# *RB1* AND BEYOND:

# DETERMINING GENETIC CAUSES OF RETINOBLASTOMA IN SOUTH AFRICAN PATIENTS

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Supervisors:
Dr. Lindie Lamola
Ms. Nkateko Mayevu

A research report in the format of a “submissible” paper submitted to the Faculty of Health Sciences,
University of the Witwatersrand, Johannesburg.
This research report is required for partial fulfilment of the requirements for the degree of Master of Science in Medicine Genomic Medicine


Johannesburg, South Africa
25 February 2024

# NATIONAL HEALTH LABORATORY SERVICE

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30 November 2023

Re: Research report submitted to the Faculty of Health Sciences by Ms. Danielle Beukman in partial fulfilment of the requirements for the degree of Master of Science in Medicine (Genomic Medicine)
I would like to thank you for agreeing to examine my research report titled "RB1 and beyond: Determining genetic causes of retinoblastoma in South African patients"

The candidate, Ms. Danielle Beukman, is submitting her research report in the format of a "submissible" format. The research report contributes 33% towards the final mark of the degree. The candidate and her supervisors have chosen to submit the paper to the journal "Human Genetics" published by Springer. The submissible paper attached is therefore written in accordance with the author guidelines of the above-mentioned journal. These guidelines have been attached as a website link for your reference. The only deviation from these guidelines is that the manuscript has been presented as one complete document (including tables, figures, and list of abbreviations) to make marking easier, rather than being submitted separately.
The research protocol is attached as an appendix for the purposes of providing an extended literature review and further context to the study. The protocol has already been assessed and approved by the Faculty of Health Sciences and is therefore not for examination.
Please refer to the Table of Contents for a complete list of the contents provided in this research report.

Yours sincerely,

Student: Ms. Danielle Beukman

Supervisor: Dr Lindie Lamola

Co-supervisor: Ms. Nkateko Mayevu

# Declaration

I, Danielle Beukman, declare that this research report, in the form of a “submissible” paper, is my own, unaided work. It has not been submitted before for any degree or examination at any other University.

This research report is required for the Degree of Master of Science Genomic Medicine at the University of the Witwatersrand, Johannesburg.

Danielle Beukman
WITS student number 719425

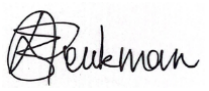
Signature of candidate, Danielle Beukman

Signed on 26 February 2024, in Johannesburg, RSA

## Contribution of the candidate to the paper

Declaration: Student's contribution to article(s) and agreement of co-authors

I, Danielle Beukman, student number 719425, declare that this Research Report, entitled "RB1 and Beyond: Determining genetic causes of Retinoblastoma in South African patients" is my own work and that I made significant contributions towards the research findings presented in this paper, intended to be submitted for publication below.



Signature of Student Date: 15 November 2023

Signature of Primary Supervisor Dr. L.Lamola

Date: 16 November 2023

**Agreement by co-authors**

By signing this declaration, the co-authors listed below agree to the use of the research d fini ngs in tended for a published article by a student as part of their Research Report.

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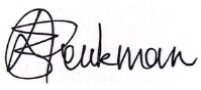
| 1st | Ms. Danielle Beukman |  | 15 November 2023 |
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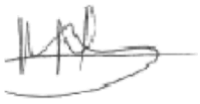
| 3rd | Ms. Nkateko Mayevu |  | 16 November 2023 |

# Dedication

I dedicate this research report to my family, in particular my beloved mother, Margaret and elder sister, Natasha. Your everlasting support has immeasurable value.

## Abstract

Retinoblastoma is the most common solid tumour of the retina, affecting mainly paediatric patients. Although loss-of-function germline variants the *RB1* gene is the tumour suppressor gene most often associated with heritable retinoblastoma, two contradictory research findings complicate the assumption that a pathogenic germline variant in *RB1* will lead to the development of heritable retinoblastoma. Firstly, heritable retinoblastoma has been observed in cohorts with biallelic wild-type *RB1* genes, secondly, biallelic loss-of-function is not always sufficient to lead to retinoblastoma tumorigenesis. By investigating germline variants in additional cancer predisposition genes to discover candidate driver genes in heritable retinoblastoma, the full germline mutational spectrum of heritable retinoblastoma can be established. In this study we investigate sequence germline variants in cancer predisposition genes beyond *RB1* in South African retinoblastoma patients with an African ancestry by performing whole exome sequencing on eight patients diagnosed with retinoblastoma. Whole exome sequencing data for genes previously associated with retinoblastoma was subjected to variant annotation by means of a variant effect predictor and filtering criteria applied manually. Variant classification was performed according to guidelines proposed by The American College of Medical Genetics and Genomics and the Association of Molecular Pathology. The highest degree of variant classification was variants of unknown significance. The absence of sequence variants capable of describing the retinoblastoma phenotype in this cohort demonstrates the necessity of looking beyond *RB1*, combining next generation sequencing with copy number analysis pipelines and additional genome mapping technologies capable of detecting structural variants. The detection of sequence variants previously reported to be possibly damaging but now considered polymorphisms highlights the importance of revisiting variant classification. In closing, this study adds genomic information from patients with a South African ancestry to mounting genomic data, ensuring that previously underrepresented populations can also benefit from future cancer predisposition and precision medicine research.

# Acknowledgements

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I feel honored to have shared this year with my classmates, Anréé, Ingrid and Racilya, your friendship made every Wednesday worth it! I cherish every moment. Dylan, Thandeka, Phophi and Gabriella, thank you for being there when I needed advice.

Most importantly, to God and my family, thank you for keeping the rent low and spirits high.

To my mom Kleintjie, thank you for your unconditional support and the endless supply of Earl Grey tea. Dankie ma.

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# Table of Abbreviations

| Abbreviation | Meaning |
|---|---|
| ACMG/AMP | American College of Medical Genetics and Genomics and Association for Molecular Pathology |
| BAM | Binary alignment map |
| CADD | Combined Annotation-Dependent Depletion |
| CMA | Chromosomal microarray |
| CNV | Copy number variation |
| HREC Medical | Human Research Ethics Committee (Medical) |
| IGV | Integrative genomics viewer |
| Indels | Insertions and Deletions |
| LOVD | Leiden Open Variation Database |
| MAF | Minor Allele Frequency |
| miRNA | Micro RNA |
| MLPA | Multiplex ligation-dependent probe amplification |
| PeCan | Paediatric Cancer |
| POLYPHEN2 | Polymorphism Phenotyping version 2 |
| RB | Retinoblastoma |
| *RB1* | Retinoblastoma 1 tumor suppressor gene |
| SNV | Single Nucleotide Variants |
| SIFT | Sort Intolerable from Tolerable |
| SANCR | South African National Cancer Registry |
| *TSG* | *Tumor suppressor gene* |
| VCF | Variant Call files |
| VEP | Variant effect predictor |
| VUS | Variant of uncertain significance |
| WES | Whole exome sequencing |

**Research report in the format of a "submissible" paper**

To be submitted to the Journal Human Mutation

# *RB1* and Beyond: Determining genetic causes of Retinoblastoma in South African patients

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## 1. Abstract for Journal Submission

**Background:** Retinoblastoma is the most common solid tumour of the retina, affecting mainly paediatric patients. Although loss-of-function germline variants the *RB1* gene is the tumour suppressor gene most often associated with heritable retinoblastoma, two confounding research findings contradict the direct genotype phenotype relationship. First, heritable retinoblastoma has been observed in cohorts with biallelic wild-type *RB1* genes, secondly, biallelic loss-of-function is not always sufficient to lead to retinoblastoma tumorigenesis. By investigating germline variants in additional cancer predisposition genes beyond *RB1* allows for discovery of candidate driver genes in heritable retinoblastoma. In this study we investigate sequence germline variants in cancer predisposition genes beyond *RB1* in retinoblastoma patients with a South African ancestry.

**Methods:** Whole exome sequencing with single nucleotide resolution was performed to establish germline sequence variants. Variant annotation, prioritization and filtering was executed to detect germline variants in cancer predisposition genes previously proposed as candidate genes in retinoblastoma malignancy and paediatric cancer predisposition syndromes. Variant prioritization excluded alleles with a minor allele frequency above 5% and variants predicted to have no impact on a protein level due to no change in the amino acid sequence and protein structure. Variants shared between individuals and variants of interest were classified according to ACMG/AMP guidelines.

**Results:** A total of 353 655 variant were annotated, 4538 of which were novel. After collating functional data, previously reported and reviewed *RB1* variants, all sequence variants in *RB1* are likely benign, (NM_000321.3:c.1574C>G, NP_000312.2:p.Ala525Gly and NM_000321.3:c.45_53del NP_000312.2:p.Ala16_Ala18del) amending outdated variants incorrectly classified as likely pathogenic. The highest degree of variant classification in genes previously associated with retinoblastoma are variants of unknown significance.

**Conclusion**: The absence of sequence variants capable of describing the retinoblastoma phenotype in this cohort demonstrates the necessity of combining next generation sequencing with analysis pipelines and additional genome mapping to detect structural variants. This study adds genomic information from patients with a South African ancestry, an underrepresented population.

**Keywords:** germline variation, cancer predisposition genes, whole exome sequencing, *RB1* gene, retinoblastoma, sequence variants

## 2. Background

### 2.1. Retinoblastoma in South Africa

Retinoblastoma (RB) is the most commonly occurring solid intraocular cancer, developing in childhood. The South African National Cancer Registry (SANCR) documented an average RB incidence of 1 in 21 641 live births for the time elapsed between 2004 and 2018 (https://www.nicd.ac.za/centres/national-cancer-registry). South African patients have a later mean age of diagnosis (2.5 years), compared to an earlier mean age of diagnosis of 1.2 years in high income countries (Stuart et al., 2022). Routine cancer surveillance is poor in South Africa due to an overburdened public health care system. As an early age of diagnosis is the strongest indicator of an improved prognosis, a later mean age of diagnosis puts South African children suffering from paediatric cancers in a disadvantageous position (Stuart et al., 2022).

Recent efforts have been made to address South Africa's later mean age of cancer diagnosis compared to global averages. The overall survival rate for RB patients in South Africa increased from 52% in 1994 (Hesseling et al., 1995) to 86.7% twenty years later (Ndlovu et al., 2023). Limiting disease for RB exhibits a 100% overall survival rate, emphasizing the importance of early detection. Children with a black South African ancestry have a disproportionately high incidence of RB compared to other racial groups (Ndlovu et al., 2023).

The majority (60%) of RB cases are sporadic and unilateral (tumors affecting a single eye), occurring at a mean age of 24 months. The remaining 40% of RB cases are heritable, bilateral (tumors affecting both eyes) RB, with a significantly earlier mean age of onset of 12 months (Bouchoucha et al., 2023; Knudson, 1971). A small proportion of RB patients present with pineoblastoma in conjunction with bilateral RB, referred to as trilateral RB. The presence of a constitutional (germline) mutation in *RB1* can either be inherited from a parent or occur de novo at an early stage of embryonic development, a mutation occurring at a later stage of embryonic development will result in mosaicism (Lin & Chintagumpala, 2021; Rushlow et al, 2009).

### 2.2. Future implications of a definitive genetic diagnosis

The detection of a disease-causing germline variant and a mutation signature can aid in treatment planning and risk assessment. Patients with a pathogenic germline variant in *RB1* have a predilection to developing further malignancies (Ketteler et al., 2020; Shinohara et al., 2014) and a recurrence risk

in offspring. A literature search into genetic variants predisposing black South African individuals to RB malignancy proved futile, revealing a gap in our understanding of hereditary and paediatric cancers in patients with a South African ancestry.

Black South Africans are greatly underrepresented genetic research, the paucity of genomic sequencing data from black South African individuals creates a challenge when cancer screening and risk assessment protocols are designed. The aim of this study was to detect sequence variants predisposing paediatric black South African patients to developing RB.

## 2.3. The genetic landscape of RB

The first tumor suppressor gene (TSG) described fully was the Retinoblastoma 1 (*RB1*) gene, by Alfred Knudson in 1971, a revolutionary finding that initiated exploration into cancer predisposition genes. More than a decade later, in 1986, the *RB1* gene position was identified by positional cloning (Lee et al., 1987). The explanation of the two-hit hypothesis pioneered our understanding of the molecular basis of cancer (Knudson, 1971). A genetic predisposition to RB malignancy is inherited if a single nonfunctional copy of *RB1* is inherited or present in the germline, a second hit in somatic tissue of the retina will then result in complete loss of function leading to uncontrolled cellular proliferation (Knudson, 1971).

The *RB1* gene exhibits allelic heterogeneity, with more than 1700 different pathogenic germline *RB1* variants logged on the Leiden Open Variation Database (LOVD) (Fokkema et al., 2011). An array of different types of germline variants in *RB1* can serve as an inherited predisposition, or “first hit” (Stenfelt et al., 2017).

Sequence variants such as single nucleotide variants (SNV) leading to a loss-of function in the *RB1* gene product as well as structural variants like whole gene deletions, copy number variation (CNV) (Xu et al., 2020) and rearrangements can render a germline copy of *RB1* non-functional (Davies et al., 2021). Chromothripsis spanning the *RB1* locus provides an additional mechanism of inactivating *RB1* (McEvoy et al., 2014).

With the emergence of sequencing technologies and increasing use thereof, two genetic anomalies with regards to RB was encountered that contradicted the *RB1* two-hit hypothesis. Firstly, in some cases the presence of a pathogenic germline variant in *RB1* is not sufficient to increase the risk to

develop RB or a retinoma neoplasm, as numerous families have a strong family history of a pathogenic variant in *RB1* without the development or RB in successive generations. A nonfunctional *RB1* copy confers genomic instability but additional genetic changes beyond *RB1* may be required for the development of RB, the number of genetic modifications required is greatly debated (Dimaras et al., 2008; Corson & Gallie, 2007). Secondly, RB has been diagnosed in cases with a family history of the disease, indicative of a pathogenic RB1 variant according to the two-hit hypothesis, yet germline sequencing revealed two wild-type copies of *RB1* (Rushlow et al., 2013), suggesting that germline mutations in other tumor predisposition genes beyond *RB1* can act as putative drivers in RB malignancy (Akdeniz et al., 2019).

## 2.4. Locus heterogeneity observed in RB predisposition

Genes other than RB1 have been linked to heritable RB aetiology. The notion of looking beyond RB1 as provided evidence to add to our knowledge of the genomic landscape of heritable RB. Table 1 summarizes cancer susceptibility genes that have been implicated in RB aetiology, paediatric cancers as well as retinal development pathways.

Copy number analysis pipelines have been designed to detect unbalanced copy number variations, such as large deletions and duplications. In future the addition of copy number analysis should be performed in an aim to detect loss of function variants due to copy number variation in the cancer susceptibility genes mentioned in table 1 (Kooi et al., 2016), bridging the gap between sequence variants and larger structural variants.

Changes at an epigenetic level have been proven to contribute to RB development, with promotor methylation of TSG beyond *RB1* greatly influencing disease progression (Benavente & Dyer, 2015; McEvoy & Dyer, 2015). Non-coding RNAs and miRNAs have been functionally linked to RB tumorigenesis (Reis et al., 2012), creating microRNA (miRNA) signatures and multiomics techniques to be used in clinical settings as both diagnostic and prognostic markers (Delsin et al., 2019; Plousiou & Vannini, 2019).

Germline loss of function mutations in the TSG *p53* has been associated with multiple cancer predisposition syndromes and RB neoplasm development (Kato et al., 1996). Due to the important role *p53* plays in regulating apoptosis in RB (Nork et al., 1997), loss of heterozygosity structural variants involving tumor protein 53 (*p53)* have been associated with RB (Kato et al., 1996). A study conducted

by Castéra, and colleagues discovered a significant contribution of E3 ubiquitin-protein ligase (*MDM2)* as a modifier gene in RB (Castéra et al., 2010) as RB progression is marked by *MDM2* signaling pathway aberrations (Xu et at., 2009). *MDM2* in conjunction with P53-Binding Protein *(MDM4)* regulates the *p53 signaling pathway*. *MDM4* variants implicated in RB oncogenesis were found in a Brazilian cohort (de Oliveira Reis et al., 2012).

The use of exome gene panels detected pathogenic variants in a heritable RB cohort from Istanbul with wild type *RB1* linking RB oncogenesis with the retinoic acid pathway. Pathogenic variants were found in Dehydrogenase [quinone] 1 (*NQO1),* Fibroblast growth factor receptor 4 *(FGFR4),* C-Type Lectin Domain Containing 7A (*CLEC7),* Apolipoprotein C3 *(APOC3),* MutY DNA Glycosylase *(MUTYH),* UDP-Glucuronosyltransferase 1-A *(UGT1A1)*, and RB disease segregated with a variant in Keratin 85 (*KRT85)* (Akdeniz et al., 2019).

The downregulation of Cyclin Dependent Kinase Inhibitor 1A (*CDK4I)*, also rereferred to as *p16INK4a*, is strongly associated with heritable RB but not sporadic RB (Indovina et al., 2009). Germline mutations in *CDKN1A* are linked to uncontrolled cellular proliferation, increasing the risk of developing a neoplasm (Carvalho et al., 2013). Pathogenic variants in oncogenes BHLH Transcription factor (*MYCN)* and Tuberous Sclerosis Complex 2 (*TSC2)* have also been proven to act as major drivers in RB oncogenesis (Francis et al., 2021).

