# Minimal disseminated disease evaluation and outcome in trilateral retinoblastoma

Ana V Torbidoni,[1,2] Claudia Sampor,[1] Viviana E Laurent,[1] Rosario Aschero,[3] Saipriya Iyer,[1] Jorge Rossi,[4] Daniel Alderete,[1] Daniel F Alonso,[2,5] Irene Szijan,[2,6] Guillermo L Chantada[1,2]

[1]Hematology-Oncology Service, Pediatric Hospital S.A.M.I.C. Prof. Dr. Juan P. Garrahan, Buenos Aires, Argentina
[2]National Scientific and Technical Research Council, CONICET, Buenos Aires, Argentina
[3]Pathology Service, Pediatric Hospital S.A.M.I.C. Prof. Dr. Juan P. Garrahan, Buenos Aires, Argentina
[4]Immunology Service, Pediatric Hospital S.A.M.I.C. Prof. Dr. Juan P. Garrahan, Buenos Aires, Argentina
[5]Molecular Oncology Laboratory, Quilmes National University, Bernal, Argentina
[6]Genetic and Molecular Biology, University of Buenos Aires, Buenos Aires, Argentina

**Correspondence to**
Dr Guillermo L Chantada, Hematology-Oncology Service, Hospital JP Garrahan, Buenos Aires C1245AAL, Argentina; gchantada@garrahan.gov.ar

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## ABSTRACT

Trilateral retinoblastoma (TRb) presents a management challenge, since intracranial tumours are seldom times resectable and quickly disseminate. However, there are no risk factors to predict the final outcome in each patient.

**Objective** To evaluate minimal disseminated disease (MDD) in the bone marrow (BM) and the cerebrospinal fluid (CSF) at diagnosis and during follow-up and reviewing its potential impact in the outcome of patients with TRb.

**Methods and analysis** We evaluated MDD in five patients with TRb, detecting the mRNA of *CRX* and/or *GD2*, in samples from BM and CSF, obtained at diagnosis, follow-up and relapse.

**Results** Treatment involved intensive systemic chemotherapy in four patients, one did not receive this treatment and died of progression of the disease. Two patients underwent stem cell rescue. Three patients had leptomeningeal relapse and died. One patient remains disease-free for 84 months. *RB1* mutations were identified in the five patients, all of them were null mutations. At diagnosis, one patient had tumour cells in the CSF, and none had the BM involved. Only one case of four presented MDD during follow-up in the CSF, without concomitant detection in the BM. On leptomeningeal relapse, no case had MDD in the BM. In all these cases, cells in the CSF were positive for *GD2* and/or *CRX*.

**Conclusion** CSF dissemination always concluded in the death of the patient, without concomitant systemic dissemination denoting the importance of increasing treatment directed to the CSF compartment. The MDD presence could indicate a forthcoming relapse.

## INTRODUCTION

Retinoblastoma (Rb) is the most common primary intraocular tumour found in children. In developed countries, it has a very high rate of disease-free survival.[1 2] One major disease-related cause of death observed in Rb is trilateral retinoblastoma (TRb). This rare condition occurs in children with the germline mutation in the *RB1* gene, presenting either with unilateral or bilateral Rb, who develop an intracranial midline neuroblastic tumour, usually in the pineal gland (in about three quarter of the cases) or in the supra/parasellar region (in the remaining quarter).[3 4] The incidence of TRb in patients with hereditary Rb has been recently estimated to be around 3.5%.[5] The influence of chemotherapy or radiotherapy in increasing or reducing the risk of developing TRb is controversial.[6] Therefore, there are no current risk factors identifiers for developing TRb.

