The Glycosphingolipid GD2 as an Effective but Enigmatic Target of Passive Immunotherapy in Children with Aggressive Neuroblastoma

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Review

The Glycosphingolipid GD2 as an Effective but Enigmatic Target of Passive Immunotherapy in Children with Aggressive Neuroblastoma (HR-NBL)

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Abstract: Neuroblastoma (NBL), the most frequent and lethal pediatric cancer of children in pre-school age, is still considered enigmatic. This is in view of its extreme heterogeneity, from spontaneous regression to incurable disease (in a larger proportion of cases, approx. 40%). NBL has an embryonal origin, not completely understood. This origin was recently discussed in view of new findings on Schwann precursor cells. Furthermore, a very complex and heterogeneous genomic landscape has so far hampered the success with so-called targeted or precision-medicine strategies. Since over three decades, the glycosphingolipid or disialoganglioside GD2 was shown to be expressed on NBL cells and utilized as target for passive immunotherapy with anti-GD2 antibodies (GD2-IMT). In 2010, a new international protocol has been established with GD2-IMT, which increases remission length and survival in aggressive NBL (HR-NBL). By reviewing the different biological and molecular aspects of NBL and GD2-IMT, this mini-review questions some of today's accepted interpretations and particularly the present lack of association between GD2-IMT and the underlying molecular landscape. An alternative model is presented involving the Micro-Foci inducing virus (MFV) that we have studied for several years. MFV infection can induce extensive genomic aberrations (such as 100X NMYC DNA-amplification). Since this family of viruses uses molecules for cell adhesion and entry similar to GD2 (i.e., GM2), it is here hypothesized that GD2 is the port-of-entry for MFV and that the great success of anti-GD2 therapies is partly/completely explained by inhibition of spreading of this clastogenic virus in aggressive NBL.

Keywords: neuroblastoma (NBL); glycosphingolipid GD2; anti-GD2 immunotherapy (GD2-IMT); NBL genomic landscape; micro-foci inducing virus (MFV); sialylated glycosides receptors

1. Introduction

Neuroblastoma (NBL) represents the most common extra-cranial solid tumor of early childhood with an estimated 800 cases/year in the USA and an incidence between 5.5 and 11.5 per million people in North America [1–3]. It shows a relatively high mortality (approximately 15% mortality rate among childhood cancers) in view of its aggressive form, which comprises approximately 50% of total NBL cases, High Risk NBL (HR-NBL) [1,4]. NBL has been extensively studied throughout the years particularly at the genetic/genomic level under different perspectives: 1. presence of specific alterations in oncogenes, especially the amplification of the MYCN gene during the 80's [5–8]; 2. detection of deletions/silencing of Tumor Suppressor Genes (TSGs) or segmental chromosomal copy number alterations (especially chromosome 1p, 11q, 17q and 5p) throughout the 90's [9–12]; 3. detection of mutations associated with NBL familial forms through Next Generation Sequencing (NGS) and Genome-Wide Association (GWA) studies in more recent years [1,4,13–17]. Despite such

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greater knowledge about molecular mechanisms in NBL, well-known molecular markers remain just associated with prognosis: they have confirmed greater heterogeneity in HR-NBL [18–21]. In low stage disease, no or few markers are present, while well-known markers of aggressive NBL (i.e., Stage 3–4) are the mentioned MYCN amplification (now utilized for initial staging in most Western Countries) and the 1p, 11q, 17q and 5p segmental alterations (Copy Number Variation or CNV) [1,18,22]. Such heterogeneity finds a parallel prognostic value, since 80–90% of patients in early stages are cured, while only approximately 40% in HR-NBL and less than 10% in NBL with relapsed/recurrent disease [1,18,23,24].

For decades, the peculiar form of NBL-4S has been studied for possible hints about this disease onset and progression: it is diagnosed in infants or very early childhood (typically < 1.5 year of age) as an apparently aggressive form with disseminated metastases throughout the body (in the peripheral blood, skin, cartilage, bone marrow, as in "Stage 4" but not inside bones) [18,22]. However, the fact that it suddenly regresses and disappears at 1–1.5 ~~y.o.~~ has led several pediatric oncologists to adopt the strategy of simply observing these cases with or without surgery, therefore without the need of adjuvant therapies (chemo- radiotherapy) [3,22,25]. Unfortunately, we still don't understand the reason of NBL-4S regression (or for its initial onset) [1,22,26].

Additional hints on NBL onset/progression were provided by considering its lineage and cell-of-origin. Robert Bolande introduced the concept of neurocristopathies (NCPs) in the '70's i.e., pathologies associated with derangement of Neural Crest cells (NCC) either phenotypically (epigenetically) or genetically [27]. Bolande's initial classification emphasizing a prominent role of NBL divided NCPs into 4 categories: 1. tumors; 2. tumor syndromes; 3. malformations; 4. all other. This was revised by Watt and Trainor [28], considering the timing of the particular pathology onset: 1. NC induction/specification; 2. NC migration; and 3. NC differentiation. The most recent classification by G.A. Vega-Lopez et al. takes into consideration only the site of origin throughout the cephalo-caudal axis: I. CRANIAL; II. CARDIAC; III. TRUNK; and IV SACRAL [27].

The GD2 presence in NBL was initially detected by Schulz and collaborators in 1984: at Scrips Clinic, they developed the monoclonal antibody 126 against NBL cell line LAN-1 [29]. This cell line is originated from a NBL tumor without MYCN amplification. 3F8 was shown to recognize GD2, a disialoganglioside, in the great majority of NBL tumors, also in melanomas, but not in unrelated tumors such as pediatric lymphomas and leukemias [29,30]. GD2 is similarly not expressed in differentiating ganglioneuromas, ganglioepitheliomas and pheochromocytomas which are considered differentiation pathways for NBL Cheung's group later demonstrated that GD2 expression is not suppressed by 3F8 addition/treatment, thus paving the way for the introduction of this (as well as other) anti-GD2 monoclonal antibody for therapeutic purposes [31,32]. The accepted immunological mechanisms in these therapies are antibody-dependent cellular cytotoxicity or complement-mediated cytotoxicity (ADCC or CMCC) [33]. These findings were recently confirmed by the group of Sartelet: therefore, treatment failure in these patients cannot be attributed to absence or post-therapy elimination of GD2 [34]. Furthermore, natural killer (NK) cells appear to exert an essential role in these GD2 targeted immuno-therapies [33]. In other words, several in vivo and ex vivo systems have shown that after addition of anti-GD2, the antitumor response is completely dependent upon presence and effectiveness of NK-cells [33,35,36].

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2. General Outline

This ~~mini-~~review wants to address and partially/potentially answer several questions on GD2 based therapies in NBL. These are the main questions that the mini review wants to clarify or answer:

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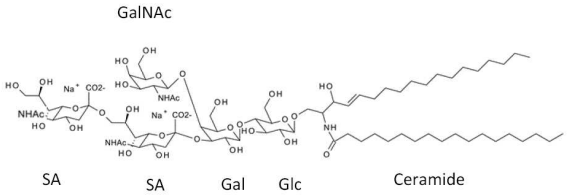
1. What is the nature of GD2 glycosphingolipid in relationship to the oncogenic program/progression of NBL cells?
2. What is the relationship between GD2 and stem cells in the neuroblastic lineage or other embryonic/stem cell lineages?

3. Why GD2 immunotherapies have been so far among the most efficient therapies in children with aggressive NBL?
4. How does GD2 immunotherapy relate to our general knowledge of genomic alterations in NBL? NBL Genomic Landscape: are there too many/moving targets in HR-NBL?
5. Do additional NBL models provide potential explicatory mechanisms for anti-GD2 immunotherapy? MFV model for NBL genomic aberrations.
6. Logical Inference: GD2 as a Port of Entry of Micro-Foci inducing Virus (MFV). Potential relationship between GD2 expression and MFV infection spread.
7. Final Discussion and Concluding Remarks.

2.1. What is the Nature of GD2 Glycosphingolipid in Relationship to the Oncogenic Program/Progression of NBL cells?

GD2 is a glycosphingolipid or disialoganglioside, in which the typical lipid part is composed by sphingosine linked to a fatty acid of variable length, while it contains two residues of sialic acid among five sugar moieties (see Figure 1). The lipid domain (ceramide) inserts into the plasma membrane, while the glycan residues are in the extracellular domain [30]. GD2 has been typically detected in mesenchymal and neural stem cells during development, while postnatally its expression is restricted in the CNS and PNS, as well as in melanocytes [37,38]. Unfortunately, the exact function of GD2 during normal development is still not understood [38]. Seminal work by Schulz et al. originally detected GD2 in pediatric neuroblastoma in 1984 [29]. The group of S. Ladish confirmed high level GD2 presence in NBL primary tumours as well as in plasma from patients with aggressive NBL by a mechanism of shedding from primary tumor cells [39,40].