Table 1: Genes implicated in the aetiology of retinoblastoma development

| Gene symbol | Full gene name | HGVS Reference transcript | Literature reference | Gene symbol | Full gene name | HGVS Reference transcript | Literature reference |
|---|---|---|---|---|---|---|---|
| *RB1* | Retinoblastoma 1 | NM_000321.3 | Knudson, 1971 | *MCCC2* | Methylcrotonyl-CoA Carboxylase Subunit 2 | NM_022132.4 | Akdeniz et al., 2019 |
| *ACADS* | Short-chain acyl-CoA dehydrogenase | NM_000017.3 | Akdeniz et al., 2019 | *MDM2* | E3 ubiquitin-protein ligase | NM_002392.6 | Epistolato et al., 2011 |
| *APC* | APC Regulator Of WNT Signalling Pathway | NM_000038.6 | Capasso et al., 2020 | *MDM4* | P53-Binding Protein | NM_001204172 | de Oliveira Reis et al., 2012 |
| *APOC3* | Apolipoprotein C3 | NM_000040.2 | Akdeniz et al., 2019 | *MLH1* | MutL Homolog 1 | NM_001258271.2 | Capasso et al., 2020 |
| *BRCA1* | BRCA1 DNA Repair associated | NM_007294.4 | Capasso et al., 2020 | *MSH2* | MutS Homolog 2 | NM_000251.3 | Capasso et al., 2020 |
| *BRCA2* | BRCA2 DNA Repair associated | NM_000059.4 | Capasso et al., 2020 | *MSH3* | MutS Homolog 3 | NM_002439.5 | Capasso et al., 2020 |
| *C2* | Complement 2 | NM_000063.4 | Akdeniz et al., 2019 | *MUTYH*[a] | MutY DNA Glycosylase | NM_001128425.1 | Akdeniz et al., 2019 |
| *CFB* | Complement Factor B | NM_001710.5 | Akdeniz et al., 2019 | *MYCN* | BHLH Transcription factor | NM_005378.4 | Francis et al., 2021 |
| *CLEC7A*[a] | C-Type Lectin Domain Containing 7A | NM_197947.2 | Akdeniz et al., 2019 | *NQO1* | Dehydrogenase [quinone] 1 | NM_000903.2 | Akdeniz et al., 2019 |
| *CDKN1A* | Cyclin Dependent Kinase Inhibitor 1A | NM_000389.5 | Carvalho et al., 2013 | *PAX6* | Aniridia type II | NM_001368894.2 | Li et al., 2011 Meng et al., 2014 |
| *CREBBP* | CREB Binding Protein | NM_001079846.1 | Kooi et al., 2016 | *PTCH1* | Patched 1 | NM_001083606.3 | Capasso et al., 2020 |
| *CX3CR1* | C-X3-C Motif Chemokine receptor 1 | NM_001171174.1 | Akdeniz et al., 2019 | *p16INK4A* | Cyclin-dependent kinase inhibitor 2A (CDKN2A) | NM_000077.5 | Indovina et al., 2009 |
| *DSPP*[b] | Dentin Sialophosphoprotein | NM_014208.3 | Akdeniz et al., 2019 | *TP53* | Tumor Protein P53 | NM_000546.6 | Epistolato et al., 2011 |
| *FGFR4* | Fibroblast growth factor receptor 4 | NM_002011.4 | Akdeniz et al., 2019 | *RHAG* | Rh Associated Glycoprotein | NM_000324.2 | Akdeniz et al., 2019 |
| *FUT6* | Fucosyltransferase 6 | NM_000150.2 | Akdeniz et al., 2019 | *RPGRIP1* | RPGR Interacting Protein 1 | NM_020366.3 | Akdeniz et al., 2019 |
| *GBE1* | Glycogen branching enzyme | NM_000158.3 | Akdeniz et al., 2019 | *SAMD11* | Sterile Alpha Motif Domain Containing 11 | NM_001385640.1 | Kubo et al., 2021 |
| *GHRL* | Ghrelin and Obestatin prepropeptide | NM_001134944.1 | Akdeniz et al., 2019 | *SERPINA1* | Alpha1AT | NM_001002235.2 | Akdeniz et al., 2019 |
| *GNPAT* | Glycerone-Phosphate O-Acyltransferase | NM_014236.3 | Akdeniz et al., 2019 | *SLC34A1* | Solute Carrier Family 34 Member 1 | NM_003052.4 | Akdeniz et al., 2019 |
| *HBD* | Haemoglobin Delta Chain | NM_000519.3 | Akdeniz et al., 2019 | *SMAD5* | SMAD Family Member 5 | NM_001001419.3 | Ueki & Reh, 2012 |
| *HFE* | Homeostatic Iron Regulator | NM_000410.3 | Akdeniz et al., 2019 | *TYR* | Tyrosinase | NM_000372.4 | Akdeniz et al., 2019 |
| *KRT85* | Keratin 85 | NM_002283.3 | Akdeniz et al., 2019 | *TSC2* | Tuberous Sclerosis Complex 2 | NM_001114382.3 | Francis et al., 2021 |
| *MBL2*[b] | Mannose Binding Lectin 2 | NM_000242.2 | Akdeniz et al., 2019 | *UGT1A1*[a] | UDP-Glucuronosyltransferase 1-A | NM_000463.2 | Akdeniz et al., 2019 |

# 3. Materials and Methods

## 3.1. Ethical considerations and consent

This study was conducted as a sub-study under the parent study, Paediatric Cancer (PeCan) Project, Inherited cancer predisposing mutations in a cohort of childhood cancer patients. Ethical clearance was obtained from the University of the Witwatersrand Faculty of Health Sciences Human Research Ethics Committee (Medical) (HREC Medical) prior to the commencement of the research. Ethical clearance certificates for both the parent study, protocol number M180855, and this sub-study, protocol number M230782 are included in appendices A and B respectively. The parents or legal guardians of the participants provided written and/or verbal consent and assent from a subset of participants; copies of the informed consent documents employed in the study are included in appendix F.

## 3.2. Clinical presentation and patient demographics

Eight paediatric patients diagnosed with RB were included in this study from the PeCan project. All eight patients are of black South-African ancestry. No family history of RB was reported in any of the patients. Table 2 summarizes the clinical features and demographics of the eight patients.

**Table 2**: Clinical presentation of RB patients

| **Patient ID** [a] | **Sex** [b] | **Age of diagnosis** | **Laterality of RB** |
|---|---|---|---|
| PC26 | Female | 1 Year 8 Months | Unilateral RB |
| PC43 | Female | 1 Year 6 Months | Unilateral RB |
| PC50 | Female | 4 Years | Unilateral RB |
| PC79 | Male | 4 Months | Unilateral RB |
| PC90 | Female | 3 Year 6 Months | Unilateral RB |
| PC104 | Female | 8 Months | Bilateral RB |
| PC120 | Male | 3 Months | Bilateral RB |
| PC123 | Male | 9 Months | Unilateral RB |

[a] Patient IDs used to anonymize patient information.

[b] Sex as assigned at birth

## 3.3. Whole exome sequencing

### 3.3.1. DNA sample preparation and sample quality control

Genomic DNA was extracted and purified from peripheral blood samples prior to the commencement of this project using the salt-out method (Maurya et al., 2013). DNA quantification commenced with assessing the concentration of double-stranded genomic DNA using the Qubit ™ High Sensitivity assay and the Qubit ™ fluorometer (ThermoFisher Scientific, Waltham MA, USA). Concentration of all samples were normalised to 10 ng/µl.

### 3.3.2. Library generation

Target amplification was performed using Ion AmpliSeq ™ Exome RDY reagents, followed by partial digestion of amplicons using the Ion AmpliSeq ™ FuPa reagent. Ligation of Ion P1 Adapters and Ion Xpress ™ Barcodes was completed as per ThermoFisher Scientific manufacturer's guidelines (ThermoFisher Scientific, Waltham MA, USA). Barcoded libraries were purified using Agencourt ™ AMPure XP Reagents (Beckman Coulter, Brea, CA, USA) followed by library amplification. Amplified libraries were purified in two rounds, again using Agencourt ™ AMPure XP Reagents (Beckman Coulter, Brea, CA, USA).

Libraries were quantified using the 4200 TapeStation ™ (Agilent technologies, Santa Clara, USA) in conjunction with the prescribed High Sensitivity D5000 ScreenTape assay. The concentration of 200-400 base pair fragments were measured for the purposes of quality control and diluted accordingly to facilitate the combination of exome libraries.

### 3.3.3. Templating and sequencing

Two Ion AmpliSeq ™ Exome RDY libraries were combined on a single Ion 540 ™ Chip, templated in the Ion 540 ™ Kit – Chef (ThermoFisher Scientific, Waltham MA, USA) as planned on Torrent Suite™ software. Sequencing was performed using the Ion Torrent GeneStudio ™ S5 Sequencer (ThermoFisher Scientific, Waltham MA, USA). Sequencing output included variant call files (VCF), binary alignment maps (BAM), index files and quality control reports.

### 3.4. Variant interpretation

VCF files produced were annotated with Ensembl variant effect predictor (VEP) tool (GRCh37 version 110) (McLaren et al., 2016) available online from Ensembl 110 Genome Browser (https://www.ensembl.org) (Martin et al., 2023). Note that the decision was made to align all exomes to the human reference genome build 19 (GRCh37/Hg19), for the sake of consistency throughout the bioinformatics workflow. Annotation outputs included variant location and variant type, i.e., single nucleotide variants (SNVs) such as missense or nonsense variants, insertions, or deletions (indels), frameshift, or splice variant (Hunt et al., 2022). Annotation output also included variant allele frequency as reported by two genomic databases, 1000 genomes project (The 1000 Genomes Project Consortium et al., 2015) and the genome Aggregation Database (gnomAD) (Chen et al., 2022). All the above-mentioned databases were accessed between August and September 2023.

First line of variant filtering was to prioritize the genes that have previously been reported to be implicated in RB predisposition and paediatric cancer predisposition. These genes are summarized in Table 1. Variants with a minor allele frequency (MAF) above 5% were filtered out. Lastly, variants that were predicted to have little to no impact on protein structure and/ or function were filtered out. The in-silico tools used to quantify variant impact on protein structure and function included Sort Intolerable from Tolerable (SIFT) (Ng & Henikoff, 2003), Polymorphism Phenotyping version 2 (POLYPHEN-2) (Adzhubei et al., 2010). Combined Annotation-Dependent Depletion (CADD) scores were allocated to SNVs and small indels based on how deleterious that variant is predicted to be (Rentzsch et al., 2019). Both SIFT and POLYPHEN-2 take the variant position's degree of evolutionary conservation into account as part of predicting the impact of the variant on a protein level. These in silico tools predict what structural and functional changes may occur in a protein due to amino acid substitution (de la Campa et al. 2017). Lastly, variants of interest were modelled on MutationTaster2021 to distinguish polymorphisms from mutations (Steinhaus et al., 2021).

Coverage and the quality of variant calling was assessed for the shortlisted variants by viewing binary alignment maps (BAM) and their index files in an integrative genomics viewer (IGV) (Robinson et al., 2011), variants with 30x coverage or greater were considered to be true variant calls. Variants were classified according to The American College of Medical Genetics and Genomics and Association for Molecular Pathology (ACMG/AMP) guidelines (Richards et al., 2015). Previously reported variants were analysed based on ClinVar submissions (Landrum et al., 2018).

# 4. Results

## 4.1. Clinical presentation of patients

The eight patients had a wide distribution of age of onset, ranging from as early as 3 months to as late as 4 years. Mean age of onset for RB cohort was 19 months with a standard deviation of 17.22 months. Both male (37.5%) and female (62.5%) patients were included in the cohort. The two patients (25%) with the earliest age of onset (3 months and 8 months respectively) were the only patients that presented with bilateral RB, while the remaining six patients (75%) presented with unilateral RB (onset age range ranging from 4 months to 48 months).

## 4.2. Variant annotation

Whole exome sequencing (WES) of eight paediatric RB patient with a black South African ancestry collectively returned 353 655 annotated variants spanning the entire genome. Figure 1 depicts the distribution of variant consequences as observed in the 8 patient cohort. The largest number of variants were 122 011 intronic variants, accounting for 34.5%. These intronic variants were filtered out immediately. The second highest proportion of variants were 54 392 synonymous variants (15.38%), followed by 38 018 missense variants (10.75%). The 25 640 downstream variants made up 7.25% of the total number of variants. A total of 21 679 variants (6.13%) were detected in regulatory regions, and 18 143 variants (5.13%) in upstream regions. Collectively, non-coding transcripts, non-coding transcript exon variants and splice site polymorphisms contributed 73 772 variants (20.86%), grouped as other in Figure 1. The variants in coding regions were mainly synonymous and missense variants as depicted in Figure 2. A total of 4 538 novel variants were annotated.

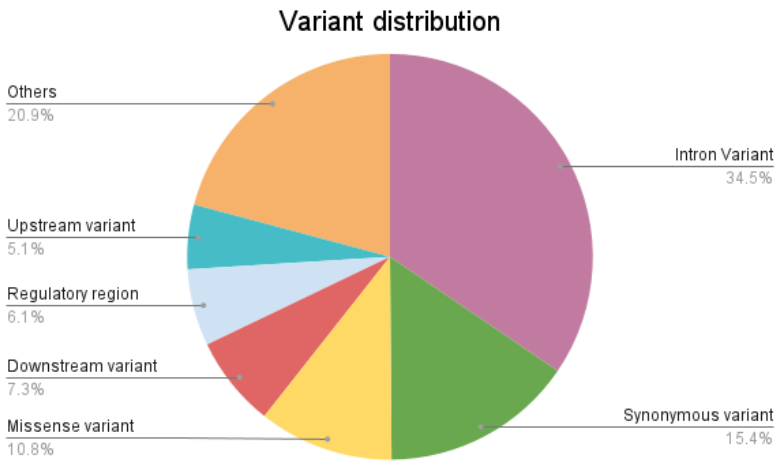


**Figure 1: Distribution of annotated variants**

The majority of the sequence variants annotated were intronic variants, 122 011 intronic variants accounting for 34.5% of all variants, followed by 54 392 synonymous variants (15.38%). Thus approximately half (49.9%) of the annotated variants were filtered out at a very early stage of variant prioritization.

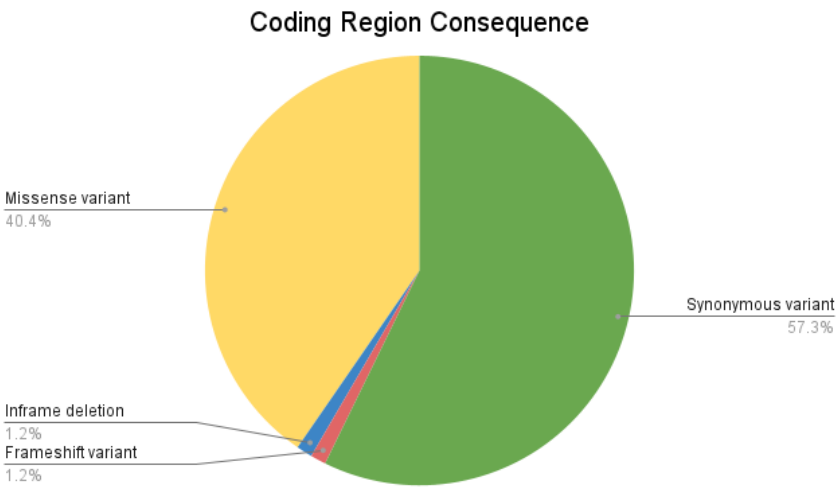


**Figure 2: Distribution of variants in coding regions**

The vast majority of annotated variants in the coding region predicted to have an impact on protein structure and function were 38 018 missense variants

## 4.3. *RB1* sequence variants

All exonic variants in *RB1* are tabulated in appendix C. Two benign *RB1* sequence variants, NM_000321.3:c.1574C>G (NP_000312.2:p.Ala525Gly) and NM_000321.3:c.45_53del (NP_000312.2:p.Ala16_Ala18del) were detected in a single patient, PC43. The beforementioned in-frame deletion of three amino acids NM_000321.3:c.45_53del (NP_000312.2:p.Ala16_Ala18del) was shared in three of the eight patients (PC43, PC50 and PC123). A single missense variant NM_000321.3:c.1574C>G (NP_000312.2:p.Ala525Gly) was found in a single patient (PC26).

## 4.4. Variants of uncertain significance

Variants of interest in each patient classified as variants of uncertain significance are summarized in Table 3, along with the ACMG/AMP codes applied to each variant. All of the variants are classified as variants of uncertain significance (VUS) because the ACMG/AMP codes applied could not categorize the variants as likely benign or likely pathogenic. Variants in blue are shared between individuals in the cohort and are discussed separately. An extended list of variants in cancer predisposing genes associated with RB, MAF < 5% are tabulated in appendix D.

A total of 12 frameshift variants predicted to have a detrimental impact on protein structure and protein folding were detected. All patients carried as least one frameshift variant. Two novel frameshift mutations were classified as VUS. A novel frameshift variant in *PTCH1* NM_001385640.1:c.2386C>T (NP_001372569.1:p.Leu796Phe) resulting in a truncated protein was detected in patient 26 and novel frameshift mutation in *PTCHD3* NM_001034842.4:c.152del, (NP_001030014.2:p.Pro51ArgfsTer69) detected in patient 120 diagnosed at a young age with bilateral tumor presentation.

