Neuroblastoma

Katherine K. Matthay[1,2], John M. Maris[3,4], Gudrun Schleiermacher[5], Akira Nakagawara[6], Crystal L. Mackall[7], Lisa Diller[8,9] and William A. Weiss[1,2,10]

Abstract | Neuroblastoma is the most common extracranial solid tumour occurring in childhood and has a diverse clinical presentation and course depending on the tumour biology. Unique features of these neuroendocrine tumours are the early age of onset, the high frequency of metastatic disease at diagnosis and the tendency for spontaneous regression of tumours in infancy. The most malignant tumours have amplification of the *MYCN* oncogene (encoding a transcription factor), which is usually associated with poor survival, even in localized disease. Although transgenic mouse models have shown that *MYCN* overexpression can be a tumour-initiating factor, many other cooperating genes and tumour suppressor genes are still under investigation and might also have a role in tumour development. Segmental chromosome alterations are frequent in neuroblastoma and are associated with worse outcome. The rare familial neuroblastomas are usually associated with germline mutations in *ALK*, which is mutated in 10–15% of primary tumours, and provides a potential therapeutic target. Risk-stratified therapy has facilitated the reduction of therapy for children with low-risk and intermediate-risk disease. Advances in therapy for patients with high-risk disease include intensive induction chemotherapy and myeloablative chemotherapy, followed by the treatment of minimal residual disease using differentiation therapy and immunotherapy; these have improved 5-year overall survival to 50%. Currently, new approaches targeting the noradrenaline transporter, genetic pathways and the tumour microenvironment hold promise for further improvements in survival and long-term quality of life.

Neuroblastoma is a tumour of early childhood and is the most common malignancy diagnosed in the first year of life, with 25–50 cases per million individuals[1]. 90% of tumours arise in children who are <10 years of age, and neuroblastoma has a median age at diagnosis of 18 months[2]. Neuroblastoma is a neuroendocrine tumour that arises in the developing sympathetic nervous system (from any neural crest element), which results in tumours in the adrenal glands and/or sympathetic ganglia.

Several genetic alterations have been shown in neuroblastoma cells, including amplification of *MYCN* (encoding the transcription factor N-MYC), mutations in *ALK* (encoding anaplastic lymphoma kinase) and segmental chromosomal alterations. The most malignant neuroblastomas have been shown to harbour amplification of *MYCN*, which is found in approximately 20% of tumours. *MYCN* amplification is usually associated with segmental chromosomal loss of the distal short arm of chromosome 1 (1p) and poor patient survival[3]. Other segmental chromosomal alterations have also been shown in neuroblastoma and are associated with adverse prognosis[4]; for example, 11q deletion negatively correlates with *MYCN* amplification, but is nevertheless associated with poor survival. In fact, the lack of mutations found in tumours at diagnosis and recurrent patterns of whole-chromosome or large segmental DNA copy number alterations suggest that neuroblastoma is a copy number-driven cancer. Germline gain-of-function mutation in *ALK* is the main driver of most familial neuroblastomas. Somatic mutations in *ALK* and mutations in members of the mitogen-activated protein kinase (MAPK) pathway provide the potential for the use of precision therapy for management[5–8].

Neuroblastoma is diagnosed using a combination of laboratory tests, radiographic imaging and pathology. After disease staging, which is based on tumour spread and the assessment of risk factors for surgical removal of the tumour, each patient is stratified as very-low-risk, low-risk, intermediate-risk or high-risk based on clinical and molecular risk factors to aid clinicians in deciding the best course of treatment. In young infants with favourable biology, many tumours spontaneously regress without the need for treatment, even if they have metastatic disease. By contrast, for children >18 months of age at diagnosis, with either metastatic or unresectable, biologically unfavourable disease

Correspondence to K.K.M. Department of Pediatrics and Helen Diller Family Comprehensive Cancer Center, University of California, San Francisco, California 94158, USA.
Kate.Matthay@ucsf.edu

Article number: 16078
doi:10.1038/nrdp.2016.78
Published online 10 Nov 2016

Author addresses

[1]Department of Pediatrics and Helen Diller Family Comprehensive Cancer Center, University of California, San Francisco, California 94158, USA.
[2]UCSF Benioff Children's Hospital, San Francisco, California, USA.
[3]Children's Hospital of Philadelphia, Philadelphia, Pennsylvania, USA.
[4]Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania, USA.
[5]INSERM U830, SiRIC Laboratoire de Recherche Translationelle en Oncologie Pédiatrique, and Department of Pediatric Oncology, Institut Curie, Paris, France.
[6]Saga Medical Center Koseikan, Saga, Japan.
[7]Department of Pediatrics, Stanford University School of Medicine, Stanford, California, USA.
[8]Department of Pediatrics, Harvard Medical School, Boston, Massachusetts, USA.
[9]Dana-Farber/Boston Children's Cancer and Blood Disorders Center, Boston, Massachusetts, USA.
[10]Departments of Neurology, Neurosurgery, and Helen Diller Family Comprehensive Cancer Center, University of California, San Francisco, California, USA.

(as defined by unfavourable pathology and/or *MYCN* amplification), intensive multidisciplinary therapy, which can include surgery, radiotherapy, chemotherapy and autologous haematopoietic stem cell transplantation (AHSCT), is needed and overall survival is much lower[9]. The occurrence of spontaneous regression and the presence of autoimmune paraneoplastic manifestations in some patients, such as opsoclonus myoclonus syndrome (OMS; a rare syndrome associated with lymphoid infiltrates in the tumour, anti-neuronal antibodies in the serum and a high survival rate[10,11]), have encouraged efforts towards harnessing the immune system to treat neuroblastoma and to activate pathways to induce differentiation of tumour cells.

The high metastatic rate and poor prognosis of advanced disease, as well as unique clinical features of neuroblastoma, have sparked intense research into its biology and the identification of new therapeutic approaches. As more information is uncovered about the molecular aberrations that characterize neuroblastoma, as well as the cellular networks leading to tumour initiation, maturation and progression, there will be a better understanding of the varying clinical phenotypes, ultimately uncovering new molecular therapeutic targets. This Primer describes the epidemiology, mechanisms, diagnosis and management of neuroblastoma. The use of the current risk-adapted therapy, which has had a major influence on improving patient survival and reducing treatment-associated toxicities[9,12–15], is also discussed.

Epidemiology

Demographics

Neuroblastoma is considered an ultra-orphan condition, with <1,000 new cases per year in North America. Indeed, the age-standardized incidence rate varies internationally; in white populations, the incidence of neuroblastoma is 9.7 cases per million but is less common in black individuals, in which the incidence is 6.8 cases per million[1]. Incidence figures in other ethnicities are not precisely known. In addition, the likelihood of developing neuroblastoma varies widely by age, with the highest number of cases detected in the perinatal period and then steadily decreasing over the first 10 years. Neuroblastoma occurs rarely in adolescents and young adults, but tends to be a much more indolent, albeit lethal, disease[16].

The phenotype of disease is highly associated with race and age. For example, patients <18 months of age are much more likely to have a tumour that will undergo spontaneous regression than older children[17]. Indeed, experience with attempts to detect neuroblastoma early using screening for catecholamine metabolites in urine demonstrated that perhaps half of all neuroblastoma that arise in the first year of life are never detected owing to complete spontaneous regression[18,19]. In addition, individuals with African ancestry are more likely to have a more malignant phenotype than individuals of European descent[20]. Neuroblastoma is more common in boys than in girls, but the genetic and epigenetic basis for this preponderance remains obscure[21]. No clearly documented and independently replicated environmental factor has been shown to influence the risk of neuroblastoma. However, environmental exposures, such as maternal drug use and the use of hair dyes during pregnancy, might still have a role in disease, but at a much lower effect size than in adult malignancies[22,23].

Genetic risk factors

In the 1970s, neuroblastoma was proposed to have a similar genetic basis as retinoblastoma, with a 'two-hit' model explaining familial disease (the first hit being a germline mutation, followed by a second hit of an acquired somatic mutation)[24]. However, unlike retinoblastoma, familial cases of neuroblastoma are exceedingly rare and are found in 1–2% of cases. In 2008, germline gain-of-function mutations in *ALK* were identified as the main predisposing factor for familial neuroblastoma[7,25]. The 'second hit' has not yet been defined, although it is not as simple as a mutation of the other *ALK* allele; amplification of the mutated allele or deletion of the normal allele is also possible. Germline loss-of-function mutations in *PHOX2B* (which encodes paired mesoderm homeobox protein 2B and is a master regulator of neural crest development) predisposes to the majority of syndromic neuroblastoma cases, in which neuroblastoma co-segregates with central congenital hypoventilation syndrome and/or Hirschsprung disease[26,27]. Additional germline mutations are also likely to predispose to neuroblastoma; ongoing sequencing efforts are designed to define the spectrum of such rare DNA variants. Many of the more highly penetrant mutations in neuroblastoma predisposition genes can either be inherited or arise *de novo* and can also affect the risk for a different malignancy in survivors. Importantly, no accepted algorithm is available to determine who should be screened for germline mutations or how to counsel families in which a child has a clear predisposition allele for neuroblastoma, especially in terms of life-long risk for cancer.

Genome-wide association studies (GWAS) have revealed that neuroblastoma is a complex genetic disease, associated with common polymorphic alleles that can influence neuroblastoma formation. Twelve highly

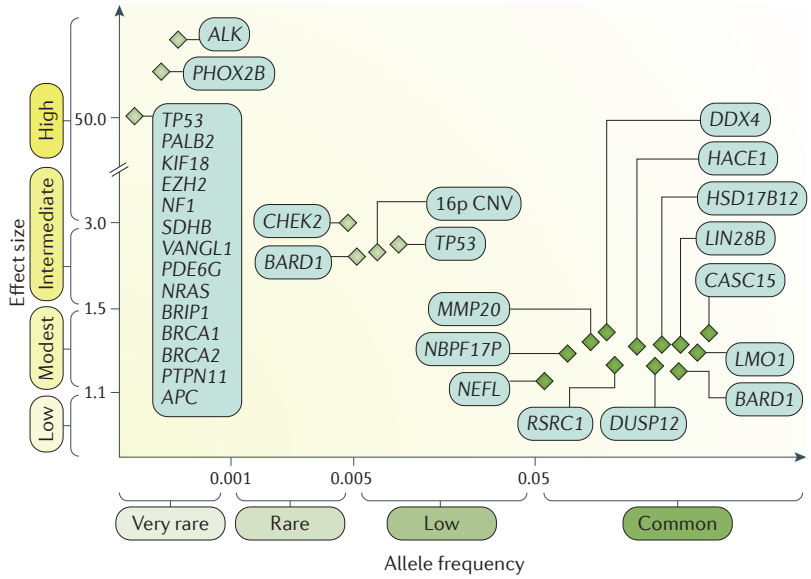

Figure 1 | **Genetic predisposition to neuroblastoma.** *ALK* and *PHOX2B* mutations cause familial neuroblastoma with high penetrance. *ALK* and *PHOX2B* mutant alleles are very rare in the population and are inherited in an autosomal dominant Mendelian manner. Other genes with damaging mutations in the germline that can predispose to neuroblastoma have been identified (such as *TP53*, *NRAS* and *BRCA2*), but the clinical relevance of many of these mutations remains to be determined. Several common polymorphisms (such as *BARD1* or *LMO1*) that individually have a relatively small effect on tumour initiation can cooperate to lead to sporadic neuroblastoma tumorigenesis. Ongoing work is identifying rare or low-frequency alleles; dozens if not hundreds of others alleles are predicted to exist, which might explain the heritability of neuroblastoma. The mechanisms of epistatic interaction of the risk alleles remain to be defined. CNV, copy number variant.

significant and validated genetic associations with neuroblastoma have been identified to date[28] (FIG. 1). Each association has a relatively modest individual effect on disease initiation (with relative risks between 1.5 and 2.5), but multiple associations can cooperate in an individual patient to promote malignant transformation during neurodevelopment. GWAS can uncover crucial cellular networks that not only participate in disease initiation but also have a role in disease progression and maintenance, and might have translational relevance. Many GWAS-defined neuroblastoma susceptibility genes have been shown to have potent oncogenic or tumour-suppressive functions in established disease[29], suggesting that the subtle effects on gene expression that cooperate at disease initiation are stochastically and/or epigenetically selected for as tumours evolve. Distal regulatory elements, such as enhancers, probably have a major role in this selection[30].

