SJRET6

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PROTOCOL FOR THE STUDY AND TREATMENT OF PARTICIPANTS WITH INTRAOCULAR RETINOBLASTOMA

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St. Jude Children's Research Hospital
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MEMORANDUM
Department of Oncology

TO: IRB

FROM: Rachel Brennan MD

DATE: September 12, 2018

SUBJECT: Clarification Memo #1 for SJRET6

This memo serves to clarify that anaphylactic reactions occurring in patients being treated on SJRET6 can be treated per institutional standards.

Section 7.7 of the protocol currently spells out specific procedures. These were put in place as guidelines should the protocol be open at other sites, but St. Jude has specific hypersensitivity kits that should be used here and can be built into the protocol specific orders.

Protocol summary

SJRET6: PROTOCOL FOR THE STUDY AND TREATMENT OF PARTICIPANTS WITH INTRAOCULAR RETINOBLASTOMA
Principal Investigator: Rachel C. Brennan, M.D.
IND holder: not applicable, non-IND study
Brief overview: The primary objective of the SJRET6 protocol is to evaluate the response rate of bilateral disease participants who have at least one eye with advanced intra-ocular retinoblastoma (stratum B) using upfront therapy with chemotherapy delivered directly to the eye. The main biology objective of the SJRET6 protocol is to improve our understanding of the biology and tumorigenesis (how tumor develops) of retinoblastoma when biology specimens are available. As clinicians, our primary goal for children with retinoblastoma is to provide optimal therapy using multiple treatment approaches [chemotherapy (into the vein and directly into membrane of the eyeball), cryotherapy (freeze and destroy tumor), thermotherapy (laser or heat to destroy tumor), radiation therapy, and surgical removal of eye if needed) in an attempt to preserve the eye and vision whenever possible, while still curing the disease. Therefore, all children with non-metastatic retinoblastoma at St. Jude will be offered enrollment on this study. Participants will be stratified into four main treatment groups, depending on whether retinoblastoma is present in one or both eyes and disease stage (early or advanced, stages I-V). Additionally, participants will be invited to participate in exploratory research objectives that address cognitive and functional development of children with retinoblastoma, and evaluation of ototoxicity, including genetic analysis.

Intervention:

Strata	Assigned interventions
Stratum A: early unilateral or bilateral retinoblastoma	Drug: vincristine IV Drug: carboplatin IV Drug: topotecan IV Drug: filgrastim or PEG-filgrastim Procedures: Focal therapies as recommended (e.g., cryotherapy, laser photocoagulation, thermotherapy and plaque radiotherapy)
Stratum B: advanced bilateral retinoblastoma or unilateral with foveal sparing (not requiring upfront enucleation)	Drug: vincristine IV Drug: topotecan IV Drug: carboplatin IV and periocular (subtenon) Drug: etoposide Drug: filgrastim or PEG-filgrastim Procedure: enucleation (surgical removal of eye) Procedure: focal therapies as recommended (e.g. cryotherapy, laser photocoagulation, thermotherapy, thermo-chemotherapy and episcleral plaque brachytherapy Procedure: external beam radiation therapy
Stratum C: advanced unilateral retinoblastoma (requiring upfront enucleation)	Drug: vincristine IV Drug: cyclophosphamide IV Drug: MESNA IV Drug: doxorubicin IV Drug: etoposide: IV Drug: carboplatin IV Drug: filgrastim or PEG-filgrastim Procedures: enucleation (surgical removal of eye)
Stratum D: bilateral retinoblastoma with upfront enucleation of one eye due to advanced disease	Drug: vincristine IV Drug: carboplatin IV Drug: topotecan IV Drug: etoposide Drug: filgrastim or PEG-filgrastim Procedure: enucleation (surgical removal of eye) Procedure: focal therapies as recommended (e.g. cryotherapy, laser photocoagulation, thermotherapy, thermo-chemotherapy and episcleral plaque brachytherapy Procedure: external beam radiation therapy

Brief outline of treatment plan: Risk stratified, multi-modality therapy based on laterality and disease stage. Participants will also undergo evaluation by occupational and speech specialists and these rehabilitative services will be provided as needed. Additionally, participants will be invited to participate in exploratory research objectives that address cognitive and functional development of children with retinoblastoma and evaluation of ototoxicity.

Sample size: The study will be open to accrual until 29 Stratum B with extensive sub-retinal (SR) seeding evaluable participants have been enrolled. We estimate that at least 200 participants will be enrolled in all four strata during the 6-8 year accrual duration.

Data management: Data management and statistical analysis will be provided locally by the Solid Tumor Division and Biostatistics Department at St. Jude Children's Research Hospital.

Human subjects: The risks to subject will be related to the toxicity of the systemic chemotherapeutic agents. Participants will be informed of this and other potential side effects during informed consent. Adverse events and treatment response will be closely monitored and reported and treated appropriately. There are also ethical considerations related to germline RB1 mutation analysis, which is a fundamental aspect of the correlative biology studies included with this study. At the time of protocol registration, the parents/legal guardians will be approached by the primary oncologist who will indicate the propensity of this disease to have an underlying genetic etiology. Each family will be referred to the genetic counselor for genetic counseling and will have a sample of the patient's blood sent for *RB1* analysis. Once the result has been issued, this information will be reported back to the genetic counselor and/or primary treating physician who will then counsel the parents as per standard St. Jude practice.

TABLE OF CONTENTS

APPENDICES

1.0 OBJECTIVES

1.1 Primary Objective

To evaluate the response (CR + PR) rate of bilateral disease participants who have at least one eye with advanced intraocular retinoblastoma and subretinal seeding (Stratum B) to two upfront courses of therapy consisting of subconjunctival carboplatin and systemic topotecan.

1.2 Secondary Objectives

1.2.1 To evaluate the ocular survival of eyes and event-free survival of participants by strata.
1.2.2 To prospectively analyze intraocular disease tissue for participants with at least one eye undergoing enucleation in order to identify the mechanism of RB1 bi-allelic inactivation. Participants may undergo upfront enucleation (due to advanced disease at diagnosis) or may receive enucleation due to progressive disease during protocol therapy.

1.3 Exploratory Biologic Objectives

1.3.1 To identify genetic lesions in intraocular disease tissue by using single nucleotide polymorphism (SNP) arrays for disease tissue and blood samples.
1.3.2 Evaluate the relationship between retinoblastoma genetic and epigenetic alterations, histopathological classification and clinical disease behavior.
1.3.3 Construct tissue microarrays that will serve as a high-throughput tool for future analysis of molecular genetic alterations in retinoblastoma.
1.3.4 Analysis of the expression of miR-191 in intraocular disease tissue and genotyping of MDM4 SNP 34091 in germline and intraocular disease tissue to identify and correlate miR expression, SNP and clinical features.
1.3.5 Analysis and characterization of the vitreous proteome from retinoblastoma patients undergoing enucleation

1.4 Exploratory Therapeutic Objectives

1.4.1 To describe the outcome of intraocular retinoblastoma with respect to the International Classification for Intraocular Retinoblastoma and the AJCC.
1.4.2 To evaluate the ocular survival of eyes, event-free survival of participants and incidence of ototoxicity in participants less than 6 months old who have early stage intraocular retinoblastoma (Stratum A) and are treated with topotecan in place of platinum therapy for 3 of 6 cycles.
1.4.3 To evaluate tumor response (tumor regression and/or calcification by RET-Cam imaging) to topotecan-containing therapy (stratum B) and focal treatments (stratum A, B or D).
1.4.4 To assess the local control and pattern of failure in patients treated with external beam radiotherapy.
1.4.5 To assess the local control and pattern of failure in patient treated with brachytherapy.

1.4.6 To observe and describe the toxicity, both acute and long term, associated with subconjunctival carboplatin therapy.

1.5 Exploratory Supportive Care Objectives

Vision

1.5.1 To describe the types of early intervention and low vision (LV) services received by children with RB.
1.5.2 To describe the visual, developmental, participation, sensory processing, language and quality of life characteristics of children referred for early intervention (EI) and/or LV services.
1.5.3 To describe parent perceived efficacy of low vision assistive devices among those who received assistive devices.

Audiology

1.5.4 To describe the results of wideband reflectance and tympanometry testing in participants receiving carboplatin therapy.
1.5.5 To describe the impact of carboplatin on changes in cochlear function that is detectable with measurement of otoacoustic emissions.
1.5.6 To describe effective methods of monitoring patients treated with carboplatin for sub-clinical changes in cochlear function.

1.6 Exploratory Pharmacology Objective

1.6.1 To assess the relationship of selected candidate genetic variants with ototoxicity in participants receiving carboplatin.

2.0 BACKGROUND AND RATIONALE

2.1 Background

2.1.1 Epidemiology of Retinoblastoma

Retinoblastoma is the most frequent neoplasm of the eye in childhood, and the third most common intraocular malignancy in all ages, following malignant melanoma and metastatic carcinoma. Retinoblastoma represents 3% of all pediatric cancers. The average incidence of retinoblastoma in the United States is 1 in 14,000 – 18,000 live births. Thus, an estimated 200-300 children develop retinoblastoma each year.[1] There is no gender or racial predilection. The human retina is differentiated before completion of gestation, but if genetic or epigenetic aberrations allow persistent cell division, hyperplasia of immature cells may result in tumor formation through the early years of childhood. Retinoblastoma is therefore a cancer of the very young and any therapeutic approach needs to consider not only the cure of the disease, but also the need to preserve vision with minimal long term effects. Retinoblastoma presents in two distinct clinical forms: 1) Bilateral or multifocal, hereditary (40% of cases), characterized by the presence of germline mutations of the *RB1* gene. Multifocal retinoblastoma may be inherited from an affected

survivor (25%) or be the result of a new germline mutation (75%) and 2) unilateral or unifocal, non-hereditary (60% of cases).

Over the past few years, epigenetic mechanisms such as hypermethylation of the RB1 promoter have been implicated in tumorigenesis.[2-4] These changes may play a larger role in altering RB1 gene expression leading to tumor formation than previously appreciated. This finding was underscored by the results of sequencing 5 tumors and matched germline samples from patients with retinoblastoma through the Pediatric Cancer Genome Project. Only one patient had loss of heterozygosity at the RB1 locus, but all had loss of *RB1* function due to other epigenetic alterations.[5] In addition, the genome was otherwise well-preserved without evidence for extensive genetic instability that could lead to tumorigenesis.

2.1.2 Biology of Retinoblastoma

Retinoblastoma is among the best understood of human neoplasms, and has served as an important model for understanding tumorigenesis. In 1971, based on the mathematical analysis of the age at presentation of hereditary and non-hereditary cases of retinoblastoma, Knudson proposed the "two-hit hypothesis", in which two mutational events in a developing retinal cell lead to the development of retinoblastoma[6]. This hypothesis was subsequently extended to suggest that the two events could be mutations of both alleles of the *RB1* gene. The RB1 gene, located in chromosome 13q14, was identified and cloned in 1986.[7,8] Its product (pRb) is a 110 kd nuclear phosphoprotein that acts by binding and inhibiting several proteins with growth-stimulatory activity. pRb is a key substrate for G1 cyclin-cdk complexes, which phosphorylate target gene products required for the transition of the cell through G1. pRb appears to function as a tumor suppressor at least in part by inhibiting cell-cycle progression past the G1-S restriction point. Thus, pRb stands as the major gatekeeper to control this critical point in growth regulation. The lack of pRb or its inactivation will remove the pRb constraint on cell cycle control, with the consequence of deregulated cell proliferation.[9]

The first hit: The RB1 gene is a large gene, containing 27 exons over about 200 kb of DNA, and mutations have been described in almost every exon. Nonsense and frame shift are the most common germline and somatic mutations, although deletions and duplications are also frequently encountered.[9] There are no mutational hotspots, although new germline mutations have an overwhelming preference for the paternal allele, suggesting that deamination of methylated CpG pairs has an important role in mutagenesis.[10]

The second hit: In both hereditary and non-hereditary retinoblastoma, the second tumorigenic event is chromosomal in nature, often as a result of mitotic recombination errors.[11] This second hit occurs at a much higher frequency than the first hit, and it is more sensitive to environmental factors such as ionizing radiation, thus the increased risk of radiation-induced malignancies in survivors of retinoblastoma.[12] After the *second hit* has taken place, retinoblastoma cells rapidly accumulate additional genetic damage.[9] However, tumorigenesis is a multistage process, and additional alterations are necessary for the development of retinoblastoma. More insight in the biology of retinoblastoma is provided in section 2.2.1.

2.1.3 Evaluation and Staging of Retinoblastoma

The diagnosis of intraocular retinoblastoma is usually made without pathologic confirmation. An examination under anesthesia with a maximally dilated pupil and scleral indentation is required to examine the entire retina. Retinoblastoma usually appears as a mass projecting into the vitreous, although the presence of retinal detachment or vitreous hemorrhage may difficult its visualization. Additional imaging studies that aid in the diagnosis include bi-dimensional ultrasound, CT scan and MRI. These imaging studies are particularly important to evaluate extraocular extension and to differentiate retinoblastoma from other causes of leukocoria. Evaluation for the presence of metastatic disease also needs to be considered in a subgroup of patients. Metastatic disease occurs in approximately 10-15% of the patients, and it usually occurs in association with distinct intraocular histologic features, such as deep choroidal and scleral invasion, or with involvement of the iris-ciliary body and optic nerve beyond the lamina cribrosa.[13] In these cases, additional staging procedures, including bone scintigraphy, bone marrow aspirates and biopsies, and lumbar puncture, must be performed. These staging studies are also recommended in patients receiving chemotherapy or radiotherapy before enucleation since a proper histologic evaluation cannot be performed. The Reese-Ellsworth (R-E) grouping system has been generally accepted as the standard for intraocular disease. This grouping system was initially designed to predict the outcome after external beam radiation therapy. It divides eyes into 5 groups on the basis of the size, location and number of lesions, and on the presence of vitreous seeding.[14] However, it does not account for the presence of subretinal seeds and it was developed before the introduction of indirect ophthalmoscopy, a technique that provides a more sophisticated view of the retina and important staging information. In recent years, a new classification has been proposed (International Classification [IC] of Retinoblastoma) that utilizes information gained from both direct and indirect ophthalmoscopy.[15-17] Therefore, this protocol will use a combination of R-E grouping and IC classification for treatment stratification purposes in order to avoid over-treating eyes that are upstaged by R-E due to the limitations of this grouping system as described above. A comparison of all retinoblastoma patients treated at St. Jude from October 1958 through January 2009 found that the R-E and IC systems agreed in 88% of cases. In the majority of cases where R-E and IC grouping disagreed, the R-E group was advanced (Group IV-V) while the IC classification was a low stage (Group A-C). Therefore, when there is a discrepancy between R-E group and IC classification, the patient will be considered for enrollment on SJRET6 according to the IC classification. Just like in RET5, the eyes will also be staged using the AJCC staging system. Continued use of both R-E and IC grouping systems will ensure an accurate comparison of treatment outcomes based on stage of disease at diagnosis when combining patients treated on RET5 and SJRET6. For patients undergoing enucleation, we use the AJCC and the St Jude staging system; a pathologic staging that incorporates other features known to influence the modality of treatment and the prognosis, such as choroidal involvement, optic nerve extension and presence of metastatic disease.[18] The different classification systems are included in Appendix I.

2.1.4 Principles of Treatment of Retinoblastoma

Treatment of retinoblastoma aims to save life and preserve useful vision, and thus needs to be individualized. Factors that need to be considered include unilaterality or bilaterality of the disease, potential for vision, and intraocular and extraocular staging.

2.1.4.1 Surgery

Enucleation is indicated for large tumors filling the vitreous for which there is little or no likelihood of restoring vision, and in cases of tumor present in the anterior chamber or the presence of neovascular glaucoma. For optimal staging, a long section (10-15 mm) of the optic nerve needs to be removed with the globe. A hydroxyapatite implant is usually fitted during the same procedure, and the extraocular muscles are attached to it. The size and type of implant are important to stimulate orbital growth.[19]

2.1.4.2 Focal treatments

Focal treatments are used for small tumors (< 3-6 mm), usually in patients with bilateral disease, and in combination with chemo-reduction. Photocoagulation with argon laser is used for the treatment of tumors situated at or posterior to the equator and for the treatment of retinal neovascularization due to radiation therapy.[20] Cryotherapy is used for the treatment of small lesions situated in the anterior retina.[21] Finally, an important focal method is transpupillary thermotherapy, which applies focused heat at sub-photocoagulation levels.[22] The use of focal treatments is especially important in conjunction with chemotherapy, and both treatment modalities appear to have a synergistic effect. The sequential administration of thermotherapy with carboplatin enhances the antitumor effect by increasing the platinum-DNA adducts, for which reason thermo-chemotherapy is a very important component in the treatment of intraocular retinoblastoma.[23,24] Also, in addition to its effect on tumor control, cryotherapy contributes to increase the intraocular penetration of carboplatin, presumably through disruption of the blood-vitreous barrier.[25,26] In general, local control rates of 70-80% can be achieved.[20,21,23]

To improve synergy, focal therapy may be administered concurrently with the first course of local (subconjunctival) chemotherapy. In the first five patients treated on SJRET6, Stratum B, three patients received concurrent cryo and/or laser therapy (“focal therapy”) with subconjunctival carboplatin (“local therapy”) in course #1.All patients who received subconjunctival carboplatin experienced periorbital edema and erythema that was managed with systemic steroids, GI prophylaxis (ranitidine), and pain control with morphine and Ativan. No patients who received concurrent focal and local therapy were hospitalized during the first course of therapy for pain control, infection, or other unexpected toxicity. Two episodes of toxicity were reported, both in patients who did NOT receive focal therapy in course 1. One patient was hospitalized after course #1 with febrile neutropenia and treated for suspected cellulitis. The other patient was delayed one week from course #1 to course #2 due to constipation and pain (abdominal).

Previous studies have already evaluated the response of intraocular retinoblastoma to single agent carboplatin,[27] multi-agent,[28,29] or combination chemoreduction/focal therapy

compared with radiation.[30] Using retina-camera (RET-CAM) imaging, a quantifiable response to chemoreduction after the first one-two cycles of chemotherapy was observed. However, these studies have not completely assessed the additional impact of focal therapies applied after two courses of chemotherapy and the impact of topotecan in chemoreduction is unknown.

2.1.4.3 Radiotherapy

Retinoblastoma is a very radiosensitive tumor. However, with the increasing use of chemo-reduction in conjunction with intensive focal therapies, external-beam megavoltage radiation therapy is usually reserved for eyes that have failed more conservative approaches, usually due to progression of vitreous and subretinal seeding, and for tumors adjacent to the optic nerve. Several techniques can be used, usually through lateral fields.[31-33] Recommended total doses are 4,000 to 6,000 cGy, in 180-200 fractions, although doses of 3,600 cGy may be effective in conjunction with other techniques.[34] Three types of tumor regression following radiotherapy have been categorized. In *type I* there is tumor shrinkage with calcium deposition, adopting a "cottage-cheese" pattern. In *type II*, the tumor is seen as a grey, homogeneous mass characterized by a partial shrinkage and loss of the pink color of capillary injection. An annulus of atrophic pigment at the base of the tumor can also be seen. In *type III* the regression pattern has features of both *type I* and *type II*, where the mass has evidence of some shrinkage, and has lost the pink color and has some nidus of calcium. Radiotherapy alone can cure 75-80% of patients. The addition of cryotherapy or photocoagulation can improve the results to 90%.[31-33] Radioactive plaque technique is very advantageous when treating localized tumors, both because the procedure time is short, and because a high dose of irradiation is delivered to the areas of interest while minimizing radiation effects to the extraocular structures. Indications for plaque therapy include solitary tumors with a diameter ranging between 6 and 15 mm, tumor thickness of 10 mm or less, and location of the lesion more than 3 mm from the optic disc or fovea. Different radioactive episcleral plaques can be used; although ^{125}I is the most widely used. Control rates of 85-90% can be achieved.[35]

2.1.4.4 Chemotherapy

Chemotherapy is indicated in patients with extraocular disease, in the subgroup of patients with intraocular disease with high-risk histologic features, and in patients with bilateral disease in conjunction with aggressive focal therapies (see below). Agents effective in the treatment of retinoblastoma include platinum compounds, topoisomerase inhibitors, cyclophosphamide, doxorubicin, vincristine and ifosfamide.[36]

2.1.4.5 Long term effects

Because their orbital growth is still in progress, children treated for retinoblastoma are at risk of functionally and cosmetically significant bony orbital abnormalities. These sequelae become evident by early adolescence, when orbital growth is largely complete, and results in the “hourglass facial deformity”.[37] Both enucleation, which causes orbital contraction, and radiotherapy, which induces arrest of bone growth, adversely affects orbital growth. In children treated for bilateral retinoblastoma, the impact of enucleation

in orbital development is not different from that of irradiation. However, final orbital volumes after enucleation correlate with the size of the prosthetic implant.[38] Patients with germline mutation of the *RB1* gene are at risk of developing second cancers.[39-41] These patients normally have multifocal, bilateral disease and are thus often treated with radiation therapy, further enhancing the susceptibility of a second cancer. The cumulative incidence of second malignancies is 4% at 10 years, 18% at 35 years and 51% at 50 years.[39,40] The tumors most commonly encountered are soft tissue sarcomas, melanomas and osteosarcomas. There appears to be an age effect on the sarcoma risk; patients receiving radiation during the first year of life are at higher risk of developing a sarcoma.[42] hus, it is important to delay or avoid the use of EBRT. Patients with non-hereditary retinoblastoma are not at an increased risk.[40,41]

2.1.5 Treatment of Patients with Metastatic Retinoblastoma

Retinoblastoma tends to metastasize to orbit, pre-auricular lymph nodes, bones, bone marrow, and central nervous system. Patients with distant metastases benefit from the use of high-dose chemotherapy and autologous hematopoietic stem cell rescue, and this approach is considered to be the standard of care.[43,44] The prognosis for patients with invasion of the CNS is very poor despite intensive therapy. Patients with metastatic retinoblastoma will not be treated on this protocol. Patients with orbital disease are curable with intensive chemotherapy and local radiation therapy.[45]

2.1.6 Treatment of Patients with Intraocular Unilateral Retinoblastoma

The majority (> 90%) of patients with unilateral retinoblastoma undergo up-front enucleation. In the United States, surgery is curative for the vast majority of patients, and no adjuvant therapy is required. However, a proportion of them will recur with either orbital or distant disease. There is no universal agreement on which patients are at risk of recurrence. However, based on the St Jude experience, and on some major series, there are some histological characteristics that appear to confer an increased risk for recurrence.[46-50] The magnitude of the risk correlates with the local invasion of the tumor, and two groups of patients may be identified:

2.1.6.1 Intermediate risk

Defined by the presence of tumor in the anterior chamber, invasion of the ciliary body/iris, massive invasion of the choroid, and invasion of the optic nerve beyond the lamina cribrosa with concomitant invasion of the choroid. Patients with these histological risk factors appear to benefit from the use of a combination of vincristine, cyclophosphamide and doxorubicin (VCD).[46,51] At St Jude, we have routinely used the VCD regimen, with excellent results. Between RET3 and the start of RET5, a total of 25 patients with intermediate risk retinoblastoma received 4 to 6 courses of VCD. Two patients have died, one of metastatic retinoblastoma to the CNS, and another patient due to the development of trilateral retinoblastoma. During RET5 therapy, 7 participants were stratified to intermediate risk (Stratum C) after enucleation; all participants are alive with no evidence of disease at a median of 3 years (range 0.5-5.8 years) after enrollment.

2.1.6.2 High risk

A much higher risk of recurrence is associated with the involvement of the sclera, and the involvement of the optic nerve at the level of the cut-end. These patients require more intensive therapy, usually with the incorporation of additional active agents, such as carboplatin and etoposide, and the use of radiation therapy to the orbit and optic nerve. For these patients we have been using alternating courses of VCD with vincristine, carboplatin and etoposide. Seven participants were stratified to the high risk treatment arm of Stratum C after enucleation on RET5; all participants are alive with no evidence of disease at a median of 3 years (range 0.5-5.8 years) after enrollment. Only one participant with high-risk disease received EBRT 4.4 months after enucleation.

2.1.7 Treatment of Patients with Multifocal Retinoblastoma

Patients with germline mutation of the RB1 gene develop multiple, bilateral retinoblastomas at an earlier age, and they are at risk of developing new tumors until the completion of retinal differentiation. Further, these patients continue to be at risk of developing extraocular tumors throughout their lives.[39-41] In the past, the treatment for patients with bilateral retinoblastoma has been enucleation of those eyes with advanced intraocular disease and no visual potential, and the use of external beam radiation therapy for the remaining eyes. However, there are several complications associated to the use of radiation therapy. Irradiation of the orbit during a period of rapid growth results in a major decrease in orbital volume, resulting in midfacial deformities. More importantly, however, is the significant increased risk for the development of a bone sarcoma in the radiation field, discussed above (section 2.1.4.5). These concerns have resulted in the development of new and more conservative approaches. In recent years, treatment of patients with bilateral retinoblastoma has thus evolved to incorporate the use of up-front chemotherapy, which intends to achieve a maximum chemo-reduction of the intraocular tumor burden early in the treatment, followed by aggressive focal therapies. The ultimate goals of this approach are to avoid or delay the use of external beam radiation therapy, and to increase the ocular salvage rate. The use of systemic chemotherapy for cytoreduction, coupled with intensive use of sequential focal therapies (cryotherapy, laser photocoagulation, thermotherapy and brachytherapy) has resulted in an increase in the eye salvage rates and in the decrease (and delay) in the use of radiation therapy. Different chemotherapy combinations are used, although the best results are achieved with a combination of vincristine, carboplatin and etoposide.[23,51-55] As discussed below (section 2.1.8), however, we have shown that a less intensive regimen with vincristine and carboplatin alone appears to be as effective as the three-drug regimen for early intraocular stages.[56] Salvage rates for Reese-Ellsworth groups I-III eyes approaches 100% using these techniques. For patients with advanced intraocular tumors (Reese-Ellsworth groups IV-V), ocular salvage rates are not better than 50%, and external beam radiation therapy is usually required.[52] However, the use of radiation therapy is usually delayed for several months, which allows for a better orbital growth and a decrease in the risk of second malignancies. A major proportion of failures occur because of progression of tumor in the vitreous, or as subretinal implants, two areas of difficult access for antineoplastic agents.[57] Carboplatin diffuses well into the vitreous.[58] Intraocular concentrations are 7 to 10 times higher when carboplatin is administered subconjunctivally,[59] and animal studies have shown a dose-dependent inhibition of

intraocular tumor growth by subconjunctival carboplatin. These encouraging preclinical data, have not been effectively translated into an improvement in the outcome of advanced eyes.[60,61] To address this, subconjunctival carboplatin was incorporated into RET5, Stratum B during courses 5, 8 and 11 for patients who developed or had persistent subretinal seeding during therapy. For patients with early administration of subconjunctival carboplatin (n=4) at course 5, three attained ocular salvage. However, for patients who did not receive subconjunctival carboplatin until course 8 or 11, ocular salvage occurred in only one of three patients. During enrollment on RET5, further preclinical data demonstrating the improved efficacy of combination of carboplatin and topotecan in preclinical models was identified.[62,63] Therefore, patients with subretinal seeding may benefit from the combination of subconjunctival carboplatin with systemic topotecan in upfront therapy. For patients who have recurrent or resistant disease despite this intervention, radiation therapy remains a valid alternative. With the incorporation of brachytherapy or EBRT early in the treatment, in a situation of minimal disease, before disease progression occurs, ocular salvage rates for R-E group V eyes may continue to improve.[53]

2.1.8 Results of the St Jude RET3 and RET5 Protocols

At St Jude, because of concerns with the systemic toxicity of the 3-drug regimen and, in particular, the risk associated with the use of etoposide, and to further investigate the true efficacy of chemotherapy, we historically elected to examine the use of vincristine and carboplatin alone and delay focal treatments until tumor progression was documented, instead of applying focal treatments early in the course of the treatment. This was the design of the RET3 protocol, which opened in January of 1996 and closed in November of 2000. Treatment included vincristine and carboplatin, given every three weeks, for a total of 8 courses, unless disease progression was documented. Focal treatments were applied as needed only after progression. Eyes failing to respond to focal therapies were treated with external radiation therapy or were enucleated.

A total of 25 participants (43 eyes) were treated.[64] All but 2 participants received all 8 planned courses of chemotherapy. One developed progressive disease after 6 courses of chemotherapy and subsequently received EBRT to both eyes; later, both eyes were enucleated. In the other participant, disease progressed in 1 peripheral lesion in 1 eye after 7 cycles of chemotherapy. The remaining 23 participants responded throughout the scheduled 8 courses of chemotherapy. Four (9.3%) eyes (R-E groups Ib, IIa, IIIa, and Vb) in 4 participants required no local ophthalmic treatments. For the remaining 39 eyes, the median time from enrollment to first focal treatment was 7 months (range, 6 to 16 months). Twenty-three eyes (53.5%) could not be salvaged with focal treatments and required EBRT, enucleation, or both. Combined chemotherapy failed in 12 of those eyes (52.2%) because of tumor progression, in 5 eyes (21.7%) because of vitreous seed progression, and in 6 eyes (26.1%) because of both. Thirteen eyes in 11 participants were enucleated (30.2%; 95% confidence interval, 17.2 to 46.1%). Nineteen eyes in 14 participants required EBRT (44.2%; 95% confidence interval, 29.1 to 60.1%). The median time to radiation from enrollment was 9.5 months (range, 6.1 to 20.9 months). All 25 patients enrolled on the RET3 protocol are currently alive at a median of 12.5 years from diagnosis (range, 10.4 to 14.5 years).

The EFS (defining event as EBRT or enucleation) estimate at 2 years for all eyes was 43.6 ± 8.5%[64]. EFS differed by R-E groups (I, II, and III versus IV and V; $p = 0.041$). Among participants with eyes classified in R-E groups I, II, and III, ocular salvage was 83%, and 63% of the eyes were saved without EBRT. These results are inferior to those obtained with the 3-drug regimens.[52,54,55,57] Our less intensive regimen may have an inferior antitumor effect. However, given the excellent early responses to chemotherapy, we believed that the inclusion of aggressive focal treatments early after chemo-reduction instead of after tumor progression could result in outcomes in eyes in R-E groups I to III similar to those of participants receiving more intense chemotherapy. First, local control for most solid malignancies is better achieved early in the course of treatment, when maximum cytoreduction has been achieved and before chemo-resistance develops. Second, the concurrent use of chemotherapy and focal therapies appears to have a synergistic effect. The sequential administration of carboplatin and thermotherapy enhances the antitumor effect by increasing the platinum-DNA adducts, for which reason thermo-chemotherapy is becoming a very important component in the treatment of intraocular retinoblastoma.[23,24] In addition to its effect on tumor control, cryotherapy increases the intraocular penetration of carboplatin, presumably through disruption of the blood-vitreous barrier.[25]

After analyzing the results of the RET3 protocol, our approach to the treatment of intraocular retinoblastoma (RET5) was modified in order to incorporate the use of focal treatments early, rather than wait until disease progression was documented. One hundred and five participants (149 eyes) were enrolled and eligible for therapy on RET5 between February 2005 and September 2010: 23 stratum A, 27 stratum B, and 55 stratum C. 49/149 (33%) eyes were classified as R-E I-III and 100/149 (67%) eyes were classified as R-E IV-V. The primary objective of the RET5 protocol was to answer the research hypothesis that the true response rate of the window therapy for RET5 stratum B patients is greater than 70%. The sample size of 53 patients was calculated based on the hypothesis with a type I error of 10% and power of 90% at true response rate of 85%. A two-stage group sequential design was adapted to give evidence of the futility or efficacy of the therapy. A total 24 of 27 stratum B patients (89%, 95% CI: 71%-97%) of RET5 responded at the first stage of interim analysis and showed early efficacy of the two initial courses therapy.

Table: Two-stage group sequential design

Stage of interim analysis	Total # of patients at the end of each group	Reject window therapy if # of responses is less than or equal to:	Accept window therapy if # of responses is greater than or equal to:
1	27	18	24
2	53	41	42

Outcome estimates were improved for RET5 patients as compared to patients treated on RET3. For stratum B patients, estimates of ocular survival per patient were 66.8% (standard error, 12.8%) for RET5 and 50.0% (14.4%) for RET3 patients [results from the 10/15/2010 DSMB report]. For stratum B patients, estimates of event-free survival per patient were 66.8% (12.8%) and 10.0% (6.7%) for RET5 and RET3 patients, respectively. None of the RET5 stratum B eyes required EBRT, compared to 73% of RET3 advanced stage eyes. Among stratum A patients, estimates of EFS were improved

for RET5 patients (2-year EFS estimate, 70.0% (14.5%)), compared to RET3 patients (45.5% (13.7%)). In addition none of the stratum A eyes required EBRT among RET5 patients, while 43% of low stage eyes in RET3 patients required EBRT. With these positive results, we will continue to treat participants based on the therapeutic approach defined in RET5.