TRb is not considered a consequence of metastatic tumour dissemination and most authors agree that it is the result of the effect of the *RB1* mutation in the neural ectoderm that shares the embryological origin with retinal cell precursors. It was considered almost invariably fatal, since intracranial tumours are seldom times resectable and quickly disseminate through the neuraxis.[7] However, intensive systemic chemotherapy has proven to be potentially curative, specifically in TRb cases with small and asymptomatic, non-disseminated intracranial tumours.[8] Nevertheless, after an initial response to treatment, leptomeningeal dissemination often occurs and represents an extremely difficult situation to treat, which generally results in the death of the patient.[9 10] Although systemic clinical dissemination is seldom times seen in patients with TRb, the current treatment protocols are based mostly on intensifying systemic treatment, with variable uses of central nervous system (CNS)-directed therapies such as radiotherapy and intrathecal or intraventricular chemotherapy.[7 8 11–15] To our knowledge, the pattern of dissemination in TRb has not been studied molecularly. We have studied minimal disseminated disease (MDD) in cases of metastatic Rb as a tool for interpreting dissemination patterns and have observed that systemic dissemination present at diagnosis usually clears quickly and completely with intensive therapy. In most cases, leptomeningeal relapse occurs as an isolated event, limited only to the cerebrospinal fluid (CSF) compartment, without systemic involvement. For those studies, we evaluated the use of the mRNA of *CRX* as the preferred biomarker for the detection of the minimal dissemination.[16] Therefore, the mRNA of *CRX* could also be a useful tool for scrutinising the dissemination status in TRb along treatment, as it has not been previously employed for evaluation of this type of patients with Rb.

Thus, our aim was to evaluate MDD of Rb cells in the bone marrow (BM) and the CSF at diagnosis and also during follow-up and reviewing its potential impact in the outcome of patients with TRb.

## MATERIALS AND METHODS

### Patients

This was a retrospective study of patients with TRb diagnosed and treated in the Pediatric Hospital S.A.M.I.C. Prof. Dr. Juan P. Garrahan (PHG) from

April of 2006 to February of 2017 in whom MDD was evaluated. The Institutional Review Board (IRB) of the PHG approved the MDD evaluation study (IRB approval number: 751) and all procedures were performed in accordance with ethical standards and with the Declaration of Helsinki. All parents/guardians signed a written informed consent allowing the MDD study.

### Diagnosis and treatment

TRb was diagnosed by imaging studies when a solid pineal or sellar mass was detected. All patients underwent head and spinal gadolinium-enhanced MRI at the time of diagnosis of TRb. No biopsy confirmation was required for diagnosis but it was attempted when felt feasible for tissue confirmation. Treatment with intensive chemotherapy followed by autologous stem cell rescue with previously reported regimens was offered for all patients with TRb with intention to cure. Cranial or craniospinal radiotherapy was scheduled only for those cases that were not achieving complete remission with chemotherapy or for palliative therapy after CNS relapse.

#### *RB1* mutation screening

Mutation screening was performed in DNA obtained from samples of peripheral blood leucocytes using the cetyltrimethylammonium bromidemethod. PCR amplification and sequencing of the 27 exons, the promoter and the intronic flanking regions were performed using an ABI 3130XL analyzer. All the mutations were confirmed using as a reference for genomic alterations the *RB1* reference sequence L11910 (GeneBank accession number). Mutations were described according to the nomenclature of the Human Genome Variation Society (HGVS) and Den Dunnen and Antonarakis.[17] Multiplex Ligation-dependent Probe Amplification assay (MLPA) was performed using the Salsa MLPA kit P047-B1 RB1 (MRC Holland) according to the manufacturer's protocol.

