Reflection paper on management of clinical risks deriving from insertional mutagenesis

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Keywords	Gene therapy medicinal products, insertional mutagenesis, integration.

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Abbreviations

AAV	Adeno-associated vectors
ADA	Adenosine deaminase
CGD	Chronic granulomatous disease
CIS	Common insertion sites
GTMP	Gene therapy medicinal products
HSC	Hematopoietic stem cells
IVIM	*in vitro* immortalization assay
LAM-PCR	Linear amplification-mediated PCR
LTR	Long terminal repeat
MAA	Marketing authorisation application
PCR	Polymerase chain reaction
Q-PCR	Quantitative-PCR
SCID	Severe combined immunodeficiency
SFFV	Spleen focus forming virus
SIN	Self-inactivating
T-ALL	T-cell lymphoid leukemia
TALEN	Transcription activator-like effector nuclease
WAS	Wiskott-Aldrich Syndrome

1. Introduction (background)

Integrating vectors are considered the tools of choice by the scientific community when aiming at treating diseases that require life-long gene expression. These vectors insert permanently into the cell genome and once the cells replicate, the integrated sequences are transmitted to its progeny. Such vector-based gene therapy medicinal products (GTMP) have been widely used to treat monogenic disorders, cancer, and infectious diseases (*1, 2*). Integrating vectors are typically employed for *ex vivo* gene transfer in different cell types, such ashematopoietic stem cells (HSC), lymphocytes, and epidermal cells (*3-12*), although they are also used for *in vivo* approaches (*13, 14*).

Insertional mutagenesis leading to oncogenesis is a recognised safety concern of vector-based GTMP. For vectors that do not efficiently integrate, such as adeno-associated vectors (AAV), plasmids or retroviral vectors modified to avoid integrations, insertions into the genome represent unintended and potentially rare events and therefore insertional oncogenesis remains, theoretically, at low risk. Insertional mutagenesis is instead more probable when using integrating vectors such as gammaretroviruses, lentivirus and transposons, which are able to stably insert a therapeutic sequence into the host genome. Gene correction/gene replacement strategies aimed at targeting specific genomic sites (i.e., exploiting sequence-specific endonucleases like artificial zinc finger or TALEN nucleases) could reduce the risk derived from random or semirandom insertions (*15*). Although these gene correction/gene replacement strategies might show some off-target activity, the risk of insertional oncogenesis is conceivably the lowest with respect to conventional approaches with integrating vectors (*16*).

This reflection paper is aimed at discussing the factors contributing to genotoxicity of vector integration, the strategies to reduce the risk associated to insertional mutagenesis and the assays to evaluate vector oncogenesis at the pre-clinical and clinical level.

1.1. Insertional mutagenesis events leading to oncogenesis in clinical experience

Serious adverse events caused by insertional mutagenesis have been reported in 12 patients with primary immunodeficiencies treated with gene-corrected HSC transduced with gammaretroviral vectors. These serious adverse events included five cases of acute T-cell lymphoid leukemia (T-ALL) in two trials for X-linked severe combined immunodeficiency (SCID-X1 (*4, 17*) occurring between 2 and 5.7 years after treatment; four cases of ALL in Wiskott-Aldrich Syndrome (WAS) (*18, 19*); and three cases of myelodysplasia in Chronic Granulomatous Disease (CGD) (*20, 21*). In all cases, transformed cell clones harbored vector insertions next to proto-oncogenes leading to their activation and that subsequently progressed into malignancy (*22*).

1.2. Insertional activation, inactivation and clonal dominance

The mechanisms of insertional mutagenesis by integrating vectors recapitulate those revealed by studies of gammaretrovirus-induced oncogenesis and exploited in oncogene capture screenings in mice (*23*). For SCID-X1, the aberrant proliferation was associated to transcriptional activation of the LMO2 proto-oncogene by viral enhancer/promoter sequences present in the vector Long Terminal Repeat (LTR). LMO2 is a transcription factor involved in hematopoietic differentiation, normally expressed in

HSCs and very early T cell precursors while is usually downregulated upon differentiation. LMO2 overexpression caused by chromosomal translocations in this locus has been causally implicated in T-cell lymphoid leukemia in humans.

