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The Ganglioside, G_{D2}, as a Circulating Tumor Biomarker for Neuroblastoma

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Abstract

Background—G_{D2} is a ganglioside that is ubiquitously expressed in the plasma membrane of neuroblastoma and is shed into the circulation.

Procedure—G_{D2} was measured with a high-pressure liquid chromatography/tandem mass spectrometry assay in serum or plasma from 40 children without cancer (controls) and in biobanked samples from 128 (73 high-risk) children with neuroblastic tumors at diagnosis, 56 children with relapsed neuroblastoma, 14 children with high-risk neuroblastoma after treatment, and 8 to 12 children each with 10 other common childhood cancers at diagnosis.

Results—The C_{18} (18 carbon fatty acid) lipoform was the predominant circulating form of G_{D2} in controls and in patients with neuroblastoma. The median concentration of G_{D2} in children with high-risk neuroblastoma at diagnosis was 167 nM (range, 16.1–1060 nM), which was 30-fold higher than the median concentration (5.6 nM) in controls. G_{D2} was not elevated in serum from children with the differentiated neuroblastic tumors, ganglioneuroma (n=10) and ganglioneuroblastoma-intermixed subtype (n=12), and in children with 10 other childhood cancers. G_{D2} concentrations were significantly higher in serum from children with *MYCN* amplified tumors (p=0.0088), high-risk tumors (p<0.00001), INSS stage 4 tumors (p<0.00001), and in children who died (p=0.034).

Conclusions—Circulating G_{D2} appears to be a specific and sensitive tumor biomarker for high-risk/high-stage neuroblastoma and may prove to be clinically useful as a diagnostic or prognostic circulating tumor biomarker. G_{D2} will be measured prospectively and longitudinally in children enrolled on a high-risk neuroblastoma treatment trial to assess its ability to measure response to treatment and predict survival.

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Keywords

Neuroblastoma; ganglioside; biomarker

INTRODUCTION

Circulating tumor biomarkers (CTBs) are essential tools in clinical practice and clinical research.[1] Clinically, CTBs are useful for screening or early detection of cancers, for diagnosis and determining prognosis, and for predicting response to treatment, monitoring response and detecting relapse or tumor progression. Research applications of CTBs parallel their clinical uses (e.g., prognostic CTBs are used to select or stratify subjects on randomized clinical trials), and CTBs that are used as clinical trial endpoints should have demonstrated clinical relevance.

G_{D2} is a ganglioside (sialic acid-containing glycosphingolipid, Fig. 1) that is found primarily on neuronal cells[2] and is also expressed on neuroblastoma tumor cells.[3,4] G_{D2} has a lipid domain (ceramide) that inserts into the plasma membrane and an extracellular glycan domain that contributes to the glycocalyx coating the cell surface. The fatty acid chain of ceramide varies in length resulting in multiple G_{D2} lipoforms. The glycan domain of G_{D2} is the target of dinutuximab, which is a therapeutic, chimeric monoclonal antibody approved by the US Food and Drug Administration for treating high-risk neuroblastoma.[5]

G_{D2} is detected on >90% of neuroblastoma specimens by immunohistochemistry,[3] and is shed from tumor cells into the circulation of patients with neuroblastoma.[6,7] Higher circulating G_{D2} concentrations were associated with higher stage, more rapid tumor progression and poorer survival.[7] In these studies, G_{D2} was measured by thin layer chromatography, which is not amenable to rapidly quantifying G_{D2} in large sample sets. We developed and validated a high-pressure liquid chromatography/tandem mass spectrometry (HPLC/MS/MS) assay for the predominant lipoforms of G_{D2} in plasma and serum,[8] and measured G_{D2} in archived serum samples from children with an array of neuroblastic tumors at diagnosis and relapse. This assay method is adaptable for clinical use. In addition, serum samples from children with other common childhood cancers and children without cancer were studied. Our ultimate goal is to determine whether circulating G_{D2} could serve as a clinical trial endpoint to measure tumor burden and response to treatment or to predict relapse and survival outcomes.

