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CLINICAL PROTOCOL

STRIM-005

Sponsor: Fondazione Telethon ETS

Protocol Version: 3.1 **Date: 08 October 2024**

EudraCT number: NA **Protocol number: STRIM-005**

Summary of Protocol Changes

This clinical investigation will be performed in compliance with the protocol, Good Clinical Practice as set forth in the ICH Guideline for Good Clinical Practice, the Declaration of Helsinki (1991), EMA guidelines for gene therapy clinical trials, and applicable local regulatory requirements.

Version	Date	Main changes
3.1	08 October 2024	Updated milestones.
3	30 April 2024	Change of the analytical method and change of the central lab. Change of PI and updated milestones. Removal of Annex 1 (ENCePP checklist for study protocols).
2.0	07 April 2023	Change of sponsorship from Orchard Therapeutics to Fondazione Telethon ETS
1.0	03 December 2020	First version

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Protocol number: STRIM-005 *EudraCT number: NA*

Protocol date: 08 Oct 2024 *Protocol version: 3.1*

SPONSOR'S SIGNATURE PAGE

CLINICAL PROTOCOL NUMBER: STRIM-005

STUDY TITLE: Methodology study to investigate the utility of retroviral insertion site analysis in samples from subjects treated with Strimvelis gene therapy

Sponsor: Fondazione Telethon ETS
Sponsor Address: Via Varese, 16b, 00185, Roma, Italia

Sponsor's Representative: [redacted]

Clinical Development Head
Fondazione Telethon ETS

On behalf of the Sponsor, I agree to this Protocol:

Sponsor's signature: **Date:**

_____ _____

Protocol number: STRIM-005 *EudraCT number: NA*

Protocol date: 08 Oct 2024 *Protocol version: 3.1*

INVESTIGATOR'S SIGNATURE PAGE

CLINICAL PROTOCOL NUMBER: STRIM-005

STUDY TITLE: Methodology study to investigate the utility of retroviral insertion site analysis in samples from subjects treated with Strimvelis gene therapy

Investigator's Name: _____

Investigator's Address: _____

My signature below confirms that I have read and approved this Protocol and assures that this clinical study will be conducted according to all the requirements in this protocol, the Declaration of Helsinki (1991), ICH Guideline for Good Clinical Practice, EMA guidelines for gene therapy clinical trials and all applicable regulatory requirements.

Investigator's signature: **Date:**

_____ _____

Protocol number: STRIM-005 *EudraCT number: NA*

Protocol date: 08 Oct 2024 *Protocol version: 3.1*

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2. List of Abbreviations

ADA	Adenosine deaminase
ADA-SCID	Adenosine deaminase severe combined immunodeficiency
$CD34^+$	Hematopoietic progenitor stem cells
CGD	Chronic granulomatous disease
CV	Coefficient of variation
DNA	Deoxyribonucleic acid
EMA	European Medicines Agency
ENCePP	European Network of Centres for Pharmacoepidemiology and Pharmacovigilance
ERT	Enzyme replacement therapy
EU	European Union
GCP	Good clinical practice
GI	Gini index
gRV-GT	Gamma retroviral gene therapy
HLA	Human leukocyte antigen
HSCT	Hematopoietic stem cell transplant
ICH	International Conference on Harmonization
IEC	Independent ethics committee
LTR	Long terminal repeat
MDS	Myelodysplastic syndrome
MLV	Murine leukemia virus
OSR	Ospedale San Raffaele
PASS	Post-Authorization Safety Study
PCR	Polymerase chain reaction
PEG-ADA	Polyethylene glycol modified bovine adenosine deaminase
RIS	Retroviral insertion site
SCID	Severe combined immunodeficiency
SD	Shannon diversity
SLIM-PCR	Sonication and Linker Mediated-PCR
SOP	Standard operating procedure
US	United States
WAS	Wiskott-Aldrich Syndrome
X-SCID	X-linked SCID

3. Responsible parties

Sponsor – responsible for study coordination and reporting

Fondazione Telethon ETS
Via Varese, 16b, 00185, Rome, Italy

Sponsor's Representative: [redacted]

Principal Investigator – responsible for subject consent and shipping samples:

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4. Abstract

Title: Methodology study to investigate the utility of retroviral insertion site analysis in samples from subjects treated with Strimvelis gene therapy.

