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Recommendations for the standardization of bone marrow disease assessment and reporting in children with neuroblastoma; on behalf of the International Neuroblastoma Response Criteria Bone Marrow Working Group

Running title: INRC BMWG Consensus Assessment Criteria

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Condensed abstract: Practical transferable recommendations to standardize quantitative reporting of bone marrow disease in children with neuroblastoma provided by the multidisciplinary INRC Bone Marrow Working Group. Wide adoption of these harmonized criteria will enhance the ability to compare outcomes from different trials and facilitate collaborative trial design.

Key words: neuroblastoma, bone marrow, quantitative, consensus, aspirates, biopsies, immunohistochemistry, immunocytology, RTqPCR.

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Author Contribution Statements:

Susan A Burchill is the corresponding author, responsible for overall content, contributed to the concept and design of the study, collection and assembly of data, data analysis and interpretation, by writing and approving the manuscript for submission.

Klaus Beiske contributed by collection and assembly of data, data analysis and interpretation, by writing and approving the manuscript for submission.

Hiroyuki Shimada contributed to data analysis and interpretation, by writing and approving the manuscript for submission.

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Michelle Haber contributed to data analysis and interpretation, by writing and approving the manuscript for submission.

Julie R Park contributed to the concept and design of the study, collection and assembly of data, data analysis and interpretation, by writing and approving the manuscript for submission.

Frank Berthold contributed to study design and concept, data analysis and interpretation, by writing and approving the manuscript for submission.

Abstract

Background: To expedite international standardized reporting of bone marrow disease in children with neuroblastoma, to improve equivalence of care.

Methods: A multidisciplinary International Neuroblastoma Response Criteria Bone Marrow Working Group was convened by the National Cancer Institute in January 2012 with representation from Europe, North America and Australia. Practical transferable recommendations to standardize reporting of bone marrow disease were developed.

Results: Consensus criteria for the collection, analysis and reporting of the percentage area of bone marrow parenchyma occupied by tumor cells in biopsies/trephines is comprehensively provided for the first time. The quantitative analysis of neuroblastoma content in bone marrow aspirates by immunocytology (IC) and reverse transcriptase quantitative polymerase chain reaction (RTqPCR) are revised. The inclusion of PHOX2b for IHC and RTqPCR is recommended. Recommendations for recording bone marrow response are provided. We endorse the quantitative assessment of neuroblastoma cell content in bilateral core biopsies/trephines and aspirates in all children with neuroblastoma, with the exception of infants where evaluation of aspirates alone is advised. Notably 5% disease is accepted as an internationally achievable level for disease assessment.

Conclusion(s): Quantitative assessment of neuroblastoma cells is recommended to provide data from which evidence-based numerical criteria for the reporting of bone marrow response can be realised. This is particularly important in the minimal disease setting and when neuroblastoma detection in bone marrow is intermittent, where clinical impact has yet to be validated. Wide adoption of these harmonized criteria will

enhance the ability to compare outcomes from different trials and facilitate collaborative trial design.

Introduction

Neuroblastoma is the most common extra-cranial solid tumor in children, and accounts for 10-15% of all cancer deaths in the first 15 years of life. Metastatic disease at diagnosis is a powerful predictor of poor outcome and is used in the International Neuroblastoma Risk Group (INRG) Staging System to select treatment for children at diagnosis.[1,2] Bone marrow is the most common site of infiltration in children presenting with metastatic disease at diagnosis,[2] and a frequent site for relapse and disease recurrence.[3,4] Persistence of neuroblastoma disease in bone marrow is predictive of poor outcome,[5,6,7] and provides a means to assess disease response without having to wait for the development of greater tumor burden.[8,9,10] Thus making it attractive as part of clinical response criteria.

Cytology of aspirates and histology of biopsies has been the gold standard to assess neuroblastoma disease in bone marrow for many years.[1,2,11] However these methods have limited sensitivity when neuroblastoma contamination is less than 10%, and could seriously underestimate the prevalence of bone marrow infiltration.[3,12] Significant improvements in the sensitivity and specificity of neuroblastoma cell detection in bone marrow aspirates have been made such that it is now possible to unambiguously detect a single neuroblastoma cell in one million normal cells using immunocytology (IC) or reverse transcriptase quantitative polymerase chain reaction (RTqPCR).[13] Furthermore these quantitative methods have shown that the level of neuroblastoma cells detected by IC[5,14] or RTqPCR[15,16,18] in bone marrow is predictive

of outcome, paving the way for their introduction into clinical practice. Although consensus criteria for the detection of neuroblastoma cells in bone marrow aspirates have previously been described,[13] there is no comparable published guidance on evaluation of bone marrow biopsies. So that the definition of bone marrow response is consistent across international studies, an International Neuroblastoma Response Criteria Bone Marrow Working Group (INRC BMWG) was convened to define consensus criteria for the standardized detection and reporting of bone marrow biopsies, and to review the criteria for analysis of aspirates building on previous international experience and exploiting new knowledge.