METHODS

Patient Samples

We measured the concentration of G_{D2} in serum or plasma samples that were previously collected and stored in biospecimen repositories for the childhood cancers listed below. The children who provided the samples that were used in this study were enrolled on a variety of IRB-approved Children's Oncology Group (COG) biology or treatment protocols unless specified.

- Serum samples from 128 children with neuroblastic tumors were provided through the COG's Biopathology Center. Samples were collected at diagnosis from patients with ganglioneuroma (GN, n=10), ganglioneuroblastoma, intermixed subtype (GNB-i, n=12), ganglioneuroblastoma, nodular subtype (GNB-n, n=16), and neuroblastoma/ neuroblastic tumor (NBL, n=90). Twenty-nine were from patients with low risk tumors (10 GN, 9 GNB-i, 3 GNB-n, and 7 NBL), 13 had intermediate risk tumors (3 GNB-i and 10 NBL), and 86 had high-risk tumors (13 GNB-n and 73 NBL) according to the COG classification system. *MYCN* was amplified in 40 of the 73 high-risk NBL samples and non-amplified in 33. International Neuroblastoma Staging System (INSS) stage was stage 1 in 18, stage 2A in 5, stage 2B in 15, stage 3 in 8, stage 4 in 80 and stage 4S in 2.
- Serum samples from 56 children who had relapsed NBL and who were enrolled on the COG phase 2 trial ANBL1221.
- Plasma samples from 14 children with high-risk NBL receiving post-consolidation immunotherapy with dinutuximab after completing induction and consolidation therapy were collected at the Children's Hospital of Philadelphia (CHOP). Patients were enrolled on an institutional IRB-approved protocol, and samples were collected prior to starting a course of dinutuximab.
- Serum samples from children with other common childhood cancers were provided through the COG's Biopathology Center and included samples from 8 children each with Wilms tumor, non-Hodgkin lymphoma, Hodgkin lymphoma, hepatoblastoma, rhabdomyosarcoma, Ewing sarcoma, and acute myelogenous leukemia.
- Serum samples from 8 children each with medulloblastoma and high-grade glioma were provided by the Children's Brain Tumor Tissue Consortium (CBTTC). Patients were enrolled on the CBTTC's biospecimen protocol.
- Plasma samples from 12 children with osteosarcoma were collected at CHOP. Patients were enrolled on an IRB-approved protocol and samples were collected prior to starting treatment.

The clinical laboratory at CHOP provided de-identified serum samples left over from clinical tests on 40 children without cancer to serve as a control sample set.

G_{D2} Assay Method

The concentrations of the 18 and 20 fatty acid carbon chain length G_{D2} lipoforms (C_{18} and C_{20}) were measured using a previously reported, validated high-pressure liquid chromatography, tandem mass spectrometry method with a lower limit of quantification for the C_{18} and C_{20} lipoforms of 3 nM.[8] The method was validated in serum and plasma. Human brain-derived G_{D2}, which is made up of approximately 60% C_{20} and 40% C_{18} lipoforms, was used to construct the standard curves for the assay. In serum/plasma from control samples and samples from children with NBL, the C_{18} lipoform was the predominant form of G_{D2}.

Data Analysis and Statistics

Median, range and interquartile range were used to describe the G_{D2} concentrations in the various subsets of samples listed above. Non-parametric methods were used to compare the G_{D2} concentrations in subsets of samples from children with NBL based on risk group, stage, *MYCN* status and survival (Mann-Whitney) and correlate the concentrations of the C_{18} and C_{20} lipoforms of G_{D2} in samples from children with stage 4 NBL (Spearman correlation).