2.1.9 Considerations for Future Therapeutic Approaches

The treatment of patients with advanced intraocular disease (R-E groups IV and V) remains a major challenge, as does minimizing acute and long-term effects of chemotherapy regimens. Chemotherapy intensification appears to correlate with outcome, and better results are obtained with protocols that include at least 6 courses of vincristine, etoposide, and carboplatin.[61,65,66] Central retinal tumors usually respond better to chemotherapy than do tumors in the peripheral retina,[67] but large central tumors may be associated with subretinal seeds, which ultimately may cause treatment failure.[61] A major proportion of failures occur because of progression of tumor in the vitreous or as subretinal implants, two areas of difficult access for antineoplastic agents.[57] In contrast to the highly protein-bound etoposide, which remains in the plasma and lacks intraocular penetration, carboplatin diffuses well into the vitreous.[59] The intraocular penetration of carboplatin is enhanced by disruption of the blood-vitreous barrier by the tumor,[58] and it is enhanced after cryotherapy.[25,26]

Therefore, after analyzing the St Jude data it appears that patients with early intraocular disease (R-E Groups I-III) can be treated with vincristine and carboplatin, and aggressive focal therapies. The results of the St Jude RET3 protocol formed the basis of the multi-institutional COG protocol for early stage intraocular retinoblastoma, as well as our approach in RET5. Attempts at increasing the intraocular concentration of carboplatin through subconjunctival injection was successfully implemented in stratum B of the RET5 protocol. As noted above, ocular salvage was successful more often in patients with early administration (course 5) of subconjunctival carboplatin (3 of 4 patients) then when subconjunctival carboplatin was not initiated until course 8 or 11 (ocular salvage in 1 of 3 patients). Therefore, patients with subretinal seeding may benefit from the combination of subconjunctival carboplatin with systemic topotecan in upfront therapy. We also pursued the use of topotecan, with good effect as 22 of 25 participants evaluated for sustained response showed at least PR in response to window therapy (vincristine/topotecan) on stratum B. The rationale for the use of topotecan is discussed below.

2.2 Rationale for the SJRET6 Protocol

2.2.1 Rationale for Biology Studies in Retinoblastoma

Tumorigenesis is a multistage process involving sequential genetic lesions. For example, pre-neoplastic cells must become growth factor independent, escape apoptosis and G2 arrest, maintain their telomeres and recruit vasculature.[68] As researchers have learned more about the genetic lesions involved in multistage tumorigenesis in different tissues it appears that there are tissue-specific and individual-specific genetic lesions that can move a cell along the course of carcinogenesis. As a result, one of the goals of targeted cancer

therapy is to use this knowledge of the genetic pathways and particular mutations for drug design and treatment suited to individual tumor types and patients. For example, genetic mutations in medulloblastoma cells lead to activation of the Hedgehog pathway ultimately leading to Gli protein activation.[69] Interestingly, in some patients and animal models, the initiating event for medulloblastoma tumor initiation involves the Hedgehog pathway but in other examples it does not. However, recent data has revealed that even in those cases when the initiation lesion does not directly involve Hedgehog pathway signaling, secondary mutations as a result of genome instability from the initiating event leads to Hedgehog pathway mutations and Gli activation. Thus, even in diseases with complex genetics and heterogeneous initiating mutations, the ultimate eventual outcome is the same – Gli activation. Medulloblastoma is a good example of how molecular genetic studies of tumor progression can lead to simplified and directed cancer therapy in the clinic. Several groups are now working on the testing the efficacy of drugs that block Gli for the treatment of medulloblastoma.

The initiating event for retinoblastoma is *RB* gene inactivation.[70] While we know a great deal about the early events that initiate retinal progenitor cell transformation, very little is known about the subsequent genetic lesions that occur over the course of retinal tumorigenesis. The only locus that has been examined in any detail in retinoblastoma is the p53 tumor suppressor gene. Like other childhood tumors of the nervous system, p53 mutations are rare in retinoblastoma. This does not necessarily mean that the p53 pathway is intact, it is possible that another member of the p53 pathway is inactivated leading to the same phenotypic alteration.

For example, genetic amplification of the *MDM2* or *MDM4* genes can lead to increased protein expression and suppression of the p53 response during tumorigenesis.[71,72] Moreover, recent data suggests that polymorphisms at the *MDM2* or *MDM4* loci may contribute to increased basal expression of these important p53 antagonists and increase cancer susceptibility.[73-75]

Bond et al. identified two novel SNPs (SNP 309 and SNP 344) in the first intron and intronic promoter of *MDM2*.[73] SNP 309 T/G was present in 40% of the healthy volunteers in their cohort and SNP 309 G/G was found in 12% of the volunteers. SNP 344 was rare and was not analyzed further. Analysis of the sequence surrounding SNP 309 demonstrated that it was part of an SP1 consensus binding site. A series of biochemical and cellular analyses demonstrated that SNP309 G/G improved SP1 binding at the intronic promoter and increased MDM2 expression. Importantly, increased MDM2 expression led to partial suppression of the p53 pathway and a subsequent acceleration of tumorigenesis in Li Fraumeni patients.[73]

In a similar study[75], Atwal et.al. analyzed the association of *MDM4* SNPs with breast and ovarian cancer risk. SNP 7 T/T was found to associate with the early onset of familial and sporadic cancer among individuals from families with elevated rates of breast and ovarian cancer.[74] Genotype data was collected for MDM4 SNP 7 among two independent cohorts of breast cancer patients (823 patients) and SNP7 T/T was associated with earlier age of onset for estrogen receptor negative breast cancers. The underlying mechanism for the association of SNP7 T/T with earlier age of onset is not known. In a more recent study, Wynendaele and colleagues identified a SNP in the 3' UTR of MDM4 (SNP34091) that

creates a putative target site for miR-191 with the C-allele.[76] The SNP34091-A allele is not efficiently recognized by miR-191 and this in turn leads to increased MDM4 protein expression and increased risk of high-grade carcinoma.[76]

Retinoblastomas have wild type p53[77,78] and cytogenetic studies have indicated that approximately 65% of retinoblastomas have genetic gain of *MDM4*.[79,80] In a very small cohort of retinoblastoma samples, there was an association between *MDM4* gain and increased mRNA and protein expression.[80] However, the sample size was much too small to provide statistical significance. MDM2 has not been analyzed for a relationship between genetic gain and gene or protein expression in retinoblastoma.

Gene expression array analysis of 52 human retinoblastomas in the lab of Dr. Dyer led to the discovery that *MDM4* was expressed at high levels in all 52 tumors irrespective of the *MDM4* copy number.[81] MDM2 was expressed at low levels in these 52 human retinoblastomas.[81] In a series of orthotopic xenografts of human retinoblastoma from our lab and Memorial Sloan Kettering Cancer Center (MSKCC)[82], MDM4 protein was expressed at high levels and MDM2 was not detected.[81] These data suggest that MDM4 expression may be elevated in retinoblastoma through mechanisms that are unrelated to the gene copy number such as the presence of small nucleotide polymorphisms. Specifically, MDM4 SNP7 T/T and/or SNP34901 A/A may contribute to tumor progression in retinoblastoma patients. It is also possible that MDM2 309 G/G contributes to tumorigenesis even though we could not detect the protein in human orthotopic xenografts. Indeed, a recent study showed an association of the MDM2 309 G/G SNP with incidence of familial retinoblastoma. There was no association with MDM4 SNP7 T/T in familial retinoblastoma. Sporadic retinoblastoma has not been analyzed for MDM2 or MDM4 polymorphisms.

To sequence the 3 SNPs that have been shown to correlate with expression of MDM2 and MDM4 and patient outcome, we will utilize DNA (tumor and blood) isolated through ST. Jude Biorepository and PCR amplify each sample with primers that are selective to each SNP and perform Sanger sequencing. We will then correlate the SNP genotype with gene expression using gene expression arrays and we will correlate the gene expression with the SNP genotype, the miRNA191 expression. Ultimately, we hope to establish a connection between the SNP genotype, the expression of MDM2 or MDM4 and the levels of miR191.

The discovery of MDM4 over-expression in retinoblastoma patients provides a unique opportunity for targeted therapy. It has been previously shown that a small molecule inhibitor of MDM2/MDMX, nutlin-3a, can efficiently kill retinoblastoma cell lines in vitro and in vivo[80,83], though it has poor penetration across the blood-ocular barrier.[84,85] In a recent preclinical trial at St. Jude Children's Research Hospital[63], an ocular formulation of nutlin-3a improved intraocular concentration and was effective in a mouse model that had an intact p53 pathway, but it was much less effective in another model lacking p53. In addition, compared with standard agents vincristine, carboplatin and etoposide, it provided the only effective therapy in a human orthotopic xenograft model. This drug is currently under consideration for further development and possible phase I study.

Concurrently with this preclinical data that provides validation for targeted therapy in retinoblastoma, availability of whole-genome sequencing data also allowed us to explore possible association between germline variation and somatic alteration. Utilizing the resources of the Pediatric Cancer Genome Project (PCGP) at St. Jude Children's Research Hospital, we have completed the whole genome sequence and epigenetic analysis of 4 primary retinoblastomas (SJRB001-4) and their matched germline DNA. One of the striking results from the analysis of the RB1 locus in SJRB001-4 was that the matching germline samples of the two tumors (SJRB001,4) with RB1 promoter hypermethylation have only 12-14 heterozygous SNP genotypes at the 178Kb RB1 locus, resulting in a regional nucleotide diversity ($0.7\text{-}0.8\text{x}10^{-4}$ per base) 10-fold lower than their genome-wide average ($7.0\text{x}10^{-4}$). By contrast, the two cases that underwent LOH have 123-128 germline heterozygous genotypes at the RB1 locus with regional nucleotide diversity ($6.9\text{-}7.2\text{x}10^{-4}$) comparable to their genome-wide average. Analysis of HapMap (http://hapmap.ncbi.nlm.nih.gov) genotype data show extended linkage disequilibrium (LD) in this region and rs1951775, a SNP assayed by SNP 6.0, was selected as the tag SNP to assess association of germline heterozygosity and RB1 LOH. All 18 cases with heterozygous genotype at rs1951775 underwent LOH in tumor at the RB1 locus. The LOH events included 14 copy-neutral LOH across the entire chromosome 13 and four somatic deletions ranging from 550Kb to 40Mb. Only seven of the 26 patients that were homozygous at rs1951775 underwent LOH that included six copy-neutral LOH and one deletion of the entire chromosome 13. These data show for the first time a strong association ($p<8\text{x}10–8$, Fisher's exact test) between a germline genetic variation and mechanism of biallelic RB1 inactivation in retinoblastoma.

The clinical significance of this observation could potentially be used to direct therapy. Retinoblastoma patients never undergo biopsy of their primary tumor before treatment. Therefore, it is impossible to use any molecular, cellular or genetic information from the tumor to direct therapy. However, the remarkable finding from the current study is that the heterozygosity of SNPs across the RB1 locus in the blood DNA could actually be used to predict LOH in the tumor. Those patients with reduced heterozygosity of SNPs across the locus are unlikely to undergo LOH and those with normal levels of heterozygosity are virtually guaranteed to undergo LOH based on our studies to date. This is important clinically, because the tumors that undergo LOH rarely if ever silence an RB1 allele by promoter hypermethylation. However, a significant proportion of those that do not undergo LOH do silence their second RB1 allele with promoter hypermethylation. Therefore, a simple analysis of the SNPs across the RB1 locus from blood of retinoblastoma patients could be used to select patients that would be most likely to benefit from targeted therapy and those that would have no benefit with respect to activation of the wild type RB1 gene transcription.

The statistical significance of the association between SNP heterozygosity across the RB1 locus and LOH is now well established with sufficient sample size and statistical power. However the proportion of patients who do not undergo LOH and have homozygous SNPs across the RB1 locus with promoter hypermethylation requires further analysis. In this protocol, we will study the association of RB1 promoter hypermethylation and RB1 locus LOH in retinoblastoma patients. We will perform SNP chip analysis on the tumor DNA and blood DNA and DNA methylation analysis using the Illumina BeadChip assay on the

tumor DNA. For comparison, we will use normal human fetal retinae from weeks 9-24 for comparison for DNA methylation analysis.

Another major hurdle in the treatment of patients with retinoblastoma is the presence of vitreous seeds. The historic teaching of vitreous seeds has been that they are semi-dormant cells with reduced proliferation and metabolic needs; this has created the challenge in developing adequate therapy. Additionally, the vitreous as micro-environment has been poorly understood. The vitreous body is a translucent medium in the eye composed of a liquid (99%) and solid (1%) phase. The solid phase is composed of collagen fibrils, halocytes, and proteoglycans while the liquid phase is primarily water but also contains inorganic salts, sugar, ascorbic acid, hyaluronic acid and soluble proteins. Recent research has identified some of these soluble proteins as growth factors that promote both homeostasis and pathologic processes, most notably diabetic retinopathy and proliferative vitreous retinopathy resulting from complex rhegmatogenous retinal detachments. Platelet derived growth factor (inhibitors) has been identified as one such key regulator.

We hypothesize that modulation of PDGF-PDGFR signaling may control vitreous seeds in RB. In a series of experiments, Dr. Morales-Tirado has documented the expression of *PDGFA* and *PDGFB* isoforms in Y79 and Weri-1 retinoblastoma cell lines. Western blot, Multiplex and flow cytometry analyses revealed a reduction in the PDGF-AB/BB isoforms (p=0.04), FLT-3L (p=0.02), and VEGF (p=0.08) after concomitant incubation of imatinib mesylate and recombinant human PDGF-AB (rhPDGF-AB), when compared to untreated and rhPDGF-AB stimulation. Cellular migration was significantly impacted in 2D and 3D culture models when RB cells were treated with imatinib mesylate. Additional studies utilizing the neutralizing antibody increased the magnitude of the angiogenic reduction. These results point to the conclusion that PDGF-PDGFR signaling sustained angiogenesis in an autocrine and paracrine fashion within the vitreous. The presence of PDGF expression in the vitreous of orthotopic xenograft RB models is under confirmation (by IHC). These studies are a step in the pursuit of our goal to identify and develop targets of immunotherapy that will control the factors sustaining retinoblastoma tumor cells seeding the vitreous, but samples of vitreous obtained at the time of enucleation could be directly tested to confirm the level of PDGF expression.

To better understand the multistage tumorigenesis of retinoblastoma, we would like to isolate fresh tumor tissue and a small sample of vitreous from St. Jude patients immediately after they have undergone enucleation. For retinoblastoma patients undergoing enucleation at St. Jude Children's Research Hospital, the eye will be processed by the ophthalmologist/pathologist Dr. Wilson immediately after enucleation with excess tumor provided first to TBANK (with consent obtained from the legal guardian) and then to Dyer lab for xenografts. A vitreous sample will be placed in a conical tube marked for further investigation in the Morales-Tirado lab. DNA, RNA, and protein will be isolated from primary tumor specimens by the St. Jude Biorepository and provided to the Dyer lab upon request for specific SJRET6 biology objectives. The portion of fresh tumor provided to the Dyer lab will be either transferred to culture medium and grown at 37°C to isolate new retinoblastoma cell lines or will be used to establish new orthotopic xenografts. The enucleated eye and remaining tumor cells will then be fixed and embedded by the Pathology department and archived as with all other

retinoblastoma cases. The fresh tumor cells will be isolated from the eye in such a way as to preserve as much of the tumor specimen in the eye as possible and not interfere with subsequent histopathological diagnosis. The uses for the RNA, protein, genomic DNA and cell lines are described in detail below. The protocols for these isolation procedures are contained with the St. Jude Biorepository.

2.2.1.1 RNA analysis

Retrospective histological analysis of retinoblastoma tumors has provided some valuable information on the types of changes in the tumor cells, the degree of intra-tumor heterogeneity and patient-to-patient heterogeneity. However, such studies are time consuming and are limited by the availability of well-characterized antibodies to individual proteins in different pathways believed to be involved in tumorigenesis. We would like to combine immunohistochemistry with a more comprehensive molecular analysis of retinoblastoma tumor samples using microarrays to obtain a clearer picture of the gene expression profiles in different tumors. By hybridizing labeled antisense RNA from mRNA isolated from fresh retinoblastoma tumors to the Affymetrix gene expression chip, we will be able to probe tens of thousands of genes and assess differences in expression levels among individuals. By comparing the gene expression profiles from different tumor samples and correlating that with histological grade we hope to better understand the genetic changes that occur in retinoblastoma samples as they progress through different stages of tumorigenesis as well as any heterogeneity among patients.

We will study the DNA repair enzymes that are required to repair topotecan induced breaks and carboplatin induced nucleotide adducts. VEGF and other endothelial growth factor will be analyzed to determine if the tumor cells themselves secrete factors that can help to recruit vasculature and promote tumor survival. Telomerase activation is believed to play an important role in tumorigenesis and thus the microarray approach will be used to study the expression of the telomerase gene in retinoblastoma. These are a subset of the questions that we will study using RNA prepared from retinoblastoma tumor samples. Similar studies on medulloblastoma have led to the identification of a set of predictor genes that are prognostic for a favorable treatment outcome.[86] While it is not feasible to obtain a biopsy from each retinoblastoma patient as with medulloblastoma, we hope to correlate molecular signatures with clinical features and then employ the most appropriate treatment. In some bilateral retinoblastoma cases, if the tumor in one eye is advanced and that eye is removed prior to treatment, this sample could in principle be used to obtain a molecular signature and inform the subsequent treatment protocol for the remaining eye.

2.2.1.2 Protein analysis

Protein purified from each retinoblastoma sample will be analyzed by SDS-PAGE and immunoblot analysis. The antibodies selected for immunoblotting will be based on the results of the microarray hybridization. That is, immunoblots will be used to verify the data from gene expression microarrays. It is important to note that many of the proteins involved in the p53 and apoptosis pathway are regulated at the post-translational level so protein analysis may identify changes not reflected at the messenger RNA level. However, due to the limited amount of protein from the lysate and the relatively small

number of antibodies available for immunoblots, this analysis will only be performed on a handful of the most relevant proteins.

2.2.1.3 Genomic DNA

Genomic DNA isolated from each retinoblastoma tumor sample by the TRCF. Additional genomic analysis will be carried out with the Affymetrix short nucleotide polymorphism (SNP) array to study loss of heterozygosity (LOH) to compare the blood DNA to the tumor DNA. In this protocol, we will study the association of RB1 promoter hypermethylation and RB1 locus LOH in retinoblastoma patients. We will perform SNP chip analysis on the tumor DNA and blood DNA and DNA methylation analysis using the Illumina BeadChip assay on the tumor DNA. For comparison, we will use normal human fetal retinae from weeks 9-24 for comparison for DNA methylation analysis.

To sequence the 3 SNPs that have been shown to correlate with expression of MDM2 and MDM4 and patient outcome, we will perform whole genome amplification of the blood and tumor DNA using the Qiagen REPLI-g service. We will then PCR amplify each sample with primers that are selective to each SNP and perform Sanger sequencing. We will then correlate the SNP genotype with gene expression using gene expression arrays and we will correlate the gene expression with the SNP genotype, the miRNA191 expression.

As a consequence of this improved understanding of the biology of retinoblastoma, patients with a particular clinical presentation may respond better to different treatments based on our understanding of the multiple stages of retinal tumorigenesis. It is impossible to predict at this time if the genetics of retinoblastoma tumor progression will converge on a single pathway as with medulloblastoma or if there will be multiple independent pathways.

2.2.1.4 DNA methylation assay

Methylation analysis will be performed using the Human Methylation 45 BeadChip and iScan system from Illumina, Inc. (San Diego, CA). Briefly, following bisulfate treatment (Zymo, Inc.), genomic DNA is amplified and fragmented and then hybridized to the BeadChip. A single-base pair extension reaction is then performed, followed by staining with a detectable fluorescent dye label. The resulting products are imaged with the iScan Reader (Illumina, Inc.). Methylation at each locus is determined by calculating the percent of total signal from each locus that is represented by the methylation probe using the GenomeStudio software (Illumina, Inc.). Quality standards for bisulfite conversion, staining, single base extension, hybridization, stringency, and non-specific binding have been verified.

A methylation state of every probe is represented by fluorescent signals from the M (methylated) and U (unmethylated) alleles. The ratio of fluorescent signals is then computed from the two alleles: $\beta = (\max(M, 0))/(|U| + |M| + 100)$. To avoid "negative signal" a small constant offset 100 is added to the denominator to regularize β value when both methylated and unmethylated probe intensities are low. The β value ranges

between 0 and 1 and under ideal conditions it has bimodal distribution with two means corresponding to methylated and unmethylated states of the interrogated CpG site.

2.2.1.5 Cell line cloning

There are currently two human retinoblastoma tumor cell lines (Y79 and Weri1) available from the ATCC but these have been cultured for decades and are believed to have undergone subsequent genetic alterations in culture. Thus, *in vitro* drug studies using these cell lines are suboptimal and a tumor cell line that more faithfully resembles the naturally occurring tumors will be valuable for *in vitro* and xenograft studies. The cells from the original tumor isolate will be cultured in RPMI medium to grow out clones of tumor cells. Dr. Dyer's lab has developed a novel xenograft system that recapitulates the clinical and histopathological features of retinoblastoma in children. This model is currently being used to test drug treatment protocols with human tumor samples as well as Y79 and Weri1 cells. With the addition of new retinoblastoma cell lines, we hope to better assess the effects of drug treatment on retinoblastoma cells that more closely mimic the original tumors. Beyond the xenograft studies, the newly isolated retinoblastoma cell lines will provide us with valuable information about the behavior of the tumor cells from individual patients. For example, we will measure the doubling time, genome instability, and apoptosis of the early passage cell lines and these phenotypic variables will be correlated with the genetics (Rb resequencing chip and LOH chip) as well as the RNA and protein analysis. Moreover, the response of each cell line to drug treatment will be tested to provide additional correlation between molecular genetic changes in retinoblastoma cells and their response to different chemotherapeutic agents.

2.2.1.6 Analysis of Vitreous Proteome

Fresh vitreous samples will be harvested from the enucleated eye through the same trephined site used to harvest fresh tumor. Under sterile conditions, 2ml of material will be transferred to a 15ml conical tube. Samples will be labeled with TBANK number and date (no direct patient identification) and snap frozen with storage by the Dyer laboratory (-80C) until analysis by the Dr. Morales-Tirado Lab at University of Tennessee, Health Science Center. In the Morales-Tirado lab, the sample will be centrifugated at 12000 RPM x 15 min x RT to separate the liquid component, as described in Yu et al, Proteomics, 2008. If debris is observed, we will filter the sample in a 0.22uM filter to clarify sample, as done by Kim et al, Proteomics, 2007. From the clarified fraction of the vitreous we will divide the sample into 2, one for protein analysis of PDGF and isoforms PDGF-A, PDGF-B and their receptors using commercially available Multiplex systems; second set for mRNA analysis of *PDGFA*, *PDGFB*, *PDGFRA*, *PDGFRB*, using commercially available primers, following the protocol for nucleic acid isolation from Chintalapudi and Morales-Tirado et al, 2015. The protocols for obtaining tumor tissue, as well as the specifics of the studies are included in Appendix II.

2.2.2 Rationale for the Use of Topotecan in the Treatment of Retinoblastoma

2.2.2.1 Use of topoisomerase inhibitors in pediatric cancer

Xenograft models have been useful for defining the optimal clinical use of camptothecin agents. Studies in mice bearing pediatric solid tumor xenografts have shown that these camptothecin analogs are among the most active of the anticancer drugs. Significant objective responses have been obtained in rhabdomyosarcoma,[55-57] neuroblastoma,[58, 59] osteosarcoma,[55] and brain tumors.[56, 57, 60 61] Moreover, studies in these xenograft models have shown that the antitumor activity is highly dependent on dose and schedule. Responses are better when the agents are given daily at low doses, for protracted periods of time. In some of these studies, this schedule of prolonged administration induced responses in xenografted tumors that had been unresponsive to intermittent administration of the agents at high doses.[56] These schedules probably produce greater antitumor activity by maximizing the number of covalent topoisomerase I-DNA complexes that are formed.

Recently, systemic topotecan was tested in genetic and orthotopic xenografts models of retinoblastoma in direct comparison with standard of care triple agent (vincristine/etoposide/carboplatin) using AUC-guided doses and clinically relevant schedules. The results in the genetic models are virtually curative and just as good as the standard agent chemotherapy regimen (no statistically significant difference).[63] This comprehensive preclinical data combined with previous *in vitro* and *in vivo* studies demonstrating the effect of topotecan in retinoblastoma[87] gives further support to using this drug in pediatric retinoblastoma.

2.2.2.2 Topotecan in the treatment of retinoblastoma

Prior to RET5, the information supporting topotecan use in retinoblastoma was based on anecdotal responses in previous Phase I and II studies. However, in combination with the *in vitro* and *in vivo* studies performed by Dr. M. Dyer at St. Jude showing that retinoblastoma cell lines and human orthotopic xenografts are very sensitive to topotecan,[62,63,87] window therapy utilizing topotecan and vincristine (V/T) was successful with 24 of 27 participants enrolled on stratum B achieving a sustained response of at least PR. The participants who responded to window therapy continued to receive alternating courses of V/T with vincristine/carboplatin, in the hope of providing a decreased risk of etoposide-induced leukemia for our patients with advanced disease.[88-90]

The mechanism for topotecan's effect in retinoblastoma may be explained by the preclinical and clinical studies that have shown that topotecan penetrates the blood brain barrier, and it has good penetration into the cerebrospinal fluid.[76] Since there are some structural and functional similarities between the blood brain barrier and the vitreoretinal barrier, the assumption that topotecan would have good intraocular penetration has been studied in rabbits and rats. The data show that topotecan consistently penetrates the vitreous in the animal eye, and that the proportion of drug that crosses the blood-vitreous barrier is similar or higher than the proportion of drug that crosses the blood-brain barrier.[91] It must be considered, however, that due to the nature of the disease, the blood-vitreous barrier is significantly disrupted in the eye with retinoblastoma, and therefore the

penetration of topotecan into the vitreous may be much higher than what the preclinical studies show.

Incorporation of topotecan into window therapy in RET5 did have anti-tumor effect and supports the continued use of this agent in retinoblastoma therapy. The primary objective of RET5 was to estimate the response (CR/PR) rate of in advanced intraocular retinoblastoma. As mentioned above, twenty-four (24) of 27 participants (88.9%) had partial responses to window therapy. One participant did not complete the window due to unacceptable toxicity and was considered a failure for response. Two participants each had PR in one eye but developed new lesions in the opposite eye prior to having sustained responses for at least 4 weeks; these 2 participants were also considered failures for response. Although longer follow-up is required, our data suggests that adding vincristine and topotecan to the backbone of vincristine/carboplatin regimen may result in improved EFS (defining event as EBRT or enucleation) for children with advanced intraocular retinoblastoma.

Rationale for adding filgrastim or peg-filgrastim

The toxicity profile for topotecan and vincristine was tolerable after adding the GCSF in RET5 (amendment 5) with skin and febrile reaction the main toxicity accompanying topotecan administration. In the RET5 protocol, patients treated with G-CSF had a significantly shorter duration of grade 4 neutropenia, a lower frequency of grade 3 or 4 non-hematologic toxicity, and less delay in completing window therapy. Therefore, filgrastim or peg-filgrastim will be incorporated into this study.[92]

In addition to efficacy data, detailed pharmacokinetic profiles for patients receiving topotecan were evaluated. In our population pharmacokinetic study of topotecan lactone, we observed that topotecan clearance changes rapidly over the first year of life (i.e., children < 6 months had lower clearance values than older children); however, this analysis included a limited number of infants and young children <1 year of age. The physiological basis of this relationship is likely renal development, since topotecan is excreted primarily by renal mechanisms. We were able to explore this relationship further in the RET5 clinical trial since the topotecan was pharmacokinetically adjusted, and many of the participants on the study were < 1 year of age. In a preliminary analysis, we studied 94 pharmacokinetic studies in 23 participants, and found that TPT lactone clearance increases with age and plateaus at approximately 1 year. As with our previous analysis, this trend mirrors the pattern of renal development. We have performed a nonlinear mixed-effects modeling analysis to revise the initial dosage recommendations based upon the observations of age-related changes in topotecan clearance. These dosage recommendations will be implemented in the present (SJRET6) clinical trial with a continued schedule of daily x 5 days.

2.2.2.3 Rationale for the use of periocular carboplatin with systemic topotecan

As discussed above, *in vitro* and *in vivo* studies performed by Dr. M. Dyer have shown that the combination of topotecan and carboplatin is the most effective against retinoblastoma.[80-82] In a recent update of their work, Dyer's group demonstrated that a protocol similar to RET5 (and the basis for the proposed SJRET6 trial) was equally

effective as the standard regimen of vincristine, carboplatin, and etoposide without increased toxicity in 2 genetic mouse models.[63] It is particularly significant that this combination was not statistically different from the standard regimen of vincristine, carboplatin, and etoposide. Ideally, the topotecan-carboplatin combination would be the one that we would like to investigate. Unfortunately, systemic administration of both agents in doses that would reach therapeutic levels in the eye results in significant systemic toxicity.[82] However, we must consider that our goal is to obtain therapeutic concentrations of both agents in the eye at the same time in order to achieve the synergistic effect. As discussed above, periocular administration of carboplatin has proven to result in high intraocular concentrations,[47] and animal studies have shown a dose-dependent inhibition of intraocular tumor growth by subconjunctival carboplatin.[19, 48] At St. Jude, we have extensive experience with this form of administration, as we routinely use it for salvage of eyes with advanced disease. The Children's Oncology Group has also used periocular carboplatin in combination with systemic administration of vincristine, carboplatin, and etoposide, in the treatment of patients with advanced intraocular disease. In RET5, periocular (subconjunctival) carboplatin was administered to patients enrolled on Stratum B starting in course 5 at the discretion of the treating team. Ocular salvage was achieved more often in patients with early administration of subconjunctival carboplatin (3 of 4 patients) vs. in patients who did not receive subconjunctival carboplatin until course 8 or 11 (1 of 3 patients). Therefore, patients with subretinal seeding may benefit from the combination of subconjunctival carboplatin with systemic topotecan in upfront therapy. For these reasons, in order to achieve therapeutic levels of topotecan and carboplatin simultaneously in the eye, for patients with subretinal seeding at diagnosis enrolled on SJRET6, Stratum B, we will administer periocular carboplatin with systemic topotecan for the initial two courses of therapy. Subsequent doses of periocular carboplatin may be administered with one or more courses of vincristine-topotecan during the consolidation phase (course 5, 8, and/or 11). Patients who fail to respond to the first two cycles of therapy with periocular carboplatin/topotecan and who will be treated with vincristine, carboplatin, and etoposide, will similarly have the opportunity to receive further doses of periocular carboplatin.

While the RET5 protocol and initial SJRET6 protocol therapy limited periocular carboplatin to 3 injections, we have identified patients that may benefit from further co-administration of periocular carboplatin with systemic topotecan. Of 7 patients enrolled on Stratum B from January 2014 to November 2014, 4 patients have completed at least 8 courses; two of the four patients had evidence of persistent vitreous seeding that is responding to therapy, but has not resolved. We are not able to identify viable vs. treated vitreous seeds with either diagnostic imaging or during direct examination under anesthesia. Persistent vitreous disease is very difficult to control following chemotherapy, so maximizing local delivery and focal therapy during the current chemotherapy regimen is of paramount importance. In both patients, the vitreous seeding is located in the eye with best potential for visual salvage during therapy. For these patients, the local toxicity of further periocular carboplatin was balanced with the potential for EBRT or enucleation if the patient has progressive or recurrent disease not amendable to current therapy. The risk of second malignancy following radiation, especially for patients on Stratum B with bilateral disease (i.e. *RB1* mutations), must also be considered and avoided if at all possible.

In both patients, a deviation to the protocol has been requested and granted as administration of additional periocular carboplatin was clinically indicated. In both patients, we have evidence of disease response to the administration of additional periocular carboplatin paired with aggressive focal therapy: over 3 months in one patient (who received 2 additional doses of periocular carboplatin) and over 3 weeks in the second patient (who has received one additional dose of periocular carboplatin).

We will not be able to differentiate the toxicity of additional doses of periocular carboplatin past the 3rd dose, as any effects are more likely due to the cumulative dose already received in the initial three doses of subconjunctival carboplatin. The main objective of this protocol, disease response to the first two courses of therapy, is not affected by the additional injection(s) of periocular carboplatin. We did not anticipate these clinical scenarios prior to drafting SJRET6, but recognize this situation may arise again in future patients and will provide additional periocular carboplatin as clinically indicated for our future patients on this protocol.

Additional doses will not be administered to patients with disease progression that meet defined criteria; they will be removed from protocol therapy as defined in Section 11.1.4.