Methods

The INRC BMWG was assembled, with representation from Europe, North America and Australia, as a component of the Neuroblastoma Clinical Trials Planning Meeting held in April 2012 in Washington DC, supported by the National Cancer Institute. Experts from the INRC BMWG presented new data on neuroblastoma cell detection using immunohistochemistry (IHC), IC and RTqPCR from the Society International of Oncology Pediatric-Neuroblastoma (SIOPEN), German Society for Pediatric Oncology and Hematology (GPOH), Children's Oncology Group (COG) and New Approaches to Neuroblastoma Therapy (NANT) cooperative groups. Between January 2012 and September 2014 the INRC BMWG of multidisciplinary experts in pediatric oncology, pathology, translational biology and statistical methods reviewed the literature, shared, sought and examined unpublished data and opinion, participating in over 35 teleconference calls.

Recommendations for analysis and reporting of neuroblastoma status in bone marrow

Sample collection, preparation for analysis and storage

Bone marrow samples from at least 2 different sites should be analyzed, usually from the right and left iliac crest.[13,17] We recommend collection of representative bilateral core biopsies for histology/IHC and bilateral bone marrow aspirates for cytology, IC and RTqPCR from all children at diagnosis, and for high-risk children at the time of response assessment at the end of induction therapy; additional time points may be specified according to trial-specific protocols. We advocate the analysis of biopsies and aspirates by central reference laboratories where disease infiltration is less than or equivalent to 5%. In very young or small infants core biopsies are not recommended, as the size and quality of the biopsy is unlikely to be adequate for robust analysis. Where feasible, we suggest that the histology/IHC of bone marrow biopsies and cytology/IC/RTqPCR of bone marrow aspirates are provided in a combined report so that any concordance or discordance is revealed.

Bone marrow aspirations from different sites should be performed using separate syringes, aspirates should not be pooled so that the heterogeneity of neuroblastoma cell infiltration can be recorded and to avoid underestimating the extent of bone marrow disease. From the first aspiration (0.1–0.3ml), 5-10 smears of aspirate from each site should be prepared, air-dried and stained with Pappenheim or modified Wright stain for initial staging using cytological examination by light microscopy. From the next 3-5ml of each sample, 0.5ml of each aspirate should be transferred immediately into RNA preserving tubes such as PAXgene^{TM} blood RNA tubes for the

extraction of RNA and RTqPCR. Samples in PAXgene™ blood RNA tubes can be stored at -80℃ for up to 5 years or at room temperature for up to 3 da ys prior to RNA extraction.[18] The remaining aspirate is transferred into anticoagulant such as ethylenediaminetetraacetic acid (EDTA) or heparin, and cells isolated using density gradient centrifugation for the preparation of cytospins for IC; cytospins should be prepared within 24 hours of aspirate collection (maintained at +4℃ to +8 ℃) and can be stored at -20℃ until analyzed. [13]

Bone marrow core biopsies should be placed immediately in fixative and decalcified. We recommend fixation in 4% buffered formalin for 18-24 hours, and decalcification by incubating in 12.5% EDTA at pH 7.0 for 4-6 hours to preserve morphology and antigenicity. The fixed, decalcified biopsy should be embedded in paraffin, and a minimum of five slides mounted with 2-3 sections of 4µm prepared. A minimum of 2 slides should be stained with hematoxylin and eosin (H&E) for histology, the remaining unstained slides can be used for IHC which is recommended.

Bone marrow quality

Only bone marrow samples of suitable quality should be investigated for diagnostic and prognostic purposes. If the sample is inadequate we recommend a repeat aspiration or biopsy, and reanalysis.