RESULTS

Table 1 lists the G_{D2} serum concentrations of the C_{18} lipoform in control samples from 40 children without cancer and in 128 pre-treatment samples from children with neuroblastic tumors. The concentration of the C_{18} lipoform of G_{D2} was below the lower limit of quantification (<3 nM) in 16 of the 40 control samples, and ranged up to 15.5 nM in the remaining control samples. The C_{20} lipoform was measurable in only 3 of 40 control samples at concentrations <5 nM.

The C_{18} lipoform was also the predominant form of G_{D2} in samples from children with neuroblastic tumors. G_{D2} concentrations were elevated in these serum samples, and concentrations were related to histology and INSS tumor stage (Table 1). In samples from children with GN and GNB-i subtype, the G_{D2} concentrations were within the range of the control samples, but G_{D2} was elevated in the pretreatment serum samples from children with GNB-n subtype and with NBL. The highest concentrations were in samples from children with metastatic (INSS stage 4) disease. The median concentration of the C_{18} lipoform of G_{D2} in the samples from children with stage 4 NBL was 30-fold higher than the median concentration in control samples.

The C_{20} lipoform was measurable in 65 of the 69 serum samples from children with stage 4 NBL, but the concentrations of the C_{20} lipoform were substantially lower (median, 15.9 nM; range, <LLQ to 68.1 nM) than the concentrations of the C_{18} lipoform. There was a correlation between the concentrations of the C_{18} and C_{20} lipoforms (R=0.81, Supplemental Fig. 1), but the ratio of the concentrations of the C_{18} to C_{20} lipoforms in children with stage 4 NBL ranged from 2.9 to 35 (median, 12).

As shown in Fig. 2, G_{D2} concentrations were significantly higher in serum from children with INSS stage 4 disease compared to stage 1–3 NBL ($p<0.0001$) and with high-risk vs. non-high-risk NBL ($p<0.0001$). In the subset of samples from children with high-risk NBL, G_{D2} concentrations were significantly higher in samples from patients whose NBL was *MYCN* amplified ($p=0.0088$) and in those who did not survive ($p=0.034$).

G_{D2} concentrations were also elevated in samples from 56 children with relapsed NBL, but at lower levels than in samples from initial diagnosis (Table 1). G_{D2} concentrations in 10 of the 11 samples taken prior to the first dose of dinutuximab from children who were in remission and scheduled to receive immunotherapy were within the control range and one sample was slightly elevated at 21.1 nM.

The serum G_{D2} concentrations in samples from children with other common types of childhood cancers are listed in Table 2. All samples from children with non-CNS cancers fell within the range of the control samples for both lipoforms (C_{18} and C_{20}). However, G_{D2} concentrations exceeded the upper range of the controls in 3 of 8 samples (maximum concentration, 35 nM) from children with high-grade gliomas and 7 of 8 samples (maximum concentration, 111 nM) from children with medulloblastoma.

DISCUSSION

An ideal CTB is produced by tumor cells and shed into the circulation or other accessible body fluid, can be accurately measured using small specimens with a cost-effective, sensitive and specific assay, reflects tumor burden and response to treatment, detects tumors at early or preclinical stages and detects minimal residual disease after treatment, and is low or undetectable in blood/body fluids of controls. G_{D2}, which is measurable in the circulation at concentrations higher than controls in all of the serum samples from children with high-risk and high-stage NBL, has many of the attributes of an ideal CTB in this retrospective study using archived serum samples. Serum G_{D2} concentration appears to clearly discriminate between high-risk/high-stage NBL and low-risk, more differentiated (GN, GNB-i) tumors. This is consistent with previous reports showing that immunohistochemical staining intensity for G_{D2} using 3A7 monoclonal antibody is dependent on the degree of differentiation.[4] Staining is most intense in stroma poor, undifferentiated NBL, and scant G_{D2} staining is seen on GNs.

Serum G_{D2} concentrations in the 73 serum samples from children with high-risk NBL were variable (range, 16.1 to 1060 nM for the C_{18} lipoform). Possible tumor determinants of the circulating G_{D2} concentration include overall tumor burden, level of G_{D2} expression on the cell surface, tumor growth rate, and the extent of tumor necrosis. The relationship of circulating G_{D2} concentration to tumor burden at diagnosis will be studied prospectively.