Previous use of subconjunctival carboplatin has identified specific ocular toxicities, including fibrosis of orbital soft tissues, orbital fat atrophy and cellulitis.[93-95] During RET5 therapy, participants with persistent vitreous disease (n=7) were well able to tolerate subconjunctival administration per protocol. Only one patient required a fat graft after enucleation of the treated eye. During SJRET6 therapy, no patient has required intervention for periocular toxicity due to subconjunctival administration of carboplatin. Two patients have now completed therapy on Stratum B, and will begin post-therapy monitoring for late effects/toxicities of periocular therapy. We plan to review the diagnostic imaging (MRI) for patients who have received periocular therapy to analyze any visualized orbital changes in response to therapy.

2.2.3 Rationale for Reduced Platinum Therapy in Infants Less than 6 Months of Age

Ototoxicity is a known toxicity of therapy with platinum agents, and is linked to total cumulative dose.[96,97] The platinum compound with the highest ototoxicity potential is cisplatin, but carboplatin at high doses has also been reported to cause serious, bilateral hearing loss.[98,99] The true incidence of ototoxicity in retinoblastoma patients is still unclear, with reports ranging from no ototoxicity[100,101] to <10%.[102-104] However, a review of 60 retinoblastoma patients treated with systemic carboplatin and vincristine at St. Jude Children's Research Hospital with comprehensive audiology testing documented that 12 patients (20%) experienced some degree of ototoxicity, with 10 patients (17%) having sustained hearing loss.[105] Age was a significant predictor of toxicity, with younger patients < 6 months more likely to have ototoxicity than older patients. It is likely that these numbers are indicative of the true incidence of ototoxicity when comprehensive evaluations are performed in this population.

Therefore, we hope to identify children at highest risk for ototoxicity (less than 6 months of age) with early stage intraocular disease (Stratum A). These participants are candidates for reduced carboplatin therapy, but still require adequate adjuvant chemotherapy to cure

disease. The combination of vincristine and topotecan was well tolerated with GCSF during window therapy on Stratum B of RET5, with almost 90% response. Therefore, participants on this subset of Stratum A (less than 6 months of age) will be treated with vincristine/carboplatin (3 courses) alternating with vincristine/topotecan (3 courses) for a total of 6 cycles of chemotherapy in combination with local ophthalmic-directed therapy.

2.2.4 Early Initiation of Therapy Services in Children with Retinoblastoma

It is known that much of a child's learning during the early years is processed through vision and that children with vision loss may experience delays in fine or gross motor skills, poor spatial awareness, interaction skills, and limited ability to participate in age appropriate occupations including self-care, and play.[106-108] Infants and toddlers (birth–36 months) who are at risk for or have disabilities should receive developmentally supportive care that addresses a broad spectrum of priorities and concerns.[109] According to the National Plan for Eye and Vision Research (2006), children with low vision may benefit considerably from coordinated and comprehensive rehabilitation services. Occupational therapy and Speech therapy are two important components of the rehabilitation and habilitation of young children with low vision.

Children with retinoblastoma have known visual deficits which may result in developmental delay, limited participation in age appropriate activities and negatively impact quality of life. Van Dijk and colleagues investigated restrictions in daily life after retinoblastoma in survivors ages 8-35 years.[110] They found that 54-55% of survivors considered themselves to be restricted in ADL skills, with the reported restrictions being mainly related to vision impairment. Specifically survivors reported learning difficulties, limited participation in leisure activities and sports activities, and mild to severe vision related difficulties at work. Even survivors with normal vision (as defined by WHO guidelines) reported functional restrictions. Forty seven percent of survivors ages 8-17 years of age considered themselves restricted from participation in team and ball sports; 67% of this group had normal vision.[110]

Currently occupational therapy and speech therapy at St. Jude has been involved with many of the children on the RET5 protocol to monitor developmental progress, educate and empower parents to advocate for resources and therapy services in their home community, and to provide direct services as needed.

Occupational therapists focus on assessment and facilitation of participation in age appropriate functional activities. In addition to developmental assessment and intervention, the child may participate in a low vision evaluation, including functional visual assessment, training and education in visual compensatory skills, as well as provision of and training in use of low vision equipment.

Speech therapists address delays and disabilities in communication, language, speech, emergent literacy, and feeding/swallowing. This may include active exploration and manipulation of objects, and interactive participation appropriate to a child's age, cognitive level, strengths, interests, and family concerns.

In addition to services at St. Jude, the occupational therapist or speech therapist may recommend and facilitate initiation of therapy services within the child's home community. Such services may include early intervention or school based services (occupational, speech, or physical therapy) and/or low vision services such as those provided by a low vision teacher or orientation/mobility specialist.

In this study, we will evaluate the indication of rehabilitation services, and the feasibility to perform the recommended services within the home community. Additionally, we will evaluate the characteristics of development, participation, and quality of life among children with retinoblastoma. Information/data obtained will be utilized to enhance direct patient care/service delivery as well as provide support/evidence for the role early initiation of therapy services in children with visual deficits.

Research participants will be evaluated by Occupational Therapy and Speech Therapy near the time of initial diagnosis, and at approximately 6 month intervals for 3 years. Additional visits within the initial 3 year period will be scheduled on an as needed basis, as per the discretion of the occupational therapist and speech therapist. Following the initial 3 year period, visits will be scheduled on an as needed basis. A scheduled long term follow up evaluation will occur at approximately 5 years after the initial evaluation. The tests and questionnaires to be performed are outlined in Appendix III.

2.2.5 Rationale for Audiology Evaluations of Carboplatin Ototoxicity

Platinum compounds are commonly used to treat retinoblastoma. While the effects on hearing of cisplatin are well documented, there is conflicting evidence concerning the potential ototoxic effects of carboplatin. While it is usually assumed that carboplatin ototoxicity is extremely rare, there are very few studies investigating cumulative incidence of and prognostic factor for ototoxicity secondary to this platinum agent. A recent study revealed 10 (17%) of 60 patients diagnosed with retinoblastoma and treated with systemic carboplatin and vincristine were diagnosed with clinically significant hearing loss post therapy.[105] These findings indicate a higher incidence of carboplatin-induced ototoxicity than previously reported, particularly in younger children diagnosed and treated under the age of 6 months.[105] Therefore, the ability to detect potential changes in cochlear function associated with carboplatin is very relevant. Monitoring ototoxicity with more sensitive methods can also enhance early detection of damage to cochlear hair cells, and enhance early intervention measures.

The main objectives of the proposed study are to a) refine existing monitoring protocols in order to detect sub-clinical changes in cochlear function that can alert the physician to the possibility of development of hearing loss as early as possible, b) to determine if wideband reflectance measurements are able to better detect the presence of middle ear pathologies and predict hearing status, and c) to assess the impact of carboplatin on changes in cochlear function that are detectable with measurement of otoacoustic emissions. We will compare measurements of middle ear status (tympanometry and wideband reflectance), outer hair cell function (otoacoustic emissions), and inner hair cell function (auditory evoked potentials) in patients receiving carboplatin chemotherapy, in order to determine if subclinical changes in cochlear function can be more efficiently detected.

Otoacoustic emissions are usually inaudible sounds produced by the healthy cochlea. These sounds can be measured non-invasively by placing a probe with a soft tip into the patient's ear canal and a miniature microphone in the probe measures otoacoustic emission levels. The measured levels of otoacoustic emissions are specifically linked to the functional status of the outer hair cells within the cochlea.[111] The ability to monitor cochlear function non-invasively is of particular interest to the pediatric oncologist, as platinum compounds often interfere with metabolic processes of cochlear hair cells. It has been shown that administration of ototoxic agents, such as cisplatin, damage outer hair cells and diminishes the measured levels of otoacoustic emissions.[112] A decrease in the measured level of otoacoustic emissions is often more sensitive in detecting early cochlear damage that may lead to permanent hearing loss compared to conventional hearing tests.[113] In an animal model, low doses of carboplatin led to exclusive loss of inner hair cells, while higher doses of carboplatin led to complete losses of inner hair cells combined with loss of outer hair cells in the middle and basal turns of the cochlea.[114] A clinical study done by Dr. Bhagat and Dr. Bass and her colleagues at St. Jude during the RET5 protocol revealed that 4 out of 10 children had a criterion decrease in DPOAE level after 3-4 courses of carboplatin.[115] Dr. Bhagat has considerable experience measuring otoacoustic emissions in humans.[115-117]

Obtaining an accurate measurement of middle ear status is important in infants and young children given the increased risk of middle ear effusion. Single-frequency tympanometry using a 226 Hz or 1000 Hz probe tone is a commonly performed measurement that is used to assess the integrity of the middle ear conductive mechanism. While tympanometry is considered a staple in the audiological test battery, previous studies have shown that 226 Hz tympanometry does not reliably identify middle ear effusion in infants younger than six months of age.[118,119] Although tympanometry at 1000 Hz has shown to be more effective in detecting middle ear pathology, particularly in infants less than six months of age, sensitivity of 1000 Hz tympanometry continues to be less than optimal when compared to wideband reflectance measurements.[120] Wideband reflectance (WBR) refers to amount of energy either reflected or absorbed by the middle ear system. WBR measurements is particularly significant for the pediatric oncology patient receiving ototoxic medications due to increased risk of otitis media with effusion from suppressed immunity as well as high frequency hearing impairment secondary to damaging effects to the cochlear structures. WBR measures a broader range of frequencies compared to conventional tympanometry, thus providing a more complete picture of the middle ear properties and improving the ability to predict hearing sensitivity[121], particularly for the high frequency region where ototoxicity is more likely to occur.

WBR energy is measured by presenting an acoustic stimulus to the ear canal via a probe encased in a soft ear tip. The response is then analyzed by commercial software provided by the equipment manufacturer. WBR is a non-invasive procedure requiring minimal test time and is easier to perform on the younger population than tympanometry due to less susceptibility to issues with probe placement, small ear canals, ear canal pressurization, and standing waves in the ear canal.[120,121] Normative data has been previously collected for wideband reflectance measures in healthy children from birth to 47 months of age[122] and will be used for this study.

2.2.6 Rationale for Pharmacogenetic Studies of Carboplatin

Ototoxicity is a common and debilitating adverse effect of platinating agents in children with retinoblastoma. In a recent study of RET3 and post-RET3 patients, 20% of children developed severe hearing damage following carboplatin treatment. Because hearing loss caused by platinating drugs has profound negative impact on the quality of life, there is an urgent need to identify clinical and biological factors associated with the risk of ototoxicity of in children with retinoblastoma. We hypothesize that inter-individual variability in susceptibility of hearing damage can be attributed to inherited genetic variation. To this end, we propose an exploratory pharmacogenetic aim within SJRET6 to test association of selected candidate genetic variants with ototoxicity. This work will be done in collaboration with Drs. Jun Yang and Clinton Stewart at Pharmacologic Sciences Department and we expect to interrogate no more than 10 genetic variants.

2.2.7 Ethical Considerations

Germline *RB1* mutation analysis is a fundamental aspect of the correlative biology studies of this study. At the time of protocol registration, the parents/legal guardians will be approached by the primary oncologist who will indicate the propensity of this disease to have an underlying genetic etiology. Permission for obtaining blood from the child will be requested, and this sample will be sent to the study laboratory for DNA isolation. Each family will be referred to the genetic counselor for genetic counseling. If the parents/guardians refuse screening, they will be asked whether they wish the samples to be retained for potential future use, or to be destroyed. This information will be communicated back to the reference biology laboratories. For those samples for which *RB1* mutation analysis is performed, once the result has been issued, this information will be reported back to the genetic counselor and/or primary treating physician who will then counsel the parents as per standard practice.

3.0 ELIGIBILITY CRITERIA AND STUDY ENROLLMENT

3.1 Inclusion Criteria

3.1.1 Newly diagnosed, untreated intraocular retinoblastoma. Participants previously diagnosed with unilateral retinoblastoma treated surgically, with focal therapy or needing chemotherapy who develop asynchronous involvement of the contralateral eye, or patients with unilateral retinoblastoma treated only with enucleation or focal therapy who develop asynchronous involvement of the contralateral eye, will be eligible for study.

3.1.2 ECOG Performance Score must be ≤ 2 within two weeks prior to registration (see Appendix V).

3.1.3 Participants must have an adequate liver function, as defined by bilirubin ≤ 3 x upper limit of normal (ULN), and SGOT and SGPT ≤ 3 x ULN.

3.1.4 Participants must have adequate renal function as defined by serum creatinine ≤ 3 x ULN for age.

3.1.5 Legal guardians must sign an informed consent indicating that they are aware of this study, the possible benefits, and toxic side effects. Legal guardians will be given a signed copy of the consent form.

Note: For inclusion on biology studies after enucleation, the patient or his/her legal guardian, as appropriate, must provide written informed consent for SJRET6 protocol within 60 days of the removal of the tissue (eye) sample.

3.2 Exclusion Criteria

3.2.1 Previously treated participants.

3.2.2 Presence of metastatic disease or gross (residual) orbital involvement.

3.2.3 Participants must not have an invasive infection at time of protocol entry.

3.2.4 Inability or unwillingness of research participant or legal guardian/representative to give written informed consent.

3.3 Research Participant Recruitment and Screening

This is a St. Jude sponsored study, to be conducted at St. Jude only. Research participants will be recruited from the principal investigator's and co-investigators' clinical practice according to standard methods within the Division of Solid Tumor and following institutional guidelines.

3.4 Enrollment On Study

A member of the study team will confirm potential participant eligibility as defined in Section 3.1-3.2, complete and sign the 'Participant Eligibility Checklist'. The study team will enter the screening and/or eligibility information into the St. Jude Clinical Trials Management System (CTMS), and a research participant-specific consent/assent form will be generated. The complete signed consent/assent form(s) must be faxed or emailed to the CPDMO at 595-6265 to complete the enrollment process.

The CPDMO is staffed 7:30 am-5:00 pm CST, Monday through Friday. A staff member from the Milli helpline is on call Saturday, Sunday, and holidays from 8:00 am to 6:00 pm. If you have a therapeutic research enrollment and need assistance releasing your consent, please call the Milli helpline (901-338-0596) on call number.

Notify the Solid Tumor CRAs at extension 3594. Enter all research participants by phoning **Dr. Rachel Brennan** (pager #1878) or **Dr. Ibrahim Qaddoumi** (pager #0650) after completing the eligibility checklist.

4.0 STRATUM AND RISK ASSIGNMENT AND CRITERIA FOR UPFRONT ENUCLEATION

The main research objective of the SJRET6 protocol is to improve our understanding of the biology and tumorigenesis of retinoblastoma when biology specimens are available. As clinicians, our primary goal for participants with retinoblastoma is to concurrently provide multi-modal therapy in an attempt to attain ocular salvage and vision preservation. Therefore, all participants with non-metastatic retinoblastoma will be enrolled on the study. Participants will be stratified into four main groups, depending on laterality and disease stage:

4.1 Stratum A: Early Unilateral or Bilateral Retinoblastoma

Participants with early bilateral or unilateral (unifocal or multifocal) retinoblastoma (R-E I-III, IC A-B; R-E IV with IC A or B; or IC C with limited sub-retinal seeding), and participants with bilateral disease in whom the advanced eye has been enucleated upfront (without any high risk histopathology) and the remaining eye has early stage disease (as defined above).

4.2 Stratum B: Advanced Bilateral or Advanced Unilateral with Foveal Sparing Retinoblastoma

Participants who are considered candidates for conservative management including:

1. Participants with bilateral retinoblastoma who have R-E IV-V and/or IC D-E in one eye

9.4.

4.3 Stratum C: Advanced Unilateral Retinoblastoma

Participants with advanced (R-E IV-V and/or IC D-E) unilateral retinoblastoma who require upfront enucleation.

4.3.1 Low Risk Stratum C Research Participants:

Participants in whom the enucleated eye does not show extra-retinal disease (see definition of intermediate and high risk below); will not receive any additional treatment.

4.3.2 Intermediate Risk Stratum C Research Participants:

Participants in whom the enucleated eye shows presence of tumor in the anterior chamber, invasion of the ciliary body/iris, massive invasion of the choroid, and invasion of the optic nerve beyond the lamina cribrosa with concomitant invasion of the choroid.

4.3.3 High Risk Stratum C Research Participants

Participants in whom the enucleated eye shows involvement of the sclera, or involvement of the optic nerve at the level of the cut-end.

4.4 Stratum D: Bilateral Retinoblastoma with Upfront Enucleation of One Eye due to Advanced Disease

Participants with bilateral retinoblastoma who may require upfront enucleation for one eye due to advanced disease (R-E IV-V and/or IC E).

4.5 Criteria for Upfront Enucleation

4.5.1 Participants with unilateral disease

Participants with advanced intraocular disease (R-E IV-V and/or IC D-E) without foveal sparing will undergo upfront enucleation, and they will be included in stratum C. Participants with early unilateral unifocal or multifocal retinoblastoma will be offered a conservative management (stratum A).

4.5.2 Participants with bilateral disease

The majority of participants with advanced (R-E IV-V) bilateral disease will be treated conservatively. However, some eyes may not be salvageable, and upfront enucleation will be performed (R-E IV-V and/or IC E). Decision regarding enucleation will be performed by the treating team at the time of the initial evaluation. In general, criteria for upfront enucleation include:

- Tumor in or on the ciliary body
- Tumor in the anterior segment
- Iris neovascularization
- Neovascular glaucoma
- Opaque media from hemorrhage
- Phthisis bulbi
- Tumor necrosis with aseptic orbital cellulitis

5.0 TREATMENT PLAN

5.1 Stratum A: Early Unilateral or Bilateral Retinoblastoma

This stratum will include mainly participants with early (R-E I-III, IC A-B, R-E IV with IC A or B, or IC C with limited sub-retinal seeding) bilateral retinoblastoma. Participants with unilateral disease diagnosed at an early stage, and participants with early multifocal unilateral disease are rare, but these participants are also candidates for conservative management. Therefore, they will be treated in Stratum A.

5.1.1 Stratum A Treatment Administration

5.1.1.1 **Children ≥ 6 months old at time of enrollment** - 8 courses of vincristine and carboplatin, given at 3-4 week intervals

Agent	Dose	Route	# Doses	Schedule
vinCRIStine (infants <12 months of age **or** < 10 kg)	0.05 mg/kg	IV*	1	Day 1
vinCRIStine (children ≥12 months of age **and** ≥ 10 kg)	1.5 mg/m^2 (max 2 mg)	IV*	1	Day 1
CARBOplatin	Given IV to achieve AUC of 6.5 mg/ml/min	IV over 60 minutes	1	Day 1

**Vincristine administered via minibag as per SJ policy*

Disease evaluation - children ≥ 6 months

Participants who demonstrate an excellent response to chemotherapy and focal treatments by exam under anesthesia may receive only 6 courses of vincristine/carboplatin after discussion with the treatment team and the family.

5.1.1.2 **Infants < 6 months old at time of enrollment -** Therapy will consist of six courses of chemotherapy; three courses of vincristine and carboplatin, given at 3-4 week intervals as described above, alternating with 3 cycles of vincristine and topotecan, given at 3-4 week intervals.

Cycles 1, 3, 5 (VCR/TOPO)

Agent	Dosage	Route	# Doses	Schedule
vinCRIStine (infants < 12 months of age **or** <10 kg)	0.05 mg/kg	IV*	1	Day 1
vinCRIStine (children ≥12 months of age **and** ≥10 kg)	1.5 mg/m^2 (max 2 mg)	IV	1	Day 1
Topotecan	TSE of 140 ± 20 ng/ml*hour	IV over 30 minutes	5	Day 1-5
G-CSF (filgrastim) or PEG-filgrastim	5 mcg/kg/day	SQ		G-CSF: daily injection starting 24-36 hours after chemotherapy for 7–10 days, until ANC is ≥2,000/µL on one occasion after the expected nadir. PEG-filgrastim: one injection 24-36 hours after chemotherapy
	100 mcg/kg *(weight < 45 kg)* or 6 mg (*weight ≥ 45 kg*)	SQ	1	

**Vincristine administered via minibag as per SJ policy*

Cycles 2, 4, 6 (VCR/CARBO)

Agent	Dosage	Route	# Doses	Schedule
vinCRIStine (infants < 12 months of age **or** < 10 kg)	0.05 mg/kg	IV*	1	Day 1
vinCRIStine (children ≥ 12 months of age **and** ≥ 10 kg)	1.5 mg/m^2 (max 2 mg)	IV*	1	Day 1
CARBOplatin	Given IV to achieve AUC of 6.5 mg/ml/min	IV over 60 minutes	1	Day 1

**Vincristine administered via minibag as per SJ policy*

Disease evaluation for infants < 6 months

Evaluation of disease response by examinations under anesthesia will be performed every three to eight weeks during treatment, at the discretion of the treating team. If there is any evidence of disease progression that is not amendable to focal treatments, the participant will resume chemotherapy as per Stratum A for patients ≥ 6 months with vincristine/carboplatin every 3-4 weeks.

Administration of topotecan for infants < 6 months

The topotecan dosage for Course 1 Day 1 will be age-adjusted per the table below, and then subsequent doses will be adjusted using a pharmacokinetically guided dosing approach to attain a target systemic exposure of 140 ± 20 ng/ml*hr.

Age	*Topotecan dosage for Cycle 1, Day 1*
0-2.99 months	2.25 mg/m^2
3-5.99 months	2.5 mg/m^2

Topotecan pharmacokinetic studies and pharmacokinetically guided dosing: see section 6.1.

5.1.2 Stratum A- Focal Treatments

Focal treatments will be administered any time after diagnosis. Focal therapies will include cryotherapy, laser photocoagulation, thermo-therapy and plaque radiotherapy.

In select cases of very early stage retinoblastoma, participants may receive focal therapies only and chemotherapy will be held at the discretion of the treating team. If there is any evidence of progression or unsatisfactory results, the participant will begin chemotherapy as per Stratum A (vincristine/carboplatin) above.

For selected participants an effort will be made to perform sequential chemo-thermotherapy. In these cases, carboplatin will be administered one or two hours prior to thermotherapy. In some participants, additional doses of carboplatin may be required after the completion of the 8 scheduled courses in order to achieve tumor control through thermotherapy.

Examinations under anesthesia will be performed every three to six weeks during treatment, at the discretion of the treating team.

5.1.3 Stratum A - Dose Modifications for Vincristine/Topotecan

Each course of chemotherapy will be given only if ANC ≥ 750/µL and platelets ≥ 100,000/µL. Chemotherapy will be delayed until requirements are met.

If chemotherapy is delayed for participants receiving only vincristine and carboplatin for more than 7 days in two consecutive courses due to ***neutropenia***, G-CSF or PEG-GCSF may be administered in subsequent courses. The recommended dose of G-CSF is 5 mcg/kg/day, daily until ANC is ≥ 2,000/µL after the expected nadir. The recommended dose for PEG-GCSF for participants <45kg is 100mcg/kg SQ x1 or for participants ≥45kg is 6 mg SQ x1.

If chemotherapy is delayed more than 7 days in two consecutive courses due to thrombocytopenia, the dose of carboplatin will be decreased to AUC of 5.5 mg/ml/min.

Participants receiving 3 cycles of vincristine and topotecan due to age < 6 months at diagnosis will receive G-CSF (5 mcg/kg/day), starting 24-36 hours after the completion of each course of topotecan chemotherapy, for 7 to 10 days, until ANC is ≥ 2,000/μL in one occasion after the expected nadir, or PEG-GCSF (participants <45kg: 100 mcg/kg SQ x1 or participants ≥45kg: 6 mg SQ x1)

5.2 Stratum B: Advanced Bilateral or Advanced Unilateral with Foveal Sparing Retinoblastoma

Stratum B will include participants with at least one R-E IV-V eye that after careful evaluation by the treating team it is considered not to require upfront enucleation (R-E IV-V and/or IC D-E). We anticipate that a proportion of participants treated on this stratum will not have advanced disease in both eyes. Some R-E I-III eyes will be treated in this stratum. The response obtained in those eyes will be compared to that of eyes treated on the stratum A. In addition, some participants with unilateral (unifocal or multifocal) retinoblastoma with R-E IV-V and IC D-E that demonstrate foveal sparing with potential for vision preservation may be considered as candidates for conservative management and will be treated on Stratum B.

5.2.1 Stratum B - Treatment Administration for Participants **Without** Extensive Sub-Retinal (SR) Seeding

Treatment will consist of **two up-front courses** of vincristine and topotecan, given at 3-4 week intervals:

Agent	Dosage	Route	# Doses	Schedule
vinCRIStine (infants < 12 months of age **or** < 10 kg)	0.05 mg/kg	IV*	1	Day 1
vinCRIStine (children ≥ 12 months of age **and** ≥ 10 kg)	1.5 mg/m^2 (max 2 mg)	IV*	1	Day 1
Topotecan	TSE of 140 ± 20 ng/ml*hr,	IV over 30 minutes	5	Day 1-5
G-CSF (filgrastim) or PEG-filgrastim	5 mcg/kg/day	SQ		G-CSF: daily injection starting 24-36 hours after chemotherapy for 7–10 days, until ANC is ≥2,000/μL on one occasion after the expected nadir. PEG-filgrastim: one injection 24-36 hours after chemotherapy
	100 mcg/kg (*weight < 45 kg*) or 6 mg (*weight ≥ 45 kg*)	SQ	1	

**Vincristine administered via minibag as per SJ policy*

Stratum B participants **without** SR seeding: examination under anesthesia (EUA)

An examination under anesthesia will be performed after each course of the up-front treatment (approximately days 21 and 42). Evaluation of response to the up-front courses will be performed after the second course with examination under anesthesia (EUA).

5.2.1.1 Stratum B participants without SR seeding: ≥ PR after 2 cycles

Participants with ≥ partial response to the upfront two cycles of vincristine and topotecan will receive three additional courses of vincristine-topotecan and six courses of vincristine-carboplatin, given at 3-4 week intervals:

Cycles 3, 4, 6, 7, 9, and 10 (VCR/CARBO)

Agent	Dosage	Route	# Doses	Schedule
vinCRIStine (infants < 12 months of age **or** <10 kg or vinCRIStine (children ≥ 12 months of age **and** ≥ 10 kg)	0.05 mg/kg	IV*	1	Day 1
	1.5 mg/m^2 (max 2 mg)	IV*	1	Day 1
CARBOplatin	Given IV to achieve AUC of 6.5 mg/ml/min	IV over 60 minutes	1	Day 1

**Vincristine administered via minibag as per SJ policy*

Cycles 5, 8, and 11 (VCR/TOPO)

Agent	Dosage	Route	# Doses	Schedule
vinCRIStine (infants < 12 months of age **or** <10 kg) or vinCRIStine (children ≥ 12 months of age **and** ≥ 10 kg)	0.05 mg/kg	IV*	1	Day 1
	1.5 mg/m^2 (max 2 mg)	IV*	1	Day 1
Topotecan	TSE of 140 ± 20 ng/ml*hr,	IV over 30 minutes	5	Day 1-5
CARBOplatin**	20 mg	Periocular (subtenon)	1	Day 1, 2, 3, 4 OR 5
G-CSF (filgrastim) or PEG-filgrastim	5 mcg/kg/day	SQ		G-CSF: daily injection starting 24-36 hours after chemotherapy for 7–10 days, until ANC is ≥2,000/µL on one occasion after the expected nadir. PEG-filgrastim: one injection 24-36 hours after chemotherapy
	100 mcg/kg (*weight < 45 kg*) or 6 mg (*weight ≥ 45 kg*)	SQ	1	

**Vincristine administered via minibag as per SJ policy*
***An affected eye with persistent vitreous disease (R-E IV-V) may receive up to three injections of subtenon CARBO, on day 1, 2, 3, 4, or 5 in course 5, 8, and 11.*

5.2.1.2 Stratum B participants **without** SR seeding and **< PR after 2 cycles VT**

Participants with < partial response to the upfront vincristine and topotecan cycles will receive 6 courses of vincristine, carboplatin and etoposide (VCE), given at 3-4 week intervals.

Agent	Dosage	Route	# Doses	Schedule
vinCRIStine (infants < 12 months of age or < 10 kg or vinCRIStine (children ≥ 12 months of age **and** ≥10 kg)	0.05 mg/kg	IV*	1	Day 1
	1.5 mg/m^2 (max 2 mg)	IV*	1	Day 1
CARBOplatin	Given IV to achieve AUC of 6.5 mg/ml/min	IV over 60 minutes	1	Day 1
Etoposide** (infants <12 months of age **or** < 10 kg) or Etoposide** (children ≥12 months of age **and** ≥10 kg)	3.3 mg/kg/day	IV	3	Days 1-3
	100 mg/m^2/day	IV	3	Days 1-3
CARBOplatin	20 mg	Periocular (subtenon)***	1	Day 1, 2, or 3
G-CSF (filgrastim) or PEG-filgrastim	5 mcg/kg/day	SQ		G-CSF: daily injection starting 24-36 hours after chemotherapy for 7–10 days, until ANC is ≥2,000/µL on one occasion after the expected nadir. PEG-filgrastim: one injection 24-36 hours after chemotherapy
	100 mcg/kg (*weight < 45 kg*) or 6 mg (*weight ≥ 45 kg*)	SQ	1	

**Vincristine administered via minibag as per SJ policy*
***Participants who develop an allergic reaction to etoposide can receive* Etopophos® *for all subsequent doses.*
**** An affected eye with persistent vitreous disease (R-E IV-V) may receive up to three CARBOplatin subtenon injections, on day 1, 2, or 3 of cycles 3, 5 and 8 (cycles 1, 3 and 6 of VCE).*

5.2.2 Stratum B - Treatment Administration: Participants **With** Extensive Sub-Retinal (SR) Seeding

Treatment will consist of two up-front courses of subconjunctival (subtenon) CARBOplatin and systemic topotecan, given at 3-4 week intervals

Agent	Dosage	Route	# Doses	Schedule
Topotecan	TSE of 140 ± 20 ng/ml*hr,	IV over 30 minutes	5	Day 1-5
CARBOplatin*	20 mg	Periocular (subtenon)*	1	Day 1, 2, 3, 4 OR 5
G-CSF (filgrastim) or PEG-filgrastim	5 mcg/kg/day	SQ		G-CSF: daily injection starting 24-36 hours after chemotherapy for 7–10 days, until ANC is ≥2,000/μL on one occasion after the expected nadir. PEG-filgrastim: one injection 24-36 hours after chemotherapy
	100 mcg/kg (*weight < 45 kg*) or 6 mg (*weight ≥ 45 kg*)	SQ	1	

Stratum B participants **with** SR seeding: examination under anesthesia (EUA)

An examination under anesthesia will be performed after each course of the up-front treatment (approximately days 21 and 42). Evaluation of response to the up-front courses will be performed after the second course with examination under anesthesia (EUA).

5.2.2.1 Stratum B participants **with** SR seeding: **≥ PR after 2 cycles**

Participants with ≥ partial response to the upfront two cycles of subconjunctival CARBOplatin and systemic topotecan, will receive three additional courses of vincristine-topotecan, and six courses of vincristine-carboplatin, given at 3-4 week intervals:

Cycles 3, 4, 6, 7, 9, and 10 (VCR/CARBO)

Agent	Dosage	Route	# Doses	Schedule
vinCRIStine (infants < 12 months of age **or** < 10 kg or vinCRIStine (children ≥ 12 months of age **and** ≥ 10 kg)	0.05 mg/kg	IV*	1	Day 1
	1.5 mg/m^2 (max 2 mg)	IV*	1	Day 1
CARBOplatin	Given IV to achieve AUC of 6.5 mg/ml/min	IV over 60 minutes	1	Day 1

**Vincristine administered via minibag as per SJ policy*

Cycles 5, 8, and 11 (VCR/TOPO)

Agent	Dosage	Route	# Doses	Schedule
vinCRIStine (infants < 12 months of age **or** < 10 kg or vinCRIStine (children ≥ 12 months of age **and** ≥ 10 kg)	0.05 mg/kg	IV*	1	Day 1
	1.5 mg/m^2 (max 2 mg)	IV*	1	Day 1
Topotecan	TSE of 140 ± 20 ng/ml*hr,	IV over 30 minutes	5	Day 1-5
CARBOplatin**	20 mg	Periocular (subtenon)**	1	Day 1, 2, 3, 4 OR 5
G-CSF (filgrastim) or PEG-filgrastim	5 mcg/kg/day	SQ		G-CSF: daily injection starting 24-36 hours after chemotherapy for 7–10 days, until ANC is ≥2,000/µL on one occasion after the expected nadir. PEG-filgrastim: one injection 24-36 hours after chemotherapy
	100 mcg/kg (*weight < 45 kg)* or 6 mg (*weight ≥ 45 kg*)	SQ	1	

**Vincristine administered via minibag as per SJ policy*
***Subtenon CARBO injection, on day 1, 2, 3, 4, or 5 to R-E IV-V eyes with persistent vitreous disease in cycle 5, 8, and/or 11 as clinically indicated.*

5.2.2.2 Stratum B **with** SR seeding **< PR after C1-2**: 6 cycles VCE

Agent	Dosage	Route	# Doses	Schedule
vinCRIStine (infants < 12 months of age **or** < 10 kg or vinCRIStine (children ≥ 12 months of age **and** ≥ 10 kg)	0.05 mg/kg	IV*	1	Day 1
	1.5 mg/m^2 (max 2 mg)	IV*	1	Day 1
CARBOplatin	Given IV to achieve AUC of 6.5 mg/ml/min	IV over 60 minutes	1	Day 1
Etoposide** (infants <12 months of age **or** < 10 kg or Etoposide** (children ≥12 months of age and ≥ 10 kg)	3.3 mg/kg/day	IV	3	Days 1-3
	100 mg/m^2/day	IV	3	Days 1-3
CARBOplatin	20 mg	Periocular (subtenon)***	1	Day 1, 2, or 3
G-CSF (filgrastim) or PEG-filgrastim	5 mcg/kg/day	SQ		G-CSF: daily injection starting 24-36 hours after chemotherapy for 7–10 days, until ANC is ≥2,000/μL on one occasion after the expected nadir. PEG-filgrastim: one injection 24-36 hours after chemotherapy
	100 mcg/kg (*weight < 45 kg*) or 6 mg (*weight ≥ 45 kg*)	SQ	1	

**Vincristine administered via minibag as per SJ policy*
***Participants who develop an allergic reaction to etoposide can receive* Etopophos® *for all subsequent doses.*
****CARBOplatin subtenon injection, on day 1, 2, or 3 of cycles 3, 5, and/or 8 (cycles 1 or 3 or 6 of VCE) to R-E IV-V eyes with persistent vitreous disease as clinically indicated.*

5.2.3 Stratum B: Administration of Topotecan

The topotecan dosage for Course 1 Day 1 will be age-adjusted per the table below, and then subsequent doses will be adjusted using a pharmacokinetically guided dosing approach to attain a target systemic exposure of 140 ± 20 ng/ml*hr.