Glc = Glucose

Gal = Galactose

SA = Sialic Acid (Neu5Ac or NANA)

GalNAc = N-Acetyl Galactosamine

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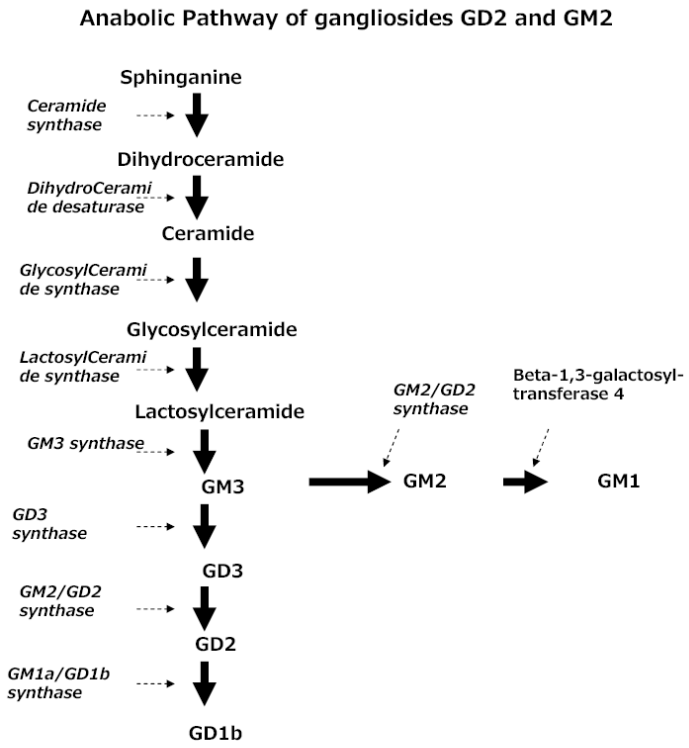

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Figure 1. Structure and Synthetic Pathway of disialoganglioside GD2

1A. Structure of the disialoganglioside GD2. Structure of GD2. Residues Glc, Gal, SA and GAlNAc are identified as Glucose, Galactose, Sialic Acid (also Neu5Ac or NANA) and N-Acetyl Galactosamine. **1B**. Synthesis of GD2/GM2. General Synthetic Pathway for GD2 and GM2. Figures 1A and 1B have been adapted from References 41, 70, 186, 187, 188 and 190 and from image material in internet (google-image).

Concomitant work led to the exploitation of this observation by Cheung et al.: In fact, they developed one of the first examples of pediatric cancer immunotherapies (CIT) [30–32] (see Section 3). Methodologies in those years were based on thin layer chromatography which requires larger sample sets [39,40]. Very recently, however, a new method based on High Pressure Liquid Chromatography and double Mass Spectrometry (HPLC/MS/MS) has been developed and optimized for NBL plasma and serum, which is quite efficient also for archived samples [41,42]. Also with this new method, GD2 excesses (average 100 and up to approximately 200 folds) were detected in aggressive forms of NBL, opening the door for new tools of diagnostics, prognostic and treatment efficacy verification [42].

One outstanding question relates on the role of such larger excesses of GD2 in NBL. Although GD2 presence was also described in mesenchymal stem cells, for example in breast cancer (see next point), in other situations it characterizes a small or minute population of stem cells, so called cancer stem cells (CSCs) [43,44]. In neuroblastoma however, GD2 expression when present is often widespread and persistent [30,33]. In a very recent paper by Terzic et al., a GD2 immunostaining Score system was applied to NBL with different stages [34]. However, the values ranged from 1.67 (S1) to 1.84 (S4) with a peak at S2 (1.90), certainly not logically obvious: Such variability could

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probably just show a trend with increasing stage/aggressiveness. Furthermore, the authors proposal that lower levels of initial GD2 levels correlate with patient resistance to GD2 immunotherapy (ADCC with anti GD2- ch14–18 humanized monoclonal- with alternating GM-CSF and IL-2 [45]), needs further verification in view of the very small number of analyzed patients (5/20) as well as the small levels of difference, as just discussed [34]. The same authors [34] conclude their discussion acknowledging the need for further studies [30,33,34]. The second point and "tenet" of anti-GD2 therapy has been the confirmed evidence for persistence of this antigen, also following immunotherapy [45]. Treatment failure therefore does not impinge on loss of- /selection for GD2 negative (GD2-) cells: The mechanism ought to be different [34]. However, the group of Berthold has recently analyzed a larger set of patients, 1261, discovering complete or partial negativity in 57 of them [46]. Once more, the finding is puzzling, since there is no difference among negatives in comparison with positives in staging, tumor sites, molecular characteristics, tumor markers, histology, overall or event-free survival [46]. In view of the generally widespread presence of GD2 in NBL, the answer should probably be found elsewhere. For example, what is the nature or function of GD2? Ohmi et al. have proposed that gangliosides including GD2 contribute to the genesis and maintenance of so called membrane microdomains [47]. Still, their exact role, especially in NBL tumor formation, progression and metastatic dissemination is currently obscure [38]. A potential clue is coming from additional studies which associate GD2 with stem cells (or Cancer Stem Cells CSC, see also next point 2.2). One of the main tenets of such hypothesis is that NBL derives from Neural Crest (NC) cells (see [27,48]). Further impetus to these ideas was given by the work of Brian Hall [49] and others [50] who recognized NC as a 4th germ layer beside the well-known 3 (endoderm, mesoderm, ectoderm), therefore with a quadroblastic rather than triploblastic embryonic structure [49]. These cells embryonically derive from the dorsal edge of the Neural Tube (NT), according to NC gene-expression programs, thus creating a series of NC cells (NCCs) [50]. However, in order to achieve their very extensive migration and dissemination program, they must initially perform an extensive and efficient Epidermal to Mesenchimal Transition (EMT). EMT was first discovered as an essential element of the metastatic process by R. Weinberg group at MIT [51–53]. It is now convincingly clear in several tumor systems (see in the next section, also discussion on breast cancer cells and GD2) that tumor cells in order to intravasate –i.e., passing by intrafiltration- into the bloodstream, travel with blood circulation and metastasize must first pass from the original epithelial form (90% of human cancers) to a related one, the mesenchymal phenotype [53,54]. This is clearly due to a genetic program switch, since the same cell can then revert –once it has found the ideal metastatic niche- into the original epithelial phenotype [55]. GD2 appears to be an essential player of such metastatic program, as well as in the normal traveling program that embryonic NCC must undertake [38,56]. It can be therefore speculated that the NBL target corresponds to an immature NC cells, which is still capable of transforming itself from the neuroectodermal more differentiate form to the undifferentiated, mesenchimal and migrant cell type, where GD2 expression appears to be very high [38]. Such program switch, i.e., the first question as initially indicated, could also explain the rather restricted window of susceptibility (in infants, young children, very rarely after 10 years of age) for NBL onset, since most likely the NCC precursor capable of such acrobatic EMT changes has limited lifespan [1,3]. The answer is apparently satisfactory, but only partially. Because the next and immediate question is: "Why such NCC precursor does not normally switch back after migration into a normal neuroectodermal and GD2-negative cell?". What is the essential trigger in NBL which maintains such high GD2 positivity and mesenchymal stemness? The situation may be similar to the carcinoma of the uterine cervix in women (CC), in which susceptibility is strongly and strictly linked to a specific site of the cervix where the squamous and columnar epithelium intersect: The so called Squamous-Columnar Junction (SCJ), also called the Transition Zone (TZ) [57]. Only those cells with staminal markers, which are exclusively present at the SCJ/TZ area, appear to be susceptible to carcinogenesis, so much so that Herfs and Crum have recentlty proposed to remove the target, i.e., the SCJ, in order to avoid carcinogenesis [58]. In this case, it is also well known which agent causes the target carcinogenesis, since the discovery of high-risk human papilloma viruses (HR-HPVs), which are now

recognized as etiological agents of cervical carcinomas [59]. H. zur Hausen was eventually awarded the Nobel Prize for such discovery [59,60]. Herfs and Crum model, however, is particularly attractive, since it also provides good evidence for HR-HPV susceptibility especially in cells of the SCJ/TZ. These cells were initially characterized by Herfs and Crum in a PNAS article (2012) by Affimetrix gene-expression profiling (GEP) as presenting markers typical of stem cells (SCs) [61]. From 77 genes, they eventually validated 5 (krt7, AGR2, CD63, MMP7 and GDA) which are typically present in SCJ/TZ cells. Such stem cell markers were present in all the most aggressive cervical carcinoma cases (56/120), but only at lower frequency in lower risk cases (19%) [61]. Therefore, the take-home message we can extrapolate from this cervical-carcinoma (CC) model is that stem cells are confirmed as targets of malignant transformation of CC, where the molecular players are also rather well studied, i.e., the gene E6/E7 of HR-HPVs [62–64]. Another good example for this general theme of cancer cell susceptibility could be prophylactic lumpectomies or oophorectomies in women with BRCA-1/2 mutations [65,66]. Also in these cases, preventive surgery has led to evident and important decrease in incidence or recurrence [67]. For both genes however, a clear link to carcinogenesis is still missing and there are only clues for their involvement in the Homologous Recombination (HR) pathway of DNA repair [68,69]. Using CC as a paradigmatic example for prospective therapies in NBL, would it be possible to eliminate or extirpate the targets for HR-NBL in young children? There are several considerations suggesting that the Herfs-Crum paradigm for HR-HPVs in CC, is not applicable to HR-NBL. Please see further discussion in 2.7 (Final Discussion and Concluding Remarks).

2.2. What is the Relationship between GD2 and Stem Cells in the Neuroblastic Lineage or Other Embryonic/stem Cell Lineages?