Mechanisms/pathophysiology

Neuroblastoma stem cell

Neuroblastoma arises from cells of the developing sympathetic nervous system (FIG. 2), probably from sympathoadrenal progenitor cells that differentiate to form sympathetic ganglion cells and adrenal chromaffin cells (the catecholamine-secreting cells of the adrenal medulla)[31]. Evidence from human tumours confirming the presence of these neuroblastoma stem cells is only now emerging[32].

Genetic alterations

Several genetic alterations have been observed in neuroblastomas, including gene amplifications, polymorphisms and chromosomal alterations.

MYCN *amplification*. N-MYC is a master regulator of transcription that can activate genes that affect cancer hallmarks, such as sustained growth, and repress genes that drive differentiation (reviewed in REF. 33). Most known genes can be activated by N-MYC, thus defining a simple downstream pathway of activation is impossible. Transcriptional targets of N-MYC that promote cell cycle progression include cyclin-dependent kinase 4 (CDK4), the serine/threonine-protein kinase cell cycle checkpoint kinase 1 (CHK1), inhibitor of DNA-binding 2 (ID2), minichromosome maintenance protein (MCM), Myb-related protein B (MYBL2) and S-phase kinase-associated protein 2 (SKP2); whereas cyclin-dependent kinase-like 5 (CDKL5) and tissue transglutaminase promote differentiation[30] (FIG. 3). The identification of *MYCN* as a transforming gene in neuroblastoma followed observations that metaphase spreads from some neuroblastomas showed cytogenetic signatures of gene amplification (homogeneously staining regions, such as regions of uniform Giemsa staining, in addition to the presence of double minute chromosomes)[34–36] and the identification of amplified *MYC* homologues in neuroblastomas[37]. *MYCN* amplification has been shown to be associated with advanced tumour stage and disease progression (independent of the stage of disease and the age at diagnosis)[38,39] and is used as a biomarker for risk stratification.

In response to DNA damage and/or the expression of mitogenic oncogenes, some cell types, such as fibroblasts, activate cell cycle checkpoints[40]. The presence of similar checkpoint activation in sympathoadrenal progenitor cells is uncertain; if analogous checkpoints are activated in response to *MYCN* amplification in these cells *in vivo*, then amplification would presumably only occur in the setting of preceding genetic mutations, which enable cells to tolerate genomic instability and prevent the prior activation of these cell cycle checkpoints. Thus, although gene amplification is generally considered a late event in most cancers, this has not been shown in tumours derived from sympathoadrenal progenitor cells. In addition, through targeting the expression of *MYCN* to sympathoadrenal progenitor cell models, the misexpression (as opposed to overexpression, as *MYCN* is not normally expressed in terminally differentiated cells) associated with *MYCN* amplification fails to recapitulate co-expression of other genes that are often co-amplified with *MYCN* in human tumours at the 2p24 amplicon, such as *ALK*. In addition, cell models fail to recapitulate the potential for titration of regulatory proteins that might bind to the amplified DNA or to the chromatin associated with amplified *MYCN*. Thus, in modelling the misexpression of *MYCN* as a surrogate for gene amplification, other amplification-specific contributors to transformation are not represented.

Despite the potential differences between amplification of *MYCN* on 2p24 in human neuroblastoma and misexpression of *MYCN* in model systems, transgenic mouse models for *MYCN*-driven neuroblastoma have been generated and are used to study neuroblastoma *in vivo*[41] (BOX 1).

The *MYCN* locus also encodes an antisense transcript: *MYCNOS* (encoding N-CYM)[42]. In human neuroblastoma, *MYCNOS* is always co-amplified and co-expressed with *MYCN* and the expression of *MYCNOS* mRNA is associated with poor clinical outcome[42,43]. N-CYM has been shown to stabilize N-MYC by inhibiting glycogen synthase kinase 3β (GSK3β)-driven degradation of N-MYC. By contrast, mice transgenic for both *MYCN* and *MYCNOS* showed frequent metastases[43], suggesting a role for N-CYM in tumour metastasis. Interestingly, transgenic mice for only *MYCNOS* did not develop neuroblastoma.

ALK *amplification.* *ALK* was identified as a predisposition gene for familial neuroblastoma[7,25], although somatic mutations in *ALK* have also been shown in approximately 14% of high-risk neuroblastomas[44]. Owing to their similar locations on 2p, *ALK* and *MYCN* can be co-amplified. The expression of *ALK* is limited to neural tissues. Once considered an orphan receptor, ALK has now been shown to have more than two ligands: heparin and members of the FAM150 protein family[45,46]. Gain-of-function mutations in *ALK* can drive neuroblastoma formation in one mouse model (controlled by the dopamine β-hydroxylase (*Dbh*) promoter), but require coincident misexpression of *MYCN* (using the tyrosine hydroxylase (*TH*) promoter) in both zebrafish and a *Th*-driven mouse model[47,48]. The basis for cooperation between *ALK* and *MYCN* might be due to ALK-mediated activation of phosphoinositide 3-kinase (PI3K) signalling, leading to stabilization and increased levels of N-MYC (FIG. 3). Why *ALK* acts as a transforming gene in the absence of *MYCN* in one animal model of neuroblastoma but not in others is not known. ALK also signals through RAS, which leads to downstream MAPK signalling; the MAPK pathway is frequently activated in neuroblastoma at relapse[49–51]. ALK upregulates the proto-oncogene tyrosine-protein kinase receptor RET and RET-driven sympathetic neuronal markers of the cholinergic lineage[52], which might correspond to the normal developmental roles of ALK, but also offers novel therapeutic entry points for combined ALK and RET inhibition of neuroblastoma[53].

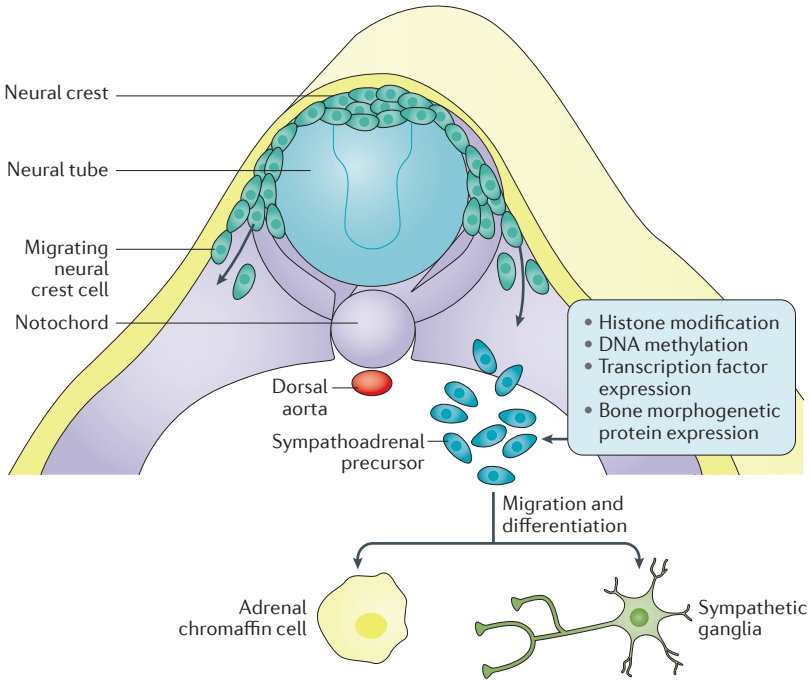

Figure 2 | **The neuroblastoma stem cell.** During development, the neural crest arises from the neural tube after tube closure. Neural crest cell specification into a wide range of cell types contributes to many anatomical structures[213] and occurs via an epithelial-to-mesenchymal transition (a process by which cells lose polarity and have reduced adhesive properties), which enables neural crest cells to delaminate and migrate from the neural tube. A complex series of epigenetic and transcriptional programmes regulate this delamination, migration and differentiation, which involves histone modification, DNA methylation and the expression of bone morphogenetic proteins and transcription factors. Activated transcription factors include those in the SOX family, which in turn activate transcriptional regulators of cellular proliferation and differentiation[214]. Neural crest cells can be broadly subdivided into four functional types: vagal and sacral, cranial, cardiac and trunk. Cells of the trunk neural crest migrate to the dorsal aorta, where they differentiate into sympathoadrenal progenitor cells, which eventually give rise to cells of the peripheral nervous system, including sympathetic ganglia and the adrenal gland — the main sites in which neuroblastoma arises. Adapted from REF. 215, Nature Publishing Group.

LIN28B *polymorphisms.* Polymorphic alleles within the *LIN28B* (encoding lin-28 homologue B) locus are highly associated with the development of high-risk neuroblastoma[54]. Amplification of *LIN28B* occurs rarely in high-risk neuroblastoma, but overexpression occurs commonly[55]. In neuroblastoma cells, misexpression of *LIN28B* leads to high levels of N-MYC. Similarly, misexpression of *LIN28B* in mice (under the control of the *Dbh* promoter) drives the development of neuroblastoma that contains high levels of N-MYC[55]. It was well known that LIN28B negatively regulates microRNA (miRNA) biogenesis through depletion of the let-7 family of miRNAs and more recently it was shown to modulate the activity of the GTP-binding nuclear protein RAN and the stability of Aurora kinase A (AURKA) in neuroblastoma cells[56]. These findings show that LIN28B–RAN–AURKA signalling drives neuroblastoma oncogenesis and that this pathway could be used for therapeutic targeting. In addition, *MYCN* can function as a competing endogenous RNA for let-7 miRNAs, demonstrating that LIN28B-dependent and LIN28B-independent mechanisms exist for let-7 depletion and miRNA deregulation in neuroblastomas[57].

Other genomic rearrangements. Genomic surveys of neuroblastoma tumours using whole-genome sequencing have identified loss-of-function genetic alterations in *ATRX* (encoding the RNA helicase, transcriptional regulator ATRX) in approximately 10% of patients and *TERT* (encoding telomerase reverse transcriptase) promoter rearrangements (resulting in enhancer hijacking) in approximately 25% of patients[58,59], although

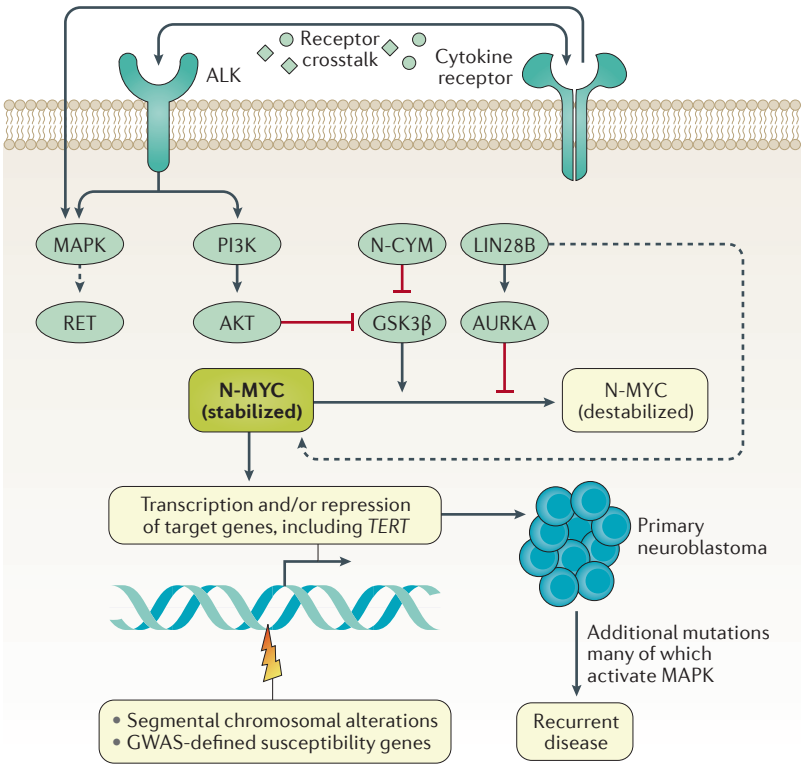

Figure 3 | **MYCN-amplified neuroblastoma initiation.** In high-risk *MYCN*-amplified neuroblastoma, multiple mechanisms converge to stabilize N-MYC. Some targets shown here, including anaplastic lymphoma kinase (ALK), RET, phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK), are potentially druggable. No simple list of target genes delineates the role of N-MYC in blocking differentiation and sustaining growth, although some relevant genes that are activated by N-MYC include *CDK4*, *SKP2*, *CHEK1*, *ID2* and the *mir-17~92* cluster gene. Relevant genes that are repressed by N-MYC include *TP53*, *INP1*, *DKK1* and *CDKL5* (reviewed in REF. 33). Factors in the tumour microenvironment can promote crosstalk between cytokine receptors and receptor tyrosine kinases including ALK, which can induce MAPK activation. Mechanisms for high-risk disease that are not driven by amplified *MYCN* are less well understood and might involve other pathways, such as telomerase reverse transcriptase (TERT) and the transcriptional regulator ATRX (not shown) as well as MAPK. Furthermore, loss of putative tumour suppressor genes in deleted chromosomal segments also occurs in tumours that are not driven by *MYCN* amplification. Solid lines indicate direct interactions; dashed lines indicate indirect interactions. AURKA, Aurora kinase A; GSK3β, glycogen synthase kinase 3β; GWAS, genome-wide association studies; LIN28B, lin-28 homologue B.