Age	Topotecan dosage for C1D1
0-2.99 months	2.25 mg/m^2
3-5.99 months	2.5 mg/m^2
6-8.99 months	2.75 mg/m^2
9-11.99 months	3 mg/m^2
12 months and older	3.5 mg/m^2

Topotecan pharmacokinetic studies and pharmacokinetically guided dosing: see Section 6.1.

5.2.4 Stratum B: Dose Modifications for Vincristine/Topotecan and G-CSF

Each course of chemotherapy will be given only if ANC ≥ 750/μL and platelets ≥ 100,000/μL. Chemotherapy will be delayed until requirements are met.

If chemotherapy is delayed more than 7 days due to thrombocytopenia after a course containing topotecan or carboplatin, subsequent courses containing the causative agent will have a 20% reduction in the dose of the agent.

If chemotherapy is delayed for participants receiving vincristine and carboplatin for more than 7 days in two consecutive courses due to *neutropenia*, G-CSF or PEG-GCSF may be administered in subsequent courses. The recommended dose of G-CSF is 5 mcg/kg/day, daily until ANC is ≥ 2,000/μL after the expected nadir. The recommended dose for PEG-GCSF for participants <45kg is 100mcg/kg SQ x1 or for participants ≥45kg is 6 mg SQ x1.

5.2.5 Stratum B: Dose Modifications for VCE

Each course of chemotherapy will be given only if ANC ≥ 750/μL and platelets ≥ 100,000/μL. Chemotherapy will be delayed until these requirements are met.

If chemotherapy is delayed more than 7 days due to neutropenia in two consecutive courses, the dose of carboplatin and etoposide will both be decreased by 20%.

If chemotherapy is delayed more than 7 days in two consecutive courses due to *thrombocytopenia, the dose of carboplatin will be decreased to AUC of 5.5 mg/ml/min.*

Participants who develop an allergic reaction to etoposide can receive Etopophos® for all subsequent doses.

5.2.6 Periocular Administration of Carboplatin

Carboplatin 20 mg will be diluted in 2 mL of NS or D5W and given by subtenon administration while the participant is under general anesthesia. Periocular inflammation is common after subtenon administration of carboplatin. Participants will receive dexamethasone 1-2 mg IV (0.1 to 0.15 mg/kg/dose) after the procedure; and oral dexamethasone may be given for 3-5 additional days afterwards.

For patients **without** subretinal seeding at diagnosis: Three injections will be considered for active vitreous disease or new subretinal disease in courses 5, 8, and 11 (for participants who responded to upfront therapy) or in courses 3, 5 and 8 (for participants who did not respond to upfront therapy) (see sections 5.2.1.1 and 5.2.1.2)

For patients **with** extensive subretinal seeding at diagnosis: Two injections in courses 1 and 2 for upfront therapy, and additional injection(s) for active vitreous or subretinal disease in course 5,8, and/or 11 (for patients who respond to upfront therapy) or course 3,

5, and/or 8 (for patients who do not respond to upfront therapy) (see sections 5.2.2.1 and 5.2.2.2) as clinically indicated.

5.2.7 Focal Therapy

Focal treatments will be administered any time after diagnosis. Focal therapies will include cryotherapy, laser photocoagulation, thermo-therapy and plaque radiotherapy.

Examinations under anesthesia will be performed every three to six weeks during treatment, at the discretion of the treating team.

5.2.8 External Beam Radiation Therapy (EBRT)

Participants with advanced intraocular disease (strata B and D) in whom it is suspected that active disease is present in the vitreous after completing therapy should be evaluated for consideration of EBRT (44-46 Gy) – see section 5.5).

5.3 Stratum C: Advanced Unilateral Retinoblastoma

Participants with unilateral (unifocal or multifocal) advanced (R-E IV-V and/or IC D-E) intraocular disease will undergo enucleation. Adjuvant therapy will be indicated in the cases described below:

5.3.1 Stratum C: Low Risk Participants:

Participants in whom the enucleated eye does not show extra-retinal disease (see definition of intermediate and high risk below); *will not receive any additional treatment, but will remain on study.*

5.3.2 Stratum C: Intermediate Risk Research Participants:

Participants in whom the enucleated eye shows presence of tumor in the anterior chamber, invasion of the ciliary body/iris, massive invasion of the choroid, and invasion of the optic nerve beyond the lamina cribrosa with concomitant invasion of the choroid, will receive 4 courses of adjuvant chemotherapy with VCD:

5.3.2.1 Stratum C intermediate risk: 4 cycles of VCD

Agent	Dosage	Route	# Doses	Schedule
vinCRIStine (infants < 12 months of age **or** <10 kg) or vinCRIStine (children ≥ 12 months of age **and** ≥ 10 kg)	0.05 mg/kg	IV*	1	Day 1
	1.5 mg/m^2 (max 2 mg)	IV*	1	Day 1
CYCLOphosphamide (infants < 12 months of age **or** <10 kg or CYCLOphosphamide (for children ≥ 12 months of age **and** ≥ 10 kg)	40 mg/kg	IV	1	Day 1
	1200 mg/m^2	IV	1	Day 1
MESNA	300 mg/m^2	IV	4	Day 1, before CYCLO and at 3, 6 and 9 hours after
DOXOubicin for infants <12 months of age **or** < 10 kg or DOXOrubicin for children ≥12 months of age **and** ≥10 kg	1.5 mg/kg	IV	1	Day 1
	45 mg/m^2	IV	1	Day 1
G-CSF (filgrastim) or PEG-filgrastim	5 mcg/kg/day	SQ		G-CSF: daily injection starting 24-36 hours after chemotherapy for 7–10 days, until ANC is ≥2,000/μL on one occasion after the expected nadir. PEG-filgrastim: one injection 24-36 hours after chemotherapy
	100 mcg/kg (*weight < 45 kg*) or 6 mg (*weight ≥ 45 kg*)	SQ	1	

**Vincristine administered via minibag as per SJ policy*

5.3.2.1 Stratum C: dose modifications VCD

Each course of chemotherapy will be given only if ANC ≥ **750/** μL, and platelets ≥ 100,000/μL. Chemotherapy will be delayed until requirements are met. If chemotherapy is delayed more than 7 days due to neutropenia in two consecutive courses, the dose of all agents will be decreased by 20%.

5.3.3 Stratum C: high risk research participants

Participants in whom the enucleated eye shows involvement of the sclera, or involvement of the optic nerve at the level of the cut-end, will be treated with 6 courses of chemotherapy, with alternating courses of VCE and VCD, as follows:

5.3.3.1 Stratum C high-risk: Cycles 1, 3 and 5 (VCE)

Agent	Dosage	Route	# Doses	Schedule
vinCRIStine (infants < 12 months of age **or** < 10 kg) or vinCRIStine (children ≥ 12 months of age **and** ≥ 10 kg)	0.05 mg/kg	IV*	1	Day 1
	1.5 mg/m^2 (max 2 mg)	IV*	1	Day 1
CARBOplatin	Given IV to achieve AUC of 6.5 mg/ml/min	IV over 60 minutes	1	Day 1
Etoposide** (infants <12 months of age **or** < 10 kg) or Etoposide** (children ≥12 months of age **and** ≥ 10 kg)	3.3 mg/kg/day	IV	3	Days 1-3
	100 mg/m^2/day	IV	3	Days 1-3
G-CSF (filgrastim) or PEG-GCSF	5 mcg/kg/day	SQ		G-CSF: daily injection starting 24-36 hours after chemotherapy for 7–10 days, until ANC is ≥2,000/µL on one occasion after the expected nadir. PEG-filgrastim: one injection 24-36 hours after chemotherapy
	100 mcg/kg (*weight < 45 kg*) or 6 mg (*weight ≥ 45 kg*)	SQ	1	

**Vincristine administered via minibag as per SJ policy*

***Participants who develop an allergic reaction to etoposide can receive Etopophos® for subsequent doses.*

5.3.3.2 Stratum C high-risk: Cycles 2, 4 and 6 (VCD)

Agent	Dosage	Route	# Doses	Schedule
vinCRIStine (infants < 12 months of age **or** < 10 kg or vinCRIStine (children ≥ 12 months of age **and** ≥ 10 kg)	0.05 mg/kg	IV*	1	Day 1
	1.5 mg/m^2 (max 2 mg)	IV*	1	Day 1
CYCLOphosphamide (infants <12 months of age **or** < 10 kg) or CYCLOphosphamide (children ≥ 12 months of age **and** ≥ 10 kg	40 mg/kg	IV	1	Day 1
	1200 mg/m^2	IV	1	Day 1
MESNA	300 mg/m^2	IV	4	Day 1, before CYCLO and at 3, 6 and 9 hours after
DOXOrubicin (infants <12 months of age **or** < 10 kg) or DOXOrubicin (children ≥12 months of age **and** ≥ 10 kg)	1.5 mg/kg	IV	1	Day 1
	45 mg/m^2	IV	1	Day 1
G-CSF (filgrastim) or PEG-GCSF	5 mcg/kg/day	SQ		G-CSF: daily injection starting 24-36 hours after chemotherapy for 7–10 days, until ANC is ≥2,000/µL on one occasion after the expected nadir. PEG-filgrastim: one injection 24-36 hours after chemotherapy
	100 mcg/kg (*weight < 45 kg*) or 6 mg (*weight ≥ 45 kg*)	SQ	1	

**Vincristine administered via minibag as per SJ policy*

5.3.3.3 Stratum C high-risk: high-risk dose modifications

Each course of chemotherapy will be given only if ANC ≥ 750/µL and platelets ≥ 100,000/µL. Chemotherapy will be delayed until requirements are met. If chemotherapy is delayed more than 7 days due to neutropenia in two consecutive courses, the dose of all agents will be decreased by 20%.

If chemotherapy is delayed more than 7 days in two consecutive courses due to *thrombocytopenia, the dose of carboplatin will be decreased to AUC of 5.5 mg/ml/min.*

Participants who develop an allergic reaction to etoposide can receive Etopophos® for all subsequent doses.

5.3.3.4 Stratum C high risk participants with extra-ocular extension: external-beam radiation therapy

High-risk participants with extra-ocular extension (i.e. tumor extending beyond the sclera/cornea or beyond the cut end of the optic nerve) will be candidates for consideration of EBRT to the entire orbit, including the optic nerve, for a total dose of 45 Gy. EBRT will be administered after 2 or 3 courses of treatment. Alternatively, patients with extra-ocular extension may be considered for enrollment on a therapeutic protocol for metastatic retinoblastoma (or best clinical management) in place of SJRET6. Participants will be evaluated on an individual basis to determine whether they might benefit from referral for proton therapy.

5.4 Stratum D: Bilateral Retinoblastoma with Upfront Enucleation of One Eye due to Advanced Disease (R-E IV-V and/or IC E)

5.4.1 Stratum D: Upfront Enucleation

Management of participants with bilateral retinoblastoma is often complex; and some participants will have one eye enucleated upfront due to advanced disease. The decision for enucleation will be made after thorough consideration by the treating team. The treatment of the remaining eye will depend on a combination of two factors: a) R-E group of the remaining eye, and b) Histology of the enucleated eye. All patients will receive one course of vincristine, carboplatin, etoposide (VCE) following enucleation so that therapy is not delayed by the final evaluation of the histopathology of the enucleated eye. Administration of subconjunctival carboplatin to the remaining eye may be considered in course #1 (as per section 5.2.4.2) if clinically warranted. Subsequent therapy will be based on the chart below. Patients with intermediate or high risk histopathology of the enucleated eye will receive an additional 5 courses of VCE for a total of 6 courses of VCE. If therapy changes to treatment on stratum A, the patient will receive an additional 7 courses to complete 8 courses of therapy. Patients who are eligible to proceed with stratum B will omit cycle 10 of vincristine/carboplatin. Eyes which have extensive tumor necrosis at the time of primary enucleation cannot be assessed for the presence/absence of high risk histopathology and will therefore be treated as “high risk.”

Though we have accumulated some information regarding the use of vincristine, cyclophosphamide, and doxorubicin in the treatment of intraocular retinoblastoma, it is not considered standard of care. Therefore, participants with intermediate and high risk features will be treated with 6 courses of VCE. Those participants in whom the enucleated eye shows only low risk histology and in whom the remaining eye is R-E IV-V, will still be eligible to proceed with stratum B therapy. Since these patients will not receive topotecan and subconjunctival carboplatin as “upfront therapy” in courses

St. Jude Children's Research Hospital
IRB NUMBER: Pro00003243
IRB APPROVAL DATE: 01/12/2021

1 and 2, they will not be eligible for final analysis of response to window therapy. They will be eligible for analysis in outcomes following stratum B therapy (EFS and OS).

Therapy for Stratum D patients:

		Histology of enucleated eye	
		Low Risk	*Intermediate and High Risk*
R-E group of Remaining eye	*R-E I-III IC A-B*	Course #1: VCE Treatment as Stratum A (age ≥6mo = VC x 7; age <6mo = alternating VT & VC x 5 total)	Course #1: VCE Treatment as unilateral high-risk (VCE x 5)
	R-E IV-V IC C-E	Course #1: VCE Treatment as Stratum B (omit course #10 VC)	Course #1: VCE Treatment as unilateral high-risk (VCE x 5)

5.4.2 Stratum D - Administration of VCE

Agent	Dosage	Route	# Doses	Schedule
vinCRIStine (infants < 12 months of age **or** < 10 kg) or vinCRIStine (children ≥12 months of age **and** ≥10 kg)	0.05 mg/kg	IV*	1	Day 1
	1.5 mg/m^2 (max 2 mg)	IV*	1	Day 1
CARBOplatin	Given IV to achieve AUC of 6.5 mg/ml/min	IV over 60 minutes	1	Day 1
Etoposide** (infants <12 months of age **or** < 10 kg or Etoposide** (children ≥12 months of age) **and** ≥10 kg	3.3 mg/kg/day	IV	3	Days 1-3
	100 mg/m^2/day	IV	3	Days 1-3
CARBOplatin	20 mg	Periocular (subtenon)***	1	Day 1, 2, or 3
G-CSF (filgrastim) or PEG-GCSF	5 mcg/kg/day	SQ		G-CSF: daily injection starting 24-36 hours after chemotherapy for 7–10 days, until ANC is ≥2,000/µL on one occasion after the expected nadir. PEG-filgrastim: one injection 24-36 hours after chemotherapy
	100 mcg/kg (*weight <45 kg*) or 6 mg (*weight ≥45 kg*)	SQ	1	

**Vincristine administered via minibag as per SJ policy*
***Participants who develop an allergic reaction to etoposide can receive Etopophos® for all subsequent doses.*
****Up to three CARBOplatin subtenon injections, on day 1, 2, or 3 of courses 1, 3 and 5 to R-E IV-V eyes with persistent vitreous disease.*

5.4.2.1 Stratum D - dose modifications for VCE

- Each course of chemotherapy will be given only if ANC ≥ 750/μL and platelets ≥100,000/μL. Chemotherapy will be delayed until requirements are met.
- If chemotherapy is delayed more than 7 days due to neutropenia in two consecutive courses, the dose of carboplatin and etoposide will be decreased by 20%.
- If chemotherapy is delayed more than 7 days in two consecutive courses due to *thrombocytopenia, the dose of carboplatin will be decreased to AUC of 5.5 mg/ml/min.*
- Participants who develop an allergic reaction to etoposide can receive Etopophos® for all subsequent doses.

5.4.3 Stratum D - Focal Therapies

Focal therapies will be used any time after diagnosis. The different modalities will be used at the discretion of the treating team. Those include cryotherapy, laser photocoagulation, thermotherapy (and thermo-chemotherapy) and episcleral plaque brachytherapy.

5.4.4 Stratum D - External Beam Radiation Therapy (EBRT)

EBRT will be considered for administration to any eye in which the disease is considered to be not controllable with focal treatments alone, and in participants with enucleated eyes in which high risk of orbital and/or CNS disease is documented histologically (high-risk group with disease extension beyond the sclera or cornea, or beyond the cut end of the optic nerve). EBRT will be administered using standard techniques practices with the objective of limiting dose to normal tissues including the hypothalamic-pituitary unit, supratentorial brain, orbit, cochleae and contralateral eye when indicated. Participants will be evaluated on an individual basis to determine whether they might benefit from referral for proton therapy.

5.4.4.1 Stratum D intraocular disease:

Participants with recurrent or progressive disease considered to be not controllable with focal treatments should receive 44-46 Gy.

5.4.4.2 Stratum D high-risk disease:

- Participants with disease extension beyond the sclera or cornea or beyond the cut end of the optic nerve should receive 44-46 Gy to the volume at risk to be defined by the treating radiation oncologist.
- Timing of radiation will be at the discretion of the treating physicians, although it is recommended after 2 or 3 courses of chemotherapy.
- Alternatively, patients with extra-ocular extension may be considered for enrollment on a therapeutic protocol for metastatic retinoblastoma (or best clinical management) in place of SJRET6.
- Participants will be evaluated on an individual basis to determine whether they might benefit from referral for proton therapy.

5.5 Radiation Therapy Guidelines

General guidelines

The radiation therapy guidelines for this study were developed specifically for salvage of children with progressive focal or disseminated intra-ocular retinoblastoma. The guidelines are meant to cover a broad range of indications and the use of a variety of treatment modalities.

5.5.1 Indications for Radiation Therapy

Participants who have developed progressive disease despite focal (non-irradiative) therapy with chemotherapy (orbital and/or regional involvement) will receive radiation therapy (RT) to refractory disease sites. Choice of external beam radiotherapy vs. brachytherapy shall depend upon the nature of the progressive or refractory disease and shall be determined by consensus between the treating ophthalmologist and radiation oncologist

5.5.2 Timing

All participants should be seen in consultation by a radiation oncologist at the time of determination of need for salvage radiotherapy. The purpose of the consultation is to participate in staging, determination of the optimal radiation modality and to review the adequacy of the initial diagnostic imaging studies that will be used for subsequent RT planning.

5.5.3 Equipment and Methods of Delivery and Verification

Equipment	Photons (any energy)	Electrons (any energy)	IMRT (4-10MV)	Protons	Brachytherapy*
Linear Accelerator	X	X	X		
Proton Beam				X	
Brachytherapy - high or low dose rate					X

*****Permanent radioactive implants are not allowed on this protocol.*

5.5.3.1 Treatment planning

CT (volumetric) based planning is required to optimize dose to the PTV while protecting normal tissues. A DVH is necessary to determine target coverage and evaluate dose to normal tissues. CT section thickness should be ≤5mm although 2-3 mm is preferred.

5.5.3.2 In-room verification of spatial positioning

Cone beam CT will be used if possible for cases requiring teletherapy and is recommended. Portal imaging with orthogonal pairs may be used if necessary. However,

conebeam CT is preferred for IMRT and 3-D CRT to verify that target volume positioning is in correct alignment relative to the patient position.

5.5.4 Target Volumes

5.5.4.1 Standard tumor and target volume definitions

International Commission on Radiation Units and Measurements (ICRU) Reports 50, 62 and 78 (www.icru.org) define prescription methods and nomenclature that will be utilized for this study. Treatment planning will be based on the following definitions and applies only to the primary tumor site:

Photons

- *Gross tumor volume (GTV)* is the volume occupied at diagnosis by visible or palpable disease.
- *Clinical target volume (CTV)* includes the GTV and sites with potential occult tumor involvement adjacent to the GTV.
- *Planning target volume (PTV*) is the CTV surrounded by a geometric margin to account for variability in set-up, breathing or motion during treatment.

Protons

- *GTV* is the same for protons and photons.
- *CTV* is the same for protons and photons.
- *PTV* is not the same as photons due to differences in beam penetration with movement and set up uncertainty. PTV varies with each individual field and coverage and will require additional adjustment to (1) the lateral margins, (2) smearing of compensator, (3) range of beam (depth of penetration) and, (4) modulation (number of required Bragg peaks). Adjustments to any of the aforementioned parameters (usually 2-7 mm) will be based on the set up error determined for the particular body site at the individual proton institution. Motion of the target volume in three dimensions (cranial, caudal, anterior to posterior, and lateral) may be determined by 4-dimensional CT, respiratory gated CT, or other accepted techniques.

Brachytherapy

- *GTV* is the same as for photons.
- *CTV* is the same as for photons.
- PTV is equal to *CTV*.

5.5.4.2 Protocol tumor and target volume definitions

GTV: GTV is defined as the visible and/or palpable disease defined by physical examination, computed tomography (CT), magnetic resonance imaging (MRI) or positron emission tomography (PET scan) prior to any surgical debulking or chemotherapy. For patients who undergo initial surgery, operative notes and pathology reports may be helpful. If the tumor has been resected or responded to chemotherapy and the normal tissues have returned to their normal positions, the GTV excludes the volume

which extends into the cavity. The GTV must include all infiltrative intra-ocular disease detected at initial presentation.

CTV: If there are no sites that warrant irradiation for potential occult tumor, then the CTV is defined as the GTV plus 5mm (but not extending outside of the patient). For some sites, the definition of CTV is modified to account for specific anatomic barriers to tumor spread.

PTV: For external beam photon techniques, the PTV is defined as the CTV plus an institutional specified margin to account for day-to-day setup variation related to the ability to immobilize the patient and physiologic motion of the CTV. The minimum margin is 3mm but does not have to be uniform in all dimensions. For proton planning, beam specific PTV expansions will be required.

5.5.4.3 Dose definition

Photon dose is to be specified in centigray (cGy)-to-muscle. For proton beam, the absorbed dose is specified in CGE, which is the same as ICRU 78 DRBE using a standard RBE of 1.10 with respect to water.

5.5.4.4 Prescribed dose and fractionation

The protocol-specified dose per fraction is 180cGy. The treatment should be limited to one fraction per day. At least two fractions should be given during the first week of treatment.

Target volumes and doses for patients with Stage 2 and 3 retinoblastoma

Stage/site	GTV	CTV	PTV	PTV doses
Stage 2 & 3 orbital involvement	Residual tumor	Orbit including GTV	CTV+3mm	4500 cGy

Definitions: orbit = internal bony orbit, optic foramen and optic nerve to chiasm; GTV = gross tumor volume; CTV = clinical target volume; PTV = planning target volume

Target volumes and doses for patients with Stage 4a retinoblastoma

	Irradiation sites	GTVx	CTVx	PTVx	PTVx Doses
4a	≥ 5mm pre-RT residual	Pre-RT residual	GTV+3mm	CTV+3mm	3600 cGy
	< 5mm pre-RT residual or CR	-	-	-	-

Definitions: GTV = gross tumor volume; CTV = clinical target volume; PTV = planning target volume; disease measurements are determined by MR or CT; x = denotes more than one metastatic site that requires irradiation and the nomenclature of GTVa, CTVa, PTVa; GTVb, CTVb, PTVb is preferred with appropriate documentation for each target volume.

5.5.4.5 Prescribed dose and fractionation

Dose uniformity: At least 95% of the protocol-specified dose should encompass 100% of the PTV1/PTV2 and no more than 10% of PTV1 (PTV2 for patients with a volume reduction) should receive greater than 110% of the protocol dose as evaluated by DVH. The 100% isodose should be equal to the prescribed dose. Virtual wedges, compensators and other methods of generating more uniform dose distributions are encouraged.

Tissue heterogeneity: Calculations must take into account tissue heterogeneity and should be performed with CT-based treatment planning to generate dose distributions and treatment calculations from CT densities.

Environment of care - interruptions, delays and dose modifications: There will be no planned rests or breaks from treatment, and once radiation therapy has been initiated, treatment will not be interrupted except for any life-threatening infection or severe hematologic toxicity defined as ANC < 300/μL or platelets <40 000/μL during the course of treatment. Under these circumstances, radiation therapy shall be delayed until the counts have recovered. Blood product support should be instituted according to institutional/protocol guidelines. There is no minimum hemoglobin level during radiation therapy. The reason for any interruptions greater than 5 treatment days should be recorded in the patient's treatment chart and submitted with the QA documentation. There should be no modifications in dose fractionation due to age or field size. If any area has been previously treated (emergently), care should be taken not to exceed normal tissue tolerance levels.

5.5.5 Treatment Technique

5.5.5.1 Beam configuration: Every attempt should be made to minimize dose to organs at risk without compromising coverage of the target volume. Three-dimensional conformal therapy (coplanar or non-coplanar) or IMRT are required to minimize dose to normal tissues.

5.5.5.2 Selection of proton beam arrangements: There are uncertainties (1-3 mm) in the distal range of the proton beam in which the RBE may be greater than 1.1; therefore, single proton beam plans that stop in a critical organ will not be allowed. Individual proton beams that are a component of a multi-field proton beam, which stop within such an organ, will be allowed.

5.5.5.3 Field shaping: Field shaping for photons will be done with either customized cerrobend blocking or multileaf collimation. The field shaping for protons will be done with either brass apertures or proton-specific multileaf collimation.

5.5.5.4 Teletherapy simulation including patient positioning and immobilization

Patient positioning: Reproducible setups are critical and the use of immobilization devices is strongly encouraged. The patient may be treated in any appropriate, stable position. Consideration should be given to implications for inter and intrafraction motion when using non-standard position approaches.

Immobilization devices: Standard immobilization devices for the head and neck are to be used. For IMRT delivery approaches, the methods used for localization and immobilization of both patient and tumor are critical. The imaging studies should provide a clear assessment of the target volume with the patient in the treatment position.

5.5.5.5 Special considerations: Anesthesia or sedation may be required in certain patients, such as very young patients, to prevent movement during simulation and daily treatments.

5.5.5.6 Motion management and margins to account for target volume and organ motion: Considering motion of normal tissues and target volumes is important. The internal target volume (ITV) is defined as the CTV surrounded by the internal motion (IM) component of the PTV and is meant to account for potential motion of the CTV. The planning organ at risk volume (PRV) includes the organs at risk (OAR) surrounded by a margin to compensate for physiologic change in the target volume. If adequate clinical data do not exist to define the IM component of the PTV or the PRV margin, the following suggestions are provided:

- For a CTV susceptible to physiologic motion, a margin of at least 5mm should be added to the CTV prior to PTV margin expansion or a PTV margin of 10mm should be chosen.
- A description of the method used and evidence (i.e., observed motion during fluoroscopy, motion of surrogate markers using camera systems, or analysis of 4-D CT) of the remaining tumor motion should be noted.

5.5.6 Organs at Risk

The organs at risk guidelines in this section are recommendations. If the recommended doses to the organs at risk are exceeded because of target volume coverage requirements or other conditions, an explanation should be included in the quality assurance documentation. In some cases, photon IMRT may be the preferred treatment method to meet these recommendations and the required target volume coverage guidelines. Normal tissue dose recommendations are the same for photons and protons (proton dose measured in CGE).

Organs at risk dose recommendations

Organ	Volume (%)	Dose (cGy)
Optic nerve	100%	5400

5.5.7 Brachytherapy

Sources used will have assay directly traceable to the National Institute of Standards and Technology (NIST).

Timing: There must be at least 6 weeks between the last local ophthalmic therapy or systemic chemotherapy (whichever was later) and the start of plaque radiotherapy.

Isotope: Iodine-125 shall be utilized for plaques.

Plaque size: The plaque size is chosen so that the tumor base is covered entirely by the plaque with a tumor free margin of 1 – 2 mm on all sides. (For example, a tumor with a basal diameter of 10 mm would be treated with a plaque of at least 12-14 mm in diameter.)

An exception is permissible if the tumor is ≤ 2 mm of the optic nerve. In this case, the plaque may be trimmed (or "notched") around the optic nerve so that the posterior edge of the plaque lies between the optic nerve and the posterior edge of the tumor.

Radiation dose: The tumor dose is prescribed to the apex of the tumor. The dose rate at the prescription point must be ≥ 40 cGy/h but ≤ 300 cGy/h.

The thickness of the tumor is defined as the distance from the surface of the sclera to the apex of the tumor. The sclera is assumed to be 1 mm thick. Thus, if a tumor is said to be 3 mm thick, the point of dose prescription is 4 mm from the surface of the radioactive plaque, i.e. 3 mm thick tumor + 1 mm thick sclera = 4 mm. If the plaque is placed over one of the ocular muscles, the plaque must be considered to be 1 mm away from the external surface of the sclera, i.e. 3 mm thick tumor + 1 mm thick sclera + 1 mm muscle thickness = 5 mm. If overlying vitreous seeds are present, the dose is designed to include those seeds.

CT-based treatment planning is optional.

The total dose to the tumor apex is 3500-4500 cGy. The minimum tumor thickness is 4mm. The maximum tumor thickness is 12mm for I-125.

Organs at risk: The location of the following critical structures, relative to the plaque, are determined by the treating ophthalmologist and radiation oncologist and noted on the retinal drawing or map.

Sclera: The dose to the sclera is estimated and should be reported at a point on the central axis of the plaque 1 mm from the surface of the plaque. This point is approximately coincident with the internal surface of the sclera near the center of the tumor.

Optic nerve: The optic nerve will generally be shielded by the lip of the plaque and exposure minimized by careful plaque positioning. The dose to the center of the optic disc should be calculated and reported.

Retina: The dose to the retina opposite the tumor should be calculated and reported. The dose is calculated 16 mm from the scleral surface at the base of the tumor measured along a diameter of the globe passing through the apex of the tumor for infants ≤ 6 months of age and 20 mm for patients > 6 months of age.

Lens: The dose to the center of the lens should be calculated and reported.

Dose calculations and reporting: Required normal tissue DVH according to primary treatment site(s)

Treatment area	Required DVH
Head	Optic nerve
	Optic chiasm

5.5.8 Definition of Minor and Major Deviations

Definitions of deviation in protocol performance will be applied to the treatment of the primary lesion only.

	DEVIATION	
	Minor	Major
Prescription dose		
External beam	Difference in prescription dose is 6%-10% of protocol-specified dose	Difference in prescription dose is > 10% of protocol-specified dose
Brachytherapy	Difference in prescription dose is 6%-10% of protocol-specified dose	Difference in prescription dose is >10% of protocol-specified dose
Dose uniformity		
External beam	> 10% PTV receives > 110% of protocol dose *or* 95% isodose covers < 90% of the PTV or < 100% but > 90% of the CTV	95% isodose covers < 90% of CTV.
Brachytherapy	95% isodose covers <100% of CTV	90% isodose covers < 100% of CTV.
Volume	CTV or PTV margins are less than the protocol specified margins in the absence of anatomic barriers to tumor invasion (CTV) or without written justification (PTV)	GTV does not encompass MR-visible residual tumor

6.0 PHARMACOLOGY STUDIES

6.1 Topotecan Pharmacokinetics

6.1.1 Sample Collection and Processing

A modified limited sampling model will be used, with blood samples (2.5 ml) obtained prior to the infusion, and at 5 minutes, 1.5 hours, and 2.5 hours after the end of the topotecan infusion. Samples must be obtained from a site separate from the site used for drug administration. If whole blood samples cannot be obtained on the scheduled day for technical reasons, then the participant will be studied in the next possible day.

Before drawing samples, page the on-call pharmacokinetics technician (beeper #0779) to have personnel available to process samples.

