Concise Review: Lessons Learned From Clinical Trials of Gene Therapy in Monogenic Immunodeficiency Diseases

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ABSTRACT

Thirty years ago, retroviral transfer of genetic material into hematopoietic stem and progenitor cells (HSC/Ps) led to predictions that this technology would transform modern medicine [Nature 1983;305:556–558; Nature 1984;310:476–480]. Studies in several immunodeficiency diseases in the past 15 years have demonstrated clear proof of principle that gene therapy can have long-lasting, potentially curative effects without the need to search for allogeneic donors and without risk of graft-versus-host disease. Improvement in gene transfer efficiency for target HSC/Ps brought to light issues of insertional mutagenesis caused by transfer vectors, resulting in oncogene transactivation and leukemias. Lessons from these adverse events have now led to a new generation of vectors, refinements in conditioning regimens, and manufacturing, which are paving the way for expanded applications of the current technology and recent emphasis on gene targeting/genome editing as the next advancements in the field. Stem Cells Translational Medicine 2014;3:636–642

XPÉRIENCE IN IMMUNOLOGICAL CLINICAL TRIALS TO DATE

X-Linked Severe Combined Immunodeficiency

Severe combined immunodeficiency (SCID) comprises a number of rare monogenic diseases with the common feature of a block in T-cell differentiation and impaired B-cell and natural killer (NK) cell immunity [1]. The most common variant of SCID results from the deficiency in expression of the common cytokine receptor γ-chain, which is shared by the receptors for interleukin (IL)-2, IL-4, IL-7, IL-9, IL-15, and IL-21. This condition is inherited in a sex-linked fashion (X-linked SCID or SCID-X1) and is characterized by abnormal development or function of T, B, and natural killer (NK) cells, although B cells are usually present in humans (so-called T-minus, B-plus SCID). Survival depends on the reconstitution of T-cell development and function by allogeneic bone marrow transplantation [2].

Two independent gene therapy trials, aimed at correcting the immunologic defect of SCID-X1 infants who lack a genotypically matched bone marrow donor, have been reported [3, 4], and another has recently been opened for accrual. To date, a total of 20 patients have been treated in the reported studies. Despite minor technical differences in the two protocols, the basic design of both previous gene therapy trials is quite similar: the complete coding region of the human γ-chain was incorporated into a first-generation γ-retroviral vector regulated by the murine leukemia virus (MLV) long terminal repeat (LTR) sequence. This vector was used to transduce bone marrow-derived CD34+ cells in vitro, and cells were subsequently infused without prior conditioning or cytoxic treatment. Results in both trials have been extremely encouraging, with a current disease-free survival of approximately 85% and more than 10-year follow-up for many patients [5, 6]. The majority of patients achieved rapid normalization of T-cell numbers and have maintained a highly diverse functional T-cell repertoire long-term, suggesting that there is ongoing thymic activity. Protective levels of antibodies, including antibody production after immunization, were achieved in some patients, and the prophylactic administration of intravenous immune globulin could be discontinued in approximately one half of the cohort. The suboptimal reconstitution of humoral immunity is in fact not unexpected as the intrinsically dysfunctional B-cell compartment is not corrected in the absence of myelosuppressive conditioning and sustained engraftment of transgenic hematopoietic stem cells (HSCs). Attempts to treat older patients with atypical presentation, or following failed allogeneic procedures, have not been so successful, most likely because of irreversible decline in thymic function over time [7, 8].