Rationale and background: Adenosine deaminase (ADA) deficiency results in severe combined immunodeficiency (SCID), a fatal autosomal recessive inherited immune disorder. Strimvelis is a gamma retroviral gene therapy (gRV-GT) that restores ADA function in hematopoietic cell lineages, and in doing so prevents the pathology caused by purine metabolites (i.e., impaired immune function). Strimvelis is intended for patients with ADA-SCID and for whom no suitable human leukocyte antigen (HLA)-matched related stem cell donor is available. The genetically modified cells of Strimvelis are made using an engineered gamma retrovirus which incorporates into deoxyribonucleic acid (DNA); a mechanism that carries the risk of causing leukemia due to the possibility of retrovirus insertion in the vicinity of putative oncogenes. Risk factors for leukemia following gene therapy are not fully understood, and there are no reliable predictive markers. It has been hypothesised that measuring the clonal dynamics of gene modified stem cells by retroviral insertion site (RIS) analysis in peripheral blood could be a useful predictor of long-term clinical outcome.

This study is a post-approval commitment to evaluate the utility and reliability of a new RIS methodology to accurately assess clonal dynamics following gene therapy with Strimvelis.

Research question and objective(s): The objective of this methodology study is to evaluate the accuracy and precision of Sonication and Linker Mediated (SLiM) - PCR for RIS analysis. The study will also describe insertion sites recovered from subjects treated with gRV-GT, how their clonal dynamics change over time and whether SLiM-PCR may have utility for investigating and predicting the potential for insertional oncogenesis in subjects treated with Strimvelis.

The primary objective of this study is to assess the accuracy and precision of SLiM-PCR methodology for RIS analysis using control insertion site DNA.

The secondary objective of this study is to describe the abundance and diversity of insertion sites in subjects treated with gRV-GT.

Study design: This non-interventional, retrospective, methodology study will use peripheral blood and bone marrow samples previously taken from subjects treated with gRV-GT at Ospedale San Raffaele (OSR). The study does not require subjects to undergo any further treatment, intervention or blood withdrawal. Eligible samples will be shipped to a central laboratory and will undergo DNA extraction prior to SLiM-PCR analysis. Each analysis run will include a control DNA sample and up to four subject samples. Each sample will be analysed in triplicate.

Population: Subjects who have previously received treatment with gRV-GT, either during clinical development (clinical trials and early access programs) or in the post-marketing setting as the approved product (Strimvelis) or under hospital exemption, and for whom at least one biological sample is available that meets the following eligibility criteria:

Peripheral blood, bone marrow, or DNA extracted from either source.

Taken at least 6 months after gRV-GT.

Stored at -20^{o}C or below since the time of sampling.

Likely to provide 500 ng (maximum)of DNA (following extraction by the central laboratory).

Variables: SLiM-PCR analysis will generate data on the following variables:

- Insertion site abundance
- Identity of the gene nearest the clone insertion site
- Shannon diversity indices

Data sources: Automated insertion site reports generated following SLiM-PCR analysis.

Study size: No formal sample size calculation has been performed. It is expected that this study will include approximately 70 eligible samples from approximately 15 subjects previously treated with gRV-GT.

Statistical analysis:

The primary objective is to determine the precision and accuracy of SLIM-PCR methodology for detecting Strimvelis vector insertion sites.

SLiM-PCR method for LV and γ-RV IS retrieval has been validated and tested its accuracy and sensitivity for clonal abundance estimations. The method on an experimental framework was applied in which the genomic DNA of monoclonal samples harboring one LV or γ-RV vector IS in known genomic positions was mixed at different ratios with the genomic DNA of samples with IS are randomly distributed in the genome. It is possible to evaluate precision and accuracy in the quantification by estimating the relative abundance of the clones with known IS versus the expected value, and the sensitivity by determining the limit of detection, also using different amounts of DNA.

Within each analytical run, positive control samples containing clones with a single known Strimvelis vector insertion site, and known abundance, will be analysed via SLIM-PCR to assess the abundance of retrieved insertion sites.

The percentage coefficient of variation (%CV) of the abundance will be used to assess the variability (precision).

The mean difference between the retrieved abundance and the expected abundance will be used to assess the accuracy.