6.1.2 Topotecan Pharmacokinetic Analysis

A two-compartment pharmacokinetic model will be used to fit the topotecan plasma concentration-time data from each participant using maximum a posteriori (MAP-Bayesian) estimation as implemented in ADAPT II. Individual pharmacokinetic parameters estimated will include volume of the central compartment (Vc), elimination rate constant (K_e), and intercompartmental rate constants (K_{cp}, K_{pc}). Systemic clearance (Cl) will be calculated using standard equations and area under the concentration time curve from time zero to infinity ($AUC_{0-\infty}$) will be calculated using the log-linear trapezoidal method.

6.1.3 Pharmacokinetically Guided Dosing

Topotecan dosage will be adjusted as necessary to attain a target plasma topotecan lactone AUC 140 ± 20 ng/ml*hr. For participants < 6months old treated on Stratum A or participants treated on Stratum B, plasma samples will be obtained with dose 1 in all participants for the first and second cycles of topotecan. If studies with the first dose of the first cycle and with the first dose of the second cycle are within the targeted range, then further pharmacokinetic sampling will not be necessary. If the topotecan AUC with the first dose is not within the targeted range, then a dose adjustment will be made and additional serial samples will be obtained with dose 2 or 3. The dose adjustments will be based on the methodology previously described.[75] Subsequent dose adjustments and repeat serial blood sampling will be performed until the AUC is within the targeted range; however, if the topotecan level is not within the targeted range after three dose adjustments, the one final dose adjustment will be made and no further pharmacokinetic samples will be obtained in that cycle. For participants treated on Stratum B, if dose adjustments are required in cycle 2, pharmacokinetic studies will be performed in cycle 5 beginning with dose 1. Similarly pharmacokinetic targeting studies will be performed with dose 1 of cycle 8. For participants <6months old treated on Stratum A, if dose adjustments are required in cycle 2 of topotecan (course 3), pharmacokinetic studies will be performed in cycle 3 of topotecan (course 5) beginning with dose 1.

6.2 Pharmacogenetics Studies

Participants treated at St. Jude Children's Research Hospital will be offered enrollment on the PGEN5 study, a non-therapeutic study that investigates the influence of genetic polymorphisms on the disposition or effects of therapeutic agents, such as topotecan. Thus, samples for pharmacogenetics studies will *not* be collected as part of the SJRET6 study.

7.0 DOSE MODIFICATIONS FOR NON-HEMATOLOGIC TOXICITY

7.1 Hepatotoxicity

If total bilirubin is > 2.0 mg/dL, obtain fractionated bilirubin. If direct bilirubin is > 0.6 mg/dL and < 1.0 mg/dL, then give half the dose of vincristine, etoposide, and doxorubicin. If the direct bilirubin is > 1.0 mg/dL, hold those agents.

For grade 3 or 4 toxicity in hepatic transaminases (SGOT, SGPT), hold chemotherapy until toxicity is less than Grade 2 and give full doses. If grade 3 or 4 toxicity develops in future cycles, reduce doses of the causative agent by 25% subsequently.

7.2 Nephrotoxicity

In this protocol, carboplatin and topotecan are administered targeting a systemic exposure. If there is an increase in serum creatinine to above the age-adjusted normal value or to twice the baseline pre-chemotherapy value, a GFR should be obtained, and the doses of carboplatin and topotecan will be adjusted accordingly. If grade 3 or grade 4 toxicities develop, both agents should be held.

7.3 Neurologic Toxicity

If severe peripheral neuropathy or ileus develops from vincristine, vincristine should be stopped or withheld until the ileus resolves or the peripheral neuropathy improves. Then, restart vincristine at 50% dose and escalate by 25% if tolerated. If neuropathy recurs on escalating dose, return to previously tolerated dose once the neuropathy has improved.

7.4 Hematuria

There will be no dose modification of cyclophosphamide for micro-hematuria, but if present, give a fluid bolus of NS 10 mL/kg over 15 minutes and increase hydration rate by 50%. If gross hematuria occurs (Grade 2 to 4) then next course of cyclophosphamide should be administered at full dose, and it is recommended that the participant be admitted, and MESNA be administered as a continuous infusion.

7.5 Audiologic

If the participant develops hearing loss $\geq$40db at 2000 Mhz or loss correctable with a hearing aid, carboplatin will be discontinued.

7.6 Allergic Reactions to Chemotherapy other than Etoposide

If an allergic reaction to etoposide or carboplatin develops, treat with diphenhydramine 1-2 mg/kg and, depending on severity, hydrocortisone 100 – 300 mg/m^2. Participants who develop an allergic reaction to etoposide can receive Etopophos® for all subsequent doses.

7.7 Etoposide Reactions

7.7.1 Cardiovascular Effects

Transient hypotension has occurred in about 1 to 2 % of participants following rapid IV administration of etoposide during clinical trials. However, hypotension has not been associated with cardiac toxicity or electrocardiogram changes. Blood pressure usually normalizes within a few hours after discontinuation of the infusion. To avoid this complication, etoposide should be infused over 30 – 60 minutes. If hypotension should occur, stop the infusion, and if necessary, give 10 mL/kg NS bolus over 15 minutes.

Repeat as necessary. Once symptoms resolve, resume infusion at ½ the previous infusion rate until the full dose administered. If hypotension recurs, stop infusion and administer 10 mL/kg NS bolus as indicated. Once hypotension resolves, resume infusion at ½ previous infusion rate until complete. Consider infusing NS at 1 – 1.5 x maintenance during remainder of infusion. For all subsequent doses, further dilute and infuse over 2 hrs.

7.7.2 Sensitivity Reactions

Anaphylactoid reactions consisting principally of chills, rigors, diaphoresis, pruritis, loss of consciousness, nausea, vomiting, fever, bronchospasm, dyspnea, tachycardia, hypertension, and/or hypotension have occurred in 0.7 – 3% of patients receiving etoposide. Other manifestations include flushing, rash, substernal chest pain, lacrimations, sneezing, coryza, throat pain, back pain, abdominal cramps, and auditory impairment. Facial/lingual swelling, coughing, diaphoresis, cyanosis, tightness in the throat, and laryngospasm have also occurred.

If an anaphylactoid reaction should occur:

1. Stop the infusion immediately and notify H/O Fellow or Attending MD
2. Administer the following as indicated:
    a) diphenhydramine 1mg/kg IV (max dose 50 mg)
    b) hydrocortisone 50 – 100 mg/m^2 IV
    c) epinephrine 0.01 mg/kg of a 1:1000 concentration for SQ administration
    d) fluid bolus 10 mL/kg NS infused over 15 minutes
3. Substitute etoposide with etoposide phosphate (Etopophos®) for all subsequent doses.
4. If anaphylaxis does recur, pre-medicate all subsequent doses with diphenhydramine 1mg/kg (max 50 mg) and hydrocortisone 50 – 100 mg/m^2. Consider slowing the loading dose to be administered over 1 hour.
5. Have at bedside all of the following for all subsequent infusions:
    a) Diphenhydramine 1mg/kg IV (max 50 mg)
    b) Hydrocortisone 50 – 100 mg/m^2 IV
    c) Epinephrine 0.01 mg/kg of a 1:1000 concentration for SQ administration

Please note: anaphylactoid reactions are still possible with etoposide phosphate. If the patient cannot tolerate the substitution, drug is contraindicated and must be discontinued.

7.8 Ocular Toxicities

Particular attention will be paid to ocular toxicities associated with the combined administration of systemic topotecan with periocular carboplatin in stratum B participants. Subtenon administration of carboplatin commonly causes transient periocular inflammation. There is extensive experience with the concurrent administration of periocular carboplatin with systemic chemotherapy (vincristine, carboplatin and etoposide), and therefore we do not expect any different toxicities in our regimen (with systemic topotecan), nor did we experience any new toxicities during RET5 therapy. Transient peri-orbital swelling is expected, usually lasting 6-8 days, and will not be considered a limiting toxicity. However, if any grade 3 or 4 ocular or skin

toxicity occurs after any periocular injection, no further doses will be given to that participant. We will also monitor for any infections, optic atrophy, conjunctival fibrosis, and fat atrophy.

7.9 Other Toxicities

For any other Grade 3 or 4 toxicities, the primary investigator should be contacted.

8.0 DRUG INFORMATION

8.1 Topotecan (Hycamtin®)

Source and pharmacology: Topotecan is a semi-synthetic derivative of camptothecin that inhibits topoisomerase I activity. Topoisomerase I relieves torsional strain in the DNA helix during replication by inducing reversible single strand DNA breaks. Topotecan binds to the topoisomerase I-DNA complex and prevents relegation of single strand breaks. This results in double-strand DNA breaks during DNA synthesis when replication enzymes interact with the ternary complex formed by topotecan, topoisomerase I and DNA. Topotecan undergoes a rapid, pH-dependent opening of the lactone ring to yield a relatively inactive, hydroxy acid in plasma. At physiologic pH, topotecan exists mainly as the hydroxy acid. Metabolism occurs via a pH dependent hydrolysis of the lactone moiety. Metabolism to an N-demethylated metabolite represents a minor metabolic pathway. Mean elimination half-life is three hours. Approximately 30% of a dose is excreted in the urine. Dosage adjustment is recommended for participants with moderate to severe renal dysfunction).

Formulation and stability: Topotecan is available in single-dose vials containing 4 mg of topotecan as a lyophilized light yellow to greenish powder and 48 mg of mannitol. The intact vials should be stored at room temperature and protected from light. Each vial may be reconstituted with 4 ml of sterile water for injection to yield a final concentration of 1 mg/ml. Because the reconstituted solution does not contain a preservative, it is recommended that it be used immediately after reconstitution. The reconstituted solution can be further diluted with 5% dextrose or 0.9% NaCl containing solutions. Once diluted for administration, the drug is stable for at least 24 hours at room temperature and ambient lighting.

Supplier: Commercially available

Toxicity: The dose-limiting toxicity is myelosuppression. Other toxicities reported commonly include nausea and vomiting, diarrhea, mucositis, abdominal pain, fever, rash, alopecia, anorexia, headache and flu-like symptoms. Toxicities reported less commonly include elevated liver function tests and paresthesias.

Dosage and route of administration: I.V. to achieve a TSE of 140 ± 20 ng/ml*hr, daily for 5 consecutive days.

8.2 VinCRIStine (Oncovin®)

Source and pharmacology: Vincristine is an alkaloid obtained from the periwinkle *(Vinca rosea)* plant. It reversibly binds to microtubule and spindle proteins causing metaphase arrest. Vincristine has poor penetration into the CSF. It is approximately 75% protein bound. Extensive metabolism occurs in the liver. Excretion is primarily in the bile. A dosage decrease is recommended in participants with a bilirubin > 3 mg/dl.

Formulation and stability: Vincristine is supplied in multiple-dose 1 mg/ml vials containing 1 ml, 2 ml and 5 ml. The intact vials should be stored under refrigeration and protected from light.

Supplier: Commercially available

Toxicity: Dose limiting toxicity is neurotoxicity. This can be characterized by constipation and/or paralytic ileus, ptosis, vocal cord paralysis, weakness, jaw pain, abdominal pain, peripheral neuropathies, loss of deep tendon reflexes and "foot drop". Peripheral neuropathy is often the first sign of neurotoxicity and is initially reversible. Other toxicities reported include alopecia, mild nausea and vomiting, SIADH, myelosuppression, orthostatic hypotension, optic atrophy, transient cortical blindness, and auditory damage. Acute shortness of breath and severe bronchospasm have been reported following the administration of vinca alkaloids. Myelosuppression is rare at usual doses. Vincristine is a vesicant and may cause severe tissue damage if extravasation occurs. NOTE: Dosing on per kg (rather than per m^2) basis has been advocated for infants in order to decrease toxicity.

Dosage and route of administration: Participants < 12 months of age or < 10 kg: 0.05 mg/kg IV day 1; participants ≥ 12 months of age and ≥ 10 kg: 1.5 mg/m^2 IV day 1 (max. dose 2 mg).

8.3 CARBOplatin (Paraplatin®)

Pharmacology: Carboplatin is an inorganic heavy metal coordination complex that has biochemical properties similar to those of a bifunctional alkylating agent. Carboplatin must undergo activation, by aquation, to form positively charged platinum complexes that react with nucleophilic sites on DNA, producing predominantly intrastrand DNA cross-links. Carboplatin is widely distributed in body tissues and fluids with highest concentrations in the kidney, liver, skin and tumor tissue. Carboplatin does not bind significantly to plasma proteins, but the activated form does bind to tissue and plasma proteins. In adults with normal renal function, the plasma elimination half-life is ≈2-3 hours. The elimination of carboplatin and its platinum-containing products is primarily dependent on glomerular filtration rate; therefore dosages may be adjusted for renal function.

Formulation and stability: Carboplatin is supplied in 20-ml amber vials containing 50, 150 or 450 mg of carboplatin as a white lyophilized powder. Vials should be reconstituted with sterile water for injection, D5W or 0.9% NaCl to give a concentration of 10 mg/ml and further diluted with D5W or 0.9% NaCl to concentration of 0.5 mg to

St. Jude Children's Research Hospital
IRB NUMBER: Pro00003243
IRB APPROVAL DATE: 01/12/2021

1.0 mg per ml. It is recommended that the reconstituted solution be discarded 8 hours after preparation.

Supplier: Commercially available.

Toxicity: Dose limiting toxicity is bone marrow suppression with thrombocytopenia being prominent. Nausea and vomiting of moderate severity are common. Hepatic dysfunction (after high doses), alopecia, ototoxicity, peripheral neuropathies, reversible renal toxicity, visual disturbances, and allergic reactions have all been reported less commonly. Pulmonary fibrosis is rare, but occurs most commonly in patients treated with cumulative doses > 1400 mg/m^2 or receiving bone marrow transplant dosages. Electrolyte abnormalities including hyponatremia, hypomagnesemia, hypocalcemia and hypokalemia can occur. Secondary cancers have been reported rarely. When given by subtenon administration, carboplatin may cause periorbital swelling. Direct injection close to the optic nerve may cause optic neuropathy.

Dosage and route of administration: IV to target AUC of 6.5; subtenon: 20 mg.

8.4 Etoposide (VP-16) (Vepesid®)

Source and pharmacology: Etoposide is an epipodophyllotoxin derived from *Podophyllum pelatatum.* It is thought to act mainly by inhibiting topoisomerase II, causing double and single strand DNA breaks. Etoposide is cell cycle, phase-specific, with activity in the G2 and S phases. Absorption of etoposide is approximately 30-40% and is highly variable and somewhat dose-dependent. It is extensively bound to serum proteins and is metabolized in the liver, including cytochrome P450 3A metabolism to several moieties that include a reactive oxidized species. Etoposide and its metabolites are excreted mainly in the urine with a smaller amount excreted in the feces. Dosage adjustments should be considered in patients with liver dysfunction, kidney dysfunction or hypoalbuminemia.

Formulation and stability: Etoposide is available in multi-dose vials containing 100mg, 150mg, 500mg and 1000mg of etoposide as a 20mg/ml solution and 30% alcohol. Etoposide is also available as a 50 mg capsule. The intact vials of etoposide solution should be stored at room temperature. The capsules should be stored under refrigeration. Etoposide solution should be diluted in D_5W or 0.9% NaCl prior to administration. Solutions with a final concentration of 0.2 and 0.4 mg/ml are stable at room temperature for 96 hours and 24 hours respectively.

Supplier: Commercially available

Toxicity: Dose limiting toxicity is myelosuppression. Nausea and vomiting (usually of low to moderate severity), diarrhea, mucositis (particularly with high doses), alopecia and anorexia are fairly common. Hypotension can occur with rapid infusions. Other side effects reported less commonly include hepatitis, fever and chills, anaphylaxis and peripheral neuropathy. Secondary leukemia has been reported.

Dosage and route of administration: Participants < 12 months of age or < 10 kg: 3.3 mg/kg/d IV x 3 days; participants ≥ 12 months of age and ≥ 10 kg: 100 mg/m^2/d IV x 3 days.

8.5 DOXOrubicin (Adriamycin®)

Source and pharmacology: Doxorubicin is an anthracycline antibiotic produced by *Streptomyces peucetius*. Doxorubicin exerts its anti-tumor effects in several different ways. Doxorubicin intercalates between base pairs of DNA causing steric obstruction, disruption of DNA function and inhibition of RNA synthesis. In addition, doxorubicin inhibits topoisomerase II, an enzyme responsible for allowing strands of DNA to pass through one another as they unwind. Lastly, doxorubicin undergoes enzymatic electron reduction to generate highly reactive species, including the hydroxyl free radical, which is thought to be responsible for the drug's cardiac toxicity, but may play a role in its anti-tumor activity as well. Doxorubicin is cell-cycle, phase non-specific. Doxorubicin is widely distributed in the tissues and plasma, but does not cross the blood brain barrier to an appreciable extent. It is metabolized to doxorubicinol, which is thought to be the major active metabolite, and aglycones. Doxorubicin and its metabolites are excreted mainly in the bile and feces (≈80%). The remainder is excreted in the urine. Dosage should be reduced in patients with liver dysfunction (bilirubin > 1.2 mg/dl) or renal dysfunction (creatinine > 3 mg/dl).

Formulation and stability: Doxorubicin is available in vials containing 10 mg, 20 mg, 50 mg and 200 mg as a 2mg/ml red-orange solution. It is also available in vials containing 10 mg, 20 mg, 50 mg, 100 mg and 150 mg of doxorubicin as a red-orange lyophilized powder. Intact vials of doxorubicin solution should be stored under refrigeration while the lyophilized product should be stored at room temperature. Both products should be protected from light. Lyophilized doxorubicin can be reconstituted by adding 5, 10, 25, 50 or 75 ml of 0.9% NaCl respectively to the 10, 20, 50, 100 and 150 mg vials to produce a final concentration of 2 mg/ml. Bacteriostatic diluents are not recommended. After reconstitution, the resultant solution should be protected from light and is stable for 7 days at room temperature and 15 days if refrigerated.

Supplier: Commercially available

Toxicity: Dose-limiting toxicities include myelosuppression and cardiotoxicity. Two forms of cardiac toxicity can occur. Acute toxicity may take the form of arrhythmias, heart block or pericarditis and may be fatal. The chronic form of cardiotoxicity is related to total cumulative dose and is characterized by heart failure. Mediastinal radiotherapy and/or other cardiotoxic drugs may increase the risk of cardiotoxicity. In general, total lifetime dosages of 450-550mg/m^2 should not be exceeded. Other toxicities include nausea and vomiting, mucositis, alopecia, diarrhea and red discoloration of the urine and other body fluids. Severe tissue damage and necrosis can occur upon extravasation. Radiation recall reactions can occur and can be severe. Rarely, allergic reactions have occurred. Typhilitis can occur when combined with cytarabine.

Dosage and route of administration: Participants < 12 months of age or < 10 kg: 1.5 mg/kg IV; participants ≥ 12 months of age and ≥ 10 kg: 45 mg/m^2 IV.

St. Jude Children's Research Hospital
IRB NUMBER: Pro00003243
IRB APPROVAL DATE: 01/12/2021

8.6 Cyclophosphamide (Cytoxan®)

Source and pharmacology: Cyclophosphamide is a nitrogen mustard derivative. It acts as an alkylating agent that causes cross-linking of DNA strands by binding with nucleic acids and other intracellular structures, thus interfering with the normal function of DNA. Cyclophosphamide is cell-cycle, phase non-specific. Cyclophosphamide is well absorbed from the GI tract with a bioavailability of > 75%. Cyclophosphamide is a prodrug that requires activation. It is metabolized by mixed-function oxidases in the liver to 4-hydroxycyclophosphamide, which is in equilibrium with aldofosfamide. Aldofosfamide spontaneously splits into cyclophosphamide mustard, which is considered to be the major active metabolite, and acrolein. In addition, 4-hydroxycyclophosphamide may be enzymatically metabolized to 4-ketocyclophosphamide and aldofosfamide may be enzymatically metabolized to carboxyphosphamide which are generally considered to be inactive. Cyclophosphamide and its metabolites are excreted mainly in the urine. Dosage adjustments should be made in participants with a creatinine clearance of <50 ml/min.

Formulation and stability: Cyclophosphamide is available in 25 and 50 mg tablets. Cyclophosphamide is also available in vials containing 100, 200, 500, 1000 and 2000mg of lyophilized drug and 75 mg mannitol per 100 mg of cyclophosphamide. Both forms of the drug can be stored at room temperature. The vials are reconstituted with 5, 10, 25, 50 or 100 ml of sterile water for injection respectively to yield a final concentration of 20 mg/ml. Reconstituted solutions may be further diluted in either 5% dextrose or 0.9% NaCl containing solutions. Diluted solutions are physically stable for 24 hours at room temperature and 6 days if refrigerated, but contain no preservative, so it is recommended that they be used within 24 hours of preparation.

Supplier: Commercially available

Toxicity: Dose limiting toxicities of cyclophosphamide are bone marrow suppression and cardiac toxicity. Cardiac toxicity is typically manifested as congestive heart failure, cardiac necrosis or hemorrhagic myocarditis and can be fatal. Hemorrhagic cystitis may occur and necessitates withholding therapy. The incidence of hemorrhagic cystitis is related to cyclophosphamide dose and duration of therapy. Forced fluid intake and/or the administration of MESNA decrease the incidence and severity of hemorrhagic cystitis. Other toxicities reported commonly include nausea and vomiting (may be mild to severe depending on dosage), diarrhea, anorexia, alopecia, immunosuppression and sterility. Pulmonary fibrosis, SIADH, anaphylaxis and secondary neoplasms have been reported rarely.

Dosage and route of administration: Participants < 12 months of age or < 10 kg: 40 mg/kg IV; participants ≥ 12 months of age and ≥ 10 kg: 1,200 mg/m^2 IV.

8.7 MESNA (Mesnex®)

Source and pharmacology: Mesna is a synthetic sulfhydryl (thiol) compound. Mesna contains free sulfhydryl groups that interact chemically with urotoxic metabolites of oxazaphosphorine derivatives such as cyclophosphamide and ifosfamide. Oral bioavailability

St. Jude Children's Research Hospital
IRB NUMBER: Pro00003243
IRB APPROVAL DATE: 01/12/2021

is ≈50%. Upon injection into the blood, mesna is oxidized to mesna disulfide, a totally inert compound. Following glomerular filtration, mesna disulfide is rapidly reduced in the renal tubules back to Mesna, the active form of the drug. Mesna and mesna disulfide are excreted primarily via the urine.

Formulation and stability: Mesna is available in 2 ml, 4 ml and 10 ml amps containing 100 mg/ml of mesna solution. The intact vials can be stored at room temperature. Mesna may be further diluted in 5% dextrose or 0.9% NaCl containing solutions. Diluted solutions are physically and chemically stable for at least 24 hours under refrigeration.

Supplier: Commercially available

Toxicity: MESNA is generally well tolerated. Nausea and vomiting, headache, diarrhea, rash, transient hypotension and allergic reactions have been reported. Patients may complain of a bitter taste in their mouth during administration. MESNA may cause false positive urine dipstick readings for ketones.

Dosage and administration: IV infusion, following current pharmacy guidelines for administration.

8.8 Etoposide Phosphate (Etopophos®)

To be used in case of etoposide reactions.

Source and pharmacology: Etoposide is an epipodophylotoxin derived from *Podophyllum pelatatum.* It is thought to act mainly by inhibiting topoisomerase II, causing double and single strand DNA breaks. Etoposide is cell cycle, phase-specific, with activity in the G2 and S phases. Absorption of etoposide is approximately 30-40% and is highly variable and somewhat dose-dependent. It is extensively bound to serum proteins and is metabolized in the liver, including cytochrome P450 3A metabolism to several moieties that include a reactive oxidized species. Etoposide and its metabolites are excreted mainly in the urine with a smaller amount excreted in the feces. Dosage adjustments should be considered in participants with liver dysfunction, kidney dysfunction or hypoalbuminemia.

Formulation and stability: Etoposide phosphate is a water-soluble ester of etoposide. The higher water solubility of etoposide phosphate than that of etoposide lessens the potential for precipitation following dilution and during administration. Etoposide phosphate is available in single-dose vials containing etoposide phosphate equivalent to 100mg etoposide. The intact vials of etoposide solution should be stored at 2 to 8 degrees Celsius. Etoposide phosphate solution should be diluted in D5W or 0.9% NaCl prior to administration. Solution is stable at room temperature for 24 hours.

Supplier: Commercially available.

Toxicity: Dose limiting toxicity is myelosuppression. Nausea and vomiting (usually of low to moderate severity), diarrhea, mucositis (particularly with high doses), alopecia and anorexia are fairly common. Hypotension can occur with rapid infusions. Other side

effects reported less commonly include hepatitis, fever and chills, anaphylaxis and peripheral neuropathy. Secondary leukemia has been reported.

Dosage an route of administration: Used in substitution for etoposide in participants that experience allergic reaction, Etopophos® will be administered (per current pharmacy guidelines).

8.9 G-CSF (Filgrastim) (Neupogen®)

Source and pharmacology: G-CSF (granulocytic colony stimulating factor), is a biosynthetic hematopoietic agent that is made using recombinant DNA technology in cultures of *Escherichia coli.* G-CSF stimulates production, maturation and activation of neutrophils. In addition, endogenous G-CSF enhances certain functions of mature neutrophils, including phagocytosis, chemotaxis and antibody--dependent cellular cytotoxicity.

Formulation and stability: G-CSF is supplied in vials containing 300 mcg and 480 mcg of G-CSF at a concentration of 300 mcg/ml. The intact vials should be stored under refrigeration. The vials can be left out of refrigeration for 24 hours, but should be discarded if left at room temperature for longer periods of time. G-CSF can be drawn up into tuberculin syringes for administration and stored under refrigeration for up to 7 days prior to usage. G-CSF can be further diluted for IV infusion in 5% dextrose. Do not dilute in saline---precipitate may form. If the final concentration of this product is < 15 mcg/ml, it is recommended that albumin be added to a final concentration of 2mg/ml (0.2%) to minimize adsorption of the drug to infusion containers and equipment.

Supplier: Commercially available

Toxicity: G-CSF causes marked leukocytosis. Adverse reactions reported commonly include bone pain, thrombocytopenia, diarrhea, nausea, rash, alopecia, fever, anorexia and pain or bruising at the injection site. Allergic reactions, MI, atrial fibrillation, and splenomegaly have been reported rarely. G-CSF is contraindicated in participants with allergy to E. coli derived products.

Dosage and route of administration: 5 mcg/kg/day, subcutaneously, until ANC is ≥ 2,000/μL after the expected nadir.

8.10 Pegfilgrastim (pegylated filgrastim, PEG filgrastim, SD-01, Neulasta®)

Source and pharmacology: Pegfilgrastim is the pegylated form of recombinant methionyl human G-CSF (filgrastim). Pegfilgrastim is produced by covalently binding a 20-kilodalton (kD) monomethoxypolyethylene glycol molecule to the N-terminal methionyl residue of filgrastim. The molecular weight of pegfilgrastim is 39 kD. G-CSF is a lineage specific colony-stimulating factor which regulates the production of neutrophils within the bone marrow and affects neutrophil progenitor proliferation, differentiation, and selected end-cell functional activation (including enhanced phagocytic ability, priming of the cellular metabolism associated with respiratory burst, antibody dependent killing, and the increased expression of some functions associated with cell surface antigens).

After subcutaneous injection the elimination half-life of pegfilgrastim ranges from 15 to 80 hours and the time to peak concentration ranges from 24 to 72 hours. Serum levels are sustained in most patients during the neutropenic period post-chemotherapy, and begin to decline after the start of neutrophil recovery, consistent with neutrophil-dependent elimination.

Toxicity: mild to moderate medullary bone pain, local irritation at the injection site, headache, elevations in alkaline phosphatase, LDH, uric acid, thrombocytopenia, low grade fever, allergic reactions, splenomegaly, splenic rupture, exacerbation of pre-existing skin rashes, sickle cell crisis in patients with SCD, excessive leukocytosis, ARDS. Fetal toxicities and teratogenic effects in humans are unknown. There is conflicting data in animals. It is unknown whether the drug is excreted in breast milk.

Formulation and stability: Supplied as a preservative-free solution containing 6 mg (0.6 mL) of pegfilgrastim (10 mg/mL) in a single dose syringe with 27 g, ½ inch needle with an UltraSafe® Needle Guard. Store refrigerated at 2º-8ºC (36º-46ºF) and in the carton to protect from light. Prior to injection, pegfilgrastim may be allowed to reach room temperature protected from light for a maximum of 48 hours. Avoid freezing.

Guidelines for administration: per current institutional and pharmaceutical guidelines.

Supplier: Commercially available from various manufacturers. See package insert for further information.

9.0 REQUIRED EVALUATIONS, TESTS, AND OBSERVATIONS

9.1 Pre-Study Evaluations

Evaluations to be obtained in all participants at diagnosis include the following:

- Detailed medical history including family history
- Physical examination with documentation of measurable lesions in both eyes:
  - Lesions will be photographed using the RET-Cam, and the images will be stored digitally.
  - During the procedure, drawings will also be performed.
- Diagnostic imaging:
  - MRI of the brain and orbits
  - US of the orbits
- Appropriate safety laboratory tests, including CBC, electrolytes, blood chemistries, and urinalysis.
- Evaluation of the germline status of the RB gene. Blood collection is recommended at diagnosis, but it can be performed at any given time since it is not a condition necessary before therapy.

In addition to the above studies, the following evaluations should be performed in participants with *advanced disease*:

- For participants with *unilateral disease*, shown to have pathological *intermediate risk* (receiving VDC therapy):
  - Bilateral bone marrow aspirates and biopsies
  - Lumbar puncture for cytology
  - ECHO/EKG

- For participants with *unilateral disease*, shown to have pathological *high-risk disease* (receiving VDC and VCE):
  - Bilateral bone marrow aspirates and biopsies
  - Lumbar puncture for cytology
  - ECHO/EKG
  - Tc^{99m}-DTPA renal clearance
  - Audiogram or BAER, and OAE

- For participants with in both strata A and B *who will be receiving carboplatin chemotherapy:*
  - Tc^{99m}-DTPA renal clearance
  - Audiogram or BAER, and OAE

- For participants with *bilateral disease* in stratum D
  - Bilateral bone marrow aspirates and biopsies
  - Lumbar puncture for cytology
  - Tc^{99m}-DTPA renal clearance
  - Audiogram or BAER, and OAE

9.2 Occupational and Speech Therapy

Research participants will be evaluated by Occupational and Speech Therapy at diagnosis, and approximately every 6 months for a period of three years. Additional visits within and after this 3 year period will be scheduled on an as needed basis as determined by the occupational therapist or speech therapist. A scheduled long term follow up evaluation will occur approximately 5 years after the initial evaluation. The tests and questionnaires to be performed are outlined in Appendix III.

9.3 Audiology Evaluations

Applies only to participants receiving carboplatin. Participants will receive audiological testing as indicated by stratum. More frequent evaluations may be considered by the audiologist based on test results and the potential risk for hearing loss.

9.3.1 Tests and Outcome Measurements

At each evaluation, the following measurements will be performed:

1. Otoscopy will be performed to determine the condition of the pinna, external auditory canal, and tympanic membrane.
2. Tympanometry using a 226 Hz and 1000 Hz probe tone and wideband reflectance (WBR) measures using a broadband chirp stimulus from 200-6000 Hz to assess integrity of the middle ear mechanism.
3. Otoacoustic emissions will be assessed to determine cochlear outer hair cell function.
4. Auditory evoked potentials will be used for patients under the age of 3 years or for older children who are unable to respond to conventional audiometric testing techniques. Click and 500-4000 Hz tone burst ABR as well as 8 kHz ASSR will be assessed at the baseline evaluation. Select frequencies will be tested at subsequent evaluations as determined by the audiologist. Bone conduction ABR will be obtained as needed to rule out a conductive component to any hearing impairment.
5. Pure tone air conduction audiometry will be obtained to assess peripheral hearing sensitivity in patients older than 3 years. Each audiogram will include air conduction thresholds at 250, 500, 1000, 2000, 3000, 4000, 6000, and 8000 Hz measured in dB HL. Bone conduction thresholds will be obtained as needed to rule out a conductive component to any hearing impairment.

Every audiogram or ABR/ASSR evaluation will be assigned a grade based on the Chang Ototoxicity Grading Scale[123] (see Appendix VII).

9.4 Evaluations During Study and at Follow-up:

9.4.1 Stratum C: advanced unilateral disease**,** low-risk

	Diagnosis	After enucleation*
History	X	Every 6 – 12 months
Physical exam	X	Every 6 – 12 months
Complete blood count with differential	X	
BUN, creatinine, electrolytes, uric acid, SGOT, SGPT, alkaline phosphatase, total bilirubin, total protein and albumin	X	
EUA[@], RET-Cam, with documentation of lesions	X	Every 3 – 6 months^ until 5 years of age.
MRI– patients with tumor and germline *RB1* mutation identified, or unable to identify mutation in tumor	X	Every 6 months until age 5 ***
MRI – patients with tumor *RB1* mutation that is not identified in the germline	X	6 months after diagnosis, one year after diagnosis, then every year until five years of age***
Ultrasound	X	
Mutational analysis of *RB1*	X**	
Biology studies	X[+]	
Occupational and speech therapy	X	At diagnosis and then approximately every 6 months for 3 years. Approximately 5 years from diagnosis***

**Clinical follow-up after enucleation will be every 6 – 12 months, depending on the age of the participant and time since enucleation.*

^ EUA after enucleation is performed for evaluation of the contralateral eye.