However, serious adverse events related to gene therapy occurring 31–68 months post-gene...
therapy were reported in a total of five patients were reported in a total of five patients [9–12]. Untoward effects of viral integration into the genome resulted in T-cell leukemia, leading to the death of one of the affected patients. Although there has been considerable speculation on a mechanistic role of the transgene during leukemogenesis, this has not been experimentally proven [13]. Indeed, the occurrence of similar mutagenesis in several other trials using LTR-intact gammaretroviral vectors points to a dominant role for mutagenesis rather than aberrant yc-mediated signaling. Proviral integration in the proximity of proto-oncogenes, particularly the LIM domain only 2 (LMO2) promoter, was identified in four of five patients and resulted in significant dysregulation of gene expression at this locus [10, 11]. LMO2 is a master regulator of human hematopoiesis, involved in stem cell growth, and is not normally expressed in T cells. However, LMO2 activation has previously been implicated in some cases of de novo human T-cell acute lymphoblastic leukemia (T-ALL). In addition, LMO2 transgenic mice have been shown to develop T-ALL within 10 months [14]. It is increasingly clear that retroviral vectors may turn on cellular proto-oncogenes adjacent to their integration site in the genome (as described in Lessons From Insertional Mutagenesis). The strong enhancer activity of the retroviral LTR element shows particular propensity to the upregulation of genes neighboring the integration site [15–17]. Interestingly, in the SCID-X1 trial, a skewing of vector integration site distribution in vivo was noted. Compared with retroviral integration sites (RIS) recovered from transduced CD34+ cells, RIS recovered from T cells in vivo 9–30 months after transplantation showed significant skewing suggestive of a selection of clones as a result of viral integration in certain growth- and survival-promoting genes [18]. In all patients there was a remarkable accumulation of other contributory genetic lesions unrelated to proviral location, presumably occurring during thymopoiesis after engraftment, and promoted by an initial proto-oncogenic insult. The exact mechanisms leading to development of frank leukemia in SCID-X1 are therefore not fully defined.

In summary, to date, 5 of 20 patients treated with gene therapy for SCID-X1 have encountered a live-threatening severe adverse event, thought to be triggered by retroviral activation of LMO2 in 4 patients. Although one patient succumbed to the disease following an unsuccessful allogeneic bone marrow transplantation, the other four patients were successfully treated and recovered good levels of functional immunity, suggesting that thymopoiesis is sustained long-term in these patients. The continued development of safety-enhanced vectors and the validation of these vectors in clinically relevant systems have emerged as a major priority in the field, and a collaborative international trial conceived through the Transatlantic Gene Therapy Consortium has recently been opened using a gammaretrovirus that is deleted of LTR enhancer elements and in which transgene expression is mediated by a cellular promoter. Early clinical data suggest that efficacy is maintained, and that, although follow-up is short, there is evidence for beneficial effects in terms of the lack of insertion site clustering at loci such as LMO2 [19].

Adenosine Deaminase Deficiency

Adenosine deaminase (ADA) is a housekeeping enzyme of the purine metabolic pathway, expressed in all tissues of the body [20]. Deficiency of this enzyme leads to a build-up of toxic metabolites with detrimental systemic effects, including neurodevelopmental deficiencies, sensorineural deafness, and skeletal abnormalities. Importantly, ADA deficiency results in the accumulation of metabolites that causes abnormal T-, B-, and NK-cell development, resulting in the SCID phenotype. As is the case with the more common SCID-X1, untreated patients generally succumb to severe opportunistic infections in the first year of life. Treatment strategies used to manage affected patients include allogeneic hematopoietic stem cell transplantation (HSCT), enzyme replacement therapy (which is imperfect), and more recently gene therapy [21, 22]. A number of gene therapy trials for ADA deficiency were initiated in the early 1990s, utilizing retroviral gene transfer into various cell types, including peripheral blood lymphocytes, umbilical cord blood, and bone marrow [23–26]. These early studies failed to produce clear efficacy. By contrast, more recent studies introduced key modifications to the gene therapy protocol, including the use of a reduced intensity myelosuppressive conditioning regimen and the withdrawal of concurrent pegylated (PEG)-ADA replacement [27, 28]. The Milan-based group of Aiuti and colleagues has reported on their initial experience with 10 children [29, 30]. Patients were conditioned with 4 mg/kg busulfan before the infusion of transduced cells. The choice of this alkylation agent to create space for engrafting cells was largely based on wide clinical experience in the setting of allogeneic HSCT. At this dosage level, the risk of toxicity (potentially arising from long-lasting myelosuppression and late effects on fertility, growth, or endocrine function) was likely to be very low. Clinical response has been robust and sustained in the majority of patients with normalization of T-cell and B-cell function [29, 30]. Similar trials have been initiated at other centers worldwide using variations on LTR-intact gammaretroviral vectors and low-intensity conditioning regimens. At the time of writing, groups in Milan, London, and Los Angeles have treated more than 40 patients with a remarkable overall survival rate of 100% and disease-free survival (without the need for allogeneic HSCT or recommencement of PEG-ADA therapy) of approximately 75% [31, 32].