ATRX and *TERT* mutations are usually not present in tumours with *MYCN* amplification[5,60]. As TERT is also a target of N-MYC, these data indicate that all neuroblastomas require a way to activate TERT (*MYCN* amplification or enhancer hijacking) or to bypass this checkpoint (*ATRX* mutation). Other genes involved in chromatin remodelling that are thought to have a role in neuroblastoma include the Polycomb complex genes *ARID1A* and *ARID1B*. Haploinsufficiency for *ARID1A* and *ARID1B* are recurrent events in high-risk neuroblastoma, but the frequency of these mutations and their effects on chromatin structure have not been defined[61]. Additional focal copy number alterations (gains or losses) also affect N-MYC target genes, for example, a focal gain in the N-MYC-regulated *mir-17~92* cluster in a neuroblastoma cell line[62]. Moreover, other focal gains and amplifications show enrichment for other N-MYC target genes[62]. These recurrent genetic lesions have yet to be modelled in mice or other organisms to understand their involvement in neuroblastoma development.

Segmental chromosomal copy number alterations. In high-risk neuroblastomas that are not driven by amplification of *MYCN* or are mutated for *ATRX*, most do not show recurrent somatic mutations in any known protein-coding gene[5]. However, the presence of recurrent somatic mutations in non-coding regions of the genome, such as enhancer elements or other regulatory regions, in these cancers remains unclear. Almost all high-risk neuroblastomas show recurrent segmental chromosomal copy number alterations; gain of 17q has been shown in over half of cases of neuroblastoma[63] and loss of 1p has been shown in one-third of cases[64]. Both gain of 17q and loss of 1p correlate with *MYCN* amplification and poor prognosis. In addition, loss of 11q has been shown in one-third of high-risk cases, is inversely correlated with *MYCN* amplification and is associated with high-risk disease[64]. Other relatively common segmental chromosomal alterations in neuroblastoma include gains of 1q and 2p and loss of 3p, 4p and 14q, but the risk of poor prognosis associated with these copy number alterations is less established than associations with 1p, 11q and 17q[5,33]. Some recurrent segmental alterations also occur at relapse, including deletions of 1p and 6q[51].

The loss of 1p and 11q and their association with high-risk neuroblastoma suggest the presence of a tumour suppressor gene on these chromosomes. However, no such gene has been identified, although several candidate tumour suppressor genes have been identified in the deleted region of 1p, including *CHD5*, *CAMTA1*, *KIF1B*, *CASZ1* and *mir-34A*[65]. Thus, the high frequency of segmental chromosomal alterations in neuroblastoma coupled with the relative rarity of recurrent mutations in known protein-coding genes suggest the loss of 1p and/or 11q as driver events and the underlying genetics signified by these losses are complex.

Immune system

No clear explanation exists for the aforementioned age-related and stage-related differences in neuroblastoma outcome, but the host reaction (for example, the immune system) to neuroblastoma might have a role. Evidence that immune surveillance recognizes incipient neuroblastoma cells comes from observations in the neuroblastoma-associated paraneoplastic syndrome OMS. Myeloid cells within the tumour microenvironment have been correlated with adverse outcomes in several cancers[66], which has largely been attributed to the effects of alternatively activated (M2) macrophages that can augment tumour growth and suppress T cell-mediated and natural killer (NK) cell-mediated immune clearance. In one study, metastatic neuroblastoma tumours had higher counts of $CD163^+$ M2 macrophages than lower stages of neuroblastoma[67].

Box 1 | ***MYCN*-driven transgenic mouse models of neuroblastoma**

A transgenic mouse model for *MYCN*-driven neuroblastoma has been used since the 1990s[41]. The model expresses human *MYCN*, driven by the rat tyrosine hydroxylase (*Th*) promoter (known as the *Th-MYCN* transgene). TH is an enzyme that converts tyrosine to dopamine[201]. Outbred mice that express the *Th-MYCN* transgene develop primary tumours. These tumours showed complex genetics, such as secondary amplification of the *Th-MYCN* transgene and endogenous *Mycn*, consistent copy number alterations (in outbred tumours), accelerated tumour formation with loss-of-function mutations in *Nf1*, *Rb1* or *Casp8* (REFS 41,202) and changes in penetrance depending on the mouse strain used[203]. The strain-specific differences in penetrance suggest that mouse modifier genes can affect tumour formation; arginase 1 (REF. 203) — which has a role in the synthesis of the neurotransmitter γ-aminobutyric acid (GABA) and in other cancer-relevant pathways — was identified as a significant modifier of tumour formation. Moreover, GABA signalling has been identified as a pathway that drives human neuroblastoma development[203].

Another model for *MYCN*-driven neuroblastoma uses the dopamine β-hydroxylase (*Dbh*) promoter, which converts dopamine to noradrenaline, to drive Cre recombinase[204]. When crossed to mice in which the expression of *MYCN* can be activated by Cre, these animals develop tumours in neural crest-derived tissues and have histology similar to those arising in *Th-MYCN* mice. Strain-specific disease modifiers might play a less prominent part in these animals than *Th-MYCN* mice, although two transgenic mice must be crossed to generate a doubly transgenic tumour-prone mouse[204].

Furthermore, patients >18 months of age who present with *MYCN* non-amplified metastatic neuroblastomas have a higher expression of inflammatory genes identified with tumour-associated macrophages (such as *CD33*, *FCGR3* (also known as *CD16*), *IL6R*, *IL10* and *CD14*) than those presenting at <18 months of age, and the tumour-associated macrophage-linked inflammatory signature was correlated with an adverse prognosis. Another study has confirmed these observations and demonstrated increased levels of prostaglandin E_2 in high-risk *MYCN* non-amplified neuroblastoma with 11q chromosomal deletions, compared with low-risk tumours. Immunofluorescence studies have implicated cancer-associated fibroblasts within the neuroblastoma microenvironment as the mediators of this immunosuppression by prostaglandin E_2 (REF. 68).

The full mechanism of how tumour-associated myeloid cells facilitate tumour growth remains to be gleaned, but IL-6 produced by infiltrating myeloid cells has been implicated in several studies[69,70]. IL-6 is produced by tumour-associated $CD68^+$ macrophages and can bind to the IL-6 receptor, which is expressed by neuroblastoma cells. IL-6 receptor binding activates a signal transducer and activator of transcription 3 (STAT3)-mediated transcriptional programme that augments tumour proliferation and drug resistance, and contributes to immune evasion[71] (FIG. 4). IL-6 has also been shown to be expressed at high levels in sites of neuroblastoma metastases, notably in bone marrow, where it has been implicated in tumour growth and local immunosuppression[72].

The immune response to cancer can be divided into responses that are mediated by antibodies, NK cells and T cells. Each of these responses has been the focus of development of immune-based therapies for neuroblastoma (BOX 2). The basis for developing adoptive NK cell therapy is owing to the consistent observation that neuroblastomas express low levels of major histocompatibility complex class I proteins, which are the main ligands for inhibitory killer-cell immunoglobulin-like receptors (KIRs), expressed on the surface of NK cells[73,74]. T cells can mediate potent and long-lasting antitumour immunity. The predominant natural targets of T cell recognition are mutated self-proteins that arise in cancers due to genetic instability. However, paediatric tumours in general, and neuroblastoma in particular, show a paucity of recurrent coding single-nucleotide variants compared with many adult carcinomas and are predicted to have a relatively low level of inherent immunogenicity[5]. To overcome this issue, engineered T cells expressing specific receptors (known as chimeric antigen receptors) that recognize specific targets on cancer cells have been designed for neuroblastoma (BOX 2).

Tumour regression

Spontaneous maturation and regression have been long described in young infants with neuroblastoma, especially those with stage 4S disease (that is, patients <1 year of age with metastasis limited to the skin, liver or bone marrow)[15,75]. Moreover, screening infants at 3 months of age for neuroblastoma, by measuring urinary catecholamines, revealed that many more neuroblastomas are detected at the time of screening than the number of tumours that manifest later in life (see Screening and prevention)[19]. The mechanisms of spontaneous tumour regression are not fully known, perhaps because these tumours regress before they manifest clinically. However, tumours in children with stage 4S neuroblastoma — known to spontaneously regress in at least half of cases — show no TERT expression[76], suggesting that telomere crisis might have a role in tumour regression. The host immunity, with generation of antibodies against the tumour, might also have a role in tumour regression, as in OMS-related neuroblastoma, which is associated with anti-neuronal antibodies, differentiated tumours and a favourable outcome. One of the likely key mechanisms underlying tumour regression is the nerve growth factor (NGF) dependency of neuroblastoma cells[77,78]. NGF depletion in the developing sympathetic neurons induces many pro-apoptotic genes, such as *TP53*, *TP63*, *E2F1*, *UNC5D*, *KIF1B* and *PRUNE2* (REF. 79); these genes are also expressed in favourable neuroblastomas, including stage 4S tumours, at markedly high levels.

Diagnosis, screening and prevention

Clinical presentation

The median age at diagnosis of neuroblastoma is 18 months, with 40% of patients diagnosed at infancy and 90% of patients at <10 years of age[2]. Age at diagnosis is highly prognostic, as patients <18 months of age have a much better overall survival than patients >18 months of age[2,12]. Adolescents and adults rarely develop neuroblastoma, accounting for <5% of all cases, but typically show a more indolent clinical course with *de novo* chemotherapy resistance. In adolescents, approximately 40% of these tumours harbour loss-of-function

mutations in *ATRX* compared with <20% in younger children and 0% in infants <1 year of age, according to a study of 104 patients[60].

The clinical signs and symptoms of neuroblastoma are directly linked to the location of the primary tumour and sites of metastatic disease. Primary tumours can form anywhere in the sympathetic nervous system, with >50% occurring in the medulla of the adrenal glands (bilateral primary adrenal tumours occur in <1% of all cases); these tumours are associated with poorer survival than primary tumours in other regions[80]. Whereas localized disease often presents as an incidental finding, large abdominal tumours can cause hypertension, abdominal distension and pain. Primary tumours in the neck might cause damage to the cervical ganglion, such as the ganglion stellatum, causing Horner syndrome, symptoms of which can include ptosis (drooping of the upper eyelid), miosis (constriction of the pupil), enophthalmos (posterior displacement of the eyeball), in addition to anhidrosis (lack of sweat). Tumours arising in the paraspinal sympathetic ganglia (also known as the paravertebral sympathetic ganglia) can grow along spinal nerves towards the spinal cord and expand into the neural foramina, which can lead to spinal cord compression.