[+]Immediately after enucleation, concurrent banking to be performed as per TBANK consent and enrollment

[@]Transfer to an awake exam in the Eye Clinic is participant- and disease-dependent. Usually this requires participant cooperation and disease stability (i.e., no focal therapy given) > 1 year.

***** Can be obtained any time after enrollment***

**** May be adjusted to coincide with other follow-up studies*

9.4.2 Stratum C: advanced unilateral disease, intermediate- and high-risk, and Stratum D: advanced bilateral disease requiring upfront enucleation and VCE therapy

	Diagnosis	During therapy	End of therapy	Follow-up years 1-2	Follow-up years 3-5
History	X	Before each course	X	q 6-12	q 6-12 months
Physical exam	X	Before each course	X	q 6-12 months	q 6-12 months
Complete blood count	X	Before each course	X		
Urinalysis	X		X	--	--
BUN, creatinine, electrolytes, uric acid, SGOT, SGPT, alkaline phosphatase, bilirubin (total and direct), total protein and albumin	X	Before each course	X		
BMA/Bx + LP	X				
ECHO/EKG***	X	Before 3rd course of VDC	X	^^	^^
Audiogram or BAER and OAE*	X	Before 3rd course VCE (Stratum D only)	X	q 12 months	q 12 months
Tc^{99m}-DTPA GFR*	X	Before 3rd course VCE (Stratum D only)	X	--	--
EUA@, RET-Cam, with documentation of lesions	X	Every 3 – 6 months^			
MRI – patients with tumor and germline *RB1* mutation identified, or unable to identify mutation in tumor	X	Every 6 months until age 5 +			
MRI – patients with tumor *RB1* mutation that is not identified in the germline	X	6 months after diagnosis, one year after diagnosis, then every year until five years of age+			
Ultrasound	X				
Mutational analysis of *RB1*	X**				
Biology studies	X#				
Occupational and Speech therapy	X	At diagnosis and then approximately every 6 months for 3 years. Approximately 5 years from diagnosis+			

^ *EUA after enucleation is performed for evaluation of the contralateral eye.*
@*Transfer to an awake exam in the Eye Clinic is participant- and disease-dependent. Usually this requires participant cooperation and disease stability (i.e., no focal therapy given) > 1 year*
** Only for high-risk participants (Stratum C) or participants on Stratum D receiving carboplatin. OAE will not be performed after end of therapy evaluation. Cystatin-C may be obtained instead of GFR to assess renal function at end of therapy and during follow-up as needed*
+ *May be adjusted to coincide with other follow-up studies*
# *Immediately after enucleation, concurrent banking to be performed as per TBANK consent and enrollment.* ** *Can be*

St. Jude Children's Research Hospital
IRB NUMBER: Pro00003243
IRB APPROVAL DATE: 01/12/2021

obtained any time after enrollment
****ECHO/EKG not required for Stratum D participants receiving only VCE*

^^ECHO/EKG q5y per LTFU guidelines or as clinically indicated

9.4.3 Strata A and B: unilateral early stage eyes undergoing ocular salvage, and bilateral disease without upfront enucleation; this includes Stratum D participants who are treated as per Stratum A or Stratum B after upfront enucleation

	Diagnosis	During therapy	End of therapy	Follow-up years 1-5
History	X	Before each course	X	q months 6-12 months
Physical exam	X	Before each course	X	q months 6-12
Complete blood count	X	Before each course	X	
Urinalysis	X		X	--
BUN, creatinine, electrolytes, uric acid, SGOT, SGPT, alkaline phosphatase, bilirubin (total and direct), total protein and albumin	X	Before each course	X	
Audiogram or BAER, and OAE±	X	After course # 4 (Stratum A) and after course # 3 of CBP (Stratum B)	X	q 12 months
Tc^{99m}-DTPA GFR	X	After course # 4 (Stratum A) and after course # 3 of CBP (Stratum B)	X****	--
EUA@, RET-Cam, with documentation of lesions	X	q 3-8 weeks*	X	As needed
MRI	X	X^	Every 6 months x 4^{+} Then yearly till age of 5 years. +	
Ultrasound	X	X^^	**X**^^	
Mutational analysis of *RB1*	X***			
Biology studies	$X^{\#}$			
Pharmacogenetics (PGEN5)**	X			
Topotecan Pharmacokinetics**	See section 6.0			
Occupational and speech Therapy	X	At diagnosis and then approximately every 6 months for 3 years. Approximately 5 years from diagnosis+		

$^{+}$ *May be adjusted to coincide with other follow-up studies*
$^{\#}$ *Immediately after enucleation in those cases undergoing enucleation at any time during treatment, concurrent banking to be performed as per TBANK consent and enrollment*
** Participants on stratum B will undergo EUA after each one of the two initial courses of therapy*
@Transfer to an awake exam in the Eye Clinic is participant- and disease-dependent. Usually this requires participant cooperation and disease stability (i.e., no focal therapy given) > 1 year
^ MRI will be performed after the second course of the up-front therapy in stratum B participants
^^ Ultrasound will be performed after the second course of the up-front therapy in stratum B participants, and at end of therapy evaluation for participants in both strata.
*** Stratum A participants < 6months old and Stratum B participants only*
**** Can be obtained any time after enrollment± OAE will not be performed after end of therapy evaluation*
*****Cystatin-C may be obtained instead of GFR to assess renal function at end of therapy and during follow-up as needed*

10.0 BIOLOGY AND PATHOLOGY STUDIES

10.1 Processing of Fresh Tumor Material

For retinoblastoma patients undergoing enucleation at St. Jude Children's Research Hospital, the eye will be processed by the ophthalmologist/pathologist Dr. Wilson immediately after enucleation (as described in Appendix II) with excess tumor provided to the St. Jude Biorepository (with TBANK consent obtained from the legal guardian) for cryopreservation (a TB-ID number will be assigned by Biorepository) and 3-5mm^3 of tumor provided to the Dyer lab for cell culture and/or creation of orthotopic xenografts. For use in the Dyer lab, a SJRET-6 Tumor Number (SJRET-6TN) will be assigned to the tumor sample (no link to MRN). The tumor sample will either be immediately washed in warm (37°C) Hanks Balanced Salt Solution, labeled with the SJRET-6TN, and sent to Dr. Dyer's laboratory for cell cloning or used in the creation of orthotopic xenografts. Dr. Dyer's laboratory must be contacted before the surgical procedure (ext. # 3487).

10.1.1 Processing of Fresh Vitreous

Fresh vitreous samples will be harvested from the enucleated eye through the same trephined site used to harvest fresh tumor. Under sterile conditions, 2ml of material will be transferred to a 15ml conical tube. Samples will be labeled with the same SJRET-6 Tumor Number as the primary tumor sample (no link to MRN). This material will be snap frozen for with storage by the Dyer laboratory (-80C) until analysis by the Dr. Morales-Tirado Lab at University of Tennessee, Health Science Center.
The protocols for obtaining tumor tissue, as well as the specifics of the studies are included in Appendix II.

Processing of the tumor material will be performed as outlined in Appendix II.

10.2 Processing of Fixed Tumor Material

10.2.1 Histopathological Evaluation

Sections of the enucleated eye will be processed for diagnostic purposes, and evaluation of invasion of ciliary body, anterior chamber, choroid, sclera and optic nerve will be noted.

10.2.2 Generation of Tissue Microarrays

Two to four 1.0 mm diameter tissue cores will be extracted from representative tissue block(s), which are located in Pathology, for the construction of tissue microarrays. For this purpose, fresh H&E-stained slides from each of the paraffin donor blocks will be utilized as guides in selecting morphologically representative areas. Two to four 1.0 mm diameter tissue cores from each donor block will be precisely arrayed in the recipient TMA block using a tissue arrayer (Beecher Instruments, Silver Spring, MD) equipped with a thin-walled stainless steel tube (punch) with a sharpened end similar to a cork borer. A stainless stylet will be used to transfer tissue cores into recipient (array) blocks at defined microarray coordinates. Staggering of the first row of cores will be used to ensure reliable orientation of tissue sections and identification of each donor sample.

Subsequently, 4 μm-thick sections from the microarray blocks will be mounted on positively-charged slides for future immunohistochemical analysis. Tissue cores will only be taken in cases where there is adequate tissue available and will not otherwise compromise the donor block for future diagnostic utility. A database will be generated identifying the microarray coordinates with individual donor cases (identifier of pathology cases only) with password protection for access by only the Pathology section coordinator. The database will allow for parallel entries of subsequent results.

10.3 Processing of Vitreous Samples

In the Morales-Tirado lab, the sample will be centrifugated at 12000 RPM x 15 min x RT to separate the liquid component, as described in Yu et al, Proteomics, 2008. If debri is observed, we will filter the sample in a 0.22uM filter to clarify sample, as done by Kim et al, Proteomics, 2007. From the clarified fraction of the vitreous we will divide the sample into 2, one for protein analysis of PDGF and isoforms PDGF-A, PDGF-B and their receptors using commercially available Multiplex systems; second set for mRNA analysis of *PDGFA*, *PDGFB*, *PDGFRA*, *PDGFRB*, using commercially available primers, following the protocol for nucleic acid isolation from Chintalapudi and Morales-Tirado et al, 2015.

11.0 EVALUATION CRITERIA

For participants with unilateral, enucleated retinoblastoma, by definition there will be no measurable disease. For participants with bilateral retinoblastoma, the primary form of evaluation of retinoblastoma is through funduscopic examination. A careful documentation of the lesions is performed in each exam using the RET-CAM, which allows storage of all the information for further comparisons. An ultrasound (US) of the involved eyes will be performed as a secondary method to evaluate response and to confirm the EUA findings. When the EUA and US are discordant, EUA result will be the method used to evaluate response.

In addition to the visual evaluation, participants will undergo an MRI of both orbits, which will be used for measuring lesions but will not be used to evaluate response. In addition, measurements of the pineal gland will be recorded.

At diagnosis, each distinct tumor mass will be numbered (during initial EUA) for tracking focal therapy administration and recurrences throughout therapy. If present, new lesions will be tracked in a similar fashion. Vitreous seeding will be tracked separately.

The “tumor status” (for each distinct tumor mass) will be tracked during EUAs. In addition, a separate note of “disease response” will be followed that takes into account the overall disease response (per eye) to therapy:

1. Unilateral disease s/p enucleation with no evidence of disease in the contralateral eye: **NED**
2. Patient who is currently receiving systemic chemotherapy and/or focal therapy: **PR vs. >PR vs. <PR**

3. Patient who has completed systemic chemotherapy (or none was ever prescribed) and in whom no focal therapy has been applied to the eye(s) with disease in > 1 year: **CR**

11.1 Response Criteria for Retinoblastoma

11.1.1 Complete Response (CR)

Complete regression, calcification or scarring of all apparent tumor masses in the funduscopic examination. Lesions which initially decrease in size and then remain unchanged (but incompletely calcified or scarred) in the absence of further focal or systemic therapy will also be considered CR. The duration of response will be recorded. The response must persist for at least 4 weeks.

11.1.2 Partial Response (PR)

Greater than 50% (but less than 100%) reduction of the tumor masses in the funduscopic examination, without the appearance of any new lesions. The response must persist for at least 4 weeks.

11.1.3 Stable Disease (SD)

Less than 50% reduction or less than 25% increase in the tumor masses in the funduscopic examination.

11.1.4 Progressive Disease (PD)

An increase of 25% in the size of all measurable tumors in the funduscopic examination or disease progression that is not amenable to local therapy and requires EBRT, enucleation, or both.

11.1.5 Recurrence

An increase in the size of a tumor mass or appearance of new tumors more than 3 months after completion of all systemic and focal therapy by funduscopic examination.

11.2 Toxicity Evaluation Criteria

Common Terminology Criteria for Adverse Events v4.0 (CTCAE): This study will utilize the CTCAE of the National Cancer Institute (NCI) for toxicity and performance reporting. A copy of the current version of the CTCAE can be downloaded from the Cancer Therapy Evaluation Program (CTEP) home page (http://ctep.info.nih.gov). Additionally, toxicities are to be reported on the appropriate data collection screens/forms.

11.3 General Assumptions Regarding Chemotherapy Administration for all Treatment Strata

- The timing and duration for administration for all commercially available agents are provided in the treatment phase sections as guidelines only. Variations in the timing and duration of chemotherapy infusions according to institutional practice or variations based on patient care needs are acceptable, as long as the treating investigator and/or PI determines that there was no impact on patient safety. These variations will not be considered protocol deviations, as long as the total dose is given within 10% of protocol specified dose.
- The term "day" does not refer to an absolute calendar day. It refers to a general 24-hour period as per St. Jude Nursing P&P.
- Medication dosing may be modified for research recipients based upon actual body weight or adjusted ideal body weight when clinically indicated. Criteria for medication calculations based on body weight/body surface area can be found in any version of the St. Jude Formulary. Medication doses may be rounded to the nearest integer or to the nearest appropriate quantity when clinically or pharmaceutically indicated as per the M.D. and PharmD.

11.4 Guidance during Times of Drug Shortages and Unavailability

Treating investigators are urged to consult with the PI or co-PI and use their best clinical judgment in optimizing therapeutic intent and ensuring patient safety in managing the protocol-specified therapy. Although these decisions may constitute "Protocol Violations," they are unavoidable and made in consideration of the best interest of an individual patient. These will not be considered monitoring/audit findings if appropriately documented. All protocol deviations must be noted in the research database and the alterations in therapy due to the agent shortage will be captured. This should be accomplished by entering "dose modified" and details noted in the comments field. These deviations will also be noted in the Deviation Log with the notation "Drug substitution/reduction due to unavoidable drug shortage/unavailability".

12.0 OFF STUDY AND OFF THERAPY CRITERIA

12.1 Off-Study Criteria

One of the aims of this study is to provide a comprehensive and multidisciplinary approach to participants with retinoblastoma, and to investigate different therapeutic, biological and cognitive questions. Participants may fail therapy due to progressive disease, but may remain in the study for follow-up and to participate in some of the secondary objectives. Therefore, off-study criteria will be defined by the following:

- If the participant or legal guardian desire to withdraw from the study
- Completion of planned follow-up at St. Jude Children's Research Hospital
- Death
- Lost to follow-up

12.2 Off-Therapy Criteria

- Life-threatening toxicity
    - Any evaluable participant who develops irreversible life-threatening non-hematologic organ system dysfunction (grade 4) considered to be primarily related to drug toxicity will have the treatment discontinued, and an alternative regimen will designed
- Progressive disease
    - Enucleated participants with unilateral disease who develop progressive disease (orbital or extra-orbital metastases) will receive salvage therapy (as best clinical management) but may remain on study at PI discretion for the secondary objectives (biology studies, cognitive development and evaluation of visual cortex)
    - The development of intraocular progressive disease is a common event as discussed above, and participants may require EBRT or enucleation. In these cases, participants will come off therapy but will not be removed from the study
- Second malignancy
- Development of unacceptable toxicity during treatment (with concurrence of PI)
- Participant/family decision to withdraw from protocol treatment at any time for any reason
- Discretion of the PI or co-PIs, such as the following
    - The researcher decides that continuing in the study would be harmful
    - A treatment is needed that is not allowed on this study
    - The participant misses so many appointments that the data cannot be used in the study
    - New information is learned that a better treatment is available, or that the study is not in the participant's best interest
- Completion of all protocol prescribed treatment
    - Participants receiving systemic chemotherapy: date of last chemotherapy
    - Participants receiving enucleation only: date of enucleation

13.0 SAFETY AND ADVERSE EVENT REPORTING REQUIREMENTS

13.1 Reporting Adverse Experiences (AEs) and Deaths On Treatment

Only "unanticipated problems involving risks to participants or others" referred to hereafter as "unanticipated problems" are required to be reported to the St. Jude IRB promptly, but in no event later than 10 working days after the investigator first learns of the unanticipated problem. Regardless of whether the event is internal or external (for example, an IND safety report by the sponsor pursuant to 21 CFR 312.32), only adverse events that constitute unanticipated problems are reportable to the St. Jude IRB. As further described in the definition of unanticipated problem, this includes any event that in the PI's opinion was:

- Unexpected (in terms of nature, severity, or frequency) given (1) the research procedures that are described in the protocol-related documents, such as the IRB-

approved research protocol and informed consent document, as well as other relevant information available about the research; (2) the observed rate of occurrence (compared to a credible baseline for comparison); and (3) the characteristics of the subject population being studied; and
- Related or possibly related to participation in the research; and
- Serious; or if not serious suggests that the research places subjects or others at a greater risk of harm (including physical, psychological, economic, or social harm) than was previously known or recognized.

Unrelated, expected deaths do not require reporting to the IRB. Though death is "serious", the event must meet the other two requirements of "related or possibly related" and "unexpected/unanticipated" to be considered reportable.

Deaths meeting reporting requirements are to be reported immediately to the St. Jude IRB, but in no event later than 48 hours after the investigator first learns of the death.

The following definitions apply with respect to reporting adverse experiences:

Serious Adverse Event: Any adverse event temporally associated with the subject's participation in research that meets any of the following criteria:

- results in death;
- is life-threatening (places the subject at immediate risk of death from the event as it occurred);
- requires inpatient hospitalization or prolongation of existing hospitalization;
- results in a persistent or significant disability/incapacity;
- results in a congenital anomaly/birth defect; or
- any other adverse event that, based upon appropriate medical judgment, may jeopardize the subject's health and may require medical or surgical intervention to prevent one of the other outcomes listed in this definition (examples of such events include: any substantial disruption of the ability to conduct normal life functions, allergic bronchospasm requiring intensive treatment in the emergency room or at home, blood dyscrasias or convulsions that do not result in inpatient hospitalization, or the development of drug dependency or drug abuse), a congenital anomaly/birth defect, secondary or concurrent cancer, medication overdose, or is any medical event which requires treatment to prevent any of the medical outcomes previously listed.

Unexpected Adverse Event:
- Any adverse event for which the specificity or severity is not consistent with the protocol-related documents, including the applicable investigator brochure, IRB approved consent form, Investigational New Drug (IND) or Investigational Device Exemption (IDE) application, or other relevant sources of information, such as product labeling and package inserts; or if it does appear in such documents, an event in which the specificity, severity or duration is not consistent with the risk information included therein; or

- The observed rate of occurrence is a clinically significant increase in the expected rate (based on a credible baseline rate for comparison); or
- The occurrence is not consistent with the expected natural progression of any underlying disease, disorder, or condition of the subject(s) experiencing the adverse event and the subject's predisposing risk factor profile for the adverse event.

Internal Events: Events experienced by a research participant enrolled at a site under the jurisdiction of St. Jude IRB for either multicenter or single-center research projects.

External Events: Events experienced by participants enrolled at a site external to the jurisdiction of the St. Jude Institutional Review Board (IRB) or in a study for which St. Jude is not the coordinating center or the IRB of record.

Unanticipated Problem Involving Risks to Subjects or Others: An unanticipated problem involving risks to subjects or others is an event which was not expected to occur and which increases the degree of risk posed to research participants. Such events, in general, meet all of the following criteria:

- unexpected;
- related or possibly related to participation in the research; and
- suggests that the research places subjects or others at a greater risk of harm (including physical, psychological, economic, or social harm) than was previously known or recognized. An unanticipated problem involving risk to subjects or others may exist even when actual harm does not occur to any participant.

Consistent with FDA and OHRP guidance on reporting unanticipated problems and adverse events to IRBs, the St. Jude IRB does not require the submission of external events, for example IND safety reports, nor is a summary of such events/reports required; however, if an event giving rise to an IND safety or other external event report constitutes an "unanticipated problem involving risks to subjects or others" it must be reported in accordance with this policy. In general, to be reportable external events need to have implications for the conduct of the study (for example, requiring a significant and usually safety-related change in the protocol and/or informed consent form).

Although some adverse events will qualify as unanticipated problems involving risks to subjects or others, some will not; and there may be other unanticipated problems that go beyond the definitions of serious and/or unexpected adverse events. Examples of unanticipated problems involving risks to subjects or others include:

- Improperly staging a participant's tumor resulting in the participant being assigned to an incorrect arm of the research study;
- The theft of a research computer containing confidential subject information (breach of confidentiality); and
- The contamination of a study drug. Unanticipated problems generally will warrant consideration of substantive changes in the research protocol or informed

consent process/document or other corrective actions in order to protect the safety, welfare, or rights of subjects or others

13.2 Recording AEs and SAEs

Adverse events (AEs) will be evaluated and documented by the clinical staff and investigators throughout inpatient hospitalizations and each outpatient visit. CRAs are responsible for reviewing documentation related to AEs and entering directly into CRIS protocol-specific database. The data to be recorded are 1) the event description, 2) the NCI CTCAE v4.0 code and grade, 3) the onset date, 4) the resolution date (or ongoing), 4) action taken for event, 5) participant outcome 6) relationship of AE to protocol treatment/interventions, 7) if AE was expected or unexpected, and 8) comments, if applicable. AEs that are classified as serious, unexpected, and at least possibly related will be notated as such in the database as "SAEs". These events will be reported expeditiously to the St. Jude IRB within the timeframes as described above. Cumulative summary of Grade 3-5 events will be reported as part of the progress reports to IRB at the time of continuing review. Specific data entry instructions for AEs and other protocol-related data will be documented in protocol-specific data entry guidelines, which will be developed and maintained by study team and clinical research informatics.

Additionally, we will capture the use of any concurrent ototoxic medications administered in this population, which could have an additive ototoxic effect. These medications include: ethacrynic acid, bumetanide, vancomycin, gentamicin, tobracycin, kanamycin, and furosemide.

The study team will meet regularly to discuss AEs (and other study progress as required by institutional DSMP). The PI will review Adverse Event reports generated from the research database, and corrections will be made if applicable. Once the information is final the PI will sign and date reports, to acknowledge his/her review and approval of the AE as entered in the research database.

14.0 DATA COLLECTION, MONITORING AND CONFIDENTIALITY

14.1 Data Collection

Electronic case report forms (eCRFs) will be completed by the St. Jude Solid Tumor CRAs. Data will be entered from record directly into a secure protocol-specific CRIS database, developed and maintained by St. Jude Clinical Research Informatics.

Data Management will be supervised by the Director of Clinical Trials Management, and Manager of Clinical Research Operations for the Solid Tumor Division, working with Dr. Brennan and her designee(s). All protocol-specific data and all grade 3-5 adverse events will be recorded by the clinical research associates into the CRIS database, ideally within 2-4 weeks of completion of study phase. All questions will be directed to the attending physician and/or PI and reviewed at regularly-scheduled working meetings. The attending physicians (or their designees) are responsible for keeping up-to-date roadmaps in the participant's primary St. Jude medical chart.

Regular (at least monthly) summaries of toxicity and protocol events will be generated for the PI and the department of Biostatistics to review.

14.2 Study Monitoring

This study is considered moderate risk for monitoring purposes. Protocol and regulatory compliance, including essential regulatory documentation, will be assessed as well as the accuracy and completeness of data points related to the primary study objective semi-annually. If the study design has strata, accrual will be tracked continuously. The first two enrollees and then 10 % of participants will be monitored semi-annually.

The PI and study team are responsible for protocol and regulatory compliance, and for data accuracy and completeness. The study team will meet at appropriate intervals to review case histories or quality summaries on participants and retain copies of the minutes which are signed by the PI.

The Eligibility Coordinators in the Central Protocol and Data Monitoring Office (CPDMO) will verify informed consent documentation of and eligibility status for 50% of the St. Jude participants within 5 working days of enrollment completion.
During semi-annual routine monitoring events, the Monitor will verify informed consent documentation and eligibility status for 50% of non-St. Jude participants as well as perform consent and eligibility verification on selected St. Jude enrollments. Overall study conduct, compliance with primary objectives, age of majority consenting, safety assessments and reporting, and the timeliness and accuracy of database entries are monitored routinely.

Study documents routinely monitored on selected participants include medical records, database entries, study worksheets, and case report forms. Study documents are monitored for participant status, demographics, staging, subgroup assignment, treatments, investigational drug accountability, evaluations, responses, participant protocol status, off-study and off-therapy criteria, and for all other specifics as detailed in a separate study-specific monitoring plan. The study-specific monitoring plan may be revised over time, to adapt monitoring frequency and/ or intensity to a changing environment when appropriate (for example: new safety signals; positive history of compliance; all participants are in long term follow-up; or the enrollment period has ended).

The recording and reporting of Adverse Events, Serious Adverse Events (SAEs), and Unanticipated Problems (UPs) to include type, grade, attribution, duration, timeliness and appropriateness will be reviewed by the Monitor/ CRM. The CRM will generate a formal report which is shared with the Principal Investigator (PI), study team and the Internal Monitoring Committee (IMC).

Continuing reviews by the Internal Review Board (IRB) and Scientific Review Committee (CT-SRC) will occur at least annually. In addition, unanticipated problems are reviewed in a timely manner by the IRB.

St. Jude affiliates and domestic collaborating study sites will be monitored on-site by a representative of St. Jude as needed. International collaborators will be monitored by a

Contract Research Organization (CRO), or other mechanism according to the study specific monitoring plan.

14.3 Confidentiality

Study numbers will be used in place of an identifier such as a medical record number. No research participant names will be recorded on the data collection forms. The list containing the study number and the medical record number will be maintained in a locked file and will be destroyed after all data have been analyzed. The medical records of study participants may be reviewed by the St. Jude IRB, FDA, and St. Jude clinical research monitors.

15.0 STATISTICAL CONSIDERATIONS

15.1 Primary Objective

15.1.1 To evaluate the response (CR + PR) rate of bilateral disease patients who have at least one eye with advanced intraocular retinoblastoma (stratum B patients) to two upfront courses of therapy consisting of subconjunctival carboplatin and systemic topotecan.

Investigators: Rachel Brennan, MD; Matthew Wilson, MD, FACS; Ibrahim Qaddoumi, MD; Barrett Haik, MD, FACS; Michael Bishop, MD; Thomas Merchant, DO, PhD; Atmaram Pai-Panankiker, MD; Jesse Jenkins, MD, Brent Orr, MD; Tracy Douglas, NP, Gena Borgman, FNP; Alison Young, FNP.
Biostatisticians: April Sykes, MPH; Natasha A Sahr, PhD

The primary objective is to evaluate the response rate of stratum B patients who had significant subretinal or vitreal seeding and received two upfront courses of therapy consisting of subconjunctival carboplatin and systemic topotecan. Stratum B patients who had no significant subtretinal seeding and received vincristine and topotecan are not evaluable for this primary objective. From previous St Jude RET5 study, a total 24 of 27 (89% with a 95%CI: 71%-97%) stratum B patients had response (CR+PR) and showed early efficacy of the two initial courses of vincristine and topotecan therapy.

The hypothesis for this trial is "the true response rate is not worse than 75% vs. 93%. The subconjunctival carboplatin and systemic topotecan treatment will be considered not worthy of continued investigation if the response rate is equal or less than 75% and worthy of further investigation if the response rate equal or exceeds 93%. Response will be assessed per patient (rather than per eye) using the worst response of two eyes. The evaluation of CR/PR status would be made only after the patient remains in CR/PR for at least 4 weeks. A total of 29 stratum B patients with seeding will be needed to detect 18% increase of response rate with 80% power and 5% type I error (East 5.4). To stop accrual early due to futility of the new treatment, the study is to be monitored by a two-stage group sequential approach with one interim analysis following evaluation of the first group of 15 patients (Table 15.1). If 11 or fewer of these patients have response (CR+PR) to two upfront courses of therapy, then consideration will be given to closing the study to accrual. If 12 or more of first stage patients have response to two upfront courses of therapy, then patients will continue until 29 patients have been treated. If 25 or fewer of the 29 patients responded, we will conclude that the response is unlikely great than 75%. The 95% confidence interval after adjusted two-stage nature will be reported for the final data analysis.

In the analysis of primary objective, patients with disease progression prior to the two upfront courses of therapy evaluation will be considered as non-responders.

For the purpose of convenience of the trial management, the study will keep enrolling patients after first 15 evaluable enrolled on the study. After response data is available on all 15 patients, the interim analysis will be done, and the trial will close or remain open based on the results of this analysis.

Table 15.1: Two-stage group sequential design (patients on Stratum B w/seeding)

Group	Number of patients enroll on each stage	Total # of patients enrolled at the end of each stage	Reject new treatment if #of response is less or equal to:
1	15	15	11
2	14	29	25

15.1.2 Accrual

This study will enroll 29 stratum B patients with seeding. Based on accrual information from previous RET5 study (Table 15.2), a total of 27 stratum B patients were enrolled on the study in approximately 6 years. Therefore approximately 4-5 stratum B participants can be enrolled on this study each year. The PI has emphasized that we can accrue at least 5-6 stratum B patients per year based on current accrual rate. Hence the study will open about 5-6 years to achieve the target 29 stratum B patients. We estimate that approximately 20 stratum A patients and 50 stratum C and D patients will be enrolled on SJRET6 study in 5-6 years.

Table 15.2: Accrual for Eligible RET5 Participants

	Stratum			**Total**
	A	**B**	**C**	**N**
Time Period				
Year 1 (02/04/2005 - 02/03/2006)	3	2	12	17
Year 2 (02/04/2006 - 02/03/2007)	2	5	7	14
Year 3 (02/04/2007 - 02/03/2008)	1	6	8	15
Year 4 (02/04/2008 - 02/03/2009)	5	6	14	25
Year 5 (02/04/2009 - 02/03/2010)	7	4	8	19
Partial Year 6 (02/04/2010 - 11/11/2010)	5	4	6	15
Total	**23**	**27**	**55**	**105**

Based on accrual rates since start of SJRET6 (Table 15.3), the accrual rate was overestimated by the previous RET5 study (Table 15.2). Based on accrual information from the current RET6 study (Table 15.3), a median of 2.5 patients (range: 0-9 patients) were enrolled per year for stratum A, a median of 4.5 patients (range: 2-8 patients) were enrolled per year for stratum B, a median of 12 patients (range: 4-15 patients) were enrolled per year for stratum C, and a median of 2 patients (range: 1-3 patients) were enrolled per year for stratum D.

Table 15.3: Accrual for RET6 Participants

	Stratum				Total
	A	B	C	D	
Time period					
Year 1: (06/19/2013 - 06/18/2014)	3	5	15	2	25
Year 2: (06/19/2014 - 06/18/2015)	2	2	12	2	18
Year 3: (06/19/2015 - 06/18/2016)	9	8	12	1	30
Year 4: (06/19/2016 - 06/18/2017)	6	4	12	2	24
Year 5: (06/19/2017 - 06/18/2018)	0	6	4	2	12
Partial Year 6: (06/19/2018 - 05/09/2019)	2	2	10	3	17
Total	22	27	65	12	126

Stratum B enrollment included patients both eligible (with significant subretinal or vitreal seeding and received two upfront courses of therapy) and ineligible (no significant subtretinal seeding and received vincristine and topotecan) for evaluation of the primary outcome (Table 15.4). Based on accrual information from the current RET6 study, a median of 4 patients (range: 0-5 patients) eligible for primary outcome evaluation were enrolled on stratum B per year.

Table 15.4: Accrual for RET6 Stratum B Participants by primary outcome eligibility

	Stratum		Total
	B, with subretinal or vitreal seeding	B, without subretinal or vitreal seeding	
Enrollment period			
Year 1: (06/19/2013 - 06/18/2014)	5	0	5
Year 2: (06/19/2014 - 06/18/2015)	2	0	2
Year 3: (06/19/2015 - 06/18/2016)	5	3	8
Year 4: (06/19/2016 - 06/18/2017)	3	1	4
Year 5: (06/19/2017 - 06/18/2018)	5	1	6
Year 6: (06/19/2018 - 05/09/2019)	0	2	2
Total	20	7	27

The rates for stratum B suggest that it is reasonable to expect 3-5 primary outcome eligible stratum B participants enrolled per year on this study. Therefore, the study should be open 6-8 years to achieve the target 29 stratum B patients. Since current enrollment of eligible stratum B as of 05/09/2019 is at 20 patients, it is expected to take 1.5-2 years beyond original estimate to enroll the remaining 9 patients in stratum B of this study. At this time, we estimate that we will enroll approximately 25 patients in stratum A, 77 patients in stratum C, and 14 patients in stratum D for this study.

As of October of 2019 we have enrolled 136 total participants, with 3 primary outcome eligible stratum B participants remaining to accrue. We therefore will increase overall accrual to 200 participants in order to meet the primary objective of 29 total stratum B patients with seeding.