Our results suggest that G_{D2} may have utility in the clinical setting as a diagnostic and prognostic CTB. G_{D2} was not elevated in the serum of children with other common, non-CNS childhood cancers (including osteosarcoma, which expresses G_{D2} on the tumor cell surface[4]), indicating that G_{D2} is specific for NBL. In addition, G_{D2} serum concentrations were significantly higher in children with high-risk, high-stage and *MYCN*-amplified NBL. G_{D2} concentrations were also higher in samples from children who had high-risk NBL and who died. Rigorous prospective studies are required to validate a new prognostic tumor biomarker.[9]

Serum G_{D2} was elevated in children with medulloblastoma, albeit to a lesser extent than in children with NBL. G_{D2} has been previously shown to be expressed on the cell surface of most high-grade gliomas and medulloblastomas.[10] Serum G_{D2} is not likely to be a sensitive CTB for medulloblastoma based on the limited number of samples studied, but we plan to measure G_{D2} in the cerebrospinal fluid of patients with medulloblastoma to determine whether it can detect and quantify leptomeningeal tumor spread.

The promising results of this retrospective study will be validated by longitudinally monitoring plasma G_{D2} over the course of treatment in COG clinical trials enrolling children with newly diagnosed NBL. This platform should also allow us evaluate G_{D2} as a potential clinical trial endpoint to measure tumor burden at diagnosis and response to treatment and to predict relapse or survival. CTBs that are validated clinical trial endpoints of treatment outcomes can improve the accuracy and sensitivity of assessing tumor response and could substantially shorten the time line of phase 3 trials if they are predictive of subsequent relapse or survival.

CTBs have potential advantages over tumor measurements from serial imaging studies in assessing tumor burden, response to treatment and monitoring for recurrence:

- CTBs that reflect tumor burden can provide a more objective continuous measure of change in total tumor burden compared to response categories (CR, PR, etc.) derived from time consuming tumor measurements taken from selected lesions that are >1 cm in diameter on imaging studies.
- CTBs are more cost effective and more convenient.
- CTBs avoid radiation exposure and risks of sedation/anesthesia.
- CTBs can be measured more frequently and do not require advanced scheduling.
- Sensitive CTBs can quantify response in patients with evaluable (e.g., MIBG positive) or undetectable disease on imaging studies and in matrix-producing tumors, which are difficult to assess by imaging.

G_{D2} is the ligand for the therapeutic monoclonal antibody, dinutuximab, and circulating G_{D2} binding to dinutuximab in plasma could block the antibody's therapeutic effect or potentially increase its toxicity through systemic complement activation. The previously reported association constant (K_A) of dinutuximab for G_{D2} is high (3.5×10^8 M^{-1} or 0.35 nM^{-1}).[11] Using this K_A, we estimated that 200 nM G_{D2} would saturate >95% of dinutuximab binding sites (assuming two binding sites per antibody molecule) at the peak dinutuximab concentration of 10 μg/mL (~70 nM) after a standard course of 17.5 mg/m^2 daily × 4 doses.[12] This G_{D2} concentration is equivalent to the median concentration in the samples from patients with stage 4, high-risk NBL, at diagnosis. We measured G_{D2} concentrations in plasma from 14 patients receiving post-consolidation therapy with dinutuximab, and G_{D2} concentrations were in the control range in all but 1 patient. The use of dinutuximab in up front induction regimens when circulating G_{D2} concentrations are likely to be higher could impact dinutuximab's efficacy, if its binding sites are occupied by circulating G_{D2}.