Although during embryonic development GD2 is mostly expressed in neural and mesenchymal stem cells, its postnatal expression is restricted to certain cells of the central and peripheral nervous system as well as MSC in peripheral blood [37,70]. For example, nerve pain fibers have high expression of GD2, which constitutes an obvious problem and limitation in GD2 antibodies-based therapies. Both GD2 and GD3 are considered excellent markers of neural cell *stemness* either in animal models or in humans [37] , so that they can characterize *bona fide* Neural Stem Cells (NSCs). Therefore, the hypothetical GD2 function in NBL has to be associated with stem cell behaviour, which is re-acquired by these tumor cells most likely by de-differentiation (or by direct transformation of a stem cell precursor) [71]. The function is probably linked to migration (embryo) and metastasis formation (HR-NBL) [56] and is also associated with several tumor types including small cell lung carcinomas (SCLCs) [72,73], osteosarcoma (OS) [74–77], glioblastoma multiforme (GBM) [78,79] and breast cancer [44,80–83] (BC, discussed later) [71,84,85]. One of the best studied models is osteosarcoma [86], where indolent cancer cells became much more aggressive, metastatic and endowed with high migration capabilities in a Boyden chamber after transfection of- and GD2 induction [86]. Another way of inducing EMT is by activating the transcription factors Slug and Twist, which are typically activated in aggressive NBL, usually through the transcription factor c-myb [87], but at least in the case of *Twist* also by the activation of MYCN [88,89]. Therefore, the emerging landscape is that of an immature/embryonic phenotype induced in NBL by upstream master regulators such as myb and especially MYCN (in view of its amplification in aggressive NBL and Twist factor activation-cascade) [83,87–89]. This immature or stem-cell character is epitomized by GD2 expression, essential for the EMT establishment, which then empowers NBL cells with highly migratory/metastatic potential [38,50,51,90]. At the molecular genetic level, this seems to be associated with embryonal precursor cells in mice, which become SOX10+ and PHOX2B+ at E10.5 [91]. These cells can be induced with a construct developed in Michael J. Bishop's lab, in which the MYCN gene is driven by the rat Tyrosyne Hydroxylase promoter [92]. Therefore, the MYCN gene is capable of efficiently causing malignant transformation: In this sense it behaves as a master regulator, since it elicits the full pattern of malignancy, differently from the more recently discovered *ALK* or *LIN28* mutations which rather display an ancillary role [93,94]. In other constructs, the Th-promoter linked to a complete set of different ALK mutations, cannot induce full transformation, although they can enhance the MYCN

driven transforming and tumorigenic effect [95]. Another essential gene that MYCN appears to recruit in its carcinogenic program is the *Enhacer of Zeste Homolog 2* (EZH2), which contains the methylase activity of the *Polycomb Repressive Complex 2* (PRC2), therefore the essential enzymatic activity of PRC2 [91,96]. In fact, work by the group of Perini and Sala showed that MYCN physically interacts with PRC2, thus recruiting this silencer to the promoter sites to be down regulated [97], data confirmed by Tsubota [91,98]. In this gene expression landscape, GD2 becomes apparently highly expressed, although with some variance as noted by Berthold. Furthermore, MYCN is probably not the only regulator for its higher expression in NBL, as GD2 is also expressed in tumors devoid of MYCN amplification (present in approximately 25% of NBLs). However, other tumors and models of ectodermal and neuro-ectodermal origin impinge on GD2 expression and function, in particular melanomas, gliomas, bladder carcinoma and breast cancer (BC). According to studies at MD Anderson Cancer Center, BC is particularly revealing for the potential function of GD2 [90]. This group analyzed BC cancer stem cells (CSC), which Al-Hajj and colleagues had initially isolated as "*bona fide*" BC-CSC [99], by showing their much increased capability of inducing tumors in NOD/SCID mice and characterized them as displaying high levels of CD44 on their membranes and low levels or absent CD24, i.e., $CD44^{hi}CD24^{lo}$ cells [90]. When Human Mammary Epithelial Cells (HMECs) were transfected with gene *Twist* and *Snail* (or treated with TGF-β), they appear to behave as Mesenchimal Stem Cells (MSC) [100]. Therefore, presence of a MSC marker such as GD2 was hypothesized and discovered on the transformed HMECs and on the $CD44^{hi}CD24^{lo}$ population, i.e., the BC-CSC [90]. GD2 is predominantly present in both these populations as confirmed by gene-expression studies [100]. Furthermore, the treatment of this population with a GD3-synthetase (GD3S) short hairpin RNA (Inhibitory *shRNA*) or with the inhibitor of GD3S *triptolide*, led to a great decrease in GD2 expression [90]. GD3S was chosen as an appropriate target, since GD3 is the precursor of GD2 (and GM3, see Figure. 1B): Its down-regulation therefore leads to a more drastic inhibition of the stem cell marker GD2 [101]. This was accompanied by a loss of the CSC properties, including abrogation of tumor formation in vivo and strong inhibition of mammospheres production [83,101]. In conclusion, in this important model of GD2 expression and correlation with its developmental function and role in cancer biology, 1. this sphingolipid presence is strongly correlated to EMT processes, thus leading to a first step in intravasation and metastatization [90][100]; 2. its presence appears to be phenotypically (i.e., epigenetically) determined since $CD44^{lo}CD24^{hi}$ cells can easily convert into $CD44^{hi}CD24^{lo}$ cells and vice-versa [90]; and 3. EMT-derived Mesenchimal GD2+ cells show all the features of aggressive BC cells and in particular of Triple Negative Breast Cancer (TNBC), as recently confirmed by several groups [44,81,83,101,102]. Initial trials for women with GD2+ and aggressive BC (mostly TNBC) utilizing dinutuximab (anti GD2 monoclonal antibodies) are scheduled to initiate soon (VL Battula, MD Anderson, personal communication, September 2019).

2.3. Why GD2 Immunotherapies Have Been So Far Among the Most Efficient Therapies in Children with Aggressive NBL (HR-NBL)?

Neuroblastoma has been known for decades to present a very heterogeneous clinical picture: from indolent disease present in Stage 1–2 to essentially untreatable forms such as Stages 3–4, despite at least 3 types of therapeutic strategies [1,103]. It should be emphasized that both the staging system (i.e., S1–S4) and the standard protocols for HR-NBL have been modified throughout the years, in order to better match patients needs and improve final outcomes [1,24].Today, therapies rely on three major phases: A. Induction with several different regimens of chemotherapies plus surgery for resecting the tumor mass; B. Consolidation with chemotherapy at high doses followed by autologous hematopoietic stem-cell rescue (AHSCT) and external beam radiotherapy and C. Post-consolidation (= Immunotherapy) with anti-GD2, GM-CSF and cis-retinoic acid (cisRA) [15,23,45]. In previous protocols, Autologous Stem Cell Transplantation ASCT was also added: It has been now questioned in MYCNA+ stage 2/3 patients in view of lack of survival advantage [104]. The various phases to reach such protocol which is now considered standard for HR-NBL are therefore described to follow:

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It should be emphasized that the beginning of NBL immunotherapy was initiated by the seminal observation of the Hellstroms et al. at Sloan Kettering, who described in 1968 how sera from nine children with NBL inhibited the growth in culture of both autologous and allgeneic neuroblatoma tumor cells [105]. As already mentioned, the initial observation by Schulz in 1984 that NBL patients tumor cells and sera bear GD2 antigen was another breakthrough [29], followed by extensive work of S. Ladisch and NK Cheung groups, leading to the first clinical studies with anti-GD2 monoclonals demonstrating a partial response in HR-NBL patients. This was due to complement dependent cytotoxicity (CDC) and Antigen Dependent Cellular Citotoxicity (ADCC) [31,39,40,106]. It was then shown in vitro that both GM-CSF and IL-2 could enhance ADCC against GD2 [107]. This set the stage for the initial Phase I trials [32], testing different combinations, especially when the chimera (i.e., humanized) anti GD2 m3F8 became available toward millennium end [108]. Phase I declared that ch14.18 plus IL2 and GM-CSF combined with cisRA after myeloablative therapy and subsequent Autologous Stem Cell Transplantation (ASCT) was a tolerable regimen [109,110]. From this good start, over ten years elapsed till completion of the Phase III randomized trial showing the clear superiority of cisRA together with ch14.18, IL2 and GM-CSF versus cisRA alone (as post-consolidation with immunotherapy) for HR-NBL cases who had already clinical response after 1. induction therapy and 2. myeloablative consolidation therapy/AHSCT [45]. This post-consolidation immunotherapy improved by approximately 20 percentiles the event free survival - EFS (from 46% to 66%) and by 10 percentile the overall survival-OS (from 75% to 86%) [45]. Further clinical trials are now improving this outcome, using dinutuximab/ch14.18 as humanized antibody and eliminating significant toxicities which include excessive neuropathic pain. Furthermore, for particular subgroups of patients (i.e., MYCNA+ stage 2/3), ASCT may be questionable in view of similar OS/EFS results in the absence of ASCT [104]. Other attempts have targeted the tumor microenvironment, which is known to dampen the NK response after ch14.18 addition: Indeed NK cells appear to be essential for antitumor response [111,112]. For example, lenalomide can at the same time activate ADCC response after the monoclonal antibody and prevent the tumor microenvironment (TME) inhibition [113]. TME accomplishes this by enhanced expression of IL-6 and TGF-β, which are generally present in HR-NBL and antagonistic to IL-2 immuno-stimulation [107,114]. In a very recent and exciting development in this direction, the group of R. Seeger in LA has developed a novel monoclonal antibody, TRC105, which targets the TME by specifically binding CD105+ cells (anti-CD105 monoclonal antibody) [114,115]. These include Mesenchimal stromal cells –MSCs- [116–118], tumor associated macrophages (TAMs) [119,120], cancer associated fibroblasts (CAFs) [121] and endothelial cells in the TME [107]. Both MSCs and TAMs have both protumorigenic and immunosuppressive properties. For example, TAMs diferentiang from monocytes can promote radio- and chemotherapy resistance in a series of tumor models and in HR-NBL (not MYCNA) are associated with quite poor EFS at 5 years [107,122]. These exciting new results await to be translated into clinical trials, which should test TRC105 (anti-CD105 monoclonal antibody) in combination with anti-GD2 monoclonals (dinutuximab) [114].