Two previously reported frameshift variants were detected in a single patient only. Frameshift variant in FUT6, rs150560931, caused by a single nucleotide insertion NM_000150.3:c.501_502insC (NP_000141.1:p.Tyr168LeufsTer230) and a frameshift variant in *CREBBP,* rs587783507, NM_001079846.1:c.5723del (NP_001073315.1:p.Pro1908HisfsTer30). The TSG *MSH3 contained a novel inframe deletion,* NM_002439.5:c.162_179del NP_002430.3:p.Ala57_Ala62del and a previously reported missense variant rs34168832 NM_002439.5:c.1817G>A (NP_002430.3:p.Ser606Asn) in two respective patients.

The vast majority of variants were missense variants, two of which were novel. The novel missense variant were detected in *APC* NM_000038.6:c.1251T>G (NP_000029.2:p.Cys417Trp) and *MBL2* NM_000242.3:c.34_35delinsGG (NP_000233.1:p.Leu12Gly). Three previously reported missense variants were predicted to be damaging by more than one in-silico prediction tool. DNA mismatch repair genes, *MSH3,* rs34168832, NM_002439.5:c.1817G>A (NP_002430.3:p.Ser606Asn) and *MLH1* rs63751244 NM_001167617.3:c.1375G>A (NP_001161089.1:p.Glu459Lys). *UGT1A1,* NM_000463.3:c.22G>A (NP_000454.1:p.Gly8Arg) was also deemed a VUS.

For patient PC43 diagnosed at 18 months, presenting with unilateral RB, a total of 14 variants in cancer predisposing genes were annotated, the greatest number of variants per patient. Twelve out of the fourteen variants were missense variants predicted to have a negligible impact on protein function. By factoring in Clinvar record for the existing missense variants, all were classified as likely benign based on codes PM2 and BP4. The frameshift variant *SAMD11* NM_001385640.1:c.1890_1891del (NP_001372569.1:p.Lys631ArgfsTer71) variant in patient PC43 is shared between individuals in the cohort and deemed a VUS. Patient PC123 also carried the frameshift variant in *SAMD11* NM_001385640.1:c.1890_1891del (NP_001372569.1:p.Lys631ArgfsTer71), along with a missense variant in *HFE* rs28934889. Table 4 contains the ACMG/AMP codes applied to shared variants.

Table 3: Subset of variants of interest as observed per patient

| Gene | Variant HGVSc and Variant HGVSp | rsID | Consequence | Impact [a] | ACMG/AMP [b] |
|---|---|---|---|---|---|
| **PC 26** | | | | | |
| *PTCH1* | NM_000264.5:c.3913del, NP_000255.2:p.Asp1305ThrfsTer67 | novel | frameshift variant | HIGH | VUS (PM2, PP3, PM4) |
| *MLH1* | NM_001167617.3:c.1375G>A, NP_001161089.1:p.Glu459Lys | rs63751244 | missense variant | MODERATE | VUS (PM2, PP3) |
| *UGT1A1* | NM_000463.3:c.22G>A, NP_000454.1:p.Gly8Arg | rs749552053 | missense variant | MODERATE | VUS (PM2, PP3) |
| **PC 43** | | | | | |
| *SAMD11* | NM_001385640.1:c.1890_1891del, NP_001372569.1:p.Lys631ArgfsTer71 | novel | frameshift variant | HIGH | VUS (PM2, PP3,PM4 ) |
| **PC 50** | | | | | |
| *APC* | NM_000038.6:c.1251T>G, NP_000029.2:p.Cys417Trp | - | missense variant | MODERATE | VUS (PM2, PP3) |
| *SMAD5* | NM_001001419.3:c.1313-1_1313insC, NP_001001419.1:p.Asn438ThrfsTer11 | - | frameshift variant | HIGH | VUS (PM2, PP3, PM4) |
| *CREBBP* | NM_001079846.1:c.5723del, NP_001073315.1:p.Pro1908HisfsTer30 | rs587783507 | frameshift variant | HIGH | VUS (PM2, PP3, PM4) |
| **PC 79** | | | | | |
| *SMAD5* | NM_001001419.3:c.1313-1_1313insC, NP_001001419.1:p.Asn438ThrfsTer11 | - | frameshift variant | HIGH | VUS (PM2, PP3, PM4) |
| *MSH 3* | NM_002439.5:c.1817G>A, NP_002430.3:p.Ser606Asn | rs34168832 | missense variant | MODERATE | VUS (PM2, PP3) |
| **PC 90** | | | | | |
| *SAMD11* | NM_001385640.1:c.1890_1891del, NP_001372569.1:p.Lys631ArgfsTer71 | novel | frameshift variant | HIGH | VUS (PM2, PP3, PM4) |
| *SMAD5* | NM_001001419.3:c.1313-1_1313insC, NP_001001419.1:p.Asn438ThrfsTer11 | - | frameshift variant | HIGH | VUS (PM2, PP3, PM4) |
| **PC 104** | | | | | |
| *SAMD11* | NM_001385640.1:c.1890_1891del, NP_001372569.1:p.Lys631ArgfsTer71 | novel | frameshift variant | HIGH | VUS (PM2, PP3, PM4) |
| **PC 120** | | | | | |
| *PTCHD3* | NM_001034842.4:c.152del, NP_001030014.2:p.Pro51ArgfsTer69 | - | frameshift variant | HIGH | VUS (PM2, PP3, PM4) |
| *SMAD5* | NM_001001419.3:c.1313-1_1313insC, NP_001001419.1:p.Asn438ThrfsTer11 | - | frameshift variant | HIGH | VUS (PM2, PP3, PM4) |
| **PC 123** | | | | | |
| *SAMD11* | NM_001385640.1:c.1890_1891del, NP_001372569.1:p.Lys631ArgfsTer71 | novel | frameshift variant | HIGH | VUS (PM2, PP3, PM4) |
| *HFE* | NM_139006.3:c.157G>A, NP_620575.1:p.Val53Met | rs28934889 | missense variant | MODERATE | VUS (PM2, PP3) |

[a] The impact of the variant of protein structure and function is a prediction based on scored collected from multiple in silico prediction tools, including SIFT, POLYPHEN-2 and CADD.

[b] Codes in brackets applied according to ACMG/AMP guidelines to classify variant

-: Previously reported variants without an rsID at the time of interpretation

## 4.5. Shared variants

Several of the genes proposed as candidate genes in RB or paediatric cancer, as mentioned in Table 1 returned variants observed in multiple patients despite the low allele frequency in the general population. Table 4 depicts the shared variants, the frequency at which the mutation occurred in the eight patients in the study cohort and the allele frequency in the general population. Two frameshift variants, *SAMD11* NM_001385640.1:c.1890_1891del (NP_001372569.1:p.Lys631ArgfsTer71) and *SMAD5* NM_001001419.3:c.1313-1_1313insC (NP_001001419.1:p.Asn438ThrfsTer11) were found in half the patients. Coverage and quality of variant calling was evaluated by visualizing BAM files on an IGV. The IGV images of a select few variants are depicted in appendix E to demonstrate the BAM images utilized to assess variant calling quality.

Table 4: Shared variants (SV)

| **Gene** | **Variant HGVSc notation and Variant HGVSp notation** | **Variant frequency [a]** | **MAF [b]** | **rsID** | **ACMG/AMP Classification [c]** |
|---|---|---|---|---|---|
| *HFE* | NM_139006.3:c.157G>A<br>NP_620575.1:p.Val53Met | 2 (25%) | 0.0002 | rs28934889 | VUS (PM2, PP3) |
| *KRT85* | NM_002283.4:c.233G>A<br>NP_002274.1:p.Arg78His | 3 (37.5%) | 0.0328 | rs61630004 | Likely benign (PM2, PP1, BP6) |
| ***SAMD11*** | **NM_001385640.1:c.1890_1891del<br>NP_001372569.1:p.Lys631ArgfsTer71** | 4 (50%) | - | Novel variant | VUS (PM2, PP3, PM4) |
| ***SMAD5*** | **NM_001001419.3:c.1313-1_1313insC<br>NP_001001419.1:p.Asn438ThrfsTer11** | 4 (50%) | - | Novel variant | VUS (PM2, PP3, PM4) |

[a] Variant frequency in cohort of eight RB patients

[b] Minor allele frequency in general population from gnomAD

[c] Codes in brackets applied according to ACMG/AMP guidelines to classify variant

Variants in bold are novel variants predicted to have a deleterious impact on protein structure and function according to in-silico prediction tools, including SIFT, POLYPHEN-2 and CADD.

The majority of variants classified as VUS were missense variants, however the variants predicted to have the largest impact on protein structure and function were the SAMD11 and SMAD5 frameshift variants shared between individuals. Both of the frameshift variants mentioned are novel variants occurring in 50% of the RB population of the study.

## 5. Discussion

The germline genetic contribution to RB aetiology has evolved beyond *RB1,* but little to no investigation has probed into the genetic landscape of RB in South African patients of African ancestry. This study aimed to address this disparity and gap in genomic knowledge by testing a cohort of eight paediatric RB patients with a South African ancestry for germline sequence variants predisposing them to RB malignancy. WES sequencing data uncovered 22 variants in cancer predisposition genes with an uncertain significance and 4 538 novel variants distributed across the genome. The lack of functional and segregation data for the novel variants in cancer predisposition genes reduced them to a VUS classification.

The correlation of the young age of onset with bilateral tumor presentation is characteristic of individuals carrying a germline mutation or "first hit" (Knudson, 1971). This correlation was observed in the cohort, patients PC104 and PC120 both presented with bilateral RB at a young age, exemplifying that the two hit hypothesis still holds true, the presence of a constitutional germline mutation in cancer susceptibility gene can be observed as bilateral RB presentation at a young age. No clear pathogenic or likely pathogenic sequence variant was observed in *RB1* in patients PC104 or PC120. In approximately half of the patients, no exonic sequence variant impacting protein structure was observed in *RB1*, as presented in appendix 3. The absence of likely pathogenic or pathogenic sequence variants in RB1 describe the RB phenotype cements the notion that variants in cancer susceptibility genes beyond *RB1* need to be investigated to determine the full germline mutational spectrum associated with heritable RB. Patient 104, who was diagnosed at 8 months with bilateral RB had a mere two sequence variants of interest in cancer predisposing genes, neither of which explain the RB phenotype.

### 5.1. *RB1* variants

The in-frame deletion NM_000321.3:c.45_53del NP_000312.2:p.Ala16_Ala18del present in three patients, has previously been described (rs572454921) and is predicted to be benign because it result in a negligible change in protein structure. One patient, PC 26 had a *RB1* missense variant rs4151539 (NM_000321.3:c.1574C>G, NP_000312.2:p.Ala525Gly) previously described in the literature as probably pathogenic, in a Brazilian RB cohort that screened for *RB1* mutations in both RB and retinoma patients (Barbosa et al., 2013). As the reporting of variants observed in sequencing data became good scientific practice, conflicting interpretations of this variant emerged. This variant is classified as likely benign.

## 5.2. Shared variants

Variants shared between the patients in the RB cohort at a higher allele frequency than the allele frequency observed in the general population are of interest. The majority of these shared variants are found in genes associated with retinal metabolism and rod photoreceptor development (Gao et al., 2009). The variants observed are yet to be linked to RB with functional studies. The shared variants are depicted in blue and summarized in Table 3, with the ACMG/AMP codes applied.

A VUS in the Homeostatic Iron Regulator (*HFE)* gene was detected in two individuals, previously identified as rs28934889, NM_139006.3:c.157G>A, NP_620575.1:p.Val53Met. Akdeniz and colleagues were the first to propose HFE as a candidate gene in RB (Akdeniz et al., 2019). The *HFE* gene is responsible for iron metabolism in the retina, optimal iron metabolism and regulation prevents oxidative damage due to iron overload (Gnana-Prakasam et al., 2010). The HFE complexed with other proteins regulate iron concentrations in the electron transport chain through the SMAD1/5/8 pathway (Gao et al., 2009).

The *KRT85* rs61630004 (NM_002283.4:c.233G>A NP_002274.1:p.Arg78His) missense mutation observed in three of the eight RB patients has been described in literature as a candidate gene in RB etiology (Akdeniz et al., 2019), suggesting the variant might segregate with disease, leading to the application of ACMG/AMP code *PP1*. A study conducted in Istanbul observed this variant in multiple individuals affected with RB, two of them in the same family, implying that the variant might segregate with disease. Due to the significantly higher frequency of variant in disease cohort compared to the general population ACMG/AMP code PM2 was applied. The prediction of a deleterious effect by an in-silico tool the variant was classified as pathogenic by Akdeniz and colleagues.

The in-silico predictors used has since become outdated, improved versions of the same in-silico tools, SIFT (Ng & Henikoff, 2003) and POLYPHEN-2 (Adzhubei et al., 2010) now predict rs61630004 (NM_002283.4:c.233G>A NP_002274.1:p.Arg78His) to have inconsequential effects on protein structure and function. MutationTaster2021 (Steinhaus et al., 2021) classified this variant as a polymorphism as opposed to a damaging mutation. The application of ACMG/AMP codes *PM2, PP1 and BP6* collectively lead to the current classification of *KRT85* NM_002283.4:c.233G>A NP_002274.1:p.Arg78His as likely benign which is in accordance with Clinvar classification.

The notably high frequency (50%) of a novel frameshift variant in *SAMD11* NM_001385640.1:c.1890_1891del brings the impact of this *SAMD11* on RB predisposition in question. The absence of this variant in population databases prompted the application of the ACMG/AMP code *PM2*. A truncated protein is produced, predicted to have a detrimental effect on protein function, as a result, ACMG/AMP codes *PP3* and *PM4* were

applied. Unfortunately, the genotype-phenotype correlation is difficult to establish. A recent functional study demonstrated that *SAMD11* is involved in establishing rod photoreceptor identity in retinal cells during retinal cell differentiation and development by forming part of the rod-specific polycomb recessive complex (Kubo et al., 2021). Dysregulation of *SAMD11* is one of the numerous genetic causes of X-linked recessive retinitis pigmentosa (Corton et al., 2016).

A second novel frameshift mutation was observed in the *SMAD5,* a gene that similar to *SAMD11* is expressed in retinal tissue. In vivo experiments provide functional proof that *SMAD5 i*s activated to repair retinal cells in response to light damage. Identical to the *SAMD11* variant discussed above, the novel nature of the *SMAD5* variant prompted the application of the *PM2* code. Furthermore, the *PP3* code was applied because in-silico tools predicted this frameshift variant to be deleterious. *SMAD5* is a component of the BMP-SMAD1/5/8 neuroprotective signaling pathway promote survival of retinal ganglion cells (Ueki & Reh, 2012). In half of the patients in the RB cohort, a germline NM_001001419.3:c.1313-1_1313insC variant in *SMAD5* was observed, predicted to cause a lack of function. Future functional studies are needed to speak to whether the inability to repair retinal cells damaged by light can create genomic instability, increasing the likelihood of future mutations and clonal evolution. Both patients PC104 and PC120 presented with an early age of onset and bilateral tumours, suggestive of hereditary RB. The lack of sequence variants capable of rationalizing the RB phenotype shines a light on the limitation of analyzing sequence variants exclusively.

### 5.3. Variants of uncertain significance

The frameshift mutation in CREB Binding Protein (*CREBBP)* NM_001079846.1:c.5723del (NP_001073315.1:p.Pro1908HisfsTer30), produces a truncated protein product. *CREBBP* has been established as a TSG in lymphoma (Zhang et al., 2017) with haploinsufficient function. Loss of function of *CREBBP* is the autosomal dominant genetic cause of Rubinstein-Taybi syndrome, a neurodevelopmental disorder associated with a predisposition to developing tumours of the nervous system (Roelfsema & Peters, 2007). Somatic *CREBBP* loss of function mutations and downregulation have been proven to be putative drivers in RB tumorigenesis (Kooi et al., 2016), however the impact of germline deleterious mutations in *CREBBP* on RB aetiology remains unknown, this variant has not been reported in combination with RB.

PC 50, diagnosed with unilateral RB at the age of 4 years, presented with a germline variant in the Adenomatous polyposis coli gene (*APC)* NM_000038.6:c.1251T>G, NP_000029.2:p.Cys417Trp, TSG inactivated in a large proportion of colorectal cancers (Sparks et al., 1998), a substructure of *APC* called *APC2* has been established as a TSG in RB (Singh et al., 2016; Beta et al., 2015).Wild-type *APC* antagonizes the WNT signaling pathway, to negatively regulate cell growth. Mutations in genes regulating the WNT signaling

pathway have been linked to vitreoretinopathies like retinopathy of prematurity (Drenser, 2016). Case studies have been reported where patients with regressed retinopathy developed RB after approximately 4 years, but due to the small sample size, a causative link between retinopathy and RB could not be established with adequate statistical power (Vasu et al., 2011). The late age of onset and unilateral RB presentation in PC 50 are indicative of sporadic RB, the absence of a driver mutation is reasonable.

From the beforementioned study by Singh and colleagues, defective mismatch repair was implicated in RB (Singh et al., 2016) by increasing genomic instability. Two DNA mismatch repair genes, *MLH1* and *MSH3*, observed in two patients, PC26 and PC79 respectively, returned variants deemed as variants of uncertain significance.

Patient PC26 presented with a frameshift mutation in Patched gene 1 (*PTCH1*), predicted to be deleterious by in-silico prediction tools (ACMG/AMP code *PP3* applied). *PTCH1* is a TSG implicated in central nervous system cancers such as medulloblastoma (Skowron et al., 2021) and carcinoma due to dysregulation of the sonic hedgehog signaling pathway leading to inappropriate cellular proliferation (Doheny et al., 2020).