The use of archived serum samples for this initial study of circulating G_{D2} allowed us to efficiently establish G_{D2}'s potential as a CTB for NBL, but the retrospective nature of the study has limitations.[13] Serum samples used in this study had been stored for varied lengths of time, and this could have contributed to variability in the serum G_{D2} concentrations. The serum samples were collected on the COG NBL biology protocol (ANBL00B1), and the patients were not treated uniformly, which limits our ability to assess its prognostic value. The available clinical information, such as measures of tumor burden at diagnosis, was also limited. It is also possible that some specimens were collected after surgical resection of the

primary mass in some low risk patients, and this could result in underestimation of the serum G_{D2} concentration. Prospective studies to validate our observations and study the clinical utility and research applications of circulating G_{D2} must be conducted, but may take up to a decade to complete. This study demonstrates the value of performing the initial evaluation of a CTB in archived samples for rare cancers like NBL.

Circulating G_{D2} appears to be a specific and sensitive CTB for high-risk/high-stage NBL. Our retrospective results will be validated by prospectively and longitudinally monitoring circulating G_{D2} over the course of frontline therapy on the current COG high-risk NBL clinical trial (ANBL1531) to further evaluate its potential clinical utility and its role as a clinical trial endpoint in quantifying tumor burden, monitoring response to therapy, detecting recurrence and predicting survival. We are also more precisely defining G_{D2} reference ranges in infants and young children without cancer and studying the pharmacokinetics of G_{D2} in an animal model.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Serum samples from children who had relapsed neuroblastoma and who were enrolled on ANBL1221 were provided from Alice Yu's laboratory from the University of California, San Diego; and serum samples from children with acute myelogenous leukemia were provided from Terzah Horton's laboratory at Baylor College of Medicine, Houston.

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Abbreviations

CBTTC	Children's Brain Tumor Tissue Consortium
CHOP	Children's Hospital of Philadelphia
COG	Children's Oncology Group
CTB	Circulating tumor biomarker
GN	ganglioneuroma
GNB-i	Ganglioneuroblastoma, intermixed
GNB-n	Ganglioneuroblastoma, nodular
INSS	International Neuroblastoma Staging System
IRB	Institutional Review Board
K_A	Association constant

LLQ	Lower limit of quantification
NBL	neuroblastoma/ neuroblastic tumor

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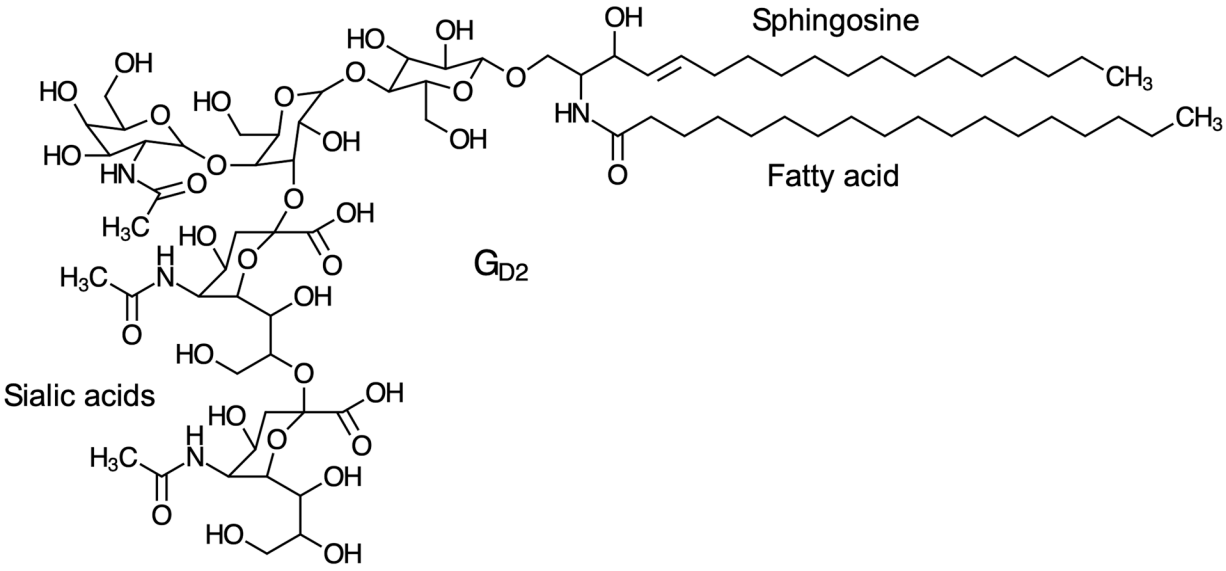

Fig 1.

