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Improving the assessment of risk factors relevant to potential carcinogenicity of gene therapies: a consensus paper

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ABSTRACT:

Regulators and industry are actively seeking improvements and alternatives to current models and approaches to evaluate potential carcinogenicity of gene therapies (GTs). A meeting of invited experts was organised by NC3Rs/UKEMS (London, March 2023) to discuss this topic. This paper describes the consensus reached amongst delegates on the definition of vector genotoxicity, sources of uncertainty, suitable toxicological endpoints for genotoxic assessment of GTs, and future research needs. The collected recommendations should inform the further development of regulatory guidelines for the non-clinical toxicological assessment of GT products.

INTRODUCTION

The therapeutic potential of gene therapies (GTs) to address hitherto untreatable conditions has led to a rapidly increasing number of candidates entering the clinic, with more than 16 market authorisations and thousands of patients treated with GTs.[1] The regulatory framework for the assessment of GT products comprises both regulations and guidance documents that cover specific jurisdictions, and requiring the interpretation of these documents by regulators and sponsors through experience gained in practice.[2-7] Approvals are given on a case-by-case basis and involve submission of a portfolio of evidence to support each investigational drug application prior to initiating clinical studies and throughout the clinical development process.

For the purposes of this paper, GTs were defined as ex vivo or in vivo therapies that modify the genome with either gamma-retroviral (γRV), lentiviral (LV) or adeno-associated (AAV) vectors or DNA transposons (e.g. PiggyBac and Sleeping Beauty). Genome editing (e.g. by CRISPR or zinc finger nucleases) or nanoparticles as delivery method are not covered. Similarly, we focused on autologous therapies (influenced by patient/disease context) as opposed to allogeneic treatments where a cell therapy is manufactured from a healthy donor, removing the influence of the pre-existing patient genome alterations. In contrast, allogeneic products have an increased exposure risk from any manufacturing-driven genotoxic event, since a higher number of patients are treated from the same product batch.

Concerns about the risk of carcinogenicity (this term is considered equivalent to tumourigenicity or oncogenicity for the purposes of this paper) associated with the use of integrating viral vectors was first raised in the early 2000s, when clinical trials of ex vivo GT for primary immune deficiencies were put on hold in the United States and France following several cases of leukaemia.[8-10] Since then, further clinical cases of leukaemia/lymphoma (and myelodysplasia) definitively linked to vector integration, as well as cases of uncertain relationship to vector integration, have occurred.[11-15] In 2023, the US Food and Drug Administration (FDA) announced an investigation of the identified risk of T cell malignancy following BCMA-Directed or CD19-Directed Autologous Chimeric Antigen Receptor (CAR) T cell immunotherapies. While noting that the overall benefits of these

products continue to outweigh their potential risks, in 2024, a class wide black box warning in their labels was issued.[16,17] One of such secondary malignancies has been recently reported.[18] Levine et al responded to the announcement of the investigation, highlighting that while safety concerns should be thoroughly investigated, existing data from follow-up studies suggest that the risk of T cell malignancies remains low when compared to existing cancer treatments.[19]

Furthermore, various RV and LV, as well as AAV vectors, in vivo and/or ex vivo, have been associated with neoplasia in mouse models or with non-oncogenic clonal expansion in dogs.[20-23] These data exemplify the potential for carcinogenicity associated with various GT vectors and have led to increased regulatory oversight and safety precautions as well as research to produce safer vectors, even though carcinogenicity has not been reported following the use of AAV vectors in large animal studies or in humans.

Guidance about genotoxicity (via insertional mutagenesis) and carcinogenicity risk assessment is generic, with no specific assays proposed. The traditional ICH (International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use) S1 test battery and life-time animal bioassays were designed for small molecules and are generally not applicable and insufficient for detection of potential carcinogenicity in humans for GTs. Therefore, more tailored approaches are actively encouraged, and a weight of evidence approach is advocated using all available data, both product specific as well as from similar or related products. However, there is still no scientific consensus on the most appropriate approaches or models to assess the risk of insertional mutagenesis and potential carcinogenicity of vector-mediated GTs for regulatory purposes. Whilst both in silico and in vitro models are recommended in guidelines, and regulatory authorities state that animal studies should be reduced and avoided where possible, small animal in vivo studies are still often used despite concerns over their relevance.[24]

There is a clear need for the development and use of approaches that can reliably detect potential carcinogenicity of GTs in humans. Ideally, these assays should be predictive, human-relevant, fast, cost-efficient and have reduced reliance on animals, and where possible, be amenable to use in the clinic.

To action these concerns and to address the clinical need to accelerate the approval rate of investigational GT products, a group of invited expert scientists from industry, academia and regulatory authorities met to explore the principles and open questions on genotoxicity and carcinogenicity hazard identification, risk assessment and risk mitigation through non-clinical testing. Scientists were selected to cover expertise in different vector types, genotoxicity assessment, in vitro and in vivo genotoxicity models, regulatory and non-clinical risk assessment of gene therapy products. A set of consensus statements were drafted by a small coordinating group prior to the meeting of the expert group where the statements were amended and finalised. The intention was to achieve a high-level constructive, scientifically acceptable framework that could be agreed by all participants. The final report highlights areas of agreement, as well as areas where a consensus could not be achieved and is intended for wide dissemination within the relevant scientific and regulatory communities to facilitate further discussion and inform new assay development.

In this paper, we describe the scientific and regulatory background that led to this discussion, and present the consensus set of principles reached among scientists during a two-day workshop held in London on 7 and 8 March 2023, hosted by the UK National Centre for the Replacement Refinement and Reduction of Animals in Research (NC3Rs) and the UK Environmental Mutagen Society (UKEMS). Representatives from the FDA CBER/OTP, MHRA, PMDA and (CBG-)MEB attended the meeting in a mainly observational capacity and provided high level comments. The participation of regulatory agency representatives does not necessarily reflect the position of their respective agencies.