The patient predicted to be genetically predisposed to RB malignancy due to early age of onset and bilateral tumours, patient (PC120) presented with a novel frameshift variant in *PTCHD3* NM_001034842.4:c.152del, NP_001030014.2:p.Pro51ArgfsTer69 where a truncated protein is produced. When investigating the germline mutational spectrum of predisposing genes implicated in colorectal cancer, *PTCHD3* was proposed as a candidate predisposition gene that may serve as a TSG (Smith et al., 2013).

## 5.4. Future prospects and limitations

The majority of final variant classifications were VUS. In the case that functional data and segregation data becomes available for a variant classified as VUS, the variant will be revisited and reclassified in accordance with ACMG/AMP guidelines. The inability to find previously reported driver mutation in this cohort, especially in the patients with a young age of onset and bilateral RB, brings the utility of analyzing only sequence variants from WES data in question. This study had several limitations, mainly the failure of WES to detect large balanced structural anomalies and large deletions and duplications.

The addition of copy number variation assays such as multiplex ligation-dependent probe amplification (MLPA) (Livide et al., 2012) or chromosomal microarrays (CMA) can complement WES sequencing data, producing a more comprehensive picture of all germline variants present in an individual. Epigenetic changes such as promotor methylation (McEvoy & Dyer, 2015) and non-coding RNA molecules (Plousiou & Vannini, 2019) that contribute to RB etiology went undetected when sequencing on a genomic level.

The underrepresentation of African populations in cancer genomics cohorts inevitably complicates variant interpretation, exemplified by the numerous novel variants found in this study. Cancer is an extremely heterogenous disease. The possibility of patients carrying a germline variant in genes not included in the curated list (Table 1) cannot be discounted. The study cohort included patients of both sexes, where the age of onset varied widely, and RB presentation was unilateral in some and bilateral in others. As the majority of RB cases are sporadic, it is reasonable to assume that some patients will not carry a predisposing constitutive variant.

## 6. Conclusion

The utmost degree of sequence variant classification according to the ACMG/AMP guidelines was variant of unknown significance. The inability to link the RB phenotype of the testing cohort to a germline sequence variant in the genes previously associated with RB supports the beyond *RB1* hypothesis. Although *RB1* is likely to remain the most likely driver in RB etiology, a wider testing strategy including CNV will yield more informative results. Understanding the full mutational landscape of RB will aid in the construction of appropriate treatment plans for RB patients and genetic counselling for family members.

Variant classification in understudied and underrepresented populations, like the black South African population is hampered by the lack of information available. The genomic data currently available is a far cry from being representative of a genetically diverse country like South Africa. This study adds to the ever-growing collection of variants found in the black South African population, a critical set piece to expanding the genomic research resources in South Africa.

**Competing interests**

The authors declare that they have no competing interests.

**Statement on data availability**

Data is not available in public domain at present. All variants will be made available on Clinvar https://www.ncbi.nlm.nih.gov/clinvar/ .

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## Appendices

**Appendix A:**

Human Research Ethics Committee (Medical), University of the Witwatersrand Ethics Certificate (M180855)

**Appendix B:**

Human Research Ethics Committee (Medical), University of the Witwatersrand Ethics Certificate (M230782)

**Appendix C:**

*RB1* sequence variants in all patients

**Appendix D**

Extended list of variants of interest

**Appendix E**

BAM images to evaluate variant coverage

**Appendix F:**

Information, Consent and Assent forms

**Appendix G:**

Title Approval and Approved Research Protocol

**Appendix H:**

Plagiarism declaration and Turnitin report

**Appendix I:**

Journal Author Guidelines for Submissible format

R14/49 Dr L Lamola, et al

# HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
## CLEARANCE CERTIFICATE NO. M180855

**NAME:** Dr L Lamola, et al
**(Principal Investigator)**
**DEPARTMENT:** School of Pathology
Department of Human Genetics
National Health Laboratory Service

**PROJECT TITLE:** Inherited cancer predisposing mutations in a cohort of childhood cancer patients

**DATE CONSIDERED:** 31/08/2018

**DECISION:** Approved unconditionally

**CONDITIONS:**

**SUPERVISOR:** Not applicable

**APPROVED BY:** CB Penny
Dr CB Penny, Chairperson, HREC (Medical)

**DATE OF APPROVAL:** 26/02/2019

**This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.**

**DECLARATION OF INVESTIGATORS**

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary on 3rd floor, Phillip V Tobias Building, Parktown, University of the Witwatersrand, Johannesburg.

I/We fully understand the conditions under which I am/we are authorised to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated from the research protocol as approved, I/we undertake to resubmit to the Committee. **I agree to submit a yearly progress report**. When a funder requires annual re-certification, the application date will be one year after the date of the meeting when the study was initially reviewed. In this case, the study was initially reviewed in **August** and will therefore reports and re-certification will be due early in the month of **August** each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature

26/02/2019
Date

**PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES**

UNIVERSITY OF THE
WITWATERSRAND,
JOHANNESBURG

R49 Ms D Beukman

# HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
## CLEARANCE CERTIFICATE NO. M230782

| | |
|---|---|
| **NAME:** (Principal Investigator) | Ms D Beukman |
| **DEPARTMENT:** | School of Pathology<br>Division of Human Genetics<br>Medical School<br>University |
| **PROJECT TITLE:** | *Beyond RB1: Determining genetic causes of retinablastoma in South African patients* |
| **DATE CONSIDERED:** | Ad hoc |
| **DECISION:** | Approved unconditionally |
| **CONDITIONS:** | Sub-study under M18/08/55 |
| **NOTE:** | If contact information regarding student study participants is required, please contact the Registrar's office - <Nicoleen.Potgieter@wits.ac.za> |
| **SUPERVISOR:** | Dr L Lamola; Ms N Mayevu |
| **APPROVED BY:** | Professor P Ruff, Chairperson, HREC (Medical) |
| **DATE OF APPROVAL:** | 2023/08/18 **EXPIRY DATE:** 2028/08/17 |

**This Clearance Certificate is valid for 5 years from the date of approval. An extension may be applied for.**

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**DECLARATION OF INVESTIGATORS**

To be completed in duplicate and **ONE COPY** returned to the Research Office secretariat on the 3rd floor, Phillip Tobias Building, Parktown, University of the Witwatersrand, Johannesburg.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated from the research protocol as approved, I/we undertake to submit details to the Committee. **I agree to submit a yearly progress report**. When a funder requires annual re-certification, the application date will be one year after the date when the study was initially reviewed. In this case, the study was initially reviewed in **July** and therefore reports and re-certification will be due in the month of **July** each year. Unreported changes to the study may invalidate the clearance given by the HREC (Medical).

Signature of Principal Investigator _____ Date _____

**Appendix C:**

*RB1* sequence variants in all patients

Appendix 3 Table 5: *RB1* Exonic variants

| Location | Consequence | HGVSc | HGVSp | Existing variant | SIFT | Polyphen-2 | CADD_ PHRED | AF [a] | CLIN_SIG [b] |
|---|---|---|---|---|---|---|---|---|---|
| **PC26** | | | | | | | | | |
| 13:48955458-48955458 | missense variant | NM_000321.3:c.1574C>G | NP_000312.2:p.Ala525Gly | rs4151539 | tolerated(0.07) | probably damaging(0.962) | 24.7 | 0.0062 | benign/likely benign, |
| **PC43** | | | | | | | | | |
| 13:48878084-48878093 | Inframe deletion | NM_000321.3:c.45_53del | NP_000312.2:p.Ala16_Ala18del | rs572454921 | | | 13.38 | 0.0108 | benign |
| 13:48955516-48955516 | synonymous | NM_000321.3:c.1632A>G | NP_000312.2:p.Arg544%3D | rs143948310 | | | 12.48 | 0.0020 | Benign, likely benign |
| **PC50** | | | | | | | | | |
| 13:48878084-48878093 | Inframe deletion | NM_000321.3:c.45_53del | NP_000312.2:p.Ala16_Ala18del | rs572454921 | | | 13.38 | 0.0108 | benign |
| **PC79** No exonic variants | | | | | | | | | |
| **PC90** No exonic variants | | | | | | | | | |
| **PC104** No exonic variants | | | | | | | | | |
| **PC120** No exonic variants | | | | | | | | | |
| **PC123** | | | | | | | | | |
| 13:48878084-48878093 | Inframe deletion | NM_000321.3:c.45_53del | NP_000312.2:p.Ala16_Ala18del | rs572454921 | | | 13.38 | 0.0108 | benign |

All exonic sequence variants in *RB1* with a minor allele frequency lower than 5 %.

[a] Allele frequency as reported in gnomAD

[b] Clinical significance as reported in Clinvar and ACMG/AMP variant classification codes

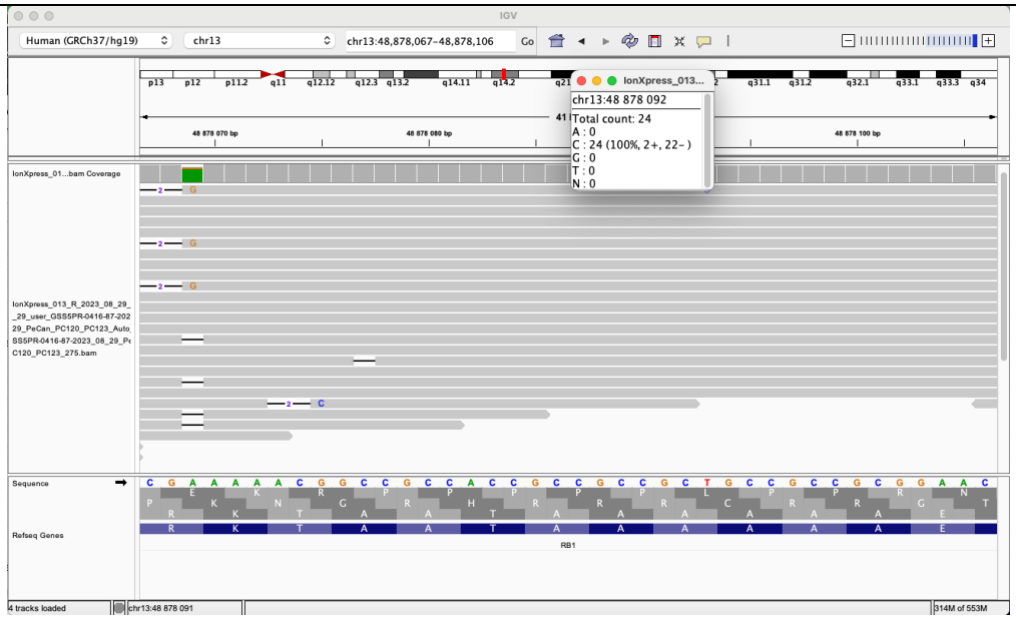
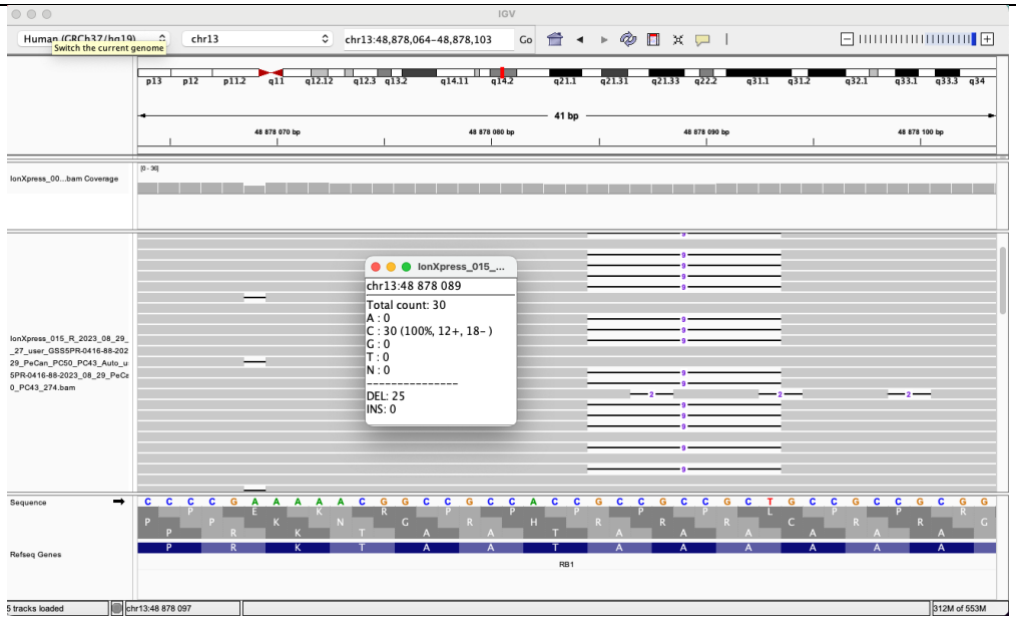
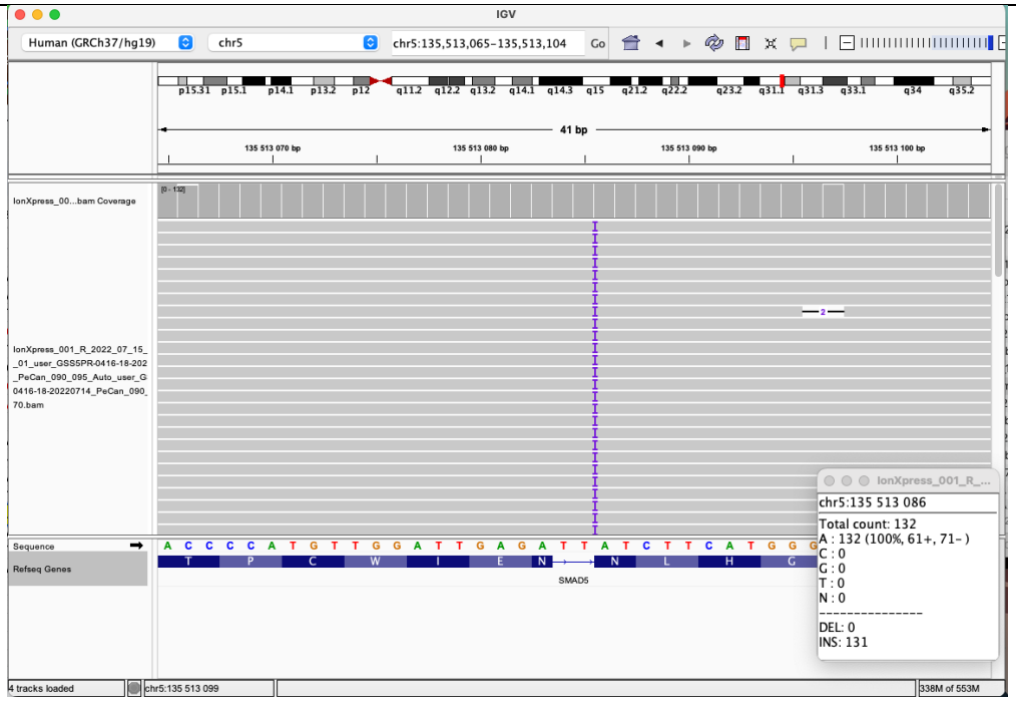
## Appendix D: Extended list of variants of interest