### Minimal disseminated disease evaluation

#### Specimen collection and preservation

Samples of BM and CSF were collected as part of a prospectively defined schedule: at diagnosis, following induction chemotherapy and in any case where relapse was suspected clinically or by imaging studies. For extent of disease evaluation and MDD detection, BM examination included samples of two biopsies and two aspirates taken from each posterior iliac crest that were sent for pathological assessment (the biopsies) and immunocytology for disialoganglioside GD2 and PCR determinations for *CRX* as previously described (the aspirates).[16 18] Immunocytology for disialoganglioside GD2 was done using the 3F8 antibody as previously reported and the expression of the mRNA of *CRX* was evaluated by real-time PCR as previously reported.[16 18] Total 3–5 mL CSF samples were collected and the first collected specimen was used for cytology, the second sample was taken for immunocytology (which was done in case cell count exceeded 3 cells/mm$^3$), and the third sample was stored at −70°C for PCR determinations as previously reported. For MDD determination in CSF, we used detection of the mRNA of *GD2* synthase as it was already reported up to it was replaced by mRNA of *CRX* detection.[16 19]

#### MDD analysis

Patients in whom the mRNA of *CRX* was detected in the BM and/or CSF or the mRNA of *GD2* synthase in the CSF and also showed the absence of detectable malignancy by microscopy in those sites, were considered to have MDD. The *CRX* mRNA positivity was expressed as relative expression levels according to our previous work.[16]*GD2* synthase results were shown as qualitative data (positive-negative).[19] The presence of MDD did not influence any treatment decision. Specimens were processed in a blind fashion since the pathology and the PCR operators did not know outcome results. Descriptive analyses were performed.

## RESULTS

### Clinical characteristics and treatment

This study included six patients with TRb, two females and four males. One of these patients was diagnosed with 'quadrilateral' Rb at 3 weeks of age. However, no MDD studies could be done due to the age of the patient and difficulties in obtaining material for MDD, so he was considered not evaluable. For the five evaluable patients, the age ranged from 10 days to 11 months at the time of diagnosis of Rb and ranged from 2 to 48 months at the time of diagnosis of TRb. Only the patient #2 was diagnosed with TRb at the same time of the diagnosis of Rb. In the other four patients, there was a median latency period of 22.75 months, ranging from 6 to 37 months. Four patients had bilateral and one patient had unilateral eye involvement. Two patients presented with clinical symptoms of increased intracranial pressure and in three, TRb was detected in a follow-up routine examination (table 1).

### Diagnosis and extent of disease evaluation

The pineal gland was involved in four patients and the sellar area in one patient (table 1). The maximal diameter of the pineal tumours ranged from 12 mm to 25 mm. A biopsy confirmation of the pineal mass was attempted in two cases. CSF examination was negative for Rb cells in four patients at the moment of diagnosis of TRb but there was imaging evidence of leptomeningeal dissemination in one case with positive cytology in the CSF confirmed by GD2 ganglioside immunocytology (table 1).

In all patients, germline mutations of the *RB1* gene were identified, they were: 1 bp deletion in exons 7 and 10 in two patients; 2 bp deletions in exons 18 and 22 in two patients (table 1). These mutations led to frameshift, premature stop codon appearance and a generation of a truncated non-functional RB protein. They occurred in short sequence-repeats in three cases: CC, AAAA, AAA (patients 5, 1 and 3) and in an environment of several short repeats in the case of a T-del (patient 2). These data suggest a slippage of DNA polymerase during DNA replication. MLPA analysis revealed a deletion of a whole *RB1* gene plus a centromeric *ITM2B5* gene and a telomeric *RCBTB2* gene in one patient causing an absence of RB protein.

### Treatment and outcome

Three patients received intensive chemotherapy for the initial treatment of TRb. Two patients achieved a complete response and continued with consolidation with autologous stem cell rescue (table 1). However, both patients had a leptomeningeal relapse at 8 and 3 months after transplantation and died of CNS dissemination shortly thereafter. The remaining patient had severe haematopoietic toxicity and infectious complications during induction chemotherapy and needed prolonged admission to the intensive care unit. Thereafter, the parents declined continuing further therapy. A stable disease status was obtained on the evaluation of response to induction chemotherapy (figure 1). This patient was enrolled into an experimental immunotherapy phase I study with Racotumomab (an anti-idiotype vaccine) and remains in follow-up without clinical evidence