In the case of CGD, an unexpected *in vivo* expansion of gene-corrected myeloid cells showing clusters of vector integrations in the MECOM (MDS-Evi1), PRDM16 and SETBP1 loci was observed. The investigators hypothesised that the overexpression of MECOM gene by insertional activation could have led to disruption of normal centrosome duplication resulting in genomic instability, monosomy 7 and clonal progression towards aberrant expansion and myelodysplasia. The strong enhancer/promoter activity of the Spleen Focus-Forming Virus (SFFV) Long Terminal Repeat (LTR) may have favored the transcriptional activation of MECOM oncogene to high levels able to trigger cell transformation, most likely through transcripts initiated from the vector LTR and spliced into the downstream exons of the MECOM gene. Because most retroviral genomes contain a strong splice donor site downstream to the 5′ LTR, vector transcripts initiated at the LTR can be spliced to endogenous gene exons downstream the vector integration site, leading to both truncation and overexpression of its gene product, a major mechanism of proto-oncogene activation. These observations provide a strong rationale for the use of self-inactivating (SIN) LTR in vector design.

On the other hand, the detection of integrations nearby cancer genes does not necessarily associate to malignant clonal expansions. Indeed, in a gene therapy trial for the adenosine deaminase-severe combined immunodeficiency, patients' cells harboring integrations targeting LMO2 or MECOM did not aberrantly expand. Adenosine deaminase-severe combined immunodeficiency (ADA-SCID) constitutes to date an example of disease correction by gammaretroviral vector in absence of long-term vector-driven genotoxicity *(5, 7)(21)*.

Although adverse events were restricted to some gammaretroviral-based clinical trials, in a beta-thalassemia patient treated by lentiviral vector-based gene therapy, the appearance of a self-limiting dominant clone in the myeloid compartment of a patient raised some concern (*10*). The investigators showed that a dominant clone harbored a vector integration within the *HMGA2* gene that generated by splicing the fusion of the third exon of *HMGA2* gene with the vector sequence. The resulting aberrant HMGA2 mRNA displayed a higher stability, which in turn led to increased protein levels. It has been suggested that this aberrant *HMGA2* transcript is involved in the clonal dominance by conferring a homeostatic advantage to transduced cells (*10*). However, the subject showing HMGA2 clonal expansion remained healthy for more than 4years in the absence of other abnormalities (*24*). A study in mice harboring a truncated form of HMGA2 showed that overexpression of the transcript could result in benign hematopoietic stem/progenitor cell expansion (*25*). Following these observations, several groups have now shown that the generation of aberrant transcripts could indeed alter the cellular transcriptome, potentially leading to haplo insufficiency of a tumor suppressor gene (*26-28*). The clinical significance of these mutagenic events, however, remains uncertain.

2. Discussion

2.1. When is there a risk for oncogenesis as a result of insertional mutagenesis?

Although insertional mutagenesis is more probable with integrating vectors, the large majority of the molecular events are clinically silent. Because of the semirandom genome-wide integration of retroviral vectors, cells with potential at-risk vector insertions (i.e., near proto-oncogenes) should be rare among a population of transduced cells. Yet, as the total number of integrations and transduced cell infused increases, the presence of cells bearing at-risk insertions becomes more likely. It appears, however, that deregulation of cellular genes as caused by vector insertions alone is not sufficiently potent to induce alterations in the cellular program that favors self-renewal replication over proliferation in connection with differentiation or senescence. It must be noted indeed that in most genetic backgrounds, failsafe mechanisms intrinsic to the host cell efficiently prevent the insurgence of cell clones harboring integrations, conferring a selective advantage, and consequently reduce the risk of insertion-mediated oncogenesis.

Indeed, premalignant cell clones harboring genotoxic integrations acquired additional genetic mutations (monosomy of chromosome 7 in CGD clinical trial or CDKN2A loss or TCR-β to the STIL-TAL1 locus translocation in SCID-X1 clinical trial (*29*)) that facilitated the progression into full malignancy.

Overall, the following cases can be envisaged:

a) Vector insertions which are biologically silent and do not interfere with host cell genome activity.

b) Vector insertions which interfere with host cell transcription or post-transcriptional activity of neighboring genes but are biologically irrelevant.

c) Vector insertions that interfere with host cell transcription or post-transcriptional activity of neighboring genes but are clinically irrelevant as they induce only transient clonal expansion or "benign" stable relative increase of a given clone, without abnormal proliferation.

d) Insertions which interfere with host cell transcription or post-transcriptional activity of neighboring genes that, upon additional mutagenic events, lead to cellular transformation (e.g., myelodysplastic condition, leukemic proliferation, solid tumor).

The most concerning events are represented by dominant gain-of-function mutations leading to activation of proximal proto-oncogenes, which are mediated by enhancer/promoter elements in the vector and/or aberrant splicing from the vector transcript. Intragenic vector insertions could also lead to loss-of-function mutations in tumor suppressor genes by causing premature termination or aberrant splicing of the endogenous transcript, or induce chromatin remodeling. These mechanisms of insertional mutagenesis, however, would be of lesser concern because they usually require a second independent mutagenic event causing loss of heterozygosity at the targeted locus to reveal their oncogenic potential.