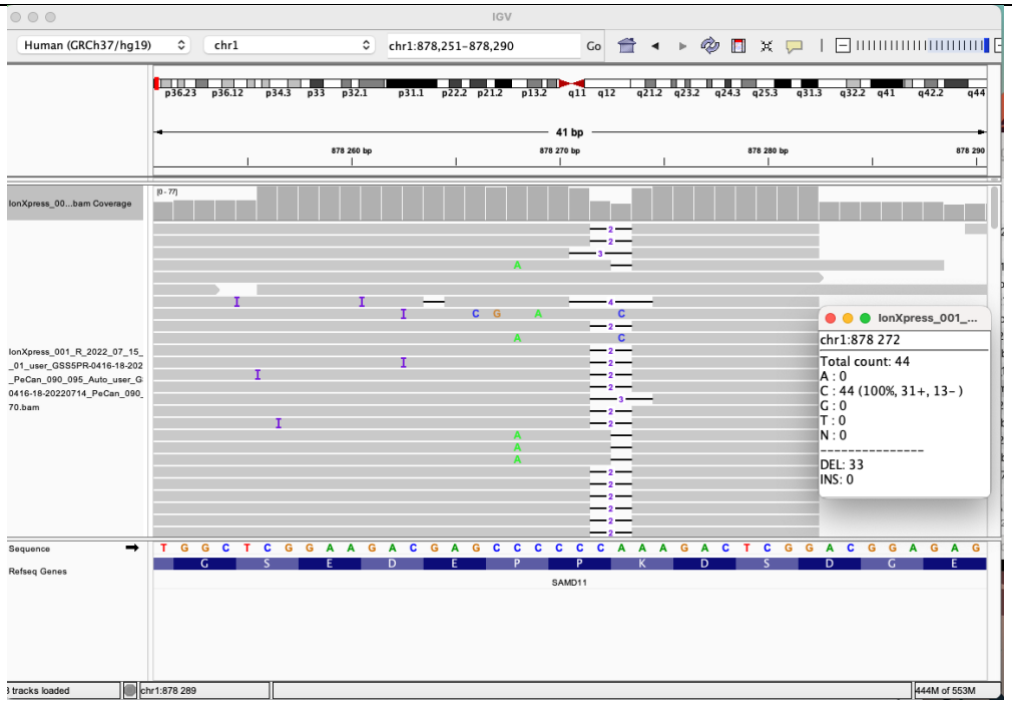
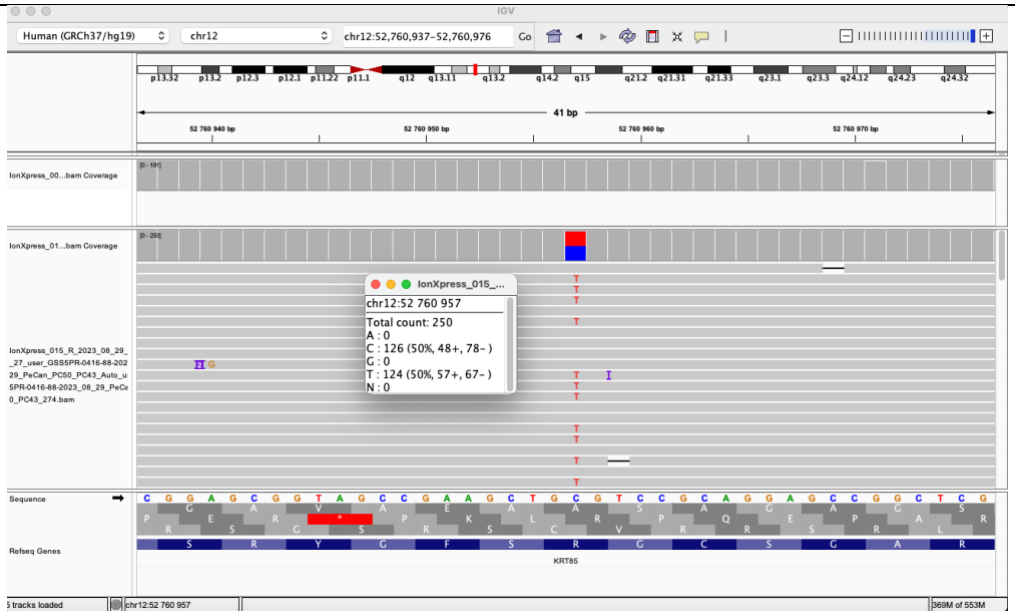
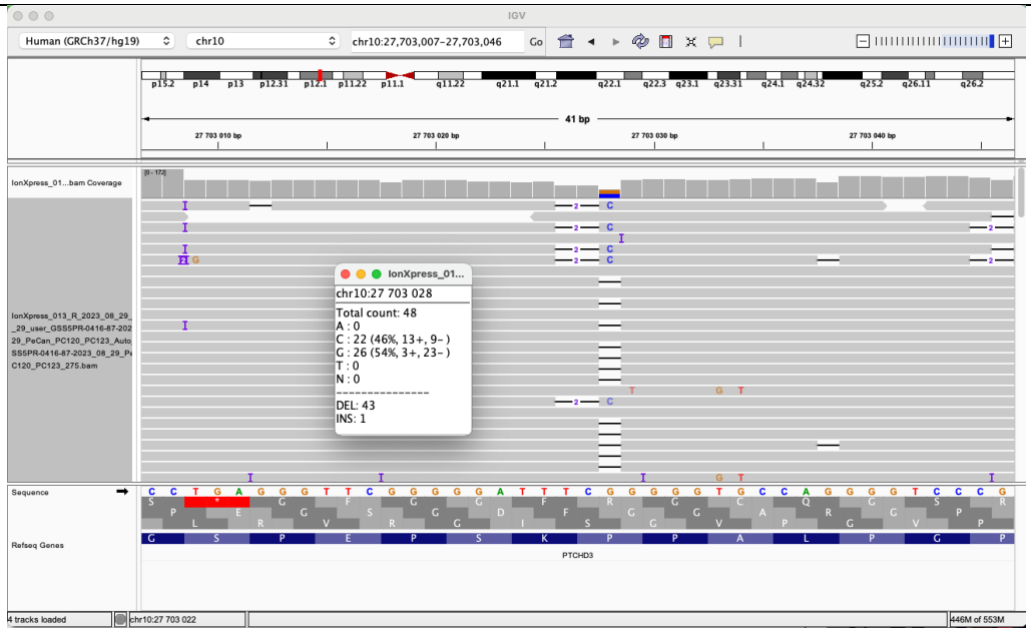
| | A | B | C | D | E | F | G | H | I | J |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | **Appendix D** | | | | | | | | | |
| 2 | Location | Allele | Consequence | IMPACT | SYMBOL | Gene | HGVSc | HGVSp | cDNA_position | Existing_variation |
| 3 | **PC26** | | | | | | | | | |
| 4 | 1:879381-879381 | T | missense_variant | MODERATE | SAMD11 | 148398 | NM_001385640.1:c.2386C>T | NP_001372569.1:p.Leu796Phe | 2895 | rs116362966 |
| 5 | 13:48955458-48955458 | G | missense_variant | MODERATE | RB1 | 5925 | NM_000321.3:c.1574C>G | NP_000312.2:p.Ala525Gly | 1736 | rs4151539 |
| 6 | 5:112173899-112173899 | T | missense_variant | MODERATE | APC | 324 | NM_000038.6:c.2608C>T | NP_000029.2:p.Pro870Ser | 2667 | rs33974176 |
| 7 | 9:98209623-98209625 | GT | frameshift_variant | HIGH | PTCH1 | 5727 | NM_000264.5:c.3913del | NP_000255.2:p.Asp1305ThrfsTer67 | 4818-4820 | - |
| 8 | 12:52760957-52760957 | T | missense_variant | MODERATE | KRT85 | 3891 | NM_002283.4:c.233G>A | NP_002274.1:p.Arg78His | 309 | rs61630004 |
| 9 | 19:5831602-5831602 | T | missense_variant | MODERATE | FUT6 | 2528 | NM_000150.3:c.977G>A | NP_000141.1:p.Arg326Gln | 1233 | rs61739552 |
| 10 | 19:5832077-5832077 | G | frameshift_variant | HIGH | FUT6 | 2528 | NM_000150.3:c.501_502insC | NP_000141.1:p.Tyr168LeufsTer230 | 757-758 | rs150560931 |
| 11 | 14:21811196-21811196 | G | missense_variant,splic | MODERATE | RPGRIP1 | 57096 | NM_001377523.1:c.1319A>G | NP_001364452.1:p.Asp440Gly | 1385 | rs17103671 |
| 12 | 3:37083760-37083760 | A | missense_variant,splic | MODERATE | MLH1 | 4292 | NM_001167617.3:c.1375G>A | NP_001161089.1:p.Glu459Lys | 1921 | rs63751244 |
| 13 | 2:234668955-234668955 | A | missense_variant | MODERATE | UGT1A1 | 54658 | NM_000463.3:c.22G>A | NP_000454.1:p.Gly8Arg | 40 | rs749552053 |
| 14 | 5:79950727-79950736 | - | inframe_deletion | MODERATE | MSH3 | 4437 | NM_002439.5:c.195_203del | NP_002430.3:p.Pro67_Pro69del | 258-266 | rs60484572 |
| 15 | 22:29130456-29130456 | A | missense_variant | MODERATE | CHEK2 | 11200 | NM_001005735.2:c.254C>T | NP_001005735.1:p.Pro85Leu | 312 | rs17883862 |
| 16 | **PC43** | | | | | | | | | |
| 17 | 1:878271-878273 | - | frameshift_variant | HIGH | SAMD11 | 148398 | NM_001385640.1:c.1890_1891 | NP_001372569.1:p.Lys631ArgfsTer71 | 2399-2400 | - |
| 18 | 1:879481-879481 | C | missense_variant | MODERATE | SAMD11 | 148398 | NM_001385640.1:c.2486G>C | NP_001372569.1:p.Gly829Ala | 2995 | rs113383096 |
| 19 | 13:48878084-48878093 | - | inframe_deletion | MODERATE | RB1 | 5925 | NM_000321.3:c.45_53del | NP_000312.2:p.Ala16_Ala18del | 199-207 | rs572454921 |
| 20 | 16:3778363-3778363 | T | missense_variant | MODERATE | CREBBP | 1387 | NM_004380.3:c.6685G>A | NP_004371.2:p.Gly2229Ser | 7482 | rs139688311 |
| 21 | 3:39307063-39307063 | A | missense_variant | MODERATE | CX3CR1 | 1524 | NM_001171172.2:c.938C>T | NP_001164643.1:p.Ala313Val | 1140 | rs137947370 |
| 22 | 4:88533730-88533730 | C | missense_variant | MODERATE | DSPP | 1834 | NM_014208.3:c.392T>C | NP_055023.2:p.Ile131Thr | 512 | rs61731009 |
| 23 | 12:52760957-52760957 | T | missense_variant | MODERATE | KRT85 | 3891 | NM_002283.4:c.233G>A | NP_002274.1:p.Arg78His | 309 | rs61630004 |
| 24 | 10:54531361-54531362 | CC | missense_variant | MODERATE | MBL2 | 4153 | NM_000242.3:c.34_35delinsGG | NP_000233.1:p.Leu12Gly | 51-52 | - |
| 25 | 14:21816432-21816432 | A | missense_variant | MODERATE | RPGRIP1 | 57096 | NM_001377523.1:c.1697G>A | NP_001364452.1:p.Gly566Glu | 1763 | rs34725281 |
| 26 | 16:2133726-2133726 | T | missense_variant | MODERATE | TSC2 | 7249 | NM_000548.5:c.3914C>T | NP_000539.2:p.Pro1305Leu | 4024 | rs45517320 |
| 27 | 13:32953529-32953529 | T | missense_variant | MODERATE | BRCA2 | 675 | NM_000059.4:c.8830A>T | NP_000050.3:p.Ile2944Phe | 9029 | rs4987047 |
| 28 | 3:37092025-37092025 | T | missense_variant | MODERATE | MLH1 | 4292 | NM_001258271.2:c.1945C>T | NP_001245200.1:p.His649Tyr | 1975 | rs2020873 |
| 29 | 2:234545330-234545330 | T | missense_variant | MODERATE | UGT1A10 | 54575 | NM_019075.2:c.162G>T | NP_061948.1:p.Glu54Asp | 208 | rs148859354 |
| 30 | 16:2097712-2097712 | A | missense_variant,splic | MODERATE | NTHL1 | 4913 | NM_002528.7:c.113C>T | NP_002519.2:p.Ala38Val | 124 | rs202082304 |
| 31 | **PC50** | | | | | | | | | |
| 32 | 1:879481-879481 | C | missense_variant | MODERATE | SAMD11 | 148398 | NM_001385640.1:c.2486G>C | NP_001372569.1:p.Gly829Ala | 2995 | rs113383096 |
| 33 | 13:48878084-48878093 | - | inframe_deletion | MODERATE | RB1 | 5925 | NM_000321.3:c.45_53del | NP_000312.2:p.Ala16_Ala18del | 199-207 | rs572454921 |
| 34 | 5:112154980-112154980 | G | missense_variant | MODERATE | APC | 324 | NM_000038.6:c.1251T>G | NP_000029.2:p.Cys417Trp | 1310 | - |
| 35 | 5:135513085-135513085 | C | frameshift_variant,spli | HIGH | SMAD5 | 4090 | NM_001001419.3:c.1313-1_131 | NP_001001419.1:p.Asn438ThrfsTer11 | 1758-1759 | - |
| 36 | 12:52760957-52760957 | T | missense_variant | MODERATE | KRT85 | 3891 | NM_002283.4:c.233G>A | NP_002274.1:p.Arg78His | 309 | rs61630004 |
| 37 | 17:41243800-41243800 | T | missense_variant | MODERATE | BRCA1 | 672 | NM_007294.4:c.3748G>A | NP_009225.1:p.Glu1250Lys | 3861 | rs28897686 |
| 38 | 16:3779210-3779217 | GGGGGG | frameshift_variant | HIGH | CREBBP | 1387 | NM_001079846.1:c.5723del | NP_001073315.1:p.Pro1908HisfsTer30 | 5921-5927 | rs587783507 |
| 39 | **PC79** | | | | | | | | | |
| 40 | 4:88534326-88534326 | A | missense_variant | MODERATE | DSPP | 1834 | NM_014208.3:c.988G>A | NP_055023.2:p.Ala330Thr | 1108 | rs201942511 |
| 41 | 9:98231233-98231233 | T | missense_variant | MODERATE | PTCH1 | 5727 | NM_001083606.3:c.1597G>A | NP_001077075.1:p.Glu533Lys | 2048 | rs62637629 |
| 42 | 5:135513085-135513085 | C | frameshift_variant,spli | HIGH | SMAD5 | 4090 | NM_001001419.3:c.1313-1_131 | NP_001001419.1:p.Asn438ThrfsTer11 | 1758-1759 | - |
| 43 | 12:52758810-52758810 | T | missense_variant | MODERATE | KRT85 | 3891 | NM_002283.4:c.565G>A | NP_002274.1:p.Asp189Asn | 641 | rs112554450 |
| 44 | 6:49587034-49587034 | G | missense_variant | MODERATE | RHAG | 6005 | NM_000324.3:c.199T>C | NP_000315.2:p.Phe67Leu | 226 | rs116356543 |
| 45 | 2:47637246-47637246 | G | missense_variant | MODERATE | MSH2 | 4436 | NM_000251.3:c.380A>G | NP_000242.1:p.Asn127Ser | 416 | rs17217772 |