A bone marrow smear is considered representative and suitable for quantitative reporting of disease when there is greater than 5% tumor cell infiltration. When infiltration with tumor is less than or equal to 5%, then three of the following four criteria must be fulfilled to allow discrimination between no disease or minimal disease (i)

presence of particles with stromal cells e.g. histiocytes, fibroblasts or osteoblasts, (ii) presence of megakaryocytes, (iii) the erythroblasts exceed 20% of the nucleated cells, and (iv) peripheral blood cells are within the range for age. i.e. the mature granulocytes and lymphocytes do not exceed 65% in infants (<1 year of age) or 50% in children >1 year of age. If these criteria are not met, this should be detailed when reporting on analysis of the smear.

Cytospins are prepared from mononuclear cells (MNCs) of the bone marrow and do therefore not contain histiocytes and fibroblasts. Macrophages, granulocytes, megakaryocytes and erythroblasts may be maintained in the MNC fraction at levels which are variable and always lower than in a bone marrow smear. Notably, erythroblasts never exceed 20% of all nucleated cells in a representative cytospin. Therefore, the above listed quality criteria for bone marrow smears do not apply to bone marrow cytospins.

The routine aspiration of larger volumes is not recommended to avoid dilution of the bone marrow with blood, which will reduce the sensitivity of analyses. Cells with disrupted cellular or nuclear membranes should not be analyzed.

For RTqPCR each aspirate should yield a minimum of 400ng of RNA, which has an optical density reading A_{260}/A_{280} ratio of >1.5 and <3. Amplification of cDNA, generated from 100ng of RNA, for a house-keeping gene such as β_2-microglobulin should produce a cycle threshold (Ct) <25. Where there is insufficient aspirate to complete all analyses, the priority for investigations is cytology, followed by RTqPCR and finally IC.

An optimal bone marrow core biopsy should preferably contain red bone marrow parenchyma at a minimum length of 1cm. This recommendation is derived from previous published work[19] and has since been supported by longstanding experience. The amount of hematopoietic and tumor tissue within the biopsy should be recorded

in mm; cortical bone, cartilage, soft tissue, blood clots or areas that are crushed are excluded from the measurement.

Criteria for analysis and reporting of neuroblastoma cell infiltration in bone marrow core trephine biopsies

- **Histology**

Metastatic tumor infiltration in bone marrow biopsies is estimated as the surface area occupied by peripheral neuroblastic tumor (PNT), as a percentage of the evaluable bone marrow spaces on each side of the biopsy e.g. 0%, ≤5%, >5-<10%, ≥10-<15%, ≥15-<20%, ≥20%-<25%, ≥25%-<30% and so on. Total marrow spaces can include areas of metastatic neuroblastoma, fibrosis and necrosis, hematopoietic components and adipose tissue. The Mitosis-Karyorrhexis Index (MKI) should not be attempted as this is usually not feasible reflecting the limited number of metastatic neuroblastoma cells in bone marrow biopsy specimens.[20] Importantly tumor histology should be classified as poorly differentiated (PD), undifferentiated (UD) or differentiating.[20] In the case of small tumor aggregates the presence or absence of neuropil (a complex network of interwoven cytoplasmic processes of nerve cells and neuroglial cells) detected by IHC for synaptophysin can help to discriminate UD and PD neuroblastoma. In those rare cases where stroma-rich and stroma-poor histology are present within a single biopsy, the amount of stroma-rich and stroma-poor tumor should be recorded as a percentage of the surface area occupied by the tumor (Figure 1).

- **Immunohistochemistry (IHC)**

IHC is frequently employed to improve the precision of neuroblastoma detection in bone marrow biopsies. We encourage IHC of multiple sections (>3 sections) from all biopsies using a minimum of two antibodies; to minimise cost 3 sections might be placed on a single slide with each antibody. Highly specific target antigens for which IHC is unambiguous include synaptophysin, tyrosine hydroxylase, chromogranin A and paired-like homeobox 2b (PHOX2B). Additional frequently used markers include CD56 and PGP9.5.[11,20,21,22,23,24] Where suspected, Schwann cells can be reliably detected by morphology and IHC for the S-100 protein.[20] Less useful markers for the detection of neuroblastoma cells in the bone marrow are neuron specific enolase (NSE) and NB84; NSE because it lacks specificity[25] and NB84 because it is rarely expressed by neuroblastoma cells in the bone marrow.[26] The quality of any immunohistological analysis should be monitored by simultaneous processing of a positive control sample. Relevant controls can be sections from multi-tissue blocks including the adrenal gland or other neuroendocrine tissues; an ideal control would have areas of positive and negative cells.