The secondary objectives, for subjects treated with gRV-GT and with peripheral blood and/or bone marrow samples taken at least 6 months after treatment, are to describe:

-The abundance and location of all unique insertion sites for each sample. The top 10 most abundant clones will be listed, including the name of the targeted gene. For subjects with data derived from both peripheral blood and bone marrow, these data will be presented separately.

Clonal diversity, as assessed using the Shannon diversity index. For individual subjects, data for each diversity index will be plotted over time. For subjects with data derived from both peripheral blood and bone marrow samples, both will be presented separately.

Other exploratory analyses will be considered on the basis of the emerging data; including (but not limited to) the listing of insertion sites located at or near known oncogenes, and (where possible) additional analyses of data and samples to further characterise the utility of SLIM-PCR methodology.

5. Amendments and updates

Amendment 2
Change of sponsorship from Orchard Therapeutics to Fondazione Telethon ETS in April 2023.

Amendment 3
Change of the analytical method and change of the central lab. Change of PI and updated milestones. Removal of Annex 1 (ENCePP checklist for study protocols).

Amendment 3.1
Updated milestones.

6. Milestones

Milestone	**Planned date**
Start of data collection	Q1 2021
End of data collection	Q2 2023
Final report of study results	Q2 2025

7. Rationale and background

7.1. Background

ADA deficiency results in SCID, a fatal autosomal recessive inherited immune disorder. ADA deficiency is an ultra-rare genetic disease, with an overall prevalence ranging from 0.22 to 0.68 per 100,000 live births or less than 50 children per year in the United States (US) and European Union (EU) combined. ADA is a ubiquitous enzyme of purine metabolism and mutations in the ADA gene lead to accumulation of deoxyadenosine nucleotides in plasma, white blood cells, red blood cells, and tissues. The main features of ADA-SCID are profound lymphopenia, impaired differentiation and function of T cells, B cells, and natural killer cells; recurrent infections; and failure to thrive (Hirschorn, 1999; Chapel, 2003). Non-immunological abnormalities may also occur as a consequence of the systemic metabolic defect and include hepatic, lung, skeletal alterations, and neurological deficits affecting motor function and hearing, and cognitive/behavioural deficits (Hirschorn, 1999; Gaspar, 2010; Sauer, 2009).

Subjects with ADA-SCID can be effectively treated with conventional bone marrow transplant (also referred to as hematopoietic stem cell transplant [HSCT]) from allogeneic donors, but the best outcomes for this intervention are only achieved when an HLA-matched family donor is available; a viable option for fewer than 25% of patients with ADA-SCID (Hirschorn, 2014). Decreased long-term survival (compared with matched sibling or matched family donor) has been observed with less well-matched donor sources of stem cells (Gaspar, 2010; Hassan, 2012). A non-transplant treatment option, for patients who are not suitable or do not have an HLA-matched donor for HSCT, is enzyme replacement therapy (ERT) in the form of polyethylene glycol modified PEG-ADA; a non-curative therapy given in frequent injections that externally corrects the metabolic defect, and is used as a stabilizing treatment in the US. ERT is not currently approved in the EU but is available via expanded access and compassionate use programs. Short-term treatment with PEG-ADA is recognized as beneficial for some patients with ADA-SCID but long-term efficacy is limited for some patients due to incomplete immune reconstitution (Chun, 1993; Lainka, 2005; Chaffee, 1992).

Strimvelis is an approved gene therapy (within the EU) that restores ADA function in hematopoietic cell lineages, and in doing so prevents the pathology caused by purine metabolites (i.e. impaired immune function). Strimvelis is intended for patients with ADA-SCID and for whom no suitable HLA-matched related stem cell donor is available.

7.2. Rationale

The genetically modified cells of Strimvelis are made using an engineered retroviral vector which incorporates semi-randomly into DNA, thereby introducing a specific novel genetic mark in each modified cell. This mechanism carries the potential risk of causing leukemia due to the potential for insertional mutagenesis leading to oncogenesis.

Malignancy due to insertional oncogenesis is currently reported as an important potential risk in the Strimvelis EU Risk Management Plan. Retrovirally mediated cell transduction introduces a risk of oncogenesis through either activation of proto-oncogenes from the vector's long terminal repeat (LTR) enhancers or the formation of de novo gene expression products secondary to aberrant splicing events or knockout of tumour suppresser gene function (Knight, 2013).