Scientific Background - Evidence for carcinogenic potential

Prior to the initiation of GT clinical trials with Long Terminal Repeat (LTR)- driven γRV, the potential for carcinogenesis was recognised, but the risk was considered to be low.[25] Yet, cases of vector-induced leukaemia were observed even in small clinical trials, when employing ex vivo haematopoietic stem cell GT with LTR- driven γRV. These included trials for X-linked severe combined immune deficiency (X-SCID), Wiskott-Aldrich Syndrome (WAS) and adenosine deaminase deficiency severe combined immune deficiency (ADA-SCID).[8-10,13] In addition, three out of 12 patients treated for chronic granulomatous disease

(CGD; also ex vivo GT using LTR-driven γRV) and three out of 64 patients treated with Skysona/eli-cel (ex vivo GT using a self-inactivating Lentiviral vector (SIN LV) with strong internal retroviral MNDU3 promoter), all with long term engraftment, showed myelodysplastic syndrome linked to activating retroviral integrations.[11,12,26] The latency to clinical evidence of leukaemia development was between nine months and 15 years after γRV GT, with an incidence varying from one out of more than 70 treated (ADA-SCID) to seven of 10 treated (WAS) patients.[8,27]

A key mechanism resulting in vector-induced carcinogenesis was determined to be enhancer-mediated activation of endogenous proto-oncogene promoters by the LTR region of the γRV- vectors or by the strong retroviral MNDU3 promoter in the internal position of a SIN LV.[10,12,28] However, other mechanisms (such as disruption of tumour suppressor genes or of genomic loci leading to aberrant splicing and/or transcriptional termination) were likely involved as well in some cases.[26,29,30] It is worth noting that clonal expansion (in the absence of overt carcinogenesis) has also been observed in both clinical trials and in non-clinical studies, driven, for instance, by aberrant splicing events.[31,32] These events were observed even with SIN LV vectors. Carcinogenesis has also been observed in clinical trials with differentiated lymphocytes transduced with DNA transposons. Two cases of iatrogenic T cell lymphoma were observed in the CARTELL clinical trial, where CAR-T cells had been transduced with a non-viral piggyBac system. There was no integration into known oncogenes and the molecular mechanisms behind the lymphoma remain unclear. However, high transgene copy number, altered genomic copy number and point mutations unrelated to the integration sites were all considered as contributory factors.[14,15] DNA transposons are mobile elements identified from Baculovirus, like piggyBac or extinct fish like Sleeping Beauty.[33,34] DNA transposons tend to integrate at TA or TTAA sequence motifs, but recent publications show lower frequency alternative integrations, with only one inverted repeat and with a distinct target sequence pattern.[35-37] As long as the transposase remains active, multiple cycles of excision and integration are possible, increasing the risk of DNA transposon insertional mutagenesis.[29] Several mutant transposases have been developed to prevent secondary re-integrations or to modify their integration pattern.[35,36]

Neoplasia has also been observed in animal models, using various GT vectors and routes of administration. For example, LTR-driven γRV (ex vivo) vectors and SIN LV vectors equipped with retroviral promoters (ex vivo) have been associated with neoplastic events in mouse models.[20,21] Neoplastic events in non-clinical models were primarily observed in tumour-prone mouse models administered early generation LTR-driven γRV or LVs with retroviral promoters that lack enhanced safety elements designed to reduce the risk of neoplastic transformation, such as the use of a four-plasmid production system and SIN LTR sequences. These modifications were introduced in the third generation LV vectors currently in use in the clinic, improving their safety profile.[1,21]

Despite the low integrative potential of recombinant adeno-associated viral vectors (rAAVs), liver tumours have been observed in mice following dosing as neonates; tumours occurred after one year or later (being a significant proportion of their life-span, compared to the follow-up in human patients given AAV GT).[22,38,39] Dosing of adult mice with rAAVs, in the presence of liver damage, also resulted in liver tumours.[40] AAV integration has also been found in liver tumors in adult OTC mice, however, tumor frequency was not elevated compared to controls, so the causal link to AAV insertional mutagenesis is unclear.[41] Oncogenic transformation due to rAAV has not been observed in healthy adult mice or any other species. In dogs, non-oncogenic clonal expansion in liver was recently reported in long-term studies in hemophilia A dogs treated with rAAV vectors.[23] Expanded clones showed integration in genes potentially associated with cell growth control. In addition, integrated vectors were generally truncated and the transgene commonly deleted. For unknown reasons, increases in Factor VIII were observed over time in some dogs. In another hemophilia dog study (using a distinct rAAV) clonal expansion was detected in some animals, and integrated vectors were also found to be extensively deleted and rearranged. The gene CCND1 was notably marked by integration in both studies.[42] In both studies, no adverse consequences of clonal expansion were reported. It is known that clonal expansion is commonly associated with ageing in dogs, which may have contributed to some of the observed cell proliferation.[42,43] Another study of two dogs treated with AAV for hemophilia A showed a multicentric lymphoma in one animal after AAV gene therapy. However, the tumor was not vector marked, indicating that integration did not contribute to transformation. Both dogs showed diverse vector integrations, and in a few cases

modest clonal expansion, but here too there was no evidence for genotoxicity based on AAV integration.[44] The American Society of Gene and Cell Therapy (ASGCT) hosted a virtual roundtable on AAV integration in 2021, and its summary white paper reviewed the evidence of rAAV integration in animal models and possible risks of insertional mutagenesis in patients.[45] Lastly, a recent very comprehensive study characterized the location, abundance and expansion of rAAV integrations in liver of a large cohort of non-human primates up to 15 years post-dosing. These were compared with the same endpoints in non-human primates (and humans) naturally exposed to wild-type AAVs (wtAAVs). Although both rAAV and wtAAV showed a higher frequency of integration sites in regions susceptible to DNA damage or near highly transcribed genes, only a small proportion of rAAV treated animals showed liver clonal expansions without signs of tumorigenicity and slightly lower than the expansions observed for wtAAVs viral infections in the same species and in humans.[46] It should also be noted that rAAV integrations have also been detected in extra-hepatic tissues, e.g. heart.[46,47]

rAAV vectors have been administered to over 3000 patients in more than 200 clinical trials with no cases of carcinogenesis attributed to the rAAV vector.[1,45,48,49] For instance, no tumour development has been linked to insertional mutagenesis in over 3000 children dosed with Zolgensma (an rAAV-based therapy for spinal muscular atrophy) with up to seven years follow-up, whereas liver tumors were observed in mice 20 months after neonatal dosing with a Zolgensma-like rAAV.[39,50] Although controversial, one group has shown that integrated wtAAV2 is observed in a rare subset of human hepatocellular carcinomas (HCC) with increased RNA expression in nearby oncogenes. [51,52,53] The relationship of wtAAV2 integration with altered RNA expression and HCC has not been definitively established and any implications of this observation for rAAV vectors are presently unclear. However, a plausible mechanism, linking a 3’ UTR enhancer–promoter element in wtAAV2 with liver gene expression/dysregulation, has been demonstrated, and this element, originally present in pSub201-derived rAAV vectors, should be excluded from rAAV vectors for clinical use.[54]

Background - *In vivo* and *in vitro* carcinogenesis assays: state-of-the-art and limitations

The difficulties encountered with the assessment of potential carcinogenicity of vector-mediated GT in a non-clinical system can be attributed to several factors. Many of the critical events within the multi-step process of carcinogenesis that are known to occur in humans are difficult to reconstruct in non-human models. There is incomplete understanding of how parameters such as dose and patient- and disease-specific factors influence the outcome, thus obscuring any causal relationships that may exist. Specific benefits and limitations of in vitro and in vivo models are shown in Table 1 and elaborated below.