15.2 Secondary Objectives

Investigators: Rachel Brennan, MD; Matthew Wilson, MD, FACS; Ibrahim Qaddoumi, MD; Barrett Haik, MD, FACS; Michael Dyer, PhD; Michael Bishop; Gena Borgman, FNP; Alison Young, FNP; Thomas Merchant, DO, PhD; Atmaram Pai-Panankiker, MD; Jesse Jenkins, MD, Brent Orr, MD; Tracy Douglas, NP.
Biostatistician: April Sykes, MPH; Natasha A Sahr, PhD; Guolian Kang, PhD

15.2.1 To evaluate the ocular survival of eyes and event-free survival of participants by strata

Ocular survival and event-free survival of both eyes and participants will be estimated and compared to that for similar retinoblastoma participants treated on the RET5 protocol by strata. Ocular survival per eye will be defined as the time interval from study enrollment to date of enucleation or to date of last contact for eyes that have not been enucleated. Event-free survival per eye will be defined as the time interval from date on study to date of first event (where an event includes external beam radiation or enucleation) or to the date of last contact for eyes without events. When estimating ocular and event-free survival per participant, the analysis will focus on time to first event for participants with two involved eyes. For participants with two early or two advanced stage eyes, the time from study enrollment to the date of the first enucleation (or the date of the first event) will be used in the analysis. If neither eye has been enucleated (ocular survival) or has had an event (EFS), then the participant will be censored at the date of last contact. Ocular survival and event-free survival will be estimated using the method of Kaplan and Meier and compared using the log-rank test.

15.2.2 To prospectively analyze intraocular disease tissue for participants with at least one eye undergoing enucleation in order to identify the mechanism of RB1 biallelic inactivation. Participants may undergo upfront enucleation (due to advanced disease at diagnosis) or may receive enucleation due to progressive disease during protocol therapy

One objective of this study is to improve our understanding of the biology and tumorigenesis of retinoblastoma when biology specimens are available. All participants with non-metastatic retinoblastoma will be enrolled on the study. Participants will be stratified into four main groups (stratum A-D, see section 4.1), depending on laterality and disease stage. This objective is designed for the participants who require enucleation at any time during therapy.

The tumor tissue samples will be obtained from participant who has at least one eye undergoing enucleation. The mechanism (or frequencies) for each RB1 biallelic inactivation in intraocular disease tissue will be summarized by descriptive statistics. Based on accrual information from previous RET5 study (Table 15.2), a total of 55 participants who have upfront enucleation (stratum C) were enrolled on the RET5 study in 6 years. Therefore, approximately 10 stratum C or D participants who will contribute the tumor tissue sample will be enrolled each year and a total of 50-60 tumor tissue samples will be obtained during 6-7 years.

15.3 Exploratory Biologic Objectives:

Investigators: Rachel Brennan, MD; Matthew Wilson, MD, FACS; Ibrahim Qaddoumi, MD; Barrett Haik, MD, FACS; Jesse Jenkins, MD, Brent Orr, MD; Michael Dyer, PhD. Biostatistician: Guolian Kang, PhD

15.3.1 To identify genetic lesions in intraocular disease tissue by using single nucleotide polymorphism (SNP) arrays for disease tissue and blood samples.

The SNP array contains very dense SNP markers and could be used to identify relatively small genetic lesions, such as amplifications/deletions of certain genomic regions.

Copy number variations (CNV) will be assessed by the reference alignment procedure in Pounds *et al*. which may have better concordance with cytogenetics than other procedures and is undergoing further improvement[124]. We anticipate methodological innovations to CNV analysis in the near future and the final analysis will employ the best methods at that time.

15.3.2 Evaluate the relationship between retinoblastoma genetic and epigenetic alterations, histopathologic classification and clinical disease behavior.

General or generalized linear models will be employed to evaluate the relationships specified in this aim. The response variable can be the histopathologic classification or clinical disease behavior, and the explanatory variables can be the genetic and epigenetic alterations. The significant genetic/epigenetic effects can be identified by testing if their coefficients are significantly different from zero. Additionally, different genetic models can also be explored to assess which one fits the data better. For example, suppose we represent the three genotypes of one SNP as AA, Aa and aa, the three genotype categories can be coded as 0, 1, and 2 for the additive scale G_1, as 0, 1, and 1 for the dominance scale G_2, and as 1, 0, 0 for the recessive scale G_3, corresponding to genotypes AA, Aa and aa, respectively. Testing the coefficients for G_1, G_2 and G_3 can be used to select the best genetic model.

15.3.3 Construct tissue microarrays that will serve as a high-throughput tool for future analysis of molecular genetic alterations in retinoblastoma.

No statistical analysis required.

15.3.4 Analysis of the expression of miR-191 in intraocular disease tissue and genotyping of MDM4 SNP 34091 in germline and intraocular disease tissue to identify and correlate miR expression, SNP and clinical features.

General or generalized linear models will be employed to evaluate the relationships specified in this aim. The response variable can be a binary variable of germline versus disease or miR expression and the explanatory variable is the genotype of MDM4 SNP 34091. The significant effect of MDM4 SNP 34091 can be identified by testing if the coefficient is significantly different from zero. Additionally, different genetic models can

also be explored to assess which one fits the data better similar to 15.3.2. The relationship between clinical features and SNP and miR expression will be descriptively analyzed.

15.3.5 Analysis of the vitreous proteome, including PDGF expression, present in vitreous samples from patients with intraocular retinoblastoma.

General models will be employed to evaluate this aim; final analysis will employ the best methods at that time.

15.4 Exploratory Therapeutic Objectives

Investigator: Rachel Brennan, MD; Matthew Wilson, MD, FACS; Ibrahim Qaddoumi, MD; Barrett Haik, MD, FACS; Michael Bishop, MD; Gena Borgman, FNP; Alison Young, FNP; Thomas Merchant, DO, PhD; Atmaram Pai-Panankiker, MD; Tracy Douglas, NP. Biostatistician: April Sykes, MPH; Natasha A Sahr, PhD

15.4.1 To study the association between the outcome of intraocular retinoblastoma and the International Classification for Intraocular Retinoblastoma and the AJCC Classification.

Cox regression model will be used to study the association between outcome (ocular and event-free survival) and disease stage according to the International Classification and the AJCC stage group.

15.4.2 To evaluate the ocular survival of eyes, event-free survival of participants and incidence of ototoxicity in participants less than 6 months old who have early stage intraocular retinoblastoma (Stratum A) and are treated with topotecan in place of platinum therapy for 3 of 6 cycles.

Ocular survival of eyes and event-free survival of participants less than 6 months old who have early stage intraocular retinoblastoma (stratum A) and are treated with topotecan in place of platinum therapy for 3 of 6 cycles will be estimated using the method of Kaplan and Meier. Ototoxicity (or hearing loss) data from this subgroup patients will be summarized from longitudinal audiology testing data. Incidence of ototoxicity in this subgroup of participants will be estimated together with a 95% confidence interval.

15.4.3 To evaluate tumor response (tumor regression and/or calcification by RET-Cam imaging) to topotecan-containing therapy (Stratum B) and focal treatments (stratum A, B or D).

The response rate to topotecan-containing therapy (stratum B) throughout therapy will be estimated together with an exact 95% confidence. The success rate off focal treatments (stratum A, B or D) will also be estimated together with an exact 95% confidence interval.

15.4.4 To assess the local control and pattern of failure in patients treated with external beam radiotherapy and 15.4.5 To assess the local control and pattern of failure in patients treated with brachytherapy.

The success of local control will be assessed by loco-regional failure rate. Loco-regional failure is defined as the time interval from date of start of external beam radiotherapy or brachytherapy to date of loco-regional failure. Distant failure or death prior to loco-regional failure will be considered competing events in the analyses. The cumulative incidence of loco-regional failure will be estimated using methods described in Kalb, Fleisch and Prentice. Loco-regional failures will be further categorized as infield, marginal or adjacent failures. The percentage of patients with each failure type will be reported along with an exact 95% confidence interval.

15.4.5 To observe and describe the toxicity, both acute and long term, associated with subconjunctival carboplatin therapy.

All toxicity will be monitored during the study, and any adverse event will be recorded. Any unexpected AEs are reportable to the IRB. The toxicity associated with subconjunctival carboplatin therapy will be specifically targeted for those: 1) optic nerve atrophy, 2) Periorbital swelling and inflammation and/or infection, 3) Fat atrophy 4) Conjunctival fibrosis resulting in restricted mobility of globe and impairing EUAs.

15.5 Exploratory Supportive Care Objectives

Vision
Investigators: Angela Eftink, MCD, CCC-SLP; Jessica Sweeney Sparrow, OTD, OTR/L.
Biostatistician: April Sykes, MPH; Natasha A Sahr, PhD

Audiology
Investigators: Johnnie Bass, AuD, CCC-A; Shaum Bhagat, PhD
Biostatistician: April Sykes, MPH; Natasha A Sahr, PhD

15.5.1 To determine the need for and the types of early intervention (EI) and low vision (LV) services received by children with RB

Types of early intervention (EI) include Occupational therapy, speech therapy, low vision service, physical therapy, etc. Number and percentage of children who are recommended to each service will be summarized descriptively. Number of services received will also be summarized descriptively.

15.5.2 To describe the visual, developmental, participation, sensory processing, language and quality of life characteristics of children referred for EI and/or LV services.

Data collected from the visual, developmental, participation, sensory processing, language and quality of life characteristics of children for EI and/or LV services will be summarized by mean and standard deviation or median and range.

15.5.3 To describe parent perceived efficacy of low vision assistive devices among those who received assistive devices

Data collected from parent perceived efficacy of low vision assistive devices among those who received assistive devices will be descriptively, such as mean and standard deviation or median and range.

15.5.4 Describe the results of wideband reflectance and tympanometry testing in patients receiving carboplatin therapy, to describe the impact of carboplatin on changes in cochlear function that are detectable with measurement of otoacoustic emissions and to describe effective methods of monitoring patients treated with carboplatin for sub-clinical changes in cochlear function

Transient-evoked otoacoustic emission (TEOAE) tests will be performed before and after several courses of carboplatin therapy. The change in cochlear function will be tested to see the potential onset of carboplatin ototoxicity. A signal processing technique, the wavelet transform (WT), will be used to analyze TEOAE waveforms in narrow-band frequency components to detect significant changes in TEOAE energy, latency, and normalized energy during the treatment.

15.6 Exploratory Pharmacology Objective

Investigators/Biostatisticians: Clinton Stewart, Pharm D, Jun Yang, PhD/ April Sykes, MPH; Natasha A Sahr, PhD

15.6.1 To assess the relationship of selected candidate genetic variants with ototoxicity in participants receiving carboplatin.

Selected candidate genetic variants will be studied the association with carboplatin-related hearing damage (hearing loss) to carboplatin related therapy. The participants' hearing damage will be assessed based on longitudinal audiology exams using both CTCAE scores and Chang scores during the treatment. The audiology exams scores will be dichotomized and Logistic regression model will be used to assess the association between selected candidate genetic variants and ototoxicity in participants receiving carboplatin.

16.0 OBTAINING INFORMED CONSENT

16.1 Consent/Assent at Enrollment

The process of informed consent for SJRET6 will follow institutional policy. The informed consent process is an ongoing one that begins at the time of diagnosis and ends after the completion of therapy. Informed consent should be obtained by the attending physician or his/her designee, in the presence of at least one non-physician witness. Initially, informed consent will be sought for the institutional banking protocol (research study), blood transfusion and other procedures as necessary. After the diagnosis of retinoblastoma is established, we will invite the participant to participate in the SJRET6 protocol.

Throughout the entire treatment period, participants and their parents receive constant education from health professionals at SJCRH, and are encouraged to ask questions

regarding alternatives and therapy. All families have ready access to chaplains, psychologists, social workers, and the St. Jude ombudsperson for support, in addition to that provided by the primary physician and other clinicians involved in their care.

We will also obtain verbal assent from children 7 to 14 years old and written assent for all participants ≥14 years of age. Participants who reach the age of majority while on study will be re-consented for continued participation on SJRET6 according to Cancer Center and institutional policy. Furthermore, before acceptance of participants for this protocol, the investigator(s) will explain the following to the participant and/or parents:

1. That treatment with cyto-reductive therapy and aggressive focal treatments may avoid or delay radiation therapy and enucleation.
2. That for participants with advanced intraocular disease, the chances of saving the eye are less than 50-60%, and that aggressive chemotherapy with vincristine, carboplatin and etoposide is considered to be an alternative therapy, and that radiation therapy is very frequently required. Intra-arterial chemotherapy is an emerging procedure that is currently used by some institutions, but this procedure has many potential complications and lacks preclinical or rigorous clinical evidence for safety at this time and is not considered standard of care. Participants with advanced disease are also at risk of developing metastatic disease since this procedure does not provide concurrent systemic therapy.
3. For participants with less advanced intraocular disease, the use of only two drugs (vincristine and carboplatin) provides similar results to the three-drug regimens.
4. That the use of vincristine and topotecan in our previous protocol (RET5) was effective and serves as the base for SJRET6. Also, topotecan has shown a significant activity against solid tumors in children in the previous studies performed in this institution and elsewhere, in particular in neuroblastoma, a disease with a similar spectrum of chemo-sensitivity to retinoblastoma.
5. That previous studies performed *in vitro* (in retinoblastoma cells) and *in vivo* (in animals) show that topotecan and the combination of vincristine and topotecan is very effective against retinoblastoma.
6. That certain side effects have been encountered, which include myelosuppression, nausea, vomiting, alopecia, skin rash and diarrhea.
7. That blood samples will be obtained over a period of time after the first dose of topotecan to adjust subsequent doses.
8. That periocular carboplatin will be administered to eyes with advanced disease.
9. That we would like to perform a mutational analysis of the retinoblastoma gene.
10. That if the participant's disease is advanced and the eye must be removed, researchers will obtain fresh tumor and a small sample of the vitreous (jelly of the eye) after enucleation in order to attempt to grow the cells from tumor tissue or blood in the laboratory or in laboratory animals. Those growing cells can then be studied in the laboratory.
11. That we would like to investigate the need for early intervention and the feasibility to obtain the services at the home community, as well as describe the characteristics of children with retinoblastoma who are referred for these services as it relates development, participation, and quality of life.

16.2 Consent When English is Not the Primary Language

When English is not the participant, parent, or legally authorized representative's primary language, the Social Work department will determine the need for an interpreter. This information documented in the participant's medical record. Either a certified interpreter or the telephone interpreter's service will be used to translate the consent information. The process for obtaining an interpreter and for the appropriate use of an interpreter is outlined on the Interpreter Services, OHSP, and CPDMO websites.

17.0 REFERENCES

1. Gurney JG, Severson RK, Davis S, et al: Incidence of cancer in children in the United States. Sex-, race-, and 1-year age-specific rates by histologic type. Cancer 75:2186-95, 1995
2. De La Rosa-Velazquez IA, Rincon-Arano H, Benitez-Bribiesca L, et al: Epigenetic regulation of the human retinoblastoma tumor suppressor gene promoter by CTCF. Cancer Res 67:2577-85, 2007
3. Tosi GM, Trimarchi C, Macaluso M, et al: Genetic and epigenetic alterations of RB2/p130 tumor suppressor gene in human sporadic retinoblastoma: implications for pathogenesis and therapeutic approach. Oncogene 24:5827-36, 2005
4. Lin W, Cao J, Liu J, et al: Loss of the retinoblastoma binding protein 2 (RBP2) histone demethylase suppresses tumorigenesis in mice lacking Rb1 or Men1. Proc Natl Acad Sci U S A 108:13379-86, 2011
5. Greger V, Passarge E, Hopping W, et al: Epigenetic changes may contribute to the formation and spontaneous regression of retinoblastoma. Hum Genet 83:155-8, 1989
6. Knudson AG, Jr.: Mutation and cancer: statistical study of retinoblastoma. Proc Natl Acad Sci U S A 68:820-3, 1971
7. Lee WH, Bookstein R, Hong F, et al: Human retinoblastoma susceptibility gene: cloning, identification, and sequence. Science 235:1394-9, 1987
8. Friend SH, Bernards R, Rogelj S, et al: A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. Nature 323:643-6, 1986
9. Brantley MA, Jr., Harbour JW: The molecular biology of retinoblastoma. Ocul Immunol Inflamm 9:1-8, 2001
10. Dryja TP, Mukai S, Petersen R, et al: Parental origin of mutations of the retinoblastoma gene. Nature 339:556-8, 1989
11. Zhu X, Dunn JM, Goddard AD, et al: Mechanisms of loss of heterozygosity in retinoblastoma. Cytogenet Cell Genet 59:248-52, 1992
12. Weinberg RA: Tumor suppressor genes. Science 254:1138-46, 1991
13. Karcioglu ZA, al-Mesfer SA, Abboud E, et al: Workup for metastatic retinoblastoma. A review of 261 patients. Ophthalmology 104:307-12, 1997
14. Reese AB, Ellsworth RM: The evaluation and current concept of retinoblastoma therapy. Trans Am Acad Ophthalmol Otolaryngol 67:164-72, 1963
15. Shields CL, Mashayekhi A, Au AK, et al: The International Classification of Retinoblastoma Predicts Chemoreduction Success. Ophthalmology 113:2276-2280, 2006
16. Shields CL, Shields JA: Basic understanding of current classification and management of retinoblastoma. Curr Opin Ophthalmol 17:228-34, 2006
17. Murphree AL: Intraocular retinoblastoma: The case for a new group classification. Ophthalmology Clinics of North America 18:41-53, 2005
18. Pratt CB, Fontanesi J, Lu X, et al: Proposal for a New Staging Scheme for Intraocular and Extraocular Retinoblastoma Based on an Analysis of 103 Globes. Oncologist 2:1-5, 1997
19. Shields JA, Shields CL, De Potter P: Enucleation technique for children with retinoblastoma. J Pediatr Ophthalmol Strabismus 29:213-5, 1992
20. Shields JA, Shields CL, De Potter P: Photocoagulation of retinoblastoma. Int Ophthalmol Clin 33:95-9, 1993
21. Shields JA, Parsons H, Shields CL, et al: The role of cryotherapy in the management of retinoblastoma. Am J Ophthalmol 108:260-4, 1989
22. Shields CL, Santos MC, Diniz W, et al: Thermotherapy for retinoblastoma. Arch Ophthalmol 117:885-93, 1999

23. Murphree AL, Villablanca JG, Deegan WF, 3rd, et al: Chemotherapy plus local treatment in the management of intraocular retinoblastoma. Arch Ophthalmol 114:1348-56, 1996
24. Lumbroso L, Doz F, Urbieta M, et al: Chemothermotherapy in the management of retinoblastoma. Ophthalmology 109:1130-6, 2002
25. Wilson TW, Chan HS, Moselhy GM, et al: Penetration of chemotherapy into vitreous is increased by cryotherapy and cyclosporine in rabbits. Arch Ophthalmol 114:1390-5, 1996
26. Murray TG, Cicciarelli N, O'Brien JM, et al: Subconjunctival carboplatin therapy and cryotherapy in the treatment of transgenic murine retinoblastoma. Arch Ophthalmol 115:1286-90, 1997
27. Abramson DH, Lawrence SD, Beaverson KL, et al: Systemic carboplatin for retinoblastoma: change in tumour size over time. Br J Ophthalmol 89:1616-9, 2005
28. Shields CL, De Potter P, Himelstein BP, et al: Chemoreduction in the initial management of intraocular retinoblastoma. Arch Ophthalmol 114:1330-8, 1996
29. Demirci H, Eagle RC, Jr., Shields CL, et al: Histopathologic findings in eyes with retinoblastoma treated only with chemoreduction. Arch Ophthalmol 121:1125-31, 2003
30. Sussman DA, Escalona-Benz E, Benz MS, et al: Comparison of retinoblastoma reduction for chemotherapy vs external beam radiotherapy. Arch Ophthalmol 121:979-84, 2003
31. Scott IU, Murray TG, Feuer WJ, et al: External beam radiotherapy in retinoblastoma: tumor control and comparison of 2 techniques. Arch Ophthalmol 117:766-70, 1999
32. Hungerford JL, Toma NM, Plowman PN, et al: External beam radiotherapy for retinoblastoma: I. Whole eye technique. Br J Ophthalmol 79:109-11, 1995
33. Toma NM, Hungerford JL, Plowman PN, et al: External beam radiotherapy for retinoblastoma: II. Lens sparing technique. Br J Ophthalmol 79:112-7, 1995
34. Merchant TE, Gould CJ, Hilton NE, et al: Ocular preservation after 36 Gy external beam radiation therapy for retinoblastoma. J Pediatr Hematol Oncol 24:246-9, 2002
35. Shields CL, Shields JA, Cater J, et al: Plaque radiotherapy for retinoblastoma: long-term tumor control and treatment complications in 208 tumors. Ophthalmology 108:2116-21, 2001
36. Schouten-Van Meeteren AY, Moll AC, Imhof SM, et al: Overview: chemotherapy for retinoblastoma: an expanding area of clinical research. Med Pediatr Oncol 38:428-38, 2002
37. Yue NC, Benson ML: The hourglass facial deformity as a consequence of orbital irradiation for bilateral retinoblastoma. Pediatr Radiol 26:421-3, 1996
38. Kaste SC, Chen G, Fontanesi J, et al: Orbital development in long-term survivors of retinoblastoma. J Clin Oncol 15:1183-9, 1997
39. Mohney BG, Robertson DM, Schomberg PJ, et al: Second nonocular tumors in survivors of heritable retinoblastoma and prior radiation therapy. Am J Ophthalmol 126:269-77, 1998
40. Wong FL, Boice JD, Jr., Abramson DH, et al: Cancer incidence after retinoblastoma. Radiation dose and sarcoma risk. JAMA 278:1262-7, 1997
41. Eng C, Li FP, Abramson DH, et al: Mortality from second tumors among long-term survivors of retinoblastoma. J Natl Cancer Inst 85:1121-8, 1993
42. Abramson DH, Frank CM: Second nonocular tumors in survivors of bilateral retinoblastoma: a possible age effect on radiation-related risk. Ophthalmology 105:573-9; discussion 579-80, 1998
43. Namouni F, Doz F, Tanguy ML, et al: High-dose chemotherapy with carboplatin, etoposide and cyclophosphamide followed by a haematopoietic stem cell rescue in patients with high-risk retinoblastoma: a SFOP and SFGM study. Eur J Cancer 33:2368-75, 1997
44. Dunkel IJ, Aledo A, Kernan NA, et al: Successful treatment of metastatic retinoblastoma. Cancer 89:2117-21, 2000

45. Doz F, Khelfaoui F, Mosseri V, et al: The role of chemotherapy in orbital involvement of retinoblastoma. The experience of a single institution with 33 patients. Cancer 74:722-32, 1994
46. Uusitalo MS, Van Quill KR, Scott IU, et al: Evaluation of chemoprophylaxis in patients with unilateral retinoblastoma with high-risk features on histopathologic examination. Arch Ophthalmol 119:41-8, 2001
47. Khelfaoui F, Validire P, Auperin A, et al: Histopathologic risk factors in retinoblastoma: a retrospective study of 172 patients treated in a single institution. Cancer 77:1206-13, 1996
48. Chantada GL, de Davila MT, Fandino A, et al: Retinoblastoma with low risk for extraocular relapse. Ophthalmic Genet 20:133-40, 1999
49. Shields CL, Shields JA, Baez KA, et al: Choroidal invasion of retinoblastoma: metastatic potential and clinical risk factors. Br J Ophthalmol 77:544-8, 1993
50. Honavar SG, Singh AD, Shields CL, et al: Postenucleation adjuvant therapy in high-risk retinoblastoma. Arch Ophthalmol 120:923-31, 2002
51. Mustafa MM, Jamshed A, Khafaga Y, et al: Adjuvant chemotherapy with vincristine, doxorubicin, and cyclophosphamide in the treatment of postenucleation high risk retinoblastoma. J Pediatr Hematol Oncol 21:364-9, 1999
52. Shields CL, Honavar SG, Meadows AT, et al: Chemoreduction plus focal therapy for retinoblastoma: factors predictive of need for treatment with external beam radiotherapy or enucleation. Am J Ophthalmol 133:657-64, 2002
53. Kingston JE, Hungerford JL, Madreperla SA, et al: Results of combined chemotherapy and radiotherapy for advanced intraocular retinoblastoma. Arch Ophthalmol 114:1339-43, 1996
54. Beck MN, Balmer A, Dessing C, et al: First-line chemotherapy with local treatment can prevent external-beam irradiation and enucleation in low-stage intraocular retinoblastoma. J Clin Oncol 18:2881-7, 2000
55. Gallie BL, Budning A, DeBoer G, et al: Chemotherapy with focal therapy can cure intraocular retinoblastoma without radiotherapy. Arch Ophthalmol 114:1321-8, 1996
56. Wilson MW, Rodriguez-Galindo C, Haik BG, et al: Multiagent chemotherapy as neoadjuvant treatment for multifocal intraocular retinoblastoma. Ophthalmology 108:2106-14; discussion 2114-5, 2001
57. Friedman DL, Himelstein B, Shields CL, et al: Chemoreduction and local ophthalmic therapy for intraocular retinoblastoma. J Clin Oncol 18:12-7, 2000
58. Abramson DH, Frank CM, Chantada GL, et al: Intraocular carboplatin concentrations following intravenous administration for human intraocular retinoblastoma. Ophthalmic Genet 20:31-6, 1999
59. Mendelsohn ME, Abramson DH, Madden T, et al: Intraocular concentrations of chemotherapeutic agents after systemic or local administration. Arch Ophthalmol 116:1209-12, 1998
60. Abramson DH, Frank CM, Dunkel IJ: A phase I/II study of subconjunctival carboplatin for intraocular retinoblastoma. Ophthalmology 106:1947-50, 1999
61. Shields CL, Honavar SG, Shields JA, et al: Factors predictive of recurrence of retinal tumors, vitreous seeds, and subretinal seeds following chemoreduction for retinoblastoma. Arch Ophthalmol 120:460-4, 2002
62. Nemeth KM, Federico S, Carcaboso AM, et al: Subconjunctival carboplatin and systemic topotecan treatment in preclinical models of retinoblastoma. Cancer 117:421-34, 2011
63. Brennan RC, Federico S, Bradley C, et al: Targeting the p53 pathway in retinoblastoma with subconjunctival Nutlin-3a. Cancer Res 71:4205-13, 2011

64. Rodriguez-Galindo C, Wilson MW, Haik BG, et al: Treatment of intraocular retinoblastoma with vincristine and carboplatin. J Clin Oncol 21:2019-25, 2003
65. Shields CL, Shields JA, Needle M, et al: Combined chemoreduction and adjuvant treatment for intraocular retinoblastoma. Ophthalmology 104:2101-11, 1997
66. Gunduz K, Shields CL, Shields JA, et al: The outcome of chemoreduction treatment in patients with Reese-Ellsworth group V retinoblastoma. Arch Ophthalmol 116:1613-7, 1998
67. Gombos DS, Kelly A, Coen PG, et al: Retinoblastoma treated with primary chemotherapy alone: the significance of tumour size, location, and age. Br J Ophthalmol 86:80-3, 2002
68. Hahn WC, Weinberg RA: Rules for making human tumor cells. N Engl J Med 347:1593-603, 2002
69. Eberhart CG: Medulloblastoma in mice lacking p53 and PARP: all roads lead to Gli. Am J Pathol 162:7-10, 2003
70. DiCiommo D, Gallie BL, Bremner R: Retinoblastoma: the disease, gene and protein provide critical leads to understand cancer. Semin Cancer Biol 10:255-69, 2000
71. Marine JC, Francoz S, Maetens M, et al: Keeping p53 in check: essential and synergistic functions of Mdm2 and Mdm4. Cell Death Differ 13:927-34, 2006
72. Danovi D, Meulmeester E, Pasini D, et al: Amplification of Mdmx (or Mdm4) directly contributes to tumor formation by inhibiting p53 tumor suppressor activity. Mol Cell Biol 24:5835-43, 2004
73. Bond GL, Hu W, Bond EE, et al: A single nucleotide polymorphism in the MDM2 promoter attenuates the p53 tumor suppressor pathway and accelerates tumor formation in humans. Cell 119:591-602, 2004
74. Kulkarni DA, Vazquez A, Haffty BG, et al: A polymorphic variant in human MDM4 associates with accelerated age of onset of estrogen receptor negative breast cancer. Carcinogenesis 30:1910-5, 2009
75. Atwal GS, Kirchhoff T, Bond EE, et al: Altered tumor formation and evolutionary selection of genetic variants in the human MDM4 oncogene. Proc Natl Acad Sci U S A 106:10236-41, 2009
76. Wynendaele J, Bohnke A, Leucci E, et al: An illegitimate microRNA target site within the 3' UTR of MDM4 affects ovarian cancer progression and chemosensitivity. Cancer Res 70:9641-9, 2010
77. Dyer MA, Bremner R: The search for the retinoblastoma cell of origin. Nat Rev Cancer 5:91-101, 2005
78. Kato MV, Shimizu T, Ishizaki K, et al: Loss of heterozygosity on chromosome 17 and mutation of the p53 gene in retinoblastoma. Cancer Lett 106:75-82, 1996
79. Dimaras H, Khetan V, Halliday W, et al: Loss of RB1 induces non-proliferative retinoma: increasing genomic instability correlates with progression to retinoblastoma. Hum Mol Genet 17:1363-72, 2008
80. Laurie NA, Donovan SL, Shih CS, et al: Inactivation of the p53 pathway in retinoblastoma. Nature 444:61-6, 2006
81. McEvoy J, Flores-Otero J, Zhang J, et al: Coexpression of normally incompatible developmental pathways in retinoblastoma genesis. Cancer Cell 20:260-75, 2011
82. Xu XL, Fang Y, Lee TC, et al: Retinoblastoma has properties of a cone precursor tumor and depends upon cone-specific MDM2 signaling. Cell 137:1018-31, 2009
83. Elison JR, Cobrinik D, Claros N, et al: Small molecule inhibition of HDM2 leads to p53-mediated cell death in retinoblastoma cells. Arch Ophthalmol 124:1269-75, 2006

84. Bai F, Zhu F, Tagen M, et al: Determination of nutlin-3a in murine plasma by liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS). J Pharm Biomed Anal 51:915-20, 2010
85. Zhang F, Tagen M, Throm S, et al: Whole-body physiologically based pharmacokinetic model for nutlin-3a in mice after intravenous and oral administration. Drug Metab Dispos 39:15-21, 2011
86. Pomeroy SL, Tamayo P, Gaasenbeek M, et al: Prediction of central nervous system embryonal tumour outcome based on gene expression. Nature 415:436-42, 2002
87. Laurie NA, Gray JK, Zhang J, et al: Topotecan combination chemotherapy in two new rodent models of retinoblastoma. Clin Cancer Res 11:7569-78, 2005
88. Gombos DS, Hungerford J, Abramson DH, et al: Secondary acute myelogenous leukemia in patients with retinoblastoma: is chemotherapy a factor? Ophthalmology 114:1378-83, 2007
89. Nishimura S, Sato T, Ueda H, et al: Acute myeloblastic leukemia as a second malignancy in a patient with hereditary retinoblastoma. J Clin Oncol 19:4182-3, 2001
90. Weintraub M, Revel-Vilk S, Charit M, et al: Secondary acute myeloid leukemia after etoposide therapy for retinoblastoma. J Pediatr Hematol Oncol 29:646-8, 2007
91. Carcaboso AM, Bramuglia GF, Chantada GL, et al: Topotecan vitreous levels after periocular or intravenous delivery in rabbits: an alternative for retinoblastoma chemotherapy. Invest Ophthalmol Vis Sci 48:3761-7, 2007
92. Qaddoumi I, Billups CA, Tagen M, et al: Topotecan and vincristine combination is effective against advanced bilateral intraocular retinoblastoma and has manageable toxicity. Cancer, 2012
93. Mulvihill A, Budning A, Jay V, et al: Ocular motility changes after subtenon carboplatin chemotherapy for retinoblastoma. Arch Ophthalmol 121:1120-4, 2003
94. Kiratli H, Kocabeyoglu S, Bilgic S: Severe pseudo-preseptal cellulitis following sub-Tenon's carboplatin injection for intraocular retinoblastoma. J AAPOS 11:404-5, 2007
95. Shah PK, Kalpana N, Narendran V, et al: Severe aseptic orbital cellulitis with subtenon carboplatin for intraocular retinoblastoma. Indian J Ophthalmol 59:49-51, 2011
96. Macdonald MR, Harrison RV, Wake M, et al: Ototoxicity of carboplatin: comparing animal and clinical models at the Hospital for Sick Children. J Otolaryngol 23:151-9, 1994
97. Bertolini P, Lassalle M, Mercier G, et al: Platinum compound-related ototoxicity in children: long-term follow-up reveals continuous worsening of hearing loss. J Pediatr Hematol Oncol 26:649-55, 2004
98. Musial-Bright L, Fengler R, Henze G, et al: Carboplatin and ototoxicity: hearing loss rates among survivors of childhood medulloblastoma. Childs Nerv Syst 27:407-13, 2011
99. Kushner BH, Budnick A, Kramer K, et al: Ototoxicity from high-dose use of platinum compounds in patients with neuroblastoma. Cancer 107:417-22, 2006
100. Lambert MP, Shields C, Meadows AT: A retrospective review of hearing in children with retinoblastoma treated with carboplatin-based chemotherapy. Pediatr Blood Cancer 50:223-6, 2008
101. Smits C, Swen SJ, Theo Goverts S, et al: Assessment of hearing in very young children receiving carboplatin for retinoblastoma. Eur J Cancer 42:492-500, 2006
102. Dean JB, Hayashi SS, Albert CM, et al: Hearing loss in pediatric oncology patients receiving carboplatin-containing regimens. J Pediatr Hematol Oncol 30:130-4, 2008
103. Jehanne M, Mercier G, Doz F: Monitoring of ototoxicity in young children receiving carboplatin for retinoblastoma. Pediatr Blood Cancer 53:1162, 2009

104. Pecora Liberman PH, Schultz C, Schmidt Goffi-Gomez MV, et al: Evaluation of ototoxicity in children treated for retinoblastoma: preliminary results of a systematic audiological evaluation. Clin Transl Oncol 13:348-52, 2011
105. Qaddoumi I BJ, Wu J, Billups CA, Wozniak AW, Merchant TE, Haik BG, Wilson MW, Rodriguez-Galindo C.: Carboplatin-Associated Ototoxicity in Children with Retinoblastoma. J Clin Oncol, 2011
106. Brambring M: Divergent development of gross motor skills in children who are blind or sighted. Journal of Visual Impairment and Blindness 100, 2006
107. Fazzi E, Signorini, S.G., Bova, S.M., Ondei, P.E., Bianchi, P.E. : Early intervention in visually impaired children, 2005 pp. 117-121
108. Rossetti L, Fogagnolo P, Miglior S, et al: Learning effect of short-wavelength automated perimetry in patients with ocular hypertension. J Glaucoma 15:399-404, 2006
109. Roles and Responsibilities of Speech-Language Pathologists in Early Intervention: Position Statement., American Speech-Language-Hearing Association., 2008
110. van Dijk J, Oostrom KJ, Huisman J, et al: Restrictions in daily life after retinoblastoma from the perspective of the survivors. Pediatr Blood Cancer 54:110-5, 2010
111. Brownell WE: Outer hair cell electromotility and otoacoustic emissions. Ear Hear 11:82-92, 1990
112. Stavroulaki P, Apostolopoulos N, Segas J, et al: Evoked otoacoustic emissions--an approach for monitoring cisplatin induced ototoxicity in children. Int J Pediatr Otorhinolaryngol 59:47-57, 2001
113. Dhooge I, Dhooge C, Geukens S, et al: Distortion product otoacoustic emissions: an objective technique for the screening of hearing loss in children treated with platin derivatives. Int J Audiol 45:337-43, 2006
114. Hofstetter P, Ding D, Powers N, et al: Quantitative relationship of carboplatin dose to magnitude of inner and outer hair cell loss and the reduction in distortion product otoacoustic emission amplitude in chinchillas. Hear Res 112:199-215, 1997
115. Bhagat SP, Bass JK, White ST, et al: Monitoring carboplatin ototoxicity with distortion-product otoacoustic emissions in children with retinoblastoma. Int J Pediatr Otorhinolaryngol 74:1156-63, 2010
116. Bhagat S: Analysis of distortion product otoacoustic emission spectra in normal-hearing adults. Am J Audiol 18:60-8, 2009
117. Bhagat SP, Carter PH: Efferent-induced change in human cochlear compression and its influence on masking of tones. Neurosci Lett 485:94-7, 2010
118. Margolis RH, Schachern PL, Hunter LL, et al: Multifrequency tympanometry in chinchillas. Audiology 34:232-47, 1995
119. Rhodes MC, Margolis RH, Hirsch JE, et al: Hearing screening in the newborn intensive care nursery: comparison of methods. Otolaryngol Head Neck Surg 120:799-808, 1999
120. Hunter LL, Bagger-Sjoback D, Lundberg M: Wideband reflectance associated with otitis media in infants and children with cleft palate. Int J Audiol 47 Suppl 1:S57-61, 2008
121. Margolis RH, Saly GL, Keefe DH: Wideband reflectance tympanometry in normal adults. J Acoust Soc Am 106:265-80, 1999
122. Hunter LL, Tubaugh L, Jackson A, et al: Wideband middle ear power measurement in infants nd children. J Am Acad Audiol 19:309-24, 2008
123. Chang KW, Chinosornvatana N: Practical grading system for evaluating cisplatin ototoxicity in children. J Clin Oncol 28:1788-95, 2010
124. Pounds S, Cheng C, Mullighan C, et al: Reference alignment of SNP microarray signals for copy number analysis of tumors. Bioinformatics 25:315-21, 2009.