A bone marrow biopsy is regarded as negative for tumor in the absence of neuroblastoma cell nests detected by H&E staining and IHC, using a minimum of two antibodies to analyse at least 3 sections. A case should only be confirmed negative after assessment of all available sections.

Criteria for analysis and reporting of neuroblastoma cells in bone marrow aspirates

- **Cytomorphology – bone marrow smears**

Smears should be viewed by light microscopy at low (e.g. 60-100x) and high (e.g. 600-1000x) magnification; at low magnification to assess cellular density and search for the presence of large tumor cell nests or clumps, and at higher magnification to identify small tumor cell nests, any potential single neuroblastoma cells and to recognize features of differentiation. Neuroblastoma nests may contain Homer Wright rosettes, with neuroblastoma cells in a characteristic ring around a center of neuropil.

Neuroblastoma cells are typically round and larger than small lymphocytes, with a high nuclear to cytoplasmic ratio. The cell nucleus can be round or oval, with a fine granular chromatin structure (so called “salt and pepper” pattern)(Figure 2). This is not always present, therefore it is not obligatory for the description of neuroblastoma. Cells in nests may range in size from large to intermediate to small, and can be polygonal in shape, producing a so-called “paving stone pattern”. A nest or clump of typical neuroblasts is regarded as the lower threshold for reporting, a nest or clump containing at least 3 neuroblastoma cells (Figure 2). Granules are not visible, although in exceptional circumstances emperipolesis, phagocytosis or inclusion bodies may be detected in neuroblastic cells. Although a rare event, neuroblastoma cells may mature spontaneously or after therapy (Figure 2); if features of differentiation are detected in more than 5% of tumor cells this should be reported as “presence of differentiating neuroblastoma cells”. Single neuroblasts detected by cytology alone cannot unequivocally be identified as neuroblastoma cells, and should not be reported as such (see below).

The evaluated cellularity per slide should be reported as this can impact on the sensitivity of cytology.[27] Single cells alone should not be scored as positive, rather we recommend such samples are reported as suspicious and the presence of neuroblastoma cells is confirmed or refuted following central review and analysis by additional methods such as IC. Where no tumor cells are revealed, this should be recorded as a tumor cell negative bone marrow aspirate.

- **Immunocytology (IC) - cytospins**

Ideally, to reach a sensitivity of 1 neuroblastoma cell in 1 x 10^6 MNCs IC should be reported on 3 x 10^6 MNCs per aspirate[28] using a monoclonal anti-GD_2 disialoganglioside antibody. We endorse the use of clone 14 G2a.[13] Bound antibody can be visualized by light microscopy following enzymatic reaction to produce a stable chromogen, or immunofluorescence when a fluorescent antibody or reporter is employed. Criteria for the reliable light microscopic identification of neuroblastoma cells on immunocytochemically stained slides are published elsewhere and summarized in Table 1.[13] Where immunofluorescence is used we recommend that digital images of positive cells with image acquisition details are stored, since the fluorescence will fade with time. Cytospins of control GD_2 positive cells must be processed in parallel to the test samples to manage any inter-assay variation; these might usefully be bone marrow smears with a moderate to high tumor cell infiltration or cytospins of a neuroblastoma cell line which contains high and low GD_2 expressing cells such as IMR-32. The number of tumor cells and total number of investigated cells should be reported, from which the percentage of tumor infiltration can be calculated.

Reverse Transcriptase quantitative Polymerase Chain Reaction (RTqPCR)

We recommend that RNA extracted from bone marrow aspirates taken at diagnosis is amplified by RTqPCR for the expression of at least the neuroblastoma mRNAs tyrosine hydroxylase (TH) and PHOX2B[9,16,18]. This is most strongly recommended within the minimal disease setting where neuroblastoma bone marrow contamination is ≤5% and heterogeneity of tumor content may have greatest impact. It is highly recommended for children with newly diagnosed high-risk disease at the time of entry into trial and times of response assessment, for example at the end of induction treatment.