Murine leukemia virus (MLV)-like vectors with slightly differing envelope proteins and/or gene expression systems to Strimvelis, have been tested in clinical trials for Wiskott-Aldrich Syndrome (WAS), X-linked SCID (X-SCID) and chronic granulomatous disease (CGD). In each of these 3 indications, leukemia or myelodysplastic syndrome (MDS) have occurred.

WAS trial: 9 of 10 patients were affected, with onset ranging between 1.5 to 5 years post-dose (Braun, 2014; Cavazzana, 2018).

X-SCID trials: 6 of approximately 20 children were affected, with an onset ranging between 1.5 years and 15 years post-dose (Six, 2017).

CGD trial: where patients achieved functional levels of engraftment, MDS was evident within 2 years (Williams, 2014).

The first treatment with autologous transduced CD34+ cells transduced with gamma-retroviral gene therapy (gRV-GT) occurred in 2000, and at the date of Sept 2022 (ESCGT 2023 Oral presentation Migliavacca et al, Gene Therapy For Adenosine Deaminase Deficiency: Long-term Outcome And Post-marketing Experience and unpublished) a total of 41 subjects have been treated in clinical studies, early access programs and in the post-marketing setting with Strimvelis. In addition, two (2) subjects have been treated under hospital exemption with a similar product that used the same gammaretroviral vector to transduce hematopoietic stem cells derived from mobilised peripheral blood rather than bone marrow. Of these 43 subjects there has been one case of lymphoid T cell leukemia (T-cell type acute leukemia) reported at 4.7 years post-gene therapy after treatment with gRV-GT as part of a named patient program (NPP) before the approval of Strimvelis. This event was reported in October 2020 as probably related to Strimvelis, associated with an LMO2-vector insertion in the leukemic clone, and the molecular event that contributed to the leukemic development, including patients' specific characteristics, are currently being investigated (ASGCT 2023 Abstract 215 Oral communication - Cesana et al. First Case of Leukaemia in Retroviral Gene Therapy for ADA-SCID - Molecular Therapy Vol 31 No 4S1, April 2023 Cesana Cicalese et al - Nat Comm. Submitted)No insertional oncogenesis or malignancies have been reported for 32 other ADA-SCID patients who have been treated with alternative gamma retroviral gene therapies in academic trials at other institutions (Candotti, 2012; Shaw, 2017; Gaspar, 2006; Gaspar, 2011; Otsu, 2015; Schmidt, 2003).

The case reports of leukemia and MDS following retroviral gene therapy in X-SCID, WAS, and CGD involved retroviral vector mediated activation of proto-oncogenes.

The reason for the different incidence of insertional oncogenesis between ADA-SCID and other diseases treated with gamma-retroviral gene therapy is not clear. Within the spectrum of viral promoters that have been tested in clinical trials, MLV LTRs are generally considered less potent than either the myeloproliferative sarcoma virus LTRs used in WAS or the spleen focus forming virus LTRs used in CGD (Baum, 1995). The genomic insertion sites are largely dependent upon the viral integrase and the developmental stage of the target cell. Therefore, the insertion sites recovered from Strimvelis-treated subjects are very similar to other MLV backbone transduced $CD34^{+}$ cell gene therapy treated subjects. What appears to differ, however, is the genomic stability of Strimvelis cell clones with inserts adjacent to proto-oncogenes such as LMO2 and CCND2. The differences between the programs may be due in part to the function of the gene of interest. In both WAS and X-SCID,

gene modified cells have the potential to provide a proliferative advantage because both the WAS protein and IL2RG gene proteins are involved in cell response to proliferative stimuli. In contrast, ADA is known as a metabolic "house-keeping" protein that is constitutively expressed in all cell types.

Nonetheless, it is important to consider long term follow-up with respect to the evaluation of the risk of insertional oncogenesis and the Committee for Medicinal Products for Human Use has requested further monitoring of this risk.

7.3. Retroviral insertion site analysis

Risk factors following gene therapy are not fully understood, and there are no reliable predictive markers. It is possible that measuring the clonal dynamics of gene modified stem cells by RIS analysis in peripheral blood could be a useful predictor of long-term health outcome. By studying the clonal dynamics of gene modified cells it may be possible to develop short-term and long-term markers for safety and efficacy monitoring of haematopoetic stem cell gene therapy.