**Table 1** Table summarising the characteristics and evaluations performed in the patients with TRb studied

| Patient (age at Rb diagnosis and at TRb diagnosis (months) | Clinical presentation | Mutation | MRI previous TRb diagnosis/ at TRb diagnosis (size: maximal diameter in mm) | Treatment prior to TRb diagnosis | Frontline treatment for TRb | Outcome | Dissemination studies at diagnosis, follow-up and relapse |
|---|---|---|---|---|---|---|---|
| *1-<br>► Bilateral Rb: 11 months<br>► TRb: 48 months | Symptomatic (intracranial hypertension) | E 10: g.64414 delA<br>p.T342fsX343<br>Frameshift<br>Stop codon | 6 months doubtful pineal lesion (10 mm)/<br>Pineal mass (20 mm) plus leptomeningeal dissemination | Bilateral enucleation followed by 8 cycles of adjuvant chemotherapy with Vincristine: 1.5 mg/m$^2$/dose day one; Idarubicin: 10 mg/m$^2$/dose day one; cyclophosphamide: 65 mg/kg/day (day 1), alternating with Carboplatin 550 mg/m$^2$/day days 1 and 2 and etoposide 100 mg/m$^2$ days 1–3 because of pathology risk factors | Induction with systemic chemotherapy (COG ARET 0321): four cycles<br>Craniospinal radiotherapy†<br>High dose chemotherapy* followed by stem cell rescue | Achieved complete response with first line therapy.<br>CNS relapse at 8 months after stem cell transplant.<br>Death at 4 months after relapse | Diagnosis:<br>CSF: Positive Immunocytology for GD2.<br>Follow-up: MDD positive for GD2 synthase.<br>Relapse: CSF positive with all the techniques.<br>BM negative in all of the evaluated moments with all the techniques. |
| *2-<br>► Bilateral Rb: 2 months<br>► TRb: 2 months | Detected at screening | E 7: g.56880 delT<br>p.L212fsX213<br>Frameshift<br>Stop codon | Sellar and suprasellar mass (12.6 mm) | None | Moderate dose chemotherapy + intrathecal topotecan‡ | Partial response achieved with first line therapy.<br>CNS progression 24 months after diagnosis.<br>Death: 16 days after progression | Diagnosis and follow-up: MDD negative in the CSF.<br>Relapse: CSF positive with all the techniques.<br>BM negative in all of the evaluated moments with all the techniques |
| *3-<br>► Bilateral Rb: 9 months<br>► TRb: 24 months | Symptomatic (intracranial hypertension) | E 22: g.162083/85delAA<br>p.N767fsX793<br>Frameshift Stop codon | Normal (11 months)/<br>Pineal mass (25 mm).<br>Hydrocephalus | Five cycles of systemic chemoreduction with vincristine 0.05 mg/kg/dose/carboplatin 18.7 mg/kg/dose/etoposide 6.6 mg/kg/dose and external beam radiotherapy 3960 cGy | Induction with systemic chemotherapy (COG ARET 0321) four cycles.<br>High dose chemotherapy§ followed by stem cell rescue | Achieved complete response with first line therapy<br>CNS relapse (leptomeningeal dissemination) 3 months after stem cell transplant<br>Death: 20 days after relapse | Diagnosis and follow-up: MDD negative in the CSF.<br>Relapse: CSF positive with all the techniques.<br>BM negative in all of the evaluated moments with all the techniques |
| *4-<br>► Unilateral Rb: 3 months.<br>► TRb: 36 months. | Detected at screening | Deletion of the whole *RB1* gene<br>g.ITM2B5-RB1-RCBTB2 del | Normal (3 months)/Pineal (14.5 mm) | Six cycles of systemic chemoreduction with vincristine 0.05 mg/kg/dose/carboplatin 18.7 mg/kg/dose/etoposide 6.6 mg/kg/dose followed by enucleation of affected eye | No treatment due to parents' decision | Dead 6 months after TRb diagnosis. | Diagnosis: MDD negative in the CSF and BM.<br>Follow-up and relapse were not evaluated due to parents' decision |
| *5-<br>► Bilateral (Metachronic) Rb: 10 days<br>► TRb: 6 months | Detected at screening | E 18: g.150057-58delCC<br>p.H585fsX793<br>Frameshift<br>Stop codon | Suspicious pineal mass (11 mm, 2 months)/Pineal mass (12 mm) | Three cycles of systemic chemoreduction with vincristine 0.05 mg/kg/dose/carboplatin 18.7 mg/kg/dose/etoposide 6.6 mg/kg/dose | Induction with systemic chemotherapy (COG ARET 0321): three cycles.<br>Immunotherapy with Racotumomab | Partial response by imaging.<br>Residual mass.<br>Alive 84 months after TRb diagnosis.<br>(figure 1) | Diagnosis and follow-up: MDD negative in the CSF.<br>BM negative in all of the evaluated moments with all the techniques |