***In vivo* assays:**

With the current state of knowledge, animal models have limitations for human carcinogenicity risk assessment in terms of specificity and sensitivity: a negative result does not exclude a human-specific genotoxic mechanism, while a positive result might not be human-relevant. In addition, in vivo studies present animal welfare concerns and they are expensive and time-consuming (especially in terms of long-term follow-up in large animals). For GTs, where in vivo animal studies are performed for general toxicity evaluation, some readouts could be informative in the context of carcinogenicity detection (i.e., detection of pre-neoplastic lesions, increased proliferation with Ki67 staining, haematology evaluation). The use of animals has the advantage of leveraging the cellular and tissue complexity (e.g. impact on engraftment and clonality of the transduced population, competition in fitness and survival, presence of the immune system), tissue microenvironment and disease context that may influence carcinogenicity risk. For example, Hernández et al. reported transformation linked to chronic inflammation in CGD-diseased animals but not in healthy wild-type animals.[55] Animal models of ex vivo GT (RV and LV vectors) may include xenotransplantation of human cells into immune-deficient mice, serial transplantation and/or use of tumour-prone mice.[56-61] Use of tumour-prone mice increases the sensitivity of the assay in some settings and have been able to predict mechanisms of genotoxicity and to demonstrate the impact of vector design on genotoxic potential.[61] The (human) host genetic background (germline or somatic mutations) is also difficult to adequately model in animals (e.g., potential impact on DNA repair, integration

profiles, inflammation or stress haematopoiesis). In summary, although animal studies have limited value in quantitative risk assessment of specific vectors in particular patient groups, they may be useful in understanding pathogenesis (e.g. through histological assessment of pre-neoplastic lesions) of vector-induced tumours and in determining the role of potential risk factors.

***In vitro* assays:**

All in vitro assays, regardless of using human or non-human cells, have the limitation that they cannot recapitulate the complexity of a living organism. Examples of in vitro assays currently used for genotoxicity assessment of GT products are shown in Table 2.

***In vitro* assays using murine cells:**

Two murine in vitro assays in haematopoietic stem cells – in vitro immortalization (IVIM) and surrogate assays for genotoxicity assessment (SAGA) have been developed with appropriate positive controls to explore the mutagenic potential of certain GT vectors and to qualitatively support human risk assessment.[62-64] Data generated from IVIM, alone or in combination with SAGA, have been accepted by regulators for LV and RV assessment to support approximately 20 programs.[63] In some cases, the use of the IVIM assay has also influenced the in vivo study design: a new vector, that differed from the parental one for only one element, was tested in vivo for a shorter time once proved to be like the parental one in the IVIM assay (personal communication from Dr. Rothe and Prof. Schambach). Currently both SAGA and IVIM assays are typically run together; although the SAGA assay seems to be more informative and robust.[63]

Additional in vitro assays have been described to evaluate insertional events in murine cells upon transduction with retroviral or lentiviral vectors. These include a double-negative 2 (DN2; CD4-CD8- CD44+ CD25+) arrest during T cell differentiation assay and an interleukin 3 (IL-3)-independency assay.[65,66] This group is unaware whether these assays have been used for regulatory purposes.

These in vitro tools are informative of mutagenic risk of GTs utilizing integrating lentiviral and retroviral vectors and could potentially be used for DNA transposons, but they have not been optimized nor used for evaluation of rAAVs. However, concerns also remain, for example, that mouse surrogates may have limited predictivity where the risk arises from a human sequence and/or is species-specific. It is also unclear if the use of cells from diseased animal models could improve human translation; though unpublished IVIM and SAGA data from specific animal disease models (e.g., recombination-activating 1 (Rag1), Rag2 knockouts) and tumour-prone models (e.g. cyclin-dependent kinase inhibitor 2A (Cdkn2a) knockout) have not demonstrated a superior translation compared to cells from wild-type animals. However, in the case of Rag1 and Rag2 knockout cells, this was due to poor cell growth and viability compared to wild-type cells; whereas in the case of Cdkn2a knockout cells excess proliferation of mock treated cells reduced the sensitivity to any further proliferative effects (personal communication from Dr. Rothe and Prof. Schambach).

***In vitro* assays using human cells:**

A variety of human cell-based in vitro assays have also been described (Table 2): hInGeTox assay, Jurkat based assay, IL-2 independent growth assay, soft agar colony forming assay (SACF), growth in low attachment assay (GILA), and long-term adverse treatment effect assay (LATE).[67-71] SACF, GILA and LATE have been recently proposed for CRISPR/Cas9 induced transformation evaluation; however, their utility for viral and transposon-based GT products remains to be assessed. The IL-2 independent growth assay has been used for genetically modified T cells, but its value is debated because of the limited predictivity, linked to the lack of appropriate controls and assays standardisation. hInGeTox is a new assay based on iPSC and iPSC-derived hepatocytes that combines phenotypic and molecular endpoints, however more published data are required to evaluate its predictivity.[67]

Background – Methods of integration site analysis (ISA)

GTs pose several unique challenges for genetic toxicologists: all negative controls in a standard genotoxicity assay have a biologically defined background signal, arising from basal mutations and chromosomal damage from normal endogenous processes and/or background exogenous sources. Typically, a positive signal is defined as a statistically significant difference over this background. When using ISA, negative control samples (i.e., non-transduced cells) have a zero value by default for toxicity assessment (apart from any technical artefacts), as even minimal insertion frequencies in treated samples are statistically significant, despite potentially only having limited biological impact in terms of mutagenesis and cancer risk. A more mechanistic approach might be to compare the extent of insertion events with controls having a more relevant "biological background", such as a clinically proven safe vector or from comparable non-oncogenic naturally circulating viruses.