Metastatic disease is detected in approximately 50% of patients at diagnosis, frequently in the regional lymph nodes, bone marrow and bone, but liver and skin metastasis are more common in young infants <18 months of age[81]. Metastatic sites can cause constitutional symptoms, such as bone pain, fever or weight loss, and symptoms of pallor or bleeding from anaemia and thrombocytopaenia. Specific metastatic bone sites can lead to localized bone pain and limping, and periorbital (meaning, surrounding the eye) metastasis can present as proptosis (protrusion of the eyeball out of the eye socket) or periorbital bruising. Metastasis to other sites of the body, such as the lungs or central nervous system, occurs rarely[81]. Respiratory distress, coagulation disorders or renal impairment might occur due to massive tumour cell infiltration of the liver, particularly in young infants before 3 months of age[82].

Rarely, neuroblastoma can occur with associated syndromes. For example, hypersecretion of vasoactive intestinal peptide by the tumour cells can lead to profuse watery diarrhoea in some patients. As previously mentioned, some patients with neuroblastoma will develop OMS, a rare paraneoplastic neurological disorder characterized by opsoclonus (rapid, involuntary, multidirectional conjugate eye movements), myoclonus (brief, involuntary twitching of a muscle or a group of muscles), severe irritability and cerebellar ataxia, which is most likely caused by an autoimmune process involving the cerebellum. OMS has been shown to affect 2–3% of children with neuroblastoma, but neuroblastoma has been shown in 50–80% of children with OMS[83].

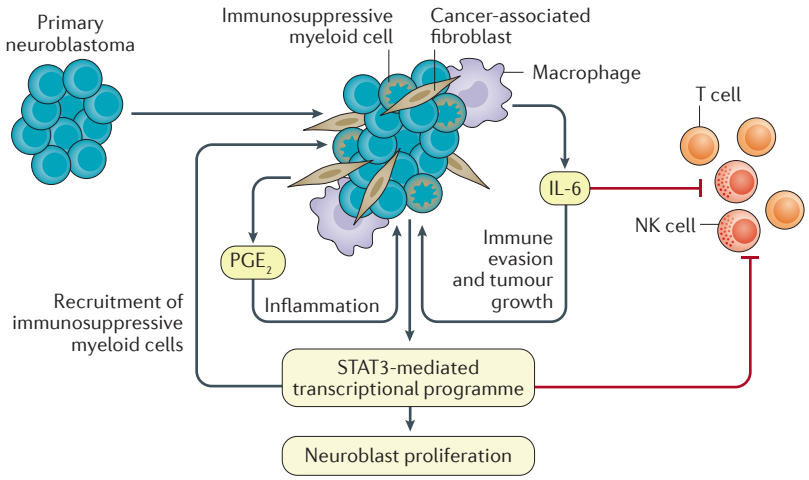

Figure 4 | **Neuroblastoma progression and the tumour microenvironment.** Once initiated, non-neuroblastoma cells within the tumour microenvironment, such as myeloid cells and fibroblasts, are recruited; these cells enhance neuroblast growth, chemoresistance and immune evasion. Tumour-associated macrophages and other myeloid-derived suppressor cells produce immunosuppressive and tumour growth-promoting mediators, such as IL-6, which induces a signal transducer and activator of transcription 3 (STAT3)-mediated transcriptional programme in the neuroblasts, contributing further to the recruitment of suppressive myeloid cells as well as direct suppression of T cells and natural killer (NK) cells. IL-6 induces numerous factors, including additional IL-6, IL-10 and vascular endothelial growth factor. Cancer-associated fibroblasts produce prostaglandin E_2 (PGE_2) via a cyclooxygenase 2-catalysed pathway. PGE_2 is a hallmark of 'tumour-promoting inflammation' that enhances neuroblast growth and chemoresistance, and recruits additional suppressive myeloid populations.

Diagnostic work-up

Confirming a diagnosis of neuroblastoma requires a range of tests, including laboratory tests, radiographic imaging and histological assessment of the tumour. The tumour stage and tumour biology are determined at diagnosis, following which patients are stratified for treatment according to the different risk groups.

Diagnostic laboratory testing. Increased levels of catecholamines or catecholamine metabolites, including dopamine, homovanillic acid (HVA) and/or vanillylmandelic acid (VMA), can be detected in the urine of 90% of all patients with neuroblastoma. The relative amounts of catecholamine metabolites in the urine is related to the degree of cellular maturation of the tumoral neural crest-derived cells, with increased dopamine levels or HVA/VMA ratio associated with biologically unfavourable disease[84]. In rare cases, increased levels of noradrenaline or adrenaline secreted by tumours can lead to arterial hypertension. Plasma-free and total normetadrenaline, metadrenaline and methoxytyramine levels represent a convenient alternative to urine markers, as adequate urine samples are sometimes difficult to obtain in infants[85]. Increased levels of catecholamines, in conjunction with the presence of typical small round blue cells in tumours or bone marrow (following bone marrow biopsy or fine-needle aspirate of tumours) with haematoxylin and eosin tissue staining, are considered diagnostic for neuroblastoma in the absence of standard histology and immunohistochemistry. Increased levels of serum markers, such as lactate dehydrogenase or neuron-specific enolase, have been described as unfavourable prognostic markers in neuroblastoma but are not specific (BOX 3).

Radiographic imaging and metastatic evaluation. Tumour imaging and metastatic evaluation of neuroblastoma includes radiological assessment of the primary tumour and osteomedullary and/or soft tissue metastases (BOX 3). Complete staging requires bilateral bone marrow aspirates (removal of bone marrow cells) and trephine bone marrow biopsies (removal of bone marrow tissue), obtained from both iliac crests, and histological examination and immunohistochemistry for a quantitative approach to detect metastatic disease[86]. Measuring neuroblastoma-specific transcripts, such as *PHOX2B* or *TH*, with quantitative reverse transcriptase PCR in blood and bone marrow aspirates can provide additional prognostic information[87].

Although ultrasonography is frequently the first imaging modality used for neuroblastoma because of its wide availability and non-invasiveness, further local assessment requires CT imaging or MRI. Preference is generally given to MRI, despite longer acquisition time and the need for sedation in younger children, based on higher contrast resolution images using T1-weighted and T2-weighted MRI sequences and lack of ionizing radiation exposure. Tumours are often heterogeneous in density and frequently present with calcifications and regional lymph node involvement. Radiographic imaging can be used to identify the presence of image-defined risk factors (IDRFs) for surgical excision of the tumour and enables staging of the tumour (see Staging and risk classification, below). IDRFs describe local extension of the primary tumour, which can consist of perivascular involvement with arterial encasement (that is, cancer surrounding an artery), infiltration of adjacent soft tissues and organs (such as the kidneys or the liver) and infiltration of the neural foramina and epidural space of the spinal canal[88].

The extent of metastatic disease is assessed by a metaiodobenzylguanidine (mIBG) scan, which uses radiolabelled mIBG (a molecule with a similar structure to noradrenaline). Iodine-123 (^{123}I) is preferred to the use of ^{131}I for the radiolabelling of mIBG because it has a lower radiation dose, shorter half-life, produces better quality images and has lower thyroid toxicity than ^{131}I (REF. 89). Approximately 90% of neuroblastomas are mIBG-avid, due to the expression of the noradrenaline transporter, which enables mIBG uptake into tumour cells[90]. mIBG scan has an estimated sensitivity of 90% and a specificity of 99% and enables the assessment of both local and metastatic soft tissue and bone marrow disease (FIG. 5). Hybrid imaging techniques, using multimodal camera systems and enabling the integration of single-photon emission CT (SPECT; which uses radiographic tracers with CT), can combine the contrast provided by tumour-avid radioactive drugs with the anatomical precision of CT[91]. Semi-quantitative mIBG-based scoring methods are currently being evaluated for their prognostic significance at diagnosis of neuroblastoma and during follow-up[92] (BOX 4).

The extent of disease in patients with mIBG non-avid neuroblastoma can be evaluated using techniques that are independent of mIBG uptake, such as technetium-99 bone scintigraphy, or, preferably, ^{18}F-fluorodeoxyglucose (FDG) PET-CT[91,93]. Other imaging techniques, such as ^{18}F-L-dihydroxyphenylalanine-PET (^{18}F-DOPA-PET) and gallium-68 (^{68}Ga)-DOTATATE-PET are also being evaluated for patients with neuroblastoma[94,95].

Pathology. Neuroblastoma pathology is an important determinant of prognosis. Peripheral neuroblastic tumours show different grades of morphological differentiation, such as neuroblastoma (predominantly composed of immature small round tumour cells), ganglioneuroblastoma (composed of both immature cells and tumour cells with terminal neuronal differentiation to ganglion cells) and ganglioneuroma (composed of tumour cells that show maturation with terminal neuronal differentiation to ganglion cells (FIG. 6)). The 1984 Shimada classification of neuroblastoma combined histopathological evaluation with biological characteristics (including patient age) and a cellular index of mitosis and karyorrexis[96]. The Shimada classification was modified by the International Neuroblastoma Pathology Committee (INPC)[97,98] to include the presence of stromal Schwannian cells, which enables the classification of neuroblastic tumours into four categories: neuroblastoma (Schwannian stroma-poor), ganglioneuroblastoma intermixed (Schwannian stroma-rich), ganglioneuroma (Schwannian stroma-dominant) and ganglioneuroblastoma nodular (composite Schwannian stroma-rich/stroma-dominant and stroma-poor). In addition, the INPC further revised the classification of nodular ganglioneuroblastoma by dividing into favourable and unfavourable subsets, according to the age of the patient, the grade of tumour differentiation and index of mitosis and karyorrexis[99].

Box 2 | **Immune-based therapies for neuroblastoma**

Antibodies against the GD2 disialoganglioside that is expressed by neuroblastoma have undergone extensive preclinical and clinical testing[205,206]. Dinutuximab, a chimeric anti-GD2 monoclonal antibody (also known as ch14.18), has shown efficacy in a pivotal phase III randomized trial when administered following autologous haematopoietic stem cell transplantation[153]. Dinutuximab in combination with cytotoxic chemotherapy has also shown early promising results in patients with bulky, refractory neuroblastoma. The anti-GD2 3F8 murine monoclonal antibody has also demonstrated promising results in patients with relapsed or primary refractory neuroblastoma[207]. Anti-GD2 antibodies exert their antitumour effects largely via cell-mediated cytotoxicity, which is heavily influenced by natural killer (NK) cell reactivity; thus, it is not surprising that patients who are predicted to have increased NK cell reactivity (determined based on killer-cell immunoglobulin-like receptor (KIR) ligand mismatch) show higher response rates to anti-GD2 antibodies[74,208,209]. To amplify the potency of anti-GD2 therapy for neuroblastoma, combined approaches using adoptive NK cell therapy with anti-GD2 monoclonal antibodies are being examined[73].

GD2 is also a T cell target; thus, a chimeric antigen receptor against GD2 was designed and tested for the treatment of neuroblastoma[210]. Administration of T cells that express the GD2-targeted chimeric antigen receptor was safe and showed signs of clinical activity; 3 out of 11 patients with measurable or evaluable disease experienced a complete response and two patients had a long-term sustained remission, but T cell persistence was transient[173]. Clinical trials are underway that incorporate co-stimulatory domains in the GD2 chimeric antigen receptors in an effort to enhance potency. However, tonic signalling, probably due to chimeric antigen receptor aggregation that leads to T cell exhaustion, is a newly recognized problem that needs to be overcome in order to achieve the T cell persistence that is thought necessary for long-term efficacy of these treatments[211].

Genomic characterization. Tumour molecular biology is also assessed at diagnosis and is routinely used for additional prognostic information. *MYCN* amplification status is frequently determined using fluorescent *in situ* hybridization, but can also be assessed using other molecular techniques. *MYCN* amplification is defined as a more than fourfold increase in the *MYCN* signal number compared with the reference probe[100] and is associated with a poor prognosis, even in localized disease or in infants[101]. An RNA-based *MYCN* expression signature, or increased expression of MYC or N-MYC, might also predict poor prognosis in tumours without *MYCN* amplification[102,103].