At the time of Strimvelis marketing authorisation application, no suitable assays had been developed or applied over the long term, and therefore the utility of RIS analysis in Strimvelis-treated patients was not fully understood. Estimates of clonal abundance and comprehensive identification of insertion site locations using early methods were compromised by several types of recovery biases and sources of variability (Brugman, 2013; Gabriel, 2009). Therefore, at the time of marketing authorisation application of Strimvelis, the existing RIS analysis methodologies were unsuitable for individual patient monitoring.

With the advent of next generation sequencing (NGS) and well-established Illumina sequencing devices, random shearing of whole genomic DNA followed by variants of conventional ligation mediated PCR may now be considered the method of choice for insertion site analysis in gene therapy.

One such method, SLiM-PCR, has been developed by SR-Tiget. The basis of this technology can be traced back to first successful insertion site analysis on multiple preclinical and clinical lentiviral-transduced samples (Scala, Nat. Comm. 2023 - PMID: 37244942; Wagner, Nat. Comm. 2022 - PMID: 35764632; Olgasi, Mol Ther Methods Clin Dev. 2021 - PMID: 34853801; Gentner, N Engl J Med. 2021 Nov - PMID: 34788506; Cesana, Nat Med. 2021 - PMID: 34140705; Milano, Sci Transl Med. 2019 - PMID: 31118293; Petrillo, Hum Gene Ther. 2019 - PMID: 31037976; Marktel, Nat Med. 2019 - PMID: 30664781; Sessa, Lancet 2016 - PMID: 27289174; Jofra Hernández, Mol Ther. 2021 - PMID: 33010230) and retroviral transduced samples (Cesana, Nat Med. 2021 - PMID: 34140705).The original method has been optimized by introducing shearing of genomic DNA by sonication and by performing individual reaction steps to achieve NGS-compliance.

Validation data indicate that the actual abundance values reported by SLIM-PCR are very close to the expected values (high accuracy), whilst CVs are low (high precision) (Cesana, Nat Med. 2021 - PMID: 34140705).

8. Research question and OBJECTIVE(S)

The objective of this methodology study is to evaluate the accuracy and precision of SLIM-PCR for RIS analysis. The study will also describe insertion sites recovered from subjects treated with gRV-GT, how their clonal dynamics change over time and whether SLIM-PCR may have utility for investigating and predicting the potential for insertional oncogenesis in subjects treated with Strimvelis.

8.1. Primary objective

To assess the accuracy and precision of SLIM-PCR methodology for RIS analysis using control insertion site DNA.

8.2. Secondary objectives

The secondary objective of this study is to describe the abundance and diversity of insertion sites in subjects treated with gRV-GT.

8.3. Exploratory objectives

Other exploratory analyses will be considered on the basis of the emerging data; including (but not limited to) the listing of insertion sites located at or near known oncogenes, and (where possible) additional analyses of data and samples to further characterise the utility of SLIM-PCR methodology.

9. Research methods

9.1. Study Design

9.1.1. Overview of design and procedures

This retrospective methodology study will use human biological samples previously taken from ADA-SCID subjects treated with gRV-GT at OSR. Subjects will not require any further treatment or intervention of any kind under this Protocol.

OSR will identify eligible samples and ensure that informed consent is provided (or has previously been given) for the use of these samples. Eligible samples will be shipped (under dry ice) to the central laboratory for preparation and RIS analysis.

Unless the DNA has not already been extracted by OSR the central laboratory will extract the DNA from all samples and will advise the Sponsor of any samples yielding less than 500 ng of DNA so that a decision can be made as to whether they should be analysed.

Samples will undergo RIS analysis by SLIM-PCR, which will be fully adapted and validated for the detection of Strimvelis vector insertion sites.

9.1.2. Scientific rationale for the design

This retrospective study has been specifically designed to evaluate the performance of SLIM-PCR as a method for RIS analysis in patients previously treated with gRV-GT. SLIM-PCR was selected for this study as it represents a state-of-the-art insertion site methodology whose utility has already been demonstrated in other gene therapy development programs.

The benefits of adopting a retrospective study design for this study include:

Reduced burden on subjects, with no requirement for additional samples to be taken.