RNA should ideally be diluted to a minimum concentration of 40ng/µl, and stored in single use aliquots (such as 10µl) in a –80℃ freezer. We recommend analysis of each RNA sample in triplicate with each replicate containing 100ng of RNA; the amount of RNA analyzed should be stated in the report. The results of RTqPCR must be reported as the cycle threshold (Ct) values so that any discrepancies between biological repeats can be identified, and using $2^{-\Delta Ct}$ or –ΔCt where the expression of the neuroblastoma mRNAs is normalised to the internal control (in this case β_2-microglobulin); ΔCt = (Ct of neuroblastoma mRNA – Ct of β_2-microglobulin).The lower Ct for reporting RTqPCR for the neuroblastoma mRNAs is a Ct value of 40. A tumor negative bone marrow has a Ct value of ≥40 for all of the neuroblastoma mRNAs examined, when amplification of the reference house-keeping gene generates a Ct value of <25. The quality of the amplification curves should be confirmed.[28,29,30]

Where possible we strongly recommend reporting results using the comparative Ct method, also known as the $2^{-\Delta\Delta Ct}$ method[31,32] which reports the fold change in ΔCt

expression of the sample relative to a calibrator sample that is analyzed in each assay (ideally on each plate), to control for variation in amplification across different platforms. We advise that the sensitivity and specificity of RTqPCR analyses across centres analyzing samples from within clinical trials is maintained by quality control to minimize inter-laboratory variability.[18]

Recommendations for reporting bone marrow response

Currently we recommend >5% bone marrow infiltration as the internationally attainable level of reliable tumor detection for reporting bone marrow response (Table 2). Importantly, prospectively the number of neuroblastoma cells and level of neuroblastoma mRNAs in bilateral bone marrow aspirates and the percentage of tumor in bilateral bone marrow biopsies should be recorded, to accumulate the data from which more precise evidence-based response criteria can be defined in the future. This is essential in the setting of minimal disease (when tumor cell infiltration is ≤5%), and when infiltration on reassessment is increased two-fold to >5% but does not reach 20%. Quantitative assessment of bone marrow infiltration is also important to more precisely evaluate the potential clinical impact of intermittent neuroblastoma cell detection. Not involved and not evaluable are clearly defined (Tables 1 and 2); where bone marrow samples were not obtained this should be recorded as 'Not done' (ND or ND_{PCR} for IC and RTqPCR respectively). In the case of discrepant results between analysis of multiple bone marrow aspirates and biopsies using any of the described methods, the sampled site with the highest level of tumor should be utilized to grade response. Where quantification is not possible locally and for assessment of

response in early phase 1 and 2 clinical trials, it is recommended that aspirates and biopsies are collected and analyzed by trial led central review.

Discussion

For the first time we describe international consensus criteria for the collection, processing and quantitative reporting of neuroblastoma cells in bone marrow biopsies. Importantly in biopsies we recommend reporting the percentage area of bone marrow parenchyma occupied by tumor cells to minimize errors that may arise when the number of tumor cells in a biopsy is very low, for example after chemotherapy. We have developed these recommendations with the ambition that they will be suitable for adoption across all centres treating children with neuroblastoma (Table 4). We anticipate that, coupled with the updated guidance for assessment of neuroblastoma cell contamination in bone marrow aspirates, they will facilitate a robust international standardization of bone marrow reporting. Previous studies have reported on the variation and inadequacy of bone marrow biopsy quality,[19,33] underlining the need for change, which we anticipate the comprehensive recommendations provided in this paper will expedite.

In response to the increased sensitivity and specificity of methods to quantify clinically relevant neuroblastoma cells in bone marrow, we advocate that children with bone marrow disease ≤5% are considered in a separate response category of minimal disease. Adoption of quantitative reporting of neuroblastoma cell number and mRNA levels in prospective clinical trials will in the future inform a more precise definition of what constitutes a response in the setting of minimal disease. This information in the

long term may inform timely introduction of emerging effective agents to treat bone marrow disease, with an anticipated improvement in outcome. International adoption of these recommendations will facilitate cross-trial comparisons and increase opportunities for collaborative trial design and research, with the expectation that this will speed the advancement of new treatments to improve outcome for children with disseminated disease.

Several studies have reported a greater frequency of neuroblastoma identification in bone marrow biopsies than in aspirates.[34,35] However, there is substantial agreement that analysis of both should be performed for the most accurate interpretation of bone marrow disease.[35,36,37] This might be particularly important in the minimal disease setting, when sequential monitoring of bone marrow disease and response evaluation may be more informative.[3,6,7] Therefore we recommend that both bilateral bone marrow aspirates and biopsies are analyzed, and their clinical value compared prospectively to inform future practice. The value of IC and IHC are both dependent on the quality of the sample, and the specificity and sensitivity of the antibodies employed. For IHC we advise using antibodies against at least two of the recommended antigens, endorsing the use of synaptophysin and chromogranin A[12] and advocating the introduction of PHOX2B.[22,23] For IC we support the use of antibodies to GD_2,[13] and commend the inclusion of a second antibody (e.g. anti-PHOX2B or anti-CD56) to control for those rare situations where GD_2 expression may be weak or negative.[38 39] We advocate the use of RTqPCR within clinical trials to quantify the level of the neuroblastoma mRNA tyrosine hydroxylase, the most widely evaluated mRNA target which has prognostic value in bone marrow,[13] in all children in the minimal disease setting and in high-risk children at trial-specific disease