In addition, the integration frequencies and patterns differ between vector types, requiring customized approaches for the interpretation of integration data. For example:

- Integration frequency is much lower with rAAVs than retroviral / lentiviral / DNA transposon-based vectors.[72] However, a recent report suggests rAAV integration might drive long-lasting transgene expression versus short-lived episomal expression which, if confirmed, would, therefore, be beneficial.[73]

- Integration patterns differ between RVs and PiggyBac transposons (promoter regions), LVs (gene bodies) and Sleeping Beauty transposons (almost random).[74,75]

- Current data suggest the integration pattern for retroviral/lentiviral/transposon-based vectors show more host genome sequence bias compared to rAAVs, the latter largely considered random and enriched for fragile DNA sites and loci with high expression levels and decreasing over time after dosing.[46,73,76] However, whilst it has been speculated that homology-directed repair (HDR) may be contributing to rAAVs integration, this appears to be a minor pathway, since even vectors designed for targeted integration achieve HDR at one tenth of random integration

efficiency.[77,78] Furthermore, the transgene is generally cloned as complementary DNA and codon optimization would further reduce the sequence homology between the vector and host genome and, as a result, HDR integration efficiency. Accordingly, human hydroxymethylbilane synthase (hHMBS) gene was not reported among the common integration sites by multiplex LAM-PCR neither in patients nor in non-human primates treated with rAAV expressing codon-optimized hHMBS.[79]

- rAAVs, in particular, frequently show integration of both concatemers and/or complete and partial vector genomes.[23,46,47,73,79]

- DNA transposons can remobilize within the genome (so long as the transposase remains active) as opposed to stable integration seen with viral vectors.[29]

ISA has high negative predictive value (i.e., lack of integration suggests safe vectors) but limited positive predictive value (i.e., evidence of integration does not mean unsafe vectors). Current ISA methods typically report hundreds to thousands of integrations per biological specimen; therefore, data interpretation is based on the identity of nearby genes and enhancer annotation should also be considered. It should be noted that a significant proportion of the human genome consists of “cancer-associated” genes when broad literature mining definitions are used.[80] Given the size of these datasets, it is likely that proto-oncogenes (and other cancer associated genes) will be identified near vector integration sites by chance alone. Therefore, in many cases, the insertional findings should be of negligible concern, unless both their location is biologically relevant, and their frequency is high enough, or expanded clones are detected. It is probable that most integration is benign, even if it is a pre-requisite for transformation leading to clonal expansion and tumorigenesis. Lastly, the age-related background level of mutations should be considered. For instance, Martincorena showed a high frequency of mutations in cancer driver genes in normal epithelial tissues of healthy adults, whose number increased with age.[81]

Rather than focusing on a single timepoint, it might be more informative to evaluate integration site (IS) profiles at different timepoints (e.g., drug product (prior to administration) and after in vivo treatment or, for in vitro study, at an early and a late timepoint) to identify any potential trends of clonal enrichment. Statistical analysis should

distinguish clusters of integration sites resulting from vector-specific integration preferences from those resulting from a selection process, potentially by combining gene integration frequency and integration site abundance. However, clonality itself does not necessarily reflect a pre-carcinogenic state. For example, for T cell products, clonal enrichment could be dependent on the presence or absence of antigen presentation: antigen-dependent expansion is likely benign and linked to physiological proliferation (Cbl and Tet2 CAR-T cases) while cases of antigen-independent expansion are either linked to tonic signaling or carcinogenicity e.g., ex vivo culture, or transposon cases.[14,15,82-84] Similarly, clonal expansions have been observed after SIN LV gene therapy in haematopoietic stem cells (HSCs) associated with integration in HMGA2 in one beta-thalassemia patient and multiple SCID patients.[31,32] In these cases, disrupted splicing appears to have resulted in a truncated messenger RNA, which removed negative regulatory sequences and resulted in cell proliferation.

The biological relevance of clonality could be carefully considered using a combination of transcription profile changes, cell growth dynamics, cancer associations reported in the literature and human data. To ultimately define the impact of a given integration (e.g., driver vs passenger role), a targeted integration could be generated, and its functional consequences (including clonal enrichment) assessed, but the feasibility and realistic throughput of such approach is currently very limited.[40,85]

Numerous ISA methods have been developed each with different degrees of sensitivity, specificity, potential sources of bias and regulatory acceptance (see Table 3 for acronyms and brief description). These methods include LAM-PCR, nrLAM-PCR*7*, LM-PCR, S-EPTS/LM-PCR and TES, among others.[42,47,86-88] Restriction enzyme- and linear amplification-based methods have largely been replaced by sonication-based and linker-mediated technologies respectively, due to reduced bias and potentially increased sensitivity. TES relies on DNA hybrid capture instead of PCR amplification, avoiding bias towards Inverted Terminal Repeat (ITR) or LTR integrations. To our knowledge, only the methods above have been used for regulatory submissions. New methods are being continuously developed, such as multiplex LAM-PCR, CreViSeq, ITR-seq, Target-seq, long read sequencing using Pacific Biosciences or Nanopore technology and INSERT-seq, but to our knowledge they

have not been fully validated nor used for regulatory submission.[79,90-95] Viral integration can also be determined by whole genome sequencing (WGS) but its sensitivity/ specificity is limited and requires high sequencing depth and coverage and should be only considered for clonal cell populations.[47] A recent paper compared TES, S-EPTS/LM-PCR and WGS for AAV integration, showing similar sensitivity of TES and S-EPTS/LM-PCR with a lower sensitivity for WGS.[47]

As for any assay validation, use of positive and negative controls is important to demonstrate assay performance and support data interpretation. Examples of technical positive control materials include cell line(s) or isolated clones with known integrations, vectors with sequences that are known to have active contributory elements that drive oncogenesis; while clinical comparators would be any vectors linked previously to clinical malignancies.[88,95,96] For applications involving detection of low frequency integrations, careful analytical validations entailing positive controls spiked into negative controls at different relative frequencies should be used to rigorously demonstrate limit-of-detection. Currently, this has been a challenge for evaluating AAV based vectors, as no clinical malignancy has been identified with rAAV GT.