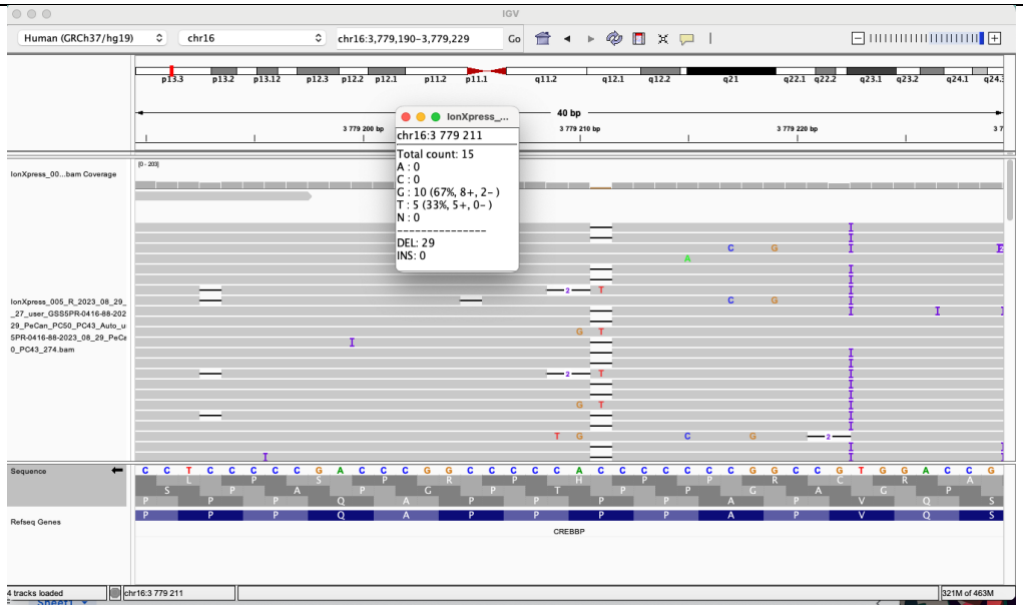
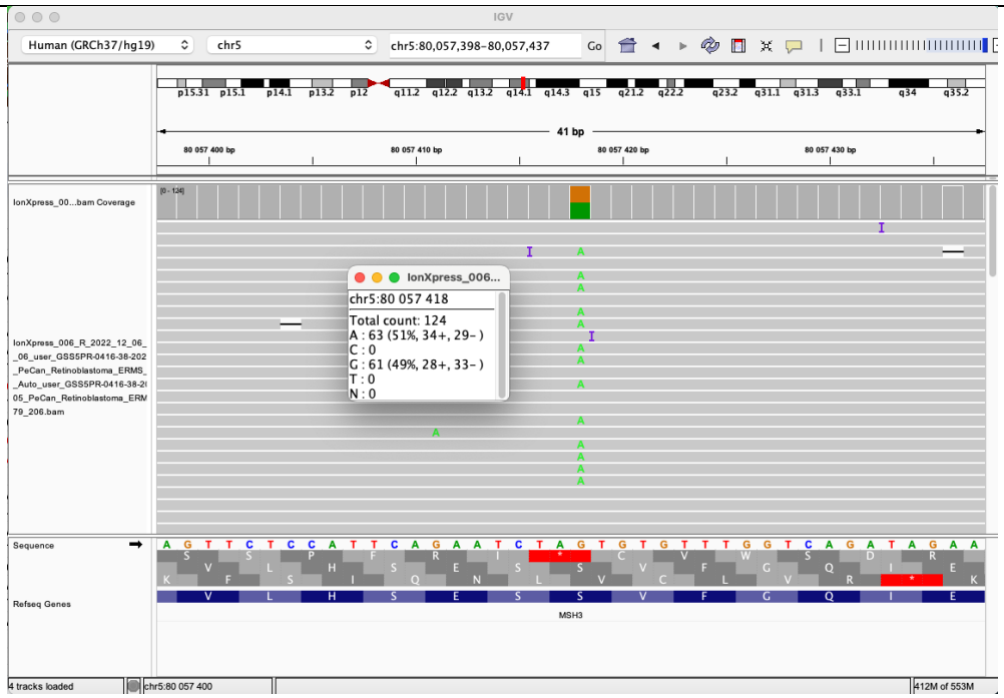
| | A | B | C | D | E | F | G | H | I | J |
|---|---|---|---|---|---|---|---|---|---|---|
| 46 | 5:80057418-80057418 | A | missense_variant | MODERATE | MSH3 | 4437 | NM_002439.5:c.1817G>A | NP_002430.3:p.Ser606Asn | 1893 | rs34168832 |
| 47 | **PC90** | | | | | | | | | |
| 48 | 1:878271-878273 | - | frameshift_variant | HIGH | SAMD11 | 148398 | NM_001385640.1:c.1890_1891 | NP_001372569.1:p.Lys631ArgfsTer71 | 2399-2400 | - |
| 49 | 7:138430019-138430019 | A | missense_variant | MODERATE | ATP6V0A4 | 50617 | NM_020632.3:c.1327A>T | NP_065683.2:p.Asn443Tyr | 1610 | rs542662856 |
| 50 | 4:88534140-88534140 | T | missense_variant | MODERATE | DSPP | 1834 | NM_014208.3:c.802G>T | NP_055023.2:p.Gly268Trp | 922 | rs61738508 |
| 51 | 5:135513085-135513085 | C | frameshift_variant,spli | HIGH | SMAD5 | 4090 | NM_001001420.3:c.1313-1_131 | NP_001001420.1:p.Asn438ThrfsTer11 | 1599-1600 | - |
| 52 | 19:5832203-5832203 | A | missense_variant | MODERATE | FUT6 | 2528 | NM_000150.3:c.376C>T | NP_000141.1:p.Arg126Trp | 632 | rs111589452 |
| 53 | 12:52758810-52758810 | T | missense_variant | MODERATE | KRT85 | 3891 | NM_002283.4:c.565G>A | NP_002274.1:p.Asp189Asn | 641 | rs112554450 |
| 54 | 17:41243502-41243502 | A | missense_variant | MODERATE | BRCA1 | 672 | NM_007300.4:c.4046C>T | NP_009231.2:p.Thr1349Met | 4159 | rs80357345 |
| 55 | **PC104** | | | | | | | | | |
| 56 | 1:878271-878273 | - | frameshift_variant | HIGH | SAMD11 | 148398 | NM_001385640.1:c.1890_1891 | NP_001372569.1:p.Lys631ArgfsTer71 | 2399-2400 | - |
| 57 | 5:79950707-79950725 | - | inframe_deletion | MODERATE | MSH3 | 4437 | NM_002439.5:c.162_179del | NP_002430.3:p.Ala57_Ala62del | 238-255 | - |
| 58 | **PC120** | | | | | | | | | |
| 59 | 5:112173899-112173899 | T | missense_variant | MODERATE | APC | 324 | NM_000038.6:c.2608C>T | NP_000029.2:p.Pro870Ser | 2667 | rs33974176 |
| 60 | 4:88533730-88533730 | C | missense_variant | MODERATE | DSPP | 1834 | NM_014208.3:c.392T>C | NP_055023.2:p.Ile131Thr | 512 | rs61731009 |
| 61 | 6:47846362-47846362 | C | missense_variant | MODERATE | PTCHD4 | 442213 | NM_001013732.4:c.2218A>G | NP_001013754.3:p.Thr740Ala | 2393 | rs113836634 |
| 62 | 10:27703027-27703028 | - | frameshift_variant | HIGH | PTCHD3 | 374308 | NM_001034842.4:c.152del | NP_001030014.2:p.Pro51ArgfsTer69 | 249 | - |
| 63 | 5:135513085-135513085 | C | frameshift_variant,spli | HIGH | SMAD5 | 4090 | NM_001001419.3:c.1313-1_131 | NP_001001419.1:p.Asn438ThrfsTer11 | 1758-1759 | - |
| 64 | 12:69218490-69218490 | T | intron_variant | MODIFIER | MDM2 | 4193 | NM_001145337.3:c.505+59G>T | - | - | rs148010327 |
| 65 | 13:32953529-32953529 | T | missense_variant | MODERATE | BRCA2 | 675 | NM_000059.4:c.8830A>T | NP_000050.3:p.Ile2944Phe | 9029 | rs4987047 |
| 66 | 2:234545898-234545898 | A | missense_variant | MODERATE | UGT1A10 | 54575 | NM_019075.2:c.730C>A | NP_061948.1:p.Leu244Ile | 776 | rs28969685 |
| 67 | 16:2096377-2096377 | C | splice_polypyrimidine_ | LOW | NTHL1 | 4913 | NM_001318193.2:c.116-10C>G | - | - | rs3211970 |
| 68 | **PC123** | | | | | | | | | |
| 69 | 1:865584-865584 | A | missense_variant | MODERATE | SAMD11 | 148398 | NM_001385640.1:c.659G>A | NP_001372569.1:p.Arg220Gln | 1168 | rs148711625 |
| 70 | 1:878271-878273 | - | frameshift_variant | HIGH | SAMD11 | 148398 | NM_001385640.1:c.1890_1891 | NP_001372569.1:p.Lys631ArgfsTer71 | 2399-2400 | - |
| 71 | 1:879381-879381 | T | missense_variant | MODERATE | SAMD11 | 148398 | NM_001385640.1:c.2386C>T | NP_001372569.1:p.Leu796Phe | 2895 | rs116362966 |
| 72 | 13:48878084-48878093 | - | inframe_deletion | MODERATE | RB1 | 5925 | NM_000321.3:c.45_53del | NP_000312.2:p.Ala16_Ala18del | 199-207 | rs572454921 |
| 73 | 6:36651889-36651889 | T | missense_variant | MODERATE | CDKN1A | 1026 | NM_000389.5:c.11C>T | NP_000380.1:p.Pro4Leu | 101 | rs4986866 |
| 74 | 4:88533730-88533730 | C | missense_variant | MODERATE | DSPP | 1834 | NM_014208.3:c.392T>C | NP_055023.2:p.Ile131Thr | 512 | rs61731009 |
| 75 | 5:176524540-176524540 | T | missense_variant | MODERATE | FGFR4 | 2264 | NM_001291980.2:c.2068C>T | NP_001278909.1:p.Arg690Cys | 2240 | rs148143006 |
| 76 | 6:26091149-26091149 | A | missense_variant | MODERATE | HFE | 3077 | NM_139006.3:c.157G>A | NP_620575.1:p.Val53Met | 169 | rs28934889 |
| 77 | 6:26091149-26091149 | A | missense_variant | MODERATE | HFE | 3077 | NM_139009.3:c.88G>A | NP_620578.1:p.Val30Met | 100 | rs28934889 |
| 78 | 9:37006494-37006494 | T | missense_variant | MODERATE | PAX5 | 5079 | NM_001280552.2:c.451G>A | NP_001267481.1:p.Val151Ile | 688 | rs115889954 |
| 79 | 14:94847262-94847262 | A | missense_variant | MODERATE | SERPINA1 | 5265 | NM_001127700.2:c.863A>T | NP_001121172.1:p.Glu288Val | 1083 | rs17580 |
| 80 | 16:2134418-2134418 | A | missense_variant | MODERATE | TSC2 | 7249 | NM_001114382.3:c.4126G>A | NP_001107854.1:p.Gly1376Arg | 4236 | rs45466399 |

**Appendix E**

BAM images to evaluate variant coverage

| | |
|---|---|
| *RB1* Missense variant<br>(Likely benign)<br>NM_000321.3:c.1574C>G<br>NP_000312.2:p.Ala525Gly |  |
| *RB1* Inframe deletion (Benign)<br>NM_000321.3:c.45_53del<br>NP_000312.2:p.Ala16_Ala18del |  |
| *SMAD5* insertion<br>Frame shift variant<br>(VUS)<br><br>NM_001001419.3:c.1313-1_1313insC<br>NP_001001419.1:p.Asn438ThrfsTer11 |  |

| | |
|---|---|
| *SAMD11* deletion<br>Frame shift variant (VUS)<br><br>NM_001385640.1:c.1890_1891del<br>NP_001372569.1:p.Lys631ArgfsTer71 |  |
| *KRT85* Missense variant<br>(Likely benign)<br>NM_002283.4:c.233G>A<br>NP_002274.1:p.Arg78His |  |
| *PTCHD3* deletion<br>Frame shift variant (VUS)<br><br>NM_001034842.4:c.152del<br>NP_001030014.2:p.Pro51ArgfsTer69 |  |

| | |
|---|---|
| *CREBBP* deletion<br>Frameshift variant (VUS)<br>NM_001079846.1:c.5723del<br>NP_001073315.1:p.Pro1908HisfsTer30 |  |
| *MSH3* Missense variant<br>(VUS)<br>NM_002439.5:c.1817G>A<br>NP_002430.3:p.Ser606Asn |  |

**Appendix F:**

Information, Consent and Assent forms

NATIONAL HEALTH LABORATORY SERVICE
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DIVISION OF HUMAN GENETICS
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Protocol: M180855

***Inherited cancer predisposing mutations in a cohort of childhood cancer patients***

**CONSENT FORM – PARENTAL CONSENT FOR CHILD PARTICIPANTS**

**PARTICIPANT STUDY ID:**

The purpose of this study, procedures to be followed, risks and benefits, have been explained to me and my child. I have been allowed to ask questions, and my questions have been answered to my satisfaction. I have been told whom to contact if I have questions, to discuss problems, concerns, or suggestions related to the research, or to obtain information or offer input about the research. I have read the attached information sheet this and agree to allow my child to participate in this research study with the understanding that I may withdraw my child at any time. To the best of my ability, I have discussed the study with my child, who agrees to be in the study. I have explained to my child that he/she may approach the researchers to be re-consented when he/she turns 18 if he/she wishes and that he/she may withdraw from the study at any time. I have been told that I will be given a signed and dated copy of this consent form if I would like one.

| | **Please initial** |
|---|---|
| I understand that the purpose of this consent form is for me to inform the study what they can or cannot do with my child's samples, clinical information and photos. | |
| I understand that all procedures/tests on the samples have been or will be approved by the Human Research Ethics Committee of the University of the Witwatersrand. I understand that every time a new study is done on my child's sample, permission will be obtained from this Ethics Committee for the study to make sure that it is used only for the purposes stated above. | |
| I understand that if I have any questions at any time, they will be answered. | |
| I understand that confidentiality will be maintained at all times through the use of a code and numbering system. | |
| I understand that I may withdraw my child from the study at any time. | |
| I have discussed the study with my child. | |

| | |
|---|---|
| I am in agreement that my child's samples may be stored and used for the purposes described in the information sheet. | |
| I understand that I can choose to have my child's sample destroyed at any time. | |
| I understand that my child may not benefit directly from the research done on their sample. | |
| | |
| If this research does identify information that is relevant to my child's cancer, I would like to be contacted to receive the information. | Yes ☐ No ☐ |
| If this research does identify genetic information that would be important or of health benefit to my family, or me, I would like to be contacted to receive the information. | Yes ☐ No ☐ |

| | |
|---|---|
| Researcher | ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐<br>Printed Name & Surname<br>_____ ☐☐/☐☐/☐☐☐☐<br>Signature/Thumbprint Date (dd/mm/yyyy) |
| Parent Participant | ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐<br>Printed Name & Surname<br>_____ ☐☐/☐☐/☐☐☐☐<br>Signature/Thumbprint Date (dd/mm/yyyy) |



Chairperson: Prof Eric Buch Acting CEO: Dr Karmani Chetty
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Practice number: 5200296

NATIONAL HEALTH LABORATORY SERVICE
School of Pathology, University of the Witwatersrand

DIVISION OF HUMAN GENETICS

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[T]: +27 11 489 9211 | [F]: +27 11 489 9226 | [E]: human.genetics@nhls.ac.za

Protocol: M180855

Dear Potential Participant,

Hello, we are Dr. Lindiwe Lamola, Dr. Candice Feben and Prof. Amanda Krause from the Division of Human Genetics at the University of the Witwatersrand and National Health Laboratory Service. We are doing research on childhood cancers. We work with other members of the Division of Human Genetics to study the genetic factors that cause childhood cancers in South Africans.

As we are trying to understand the genetic causes of cancer, we would like to invite your child to take part in this study, because your child has cancer. Participation in the study will mean answering some questions about the health and background of your child and giving a blood and/or saliva sample for genetic studies. We will also examine your affected child, review their clinical file and investigations, and take a blood and/ or saliva sample from them.

**What are childhood cancers?**

Childhood cancers are the cancers that develop in children from birth to 18 years of age. Childhood cancer is not just one disease, but a group of several major types of disease. There are many types of childhood cancers; these may include; neuroblastoma, retinoblastoma, Wilms tumor, and cancers of the brain, bone, and soft tissue. The childhood cancers are not common and differ from adult cancers in the way they grow, spread and how they are treated.

**What is cancer?**

Cancer is an abnormal growth of cells. Cells are small basic building blocks of all living things. All parts of the body are made up of cells. Cancer develops when damaged cells grow and spread faster than normal healthy cells.

**What causes childhood cancers?**

Childhood cancers are complex diseases and doctors sometimes aren't sure why some children get cancer and others don't. However, about 10% (1 in 10) of all cancers in children are caused by inherited changes in your genes – inherited means these changes can be passed from parents to children. For this study, we are interested in studying the genetic causes of childhood cancers in order to understand their role in the development of cancer.

***What will the study involve?***

In this study, we would like to understand the factors which may have played a part in your child developing cancer. To do this, we want to learn more about your family, specifically whether other family members have had cancer, and to learn more about your child, specifically related to his/her type of cancer, how old the child was when the cancer was diagnosed and whether there are any other problems which your child may have been born with or may have developed over time.

We would like to take a full family history from you and then perform a clinical examination of your child, looking at his/her weight and growth, skin, chest, abdomen, limbs and spine. The family history and examination will take approximately half an hour. We would like to look at the records in your child's hospital file to make sure that we collect all the relevant information

In order to study the genes, we will take a sample of blood and/or saliva from your child. Our blood and saliva contains cells. All the cells in our bodies contain a chemical called DNA that we inherit from our parents. DNA carries the instructions that your body must follow in order to exist and function. It contains all your genes. Your cells also contain a chemical called RNA. RNA is formed from DNA and helps DNA to do its job.

We can take the DNA and RNA out of the cells that are in the blood or the saliva sample that we take from your child. Once we have isolated the DNA and/or RNA we can study the genes. In this study, we may need to examine all of the genes to try and find the gene that may be the cause of your child's cancer.

***What can you expect from the study?***

Once you understand what the study is about and are happy to take part, we will ask you to sign a consent form on behalf of your child to join the study. If your child is old enough and able to understand the study, they may sign an assent form. A copy of these forms will be given to you to keep.

Once you have signed the consent form and your child has signed the assent form we will ask you some questions about your child's background and health. A complete clinical examination will be performed on your affected child. We may ask to access data relevant to your child's diagnosis from his/her clinical file or test results. This information will help us to understand your child's condition better and to interpret the data generated by our research.

A nursing sister or doctor will take up to three teaspoons (15ml) of blood from your child. Venupunctures (drawing blood) are normally done as part of routine medical care and present a slight risk of discomfort. Drawing blood may result in faintness, inflammation of the vein, pain, bruising or bleeding at the puncture site. Your protection is that experienced staff perform the procedures under sterile conditions.

We will also ask for a saliva (spit) sample from your affected child. A nursing sister or doctor will use a small, soft sponge to collect the saliva sample. The sponge is placed inside your child's mouth against the cheek and gently moved along the gums and cheek to soak up as much saliva as possible. He/she will then place the saliva in a special container and might repeat the process if we do not get enough saliva the first time. This procedure is painless and harmless.

***Long-term storing of samples and data***

Once we have done the research that we are planning for this project, we would like to store your child's DNA, genomic information and data on their health history and laboratory test results for future research on childhood cancer, and we will ask you to sign a separate consent form to give us permission to do this. The sample will be stored together with other participants' samples. The samples will be used in future childhood cancer studies.

***Confidentiality***

Your child's samples will be stored in a locked fridge and the personal information will be stored on a secure computer in a locked office. Only the investigators of this study will have access your child's name and personal details. Each participant will be given a code and number for the study so that no one knows who you are. The blood, saliva, RNA and DNA samples and the associated data will not be attached to your names – they will only have their code and number on them.

***What are my rights as a participant?***

You and your child do not have to join this study. Whether he/she decides to join this study or not will not affect your treatment in our clinic. You can also decide to leave the study at any time. You may choose to have your child's biological samples (blood, DNA, RNA and saliva) destroyed at any time. If you do not want your child to be part of this study or decide to leave the study, it will not affect your child's regular treatments or medical care in any way. We invite your child to approach the researchers to be re-consented when he/she turns 18 if he/she wishes. He/she can also decide to leave the study at any time.

You may not get any benefit directly from this study, but we hope it may help others who are affected by childhood cancers in the future. We may contact you in future if we believe we have identified the cause of your child's condition if you give your permission for this. Please understand that what we are trying to do is very difficult and could take a long time.

***Contact details***

If you have any problems you can contact us at any time on this telephone number: Dr Lindiwe Lamola, Dr Candice Feben or Prof Amanda Krause – 011 489 9223.