Chemical structure of the C_{18} lipoform of G_{D2}. The lipid domain, ceramide, is composed of sphingosine and a fatty acid with variable chain lengths. The glycan domain includes 2 sialic acids.

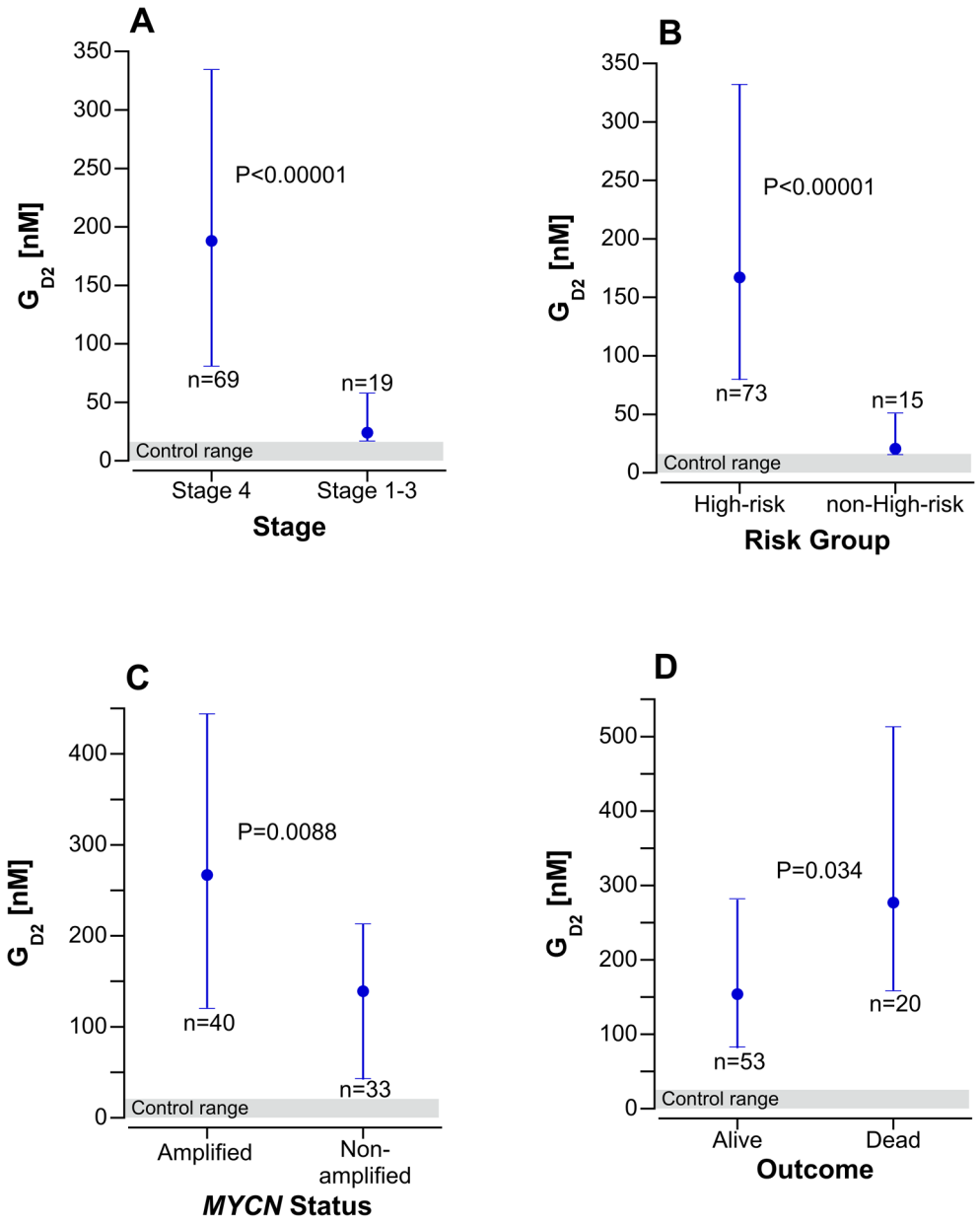

Fig 2.

Serum G_{D2} concentrations at diagnosis in children with neuroblastoma by stage (A), risk group (B), *MYCN* status (C) and survival outcome (D). The points are the median value and the error bars are the interquartile range. Panels A and B include 88 children with the histological diagnosis of neuroblastoma, peripheral neuroblastic tumor, NOS, or neuroblastic tumor, NOS, excluding the 2 children with stage 4S neuroblastoma. Panel C and D includes only 73 children with high-risk NBL. The shaded area at the bottom of each graph is the serum G_{D2} concentration range in 40 control subjects.

TABLE 1.

G_{D2} concentrations in control samples and samples from children with neuroblastic tumors

Group	N	C_{18} Lipoform of G_{D2} [nM]		
		Median	Range	Interquartile Range*
Controls	40	5.6	<LLQ – 15.5	<LLQ – 9.0
Ganglioneuroma	10	4.1	<LLQ – 9.4	<LLQ – 5.2
Ganglioneuroblastoma – Intermixed	12	6.4	<LLQ – 15.3	4.1 – 9.0
Ganglioneuroblastoma – nodular	16	39.1	<LLQ – 258	8.4 – 136