*Carboplatin (350 mg/m$^2$/day for 5 days), Cyclophosphamide (1.6 g/m$^2$/day for 4 days), Etoposide (350 mg/m$^2$/day for 5 days).
†Craniospinal radiotherapy (2520 cGy. Boost pineal area: 4500 cGy).
‡Six cycles with vincristine 0.05 mg/kg/dose/carboplatin 18.7 mg/kg/dose/etoposide 6.6 mg/kg/dose+10 cycles of intrathecal topotecan (0.4 mg/dose).
§Busulfan 120 mg/m$^2$/day × 5 days+Melphalan 140 mg/m$^2$/day × 1 day.
CNS, central nervous system; del, deletion; E, Exon; MDD, minimal disseminated disease; RMI, resonance magnetic imaging.

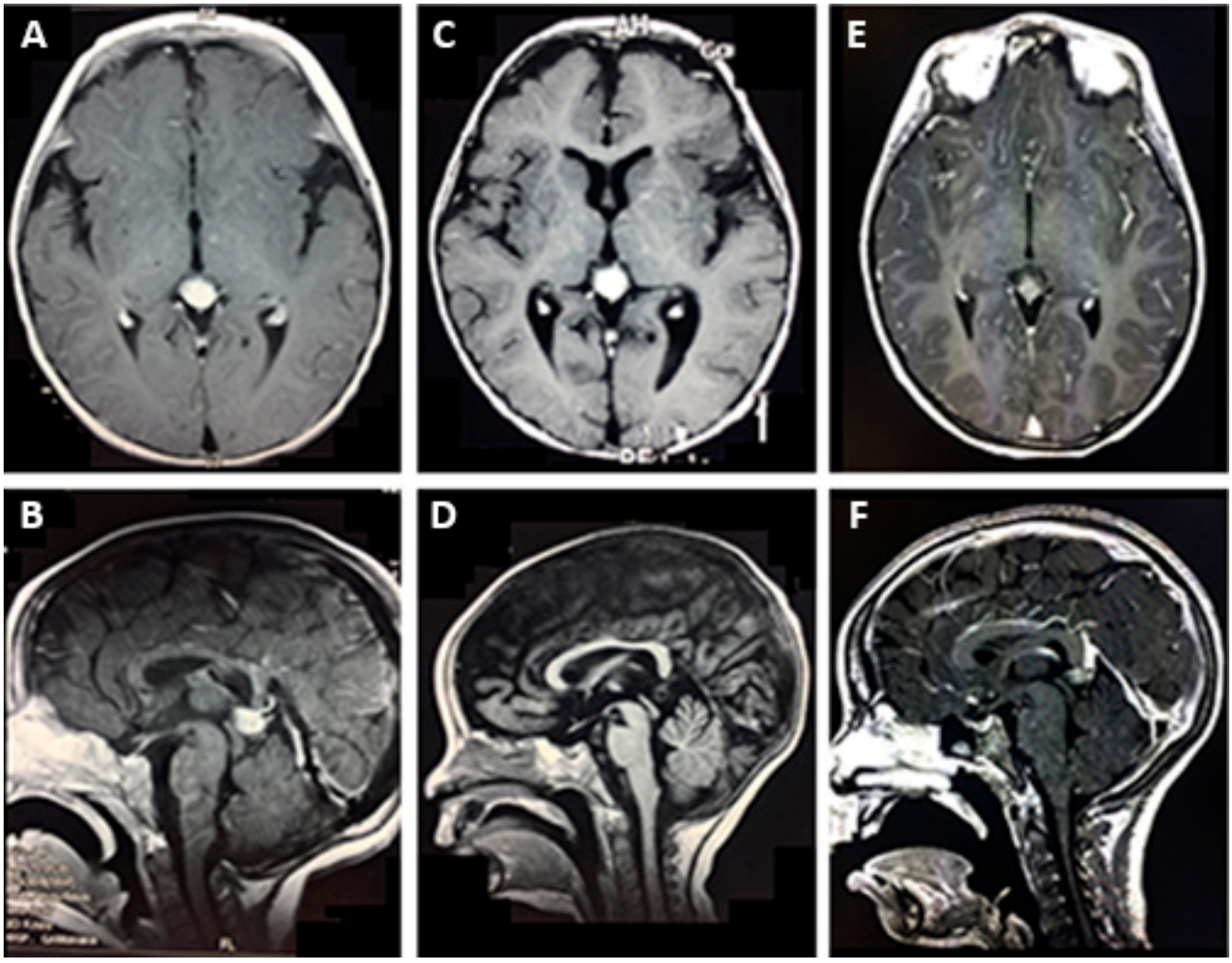


**Figure 1** (A–F) MRI of patient 5. (A–B) MRI at diagnosis of TRb. Contrast-enhanced T1-weighted images demonstrate the enlarged pineal gland with nodular homogeneous enhancement size:12 mm in maximum diameter. (C–D) MRI after induction chemotherapy. Contrast-enhanced T1-weighted images demonstrate the enlarged pineal gland without significant changes compared with diagnostic images. (E–F) MRI of the most recent evaluation, 7 years after the diagnosis. Contrast-enhanced T1-weighted images demonstrate pineal lesion decreased in size (size: 7.8 mm in maximum diameter) with relative decrease in the intensity of contrast enhancement.

of disease for 84 months. The size of her pineal mass reduced over time measuring a maximal diameter of 7.8 mm in the last follow-up (figure 1).[20] One patient had severe complications during the biopsy and her parents declined further therapy. She died of leptomeningeal dissemination 6 months thereafter. The remaining patient was treated with moderate dose of systemic and intrathecal chemotherapy as per parental request and had CSF dissemination after 24 months of diagnosis and died within 16 days after dissemination.

### Dissemination studies

Only one patient presented malignant cells in the CSF at diagnosis of TRb, the other four patients had negative CSF cytology and MDD at the moment of diagnosis of TRb. BM was not involved in any case and MDD was also negative at all-time points. One patient had MDD in the CSF during follow-up. In this case, MRI imaging studies after completion of therapy showed a residual image of uncertain interpretation. Nine months after this evaluation, overt leptomeningeal relapse occurred in this child. The remaining patients were also negative during response to treatment evaluation (table 1).

There were two patients who had a leptomeningeal relapse with positive cytology for Rb. In these cases, malignant cells were positive for *GD2* synthase and/or *CRX*. In one case, CSF and BM evaluation at the moment of relapse was not performed because of parents' choice. BM was negative for MDD at the moment of CSF relapse in all three patients studied (table 1).

Four patients died of progression-relapse of the disease and one patient (described above) is alive with a follow-up of 84 months after the diagnosis of TRb (table 1).