Hospital Street, Johannesburg, 2001 | PO Box 1038, Johannesburg, 2000
[T]: +27 11 489 9211 | [F]: +27 11 489 9226 | [E]: human.genetics@nhls.ac.za



**PLEASE NOTE!**

*Some patients may experience psychological distress should a complication be detected. You can contact the Genetic Counselling Unit at the Division of Human Genetics, NHLS Braamfontein to arrange a session with an HPCSA-registered genetic counsellor to discuss these issues. They are experienced and qualified counsellors who are able to offer distress counselling, decision support, address concerns associated with genetic disorders and testing and explain medical terminology. Appointments can be arranged by contacting Ms Janet Robbie on (011) 489 9223 or Ms Shelley Macaulay on (011) 489 9243.*

*If you have any complaints or problems with this research you may report them to the Chair of the Human Research Ethics Committee at the University of the Witwatersrand on this telephone number – (011) 717 1234.*

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Protocol: M180855

***Inherited cancer predisposing mutations in a cohort of childhood cancer patients***

**ASSENT FORM FOR CHILD PARTICIPANTS**

**PARTICIPANT STUDY ID:**

The purpose of this study, procedures to be followed, risks and benefits, have been explained to me. I have been allowed to ask the questions I have, and my questions have been answered to my satisfaction. I have been told whom to contact if I have questions, to discuss problems, concerns, or suggestions related to the research, or to obtain information or offer input about the research. I have read this assent form (or had it read to me) and agree to participate in this research study with the understanding that I may withdraw at any time. I understand that that I may approach the researchers to be re-consented when I turn 18 if I want to and that I may withdraw from the study at any time. I have been told that I will be given a signed and dated copy of this consent form if I would like one.

| | |
|---|---|
| Researcher | ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐<br>Printed Name & Surname<br>_____ ☐☐/☐☐/☐☐☐☐<br>Signature/Thumbprint Date (dd/mm/yyyy) |
| Parent or Legal Guardian | ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐<br>Printed Name & Surname<br>_____ ☐☐/☐☐/☐☐☐☐<br>Signature/Thumbprint Date (dd/mm/yyyy) |
| Child Participant | ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐<br>Printed Name & Surname<br>_____ ☐☐/☐☐/☐☐☐☐<br>Signature/Thumbprint Date (dd/mm/yyyy) |
| Translator<br>(if applicable) | ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐<br>Printed Name & Surname<br>_____ ☐☐/☐☐/☐☐☐☐<br>Signature/Thumbprint Date (dd/mm/yyyy) |

# Information sheet – Child Participant

## Inherited cancer predisposing mutations in a cohort of childhood cancer patients

Protocol: M180855

NATIONAL HEALTH LABORATORY SERVICE
UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

Division of Human Genetics
University of the Witwatersrand & National Health Laboratory Service
P.O. Box 1038
Braamfontein
Johannesburg
2000
Tel: +2711 489 9211
Fax : +2711 489 9226

**Dear Potential Participant,**

Hello, we are Dr. Lindie Lamola, Dr. Candice Feben, Prof. Amanda Krause and Dr Njabulo Mabaso from the Division of Human Genetics at the University of the Witwatersrand and National Health Laboratory Service. We are doing research on childhood cancers. We work with other members of the Division of Human Genetic to study the genetic factors that cause childhood cancers in South Africans.

### *What is cancer?*

Cancer is an abnormal growth of cells. Cells are small structures which our bodies are made up of. Sometimes our cells get damaged and they start to grow differently and become big and abnormal – when they grow like this, they become what we call cancer.

### *What causes childhood cancers?*

Cancer in children is difficult and sometimes doctors are not sure why some children get cancers and others don't.

For this study, we would like to look inside your DNA and find what is making your cells grow so much to cause cancer. By doing so, we will be able to understand how and why children like you have cancer.

### *What do I have to do?*

Once you understand what the study is about and are happy to take part, we will ask you to sign a form to join the study. A copy of these forms will be given to you to keep.

Once you have become a participant in the study the doctor will do some checks like how heavy you are and how tall you are

The doctor will need to take some of your blood and/ or buccal swab which we will use to get your DNA.

Our work in this study is to test your DNA to find what caused your cancer

We will ask your Dr for permission to access your hospital file. This information will help us to understand your condition better

***Long-term storing of samples and data***

Your sample will be stored safely together with other participants' samples. The samples will only be used in the childhood cancer study and not in any other research.

***Confidentiality***

You will be given a code and number for the study so that no one knows your real name and who you are. All samples and the data will not be attached to your name.

You do not have to join this study. Whether you decide to join this study or not will not affect your treatment in our clinic.

***What will I benefit from being part of this study?***

You may not get any benefit directly from this study, but we hope it may help others who are affected by childhood cancers in the future. As it might help us to understand why you got cancer. We may contact you in future if we believe we have identified the cause of your condition.

***Contact details***

If you have any problems you can contact us at any time on this telephone number: Dr Lindie Lamola, Dr. Candice Feben, Dr Njabulo Mabaso or Prof Amanda Krause – 011 489 9223.

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Protocol: M180855

***Inherited cancer predisposing mutations in a cohort of childhood cancer patients***

**CONSENT FORM – PARENTAL CONSENT FOR CHILD PARTICIPANTS**

**PARTICIPANT STUDY ID**

| **PERMISSION FOR PHOTOGRAPHS** | Please initial |
|---|---|
| I agree to the use of deidentified photographs of my child for medical records ONLY. | |
| I agree for deidentified photographs my child to be used for teaching purposes AND to be used for medical records but NOT for medical publication. | |
| I agree for deidentified photographs of my child be used in medical publications, including medical journals, textbooks and electronic publications AND to be used for teaching purposes AND to be used for my child's medical records.<br><br>I understand that the images may be seen by members of the general public, in addition to scientists and medical researchers that use these publications in their professional education. Although these photographs will be used without identifying information, such as my child's name, I understand that it is possible that someone may recognize my child. | |

| | |
|---|---|
| Researcher | ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐<br>Printed Name & Surname<br>_____ ☐☐/☐☐/☐☐☐☐<br>Signature/Thumbprint Date (dd/mm/yyyy) |
| Parent Participant | ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐<br>Printed Name & Surname<br>_____ ☐☐/☐☐/☐☐☐☐<br>Signature/Thumbprint Date (dd/mm/yyyy) |

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Protocol M180855

***Inherited cancer predisposing mutations in a cohort of childhood cancer patients***

**CONSENT FORM – PARENTAL CONSENT FOR CHILD PARTICIPANTS**

**PARTICIPANT STUDY ID**

| **PERMISSION FOR STORAGE** | | |
|---|---|---|
| I agree to have my samples stored in the research facility for future childhood cancer studies only without any identifiers. | Yes ☐ | No ☐ |
| I agree to have the data generated from my samples stored without any identifiers and be reanalysed if need be. | Yes ☐ | No ☐ |

| **PERMISSION FOR REUSE** | | | | |
|---|---|---|---|---|
| | **Samples** | | **Data** | |
| I agree that my child's samples & data (including clinical data) can be shared with other investigators for research *related to childhood cancers* once the ethics committee has approved the new study. My child's samples & data will be made available without any identifiers. | Yes ☐ | No ☐ | Yes ☐ | No ☐ |

| | |
|---|---|
| Researcher | ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐<br>Printed Name & Surname<br>_____ ☐☐/☐☐/☐☐☐☐<br>Signature/Thumbprint Date (dd/mm/yyyy) |
| Parent Participant | ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐<br>Printed Name & Surname<br>_____ ☐☐/☐☐/☐☐☐☐<br>Signature/Thumbprint Date (dd/mm/yyyy) |

**Appendix G:**

Title Approval and Approved Research Protocol

UNIVERSITY OF THE
WITWATERSRAND,
JOHANNESBURG

Private Bag 3 Wits, 2050
Fax: 027117172119
Tel: 02711 7172076

Reference: Mrs Sandra Benn
E-mail: sandra.benn@wits.ac.za

20 July 2023
Person No: 719425
PAG

Ms D Beukman
0C Montpark drive
Magalies unit 5
Randburg
2195
South Africa

Dear Ms Danielle Beukman

**Master of Science in Medicine: Approval of Title**

We have pleasure in advising that your proposal entitled *RB1 and beyond: Determining genetic causes of Retinoblastoma in South-African patients* has been approved. Please note that any amendments to this title have to be endorsed by the Faculty's higher degrees committee and formally approved.

Yours sincerely

Mrs Sandra Benn
Faculty Registrar
Faculty of Health Sciences

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

FACULTY OF HEALTH SCIENCES

| | | |
|---|---|---|
| CANDIDATE'S<br>SURNAME:<br>**BEUKMAN** | FIRST NAME/S:<br>**DANIELLE** | STUDENT<br>NUMBER:<br>**719425** |
| CURRENT<br>QUALIFICATIONS: **BSc Hons Experimental Physiology** | | |

| | | | |
|---|---|---|---|
| TEL: N/A | CELL: **0845834985** | E-MAIL: **beukmandanielle@gmail.com** | FAX: n/a |

| | | |
|---|---|---|
| DEGREE FOR WHICH PROTOCOL IS BEING SUBMITTED: **MSc Med Genomic Medicine** | | |
| PART-TIME OR FULL-TIME: **FULL TIME** | | |
| FIRST REGISTERED FOR THIS DEGREE: | TERM : **1** | YEAR: **2023** |
| DEPARTMENT: **HUMAN GENETICS** | | |
| TITLE OF PROPOSED RESEARCH:<br>**RB1 and beyond: Determining genetic causes of Retinoblastoma in South-African patients** | | |
| CANDIDATE'S SIGNATURE:<br>*BBeukman* | | DATE: **7 July 2023** |
| SUPERVISOR 1 (NAME & SURNAME)**: Dr. Lindie Lamola** | | 50 % Supervision |
| SUPERVISOR'S QUALIFICATIONS **PhD** | | |
| SUPERVISOR'S DEPARTMENT **Human Genetics** | | |
| SUPERVISOR'S ADDRESS / TEL / E-MAIL: **lindie.lamola@wits.ac.za** | | |
| SUPERVISOR 2 (NAME & SURNAME): **Nkateko Mayevu** | | 50 % Supervision |
| SUPERVISOR'S QUALIFICATIONS: **MSc** | | |
| SUPERVISOR'S ADDRESS / TEL / E-MAIL: **nkateko.mayevu@wits.ac.za** | | |

*SYNOPSIS OF RESEARCH*:

Introduction:

Retinoblastoma (RB) is the most common intraocular malignancy in pediatric patients. Up to 31% of RB develops due to an inherited germline variant or mutation which predisposes an individual to RB development at a younger age. The most notable predisposing heritable mutation is found in the Retinoblastoma 1 (*RB1)* gene, however recent international genomics studies have identified other driver genes implicated in heritable RB.

Aim:

Identify causative germline variants predisposing pediatric South-African patients to RB.

Justification for study:

There is a lack of studies investigating mutations causing individuals with an African ancestry to retinoblastoma. From international studies, mutations in more than 43 genes have been proved to increase susceptibility of developing RB. The proposed study will determine if the South African RB patients also have likely pathogenic or pathogenic variants in these genes previously found to be associated with RB.

Proposed methodology:

This is a sub-study under the larger parent Pediatric Cancer (PeCan) project utilizing a cohort of eight pediatric RB patients recruited under the PeCan project. DNA from these patients will be used to perform whole exome sequencing (WES). The data obtained from sequencing will be analyzed using bioinformatics and in silico tools, to identify if they have germline variants in genes previously reported to be associated with RB development.

Expected results:

The majority of RB cases occur sporadically, heritable RB accounting for approximately 31% of RB cases. With a small cohort of eight patients, I may only find two to three germline variants. As with any genetic testing, if a germline variant is found it could be useful to family members of the patients.

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

FACULTY OF HEALTH SCIENCES

| | | |
|---|---|---|
| WITS ETHICS NOT REQUIRED: Yes No<br>WITS ETHICS PENDING: Yes No<br>WITS ETHICS APPROVED: Yes No<br>(circle appropriate symbol)*<br><br>***Please note the final human ethics clearance certificate or animal ethics certificate must be available prior to starting research** | | IF Yes SUPPLY ETHICS CLEARANCE CERTIFICATE AS ATTACHMENT AND INCLUDE ETHICS NUMBER HERE:<br>M180855<br>(M230782) |
| **As supervisor/s, I/we confirm that I have read the protocol which has been submitted for assessment.** | | |
| SIGNATURE OF SUPERVISOR/S: | ……  …… | ……  …… |
| SIGNATURE PG OFFICE STAFF<br><br>…….. | REGISTERED<br><br>YES….. NO….. | STAMP |

# *RB1* and beyond:

# Determining genetic causes of retinoblastoma in South African patients

Danielle Beukman WITS

Student number 719425 MSc

(Med) Genomic Medicine

Supervisors:

Dr Lindie Lamola

Ms. Nkateko Mayevu


## Table of Contents

# 1. INTRODUCTION

## 1.1 Retinoblastoma prevalence

Retinoblastoma (RB) is a solid intraocular cancer, most often developing in childhood. The South African National Cancer Registry (SANCR) recorded an average RB prevalence of 1 in 21 641 live births for the period of 2004 to 2018 (Stuart et al., 2022). The mean age of diagnosis in South Africa is 2.5 years, significantly later than mean age of diagnosis in high income countries, 1.2 years (Stuart et al., 2022). This is startling as the age of diagnosis is the strongest indicator of prognosis and whether an RB patient needs to undergo enucleation (Stuart et al., 2022).

## 1.2 Genetic landscape of retinoblastoma

The majority (65 - 68.2 %) of RB cases are classified as sporadic, occurring at a mean age of 24 months, presenting with unilateral tumors (Knudson, 1971). Heritable retinoblastoma accounts for the remaining 31.8% - 35% of RB cases, developing mostly bilateral tumors, with an earlier mean age of onset of 12 months (Knudson, 1971). In the case of heritable RB, an individual is predisposed to developing RB due to a likely pathogenic or pathogenic germline variant in the Retinoblastoma 1 gene. The first gene associated with RB predisposition was the Retinoblastoma 1 *(RB1)* tumor suppressor gene (TSG), discovered by Alfred Knudson (Knudson, 1971). To date more than 1700 germline pathogenic variants in *RB1* have been reported on the Leiden Open Variation Database (LOVD), which is an open-source database logging research-based DNA variation (Fokkema et al., 2011).

### 1.3 Locus heterogeneity observed in retinoblastoma

Next generation sequencing (NGS) such as whole exome sequencing (WES) of European and Chinese cohorts with heritable RB has identified pathogenic variants in genes other than *RB1* that contribute to RB development (Kooi et al., 2016). NGS performed on blood samples from patients with RB from Istanbul revealed this locus heterogeneity clearly. All patients reported a family history of RB, yet wild type *BR1* was observed in six individuals. When exome sequencing was performed on the six *RB1*-negative patients, twenty-seven significant variants (likely pathogenic or pathogenic) were found in genes acting in different biochemical pathways, such as retinoblast formation and DNA repair. Variants with the highest significance are found in the following genes: *FGFR4, NQO1, ACADS, KRT85, GBE1* and *CLEC7A* (Akdeniz et al., 2019).

Mutations in the TSG *p53* have long been associated with heritable cancer syndromes. In a large study with 111 retinoblastoma patients with a family history of RB of Italian ancestry, single nucleotide variants or mutations were found in *p53* and *MDM2* (Epistolato et al., 2011). The TSG *p16INK4A* is involved in cellular regulation preventing inappropriate cellular proliferation, halting the cell cycle at the G1-S checkpoint by encoding a CDK inhibitor (Epistolato et al., 2011). In a study of 29 Italian patients with RB, promoter methylation and downregulation of *p16INK4A* was strongly associated with heritable RB but not with sporadic RB (Indovina et al., 2009). Confirming the heritable aspect of an epigenetic susceptibility marker and reaffirmed the significance of *p16INK4A* in heritable RB (Indovina et al., 2009).

Together MDM2 and MDM4 regulate p53, due to the interaction in a common biochemical pathway. *MDM2* was confirmed to be a modifier gene in families with

RB malignancies (Castéra et al., 2010). Single nucleotide variants in both *MDM2* and *MDM4* were strongly implicated in heritable RB. This study also found that the *MDM4* rs116197192G allele frequency was significantly higher in their Brazilian RB cohort compared to controls (de Oliveira Reis et al., 2012).

A study by Carvalho and colleagues from 2013 investigated samples from 141 RB patients and 120 controls, this study revealed pathogenic variants in *CDKN1A* gene in the presence of wild type *RB1* gene. *CDKN1A*, when mutated, contributes to uncontrolled cell proliferation (Carvalho et al., 2013).

*PAX6* gene encodes the PAX6 transcription factor that regulates retinogenesis. Inactivation or suppression of *PAX6* provides retinoblasts a molecular mechanism to obtain two cancer hallmarks, resisting cell death by inhibiting apoptosis and sustained proliferative signaling. Pathogenic or likely pathogenic germline variants in *PAX6* have been reported in RB patients with wildtype *RB1* (Meng et al., 2014). In this instance *PAX6* acts as a TSG, yet in other studies *PAX6* was overexpressed in pancreatic tumors, indicating that PAX6 can be classified as an oncogene (lang et al., 2008). The molecular duality of the *PAX6* gene complicates the classification of variants but is believed to differ according to the type of cancer (Lang et al., 2008).

Pathogenic germline variants in oncogenes have been found to be major driver genes in RB development (Afshar et al., 2020). Likely pathogenic or pathogenic germline variants were reported in the following oncogenes: *MYCN* and *TSC2* (Francis et al., 2021).

The genes implicated in RB malignancy are summarized in Table 1 below.