Necessary prerequisites for the definition of vector-driven aberrant clonal expansions are the following: a) the clone bears an at-risk vector insertion within a cancer-associated gene that may cause activation of its transforming potential; b) the vector-marked clone is long-lived; c) the clonal contribution is sufficiently high and increasing overtime at the expense of the other engrafted clones.

2.2. Which factors contribute to the risk?

Several factors are thought to be important in contributing to the risk of oncogenesis:

  a) Vector design (including backbone and regulatory elements)

  b) Insertion profile

  c) Vector dose

  d) Transgene product

  e) Target cell population/organ.

a) Vector design

A major feature contributing to the genotoxic risk of an integrating vector is the presence of active promoter (e.g. LTR) bearing strong enhancer and promoter sequences capable of *trans*-activating neighboring genes once the vector has inserted in the genome. Whereas SIN LTRs abrogate such risk, one should then consider the transactivation potential of enhancer/promoter sequences placed internally within the vector to drive transgene expression. Current knowledge would indicate that the choice of moderately active versus strong promoters further decreases the risk. The presence of splice donor/acceptor sites and polyadenylation signals within the vector, such as those present in integrating gene therapy vectors, may favor the generation of alternative transcripts potentially impacting the safety (*26-28*). The potential inherent genetic instability of a vector should also be taken in account for safety monitoring.

b) Insertion profile

The tendency of a vector to insert in specific genomic regions, such as near transcriptional start sites as observed with gammaretroviral vectors, could increase the probability of enhancing gene expression. On the other hand, the integration profile of lentiviral vectors appear to be safer after testing on *in vitro* and *in vivo* models for genotoxicity (*30, 31*). Depending on the locus targeted, the risk is expected to be reduced with vectors aimed at gene correction or gene replacement strategies in specific genomic sites mediated by sequence-specific endonucleases. However, careful evaluation of the specificity and "off target" activity of these vectors should be included in the risk evaluation.

c) Vector dose.

The risk of genotoxic events increases with the total number of integrations/transduced cells infused in a patient. The risk might be further increased if the number of vector integrations per cell also increases, but this correlation is at present unclear, at least as experienced from the range of vector copy number per genome encountered in current practice.

d) Transgene product

Another important point to consider is the transgene product, its mechanism of action and whether the product requires a regulated gene expression. The risk may be higher if products involved in regulation of cellular growth are expressed inappropriately (i.e., at levels higher than normal or lacking lineage restriction), accelerating the occurrence of additional mutations.

d) Target cell population/organs

These are also known contributing factors to the overall risk. In general the risk of oncogenic events appears inversely related to the maturity of cells/ tissues. For example, gammaretroviral vectors are known to induce oncogenic events in HSC but not in mature lymphocytes, likely because of the different genetic program of the two cells types (*32*). *In vivo* gene transfer into fetal tissues has been associated with a significant risk of oncogenesis as compared to their adult counterpart (*33*). The process of cell transformation may be also favored by a predisposition to acquire additional somatic or genetic mutations pre-existing before gene transfer. For *ex vivo* gene transfer, the conditions to which cells are exposed *in vitro* (e.g., duration of culture, exposure to growth factors), and *in vivo* following administration of genetically modified cells (e.g., "stressed" hematopoiesis), should be also considered. Finally, epigenetic factors (e.g. methylation status) in controlling the transcriptional activity of vector and transgene sequences could play a role in influencing the process of vector-induced oncogenesis.

2.3. When are integration studies needed?

Overall, if a particular vector system is deemed to have a capacity to permanently integrate into the host cell and to last for long time in treated individuals, it could by definition carry an intrinsic risk of generating insertional mutagenesis that should be evaluated in nonclinical and clinical integration studies. Several techniques are now available to characterise the genomic distribution according to which the vector integrates and study clonality at the molecular level, in both nonclinical and clinical samples. The need and extent of integration studies in patients should depend on scientific experience with a particular vector system and the results from previous nonclinical/clinical studies.
In the initial feasibility studies for a novel vector, insertional profiling can provide important information to understand the biology of the vector and improve its safety profile. For example, for nucleases-based or zinc finger-based vectors that are intended to target a specific site, integration studies are an essential tools to determine the specificity of insertions. In the context of nonclinical toxicology or biodistribution, insertion studies could increase the robustness of the models by providing analyses of the vector profile *in vivo* and the clonal repertoire of transduced cells. These studies are of interest for integrating vectors used both for *ex vivo* and *in vivo* approaches and should be performed in the relevant target tissues/organs. In the clinical setting, integration studies may be required for the monitoring of potential safety issues identified in nonclinical studies. Such studies could complement the overall safety monitoring of patients and aide the assessment as to whether or not a particular integration profile is more or less prone to cause adverse events. Retrieving integrations from tissues/organs after *in vivo* injection of vectors requires invasive procedures that could be combined to studies aimed at verifying vector persistence and transduction levels, when feasible and acceptable. In the case of adverse events, vector integration studies are important in determining the potential causative role of vector integration as well as in detecting minimal residual disease after treatment.