GNB–n, stage 1–3	5	6.0	<LLQ – 167	
GNB–n, stage 4	11	51.4	8.6 – 258	22.7 – 175
GNB–n, high risk	13	47.7	4.5 – 258	13.0 – 140
Neuroblastoma (NBL)	90	147	7.8 – 1060	45.4 – 300
NBL by stage				
Stage 1–2	14	21.3	7.8 – 880	15.4 – 51.1
Stage 3	5	35.5	16.8 – 330	
Stage 4	69	188	16.1 – 1060	82.0 – 333
Stage 4S	2		51.5 – 262	
NBL by risk group (excluding 4S)				
Low risk	6	21.3	7.8 – 880	
Intermediate risk	9	20.6	7.9 – 68.3	15.4 – 51.1
High risk	73	167	16.1 – 1060	79.8 – 330
Relapsed neuroblastoma	56	28.2	3.0 – 389	16.3 – 47.4
NBL post-consolidation	11	8.2	3.0 – 21.1	5.8 – 11.2

*Interquartile range derived for datasets with at least 7 samples

TABLE 2.

G_{D2} concentrations in samples from children with other childhood cancers

		C_{18} Lipoform of G_{D2} [nM]		
Group	**N**	**Median**	**Range**	**Interquartile Range**
Controls	40	5.6	<LLQ – 15.5	
Medulloblastoma	8	34	6 – 111	25 – 42
High-grade glioma	8	11	<LLQ – 35	
Osteosarcoma	12	3.0	<LLQ – 13	
Ewing sarcoma	8	<LLQ	<LLQ - <LLQ	
Rhabdomyosarcoma	8	4.5	<LLQ – 10	
Wilms tumor	8	6.5	<LLQ – 8.0	
Hepatoblastoma	8	6.5	<LLQ – 10	
Hodgkin lymphoma	8	<LLQ	<LLQ – 6.0	
Non-Hodgkin lymphoma	8	<LLQ	<LLQ – 7.0	
Acute myelogenous leukemia	8	<LLQ	<LLQ - <LLQ	