Consensus Statements

Based on the current state of the science and agreed amongst all experts, the group proposes the following **scientific principles and experimental approaches for the assessment of risk factors relevant to potential carcinogenicity of GTs:**

***In vivo* and *in vitro* assays**

1. **The existing regulatory approval process for GTs should continue to develop to ensure detection of potential causes of carcinogenicity that are relevant to humans. Areas of uncertainty in current non-clinical models will require considerable research efforts in the future. However, these efforts to improve on existing suboptimal / incomplete non-clinical assays should not delay the development of novel therapies.**

2. **Current non-clinical assays should be evaluated with clinically relevant products, ideally back-translating real-world examples and confirming negative and positive predictivity.**

3. **For all assays, either based on human or non-human cells, the low throughput and lack of broad access are limitations that need to be addressed.**

4. **Appropriate negative and positive controls, protocol standardisation and assay validation are required for wider acceptance and to support routine regulatory decision making.** Because of the current knowledge gaps and the ever-evolving technologies for the assessment of the carcinogenicity potential of GTs, when a validated assay is not available or validation is not achievable, the use of exploratory, in-development, research-grade, non-centralised assays should be considered by regulatory decision makers provided they are scientifically sound.

5. **Whenever possible, the focus should be on the "3Rs" principles of reducing animal usage, replacing them by alternative human-relevant non-clinical systems and, where that is not possible, using the information from *in vitro* assays and *in silico* tools to refine them to improve animal welfare.**

Integration Site Analysis

6. **Alongside technical controls (untransduced cells, non-integrating vectors), we suggest different approaches to define clinically negative controls for ISA, according to the vector type:**

    - For any vector types (LV, γRV, rAAV, Sleeping Beauty and piggyBac transposons), a comparison with similar vectors proven to be clinically safe for ≥15 years (or as long as feasible) and ideally in the same target cell type.
    - For LV, comparing to naturally occurring HIV infections in matching cell types (e.g., T-cell or macrophages).[97]

- For γRV, comparing to murine leukaemia virus infections in dividing B or T-cells or similar cell lines could be explored, but species differences (mouse vs human) limit this approach.[98]
- While wtAAVs are unlikely to be carcinogenic, their value as negative controls for comparison with rAAV clinical data is less clear as wtAAVs express viral proteins, including Rep protein which is responsible for integration *in vitro*.[99] However, the frequency of Rep-driven *AAVS1* integration hotspot identified *in vitro* for wtAAVs is variable across cell types and has not been confirmed consistently in human tissues, while other integration sites have also been identified.[46,52,53,93] Since rAAVs lack Rep protein, and although the relevance of Rep on wtAAV clinical integration might be limited, the value of any comparison remains debatable and should be justified case by case. Furthermore, for rAAVs, it is unclear how *in vitro* and clinical integration patterns correlate, given the discrepancy observed for wtAAVs. Nevertheless, clinically proven safe vectors or technical controls remain suitable comparators.
- For transposons, there are no naturally occurring integrations from currently used Sleeping Beauty and piggyBac. There are DNA transposons and inverted repeats throughout the human genome, including PGBD5 and RAG1/2 which may be involved in development of some tumours.[100,101] The sequence of the RAG1, RAG2 and PGBD5 inverted repeats differ from those in Sleeping Beauty and piggyBac transposons and there is no evidence of cross reactivity. However, any newly developed transposon systems should be assessed for functional and IS analysis cross reactivity with endogenous DNA transposons.

7. **If ISA data are available for vector(s) with the same core vector backbone, then further ISA with other transgenes or after vector optimization (e.g. removal of cryptic splice sites, codon optimisation, changes in producer cell line) <u>may</u> not be required.** This is further de-risked for vectors with equal or lower mutagenesis in the IVIM/SAGA assays than the ISA-characterised one. However, any potential effects on IS pattern due to a specific disease or peculiarities of the patient population should be considered. If the vector modification has the potential to influence in vivo selection

(e.g. changes to enhancer/promoter sequences, transgene with growth promoting properties) ISA should be done if appropriate animal models are available (e.g. humanized mouse models of HSC GT). Where clinical cases of carcinogenesis are observed, it is important to assess baseline samples, where feasible, for pre-existing cancer-driving mutations to better understand the pathogenesis and its relationship to GT.

8. **DNA transposons' IS profiles should be assessed at multiple timepoints or at a single timepoint after which transposase has been shown to be no longer active.** The mechanism remains unknown for the two cases of piggyBac insertional mutagenesis that were identified in the CARTELL clinical trial.[14,15] To understand the causal role of transposon integration, it has been suggested to identify sequences such as inverted repeats or poly-TA motifs (indicative of transposon re-mobilization) near copy number variant alterations or chromosomal abnormalities.[29]

Approaches to Risk Assessment

9. **All vectors have an integration risk, however, the frequency of integration events as well as the integration pattern vary between vectors of the same class or among different types of viral vectors.**

10. **As a general rule, the risk assessment package should be designed case-by-case taking into account several factors, including but not limited to type of vector, vector design, target disease/tissue and patient population, vector dose, route of administration and tissue distribution.** It should be borne in mind that depending on vector serotype, dose and route of administration, tissue distribution beyond the intended (therapeutic) target tissue could occur and this should be considered when selecting tissues for ISA and for the overall risk assessment.

11. **For many current GTs, often targeting severe diseases with limited treatment options, the risk of carcinogenesis is clearly outweighed by the therapeutic benefit** (e.g. FDA and EMA approvals for eli-cel/Skysona even with 3/64 MDS cases and 11/64 oligoclonality cases.[12]

12. **When the field progresses into less severe diseases or those with existing treatment options, the risk/benefit analysis will require a more "quantitative" understanding of the risk (for instance, by classifying GT products into broad "risk categories").**

13. **Despite the inherent mutagenic potential of integrating vectors, only a limited number of integrations have been associated with tumour formation.** The binary presence/absence of integration is insufficient to predict the likelihood and risk of eventual carcinogenesis in any specific context. Therefore, lack of integration has high negative predictive value, but a positive result requires further evaluation.

14. **Several factors plausibly influence the risk for carcinogenesis following one or more genomic integrations in a target cell.** These include: nature of the target cell (lineage, state of differentiation, epigenetic status, previous natural viral infections), vector type, vector design (e.g. tropism, nature of transgene and promoter/enhancer (strength, transactivation level), presence of LTRs, SIN design, splice sites), dose of vector, route of administration, disease background, individual patient factors (age, pre-existing conditions, genetic predisposition, co-medications, life-style factors) and proliferation rate in the target cell population following transduction. It is also important to consider that cell types vary in the number of mutations required to drive cancer formation and in the latency to cancer formation.[102] A useful review of risk factors in the context of HSC GT has recently been published.[103]

15. **Non-clinical carcinogenicity risk assessment for a novel GT product based on any vector (given *ex vivo* or *in vivo*) with the ability to integrate into host DNA (by design or incidentally) would benefit from a standardized "weight-of-evidence" approach as shown in Table 4.** This approach could be applicable as the field progresses to incorporate other viral vectors.[72]

16. **Risk factors considered to increase risk (of carcinogenesis) should be weighed against those that mitigate against. This can be further supported using product-specific assays assessing biologically-relevant endpoints where available (Table S1).** Given the divergent limitations of both *in vitro* and *in vivo* approaches, a tiered approach starting with *in vitro* assays followed by appropriate and 3Rs driven *in vivo* experiments should be adopted as a standard approach.