2.4. What information is expected from integration studies?

Methods to retrieve vector integrations should be firstly aimed at providing a representative sampling of the engrafted population of transduced cells and identify any relevant aberration in its clonal composition (such as dominance, expansion or enrichment of clones carrying integrations within a

specific subset of at-risk genes). More in-depth integration site analysis may be designed to quantify the contributions of individual vector-containing clones. Techniques such as linear-amplification-mediated polymerase chain reaction (LAM-PCR), in combination with high-throughput sequencing, are able to detect from hundreds to thousands of integration sites from a single sample depending on the abundance (integrated vector copies per cell) and clonality (different cell clones marked by distinctive vector integrations) in the tested genomic DNA. However, current techniques have still important limitations in terms of sensitivity, accurate quantification and data interpretation. Large-scale analyses of vector integration sites have been widely carried out through the use restriction enzymes to fragment the DNA of interest that may produce biases in the representation of specific PCR products with respect to others or hamper the retrieval of some integration events. To reduce the bias associated to enzyme restriction and improve accuracy of quantification, novel nonrestrictive assays for insertion retrieval have been generated and their efficiency is currently under evaluation.

The most relevant information deriving from safety assessment of nonclinical and clinical studies concerns:

a) the presence of clusters of insertions *in vivo*, and

b) the relative contribution of each integrant to the pool of transduced cells.

a) Common insertion sites (CIS). Insertional mutagenesis studies in mice use oncoretroviruses (*34*), transposons (*35*) and, more recently, modified lentiviral vectors (*36, 37*) to deregulate cancer genes and trigger oncogenesis in vivo. Cancer cells arising from these screenings harbor vector insertions recurrently clustered in specific regions called common insertion sites (CIS) nearby the gene culprit of oncogenesis. Similarly, in leukemic/myelodysplastic cells found in the blood of patients subjected to gammaretroviral gene therapy harbored CIS nearby *LMO2, MECOM, CCND2* and other cancer genes. Therefore, in certain conditions, CIS appear to be the heralds of insertional mutagenesis also in humans. However, the interpretation of data deriving from CIS from nonclinical and clinical samples should be considered with caution since CIS may not necessarily be enriched as the result of oncogenic selection but rather by a biased vector integration profile towards specific chromosomal regions. Comparative studies between vector distribution *in vitro* at the time of transduction and after *in vivo* engraftment of vector-positive cells are required to distinguish between these possibilities (*22, 32, 38, 39*). CIS analyses provide relevant information on the overall genotoxic risk of a vector throughout the study population but may not be currently used to predict oncogenesis for any given individual within the study

b) Clonal quantification of gene corrected cells. Molecular techniques such as LAM-PCR can provide an estimate quantitative assessment of clonal contribution of each gene corrected cells based on the number of sequence reads belonging to each vector-genome junction. However, the measurement of the frequency of each clone in the analyzed pool by integration site sequence counts remains influenced by technical constraints. The clonal diversity of gene corrected cells in a patient sample was initially estimated on the basis of the appearance of LAM-PCR products on electrophoreses gel. The presence of few bands was considered representative of an oligoclonal repertoire, while a smear of many bands would indicate a great number of different integration sites in the analyzed sample that could be then marked as polyclonal. After the introduction of high-throughput sequencing,

the relative number of reads obtained for each integrant has become arelevant information. For example, the sequence counts of vector-genome junctions correlated well with the dynamics of the leukemic clones bearing LMO2 integrations in the high-throughput analysis of samples from one *SCID-X1* gene therapy trial in patients before and after chemotherapeutic treatment. In addition, it was useful in determining the relative abundance of a clone carrying an insertion in the HMGA2 locus in the β -thalassaemia patient treated. However, the LAM-PCR technique has specific technical biases linked to the use of restriction enzymes. In the absence of self-evident clonal expansions, discrepancies have been observed between insertion site frequency measured by sequencing and the results of specific quantitative PCR designed on the same vector-genome junctions. To avoid overestimation of clones in samples with low transduction or from rare cell populations, sequence counts of vector-genome junctions should also be taken into account for the frequency of transduced cells in the analyzed sample.