Considering anti-GD2 therapy, a slightly different approach has been followed by the recent work of Cheung's group. Brian Kushner at al. have recently reported on the results of Stage 2/3 NBLs with MYCN amplification (MYCNA) after long-lasting treatments with 3F8 anti-GD2 monoclonal antibody associated with granulocyte macrophage colony stimulating factor (GM-CSF) [104]. Patients were classified as Stage 2/3 disease (previous classification before revision by INSS, [23,123]: They are in fact HR-NBL in view of the mentioned MYCNA [1]. Beside two patients who died early (one after achieving partial remission and one who did not receive the immunotherapy- "consolidation therapy"), 18/20 patients achieved Complete Remission or Very Good Partial Remission (CR/VGPR). Their 5 years EFS/OS is now 82% and 94% with two patients who experienced a relapse, but were put in 2nd Complete Remission (CR) with 3F8/GM-CSF [104]. These results are obviously exciting with values very similar to early stage disease and also question a re-evaluation of ASCT for HR-NBL or for subgroups among them [104]. Finally, these clinical data hint to a special role of the genomic aberration: MYCNA.

Currently therefore, NBL cases (approximately 1000 cases only in North America with an incidence of approx. 10^{-5} live births) have been stratified in 2009 in 4 new categories of Risk: 1. very low; 2. low; 3. intermediate and 4. high [1]. While categories 1–2 have a very good prognosis and category 3 may also present cases with up to 75% PFS at 5 years, patients in the high-risk category 4 are the most common diagnosis (50%) and still present a rather low PFS and OS at 5 years (approx. 40%) [23,123]. This very rapid snapshot at NBL prognosis and therapeutic options underlines the potential importance of new landscapes, interpretations and future strategies in HR-NBL.

2.4. NBL Genomic Landscape. Are There Too Many/Moving Targets in HR-NBL?

In order to find an explanation for such a widespread difference between low and HR-NBL, only recently and with the aid of Next Generation Sequencing (NGS) technology [14,16,124,125] and with initial studies of GWA (GWAS, thus associating NBL risk with particular SNPs [126]), we have obtained sufficient data in order to explain such an extremely different biological and pathological behavior also at the molecular level. The most recent studies clearly indicate that NBL tumors 1. display extensive heterogeneity in their genome make-up, 2. a relative scarceness of new tumor-specific mutations and 3. that recurrent mutations typically do not overcome the 10% threshold level in general surveys 4. that several, if not the majority, of genomic variation entails segmental chromosomal alteration, so much so that aggressive NBL could be defined a disease of copy number variation (CNV) or segmental chromosomal aberrations (SCA) (for reviews, see: [1,14–17,20,30,103,126,127]). In this respect therefore, MYCN amplification, studied for over 35 years, still remains the most relevant hit in the general and common NBL form (infants and children) [13,14]. Significant mutation frequencies were obtained by Maris group for ALK (9.2%), although it is also known that ALK is co-amplified with MYCNA, since it is located in close proximity on 2p [14]. Additional alterations include ATRX (2.5 with an additional 7.1 with focal deletions, for a total of 9.6%, see also later discussion on older patients) and TERT abnormalities [128–130], Additional mutations are: MYCN (1.7%, these are just mutations obviously, since MYCNA has been evaluated up to 32.1% [14]) and NRAS (~~0.83%~~ .83%, but this value may strongly increase in recurrent/relapsing NBL [131–133]).

Comment [M19]: Please consider this suggested change. We consider it may should not be" .83%,". YES = .83%

Comment [PUR20]: The value is very small = less than 1% (it strongly increases in relapses), PLEASE use .83%

The locus *LIN28B* is particularly interesting, since it affects regulation of miRNAs, by depleting the let-7 family of miRNAs and also stabilizing the Aurora kinase A (AURKA). At the same time, amplification or over-expression of *LIN28B* augments MYCN activity through inhibition of let-7 miRNA and by stabilization and MYCN [134,135]. Finally, MYCN acts as decoy (sponge) for let-7 miRNA, thus inhibiting its tumor suppressive function, and at the same time creates a regulatory loop with *LIN28B*, showing the presence of a converging pathway by both *LIN28B* and MYCN, which then also act on nuclear proteins such as RAN or AURKA [134,135].

Studies by J. Maris group have documented the importance of GWAS for identifying genes affected in HR-NBL: Some of these have been characterized by the same group in Philadelpia (i.e., BARD1, CASC15 lnRNA) or by other groups [136]. At least three categories of SNP variants could be identified: 1. the ones associated with low risk NBL; 2. those present in HR-NBL (CASC15/14; BARD1; LIN28B; LMO1); and 3. genes associated with CNV/SCA. Some of the HR-NBL genes are here detailed (categories 2–3). Maris group identifies important SNPs on 6p22, especially the allele rs693940, which gives an increased risk of 1.97 (higher value in GWAS studies) [136]. Subsequently, the same group identified a shorter isoform for long non coding RNA (S-CASC15) as the relevant genetic element with apparent function of tumor suppressor gene (TSG) [137]. The groups of Devoto and Maris reported several SNPs on chromosome 2q35 from 397 high-risk cases compared to 2043 controls, showing an association with BARD1 gene and higher odds values in another independent series of HR-NBL patients (for significant alleles: 1.68 and 2.74) [138]. Differently from the associated gene BRCA1, BARD1 should be considered an oncogene and NGS studies have disclosed germ-line mutations in BARD1 in NBL cases [14]. A 2012 paper in Cancer Research by the same Philadelphia group describes how BARD1β isoform behaves as a transforming oncogene in *in-vitro* models, stabilizes the Aurora kinase A (AURKA) and how therapeutic attempts were made to target AURKA

as a druggable gene [126,139]. Similarly identified through GWAS at 11p15 and considered an oncogene, LMO1 was initially identified by J. Maris group by comparing 2251 patients and 6097 controls with an odds ratio of 1.34 [140]. It belongs to a family of transcription factors already involved in other human cancers (members 2–4) and additional work by the same group in collaboration with TA Look has disclosed the presence of polymorphism: SNP rs2168101 G>T, which gives an odds ratio of 0.63 (protective) and is located in the first intron of LMO1 as a "super-enhancer" with extensive acetylation of Histone H3 Lysine27 as probable binding site for GATA factors. Therefore the G allele abrogates GATA3 binding and decreases LMO1 expression, thus explaining the protective effect, also confirmed by additional GWAS studies [141]. In a most recent effort by the groups of T. Look and J. Maris, the function of LMO1 has been clarified in a zebra-fish (ZF) model of NBL [142]: Since transgenic vectors containing LMO1 under the dopamine-β-hydroxilase (expressed in the PNS) did not induce tumors, these authors crossed the LMO1-ZF with a MYCN-ZF transgene, thus showing acceleration of tumor formation with LMO1 + MYCN (80%) vs the MYCN construct (25%) [142]. Furthermore they could document that the addition of the LMO1 transgene was 1. capable of inducing hyperplasia and overcoming the MYCN associated apoptosis and 2. causing the presence of metastases at distant sites, while metastases were not present in MYCN or in the MYCN + ALK constructs. These experiments may therefore clarify the metastatic potential of HR-NBL cells, already described in association with embryological properties of hijacking embryonic pathways [142,143]. As final example of the GWAS discoveries, Neuroblastoma breakpoint family-23 (NBPF23) was identified on chrom. 1q21 not through polymorphism but in view of CNV in this region [15]. Still as an effort of Maris group, the gene was isolated by comparing sequence variation between 846 NBL patients and 803 controls, thus showing it belongs to the NBPF group of genes, number 23 [144,145]. Another member of this family, NBPF1 had been discovered by van Roy group in an NBL case with constitutional translocation t(1;17)(p36.2;q11.2) [146]. The exact function of NBPF23 and NBPF1 genes in HR-NBL is still unknown, although they appear to be as a family transcription factors regulated by NF-kB [147].