Strikingly, no adverse events have been reported in any patient with ADA-SCID treated with LTR-intact gammaretroviral vectors despite a similar RIS pattern observed in this patient group compared with the previously mentioned SCID-X1 trials [29]. Aiuti et al. [29] published a comprehensive genome-wide analysis of retroviral integration sites (RIS) of five patients treated in Milan. This paper analyzed the RIS patterns in CD34+ cells before infusion as well as RIS in vivo, up to 47 months post-gene therapy. As anticipated, a nonrandom proviral integration pattern, favoring transcription start sites (TSS) and gene-dense regions, was observed in the pretransplant cells. RIS observed in vivo in T cells were additionally enriched for TSS, suggesting the occurrence of in vivo selection. More recently, Aiuti and colleagues [33] have demonstrated that cellular genes in the proximity of the proviral integration site are subject to moderate dysregulation in gene-modified T-cell clones isolated from patients. However, in contrast to the SCID-X1 trial, no in vivo skewing toward RIS in genes affecting survival, cell cycling, signal transduction, or proliferation was observed, making a clonal dominance effect appear less likely. Interestingly, in the London trial in which the LTR was derived from the spleen focus-forming virus, there was significant clustering at the myelodysplastic syndrome-ecotropic virus integration site-1 (MDS-EVI1) locus, a proto-oncogene implicated in the development of myelodysplasia in the human chronic granulomatous disease gene therapy trial (as described in Chronic Granulomatous Disease), but again no clonal dominance demonstrated
stimulating factor and collected. Gene transfer was performed using gene-modified cells. However, 2 years later, low-dose busulfan in Frankfurt, Germany, initiated a gene therapy trial of X-CGD [39]. Cells remained low [38]. In 2002, the group of Grez and colleagues for previous clinical gene therapy trials conducted without myeloablative conditioning for those without particularly good donor matches. In addition, the application of tailored dosing of conditioning agents such as busulfan for individual patients has markedly improved survival, the development of a gene therapy approach that uses autologous HSCs provides an important therapeutic advance for this patient group and for those without particularly good donor matches. In clinical previous gene therapy trials conducted without myeloablative conditioning, the engraftment level of gene-modified cells remained low [38]. In 2002, the group of Grez and colleagues in Frankfurt, Germany, initiated a gene therapy trial of X-CGD [39]. The initial patients received a mild immunosuppressive preparative regimen and failed to engraft significant numbers of gene-modified cells. However, 2 years later, low-dose busulfan—modeled on the successful gene therapy trial for ADA-SCID [27]—was incorporated into the preparative regimen, and two additional patients were treated [39]. This group of patients was followed with unprecedented sophistication by the prospective monitoring of integration sites that mark each hematopoietic cell before transplantation and then allow the tracking of these cells in vivo [39]. In the initial two patients, autologous peripheral blood CD34+ cells were mobilized with granulocyte colony-stimulating factor and collected. Gene transfer was performed using a gammaretroviral vector SF71gp91^pox containing the spleen focus-forming virus long terminal repeat (LTR) elements (in contrast to the Moloney murine leukemia virus LTR in the two SCID trials), which was chosen for its ability to achieve high expression levels in transduced hematopoietic stem cells [40]. Initial engraftment rates detected in the peripheral blood were 12%–13%, and significant improvement in the previously refractory infections was noted 50–60 days after therapy. Surprisingly, given that transduced cells were not expected to exhibit a natural growth or survival advantage, a gradual increase in the number of functional gene-corrected cells, up to 50%–60% of all peripheral blood cells, was observed, starting at approximately day 150 post-transplant. These events were accompanied by a selective outgrowth of progenitors carrying vector insertions that activated one of three oncogenes, PRDM16, SETBP1, and most notably MDS-EVI1. Although all three genes are well-known cancer-associated genes, most clonal outgrowths were exhausted after a few months, with the exception of those containing MDS-EVI1, which increased to high levels in both patients approximately 1 year post-cell infusion. Of note, the dominant MDS-EVI1 clones initially did not transgress the boundaries of the normal myeloid pool, as these cells remained cytokine-dependent in vitro and failed to engraft in immunocompromised mice [39].