At current state, purely molecular data seem not to be sufficient; it is recommended to combine with data obtained through other assays (e.g., biological and immunological) and clinical observation. To follow up of patients at different time points after gene therapy, it is recommended to test the persistence and contribution of identical integration sites in a given lineage overtime.

The recent development of assays for insertion retrieval independent from restriction could reduce the factors that impair the quantification of integration sites. Once these and other new nonrestrictive platforms will be consolidated, they should provide the techniques of choice for future vector integration studies (*22*).

It is important that in these assays internal controls for the validation of the assays are implemented to assess the reproducibility, sensitivity and specificity of the methods (*40*). During validation, different sampling of cells at the same time point and repetition of deep sequencing should also be performed to assess the influences of the biases linked to sample preparation and sequencing procedures on resulting sequence reads. This assay could also help to estimate how close the assays are to cover the totality of integration sites present in each sample.

2.5 What is a desirable integration profile in patients treated with integrating-vector based GTMP?

The profile of a given vector reflects first the biological properties of the wild-type virus from which they have been derived. Gamma-retroviral vectors show a strong preference for promoter regions, lentiviral vectors integrate preferentially inside transcriptional units, whereas alpha-retroviruses may show a more random distribution. It should be considered that a proper comparison between *in vitro* and *in vivo* data from patients treated with genetically modified cells is essential to draw conclusions on vector integration profile in repopulating cells. In any case, integrating vectors are expected to hit all potentially dangerous loci in the human genome. In principle, a desirable integration profile in patients who received a GTMP should be highly polyclonal, although an oligoclonal pattern may occur following low transduction efficiency/engraftment. Mathematical models of hematopoiesis predict that oligoclonality could physiologically occur, particularly in the progenitor compartment, at longer follow-up in absence of any aberrant selection. Lack of persistent clonal expansion is considered a favourable factor, but a transient rise in clones may reflect different waves of stem cell activities or of immune

responses to pathogens.

2.6 Which strategies may reduce the risk deriving from an insertional event?

The choice of vector backbone influences the insertion profile, as seen comparing gamma-retroviral and lentiviral vectors, but also comparing different transposons with varied preferences for insertion (being the Sleeping Beauty the one that seems to have less bias in insertion preferences).
SIN gamma-retroviral and lentiviral constructs can reduce the probability of vector-mediated transactivation of neighboring genes by the elimination of the enhancer/promoter sequences from viral LTRs.
In the context of SIN vectors, the use of relatively weaker promoter/enhancer such as cellular regulatory elements is preferable to viral promoter/enhancers which have been described in preclinical models to substantially increase the risk of oncogenicity. This has to be considered in the context of the level of protein product required to achieve a sufficient therapeutic effect. Chromatin insulators are boundary elements that could prevent enhancer-promoter interactions, when placed between these elements, as well as protect transgene cassettes from silencing due to positional effects. Overall, it has been suggested that insulators could improve safety by blocking the interaction between an integrating vector and the target cell genome. However their efficacy could be limited in case of read-through transcription events generating aberrant fusion transcripts between vector and host genome and additional improvement in vector design could be required to deal with this issue. Removal of splicing acceptor/donor sites in the vectors should be considered to avoid aberrant splicing events with neighboring genomic regions upon integration. In spite of all the experience accumulated with these genetic elements, each viral vector with a particular choice of genetic elements should be carefully studied on its own right, as many factors and the different interactions between particular arrangements of these elements influence the overall genotoxic risk. Novel sequence-specific endonucleases (artificial zinc fingers nucleases, TALENs etc.) may provide a framework for gene transfer in predetermined sites (like AAVS1, CCR5) that can be used for site-specific gene correction or addition (*15, 41*). Such gene transfer in "safe harbors" or specific sites could significantly improve safety in therapeutic applications, but the potential risks associated with induction of DNA double strand breaks and repair as well as the genome-wide specificity of artificial endonucleases in target cells need to be investigated in depth.

2.7 Nonclinical models aimed at assessing the risk of insertional oncogenesis

Several nonclinical assays aiming at addressing safety issues of integrating vector-based GTMP have been developed (*24*).