In neuroblastoma, diploidy, assessed by flow cytometry, is associated with a poorer outcome than triploidy. However, cut-offs for the definition of diploidy versus triploidy or hyperdiploidy remain controversial; a DNA index of strictly 1, or higher cut-point values such as 1.2, has been shown to be prognostically significant[100]. Single-nucleotide polymorphism arrays can inform about copy number and allelic status across the whole genome, including identification of potential regions of copy-neutral loss of heterozygosity (that is, loss of heterozygosity without a change in copy number), and are likely to replace the prognostic value of ploidy in future classification systems[104,105]. Neuroblastoma with gains or losses of whole chromosomes (known as numerical chromosome alterations) are associated with excellent survival, but the presence of segmental chromosome alterations are associated with a poorer survival; these features are now being incorporated into patient risk stratification[4,106].

Owing to the recent demonstration of at least the potential for patient benefit from precision therapy for those with gain-of-function mutations in *ALK* and/or RAS pathway proteins[49–51], next-generation sequence analysis is being incorporated into diagnostic evaluations for neuroblastoma. Gene panels provide the range of coverage necessary to detect subclonal mutations, especially if the tumour specimen is highly admixed with host-derived stromal elements, such as Schwannian cells and fibroblasts. Exome sequencing and whole-genome sequencing approaches, along with RNA sequencing, are rapidly advancing in terms of quality and cost efficiency. These methods provide the potential to assess amplifications (for example, in *MYCN*, *ALK* and *CDK*), segmental chromosomal alterations, homozygous deletions (for example, of *CDKN2A*) and other relevant germline and somatic mutations, simultaneously from a single small tumour biopsy sample.

Staging and risk classification

Multiple staging systems for neuroblastoma have been used in prior studies, but the most widely accepted system used in reporting studies for the past three decades was the International Neuroblastoma Staging System (INSS), which is based on the extent of surgical excision at diagnosis and metastases[107] (BOX 5). The International Neuroblastoma Risk Group (INRG) Staging System (TABLE 1) was designed to identify homogeneous pretreatment risk groups to enable the comparison of clinical trials conducted by different cooperative groups internationally[12].

The extent of disease is determined by the presence or absence of IDRFs and/or metastatic disease at the time of diagnosis (before any treatment or surgery), defining disease stages as local (L1 and L2) or metastatic (M and MS)[88]. Localized disease is classified as L1 (the tumour is restricted to one body compartment, such as the neck, thorax, abdomen or pelvis, and the absence of any IDRFs) or L2 (the presence of one or more IDRF)[88]. The absence or presence of IDRFs has no direct effect on risk group, with some patients with L2 neuroblastoma having low-risk disease and other patients with L2 neuroblastoma having intermediate-risk disease. Metastatic disease is classified as M (that is, distant metastatic disease located away from the primary site) or MS (metastatic disease in infants (<18 months of age) with deposits restricted to the liver, skin and bone marrow). The disease stage is combined with other prognostic factors, including age at diagnosis, pathology and genomic characterization (including *MYCN* amplification, 11q status and ploidy) to define pretreatment risk groups. Ultimately, this system enables patients to be grouped into very-low-risk, low-risk, intermediate-risk or high-risk groups, which dictates the treatment plan (see Management, below). With the evolution of molecular techniques for tumour characterization, most cooperative groups now integrate pan-genomic rather than single-locus genomic data for risk stratification at diagnosis, in particular, for low-risk

Box 3 | **Diagnosis and risk stratification**

Laboratory

- Complete blood count and platelet count
- Prothrombin time and partial thromboplastin time
- Electrolyte, creatinine and uric acid levels and liver function
- Ferritin and lactate dehydrogenase levels
- Urine vanillylmandelic acid, homovanillic acid and dopamine levels

Imaging

- CT or MRI of the primary site, chest, abdomen and pelvis
- CT or MRI of the head and neck if clinically involved
- ^{123}I-metaiodobenzylguanidine (mIBG) scan and then ^{18}F-fluorodeoxyglucose-PET scan if the tumour is not mIBG-avid

Pathology

- Tumour biopsy with immunohistochemistry and the International Neuroblastoma Pathology Committee classification
- Fluorescence *in situ* hybridization for *MYCN*
- Array comparative genomic hybridization or other study for segmental chromosomal alterations
- DNA index (ploidy)
- Bilateral bone marrow aspirate and biopsy with immunohistochemistry
- Optional: genomic analysis for *ALK* mutations

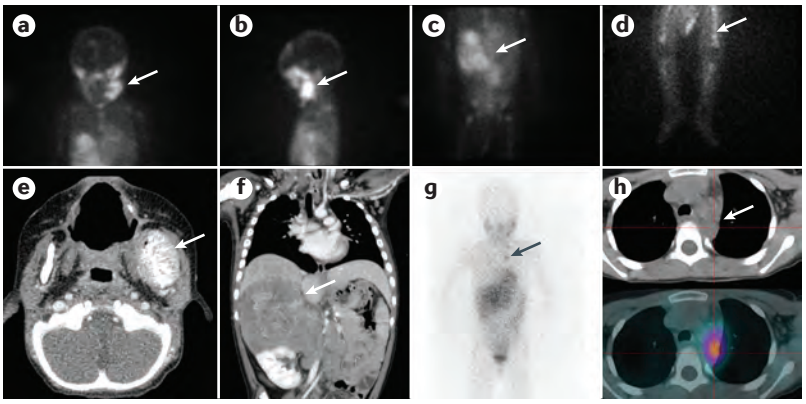

Figure 5 | **mIBG and CT imaging for the diagnosis of neuroblastoma.** Radiographic imaging of a boy 18 months of age with a primary adrenal tumour and metastasis to multiple bones, including the mandible with an associated mass. **a–d** | Metaiodobenzylguanidine (mIBG) scan is positive in multiple axial and appendicular skeletal sites, including a large metastasis in the left mandible (arrows; part **a** and part **b**), a right adrenal primary tumour (arrow; part **c**) and bilateral lower extremities (arrow; part **d**). **e**,**f** | CT of the head showing a large left mandibular metastasis with calcifications (arrow; part **e**) and a large right adrenal primary tumour (arrow) with encasement of central abdominal vessels (part **f**). **g**,**h** | mIBG planar imaging (part **g**) and CT and fused single-photon emission CT (SPECT)-CT imaging (part **h**) of a girl 6 years of age with residual active tumour (arrows) after partial resection of the mediastinal primary tumour 2 months earlier. This highlights the importance of SPECT for the accurate detection of residual tumours.

and intermediate-risk disease. With higher resolution genomic techniques and integration of next-generation sequencing at a DNA and RNA level, it is likely that risk groups will be further refined based on the tumour molecular profile.

Screening and prevention

Early detection of neuroblastoma was speculated to be important for curing this deadly disease of infancy and childhood. To aid early detection of neuroblastoma, several screening programmes have been evaluated around the world. Using a urine spot test to detect increased levels of VMA was assessed in Japan in infants 6 months of age[108]. The sensitivity and specificity of the urine spot test were inadequate, so screening with high performance liquid chromatography was later introduced to measure VMA, HVA and creatinine levels in urine[109]. No change in the age distribution of patients with neuroblastoma was evident after the introduction of screening in Japan[110]. Moreover, large-scale retrospective analyses suggested the presence of possible overdiagnosis, although the overall mortality rate of neuroblastoma decreased in the screened group[111,112]. Interestingly, the cessation of the neuroblastoma mass screening programme in Japan has not resulted in an increased mortality or in the incidence of advanced-stage disease[113].

In a North American population-based screening study, one cohort with and one without screening (using thin layer chromatography to detect the levels of HVA and VMA in urine at 3 weeks and 6 months of age) were compared. Neuroblastomas detected by screening showed favourable prognostic factors, including non-amplified *MYCN* and DNA hyperdiploidy[19], similar to findings in the Japanese screening cohort[114]. However, screening did not change the incidence of advanced-stage disease in patients >1 year of age, and children who later presented with neuroblastoma (after screening negative) showed unfavourable genomic indicators, such as *MYCN* amplification and DNA diploidy. The findings subsequently led some to hypothesize that postponing screening to older infants (>6 months of age) might be more effective in preventing advanced-stage disease than screening younger infants. However, screening 7–12-month-olds resulted in more than one-third of the patients with neuroblastoma who were identified by screening showing unfavourable tumour genetic markers, although without a significant reduction in mortality[113,114].

In a large German study, screening (the detection of catecholamine metabolites in urine) for neuroblastoma at around 1 year of age was performed in 1,475,773 children and neuroblastoma was detected in 149 children[18]. However, 55 children with negative screening results had a subsequent diagnosis of neuroblastoma. Moreover, a similar incidence of stage 4 neuroblastoma was detected in the screened and the control groups and similar mortality rates were observed between the two groups[115]. Screening also resulted in overdiagnosis of neuroblastoma, which represented children with neuroblastoma who would not benefit from earlier diagnosis and treatment. Thus, these data did not support the usefulness of general screening for neuroblastoma at 1 year of age and the screening programme was abandoned.

Management

Clinical and biological risk factors are used to define distinct risk strata (as per the INRG classification; TABLE 1) and to determine treatment plans for patients with neuroblastoma. Treatment of neuroblastoma can include observation only, cytoreductive surgery, chemotherapy, radiotherapy, AHSCT, differentiation therapy and immunotherapy.

Management according to risk stratification

Very-low-risk and low-risk neuroblastoma. Very-low-risk and low-risk neuroblastomas (INRG stages L1, L2 and MS with favourable genomic features) account for nearly 50% of all newly diagnosed neuroblastoma[12,116]. Treatment decisions aim to deliver the minimum therapy while maintaining excellent patient survival. Infants who are <1 year of age with adrenal masses (measuring <5 cm in diameter) that are presumed to be neuroblastoma can probably be observed safely without obtaining histological confirmation of neuroblastoma or surgical cytoreduction, unless the tumours grow. Observation includes monitoring using physical examination, urine catecholamine levels and tumour imaging with either ultrasound or MRI initially at 6 and 12 weeks to rule out rapid tumour growth, then every 3 months for the first year and every 6 months during the second year after diagnosis. Further metastatic evaluation is not indicated without clinical or imaging signs of progression, owing to the low-risk nature of this group[117]. This observational approach avoids potential complications of surgery in

the young infant, such as haemorrhage, vascular damage, intestinal obstruction or damage to a vital organ, such as the kidney or liver. For patients <18 months of age with low-risk disease, stages L1, L2 or MS, without amplification of *MYCN*, in the absence of clinical symptoms or tumour progression, observation only might be adequate[15,75,118,119]. Cooperative group multicentre trials are ongoing to document the feasibility of observation only in these patients.

An observational approach has also been proposed for patients <18 months of age with larger tumours and regardless of primary tumour site after histological confirmation and after confirmation of the absence of other risk factors, including *MYCN* amplification or an unfavourable genomic profile[117,119,120]. This approach is undergoing investigation in a series of international prospective studies, such as COG ANBL1232 (REF. 121) and SIOPEN LINES[122].

For patients beyond infancy (>1 year of age) with localized disease that seems to be resectable, based on the absence of IDRFs (INRG stage L1) or based on the evaluation of the surgeon, the tumour should be resected. In the absence of *MYCN* amplification, any residual disease following surgical resection of the tumour is not considered a risk factor for relapse, with an event-free survival (EFS) of >90% and an overall survival of 99–100%[15,120].

For children with low-risk neuroblastoma (INRG stage L2 or MS with favourable genomic features), the overall treatment strategy depends on the manifestation of clinical symptoms. In the presence of clinical symptoms, treatment with chemotherapy is indicated, but with limiting the number of cycles until the resolution of clinical symptoms. Neither complete resection of the primary tumour nor radiotherapy is indicated in these patients[14,15,75].

Intermediate-risk neuroblastoma. Intermediate-risk neuroblastoma refers to *MYCN* non-amplified INRG stage L2 disease, INRG stage M in patients <18 months of age and INRG stage MS with unfavourable genomic features, according to the specific features shown in TABLE 1. Although INRG stage MS disease with unfavourable genomic features (segmental chromosomal alterations) has been considered a high-risk group in patients >12 months of age, some cooperative groups now propose treatment of these infants according to intermediate-risk rather than high-risk schedules, as their prognosis might be more akin to patients 12–18 months of age with stage 4 disease without *MYCN* amplification[123].