assessment time-points. In addition we now recommend the adoption of RTqPCR for PHOX2B mRNA in clinical trials, as this is reported to be a highly specific marker for the detection of disseminating neuroblastoma cells[16] and in combination with tyrosine hydroxylase may allow the identification of children with drug refractory disease.[9] (Figure 3). Additional methods may serve to improve the sensitivity and specificity of quantitative bone marrow analysis and reporting using IC or RTqPCR, including automatic immunofluorescence plus FISH (AIPF)[39] and assessment of the RNA integrity number (RIN)[40] respectively. However the dependency of these tests on specialist equipment prohibits their inclusion as standard recommendations for assessment of disease. Whilst flow cytometry has been utilized to quantify neuroblastoma cell content in bone marrow aspirates, a requirement to analyze large numbers of cells reduces the sensitivity of this approach which is not recommended in the clinical setting.[41]

In summary, consensus methods to detect neuroblastoma cells and mRNAs in bone marrow aspirates and biopsies have been described (Figure 3). The future challenge will be to empower centres to improve the quality of bone marrow collection from children with neuroblastoma, and to assess whether these recommendations have changed practice. Adoption of these consensus recommendations by the international community will enhance the comparison of results from clinical trials to expedite trial led change in response assessment, to improve outcome for children with neuroblastoma.

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Figure 1. Histomorphological features of differentiation.

A. Trephine biopsy after chemotherapy of an initially poorly differentiated neuroblastoma demonstrating non-neoplastic Schwann cell rich stroma.

B. Contra-lateral trephine biopsy from the same trephine in A. In addition to sheets of Schwann cells (not shown), only 2 foci of tumor cells are found (areas marked with dotted lines) which include a sufficient number of differentiating neuroblasts with abundant cytoplasm (arrows) to fulfill the criteria of a differentiating neuroblastoma.

C. Trephine biopsy after chemotherapy shows proliferation of non-neoplastic Schwann cell stroma encasing a few differentiating neuroblasts/ganglion-like cells either as single cells (long arrows) or in small clusters (short arrows and inset down left) resembling maturing ganglioneuroma.

Figure 2. Cytomorphological features of differentiation.

A, B. Neuroblastoma cell nests with poorly differentiated neuroblasts

C. Neuroblastoma cell clump with varying features of differentiation: increasing cell size, larger nuclei with bluish nucleoli, more distinct nuclear membrane, coarser chromatin, polychromatic cytoplasm, neuropil between cells.

D. Differentiating cells increase even more in size, more compact and coarse granular chromatin, nuclear membrane like pencil-delineated, few bluish nucleoli, one bi-nucleated cell

E. Tumor cell clump with 5 differentiating cells: large nuclei with big nucleoli, extensive polychromatic cytoplasm

F. Tumor cell nest with undifferentiated cells and one differentiated giant ganglionic-like cell with abundant cytoplasm and two nuclei and extra chromatin. Both nuclei contain 3-4 bluish nucleoli.

Figure 3. Consensus recommendations for analysis of bone marrow biopsies and aspirates

Analysis of both bilateral bone marrow biopsies and aspirates is recommended. We advise IHC of multiple sections using antibodies against at least two of the recommended antigens and advocate the inclusion of PHOX2B. For IC we support the analysis of at least 3 x 10^6 MNCs per aspirate using a monoclonal anti-GD_2 disialoganglioside antibody; a second antibody may be included to control for those rare situations where GD_2 expression is weak or negative. Within clinical trials we recommend RTqPCR of RNA extracted from bone marrow aspirates to quantify the level of the neuroblastoma mRNAs tyrosine hydroxylase and PHOX2B. The results of RTqPCR must be reported as the Ct value and using $2^{-\Delta Ct}$ or $-\Delta Ct$, where the expression of the neuroblastoma mRNAs is normalised to an internal control; we recommend β_2-microglobulin.