The *in vitro* immortalization assay (IVIM), aimed at measuring the genotoxic potential of different vector constructs, is based on *in vitro* culture of murine bone marrow lineage depleted cells. The replating frequency of transduced cells is considered the parameter of transforming potential and is determined by limiting dilution cloning and normalised per vector copy number. The sensitivity of the technique is 1-2 mutants/10^5 cells using a positive control vector with a strong viral LTR. In general, a weaker enhancer in the vector tends to reduce the fitness of the insertional mutants, possibly by lowering proto-oncogene upregulation levels. Limitations of this approach are that it only readouts few

types of genotoxic events at selected loci, is exclusively based on the use of murine cells and myeloid lineages and the culture conditions impacts on its reproducibility.

In vivo assays for tumorigenicity should be able to detect oncogenic events caused by integrating vector. Transplant of genetically modified hematopoietic progenitor or direct injection approaches of cells provide a potentially useful model. The use of wild-type mice as recipient of HSC transduced with the vector of interest has been explored as a common platform for different vectors and transgenes. However, the occurrence of leukemia/lymphoma has been reported to be low even with vectors known to be associated with development of leukemias in clinical studies, and easily confounded by the spontaneous rate of leukemia/lymphomagenesis observed with long-term follow-up of most mouse strains. Thus, the number of mice required to obtain predictive risk calculations would be very large and the studies require long times. Serial transplantations have been proposed to increase clonal selection in wild-type mice, but this could artificially stress hematopoiesis drifting the study from the actual gene therapy protocol. The evaluation of clonal skewing of integrants near genes involved in cell growth, proliferation and survival has been proposed as a surrogate marker of leukemogenesis, but its significance for determining the oncogenetic risk within a clinical trial is currently to be determined.

Genotoxicity studies performed in disease animal models are potentially more relevant since they offer the advantage of combining analyses of vector integration with the transgene effect and general disease background. In some cases, even assays on mice reproducing the disease environment were not predictive of vector driven tumorigenicity, as in preclinical studies for *SCID-X1* gene therapy. Additionally, experiments on disease mouse models share some of the above reported limitations with genotoxicity studies on normal mice.

Another option is represented by the use of tumor-prone mouse models carrying a background potentially pre-sensitized to the oncogenic activation by vector integration. These mice are suitable for both *in vivo* and *ex vivo* tumorigenicity studies and should allow a faster and significantly more consistent assessment of genotoxicity compared with normal mice or disease models. Indeed, in the context of tumor-prone Cdkn2a-/- mice, SIN LTR and internal moderate promoter were shown to be superior in terms of safety with respect to other vector design. There are however some caveats linked to the use of these models (*24*). First, their inherent oncogenesis could mask the residual vector-driven genotoxicity associated to novel vector designs with a low tumorigenic potential. Additionally, the genetic and phenotypic background of this model is different from the one belonging to the disease for which the vector is designed. For example, these mice may differ from immunodeficient animal models in their ability to counteract vector-driven tumorigenesis through immune response. Crossing tumor-prone and disease mice could provide a further option for tumorigenicity studies combining acceleration of vector-driven oncogenesis with the infusion of transduced cells in the disease environment of interest. However, the interaction between the two genetic backgrounds is a long process and could generate a mouse strain with peculiar features that may hamper their use in gene therapy nonclinical studies.

Finally, differences in the genome, background and mechanisms of tumorigenicity of the various animal models should be considered when studying vector integrations in these models.

2.8 Clinical aspects

At present the available clinical and experimental data are limited to make meaningful prediction of the risk of insertional mutagenesis and oncogenesis. Employing the many available scientific tools as described above can help to make a risk assessment and ensure adequate risk-management and mitigation measures to be applied in clinical development of gene therapy. The reported cases indicate that it can take up to several years for tumors to develop. A long latency to tumor development also suggests a multistep progression to oncogenesis and highlights the fact that most genotoxic vector insertions actually predispose rather than directly cause tumor development.

Plans for long-term follow-up should be foreseen for all patients who receive vector-based gene therapy. Depending on the level of risk identified, the length of follow-up, frequency and intensity of clinical evaluation and associated laboratory investigations need to be planned both for clinical trials and at the postmarketing level. Investigators and applicants for MAA should be aware that the risk of oncogenesis, potential or real, will be weighed against the observed/perceived benefit attributable to the therapy. A decision on marketing authorization (and likely clinical trial authorisation, which is a national competent authority decision), will take into account the overall benefit-risk profile of the product for the sought indication.

3. Conclusions

Integrating vectors represent a promising tool for *ex vivo* or *in vivo* gene addition/correction for the treatment of inherited and acquired disorders. Risks associated to insertional genotoxicity should always be carefully taken into account and considered during the development of a gene therapy product. The overall evaluation should include factors which might favour the transition from a mutagenic to an oncogenic event, such as the vector insertion profile, the presence of elements favouring insertional activation or inactivation, the biology of the transgene, the specific target cell/tissue, and other predisposing factors should be taken in account.