The assessment of Strimvelis clonal dynamics over time will be enhanced by the inclusion of clonal abundance and diversity data that may extend beyond 10 years post-treatment for some subjects.

The analysis of peripheral blood and bone marrow samples from the same subject, and often at the same time point, will allow comparison of the insertion site profiles from these different cell sources.

Data generated by this study will be available significantly earlier than would be possible with a prospective study. The early availability of the data is important as the ultimate goal is to establish the utility of RIS analysis in identifying factors that may be predictive of the onset of insertional mutagenesis so that patients can receive appropriate follow-up as early as possible. The predictive value of SLIM-PCR has become already evident with the first case(s) of insertional oncogenesis as reported in the ASGT reference mentioned below:

ASGCT 2023 Abstract 215 Oral communication - Cesana et al. First Case of Leukaemia in Retroviral Gene Therapy for ADA-SCID - Molecular Therapy Vol 31 No 4S1, April 2023, Cesana, Cicalese et al - Nat Comm. Submitted

ASGCT 2023 Abstract 7 Oral communication – Migliavacca et al. Gene Therapy for Adenosine Deaminase Deficiency: Long-Term Outcome and Post-Marketing Experience - Molecular Therapy Vol 31 No 4S1, April 2023 Paper Clinico: Migliavacca et al - Nat Med. Accepted.

ESCGT 2023 Oral presentation Migliavacca et al, Gene Therapy For Adenosine Deaminase Deficiency: Long-term Outcome And Post-marketing Experience

Moreover the predictive value of this methodology can also be demonstrated by the clonality study carried on the remaining patients.

A successful demonstration of the utility of SLIM-PCR RIS analysis in Strimvelis-treated patients may help to establish this method as part of the standard of care following Strimvelis treatment and the sooner this is achieved the more RIS data will be contributed to the ongoing Strimvelis registry (STRIM003).

For patients who have not had RIS analysis as part of standard of care, this study will establish the feasibility of using stored samples of peripheral blood or bone marrow in order to retrospectively build a profile of clonal dynamics over time.

9.2. Setting

This study will use biological samples previously taken from ADA-SCID subjects treated with gRVGT at OSR. Subjects must have received gRV-GT as part of a clinical trial, expanded access program, as an approved product (Strimvelis) or under a post-approval hospital exemption, and must have at least one retained biological sample that meets the following eligibility criteria:

Peripheral blood, bone marrow, or DNA extracted from either source.

Taken at least 6 months after gRV-GT.

Stored at -20^{o}C or below since the time of sampling.

Likely to provide 500 ng (maximum) of DNA (following extraction by the central laboratory).

There is no restriction on the number of samples from a single subject, or on the time that has elapsed since sampling.

Review of the retained human biological samples documentation at OSR indicates that approximately 15 subjects may have eligible samples.

9.3. Variables

The date of informed consent will be recorded for each subject.

- Sample data will include:
- Subject number (unique identifier, as allocated at the time of gRV-GT treatment)
- Date of gene therapy (Note: this will be obtained from the appropriate clinical database or from OSR if the subject has not been enrolled in any previous study).
- Date of sample
- Cell source (peripheral blood or bone marrow)

SLIM-PCR data will include:

- Date of analysis
- DNA quantity used
- Number of DNA sequences (yield)
- Number of unique insertion sites
- Insertion site abundance
- Identity of the gene nearest the clone insertion site for the ten clones with the highest abundance (only for subject samples)
- Shannon diversity index (only for subject samples)

Diversity indices are useful in summarising the composition of a population of clones as they take into consideration the number of unique clones that are present in a sample as well as what the relative abundances of each of those clones are. This is particularly useful in determining if a dominant clone is present within the population as this could indicate a clonal expansion that is indicative of leukemia.

Shannon diversity (SD) is a measurement that represents the uncertainty about the identity of a single species within a population. Therefore, the greater number of unique species within a population, the less certain the measure is of the "identity" of any one species, resulting in a higher value of SD. Likewise, the less complex the population, the lower the value of SD (Shannon, 1948). When SD is calculated, it takes into account the number of distinct clones ("species") as well as the abundance of each clone. A low value of SD has been previously reported in the literature as being associated with clonal expansion and a reduction in overall clonal diversity (Braun, 2014).