Especially neuritogenesis genes were disclosed by the work of Rogier Versteeg and Akira Nakagawara's groups in smaller patient populations (87 and 64 NBLs, respectively) [148,149]. The Dutch group paper is particularly interesting, because chromothripsis, i.e., the "shattering" of chromosomes which are then re-pasted in aberrant fashion, was discovered with relatively high frequency (18% of HR-NBL) [148]. Two additional studies added new light to this overall picture of HR-NBL genome heterogeneity: Two groups from St. Jude Children's Hospital and Washington University reported ATRX mutations with high frequency (100% in one population sample) in NBL cases coming from adolescents/older children [150]. ATRX is part of a nucleosome remodeling complex, which indeed assembles nucleosomes and maintains telomere integrity (as a pathway alternative to TERT). ATRX was finally assessed in approximately 50% of such adolescent NBL cases but in 0% of infant NBLs, clearly showing an age related effect [150]. However, data with younger children were obtained at the University of Hiroshima: 11/11 (100) of NBL cases with elongated telomeres had either a DAXX (1/11) or an ATRX (10/11) alterations [151]. Both genes are involved in to the so-called ALT pathway which produces elongated telomeres [151,152]. However, ATRX may not be always involved in adolescents/older children, as one study from Spain reports no ATRX alterations: geographical, methodological, age or sample size (only 31 patients) differences may explain this discrepancy [153]. On a similar wavelength, the group of Johns Hopkins (B. Vogelstein) discovered from a limited number of patients (71) a relatively high frequency of ARID1A and ARID1B mutations (11%): these two genes are also involved in chromatin remodeling, therefore emphasizing epigenetic aspects of NBL [154].

This extremely condensed snap-shot of NBL genomic derangements underlines its high *inter-tumor* heterogeneity (however typical of most aggressive human cancers): On this basis, treatment failures are also predictable, since targets are difficultly detected and each one can become a "moving-target" in view of the extensive *intra-tumor* heterogeneity (as alluded to for the new RAS mutations at relapse/recurrencies) [124]. Finally, the high level of chromothrpsis discovered by the Dutch group

Comment [PUR21]: A more complete (micro-cytological) definition has been added: (p36.2;q11.2)

Comment [M22]: Please confirm if this is correct.

Comment [PUR23]: Corrected: NBPF1 , thanks !

Comment [M24]: Is this "!"necessary? Can this be deleted.? deleted

emphasizes heterogeneity also for mechanistic models of NBL origin. Recently, Sottoriva-Darryl-Curtis have proposed models of "big bang" tumor initiation and chromotripsis could certainly be a molecular equivalent [26,155]. One take-home message could be that at least two HR-NBL subtypes exist: 1. those in which MYCN is involved either as MYCNA or without amplification but with its over-expression, typically accompanied by 1p deletions and TERT gene-expression activation (not by mutations/rearrangements); 2. tumors without MYCNA, have typical involvement of 11q deletions; the ALT pathway with either ATRX/DAXX or other alterations is preferentially present in Δ-11q pathway (not in MYCNA), while Chrm 17q gains (the most frequent, approx 50% in NBL) are ubiquitous in both categories. However, additional segmental chromosomal aberrations (SCA) as well as the growing group of genes discovered by GWAS (see previous discussion) still need to be clarified and associated to either (or additional) pathway. A recent review-article by the group of Murray Norris epitomizes the sense of frustration that clinicians and scientists feel in addressing a proliferating number of genes and pathways in HR-NBL [156]. Still, the NBL situation is emblematic of a more general status in our present understanding of cancer molecular mechanisms as addressed by several colleagues and myself in the past [26,124,157–159].

Comment [M25]: Is this "?"necessary? Can this be deleted.? deleted

How this then relates to the importance of GD2 as molecular target for CIT (cancer immunotherapy) is still far from clear. GD2 appears to be one of the few stable markers NBL cells possess and despite genes and genomes being shattered throughout disease onset, progression and even relapse/recurrence. High presence and frequency of GD2 despite genomic chaos and deluge, should be then analyzed under another perspective, one of Darwinian selection [124,158]. In order to do so, a new explicatory model will be introduced for NBL onset and progression.

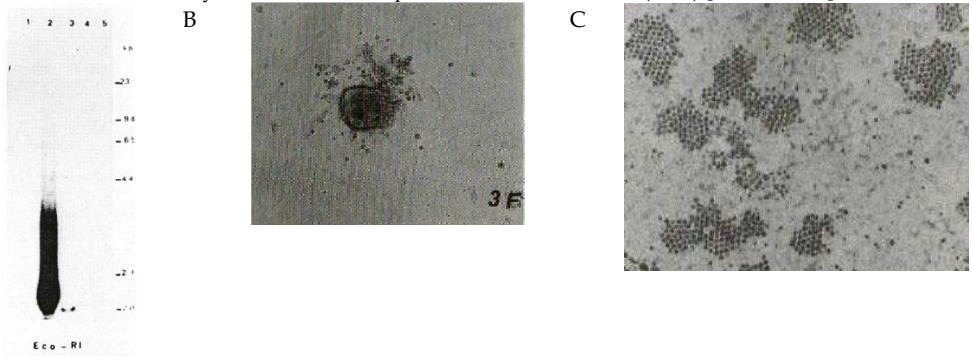
2.5. Do Additional NBL Models Provide Potential Explicatory Mechanisms for Anti-GD2 Immunotherapy? MFV Model for NBL Genomic Aberrations.

The extensive molecular biology knowledge on NBL with accurate description of genes affected (see previous section), typically in its aggressive but not in the indolent form, is in striking contrast with molecular biology on GD2 expression in HR-NBL and molecular data on its potential function [34], while a correlation between GD2 and the general genomic landscape is still lacking. The two research areas appear to be independent and impermeable and are still not unified. Yet and starting from the pioneering work of Cheung over 35 years ago at Sloan Kettering, passive immunotherapy with monoclonal antibodies against GD2 has been employed saving lives or delaying progression in HR-NBL [30]. Furthermore, additional NGS strategies will most likely increase the already detailed list of molecular disruptions, which however appear to be present as recurrent events in a limited percentage (i.e., >10%) of cases of HR-NBL ([1,124] see previous section). This dichotomy between molecular and clinical understanding has been already witnessed in science. As described in the book by Siddartha Mukherje: *"The Emperor of all Illnesses"* clinical sciences and molecular biology did not dialogue for several decades and it was only with the advent of so called Targeted Gene Therapies (TGTs) that this dialogue began (for a critical review, see [124]).

One alternative explicatory model has been proposed for NBL, starting from studies of a so called cancer-cluster, i.e., a time and geographical association of NBL cases [160]. Cancer clusters have been described for childhood cancers -especially clusters of childhood leukemia cases- for over 50 years [161–169]. In one of the most dramatic of such events, associated with the construction and inauguration of the Sellafield nuclear power plant, Leo Kinlen proposed that childhood leukemia excesses were not due to nuclear fallout or contamination (later confirmed as inexistent), but rather by a phenomenon of "Population-Mixing" [167]. In other words, the isolated children population of Seascale (next to Sellafield powerplant) –having been secluded from contact with major metropolitan areas, such as London, Liverpool etc- did not possess a specific herd-immunity against a widespread but unknown pathogen carried by the immigrant population [167,168]. Sudden exposure to and diffusion of such pathogen (called by Kinlen: Virus X) would therefore be associated with a dramatically higher risk for leukemias (and other forms of pediatric cancers) [167,169].

In the same years of Kinlen studies of Sellafield cluster, a cluster of Neuroblastoma cases was evidenced in Southern Louisiana (USA) in the town of Morgan City and extensively studied in my laboratory in New Orleans [170]. All cases displayed high/very high MYCN amplification with in one case MYCNA at 1000X (see Figure 2A) and genetic instability/heterogeneity, as well as the presence of small nodules of neuroectodermal cells *(Micro-Foci)* growing on monolayers of mesenchimal/Schwann-like cells [170] (see Figure 2B). All materials and methods have been previously described and detailed in the appropriate references: [160,170,171], as well as in more general methods collections: [172,173]. Additional methods for the cloning and sequencing of MFV genome and of the gene δ1 are detailed in Section 2.6 – 9). More recent Next generation Sequencing (NGS) technology, employed today in these studies, has been also extensively reviewed [26,124]. In view of their stem cell markers, *Micro-Foci* can be considered similar to *spheroids* or *organoids*, also studied in other tumour systems and *MFV* a potential stem-cell virus (*SCV*) [160,174,175].

A B C



2A **2B** **2C**

2. A. MYCN amplification 2B. Micro-Focus on top of mesenchimal -- 2C. MFV cytoplasmic particles by TEM.

Figure 2. **2A.** An extensive amplification of the MYCN locus was present in this tumor from an HR-NBL patient in a cancer cluster in Louisiana (lane 1). Comparison with control PBL DNA (lane 2) indicates a MYCN amplification of approximately 1000 folds. **2B.** An example of Micro-Focus composed by neuroectodermal cells growing on top of mesenchimal (Schwann) cells; **2C.** Micro-Foci inducing Virus (MFV) particles detected by Transmission Electron Microscopy (TEM) in the cytoplasm of tumor cells (MFV diameter size: 65-69 nm; magnification 18,000X)

Since *MFV* was initially detected in a cancer-cluster of pediatric neuroblatoma (i.e., geographical and chronological association [170]), we also hypothesized that this virus may correspond to the/one of the X-factor(s) proposed by L. Kinlen, H. Zur Hausen and others as responsible for pediatric cancer excesses according to a model of *"population-mixing"* [60,160,169].