For children with intermediate-risk neuroblastoma, two to eight cycles of chemotherapy are prescribed. Surgical resection of the residual primary tumour is performed when possible, as determined by imaging, but complete resection is not essential[14,124–126]. However, treatment with chemotherapy alone for children >12–18 months of age with INRG stage L2 unresectable neuroblastoma (with unfavourable histology or unfavourable genomic profile and without *MYCN* amplification) might not be sufficient; these children have lower survival than similar patients with favourable biology, indicating that a more intensive treatment regimen, including radiotherapy, is warranted[14,124,125,127]. Treatment of patients in the intermediate-risk group should now be adapted to include the intensity and length of therapy based on response to therapy, further genetic criteria (including genomic copy number profile) and histology. Based on these treatment approaches, the estimated overall 5-year survival of intermediate-risk neuroblastoma is >90% for infants with INRG stage M disease but only 70% of children >18 months of age with INRG stage L2 disease[14,125].

High-risk neuroblastoma. The majority (>80%) of patients with high-risk neuroblastoma are >18 months of age with INRG stage M disease, as well as children 12–18 months of age with INRG stage M disease, whose tumours have unfavourable biological features (*MYCN* amplification, unfavourable pathology and/or diploid). The remaining 15–20% of high-risk patients are any age and stage of disease with *MYCN* amplification[12]. Some cooperative groups also consider patients who are >18 months of age with INRG stage L2 tumours with unfavourable pathology as high risk. The 5-year overall survival probability for patients 0–30 years of age with high-risk neuroblastoma has been estimated as 29% (patients diagnosed between 1990 and 1994; $n = 356$), 34% (patients diagnosed between 1995 and 1999; $n = 497$), 47% (patients diagnosed between 2000 and 2004; $n = 1{,}015$) and 50% (patients diagnosed between 2005 and 2010; $n = 1{,}484$)[116]. This increase in overall survival has been attributed to the introduction of myeloablative therapy and immunotherapy. Although the outlook for patients with high-risk neuroblastoma has improved, further major advances in treatment are imperative[116].

The current approach for high-risk neuroblastoma incorporates induction chemotherapy (to reduce tumour burden by shrinking the primary tumour and reducing metastases) using a combination chemotherapy

Box 4 | **Semi-quantitative mIBG-based scoring methods**

Curie scoring system

The Curie scoring system divides the skeleton into nine segments, each of which is ascribed a score of 0–3, depending on the extent of disease activity (no disease focus, the presence of one focus of activity, two or more discrete foci or diffuse involvement of a bone segment). Soft tissue involvement is also scored from 0–3 then the total score is calculated[89]. The Curie scoring system has been shown to be prognostic after a therapeutic intervention in relapsed, as well as newly diagnosed, neuroblastoma. For example, the use of the Curie scoring system as a prognostic marker in patients with metaiodobenzylguanidine (mIBG)-avid high-risk stage 4 neuroblastoma and a Curie score of >2 after six cycles of induction therapy had a significantly worse 3-year event-free survival (EFS; 15.4 ± 5.3%) — defined as relapse, progressive disease, secondary cancer or death — than those with scores of ≤2 (EFS: 44.9 ± 3.9%)[92].

SIOPEN scoring system

The International Society for Pediatric Oncology Europe Neuroblastoma group (SIOPEN) scoring system divides the skeleton into 12 segments, with a score per segment of 0–6. Use of the SIOPEN scoring system indicated that higher mIBG scores at diagnosis and occurrence of any residual mIBG-positive metastases after four cycles of chemotherapy predicted unfavourable outcomes in patients with stage 4 neuroblastoma[212].

regimen, followed by delayed surgery to remove the primary tumour and subsequent myeloablative chemotherapy supported with AHSCT. Myeloablative chemotherapy is followed by maintenance therapy for minimal residual disease with anti-GD2 monoclonal antibody and cytokine immunotherapy, in addition to differentiating therapy with isotretinoin[9] (FIG. 7).

Types of treatment

Induction chemotherapy. Patients achieving a complete or very good partial remission by the INRC criteria (meaning, cases in which no signs of active neuroblastoma by mIBG scan remain after treatment, but some abnormalities on CT imaging or MRI in the primary tumour site persist, preventing a classification of 'complete remission') at the end of induction chemotherapy have a significantly greater EFS than patients with a partial or less than partial response to chemotherapy[13,92,128]. This finding has led to an increasing dose intensity used in induction chemotherapy, with current regimens incorporating multiple rotating pairs or triplets of active drugs (for example, vincristine, vindesine, etoposide, cisplatin, carboplatin, dacarbazine, doxorubicin, cyclophosphamide, ifosfamide and topotecan).

Importantly, only the most recent of the cooperative group phase III trials (COG A3973, HR-SIOPEN and COG ANBL0532)[92,129,130] prospectively included more-stringent definitions of response to chemotherapy, including mIBG semi-quantitative scores, rather than just observer estimates of whether activity changed by >50% on mIBG scan or bone scan. Regardless of the multi-agent regimen used, only small differences in overall induction response rates (complete response, very good partial response or partial response) were shown in cooperative trials with documented results of >100 patients accrued from 1990 to 2012, with 71–85% of patients showing either a complete or partial response rate[129–134]. However, one trial showed a significant improvement in 5-year EFS from 18% in patients who were randomly assigned to an induction regimen of cisplatin, vincristine, carboplatin, etoposide and cyclophosphamide (COJEC), up to 30.2% in patients receiving a more rapid regimen with a higher dose intensity (the same cumulative doses of each drug were administered but over a shorter time period)[132]. However, given the higher 5-year EFS (40%) observed in the most recent COG trial[134] (using the less dose-intensive N7 induction, followed by myeloablative therapy), a randomized comparison of rapid COJEC induction with N7 induction is ongoing in Europe[135]. For the 10–15% of patients with high-risk neuroblastoma who are refractory to standard induction therapy as administered in any of the recent reports from the past two decades, a combination of topotecan, vincristine and doxorubicin[136], or irinotecan and temozolomide[137] depending on the prior chemotherapy, has been shown to achieve a good response in some refractory or progressive patients. Another approach has been to use ^{131}I-mIBG therapy (as a radiotherapeutic metabolic agent) in these patients, which has been shown to have a >30% response rate in refractory and relapsed disease (see below)[138,139].

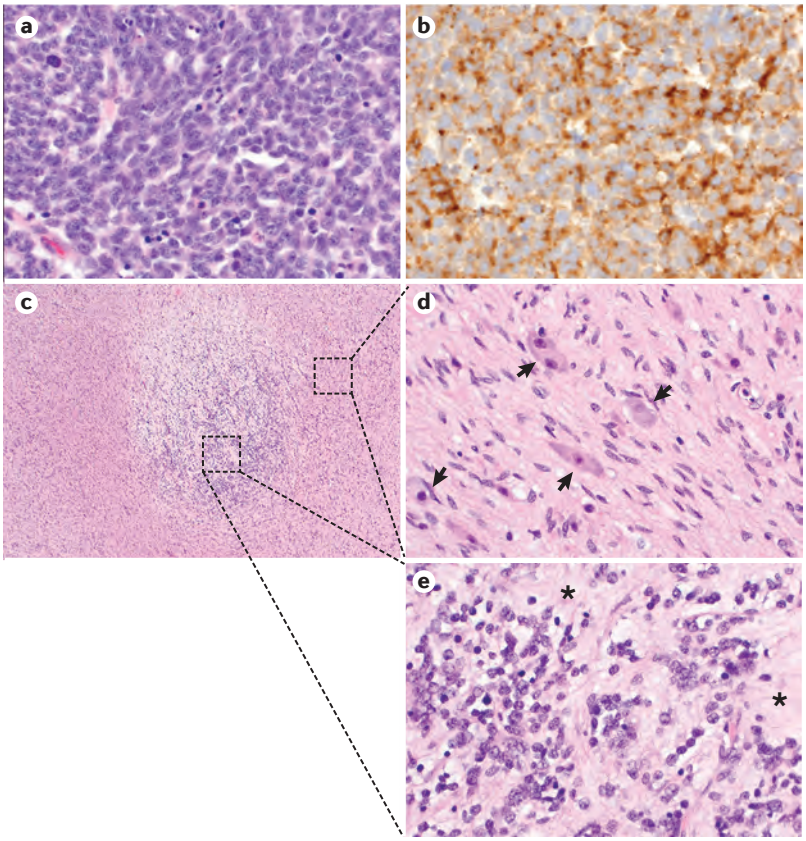

Figure 6 | **Pathology of neuroblastoma and ganglioneuroblastoma. a**,**b** | Diagnostic biopsy from a patient 3 years of age with undifferentiated neuroblastoma at 400× magnification. **a** | The neuroblastoma is composed of small, round, monomorphic cells with nuclear hyperchromasia (excess pigmentation) that are characteristic of malignant cells, granular chromatin (indicative of neuroendocrine tumours), micronucleoli and scant cytoplasm that is arranged in well-vascularized sheets and nests. Slightly larger tumour cells with paler cytoplasm and prominent nucleoli can also be observed. No ganglionic differentiation is seen. The mitosis-karyorrhexis index (MKI) is high (>4%). **b** | Immunohistochemistry shows that the tumour cells are positive for synaptophysin. **c–e** | Diagnostic biopsy from a patient 12 years of age with ganglioneuroblastoma, intermixed. The central area shows histology indicative of neuroblastoma (part **c**), with regions of peripheral ganglioneuroma (arrows indicate mature ganglion cells; part **d**). Regions of neuropil can also be observed (denoted by *; part **e**). Part **c** at 40× magnification; part **d** at 400× magnification; and part **e** at 400× magnification.

Local control. Surgical gross total resection (complete removal of the visible tumour) of the primary tumour is often difficult in patients with high-risk neuroblastoma, even after chemoreduction, owing to the frequent encasement of renal and abdominal vessels or infiltration of the neural foramina by tumours. Multiple retrospective analyses have not been able to determine if gross total resection improves the outcome of patients with metastatic neuroblastoma, owing to the logistical difficulty of conducting a randomized trial of surgery and the frequent failure of surgery to eradicate metastatic deposits in bone and bone marrow, which are common sites of relapse[140]. The routine

administration of radiotherapy to the primary tumour bed (the vasculature and connective tissue surrounding a tumour) after myeloablative therapy might also obscure the effect of surgical resection[141]. Although in biologically high-risk INRG stage L2 tumours gross total resection has been shown to significantly improve outcome[127,142,143], in a large series of patients with INRG stage M cancer, no difference was found in EFS or overall survival, regardless of whether resection was complete or incomplete[144]. A systematic review of the literature showed that the odds ratio for overall survival following gross total resection for patients with INRG stage L2 disease was 2.4 (95% CI: 1.19–4.85) compared with incomplete resection, but there was no significant survival benefit for gross total resection in patients with INRG stage M disease[140]. The addition of radiotherapy (in doses of 21–36 Gy) to the preoperative tumour bed (as it was immediately preoperatively) after delayed surgical resection and AHSCT has been shown to decrease the local recurrence of neuroblastoma in several single-arm studies and is currently accepted as standard care for high-risk neuroblastoma, although a few centres only radiate measurable residual disease[141,145].

Myeloablative therapy with AHSCT. The first improvement in EFS for patients with high-risk neuroblastoma occurred with the use of myeloablative chemotherapy (usually using melphalan-containing conditioning regimens), which was demonstrated to be superior to no further therapy or ongoing consolidation chemotherapy in three randomized trials[13,131,146]. Initial use of total body irradiation with myeloablative chemotherapy was replaced with higher doses of chemotherapy, after equivalent results but without late adverse effects were obtained. Indeed, total body irradiation is associated with infertility, growth failure and secondary malignancy (see Quality of life). Trials to identify the best myeloablative chemotherapy regimen for patient survival and the reduction of late effects are ongoing. One trial has shown an increase in EFS with busulfan and melphalan (BuMel) compared with carboplatin, etoposide and melphalan (CEM)[147]. However, the EFS of the BuMel regimen was not different to EFS values that were obtained in a COG trial using CEM, possibly owing to the different induction regimens used in these trials[134]. Other trials, including pilot trials[148,149] and a completed randomized trial[130], have evaluated the use of tandem AHSCT (that is, myeloablative chemotherapy with AHSCT given twice 6–12 weeks apart) for the treatment of high-risk neuroblastoma. One trial, comparing tandem transplantation (using thiotepa and cyclophosphamide followed by CEM 6 weeks later for myeloablation) and single transplantation (using CEM alone for myeloablation) showed a significant improvement in EFS with the tandem regimen, further validating the importance of myeloablative therapy in treatment of high-risk neuroblastoma[130]. In addition, pilot studies are testing the use of ^{131}I-mIBG to eliminate residual metastatic disease before AHSCT[150,151].