## DISCUSSION

As opposed to our findings in metastatic Rb, where systemic dissemination was present in a majority of patients at diagnosis, there was no evidence of clinical or molecular dissemination at the time of the diagnosis of TRb.[16] The pattern of MDD that we observed in patients with TRb at diagnosis in our study was more comparable with that seen in relapsed Rb in the CSF where the tumour manifestations were limited to the CSF compartment without concomitant systemic dissemination.[21] Even though clinical observations usually report no systemic dissemination in these cases, experimental reports of other paediatric CNS

tumours show that occult molecular systemic dissemination may contribute to CNS dissemination.[22] However, this does not seem to be the case in TRb as no systemic evidence of MDD was seen in our five patients with TRb at diagnosis and also at the event of relapse in three of our patients. This suggests that in cases of TRb, failure to control leptomeningeal dissemination acting as a sanctuary site was the most likely the cause of relapse in our patients coincident with previous clinical reports.[7 12 23] Current strategies for the treatment of TRb are focused on the use of high-dose systemic induction chemotherapy, with variable use of CNS-directed therapies followed by myeloablative chemotherapy with stem cell rescue, in order to achieve a maximum effect in this chemo sensitive tumour.[8] Although this strategy leads to high response rates, there are still an important percentage of patients (56% and 43%, patients with pineal and non-pineal lesion) that succumb.[4] In our series, four of five children had leptomeningeal relapse and died. Leptomeningeal involvement at diagnosis is a poor prognostic factor and leptomeningeal relapse is virtually incurable.[4] The blood-brain barrier limits the penetration of chemotherapy drugs to the CNS, probably being a major determinant of treatment failure in these cases. The chemotherapy doses used in those strategies are already maximal in terms of toxicity, so it is not possible to increase them in order to improve results. Our study shows that since there is no detectable systemic disease at any time during TRb disease course, one way of delivering chemotherapy with potential activity to the compartment where most of the relapses occur would be intraventricular or intrathecal chemotherapy. Intraventricular chemotherapy has been used for TRb, with encouraging results in at least two reports.[13 15] Topotecan and thiotepa, two drugs with proven activity for Rb, have been used by this route and may become possible options.[13] As we found for metastatic and non-metastatic Rb, a positive MDD test in the CSF during follow-up in an asymptomatic patient may herald the occurrence of overt relapse also in TRb.[21] The use of our molecular markers also helped to confirm the malignant nature of the CSF cells, since the specificity of conventional cytology in asymptomatic patients with a positive CSF has not been studied.

It is noteworthy that the only surviving patient had localised TRb resistant to chemotherapy but despite the solid appearance of the pineal mass on the MRI (figure 1), it was not biopsied, so its malignant nature could not be confirmed. However, tumour size decreased gradually from an initial maximal diameter of 12 mm to the current diameter of 7.8 mm after an experimental immunotherapy treatment. This immunotherapy was designed to induce an immune response against the N-glycolil GM3 ganglioside and it was already tested in phase I study in patients with neuroblastoma.[20] We have previously demonstrated that Rb cells showed important amount of this ganglioside.[24] Immunotherapy is under consideration for other brain tumours, so we could not rule out the possibility of a contribution of the immune system in limiting intracranial tumour progression in this case.[25]

In conclusion, in the TRb cases studied, leptomeningeal dissemination without concomitant molecular systemic dissemination was the principal cause of death, and MDD detection might herald leptomeningeal relapse.

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**Contributors** AVT and GLC conceived and designed the study. AVT and VEL developed and validated the molecular biology technique. AVT, VEL, RA, JR and IS processed the samples. CS and DFA participated in subject enrolment and sample collection. SI and CS drafted the work and revising it critically for important intellectual content. AVT, DFA and GLC designed the study and wrote the paper. All authors contributed to the review of the literature, in drafting the manuscript and in approving the final manuscript.

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**Patient consent** Guardian consent obtained.

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