MFV induces a dramatic syndrome, which we initially witnessed in rat models (Fisher 344 and Sprague Dowley rat strains): In 11/11 litters from MFV infected mothers (but 0/8 control mock-infected litters), we witnessed in pups (100% in Fisher 344, 80% in SD) extensive neuroectodermal tumours (neuroblastomas), which were present in their abdomen, ataxia, opsoclonus, seizures, cyanoses at the extremities. *Opsoclonus/myoclonus* syndrome is also present in about 3% of children with neuroblastoma. These neurological symptoms could have been caused by MFV itself or one of its subspecies. Finally, we have also shown that human neuroblasts in culture, when infected by MFV, become transformed in vitro (Figure. **3A** and **3B**) and create large tumoral masses in nude-mice (i.e., athymic) in vivo (Figure. **4B**).

Comment [PUR26]: Instead of adding a subsection 2.6.1 as you kindly suggested, I prefer here to maintain the Logical Inference that I described in 2.6 → In fact, I noticed that you changed the scheme of 2.6 as an "outline" of 8+1 points (well taken). I am now listing such points as 1), 2) …etc . 9) to make it more clear. Although 2.6 – 9) is quite longer (1 page) it is the conclusion of the same logical reasoning initiated with 2.6 – 1). For point 2.6 – 9) the subtitle *An alternative landscape for GD* was added .

Comment [M27]: Please double check if this is a section citation, because it seems there's no section 2.6.9. in this manuscript
We kindly suggest you to add a 2.6.1 as subtitle of 2.6 for citation here 2.6 – 9). Please see my previous comment PUR26

Comment [PUR28]: Figure 2 has been modified: please keep the new structure

Comment [PUR29]: Legend for 2A, 2B and 2C has been detailed (also in text): **Figure 2. . 2A.** . An extensive amplification of the MYCN locus was present in this tumor from an HR-NBL patient in a cancer cluster in Louisiana (lane 1). Comparison with control PBL DNA (lane 2) indicates a MYCN amplification of approximately 1000 folds. **2B.** An example of Micro-Focus composed by neuroectodermal cells growing on top of mesenchimal (Schwann) cells; **2C.** Micro-Foci inducing Virus (MFV) particles detected by Transmission Electron Microscopy (TEM) in the cytoplasm of tumor cells ... [1]

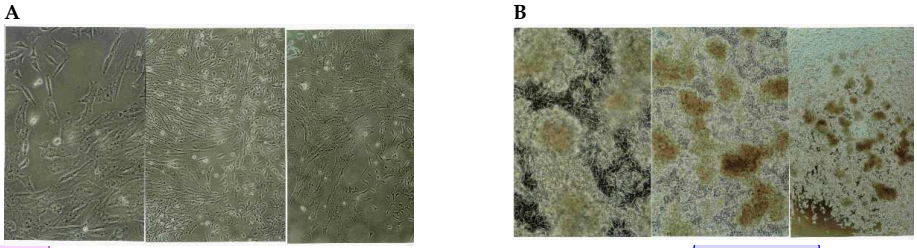


Figure 3. Caption. A. Normal Neuroblasts; B. Neuroblasts after MFVinfection

Figure 3: Mesenchimal Schwann cells before (3A) and after (3B) infection and transformation with MFV. Fig. 3A: mesenchimal mock-infected Schwann cells were grown in culture with low (2%) serum. Magnification 200X. Fig. 3B: The same cells 2.5 weeks after infection/transformation with MFV Magnification 200X.

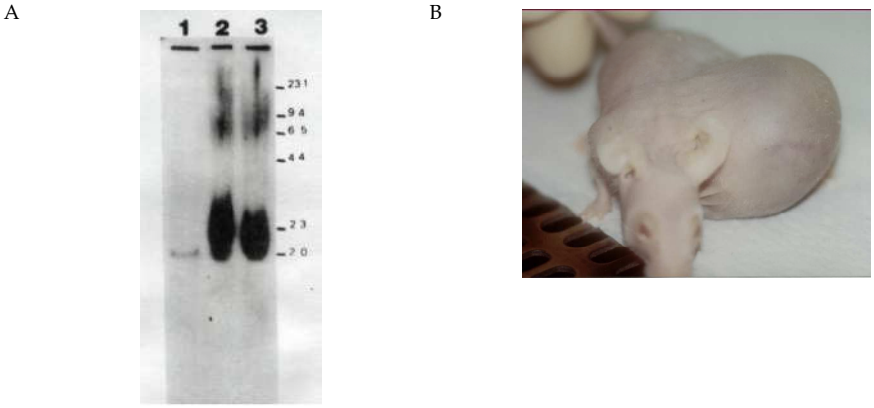


Figure. 4 Molecular and *in vivo* effects of MFV infection on neuroectodermal cells.

4A, Experimentally infected neuroectodermal cells (SK-N-AS and VA-N-BR), initially normal and diploid at the MYCN locus (lane 1, SK-N-AS), become highly transformed after MFV infection and display high MYCN amplification (100 X; lane 2 = MFV-SK-N-AS and lane 3 = MFV-VA-N-BR). 4**B,** human neuroectodermal cells initially non-tumorigenic and diploid (SK-N-SH), were infected with MFV: Transformed cells (see Figure. 3B**)** were then injected in the left flank of a *nu/nu* mouse (mock-infected cells –see Figure. 3A- in the controlateral –right- flank).

The findings on MFV were recently summarized [160,176] and allow do discriminate among recent Cancer Modeling [26,124].

Finally, these data also suggest that such aberrations may themselves have a cause/origin: MFV generates high levels of dsDNA breaks, thus causing:

1. Cell lethality (especially apoptosis, but also necrosis) [170];
2. The above described genomic aberrations especially at the MYCN amplicon with extremely high DNA-amplification levels in the original HR-NBL patient (1000X) and induced DNA-amplification levels in the infected/transformed cells of approximately 100X [26,160,170,171].
3. High level of inflammation: this is particularly due to its dsRNA genome, which activates the IFN pathway, 3'-5'-A synthetase, RNase-L etc. [160,176].

Comment [M30]: Please also help to add a general Figure 3's caption here and 2 specific captions of "A" "B" if possible

Comment [PUR31]: General caption Fig. 3 Figure 3: Mesenchimal Schwann cells before (3A) and after (3B) infection and transformation with MFV.

Comment [PUR32]: Caption 3A/3B Fig. 3A: mesenchimal mock-infected Schwann cells were grown in culture with low (2%) serum. Magnification 200X. Fig. 3B: The same cells 2.5 weeks after infection/transformation with MFV Magnification 200X.

Comment [M33]: Please also help to add a general Figure 4's caption here 4

Comment [PUR34]: General Fig. 4 caption: Molecular and *in vivo* effects of MFV infection on neuroectodermal cells.

Comment [PUR35]: Captions for 4A and 4B have been corrected with additions (corrections/additions are highlighted in red): **4A**, Experimentally infected neuroectodermal cells (SK-N-AS and VA-N-BR), initially normal and diploid at the MYCN locus (lane 1, SK-N-AS), become highly transformed after MFV infection and display high MYCN amplification (100 X; lane 2 = MFV-SK-N-AS and lane 3 = MFV-VA-N-BR). 4**B,** human neuroectodermal cells initially non-tumorigenic and diploid (SK-N-SH), were infected with MFV: Transformed cells (see Figure. 3B**)** were then injected in the left flank of a *nu/nu* mouse (mock-infected cells –see Figure. 3A- in the controlateral –right-flank).

2.6. Logical Inference: GD2 as a Port of Entry of Micro-Foci inducing Virus (MFV). Potential Relationship between GD2 Expression and MFV Infection Spread.

In this necessarily short overview of anti-GD2 monoclonal antibodies based therapies for HR-NBL, several elements were associated to this enigmatic disease (NBL)

1) NBL certainly shows a strong genetic component in approximately 25% of HR-NBL, in view of the involvement of MYCN oncogene pathway (MYCNA), as well as scattered aberrations discovered in both the MYCNA positive and negative cases (see previous Section 2.4).
2) This genetic involvement with MYCNA is sporadic (not hereditary), although a few constitutional mutations were detected in rarer familial forms of NBL [13,177,178].
3) So far, the typical strategy of Targeted Gene Therapy (TGT) has not met with success in NBL, at least with the gene-targets tested so far [26,156].
4) The protocol accepted today for HR-NBL post-consolidation therapy belongs to the arsenal of so called Cancer Immunotherapies (CIT) and is based upon work pioneered by Stephan Ladish and Nai-Kong Cheung since 35 years ago. However, the explicatory mechanisms for such excellent clinical breakthrough are not clear, especially in view of the high variability of the described genomic aberrations (Section 2.4).
5) Recent studies by the group of Kushner-Cheung have documented an augmented benefit of anti-GD2 therapy in MYCN amplified cases in stage 2/3 [104], thus confirming this may be a rather peculiar form of this pediatric cancer~~:~~ .
6) A valid explicatory mechanism for the high frequency of MYCNA in pediatric NBL was never obtained. The idea of sudden "spontaneous" amplifications up to 100–1000 times of this oncogene appears unlikely [26,179].
7) Since years ago, we have isolated a novel double stranded RNA virus from a cancer-cluster of HR-NBL and from a young HR-NBL patients with 1000X DNA-amplification of MYCN: The virus, called Micro-Foci inducing Virus (MFV) was briefly described also here [160,171].
8) Cancer-clusters for pediatric malignancies –like the NBL cluster we studied in Southern Louisiana- are rare but not extremely so [180]. In particular, Colin Murray and colleagues have defined the NBL cancer-cluster instances identified in UK as "*mini-epidemics*" [180].