More recent follow-up on these two study patients has been provided [41, 42]. Indeed, whereas gene marking remained high in both patients, downregulation of transgene expression was noted as a result of CpG methylation in the viral promoter. Interestingly, this silencing spared the powerful duplicated enhancer elements. As a consequence, gp91^pox expression was suppressed, but the capacity of the LTR-encoded enhancers to transactivate nearby genes remained intact [43]. Thus, the molecular events led to a loss of therapeutic efficacy with continued presence of insertional transactivation of potentially oncogenic genes. One patient died 2.5 years post-therapy of severe sepsis associated with myelodysplasia [44]. The second patient developed monosomy 7 and myelodysplastic syndrome (MDS), a pre-leukemia condition associated with peripheral cytopenias and dysplastic changes in bone marrow elements, and died following an unsuccessful unrelated donor bone marrow transplantation [41]. Other patients treated on the trial have subsequently developed similar complications but have undergone successful HSCT (M. Grez, personal communication). Of note, the ecotropic virus integration site-1 (EVI1) locus has previously been identified as a common target of retroviral oncogenesis [15, 45]. Dysregulated expression of Evi1 (which is not normally detected in hematopoietic cells) has also been associated with myeloid leukemia and MDS in both humans and mice [46–48]. Together these findings suggest that dysregulated expression is associated with genomic instability and the acquisition of additional somatic mutations that drive a preleukemic and eventually leukemic process. Despite these adverse molecular events, the infusion of gene-corrected CD34+ cells was highly effective with regard to clearing refractory pyogenic infections in these patients [39], demonstrating proof of principle that gene therapy can be used to treat active disease, even if only as a bridge to definitive therapy such as HSCT. Even so, the development of mutagenesis and silencing of transgene expression have provided a significant impetus to develop more sophisticated and safer vector systems in CGD, for example, through use of lineage-specific regulatory elements. The question of optimal conditioning for these patients also remains unclear as significant myeloablation will be required to achieve levels of chimerism of gene-modified cells sufficient to correct the disease phenotype. Currently, these regimens are based on alkylating agents such as busulfan, which have associated toxicities, although stem cell-targeted serotherapy could eventually provide a safer alternative.

Wiskott-Aldrich Syndrome

Wiskott-Aldrich syndrome (WAS) is an X-linked immunodeficiency that is caused by inactivating mutations in the WAS protein (WASP). WASP plays a regulatory role in cell signaling and cytoskeletal reorganization in hematopoietic cells [49]. The classical form of the disease is fatal and is characterized by immunodeficiency, thrombocytopenia, elevated frequency of tumor formation,
eczema, and autoimmunity [50]. The only currently available curative therapy for WAS is HSCT, but, with the other primary immunodeficiencies, the availability of suitably matched donors is limiting [51]. A clinical trial for the genetic correction of WAS via LTR-intact gammaretroviral delivery of the WASP cDNA into autologous CD34+ cells was recently reported and eventually recruited 10 patients [52]. A combination of a relatively high cell dose and transduction efficiency led to gene marking across both myeloid and lymphoid lineages. A marked clinical benefit from gene therapy has been reported in several of the patients. However, this has been associated with high levels of mutagenesis and leukemia associated with dysregulation of LMO2 in the majority of patients [53]. Furthermore, some patients have developed secondary myeloid malignancy as a result of insertions in the MDS-Evi1 locus (C. Klein, personal communication). At the same time, other investigators have pursued the development of strategies to reduce potential toxicity by using a third-generation lentivirus vector to effect gene transfer into hematopoietic stem and progenitor cells (HSC/Ps). Expression of the WAS cDNA was controlled by cis-regulatory elements of the WAS gene in an attempt to restrict transgene expression to the target cells required for phenotypic correction [54, 55]. Clinical studies with this vector are underway in several centers worldwide. The initial findings from one center have recently been reported [56]. Three patients received infusion of autologous genetically modified HSC/Ps after reduced intensity busulfan-based conditioning to enhance engraftment of these cells. This treatment resulted in improved (but not normal) platelet counts with increased platelet size and reduced bleeding, allowing the patients to become platelet transfusion-independent. Moreover, improved immune function and reduced autoimmune symptoms were observed. Similar clinical effects have been reported from other centers (M. Cavazzano-Calvo, personal communication and our own unpublished data).