17. **Any product-specific assays used should ideally possess the characteristics described in Table S1.** Whilst this group is not prescribing specific assays to be used for any particular product, it is hoped that the use of a standardised overall approach would allow meaningful comparisons (of risk) within specific groups of GT products. It is recognised that many of the items in Table S1 are aspirational/forward-looking, with the aim of producing the most human relevant / predictive assays.

18. **As knowledge and experience increase, a more deterministic approach, such as an "Adverse Outcomes Pathway (AOP)" analysis, could be used to illustrate potential pathogenesis of GT-induced carcinogenesis.**[104] Initially, AOP analysis on existing (non-clinical and clinical) cases of GT-induced carcinogenesis could be used to graphically demonstrate possible pathway(s) from a "molecular initiating event" (MIE; in this case integration of vector into host DNA), through a series of "key events" (KE), to a potential "Adverse Outcome" (AO; e.g. lymphoma following X-SCID or WAS GT; liver tumours following GT of neonatal mice). Further details on the AOP approach (with an example of a regulatory approved (non-GT) AOP) are given in supplementary materials, along with a hypothetical AOP framework for GT-driven carcinogenesis, and how this might be used prospectively.

Future developments / Research needs

Areas identified by the group for future development and focus were:

19. The vectors that have caused the leukaemia cases observed in the clinic with X-SCID, WAS and ADA-SCID are relevant positive controls. Their insertional mutagenesis mechanism is reasonably well known, even if the exact contribution of some critical product attributes and potential individual risk factors to the eventual carcinogenic outcome are not fully understood.

20. As data and experience are gained through more standardized approaches, it may become possible to set ranges for certain parameters (such as vector copy number (VCN), multiplicity of infection (MOI) or vector dose). For instance, for *ex vivo* GT, determining average VCN per transduced cell is considered a more accurate determinant of product risk.[4,105]

21. More effective risk assessment of GT could be achieved by closing certain knowledge gaps. The following, non-comprehensive, list includes current knowledge gaps in the GT field identified by the authors which should be subject to further research.

    a. Integration site analysis:

        - Insufficient sensitivity of ISA methods to detect very low numbers of genomic integrations in a cell population.
        - Difficulty in detection of partial (e.g., without LTR/ITR sequence) or rearranged integrations by most methods.
        - Unknown thresholds for safe integration in/near known oncogenes, whether previously associated with vector-driven transformation (e.g., *LMO2*) or not.
        - Lack of harmonisation/cross-validation of ISA methods/platforms, and reporting, to facilitate historical comparisons across vectors/companies.
        - Cancer-associated gene lists are currently inconsistent across different laboratories and are based on varying criteria used to define an “oncogene”. These lists may also vary between species.
        - Different vector types show distinct genomic integration patterns (promoters *vs* gene regions *vs* semi-random, often dependent on e.g., GC content, epigenetic marks); however, correlation of these differences with safety across different vector types is only partially understood.
        - The impact of random integration versus targeted integration (integration “hotspots”) and quantitative risk is not well defined.

    b. Clonal tracking:

        - Lack of agreement on a threshold that defines clonal outgrowth and on the cell population to be used as denominator (e.g. total white blood cells vs individual blood cell lineages).

- Poor understanding of the relationship between (oligo-/mono-) clonality arising within a population of transduced cells and potential neoplastic transformation.
- Limited understanding of the relative differential risk that should be attributed to different mutations that define these clones.
- Interpretation of clonal dominance when confounded by occurrence of multiple integrations within the same cell.[106]
- Difficulty of clonal tracking over time in solid tissues in the clinic, although approaches to this issue with cell-free DNA are in development.[107]

c. Epigenetics / Transcriptomics:

- Role of epigenetic status on integration of specific GT vectors.
- Impact/predictivity of GT vector-induced epigenetic and transcriptomic changes on/for risk of neoplasia.

d. Statistical Methods:

- Interpretation of ever-expanding datasets.
- Lack of statistical/mathematical approaches to model dynamics of potentially adverse clonal outgrowth in vivo e.g. by combining gene integration frequency and integration site abundance.

e. *In vitro* assays:

- Current in vitro models limited by short-term follow up and restricted ability to simulate competitive selection/clonal outgrowth.
- Lack of qualified/validated assays for rAAVs.
- Lack of availability of positive controls for some vectors (e.g. rAAV) and/or some cell types.
- Validation, standardisation, availability of assays in general.

f. Dose relationships:

- For rAAVs *in vivo*, the relationship (linear, with threshold) between VCN in a sample and integration frequency.
- For any cell population exposed to a fixed number of integrations (fixed bulk VCN) there is poor understanding of the relative risk between integrations spread across all/ the majority of the cells (high transduction efficiency) or integrations occurring in a small proportion of cells (low transduction efficiency).
- Allowing for differing frequencies of integration (of differing vector types), the relationship between the total number of cells carrying integrations and risk is unclear.

g. Target tissues:

- The sensitivity/resistance of different target (intended or unintended) tissues to neoplastic transformation following integration of GT vectors.
- Absolute resistance of differentiated cells to neoplastic transformation.
- The quantitative relationship between rate of cell turnover (in any given tissue) and sensitivity to neoplastic transformation.[108]

h. Patient factors:

- Impacts of age, disease background (e.g. WAS, liver fibrosis, Fanconi anemia, clonal hematopoiesis of indeterminate potential), individual patient genotype, co-medication and lifestyle factors.[109-112]

i. Non-integrating GT vectors:

- Potential risks associated with non-integrating GT vectors carrying strong promoters, regulatory elements or transgenes with growth-promoting effects.

j. Basic biology of rAAV vector-cell interactions:

- Comparison of wtAAV with rAAV.
- rAAV integration patterns in animals compared to human. The authors propose that a publicly available database containing rAAV integrations in non-clinical species, and from clinical settings must be set up. This must be associated with a minimally agreed set of metadata (genome coordinates and some annotation (e.g., nearby genes)) in order to maximise the value of these data for others in the field and characteristics of the associated rAAV
- Understanding the molecular mechanisms of rAAV-induced liver tumours in mice, from Rian locus integration to tumour formation.
- Understanding rAAV integration frequency and pattern in non-hepatic tissue and associated risks.

