described in the present review, not only would the genotype condition phenotypic peculiarities, but also the epigenome seems to play a key role, although to date, studies in this field are scarce.

The current knowledge of LFS makes it difficult to state individual recommendations adapted to the risk at all levels of clinical care (genetic counseling in assisted reproductive treatments, pediatric or medical oncology). Therefore, it is urgent to increase the understanding of this devastating entity. The systematic and coordinated study of all the elements involved in LFS is the only way to move forward.

## Compliance with ethical standards

**Conflict of interest** The authors declare to have no conflict of interest.

**Ethical approval** This work is not a research involving human participants and/or animals.

**Informed consent** Not applicable.

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