Box 5 | **International Neuroblastoma Staging System**

Stage 1
Localized tumour with complete gross surgical excision and no metastasis to the representative ipsilateral lymph nodes that were not attached to tumour.

Stage 2A
Localized tumour with incomplete gross surgical excision and no metastasis to the lymph nodes.

Stage 2B
Localized tumour with or without complete gross surgical excision, with tumour metastasis to the ipsilateral lymph nodes but no tumour metastasis noted in any enlarged contralateral lymph nodes.

Stage 3
Unresectable, unilateral tumour infiltrating across the midline, with or without regional lymph node metastasis, or localized unilateral tumour with contralateral regional lymph node metastasis, or midline tumour with bilateral infiltration or lymph node involvement.

Stage 4
Any primary tumour with metastasis to distant lymph nodes and/or other organs, except as defined for stage 4S.

Stage 4S
Localized primary tumour (stages 1, 2A or 2B) in patients <1 year of age, with metastasis limited to the skin, liver or bone marrow (<10% tumour involvement).

Adapted from REF. 107.

For harvest of cells for subsequent transplantation, several trials have shown that autologous peripheral blood stem cells can be successfully collected after only two cycles of induction chemotherapy with good yield, without substantial tumour contamination and with satisfactory engraftment[134,152]. However, some cooperative groups recommend the harvest of peripheral blood stem cells at the completion of induction chemotherapy, once the maximum bone marrow remission has been achieved. Based on previous studies, purging of peripheral blood stem cells to remove tumour cells before engraftment does not improve outcome[134]; instead, efforts are ongoing to further improve *in vivo* purging with better induction therapy and maintenance therapy after AHSCT.

Maintenance therapy. Despite the improvement in EFS with myeloablative chemotherapy followed by AHSCT, 50% of children relapse months to years after transplantation[13]. The addition of an oral differentiation treatment, isotretinoin — which has been shown to reduce proliferation and induce differentiation of neuroblastoma cells — after AHSCT or consolidation therapy showed a significant improvement in EFS, but not in overall survival in a randomized controlled trial[13]. The development of anti-GD2 monoclonal antibodies has led to further improvement in EFS after intensive induction chemotherapy and myeloablative therapy. For example, a large randomized trial showed a significant improvement in EFS in patients receiving immunotherapy following AHSCT (consisting of the

Table 1 | **Modified International Neuroblastoma Risk Groups**

Risk group for treatment	INRG stage	IDRFs in primary tumour	Distant metastases	Age (months)	Histological category	Grade of differentiation	*MYCN* status	Genomic profile	Ploidy
Very-low	L1	Absent	Absent	Any	GNB nodular, NB	Any	–	Any	Any
Very-low	L1 or L2	Any	Absent	Any	GN, GNB intermixed	Any	–	Any	Any
Low	L2	Present	Absent	<18	GNB nodular, NB	Any	–	Favourable	Any
Low	L2	Present	Absent	≥18	GNB nodular, NB	Differentiating	–	Favourable	Any
Low	MS	Any	Present	<12	Any	Any	–	Favourable	Any
Intermediate	L2	Present	Absent	<18	GNB nodular, NB	Any	–	Unfavourable	Any
Intermediate	L2	Present	Absent	≥18	GNB nodular, NB	Differentiating	–	Unfavourable	Any
Intermediate*	L2	Present	Absent	≥18	GNB nodular, NB	Poorly differentiated, undifferentiated	–	Any	Any
Intermediate	M	Any	Present	<18	Any	Any	–	Any	Hyperdiploid
Intermediate	M	Any	Present	<12	Any	Any	–	Unfavourable and/or diploid	
Intermediate	MS	Any	Present	12–18	Any	Any	–	Favourable	Any
Intermediate	MS	Any	Present	<12	Any	Any	–	Unfavourable	Any
High	L1	Absent	Absent	Any	GNB nodular, NB	Any	+	Any	Any
High	L2	Present	Absent	≥18	GNB nodular, NB	Poorly differentiated, undifferentiated	+	Any	Any
High	M	Any	Present	12–18	Any	Any	–	Unfavourable and/or diploid	
High	M	Any	Present	<18	Any	Any	+	Any	Any
High	M	Any	Present	≥18	Any	Any	Any	Any	Any
High	MS	Any	Present	12–18	Any	Any	–	Unfavourable	Any
High	MS	Any	Present	<18	Any	Any	+	Any	Any

These criteria have been modified from the original report[12] to account for emergent genomic data and current treatment approaches[4,130]. Favourable corresponds to absence and unfavourable to the presence of segmental chromosome alterations. +, amplified; –, non-amplified; GN, ganglioneuroma; GNB, ganglioneuroblastoma; IDRF, image-defined risk factor; INRG, International Neuroblastoma Risk Groups; NB, neuroblastoma. *Some clinical trial groups consider that patients with stage L2 neuroblastoma with unfavourable pathology who are >18 months of age should be treated as high-risk, as excellent prognosis was achieved with intensive chemotherapy, often followed by radiation and autologous haematopoietic stem cell transplantation.

chimeric anti-GD2 antibody (also known as ch14.18), IL-2, granulocyte–macrophage colony-stimulating factor (GM-CSF) and isotretinoin) compared with patients receiving isotretinoin alone[153]. Combined anti-GD2 antibody (ch14.18), IL-2, GM-CSF and isotretinoin is now the standard US FDA-approved therapy in North America for patients with neuroblastoma after AHSCT.

Ongoing trials[135,154] are testing whether IL-2 (which can induce adverse effects in patients) is crucial for the efficacy of the regimen and whether increasing the length of the antibody infusion can also reduce the adverse effects, which include nerve pain, hypotension, respiratory toxicity and fluid retention. Moreover, a humanized anti-GD2 monoclonal antibody with a single point mutation (K322A) that reduces complement-dependent cell lysis is also being evaluated in trials to attempt to retain efficacy but reduce treatment-associated toxicity[155]. Other therapies under investigation for maintenance therapy, which have shown preclinical activity and some modest activity in early-phase trials, include the anti-GD2 immunoconjugate Hu14.18–IL2 (REF. 156), fenretinide[157], vaccines containing the neuroblastoma antigens GD2 and GD3 (REF. 158), and difluoromethylornithine (DFMO), an inhibitor of ornithine decarboxylase (a *MYC* target gene)[159].

Relapsed neuroblastoma

In patients with relapsed neuroblastoma, survival for >1–3 years without further recurrence of disease, or without death, is rarely possible, although more recent chemotherapy combinations have been successful in eliciting a partial or complete remission. The most effective current salvage treatments for relapsed neuroblastoma are either topotecan with cyclophosphamide, irinotecan with temozolomide[137,160,161] or topotecan with temozolomide[162]. ^{131}I-mIBG therapy can also be used as salvage treatment and has shown a 30–40% response rate in both refractory and relapsed disease[138,139,163]. ^{131}I-mIBG therapy is also being tested in pilot trials during induction chemotherapy and in combination with radiosensitizers (drugs that can increase the sensitivity of tumours to radiotherapy)[164,165].

Combinations of molecularly targeted therapies, combined with chemotherapy, are also under investigation. For example, crizotinib (an ALK inhibitor) has been investigated in a phase I trial alone[49] and is undergoing testing in combination with topotecan and cyclophosphamide in patients with somatic *ALK* mutations[166]. Improved third-generation ALK inhibitors are also in paediatric trials internationally[167] and a new ALK inhibitor, designed to overcome treatment-resistant

mutations, will soon be evaluated in clinical trials in children[168]. Aurora kinase inhibitors have been combined with chemotherapy for the treatment of neuroblastoma, as they have been shown to destabilize N-MYC, as well as induce G2/M cell cycle arrest, with promising response rates in phase I studies[169]. Other treatments currently in phase I or phase II studies include immune checkpoint inhibitors; bromodomain and extra-terminal motif (BET) bromodomain inhibitors (for their inhibition of *MYCN*), although this trial is not yet open for children[170,171]; polyamine antagonists[172]; anti-GD2 antibody with NK cells[73]; anti-GD2 vaccines[158]; and T cells engineered to express chimeric antigen receptors targeting GD2 (REF. 173).

Relapse in the central nervous system has become more common with the longer survival that is evident with more-intensive therapy and might be a sanctuary site — an area of the body that is weakly penetrated by drugs — for many of the standard neuroblastoma treatments[174,175]. Attempts at salvage rescue therapy have been most successful with the combination of surgery, neuraxis radiation and, in some cases, investigational intrathecal ^{131}I anti-GD2 antibody[176].

Special disease complications

Spinal cord compression occurs in approximately 5–10% of all patients with neuroblastoma and constitutes a medical emergency[177]. Immediate treatment must be given to increase the chances of neurological recovery, especially when symptoms, for example, loss of sensation and motor function, are present. Treatment options for spinal cord compression include symptomatic treatment, for example, using high-dose corticosteroids, in addition to chemotherapy or neurosurgical intervention (either by laminectomy (surgical removal of the entire lamina — the bones found at the back of the spinal cord) or laminotomy (surgical removal of part of the lamina)), with removal of a part of the tumour if that can be done without risking further nerve damage. Although many investigators have preferred the use of chemotherapy, no data have shown chemotherapy to be superior to surgery for the treatment of neuroblastoma-associated spinal cord compression, with respect to long-term outcomes. Detailed, multidisciplinary discussions that include oncologists, neurosurgeons and radiologists are necessary to enable decisions aiming to decrease the risk of paralysis and other sequelae[177].

OMS is a debilitating syndrome, which is associated with a high rate of long-term neurological impairment, despite the favourable outcome for the neuroblastoma[11]. Owing to the postulated underlying autoimmune mechanism, immunosuppressive treatments, such as adrenocorticotropic hormone (also known as corticotropin), high-dose steroids, cyclophosphamide, intravenous gamma globulin, rituximab, immunomodulatory agents such as mycophenolate or, more rarely, in severe cases, plasmapheresis, can be used to treat the symptoms associated with OMS[11,83,178].

Vasoactive intestinal peptide secretion with debilitating secretory diarrhoea often requires symptomatic treatment with fluid and electrolyte replacement therapy. Symptoms often resolve with resection of the primary tumour, but octreotide (a somatostatin analogue that inhibits secretory diarrhoea from a multitude of causes) infusion might be indicated in case of unresponsiveness to tumour removal. The vasoactive intestinal peptide syndrome is usually associated with more-differentiated tumours, such as ganglioneuroblastoma, and these tumours rarely prove fatal[179].

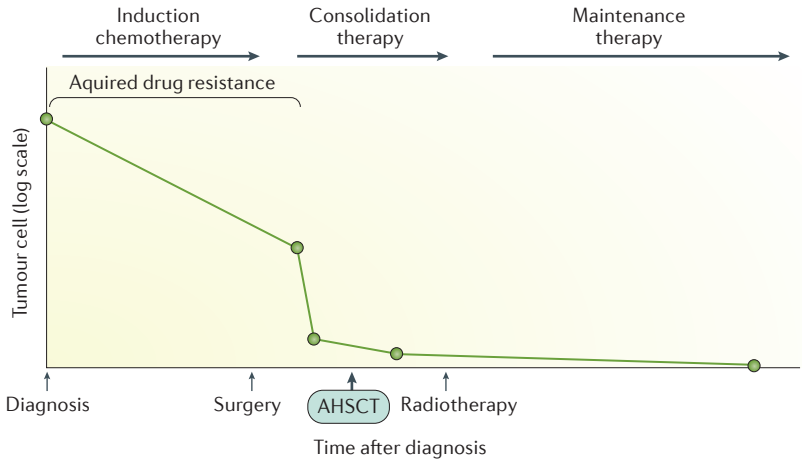

Figure 7 | **Overall treatment approach for high-risk neuroblastoma.** Induction therapy includes combination chemotherapy with four-to-six agents (commonly carboplatin, cisplatin, cyclophosphamide, doxorubicin, vincristine and topotecan) and a peripheral blood stem cell harvest. During induction therapy, clonal evolution and drug resistance can occur (acquired drug resistance), which can lead to relapse of neuroblastoma if the tumours are not eliminated by myeloablative therapy and maintenance therapy. Cytoreductive surgery is attempted after four-to-five cycles of chemotherapy. High-dose myeloablative chemotherapy with autologous haematopoietic stem cell transplantation (AHSCT) is used to eliminate remaining disease, followed by radiotherapy to the primary tumour bed and finally maintenance therapy for minimal residual disease using anti-GD2 antibody, cytokines and isotretinoin.