~~2.6.1. subtitle~~

2.6 -9) ~~9)~~ *An alternative landscape for GD2* ~~In order to discuss~~ the potential association between MFV and anti-GD2 therapies, we will have to briefly consider the present knowledge on MFV and other viruses of this family, i.e., the double stranded RNA virus Family of Reoviridae. These are segmented viruses with 10–11 segments and a total genome of approximately 18–19 Kbs. They are non-envelped and the genome segments are contained in two concentric protein shells [181,182]. In *H. Sapiens*, *Reoviridae* infections occur in early childhood. Adults have been already exposed and are immunized: Disease occurs in the young/ very young [183–185]. In the past several years, the groups of Thilo Stehle and of Terence Dermody have extensively characterized the binding and infection of eukaryotic cells by these viruses [186–189]. Although the final step of virus attachment and entry is through engagement with the Junctional Adhesion Molecule (JAM) present in tight junctions and some lymphocytes, their initial and discriminatory attachment appears to be dictated in T1L (Type 1 Lang strain) by a ganglioside closely related to GD2: GM2 [189]. This recognition, which in fact establishes the specificity of the virus binding to target cells is determined by the gene δ1 [190]. δ1 extends as a trimeric fiber from a molecular torret formed by 5 elements of the λ✾ gene product, in a configuration which appears to be also present in adenoviruses attachment protein (fiber). Three discrete domains are recognized in the δ1 structure: A tail encompassing residues 1–160 and which is predicted to form an α helical coiled-coil structure; a body from residue 170 to 309 with β spirals domains and a head from residues 310 to 455, displaying 8 stranded β barrels [189]. Dermody and Stehle have determined the specificities due to single aminoacid substitutions in the δ1 gene structure.

Comment [PUR36]: Section corrected as 2.4

Comment [M37]: Please double check if this is a section citation, if so, it seems there's no section 4. in this manuscript

Comment [PUR38]: Section corrected as 2.4

Comment [M39]: Please double check if this is a section citation, if so, it seems there's no section 4. in this manuscript

Comment [PUR40]: Yes, ref.104 is correct = Section 4 has been previously corrected into 2.4

Comment [PUR41]: Yes full stop: cancer.

Comment [M42]: "cancer:" ? Please help to confirm if this is the end stop not colon

Comment [M43]: We kindly suggest you to add a 2.6.1 as subtitle of 2.6 for citation here And then you can delete No "9" Please see my discussion in PUR 26 answering your previous comment in M27 .

Comment [PUR44]: 2.6 -9) see previous point, PUR 26 and M27

Comment [PUR45]: I suggest an alternative heading here: "*An alternative landscape for GD2*"

Comment [M46]: Please consider this suggested change (9 deleted)

Comment [PUR47]: Add: For discussing

For example, the ganglioside GM2 specific binding site is present in the head portion of the δ1 protein and it is typical of Type 1 Lang strain (T1L) [189,191]. The other well studied strain, i.e., type 3 Dearing (T3D) does not have such specificity and possesses instead one for different sialilated-glycans in its body part (not the head) [189]. Both gangliosides GM2 and GD2 belong to the same biosynthesis pathway with GM2 being upstream of a couple of steps [78,192,193] (see Figure 1B). Since they finally depend on GD3 synthase, the previously made observations on the essential role of GD3S in both breast cancer (BC) and NBL are still valid here (see Section 2). In other words, cells capable of making GM2 ought to be capable of synthesizing, in the presence of GD3S –the key regulatory enzyme- also GD2. Also in view of the very detailed 3D structure studies of Stehle and Dermody, which associated differences in binding to GM2 or other sialilated glicans to the presence of specific aminoacid variants [189], we ought to propose that also MFV binds to a sialylated ganglioside, but in this case to GD2. MFV sequence of the δ1 gene has been determined in 2004 through a collaboration with Genelabs Inc [160,171]. We obtained the initial probes by retro-transcribing the mRNA extracted from SK-N-SH cells, which had been acutely infected with MFV. We reasonably assumed—from massive cytopathic effects elicited in 48–72 h and from running the extracted RNA on 2.2 agarose gels—that the majority of mRNA produced had viral origin. We isolated and studied several clones in more detail. Automatic sequencing with universal primers was performed at Genelabs employing ABI sequencers. Additional sequencing with ABI machines and reagents was performed at Children's Hospital of Zurich (Kinderspital, KISPI) in the following years. Different regions of the δ**1** gene of Micro-Foci inducing virus (MFV) display an homology of 75–85% with the Dearing strain or T3D, the closest relative in terms of sequences. Therefore, such an homology with a variation of 25–35% of nucleotide sequences, leads to the hypothesis of a different ganglioside as ligand and specifically of GD2 for this MFV [160,171].

Alternatively, since it is now known and accepted that both GD2 and GM2 are synthesized through the same anabolic pathway (see before and Figure. 1B) and also employing the identical final enzyme (see to follow and Figure.1B), it is conceivable that cells expressing GD2 should also contain GM2. Therefore, these cells could allow MFV-infection through a canonical port-of-entry for Reoviridae (although this has been demonstrated so far for T1L strains [191]). The enzyme in question is β1,4-N-acetylgalactosaminyltrasferase (also called GM2/GD2 synthase; EC 2.4.1.92) gene [194]). Work both in Japan and at MSKCC has shown its essential role for the synthesis of both mono- and di-sialilated glycosphingolipids, thus becoming an interesting biomarker in both neuroblastoma and small cell lung carcinoma (SCLC) [194–196].

The hypothesis of GD2 as port-of-entry is proposed also in view of the peculiar association of MFV with dramatic genomic aberrations, such as MYCNA induced up to 100X in experimental systems, in which MFV infects and malignantly transforms neuroblasts [160,171] (Section 2.5). In this newly proposed landscape, one of the main functions of anti-GD2 monoclonal antibodies would be to prevent or attenuate, by killing GD2 positive cells (which may also contain the monosialylated ganglioside GM2), the binding and therefore the spreading of MFV to neighbor neuroectodermal cells. This would allow to avoid genotoxic damage, genomic aberrations and further progression of the disease [26]. Since HR-NBL patients, who relapsed after treatment often display a new mutation spectrum especially in the RAS/ALK pathway [131–133], blocking/attenuating a clastogenic agent such as MFV, could also prevent progression and the previously described chromotripsis [148,197,198].

2.7. Final Discussion and Concluding Remarks.

This ~~mini~~ review article has been presented as a general overview or a rapid journey through the evidence on the disialilated ganglioside GD2 role in neuroblastoma therapies. While there are today several excellent review articles on this subject, which we have quoted and recommended to the readers (see sections 1–4), the purpose of this ~~mini~~ review was to focus on less traveled avenues:

Comment [PUR48]: This review

Comment [PUR49]: this review

I. Analysis of stem/embryonic pathways, since NBL is an embryonic tumor;

II. Analysis also of newly discovered pathways both for NBL potential origin and GD2 function;
III. Brief overview of the Genomic Landscape of NBL in an attempt to reconcile it with anti-GD2 clinical results;
IV. To discuss them also in relationship to the MFV model, that we have previously developed;
V. To analyze a potential link between anti-GD2 therapies, the MFV model and genomic alterations, which are typical of HR-NBL.