Table 1: Genes implicated in RB development.

| Gene Symbol | Full gene name | Reference |
|---|---|---|
| *RB1* | Retinoblastoma | Knudson, 1971 |
| *ACADS* | Short-chain acyl-CoA dehydrogenase | Akdenizet al., 2019 |
| *CLEC7A* | C-Type Lectin Domain Containing 7A | Akdenizet al., 2019 |
| *CDK* | Cyclin-dependent kinases | Epistolato et al., 2011 |
| *CDKN1A* | Cyclin Dependent Kinase Inhibitor 1A | Carvalho et al., 2013 |
| *FGFR4* | Fibroblast growth factor receptor 4 | Akdenizet al., 2019 |
| *GBE1* | Glycogen branching enzyme | Akdenizet al., 2019 |
| *KRT85* | Keratin 85 | Akdenizet al., 2019 |
| *MDM2* | E3 ubiquitin-protein ligase | Epistolato et al., 2011 |
| *MDM4* | P53-Binding Protein | de Oliveira Reis et al., 2012 |
| *MYCN* | MYCN Proto-Oncogene, also called BHLH Transcription Factor | Francis et al., 2021 |
| *NQO1* | Dehydrogenase [quinone] 1 | Akdenizet al., 2019 |
| *PAX6* | Aniridia type II | Lang et al., 2008<br>Meng et al., 2014 |
| *p16INK4A* | Cyclin-dependent kinase inhibitor 2A | Indovina et al., 2009 |
| *p53* | TP53 | Epistolato et al., 2011 |
| *TSC2* | Tuberous Sclerosis Complex 2 | Francis et al., 2021 |

## 2. RATIONALE AND PURPOSE OF STUDY

Currently there is a great gap in the literature with regards to genetic variants contributing to pediatric cancer development in patients with a South African ancestry, this includes RB. Studies performed in developed countries have found numerous candidate genes that potentially contributing to an individual's likelihood of developing hereditary RB, similar studies have not been conducted in Africa. The purpose of this study is to determine if the South African cohort of RB patients have likely pathogenic or pathogenic variants in genes previously reported to be associated with RB.

## 3. AIM AND OBJECTIVES

### 3.1 AIM:

Identify causative germline variants implicated in RB development and progression in pediatric South-African patients.

### 3.2 OBJECTIVES:

- Perform whole exome sequencing (WES) on four DNA samples from South- African patients affected with RB.
- Perform bioinformatics analysis of the exome sequence data from 8 patients with RB to identify candidate likely pathogenic and pathogenic variants associated with RB development and progression. The genes to be analyzed are listed in Table 1.

# 4. METHODS

Eight pediatric patients diagnosed with RB were recruited as part of the larger parent study, Pediatric Cancer Project (PeCan). All patient's personal information accessed as part of this sub-study will be anonymized using patient codes. Patient information is encrypted, and password protected. Patient history includes race, age of diagnosis, sex, and tumor presentation. Patient information is saved on the REDCap database, accessible to researchers, I have been granted access by the gatekeeper. Clinical data will be used in conjunction with WES data as part of result interpretation. DNA was isolated from peripheral blood samples. Of the eight patients, WES has been performed on four samples (1-4), for the remaining four samples (5-8) WES will be performed as a step in this project.

## 4.1 Whole Exome Sequencing Workflow

The stepwise WES workflow is summarized in figure 1.

### *4.1.1. Assess DNA Quality:*

Initial DNA quantification will be performed using the Thermo Scientific Nanodrop Spectrophotometer ™ to assess DNA concentration, this densitometric method will be followed by using The Thermo Scientific Qubit ™ fluorometer to assess double stranded DNA concentration, as this will be the starting material in WES.

### *4.1.2. Library Preparation and Amplification:*

Library preparation and amplification will be conducted as per manufacturer's instructions provided in the Ion AmpliSeq™ manuals (ThermoFisher Scientific, Waltham MA, USA). Library preparation entails target amplification, amplicon partial digest and ligation of IonCode™ Barcode adapters. At this stage the unamplified library will be purified. Subsequently, purified libraries will be amplified and further purified.

#### *4.1.3. Library Quantification and dilution:*

Quantification of the amplified library will be performed using a Qubit™ 3 fluorometer (ThermoFisher Scientific, Waltham MA, USA). This will be followed by Tape Station™ (Agilent technologies, Santa Clara, USA) which will quantify the libraries and determine concentration based on fragment sizes of interest, between 200- 400 bp. This allows for the appropriate dilutions to be made prior to templating.

#### *4.1.4. Templating and Sequencing:*

Templating of the libraries occur in a flow cell, the ion sphere particles contain primer sequences that will bind to complementary single stranded DNA, this is facilitated by the barcode adapters. Templating creates a sphere covered with multiple copies of the same amplicon, ensuring that the signals produced during sequencing are strong enough to be detected. Templating will be performed using the Ion 540™ Kit – Chef (ThermoFisher Scientific, Waltham MA, USA), followed by exome sequencing performed in by the Ion torrent S5 semiconductor™ sequencer machine (ThermoFisher Scientific, Waltham MA, USA).

This proposed whole exome sequencing workflow is illustrated in Figure 1.

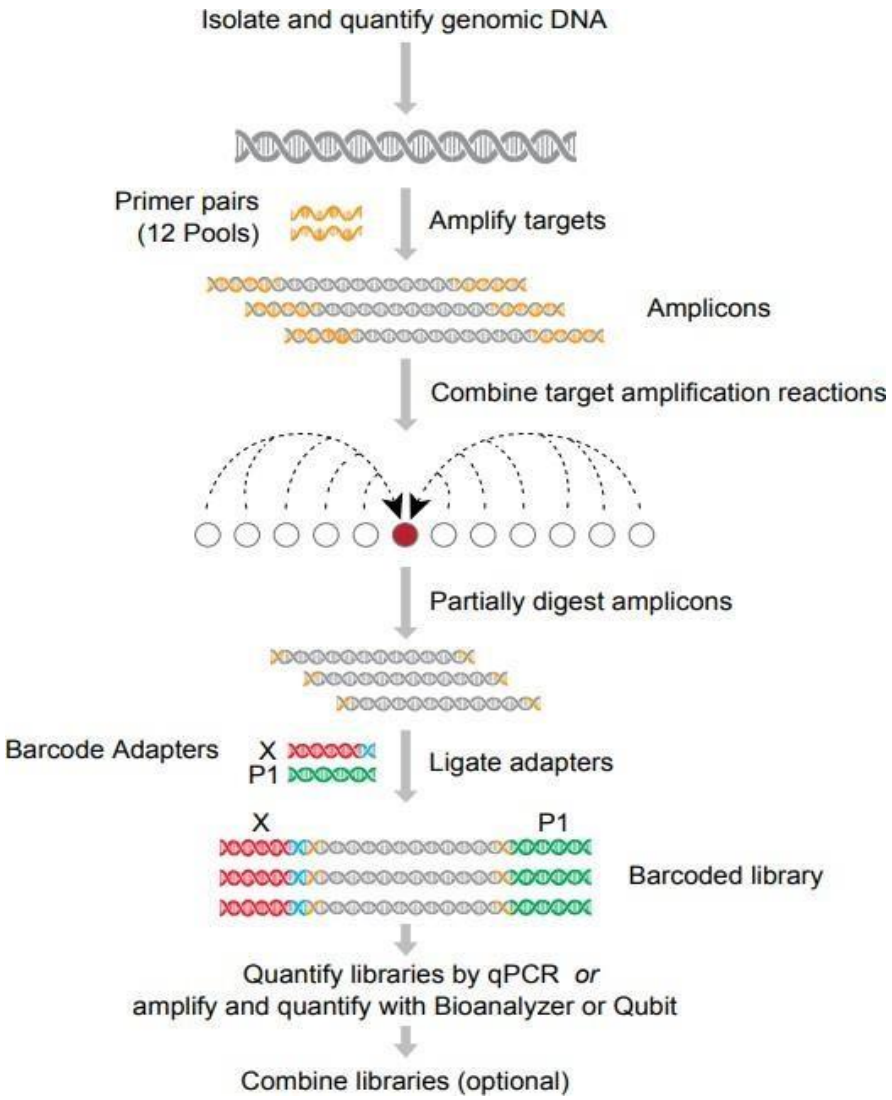


Figure 1: Ion AmpliSeq™ Exome RDY library preparation (ThermoFisher Scientific manual MAN0010084 version E.0, 2018). Xand P1 represent the barcoded adapters that get ligated to make each amplicon unique and identifiable.

### 4.2. Bioinformatics Analysis:

Once all raw data has been generated from whole exome sequencing, bioinformatic analysis will be employed to search for variants of interest. WES raw data is generated as a variant call file (VCF file) by aligning the sequences in question to a human reference genome

GRCh37/Hg19 and identifying sequence differences between the two. The GRCh37/Hg19 reference genome will be used as reference genome as the reagents (Ion AmpliSeq ™ Exome RDY kit) in stock contains primers designed according to the GRCh37/Hg19 reference genome. The sequencer instrument (Ion GeneStudio ™ S5 Instrument) software currently in use by the division utilizes analysis pipelines created for GRCh27/Hg19. A variant effect predictor (VEP) from Ensamble Genome Browser will be used to annotate the variants found in genes and transcripts (McLaren et al., 2016). Annotation output will include the following results: Variant location (chromosome, gene, and exon) and the type of variant detected (i.e., missense, nonsense, and frameshift) (Hunt et al., 2021). The frequency of the variants will be ascertained from two genomics databases, 1000 genomes project (The 1000 Genomes Project Consortium et al., 2015) and gnomAD (Gudmundsson et al., 2022).

Variant prioritization is essential to reduce the large number of variants generated and identify possible disease-causing variants. First line of variant prioritization involves selecting genes previously reported to be involved in RB development, as mentioned in Table 1. Variants with a minor allele frequency higher than 5% will be filtered out (this figure is subject to change depending on the number of results that get returned) as well as variants that have no effect on protein function or structure as predicted by in silico prediction tools such as Sort Intolerable from tolerable (SIFT) (Ng & Henikoff, 2003) and Polymorphism Phenotyping version 2 (POLYPHEN-2) (Adzhubei et al., 2010). These in silico tools predict what structural and functional change will occur in a protein due to an amino acid change (de la Campa et al. 2017).If the ClinVar database has available data on the variants of interest, the variant and gene-associated phenotype will be assessed, to aid in filtering out variants that have been reported as benign or likely benign.

A Bam.file and the shortlisted variants will be viewed in an integrative genomics viewer (IGV) to assess the quality of variant calling and coverage. The remaining shortlisted variants will be classified according to The American College of Medical Genetics and Genomics and Association for Molecular Pathology Guidelines (ACMG/AMP) which aggregates variant data from different predictors and research-based findings into a score (Richards et al., 2015).

All possible causative variants will be reported, these include variants of uncertain significance, likely pathogenic variants, and pathogenic variants. If a reportable variant is found, this information will be passed on to the Principal Investigator of the PeCan project parent study, Dr. Lindie Lamola. As part of the parent study these variants will be validated. Information will be shared with the relevant clinicians and genetic counselors to communicate findings in accordance with the ethical permissions obtained at the time of sample collection.

## 5. ETHICS

Ethics approval for the parent study, has been acquired (M180855). Ethical clearance for this project has been approved conditionally by the Wits Human Research Ethics Committee (Wits HREC) pending protocol approval by the Faculty of Health Sciences. Parent study ethics study is attached in Appendix I.

## 6. TIMING AND WORK PLAN

Study to commence as soon as ethics clearance is granted. Study to be completed by 30 Nov 2023.

| | Mar | Apr | May | Jun | Jul | Aug | Sep | Oct | Nov | Dec |
|---|---|---|---|---|---|---|---|---|---|---|
| Literature review | | | | | | | | | | |
| Ethics application | | | | | | | | | | |
| Preparing protocol | | | | | | | | | | |
| Protocol assessment | | | | | | | | | | |
| NGS Lab (WES) | | | | | | | | | | |
| Data Analysis | | | | | | | | | | |
| Paper write-up | | | | | | | | | | |

## 7. FUNDING AND BUDGET:

All costs of this study will be covered by funding already obtained for the parent study,The PeCan Project and is managed by the principal investigator Dr. Lindie Lamola. Funding has been obtained from the following partners: National Research Foundation (NRF), South African Medical Research Council Self-Initiated Research (SAMRC SIR), and Female Academic Leadership Fellowship (FALF).

Table 2: Exome sequencing budget

| Exome sequencing | |
|---|---|
| ION AMPLISEQ EXOME RDY KIT | R16775 |
| ION 540 CHIP KIT EACH x2 | R 25375 |
| ION 540 KIT-CHEF (2/INIT) | R28900 |
| DNA and library quantification | |
| Qubit reagents | R1500 |
| Tape station reagents | R2800 |
| Total | R 75350 |

## 8. PROBLEMS AND LIMITATIONS

A small sample size of 8 RB patients increases the likelihood of no significant variants being found. Short read sequence data will be obtained which may lead to alignment issues. Due to the lack of African genomics data and European bias, variants common in African populations but rare in European populations might be misinterpreted as likely pathogenic or pathogenic. The in-silico predictors that will be utilized are notorious for reporting contradictory and conflicting results. We are not using the latest reference genome because at the time of sequencing the first four exomes, Hg19 was used as the reference genome.

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## 10. Appendix I

R14/49 Dr L Lamola, et al

**HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)**
**CLEARANCE CERTIFICATE NO. M180855**

| | |
|---|---|
| **NAME:** (Principal Investigator) | Dr L Lamola, et al |
| **DEPARTMENT:** | School of Pathology<br>Department of Human Genetics<br>National Health Laboratory Service |
| **PROJECT TITLE:** | Inherited cancer predisposing mutations in a cohort of childhood cancer patients |
| **DATE CONSIDERED:** | 31/08/2018 |
| **DECISION:** | Approved unconditionally |
| ***CONDITIONS:*** | |
| **SUPERVISOR:** | Not applicable |
| **APPROVED BY:** | Dr CB Penny, Chairperson, HREC (Medical) |
| **DATE OF APPROVAL:** | 26/02/2019 |

**This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.**

**DECLARATION OF INVESTIGATORS**

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary on 3rd floor, Phillip V Tobias Building, Parktown, University of the Witwatersrand, Johannesburg.

I/We fully understand the conditions under which I am/we are authorised to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated from the research protocol as approved, I/we undertake to resubmit to the Committee. **I agree to submit a yearly progress report**. When a funder requires annual re-certification, the application date will be one year after the date of the meeting when the study was initially reviewed. In this case, the study was initially reviewed in **August** and will therefore reports and re-certification will be due early in the month of **August** each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature

26/02/2019
Date

**PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES**

**Appendix H:**

Plagiarism declaration and Turnitin report

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FACULTY OF HEALTH SCIENCES

**PLAGIARISM DECLARATION TO BE SIGNED BY ALL HIGHER DEGREE STUDENTS**

SENATE PLAGIARISM POLICY: APPENDIX ONE

I Danielle Beukman (Student number: 719425) am a student registered for the degree of MSc Med Genomic Medicine in the academic year 2023.

I hereby declare the following:

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- I confirm that the work submitted for assessment for the above degree is my own unaided work except where I have explicitly indicated otherwise.
- I have followed the required conventions in referencing the thoughts and ideas of others.
- I understand that the University of the Witwatersrand may take disciplinary action against me if there is a belief that this is not my own unaided work or that I have failed to acknowledge the source of the ideas or words in my writing.
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1. Abstract for Journal Submission

**Background:** Retinoblastoma is the most common solid tumour of the retina, affecting mainly paediatric patients. Knudson formulated the "two-hit" hypothesis on the grounds of the Retinoblastoma 1 (*RB1*) gene, the first tumour suppressor gene discovered, laying the foundation to investigate how germline variation in cancer predisposition genes can genetically predispose an individual to malignancy. Although loss-of-function germline variants the *RB1* gene is the tumour suppressor gene most often associated with heritable retinoblastoma, two confounding research findings complicate the assumption that a pathogenic germline variant in *RB1* will lead to the development of heritable retinoblastoma. First, heritable retinoblastoma has been observed in cohorts with biallelic wild-type *RB1* genes, secondly, biallelic loss-of-function is not always sufficient to lead to retinoblastoma tumorigenesis. The "beyond *RB1*" concept addresses these contradictions by investigating germline variants in additional cancer predisposition genes to discover candidate driver genes in heritable retinoblastoma. In this study we investigate sequence germline variants in cancer predisposition genes beyond *RB1* in retinoblastoma patients with a South African ancestry.

**Methods:** Peripheral blood samples from eight paediatric patients of South African ancestry diagnosed with retinoblastoma was used to extract genomic DNA. Whole exome sequencing with single nucleotide resolution was performed to establish germline sequence variants. Variant annotation, prioritisation and filtering was executed to detect germline variants in cancer predisposition genes previously proposed as candidate genes in retinoblastoma malignancy and paediatric cancer predisposition syndromes. Variants shared between individuals and variants of interest were classified according to ACMG/AMP guidelines.

**Results:** A total of 353 655 variant were annotated, 4538 of which were novel. After collating functional data, previously reported and reviewed *RB1* variants, all sequence variants in *RB1* are likely benign, amending variant classification based on updated in-silico predictors. The extended list of cancer predisposing genes revealed variants of uncertain significance.

**Conclusion:** The absence of sequence variants capable of describing the retinoblastoma phenotype in this cohort demonstrates the necessity of combining next generation sequencing with analysis pipelines and additional genome mapping to detect structural variants. This study adds genomic information from patients with a South African ancestry to mounting genomic data, ensuring that previously underrepresented populations can also benefit from future cancer predisposition and precision medicine research.

**Keywords:** genetic predisposition, germline variation, cancer predisposition genes, paediatric tumours, whole exome sequencing, *RB1* gene, retinoblastoma, sequence variants

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*by* Danielle Beukman

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