Quality of life

Patients with neuroblastoma are at risk of substantial disease-related and treatment-induced toxicity (BOX 6). At the time of diagnosis, children with neuroblastoma can present with neurological symptoms, or other symptoms, that can have long-term health consequences after completion of therapy. For example, spinal cord compression can result in permanent lower extremity weakness, as well as loss of bladder function, and cranial nerve involvement in skull-based tumours can result in neuropathy and blindness[180,181]. Moreover, some patients with neuroblastoma can develop OMS, which, despite the good prognosis for neuroblastoma-free survival of these patients, is associated with poor neurological outcomes. The majority of patients with OMS have long-term developmental and behavioural deficits. Intensive immunosuppression has been effective in reducing the severity of symptoms in some patients with OMS, but does not necessarily improve the long-term developmental outcome[11,178].

Nearly all patients treated for high-risk neuroblastoma experience substantial treatment-associated acute toxicity, including severe transient myelosuppression, chemotherapy-induced renal dysfunction and poor weight gain, which requires nutritional supplementation.

Box 6 | **Acute and late effects of neuroblastoma**

Acute effects

- Pain
- Neurological symptoms (for example, blindness, spinal cord compression syndrome or opsoclonus myoclonus syndrome)
- Frequent hospitalization
- Nausea, vomiting and risk of infection
- Mucositis
- Veno-occlusive disease
- Electrolyte imbalance
- Growth delay
- Social isolation
- Risk of toxic death

Late effects

- Impaired growth and poor weight gain
- Delayed or impaired puberty; infertility
- Hypothyroidism
- Hearing loss
- Chronic diarrhoea
- Pulmonary fibrosis
- Ongoing neurological impairment
- Scoliosis
- Dental abnormalities
- Benign neoplasms (osteochondroma and focal nodular hyperplasia)
- Chronic kidney disease
- Secondary malignant neoplasm
- Ongoing risk of late relapse from neuroblastoma

After the completion of therapy, chronic treatment-related conditions are an important concern. In a report of the health outcomes of 954 patients treated before 1986, survivors (at least 5 years post-treatment) were shown to have markedly increased risks of self-reported neurological and musculoskeletal conditions, including extremity weakness and scoliosis compared with a sibling cohort[181]. The risk of secondary malignancy after 25 years of follow-up was 3.6%, with increased relative risks of solid tumours, including thyroid, renal and soft tissue malignancies, as well as acute leukaemia. Of interest, an increased risk of renal carcinoma, a relatively uncommon secondary malignancy, has been reported in adults who survived neuroblastoma[182]. Multivariate analysis has identified exposure to etoposide and radiotherapy as risk factors for secondary cancers[181].

Studies of patients who survived high-risk neuroblastoma who were treated with contemporary therapy (intensive chemotherapy, surgery, radiotherapy and multi-agent myeloablative regimens) suggest a high prevalence of significant late effects, including secondary malignancy, endocrinopathy, renal dysfunction and hearing loss[183–185]. Growth failure and short stature are associated with exposure to total body irradiation, as are poor weight gain and chronic diarrhoea. Individuals with growth failure exhibit sub-optimal responses to growth hormone therapy[186], possibly caused by premature epiphyseal closure, which has been associated, in a small series of patients, with treatment with isotretinoin[187]. The high prevalence of hypothyroidism in patients who survived neuroblastoma reflects thyroid damage by ^{131}I-mIBG, as well as by external beam radiotherapy[188]. Diabetes mellitus and metabolic syndrome in patients who survived high-risk neuroblastoma have also been associated with exposure to abdominal and/or pancreatic radiation[189,190]. Women who survived high-risk neuroblastoma in childhood have a high rate of abnormal pubertal progression and premature ovarian failure (in one study, 75% of girls expected to be pubertal had primary ovarian failure)[183], and men will be expected to have azoospermia (the absence of viable sperm in semen) or oligospermia (decreased sperm numbers in the semen) due to alkylating agent exposure. Developmental outcomes, including school performance, have not been well characterized.

The avoidance of late toxicities can be enhanced by the identification of patients who do not need AHSCT, or other intensive therapy, to achieve long-term cure. For example, patients 12–18 months of age who have tumours without adverse biological features can achieve adequate cure rates with chemotherapy alone[123]. This strategy of using clinical, biological and demographical features at diagnosis to determine which patients might need less therapy will result in an overall decrease in burden of late toxicity.

Outlook

The outlook for neuroblastoma depends on a deeper understanding of the genetic basis of disease initiation and progression, a thorough understanding of the epigenetics of healthy sympathoadrenal development and how this is deregulated during tumorigenesis, and better animal models to elucidate pathogenesis and identify new therapies.

Disease models

Immune-competent models that are tractable and recapitulate the disease are crucially needed to leverage the rapidly growing immunotherapeutic armamentarium. A new generation of disease models might address unanswered questions, such as if single or multiple genes show epistasis, if genes with loci in chromosomal intervals that are recurrently altered in neuroblastoma have a concerted effect in cancer development and if segmental chromosome alterations can serve as biomarkers for cancer therapy (possibly using a synthetically lethal — in which a mutation in one gene does not affect cell viability, but when combined with another mutation, can lead to cell death — approach)[191]. For example, deletions of non-coding genomic regions that can act as regulatory elements could influence the expression of distant cancer genes (*trans* effects) or of adjacent genes that lie outside of the deleted non-coding region (*cis* effects). New models might also address how haploinsufficiency arising from hemizygous deletions affects the transcription of cancer genes in retained chromosomal regions and/or segments. However, the engineering of genomic deletions in mouse cells is cumbersome, involving multiple rounds

of homologous recombination, antibiotic selection and single-cell cloning. The use of CRISPR–Cas9 technology simplifies chromosome engineering, although the position and order of genes within any given chromosomal segment differ extensively between humans and mice. Thus, the generation of a pure mouse model to perfectly replicate the large-scale genomic changes of human tumours and their precise gene context is challenging, if not impossible. The ability to understand how chromosomal copy number changes contribute to neuroblastoma development and to identify druggable genes and pathways await the development of models that are engineered in human cells and for technologies that can adequately recapitulate chromosome gain as well as loss.

Genetic alterations

The rapid advances in next-generation sequencing and Team Science approach to the molecular analysis of these tumours are advancing knowledge of the clonal evolution of tumours that leads to new mutations in relapsed patients; this might enable the identification of targetable mutations. For example, although mutations in genes involved in the RAS pathway are rare at diagnosis[5], analysis of paired diagnosis and relapse specimens has shown clonally enriched somatic mutations in the relapse specimen (and also in 60% of studied neuroblastoma cell lines). These RAS pathway mutations were predicted to activate the mitogen-activated protein kinase (MAPK) pathway, and included not only aberrations in *ALK* but also in *NRAS*, *KRAS*, *HRAS*, *BRAF*, *PTPN11* and *FGFR*[51,192]. An increase in mutational burden, clonal evolution and in the emergence of other new mutations has been reported[192]. The observation of emergence of *ALK* mutations, in addition to other druggable mutations after relapse, probably justifies a new tumour biopsy after relapse when searching for actionable targets[193].

Biomarkers

New tools are being developed for use in diagnosis and to determine treatment plans for patients with neuroblastoma, such as the use of circulating tumour DNA for genomic characterization of tumours. Such genomic biomarkers can be used to tailor therapies to individual patients. Almost all tumours treated with any single drug eventually acquire resistance to treatment as a result of tumour heterogeneity, clonal evolution and clonal selection[194]. As therapy-related markers might change throughout tumour progression, biomarker investigations at multiple time points might provide crucial information for patient management. Studies of many adult malignancies have shown that the tumour genome can be reconstructed from circulating tumour DNA, providing a feasible non-invasive 'liquid biopsy' (REF. 195). Studies are in progress to identify molecular aberrations in circulating tumour DNA using a comprehensive gene panel or even next-generation sequencing. Thus, circulating tumour DNA has the potential to become a tumour biomarker for response and to identify the evolution of new genetic aberrations in tumours and, subsequently, provide new therapeutic targets.

The use of new imaging approaches, such as FDG-PET-MRI, ^{124}I-mIBG PET-CT or ^{68}Ga-DOTATATE, will result in more-precise tumour localization and prioritize targets for radiopharmaceutical therapy, including ^{131}I-mIBG, lutetium-277 (^{277}Lu)-DOTATE and astatine-211 (^{211}At)-mIBG[95,196]. Molecular imaging is also under development and could be used as a pharmacodynamic marker of inhibitor therapy effect, such as BET bromodomain inhibitors on MYC pathways using zirconium-89 (^{89}Zr)-transferrin[197].

Management and associated toxicity

Inhibitors that are under development to target activated pathways in neuroblastoma include PI3K inhibitors, Aurora kinase inhibitors[198], BET bromodomain inhibitors[170] and new histone deacetylase inhibitors. As outlined above, newer immunotherapeutic approaches in preclinical studies or phase I trials include chimeric antigen receptor T cell therapy, expanded and activated NK cells, KIR mismatch allogeneic cell transplantation, and new humanized antibody conjugates and vaccines. Combining these immune therapies with targeted therapies might also require a new generation of immunocompetent animal models, for both *MYCN*-driven disease and for neuroblastoma that arises through *MYCN*-independent pathways. New clinical trial design will incorporate adaptive designs and attempt to enrich patient cohorts for those who are likely to respond to specific therapies based on the presence of validated patient-specific biomarkers, such as the use of basket trials (that is, the inclusion of patients with specific tumour mutations but distinct histologies). Indeed, clinical trials must enrich patient cohorts for patients who are likely to respond to tailored therapies to identify active agents for incorporation into front-line therapies.

Genome-based and pharmacogenomic studies will be important tools for explaining the variability of acute and late toxicity that are exhibited by patients treated for neuroblastoma and are likely to become important in determining therapy choice in the future. For example, several putative polymorphisms associated with hearing loss and other toxicity after cisplatin exposure have been identified, but further collaborative work using patient samples and clinical outcome data is needed to develop clinically validated markers to predict which patients will experience hearing loss following treatment with platinum derivatives[199,200]. A cross-sectional study of >300 patients who survived high-risk neuroblastoma is underway through the Children's Oncology Group, which will be the first study to describe the late-effect profile of patients treated in a modern era. This study will enable the analysis of newer treatments, such as tandem AHSCT, isotretinoin exposure and antibody and/or cytokine therapy, as well as provide annotated germline biological specimens for potential risk factor analyses.

By incorporating new knowledge about the molecular biology and pathogenesis of neuroblastoma, new therapeutic targets and improved understanding of resistance, we will be able to increase treatment precision to improve patient survival and quality of life.

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Acknowledgements
The authors thank S. Cho, University of California, San Francisco, USA, for preparing the photomicrographs used in Figure 6.

Author contributions
Introduction (K.K.M.); Epidemiology (J.M.M.); Mechanisms/pathophysiology (C.L.M. and W.A.W.); Diagnosis, screening and prevention (K.K.M., G.S. and A.N.); Management (K.K.M. and G.S.); Quality of life (L.D.); Outlook (K.K.M. and J.M.M.); Overview of the Primer (K.K.M.).

Competing interests
The authors declare no competing interests.