**LESSONS FROM INSERTIONAL MUTAGENESIS**

From an early stage in the development of retroviral vectors for gene therapy applications, there has been a concern that recombinant vectors could elicit cellular transformation by altering expression of either cellular proto-oncogenes or tumor suppressor genes that are proximal to the genomic integration site. This phenomenon, referred to as insertional mutagenesis, was characterized as a property of wild-type retroviruses, all of which are characterized by insertion into the chromosomal DNA of infected cells [57]. Having greatly reduced the likelihood that retroviral gene therapy vectors could generate replication-competent virus, the risk of a recombinant vector being able to transform a cell via insertional mutagenesis was initially perceived by many investigators to be very low [58]. The lack of efficacy in preclinical models using human hematopoietic stem and progenitor (CD34+) cells also somewhat shifted emphasis further away from the risks of insertional mutagenesis. However, soon after the publication detailing the efficacy of the Paris-based SCID-X1 trial, work emerged from the group of Baum and colleagues [15] that would re-establish the importance of insertional mutagenesis as a significant risk factor in the retroviral-mediated genetic correction of hematopoietic cells, and that ultimately predicted the outcome of leukemia also in the CGD and WAS gene therapy trials (see above). Baum demonstrated for the first time that a replication-incompetent retroviral vector backbone designed for human gene therapy applications could cause cellular transformation via insertional mutagenesis in the context of a transplant model of transduced murine hematopoietic stem cells [15]. In this and a subsequent study, it was found that a single retroviral insertion in the vicinity of the Evi1 gene or the related Prmd16 gene resulted in their overexpression most likely because of the influence of the long terminal repeat viral enhancer elements and was sufficient to initiate a cascade of events resulting in leukemic transformation in vivo [15, 59]. Furthermore, a high copy number infection of murine bone marrow with recombinant retroviral vectors was able to facilitate combinatorial hits that caused leukemogenesis [60]. The pattern of cellular genes that combine to promote cellular transformation demonstrated a significant overlap with those that are dysregulated in experiments, which used replication-competent retrovirus vectors to provoke the development of leukemia [60]. Although murine hematopoietic stem cells most likely represent a more readily transformed target than their human counterparts, these studies formally established the mutagenic potential of recombinant retroviral vectors intended for gene therapy applications. Baum’s group [17] then made the seminal observation that, at low copy number, retroviral-transduced murine hematopoietic stem cells are selectively expanded during transplant dependent upon proviral insertion site. These nonmalignant dominant clones are enriched for proviral integration sites in the locale of genes encoding signal transduction molecules and growth-promoting genes [17, 61]. Analysis of mRNA expression levels in these clones revealed that the proviral insertion did indeed alter transcriptional regulation of genes proximal to the integration site and led to the hypothesis that this was a powerful method to identify proengraftment genes through positive selection. These observations were found to have direct translational relevance in the gene therapy trial for CGD, in which, as noted above, the initial nonmalignant expansion of dominant retroviral transduced clones in two patients was found to correlate with insertional upregulation of growth-promoting genes [39]. Subsequent experience with γ-retroviruses used in the clinical trial for WAS confirmed that insertional leukemogenesis in the SCID-X1 trial was not a disease-specific side effect, although the absence of clinically manifesting mutagenesis in trials for ADA-SCID indicates that disease-specific factors can play an influential role. To date, leukemias associated with replication-incompetent retrovirus vectors are associated with insertional activation of known proto-oncogenes by viral promoter/enhancer sequences (reviewed in [62]).