22. Concerted research efforts (e.g., multi-site studies via public consortia) are recommended to characterise/qualify assay performance, ideally leading to validation. Some human-relevant alternative assays, models and tools for the study of potential carcinogenicity already exist but many of these have not been taken forward into an assay validation process. A recently initiated multi-site study led by HESI CT-TRACS to evaluate the IL-2 independence assay for T-cell transformation is a good model on which such initiatives could be based.

23. In addition, suitable assays are still missing for some mechanistic aspects of carcinogenicity and dedicated research projects for assay development are needed to fill these gaps. The development of human-relevant novel *in vitro* assays to assess tumourigenicity of genetically modified T-cells (CRACK-IT challenge T-Alert) and of genetically modified HSCs (CRACK-IT Challenge Clean Cut) are current examples of industry / academic collaborations, both led by the NC3Rs, working to develop new assays in this area. Wider testing of novel assays is also important to ensure their

fitness for use and initiatives such as the HESI CT-TRACS who will perform a multi-site study on the T-Alert Challenge assay(s) are highly warranted.

Summary

This article outlines a set of high-level principles that reflect the current state of the art and expert knowledge, to guide and inform the assessment of potential vector mediated genotoxicity / potential carcinogenicity of the GT products described. The GT field is rapidly evolving, and it is expected that as progress is made, new knowledge and understanding can be integrated into these principles. Data transparency will be essential and the authors specifically propose that data generated from viral integrations site studies in non-clinical species, and from clinical settings, are made publicly accessible (through for example, databases and patient registries) . There is a clear need to develop improved non-clinical assays and the potential for non-animal, more human-relevant approaches will deliver significant advances in our understanding, even if there will remain in the near-term, a need to use animal models.

Key to realizing these advances is collaboration and engagement across the sectors, capitalising on opportunities to build on successful academic and industry collaborations through funding initiatives like the NC3Rs CRACK IT program and the HESI technical committees.

The increasing role of GT in medicine mandates a better understanding of vector mediated safety concerns to allow the full potential of GT to be exploited.

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Author contributions:

SJ and SL conceived the original idea for this work. JCK, ADRE, SL, SJ, PC, MDF, AML, HJM and AZ wrote the first draft of the paper. IK chaired the face-to-face meeting. All authors contributed to the meeting and to commenting on the first draft of the paper. Following the meeting JCK, ADRE, SL, PC, MDF, AML, HJM, CV and AZ wrote the second draft of the paper. All authors reviewed and commented on the second draft of the paper.

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J.C.K. is founder of Cornelis Consulting Ltd and serves as a consultant to Apollo Therapeutics, AviadoBio, Orchard Therapeutics and Sangamo Therapeutics; he is an ex-employee and equity stake holder of GSK and is on the scientific advisory board of Testavec. He received no financial reward for his participation in this work.

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C.V. declares no competing interests

A.Z. owns stock of F. Hoffmann - LaRoche Ltd.

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Table 1. Advantages/disadvantages of current in vitro vs in vivo genotoxicity assays.

***In vitro* models**	***In vivo* models**
Strengths • 3Rs benefit • Can use human cells • Can evaluate different assay conditions • Cost-effective • Scalable • Can be used for screening (e.g. of different vector designs) • Faster	Strengths • Can recapitulate all stages of carcinogenesis – from molecular changes, through clonal expansion to tumour metastasis • Endpoints have clinical significance (not surrogate) • Certain models can use human cells (e.g. HSCs for *ex vivo* GT)
Limitations • Endpoints are surrogates for tumour pathology *in vivo* • May not reflect physiological conditions (including impact of the immune system) • Primary cells difficult to transform – lower sensitivity • Cell lines with confounding genetic abnormalities • Limited lifespan of cell models to capture the carcinogenic process	Limitations • Impact on animal welfare • Differing tropism of GT vectors for animal cells • Different integration profile of GT vectors into animal DNA • Lack of suitable animal models able to recapitulate the effects of disease background on carcinogenicity • Time-consuming • Expensive

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Table 2. In vitro genotoxicity assays.

Assay	Cell type	Principle	References
IVIM (In vitro Immortalization assay)	primary murine cells	Murine hematopoietic progenitor cells are transduced with retroviral vectors and grown in myeloid differentiation medium for ~21 days. From day 15 post transduction, cells are seeded at very low density. Only transformed mutants will proliferate in these restrictive conditions, while non-immortalized cells will stop proliferating.	*62, 64*
SAGA (Surrogate assay for genotoxicity assessment)	primary murine cells	Murine hematopoietic progenitor cells are transduced with retroviral vectors as for the IVIM assay. Machine learning is used to recognise a specific transcriptome signature from cells collected at day 15 after infection.	*63*
DN2 block	primary murine cells	Early murine thymic precursor cells are grown on OP9-DL1 stromal cells,	*65*

		a system that allows thymic differentiation through the four stages of double negative $CD4^-CD8^-$ (DN1-4), double positive ($CD4^+CD8^+$), and single positive stages. Cells infected with oncogenic viruses will arrest in the DN2 stage ($CD44^+CD25^+$)	
IL-3 independency assay	Bcl15 murine cell line	Bcl15 cells are transduced with viruses in restrictive conditions in the absence of IL-3. The assay is based on the assumption that oncogenic viral integration will render these cells IL-3 independent.	*66*
Jurkat based assay	Jurkat cells	The activity of potential LTR insulator elements is evaluated in a Jurkat derived cell clone in which LTR is integrated in the P5 locus and drives mCherry expression.	*68*
hInGeTox	human iPSCs and iPSCs derived hepatocytes	human iPSCs and iPSCs derived hepatocytes are infected and several parameters are evaluated	*67*