APPENDIX I
STAGING OF RETINOBLASTOMA

Orientation of the extent of disease, using the Reese-Ellsworth Groupings, Original St. Jude and Modified Systems, and American Joint Commission Schemes, will be employed. The relative merits of the several systems include historical significance and common usage, as well as prognostically relevant staging information. The systems relate either to the probability of ocular disease control (primarily with radiation therapy, e.g. Reese-Ellsworth), or to outcome probabilities based on clinical (St. Jude, AJCC) or histologic criteria (Original and Modified St. Jude, AJCC schemes).

I. Reese-Ellsworth Grouping for Suitability for Treatment of Retinoblastoma by Radiation Therapy (Before Enucleation)

Ia Solitary tumor <4 dd at or behind equator
Ib Multiple tumors, none >4 dd, all at or behind equator

IIa Solitary tumor 4-10 dd, at or behind equator
IIb Multiple tumors, 4-10 dd, at or behind equator

IIIa Any lesion anterior to equator
IIIb Solitary tumor >10 dd behind equator

IVa Multiple tumors, some >10 dd
IVb Any lesion extending anteriorly to ora serrata

Va Massive tumors involving >50% of retina
Vb Vitreous seeding

II. SJCRH Original Staging

Disease confined to retina

IA Unifocal or Multifocal disease of 1 quadrant or less
IB Unifocal or Multifocal disease of 2 quadrants or less
IC Unifocal or Multifocal disease of >50% of retinal surface

Disease confined to the globe

IIA Unifocal or Multifocal disease with vitreous seeding
IIB Unifocal or Multifocal disease with extension to optic nerve head
IIC Unifocal or Multifocal disease with extension to choroid
IID Unifocal or Multifocal with extension to choroid & optic nerve head
IIE Unifocal or Multifocal disease with extension to emissaries

Extraocular extension of disease-regional

- IIIA Extension beyond cut end of optic nerve
- IIIB Extension through sclera into orbital content
- IIIC Extension to choroid & beyond cut end of optic nerve (including subarachnoid extension)
- IIID Extension through sclera into orbital contents & beyond cut end of optic nerve including subarachnoid extension)

Distant metastases

- IVA Extension via optic nerve to brain
- IVB Blood-borne metastases to soft tissues, bone
- IVC Bone marrow metastases

III. SJCRH Modified Staging

Tumor confined to retina

- IA Solitary; <6 dd
- IB Multiple; all <6 dd
- IC Solitary or multiple; involving <50% retinal surface behind equator
- ID >50% retinal involvement; behind equator
- IE >50% retinal involvement/or with involvement anterior to equator

Tumor confined to globe/extra retinal*

- IIA Extends to optic nerve head
- IIB1 Extends to choroid
- IIB2 Extends to choroid with replacement
- IIC Anterior chamber involvement
- IID Extends to choroid (1 or 2) and optic nerve

Extra choroidal extension (regional)

- IIIA Extends to emissaries
- IIIB Extends beyond cut end of optic nerve (including subarachnoid)
- IIIC Extends through sclera into orbital contents
- IIID Extends to choroid (1 or 2) and beyond cut end of optic nerve (includes subarachnoid)
- IIIE Extends through sclera and cut end of optic nerve

Distant disease

- IVA Through optic nerve into brain (includes SF+)
- IVB Blood borne metastases to soft tissue, node or bone
- IVC Bone marrow metastases

* Tumors that are confined to the globe or extra retinal but DO NOT meet criteria for IIA-D will be classified as "II."

IV. International Classification for Intraocular Retinoblastoma

Group A

Small tumors away from foveola and disc

- Tumors ≤ 3 mm in greatest dimension confined to the retina, *and*
- Located at least 3 mm from the foveola and 1.5 mm from the optic disc

Group B

All remaining tumors confined to the retina

- All other tumors confined to the retina not in Group A
- Subretinal fluid (without subretinal seeding) ≤3 mm from the base of the tumor

Group C

Local subretinal fluid or seeding

- Local subretinal fluid alone >3 to ≤ 6 mm from the tumor
- Vitreous seeding or subretinal seeding ≤ 3 mm from the tumor

Group D

Diffuse subretinal fluid or seeding

- Subretinal fluid alone >6 mm from the tumor
- Vitreous seeding or subretinal seeding > 3 mm from tumor

Group E

Presence of any or more of these poor prognosis features

- More than 2/3 globe filled with tumor
- Tumor in anterior segment
- Tumor in or on the ciliary body
- Iris neovascularization
- Neovascular glaucoma
- Opaque media from hemorrhage
- Tumor necrosis with aseptic orbital cellulitis
- Phthisis bulbi

V. American Joint Committee on Cancer (7th Ed., 2010) Clinical Classification (cTNM)

Primary tumor (T)

- TX Primary tumor cannot be assessed
- T0 No evidence of primary tumor
- T1 Tumors no more than 2/3 the volume of the eye with no vitreous or subretinal seeding.
- *T1a* No tumor in either eye is greater than 3 mm in largest dimension or located closer than 1.5 mm to the optic nerve or fovea.
- *T1b* At least one tumor is greater than 3 mm in largest dimension or located closer than 1.5 mm to the optic nerve or fovea. No retinal detachment or subretinal fluid beyond 5 mm from the base of the tumor.
- *T1c* At least one tumor is greater than 3 mm in largest dimension or located closer than 1.5 mm to the optic nerve or fovea, with retinal detachment or subretinal fluid beyond 5 mm from the base of the tumor.
- T2 Tumors no more than 2/3 the volume of the eye with vitreous or subretinal seeding. Can have retinal detachment.
- *T2a* Focal vitreous and/or subretinal seeding of fine aggregates of tumor cells is present, but no large clumps or "snowballs" of tumor cells.
- *T2b* Massive vitreous and/or subretinal seeding is present, defined as diffuse clumps or "snowballs" of tumor cells.
- T3 Severe intraocular disease
- *T3a* Tumor fills more than 2/3 of the eye
- *T3b* One or more complications present, which may include tumor-associated neovascular or angle closure glaucoma, tumor extension into the anterior segment, hyphema, vitreous hemorrhage, or orbital cellulitis
- T4 Extraocular disease detected by imaging studies
- *T4a* Invasion of optic nerve
- *T4b* Invasion into the orbit
- *T4c* Intracranial extension not past chiasm
- *T4d* Intracranial extension past chiasm

Regional lymph nodes (N)

- NX Regional lymph nodes cannot be assessed
- N0 No regional lymph node involvement
- N1 Regional lymph node involvement (preauricular, submandibular, or cervical)
- N2 Distant lymph node involvement

Distant metastasis (M)

- MX Presence of distant metastasis cannot be assessed
- M0 No distant metastasis
- M1 Systemic metastasis
- M1a Single lesion to sites other than CNS
- M1b Multiple lesions to sites other than CNS
- M1c Prechiasmatic CNS lesion(s)
- M1d Postchiasmatic CNS lesion(s)
- M1e Leptomeningeal and/or CSF involvement

Pathologic Classification (pTNM)

Primary tumor (pT)

pTX Primary tumor cannot be assessed
pT0 No evidence of primary tumor
pT1 Tumor confined to the eye with no optic nerve or choroidal invasion.
pT2 Tumor with minimal optic nerve and/or choroidal invasion.
pT2a Tumor superficially invades optic nerve head but does not extend past lamina cribrosa *or* tumor exhibits focal choroidal invasion.
pT2b Tumor superficially invades optic nerve head but does not extend past lamina cribrosa *and* exhibits focal choroidal invasion.
pT3 Tumor with significant optic nerve and/or choroidal invasion:
pT3a Tumor invades optic nerve past lamina cribrosa but not to surgical resection line *or* tumor exhibits massive choroidal invasion.
pT3b Tumor invades optic nerve past lamina cribrosa but not to surgical resection line *and* tumor exhibits massive choroidal invasion.
pT4 Tumor ivades optic nerve to resection line or exhibits extra-=ocular extension elsewhere *pT4a* Tumor invades optic nerve to resection line but NO extra-ocular extension identified
pT4b Tumor invades optic nerve to resection line and extra-ocular extension identified

Regional lymph nodes (pN)

pNX Regional lymph nodes cannot be assessed
pN0 No regional lymph node metastasis
pN1 Regional lymph node metastasis
pN2 Distant lymph node involvement

Distant metastasis (pM)

pMX Presence of distant metastasis cannot be assessed
pM0 No distant metastasis
pM1 Metastasis to sites other than CNS
pM1a Single lesion
pM1b Multiple lesions
pM1c CNS metastatsis
pM1d Discrete mass(es) without leptomeningeal and/or CSF involvement
pM1e Leptomeningeal and/or CSF involvement

APPENDIX II
BIOLOGY STUDIES

Michael Dyer, Ph.D.
Department of Developmental Neurobiology

EYE GLOBE MANIPULATION AFTER ENUCLEATION

For those participants enrolled on the biology studies, the eye will be processed immediately after enucleation in order to obtain fresh tissue before fixation.

Procedure: The landmarks of the enucleated eye will be preserved when possible, and the ophthalmologist will mark the superior part of the eye with a suture at 12 o'clock. The pathologist will have been provided information regarding the location of the tumor/s, and will better assess the location of the tumor/s by transillumination. A small incision will be performed in an area not involved by disease, and this area will be marked with ink. Fresh samples of tumor and vitreous will be obtained.

BIOLOGY STUDIES

RNA, DNA and protein isolation provided by the St. Jude Biorepository – identified by TB-ID number only

The RNA will be tested for purity, concentration, and integrity. From there, the RNA will be submitted to the Hartwell Center for linear RNA amplification, antisense RNA synthesis, biotin labeling, and hybridization to the Affymetrix human gene chip. Data will be stored and processed by the Hartwell center. Individual samples will be compared to each other and to fetal retinal progenitor cell expression profiles to determine what changes occur in retinal progenitor cells that lead to retinoblastoma. Purified protein will be resuspended in protein loading dye containing 2% SDS and separated by SDS-PAGE. The proteins will then be transferred to nitrocellulose and probed with antibodies to proteins believed to be involved in retinoblastoma tumorigenesis and chemotherapeutic response such as p53, MDM2 and Arf.

Purified genomic DNA will be PCR amplified using primer pairs that we have designed and characterized corresponding to the coding region of Rb and the upstream regulatory region that most often contain mutations in participants with retinoblastoma. The collection of Rb genomic amplicons will then be sent to the Hartwell Center for hybridization to our custom Affymetrix resequencing chip. The total genomic DNA sample will also be processed by the Hartwell Center for hybridization to the loss of heterozygosity (LOH) chip (SNP6.0). Similar analysis will be carried out with the DNA prepared from the blood of each Rb participants to identify the genomic alterations that are specific to the tumor cells.

DNA methylation assay

Methylation analysis will be performed using the Human Methylation 45 BeadChip and iScan system from Illumina, Inc. (San Diego, CA). Briefly, following bisulfate treatment (Zymo, Inc.), genomic DNA is amplified and fragmented and then hybridized to the BeadChip. A single-base

pair extension reaction is then performed, followed by staining with a detectable fluorescent dye label. The resulting products are imaged with the iScan Reader (Illumina, Inc.). Methylation at each locus is determined by calculating the percent of total signal from each locus that is represented by the methylation probe using the GenomeStudio software (Illumina, Inc.). Quality standards for bisulfite conversion, staining, single base extension, hybridization, stringency, and non-specific binding have been verified. A methylation state of every probe is represented by fluorescent signals from the M (methylated) and U (unmethylated) alleles. The ratio of fluorescent signals is then computed from the two alleles: $\beta = (\max(M, 0))/(|U| + |M| + 100)$. To avoid "negative signal" a small constant offset 100 is added to the denominator to regularize β value when both methylated and unmethylated probe intensities are low. The β value ranges between 0 and 1 and under ideal conditions it has bimodal distribution with two means corresponding to methylated and unmethylated states of the interrogated CpG site.

Cell line cloning

Immediately following isolation of the fresh tumor sample, the cells will be washed in warm (37°C) Hanks Balanced Salt Solution and gently triturated to break up large clusters of cells. Harsh enzymatic treatment such as trypsin treatment will be avoided if possible to prevent any undue stress to the cells. Immediately following the wash, the cell pellet will be resuspended in RPMI medium containing 10% fetal calf serum, antibiotics (Penn/Strep) and L-glutamine. The cells will be counted and stained with trypan blue and then the volume will be adjusted to achieve approximately 100,000 viable cells per ml. This is the optimal growth density for the retinoblastoma cell lines Y79 and Weri1 (Dyer, MA, unpublished). Cells will then be cultured at 37°C with 5% carbon dioxide and monitored weekly for growth. At least once a week the medium will be changed but more frequent changes may be required depending on the growth rate. Once cells are actively growing and they have gone through several generations, 10 batches will be frozen for long term storage in liquid nitrogen in 90% fetal calf serum and 10% DMSO for future use. Each cloned cell line will be extensively characterized at the minimal passage number to reduce the possibility of cellular or genetic changes as a result of the culture conditions. We will study the double time of each cell line, the apoptosis frequency, gene expression profile by microarray hybridization, and their response to drug treatment (carboplatin, vincristine, topotecan). Individual retinoblastoma cell lines will be genetically modified to carry the GFP gene for xenograft studies. All of these data will be compared to the data we have already obtained for the Y79 and Weri1 cell lines and the freshly isolated tumor samples.

Orthotopic xenografts

The fresh primary tumor specimen will be dissociated in trypsin/PBS at 37°C until a single cell suspension is obtained. Total cell number will be determined using a hemacytometer and the viability will be estimated using a Guava cell analyzer. The cells are collected by centrifugation and resuspended in warm RPMI with 10% FCS at approximately 10,000 cells/microliter. 5 microliters (~50,000 cells) will be injected per eye of SCID mice (B6.CB17-Prkdc<scid>SzJ; Jackson Labs) under anesthesia.

Analysis of the vitreous proteome

The sample will be flash frozen following enucleation, and maintained at -80C until analysis. After thawing, the sample will be centrifugated at 12000 RPM x 15 min x RT to separate the liquid component, as described in Yu et al, Proteomics, 2008. If debri is observed, we will filter the sample in a 0.22uM filter to clarify sample, as done by Kim et al, Proteomics, 2007. From the clarified fraction of the vitreous we will divide the sample into 2, one for protein analysis of PDGF and isoforms PDGF-A, PDGF-B and their receptors using commercially available Multiplex systems; second set for mRNA analysis of *PDGFA*, *PDGFB*, *PDGFRA*, *PDGFRB*, using commercially available primers, following the protocol for nucleic acid isolation from Chintalapudi and Morales-Tirado et al, 2015.

APPENDIX III
OCCUPATIONAL AND SPEECH THERAPY

Department of Rehabilitative Medicine
Jessica Sweeney Sparrow, OTD, OTR/L
Angela Eftink, MCD, CCC/SLP

1. The children will be followed on a scheduled basis for 60 minute encounters to include a variety of evaluations and questionnaires as well as referral for or tracking of intervention and services within the home community. Patients must have English as their primary language to participate in the evaluations, with the exception of the Sensory Profile and PedsQL as noted below.

2. Scheduled encounters will occur at initial visit and approximately every 6 months following that time for a period of 3 years. Additional visits within and after this 3 year period will be scheduled on an as needed basis as determined by the occupational therapist or speech therapist. A scheduled long term follow up evaluation will occur 5 years post initial evaluation.

3. Evaluations that will occur at speech therapy or occupational therapy encounters include the following:

   ☐ **Battelle Developmental Inventory (2nd Ed)**
   Type: Developmental assessment for early childhood (norm referenced); direct assessment
   Purpose: screening, diagnosis, and evaluation of early development
   Measures: Personal-social, adaptive, motor, communication, & cognitive ability
   Ages: birth to 7-11 years

   ☐ **Sensory Profile (Infant Toddler Sensory Profile & Sensory Profile)**
   Type: Likert scale questionnaire filled out by caregiver or, if needed, with assistance from therapist
   Purpose: Detect children's difficulties in engaging and adapting to his/her environment
   Measures: To articulate the sensory processing of both individuals with or without disabilities. Available in both English and Spanish, with the appropriate version utalized being determined by the parent's primary language.
   Ages: Infant Toddler Sensory Profile 0-36 months
   Sensory Profile 3 years to 10 years

   ☐ **Pre-school Activity Card Sort**
   Type: Card sort/Interview
   Purpose: Measures participation in 85 preschool-aged activities of children.
   Measures: Activities are categorized into six domains of function: domestic chores, personal care, leisure (vigorous and sedentary, social interactions, education, and community mobility
   Ages: 3 – 6 years.

☐ **Pediatric Quality of Life Inventory – PedsQL V.4**
Type: 23 item Likert-type scale questionnaire
Purpose: Measures health related quality of life in children and adolescents. The Parent Report for Young Child Report (ages 5-7), the Parent Report for Toddlers (ages 2-4), Infant Scales (12-24 months) and the Infant Scales (ages 1-12 months) will be utilized. Clinical validity has been established and results of assessment using the SF-15 were found to be comparable results using the original full instrument. The scales are available in both English and Spanish languages and the appropriate version will be utilized based on the parent's primary language.
Measures: Subscales on the PedsQL V.4 measure the research participant's physical, emotional, social, and school functioning.
Ages: This instrument has parallel forms for the participant and parent report.

☐ **Rehabilitation Services Questionnaire**:
Type: Questionnaire administered by the occupational therapist or Speech Therapist
Purpose: Monitor the type and frequency of rehabilitation services the child receives
Measures: Services received and/or recommended in home community and at St. Jude. Data regarding low vision device use will also be collected
Ages: any

☐ **Clinical Evaluation of Language Fundamentals Preschool- 2nd edition (CELF Preschool 2)**
Type: Norm referenced; direct assessment
Purpose: Comprehensive assessment of language skills of preschool children who will be in an academic-oriented setting
Measures: Semantics, morphology, and syntax skills
Ages: 3-0 years to 6-11 years

☐ **Receptive- Expressive Emergent Language Test- 3rd edition (REEL-3)**
Type: Norm referenced; Parent interview/report, observation, and elicitation of skills
Purpose: Identification of babies/toddlers with delays in language acquisition and discrepancies between receptive and expressive processes of emergent language. This tool can also be used to document the child's progress in language as a consequence of special intervention programs.
Measures: Receptive language and Expressive language
Ages: Birth to 36 months

☐ **Expressive Vocabulary Test- 2nd edition (EVT-2)**
Type: Norm Referenced; direct assessment
Purpose: Evaluate English Language Learners' vocabulary acquisition and measure words they use in everyday language, and assess oral skills as a foundation of writing skills.
Measures: Expressive vocabulary and word retrieval skills in Standard American English
Ages: 2:6 to 90+ years

☐ **Peabody Picture Vocabulary Test- 4th edition (PPVT-4)**
Type: Norm Referenced; direct assessment
Purpose: Evaluate receptive vocabulary without need for reading or writing skills
Measures: Receptive vocabulary. PPVT-4 is co-normed with EVT-2 to allow direct comparison of expressive and receptive vocabulary performance.
Ages: 2:6 to 90+ years

Schedule of Occupational Therapy assessments

Months following diagnosis								
OT evaluation	Initial	6	12	18	24	30	36	5 year F/U
Battelle Developmental Inventory	X		X		X		X	X
Rehab Services Questionnaire	X	X	X	X	X	X	X	X
PedsQL	X		X		X		X	X
Pediatric Activity Card Sort				X		X*		X
Sensory Profile		X				X		X

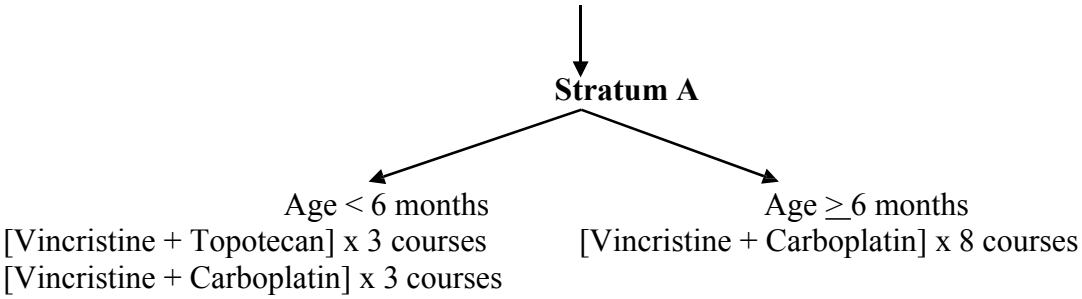
**The Pediatric Activity Card Sort is to be completed at 30 months only if not completed at 18 month visit.*

Schedule of Speech Therapy assessments

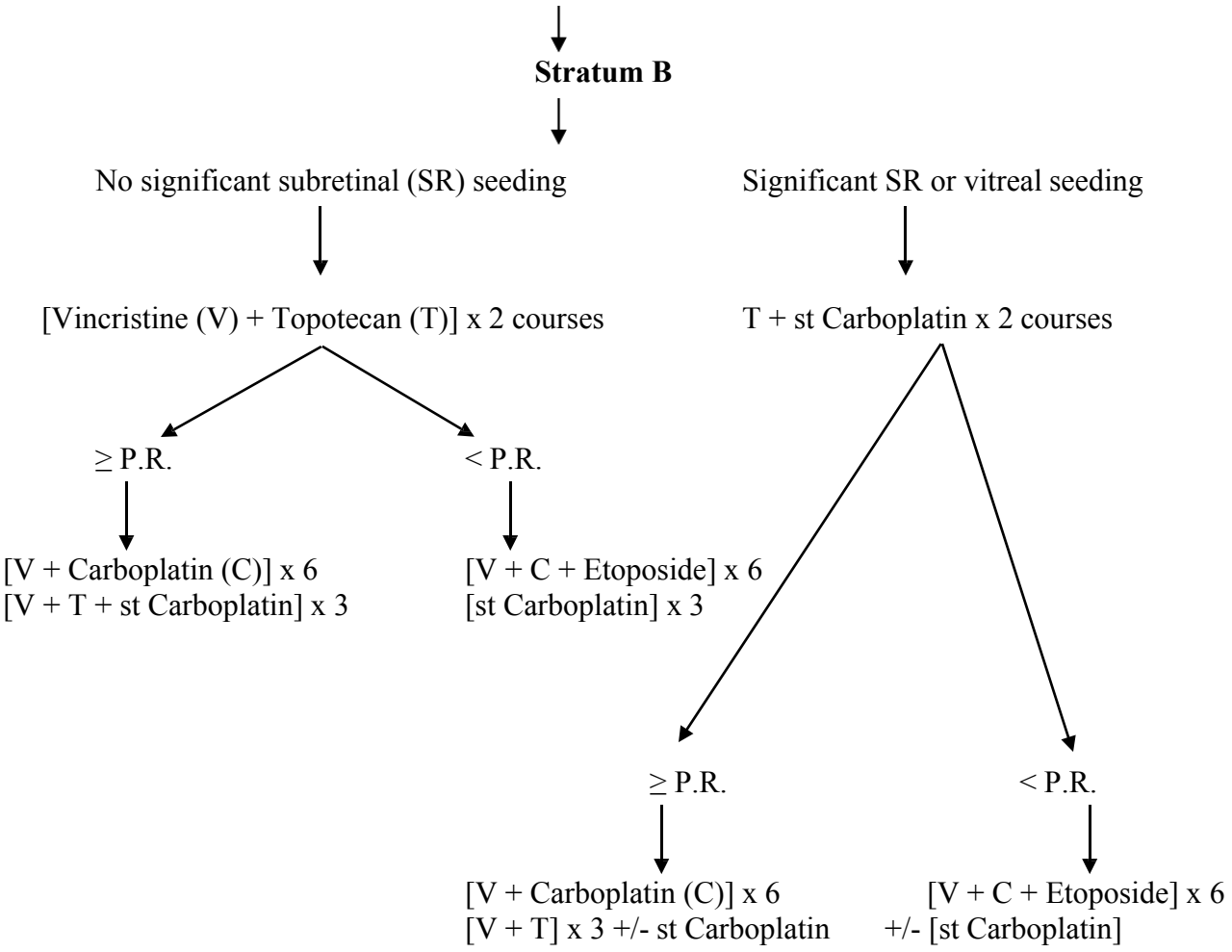
Months following diagnosis								
Speech evaluation	Initial	6	12	18	24	30	36	5 year F/U
Battelle Developmental Inventory	X		X		X		X	X
Receptive- Expressive Emergent Language Test- 3		X		X		X		
Clinical Evaluation of Language Fundamentals- Preschool 2		X		X		X		X
Expressive Vocabulary Test- 2		X		X		X		X
Peabody Picture Vocabulary Test- 4		X		X		X		X

Appendix IV
TREATMENT SCHEMA/OVERVIEW

R-E I-III + IC A or B; R-E IV + IC A or B; IC C + limited subretinal seeding
Unilateral or Bilateral



R-E IV-V, IC D (any eye): Bilateral
R-E IV-V + IC D or E with foveal sparing: Unilateral



R-E IV-V, IC D-E Unilateral

↓

Stratum C

Low Risk	Intermediate Risk	High Risk
↓	↓	↓
No treatment	VCD x 4	VCD x 3 VCE x 3 EBRT prn

Stratum D
R-E IV-V, IC E (any eye) Bilateral

↓

Upfront Enucleation

		Histology of enucleated eye	
		Low risk	***Intermediate and high risk***
R-E group of Remaining eye	***R-E I-IIIIC A-B***	Course #1: VCE Treatment as Stratum A (VC x 7 courses)	Course #1: VCE Treatment as unilateral high-risk (VCE x 5)
	R-E IV-VIC C-E	Course #1: VCE Treatment as Stratum B (omit course #10 VC)	Course #1: VCE Treatment as unilateral high-risk (VCE x 5)

Appendix V

ECOG PERFORMANCE SCORE

Grade	ECOG
0	Fully active, able to carry on all pre-disease performance without restriction
1	Restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature, e.g., light house work, office work
2	Ambulatory and capable of all self-care but unable to carry out any work activities. Up and about more than 50% of waking hours
3	Capable of only limited self-care, confined to bed or chair more than 50% of waking hours
4	Completely disabled. Cannot carry on any self-care. Totally confined to bed or chair
5	Dead

*As published in Am. J. Clin. Oncol: *Oken, M.M., Creech, R.H., Tormey, D.C., Horton, J., Davis, T.E., McFadden, E.T., Carbone, P.P.: Toxicity And Response Criteria Of The Eastern Cooperative Oncology Group. Am J Clin Oncol 5:649-655, 1982.*

Appendix VI - EVALUATIONS/INTERVENTIONS FOR STANDARD OF CARE AND FOR RESEARCH

Standard of care

- Detailed medical history
- Physical examinations (including visual acuity and examination under anesthesia (EUA) with documentation/photographs of retinoblastoma lesions)
- MRI of the brain and orbits
- US of the orbits
- Hematology (CBC with differential)
- Blood chemistries for safety
- Urinalysis
- Audiogram or BAER, and OAE
- Bilateral bone marrow aspirates and biopsies
- Lumbar puncture for cytology
- ECHO/EKG
- Tc^{99m}-DTPA renal clearance
- Occupational and speech therapy: participant early intervention services as needed
- Mutational analysis of RB1 and genetic counseling
- Topotecan clinical pharmacokinetics
- Chemotherapy agents and administration
- External beam radiation therapy
- Focal therapies as clinically indicated

Research

- Enucleation sample (if applicable) and tumor tissue for biology studies (see Appendix II)
- Pharmacogenetics (per PGEN5)
- Occupational and speech therapy parental questionnaires only.
- Audiology: Wideband reflectance (WBR)

APPENDIX VII
CHANG OTOTOXICITY GRADING SCALE

Chang Grade	Sensorineural Hearing Threshold (dB HL): bone conduction or air conduction with normal tympanogram
0	≤ 20 dB at 1, 2, and 4 kHz
1a	≥ 40 dB at any freq 6 to 12 kHz
1b	> 20 and < 40 dB at 4kHz
2a	≥ 40 dB at 4 kHz and above
2b	> 20 and < 40 dB at any freq below 4kHz
3	≥ 40 dB at 2 or 3 kHz and above
4	≥ 40 dB at 1 kHz and above