Wild-type and recombinant retroviral vectors (including α, γ, spuma, and lenti) integrate into the host genome in a semirandom manner and demonstrate insertion site biases that are dependent upon the accessibility of the insertion site in the target cell and variations in the viral integrase enzyme that depend upon retroviral genus [63–66]. This is likely to have a significant impact on mutagenic potential. For example, gammaretroviral vectors have been shown to integrate in or near a number of proto-oncogenes that are actively expressed in human CD34+ cells. When human CD34+ cells were transduced with retroviral vectors ex vivo, 21% of retroviral integrations occurred at recurrent insertion sites (“hot spots”), which were highly enriched for proto-oncogenes and growth-controlling genes [67]. Gammaretroviruses such as MLV have been shown to exert a clear preference for integration in the region immediately surrounding the TSS of actively transcribed genes [65, 68–70]. This has been borne out in clinical studies in which there is a greater than random frequency of
vector integrations near the TSS of genes that are active in hematopoietic stem cells [18, 29, 71]. Whereas lentiviral vectors also demonstrate a preference to integrate within the loci of actively transcribed genes, their integration profile favors sites that are downstream of the TSS within the body of the primary transcript [65, 67, 68, 72–75]. Using viral chimeras, it has been shown that incorporation of MLV integrase into a HIV-1-based vector alters the integration pattern of the lentivirus to more closely resemble that associated with a gammaretroviral vector [76]. It is now known that viral integrase proteins use cellular cofactors to assist the integration process. For example, lens epithelium-derived growth factor targets lentiviral integration toward transcription units, whereas binding of the MLV integrase with bromo- and extraterminal domain chromatin regulators favors integration at TSS and regulatory regions [77–79]. This work clearly demonstrates that gammaretroviral and lentiviral vectors have developed distinct mechanisms of integrase-dependent integration that may have an impact upon the mutagenic potential of recombinant retroviral vectors but also offers the potential to redesign vectors in ways that favor neutral or even targeted integration patterns [80]. To date, it remains unclear whether the differences in integration site preferences that reach statistical differences in model systems have either biological or translational relevance because these differences are relatively small and enhancers exert effects at very great distances, potentially reducing biological impact. As recently pointed out, direct comparisons in human studies have not been previously done but are now underway [81].

If one considers the possibility of integrating vectors upregulating oncogene expression via either read-through transcription or enhancer effects on the endogenous promoter, then gammaretroviral vectors could be considered as potentially more mutagenic than lentiviral vectors in this context, because of their preference for integration near the TSS. Conversely, preferential integration within the body of the primary transcript may result in lentiviral vectors having a higher probability of interrupting, for example, tumor suppressor gene expression or, as noted in the treatment of one patient with thalassemia, in altering normal gene splicing [82, 83]. Progress has been made in the development of model systems to functionally evaluate the relative mutagenic potential of different vector systems. However, the model systems developed to date have a clear preference to detect mutagenesis mediated via upregulation of oncogene transcription. It is not clear whether this is a reflection of tumor suppressor gene inactivation being consequential as a mechanism of insertional mutagenesis, or is a result of bias within the model system. As noted above, other related retroviral vector systems have also been shown to have an integration pattern that is distinct from gammaretroviral vectors and as such may represent a safer vector configuration. Recombinant foamy virus vectors do not preferentially integrate within genes, and their integration pattern does not significantly correlate with actively transcribed genes [84]. Likewise, avian sarcoma leukemia virus vectors do not favor gene-rich regions or TSS as preferred integration sites [85]. However, these novel vector systems have not been as well-characterized as gammaretroviral or lentiviral vectors with regard to safety and efficacy, and have not yet been translated to clinical use. Overall, it seems that the integration profile of different vectors may play an important role in the mechanism of mutagenesis and may also be manipulatable. However, in terms of reduction of risk, the adoption of safer vector configurations through elimination of potent enhancer sequences such as those found in gammaretroviral LTRs may prove to be the most important technological advance in the setting of any semirandom integration process [86, 87]. Of course, this also leaves some challenges with respect to design of alternative regulatory elements that achieve sufficient gene expression yet retain safety.

**CONCLUSION**

The last decade has seen the field of gene therapy for hematopoietic diseases take huge strides. Several clinical trials have demonstrated clear proof of principle that this treatment modality can have long-lasting beneficial clinical effects in patients with a variety of different genetic conditions. However, the improvements in efficiency that were required to achieve this goal have revealed toxicities arising from insertional mutagenesis primarily because of technological limitations in terms of vector design. Lessons learned have led to refinement of vector configuration and the implementation of new trials that promise to extend the applicability of gene therapy to many other patients and a wider range of inherited and acquired conditions. Significant challenges still remain before these strategies can be widely accepted as standards of care. However, the development of less toxic conditioning regimens, the refinement of bioprocessing, and manufacturing methodologies are likely to drive gene therapy into clinical mainstream in the very near future. In the future, application of genome editing together with the clinical usage of induced pluripotent stem cells and their progeny may have a significant impact on the field by allowing patient-specific mutation correction, use of natural regulatory elements, targeting of “safe sites,” and molecular analysis at the clonal level to evaluate genome integrity before treatment.

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**AUTHOR CONTRIBUTIONS**

D.A.W. and A.J.T.: conception and design, administrative support, manuscript writing, final approval of manuscript.

**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

The authors indicate no potential conflicts of interest.

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