There is ample consensus that Neuroblastoma is an embryonal tumor, in the sense that it targets cells with stem cell features and behavior. In this mini-review, we have mostly focused on the development-window in which NBL targets are available for the transformation process. Comparison was made with potentially similar carcinogenetic processes, such as the Squamous-Columnar Junction (JCV) of the uterine cervix in women infected by HR-HPVs and developing cervical carcinoma (CC) [58,61] (Section 2). Using CC as a paradigmatic example, would it be possible to eliminate or extirpate the targets for HR-NBL in young children? There are several considerations suggesting that the Herfs-Crum paradigm for HR-HPVs in CC, is not applicable to HR-NBL. 1. First of all, the described additional paradigm, present in NBL cases, i.e., the NBL-4S, tells us that the situation in NBL may be different and disconnected to the GD2 marker. Indeed GD2 is also present in NBL-4S, although data by Terzic et al. suggest that its expression may be lower (and according to their model, this should infer a worst prognosis, the opposite of what is happening) [34]. NBL-4S could be described as a situation of advanced and metastasizing NBL (to the skin, periphel blood, bone marrow, peripheral ganglia, but not bone), which suddenly regresses and disappears, as if the child immune system had awakened and got ridden of the unpleasant guest [22,199]. Several attempts have been made to define the immune response nature and signature of NBL rejection in NBL-4S, but a clear answer is still elusive, although NK cells are most likely involved [200]. 2. A second observation derives from very recent and excellent work initially published by Furlan et al. in 2017 [201]. In Section 2, the developmental processes leading to adrenal cells formation –and therefore also to the precursors of NBL/HR-NBL- has been summarized by emphasizing the role of NCC as precursor cells, which are capable of extensive migration [38]. This also suggests that NBL cells have hay-jacketed this migratory capabilities, thus creating the basis for their extensive and rapidly-acquired metastatic potentials [50]. In terms of cell-targets, adrenals contain two major cell types: Cells of the sympathetic ganglia and adrenal chromaffin cells, so that a unique precursor, named the sympathoadrenal (SA) lineage has been hypothesized for years although not proved [98]. Furlan et al. (2017) and additional work with Igor Adameyko's group (2019) have extensively utilized so-called genetic-tracing experiments [201,202]. Such experiments strongly indicate that the origin of the two cell types may be distinct. NCC certainly constitute a main source of sympathetic ganglia precursors (ME Kastriti et al. 2019), thus leading to the formation of the supra-renal ganglion and other sympathetic ganglia. On the other end, adrenal chromaffin cells derive from the so-called Schwann Cell Precursor (SCP), which travels to the adrenals following the "tracks" of peripheral nerves [201–203]. Adrenal chromaffin cells are extremely important, since they are responsible for the release of adrenalin/noradrenalin, i.e., the circulating catecholamines, the hormones essential for our response to stress stimuli [201,203]. Finally, very recently, Adameyko's group with Furlan as co-author showed that SCP is also responsible for the majority of chromaffin cells in the Zuckerkandl Organ (ZO). ZO is situated in close proximity to the adrenal gland and present only during embryogenesis, since it then disappears at birth through autophagy [202]. Although these recent findings are very exciting and could re-position our general understanding of NBL origin, aggressiveness or demise (for example, in the case of NBL-4S), this ~~mini~~ review does not have sufficient space to describe and discuss in detail the data of Furlan and Adameyko [201–203]. Suffice to say that Furlan-Adameyko's data could be associated and explain previous studies on neuroblastoma by the group of R. Ross, B. Spengler and J. Biedler and our group as well. RSB group demonstrated that two –and possibly three- types of targets exist in HR-NBL: neuronal cells called *N cells* (with scanty cytoplasm, neuritic and neurofilament processes and neuronal markers), Schwann like cells, termed *S cells*, which present much larger cytoplasms, stronger substrate attachment and are positive for vimentin and CD44 and an

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intermediate type, called *I-cells* cells, capable of differentiating toward both *N* and *S* cell types [204–207]. In the future, RBS data ought to be revised through the new magnifying lenses of Adameyko and Furlan results. They could also interface with our data (presented in Figures 2–4) and our model for the origin of extensive genomic aberrations (such as MYCNA of 1000X). In Figure 2B, a Micro-Focus is presented, as obtained from 1ary cultures of a larger HR-NBL tumor (diameter 10 cm) with high MYCNA (1000X): It is formed by neural cells packed in what could be called today a *"neurosphere"* [208]. These cells have neural markers, show at the DNA level the extensive and dramatic (1000X) MYCN amplification (MYCNA) as described and could be also assimilated to the *N* cells of RSB [204]. The underlying monolayer in the same micrograph is formed instead by very flat mesenchimal looking cells with large cytoplasms and with strong vimentin and CD44 positivity: They could be equalized to the so called *S* cells of RSB. Finally, and as described [170], the original discovery of Micro-Foci inducing Virus (MFV) was obtained by treating monolayers of *S*-type cells with ultra-filtered supernatants from cultures containing several Micro-Foci (MFs) and thus demonstrating the induction of hundred of new MFs. These experiments could be explained by hypothesizing that *I-cells* (according to RSB) were originally present in these cultures -although certainly not obvious or visible-. Alternatively, that an SCP targets could be present in the original cultures and induced to produce aberrant *N*-type cells with high MYCNA by the infection with MFV particles [160,170,203,204]. Finally, these data may also find a molecular connection with very recent discoveries by R. Versteeg group: They initially identified a super-enhancer activity associated with the enhancer of zeste homologue 2 (EZH2) gene (see Section 2.2), thus explaining EZH2 activity in constructs lacking and therefore independently from its methyltransferase activity ~~[103,210].~~ Most recently, they identified two super enhancer differentiation states: One present in mesenchimal cells of NBL (possibly as the one we and RSB described, see Figures 2–3, possibly Furlan's SCP) ~~[103,211]~~ and the other corresponding to committed adrenergic populations, that we described as MFs. Since Versteeg's group finds them associated with a network of Transcription Factors (i.e., PRRX1 in mesenchymal cells), further work in the future may allow to clarify this area ~~[103,211].~~

3. The Main Purpose of This ~~Mini~~ Review Was However to Associate the Exciting Clinical Findings of Anti-GD2 Therapies with the Molecular Landscape of HR-NBL.

The two areas have long-lasting starting points of over 35 years, but only recently started a more proficuous although still preliminary dialogue [33,103]. GD2 is still considered as a general marker for NBL, although attempts made to correlate levels of its expression with clinical responses [34,46] did not provide clear results. Furthermore, these appear to be the only published hypotheses so far for GD2 function in anti-GD2 therapy [34,46,193]. It was here underlined the very interesting clinical report by Kushner-Cheung that MYCNA cases with intermediate risk respond as low risk (essentially benign) tumors [104]. In other words, MYCNA seems to characterize a special subclass or category of HR-NBL in which anti-GD2 immunotherapy is particularly effective [104]. Several explicatory pathways have been chosen for this and similar observations. For example, the group of R. Seeger at Children's Hosp. in Los Angeles has emphasized the potential presence of inhibitory tumor micro-environment (TME) with interesting result presented in Section 2.3. However, TME effects appear to be widespread and certainly present also in tumors without MYCNA, as also shown by this group [107]. Our contribution to the discussion throughout the years has been focalized mostly on MYCNA+ tumors, because Micro Foci inducing Virus (MFV) 1. was isolated from HR-NBL with very high MYCNA values and diagnosed in a cancer-cluster of HR-NBL cases; 2. Especially in view of the fact that MFV is capable of inducing extensive MYCNA (20–100 folds) in cells originally diploid at the MYCN locus (SK-N-AS, VA-N-BR, ~~Sk-N-SH~~ SK-N-SH, etc., see Figure. 4A). Although the carcinogenetic mechanism is still partially unclear, the strong initial induction of dsDNA breaks immediately after infection (leading to extensive/dramatic apoptosis [170]) suggest that MFV could be a "radiomimetic" virus, with potential capability of inducing extensive genomic aberrations, such as "rogue cells", chromotripsis and chromoplexis, etc. (U. Rovigatti & A. Vannacci, manuscript in preparation). 3. MFV infections appear to recapitulate the whole landscape of pediatric HR-NBL in

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several animal models (in rats: Fisher 344, Sprague-Dowley, nu/nu mice etc.), including the *opsoclonus-myoclonus* syndrome (present in approx. 3 of NBLs, otherwise it is extremely rare = 10^{-7}), the appearance of ataxia, extensive diarrhea (tumor VIP, vaso-intestinal peptide) etc. [160,170,171]; 4. In connecting with anti-GD2 therapies, this ~~mini~~ review wanted to emphasize also elements that may explain its great success (despite the dismal prognosis of HR-NBL cases) with 20% increased EFS at 5 years [45]. Since this family of viruses and MFV in particular strongly rely on membrane receptors for entering, infecting and malignantly transform human cells, an analysis of what is today known about Reoviridae/MFV receptors has been presented. Although JAM is the final essential element for cellular penetration, gangliosides similar to GD2 and belonging to the same anabolic chain (i.e., GM2, two metabolic steps upstream, Figure. 1B) appear to confer infection specificity [186–189]. It is therefore hypothesized that GD2 may be the specific receptor for MFV, allowing MFV entrance into specific targets. These are generally believed to be part of the Neural Crest (i.e., NCC), but today are also discussed as possible Schwann Precursor Cell (SPC) or as mesenchymal cells (in Figure. 2B), or the *I* cell of Ross-Spengler-Biedler [160,203,204]. Our previous sequencing data on MFV [160,171] with 15–25% difference from Ortho-Reoviridae are at least compatible with a different ganglioside specificity (from GM2), in view of previous examples associated with AA modifications [189] or with co-presence of GM2 [72]. However, the final test or Occam Razor, as it always happens in science, will be experimental, as dinutuximab is employed for NBL therapy and available for experimentation.

Although neuroblastoma has been considered as a "conundrum" or as a "Tough nut to crack" [103,209], it is certainly hoped that this discussion and considerations will eventually provide new light and possibly new effective therapeutic strategies for patients with HR-NBL. Especially for patients -like the one with HR-NBL/very high MYCNA from which MFV was originally isolated- who still face suffering and a dismal end in view of poor prognosis.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: title, Table S1: title, Video S1: title.

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Legend for 2A, 2B and 2C has been detailed (also in text): **Figure 2. 2A.** An extensive amplification of the MYCN locus was present in this tumor from an HR-NBL patient in a cancer cluster in Louisiana (lane 1). Comparison with control PBL DNA (lane 2) indicates a MYCN amplification of approximately 1000 folds. **2B.** An example of Micro-Focus composed by neuroectodermal cells growing on top of mesenchimal (Schwann) cells; **2C.** Micro-Foci inducing Virus (MFV) particles detected by Transmission Electron Microscopy (TEM) in the cytoplasm of tumor cells (MFV diameter size: 65-69 nm; magnification 18,000X)

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yes, they should be renumbered (maintaining the corresponding references !) (see also PUR 51,52,54)