Vector integration and clonality assays are important tools in the comprehensive safety evaluation of a product when performed in the context of nonclinical and clinical studies. The limitations of such studies should be considered in data interpretation until the assays are further refined and validated. The initial oncogenic events in clinical trials have prompted a rapid evolution of the technological platform, as novel vectors with potential safer profile have entered clinical applications and additional new approaches to mitigate the risk are currently being investigated.

In case of appearance, accurate follow-up of expanded clones in patients is necessary to exclude the generation of potential clonal aberrancies and malignancies.

As the experimental technology available to evaluate the risk of insertional mutagenesis/ oncogenesis is in evolution, it is not possible to provide a comprehensive list of investigations on which to base the clinical risk assessment. Scientific advice from regulatory authorities might be needed to take account of further scientific developments.

4. References

1. L. Naldini, Ex vivo gene transfer and correction for cell-based therapies. Nat Rev Genet 12, 301 (2011).
2. J. J. Rossi, C. H. June, D. B. Kohn, Genetic therapies against HIV. Nat Biotechnol 25, 1444 (Dec, 2007).
3. A. Aiuti *et al.*, Gene therapy for immunodeficiency due to adenosine deaminase deficiency. *N Engl J Med* **360**, 447 (2009).
4. S. Hacein-Bey-Abina *et al.*, Efficacy of gene therapy for X-linked severe combined immunodeficiency. *N Engl J Med* **363**, 355 (2010).
5. H. B. Gaspar *et al.*, Hematopoietic stem cell gene therapy for adenosine deaminase-deficient severe combined immunodeficiency leads to long-term immunological recovery and metabolic correction. *Sci Transl Med* **3**, 97ra80 (2011).
6. K. Boztug *et al.*, Stem-cell gene therapy for the Wiskott-Aldrich syndrome. *N.Engl. J. Med.* **363**, 1918 (2010).
7. F. Candotti *et al.*, Gene therapy for adenosine deaminase-deficient severe combined immune deficiency: clinical comparison of retroviral vectors and treatment plans. *Blood* **120**, 3635 (Nov 1, 2012).
8. M. G. Ott *et al.*, Correction of X-linked chronic granulomatous disease by gene therapy, augmented by insertional activation of MDS1-EVI1, PRDM16 or SETBP1. *Nat Med* **12**, 401 (Apr, 2006).
9. N. Cartier *et al.*, Hematopoietic stem cell gene therapy with a lentiviral vector in X-linked adrenoleukodystrophy. *Science* **326**, 818 (2009).
10. M. Cavazzana-Calvo *et al.*, Transfusion independence and HMGA2 activation after gene therapy of human β-thalassaemia. *Nature* **467**, 318 (2010).
11. F. Mavilio *et al.*, Correction of junctional epidermolysis bullosa by transplantation of genetically modified epidermal stem cells. *Nat Med* **12**, 1397 (Dec, 2006).
12. F. Ciceri *et al.*, Infusion of suicide-gene-engineered donor lymphocytes after family haploidentical haemopoietic stem-cell transplantation for leukaemia (the TK007 trial): a non-randomised phase I-II study. *Lancet Oncol* **10**, 489 (May, 2009).
13. K. S. Balaggan, R. R. Ali, Ocular gene delivery using lentiviral vectors. *Gene Ther* **19**, 145 (Feb, 2012).
14. H. M. Guy, L. McCloskey, G. Lye, K. Mitrophanous, T. Mukhopadhyay, Characterization of Lentiviral Vector Production using Microwell Suspension Cultures of HEK293T-derived Producer Cells. *Hum Gene Ther Methods*, (Mar 6, 2013).
15. A. Lombardo *et al.*, Site-specific integration and tailoring of cassette design for sustainable gene transfer. *Nat Methods* **8**, 861 (2011).
16. R. Gabriel *et al.*, An unbiased genome-wide analysis of zinc-finger nuclease specificity. *Nat Biotechnol* **29**, 816 (Sep, 2011).
17. H. B. Gaspar *et al.*, Long-term persistence of a polyclonal T cell repertoire after gene therapy for X-linked severe combined immunodeficiency. *Sci Transl Med* **3**, 97ra79 (2011).
18. L. W. Seymour, A. J. Thrasher, Gene therapy matures in the clinic. *Nat Biotechnol* **30**, 588 (Jul, 2012).
19. M. Witzel et al, Hematopoietic stem cell gene therapy for Wiskott Aldrich Syndrome (abstract). *J Clin Immunol* **32**, 9 (2012).
20. S. Stein *et al.*, Genomic instability and myelodysplasia with monosomy 7 consequent to EVI1 activation after gene therapy for chronic granulomatous disease. *Nat Med* **16**, 198 (2010).
21. A. Aiuti, R. Bacchetta, R. Seger, A. Villa, M. Cavazzana-Calvo, Gene therapy for primary immunodeficiencies: Part 2. *Curr Opin Immunol* **24**, 585 (2012).
22. L. Biasco, C. Baricordi, A. Aiuti, Retroviral Integrations in Gene Therapy Trials. *Mol Ther* **20**, 709 (2012).
23. A. G. Uren, J. Kool, A. Berns, M. van Lohuizen, Retroviral insertional mutagenesis: past, present and future. *Oncogene* **24**, 7656 (Nov 21, 2005).
24. J. Corrigan-Curay *et al.*, Challenges in vector and trial design using retroviral vectors for long-term gene correction in hematopoietic stem cell gene therapy. *Mol Ther* **20**, 1084 (2012).