		(integration sites, clonality, presence of truncated transcripts, transcriptomics and methylomics). Each parameter is given a score, generating a final score that is compared to the one obtained by oncogenic viruses.	
Cytokine independent growth	human primary T cells	The assay relies on the assumption that normal human primary T cells will stop growing in the absence of IL-2, while transformed cells will grow regardless	*69*
SACF (Soft Agar Colony Forming assay)	human immortalized MCF10A and THLE	CRISPR/Cas9 edited cells are seeded in semisolid medium at very low density. Colonies are counted after ~4 weeks. The assay relies on the assumption that only transformed cells will form colonies in soft agar, while normal adherent cells will not be able to grow anchorage independently. This assay	*70*

		is not suitable for cells in suspension and has not been tested with oncogenic viruses.	
GILA (Growth in Low Attachment)		CRISPR/Cas9 edited cells are seeded in low attachment plates. Cell number is indirectly evaluated by ATP measurement two weeks after seeding. The assay is based on the assumption that only transformed cells will be able to grow in these conditions, while normal adherent cells will not be able to grow anchorage independently. Assay is not suitable for cells in suspension and has not been tested with oncogenic viruses.	
LATE (Long-term adverse treatment effect)	primary human newborn foreskin fibroblasts, MSCs and RPE-1 cells	Primary cells are transduced with a lentivirus expressing GFP, Cas9 and the sgRNA against gene of interest. Enrichment over time of GF positive cells indicates that the CRISPR/Cas9-	*71*

		induced modification of the target gene has induced a proliferative advantage. Assay has not been tested with oncogenic viruses.	

Table 3. Integration Site Analysis Methods.

Acronym / Method	Description	References
LAM-PCR	Linear amplification- mediated polymerase chain reaction: ssDNA is amplified by linear PCR with biotinylated primer allowing enrichment with streptavidin, dsDNA synthesized and digested with restriction enzyme(s), ds-linker ligated and amplified in two nested exponential PCRs and third PCR adding adapters. Short read NGS sequencing	*86*
nrLAM-PCR	Linear amplification-mediated polymerase chain reaction plus non-restricted linear amplification polymerase chain reaction: ssDNA is amplified by linear PCR with biotinylated primer allowing enrichment with streptavidin, ss-linker ligated and amplified in two nested exponential PCRs with adapters. Short read NGS sequencing	*87*
LM-PCR	Ligation or linker-mediated polymerase chain reaction 2009-2013 version: dsDNA is digested with restriction enzymes, primer extension, ds-linker ligation, two nested exponential PCR amplifications with adapters. Short read NGS sequencing 2017 version: dsDNA is sheared, followed by ds-linker ligation, adding a blocking oligonucleotide for internal sequence in exponential PCRs	*88*
S-EPTS/LM-PCR	Shearing extension primer tag selection ligation-mediated polymerase chain reaction: dsDNA is sheared, primer extension with biotinylated primer allowing enrichment with streptavidin, linker ligation,	*89*

	two nested exponential PCR including adapters for library preparation. Short read NGS sequencing.	
TES	Target Enrichment Sequencing: dsDNA is fragmented and adapters ligated, PCR amplified, hybridised with biotinylated capture probes along the vector for enrichment with streptavidin, second PCR amplification and short read NGS sequencing.	*42, 47*
Multiplex LAM-PCR	Multiplex Linear amplification-mediated polymerase chain reaction: same protocol as LAM-PCR above but with five primer pairs multiplexed along the vector for each PCR step. Short read NGS sequencing	*79*
CreViSeq	CRISPR-enhanced Viral Integration Site Sequencing: dsDNA sheared, in vitro circularization with T4 ligase, cleavage of LTR sequence with CRISPR, ligated adapters and short read NGS sequencing	*90*
ITR-Seq	Inverted Terminal Repeats Sequencing : dsDNA sheared, end-repair and Y-adapter ligation, two nested exponential PCR with adapters, and short read NGS sequencing	*91*
Target-seq	dsDNA enzymatic digestion, adapter ligation, first exponential PCR with 5'-phosphorylated target primer, barcode ligation to 5'-P product, second exponential PCR with adapters. Short read NGS sequencing	*92*
Long read sequencing using Pacific Biosciences	dsDNA digested with restriction enzymes, ds-linker ligation, two nested exponential PCR with biotinylated primer allowing streptavidin enrichment, second PCR amplifications with barcodes. Long read NGS sequencing	*93*

Long read sequencing using Nanopore	For on-target integration. dsDNA isolated by gravity-flow, shearing at 20kb and size selection at 4kb, dephosphorylated, Cas9 cleaved with 2-4 gRNAs, adapters ligated and long read sequencing.	*94*
INSERT-seq	dsDNA is sheared, end-repair and Y-adapter ligation, exponential PCR with exonuclease digestion non-amplified dsDNA, nested exponential PCR, adapter ligation and long read sequencing.	*95*
WGS	Whole genome sequencing. dsDNA fragmentation and ds-adapter ligation. Short read sequencing.	*47*

Table 4. Generic risk assessment for a novel (integrating) GT product.

Parameter@	Likely to increase risk	Likely to reduce or mitigate risk
Vector design	Presence of full LTR (LV, γRV) only) Presence of strong promoters or enhancers Multiple splice sites Vector instability Ubiquitous promoters	Use of "SIN design" (LV, γRV only) Use of weak promoters Codon optimisation Splice donor/acceptor sites removal Tissue specific promoters Safer regulatory elements Kill switch
Transgene product	Transgene with pathway-related risks (e.g., growth promoting properties, altering genomic stability); especially if expressed at supra-physiological levels	Transgene with no known pathway-related risks
Vector producing cells	Presence of potential vector sequence modifying enzymes in cell line e.g. Apobec3c	Absence of potential vector sequence modifying enzymes in cell line
"Dose"	High VCN	Low VCN
Route	*In vivo* GT using vector pseudo/serotype with low tissue specificity	*Ex vivo* GT eliminates/reduces risk to non-intended tissues Use of tissue specific vector pseudo/serotype and/or promoter reduces risk to non-intended tissues
Target* cells	Target cells with multipotent potential (stem or progenitor cells) Target cells rapidly dividing	Target cells post-mitotic / terminally differentiated

Integration profile/sites	Integrations biased towards promoters, transcription start sites e.g., RV or PiggyBac transposons (LV, γRV DNA transposons only)	Random integration e.g., LV or Sleeping Beauty transposons Targeted integration in safe loci (LV, γRV DNA transposons only)
Patient factors	Disease background known to carry higher risk of carcinogenesis Young patients – especially neonatal, paediatric	No known disease/genetic background risk Adult patients

* Includes cells unintentionally transduced by GT vector due to unavoidable biodistribution. @ Each parameter should be assessed qualitatively (and where possible quantitatively) to produce a weight-of-evidence based risk assessment.