25. K. Ikeda, P. J. Mason, M. Bessler, 3'UTR-truncated Hmga2 cDNA causes MPN-like hematopoiesis by conferring a clonal growth advantage at the level of HSC in mice. *Blood* **117**, 5860 (Jun 2, 2011).

26. D. Cesana *et al.*, Whole transcriptome characterization of aberrant splicing events induced by lentiviral vector integrations. *The Journal of clinical investigation* **122**, 1667 (May 1, 2012).

27. A. Moiani *et al.*, Lentiviral vector integration in the human genome induces alternative splicing and generates aberrant transcripts. *The Journal of clinical investigation* **122**, 1653 (May 1, 2012).

28. D. Heckl *et al.*, Lentiviral vector induced insertional haploinsufficiency of Ebf1 causes murine leukemia. *Molecular therapy : the journal of the American Society of Gene Therapy* **20**, 1187 (Jun, 2012).

29. S. J. Howe *et al.*, Insertional mutagenesis combined with acquired somatic mutations causes leukemogenesis following gene therapy of SCID-X1 patients. *J Clin Invest* **118**, 3143 (2008).

30. D. Zychlinski *et al.*, Physiological promoters reduce the genotoxic risk of integrating gene vectors. *Molecular therapy : the journal of the American Society of Gene Therapy* **16**, 718 (Apr, 2008).

31. E. Montini *et al.*, Hematopoietic stem cell gene transfer in a tumor-prone mouse model uncovers low genotoxicity of lentiviral vector integration. *Nat Biotechnol* **24**, 687 (2006).

32. L. Biasco *et al.*, Integration profile of retroviral vector in gene therapy treated patients is cell-specific according to gene expression and chromatin conformation of target cell. *EMBO Mol Med* **3**, 89 (2011).

33. M. Themis *et al.*, Oncogenesis following delivery of a nonprimate lentiviral gene therapy vector to fetal and neonatal mice. *Molecular therapy : the journal of the American Society of Gene Therapy* **12**, 763 (Oct, 2005).

34. J. Kool, A. Berns, High-throughput insertional mutagenesis screens in mice to identify oncogenic networks. *Nat Rev Cancer* **9**, 389 (Jun, 2009).

35. N. G. Copeland, N. A. Jenkins, Harnessing transposons for cancer gene discovery. *Nat Rev Cancer* **10**, 696 (Oct, 2010).

36. M. Ranzani *et al.*, Lentiviral vector-based insertional mutagenesis identifies genes associated with liver cancer. *Nat Methods* **10**, 155 (Feb, 2013).

37. E. Montini *et al.*, The genotoxic potential of retroviral vectors is strongly modulated by vector design and integration site selection in a mouse model of HSC gene therapy. *The Journal of clinical investigation* **119**, 964 (Apr, 2009).

38. A. Biffi *et al.*, Lentiviral vector common integration sites in preclinical models and a clinical trial reflect a benign integration bias and not oncogenic selection. *Blood* **117**, 5332 (May 19, 2011).

39. A. P. Presson, N. Kim, Y. Xiaofei, I. S. Chen, S. Kim, Methodology and software to detect viral integration site hot-spots. *BMC Bioinformatics* **12**, 367 (2011).

40. C. Baum, U. Modlich, G. Gohring, B. Schlegelberger, Concise review: managing genotoxicity in the therapeutic modification of stem cells. *Stem Cells* **29**, 1479 (Oct, 2011).

41. C. Mussolino, T. Cathomen, RNA guides genome engineering. *Nat Biotechnol* **31**, 208 (Mar, 2013).