9.4. Data sources

This study will collect primary data only.

The central laboratory will provide the sponsor with a comprehensive SLIM-PCR report for all analyzed subjects and also an electronic format of the data included in the reports.

Source documents at OSR will provide evidence for the status of the subjects and substantiate the integrity of the data collected.

9.5. Study size

No formal sample size calculation has been performed. It is expected that this study will include approximately 70 eligible samples from approximately 15 subjects previously treated with gRV-GT.

9.6. Data management

All subject data related to the analysis and reporting of insertion site analysis will be managed within the Strimvelis SLIM-PCR bioanalytical pipeline.

All subject data relating to the samples collected at OSR and transfer to the central laboratory will be used to identify the subject and sample in the SLIM-PCR reports.

The investigator is responsible for verifying that data entries on the sample transfer form are accurate and correct by signing the sample transfer form. The investigator must retain this responsibility when delegating the signing of the form to qualified study team member.

Data reported on the sample transfer form that are transcribed from source documents must be consistent with the source documents or the discrepancies must be explained. The investigator must maintain accurate documentation (source data) that supports the information entered in the sample transfer form.

The sponsor or designee is responsible for the data management of this study including quality checking of the data.

9.7. Data analysis

The primary objective is to determine the precision and accuracy of SLIM-PCR methodology to characterise insertion sites. Replicate measurements and control DNA with single known insertion sites, and with known abundance values, will be used to allow determination of the bias/accuracy and variability/precision, where small bias indicates high accuracy and low variability (high precision) indicates good reproducibility. Precision and accuracy will be evaluated for each sample run.

Precision will be determined by the variability (%CV) of the abundance data.

Accuracy will be determined based on the difference between the mean retrieved abundance and the expected abundance of control DNA.

Due to the nature of deep sequencing methodology, small variations in the number of DNA sequences retrieved for each sample are expected. Analysis of the effect of DNA sequences (yield) per sample and its relation to accuracy and reproducibility will provide information about the number of sequences needed to reach a specific sensitivity.

9.7.1. Secondary analyses

The secondary objective is to describe the abundance and diversity of insertion sites in subjects treated with gRV-GT.

The abundance and location of all unique insertion sites will be determined, and the top 10 most abundant will be used to explore clonal dynamics over time and help identify the development of prominent clones. For subjects with data derived from both peripheral blood and bone marrow, these data will be presented separately.

Diversity will be assessed using the Shannon diversity index. For individual subjects, data for each diversity index will be plotted over time. For subjects with data derived from both peripheral blood and bone marrow samples, both will be presented separately.

9.7.2. Exploratory Analyses

Other analyses will be considered on the basis of the emerging data; including (but not limited to) the listing of insertion sites located at or near known oncogenes, and (where possible) additional analyses of data and samples to further characterise the utility of SLIM-PCR methodology.

9.8. Quality control and quality assurance

9.8.1. Quality control

All procedures will be performed according to the protocol and will be documented appropriately.

Quality control processes related to the insertion site analysis method and reporting of data will be governed by the Quality Management System in place at the central laboratory at the time the samples are analysed.

9.8.2. Quality assurance

Monitoring

Study monitors will oversee the progress of the study and of ensure that it is conducted, recorded, and reported in accordance with the protocol, Standard Operating Procedures (SOPs), Good Clinical Practice (GCP), and the applicable regulatory requirement(s).

Before the investigational site can enter a subject into the study, the study monitor will discuss with the investigator(s), and other personnel, their responsibilities with regard to protocol adherence, and the responsibilities of the sponsor or its representatives. This will be documented in a Clinical Study Agreement between the sponsor (or delegate) and the investigator.

During the study, the study monitor will have regular contacts with the investigational site, for the following:

- Provide information and support to the investigator(s)
- Confirm that the investigational team is adhering to the protocol
- Perform source data verification to confirm that data entered into the sample transfer form by authorized site personnel are accurate, complete, and verifiable from source documents and that rights of subjects are being protected.
- Checking laboratory process validation documentation, the informed consent process, and the management of essential documentation. Monitoring will require direct access to original records for each patient.
- Record and report any protocol deviations not previously reported to the sponsor or delegate.

The monitor will be available between visits if the investigator(s) or other staff needs information or advice.

The investigator must permit study-related monitoring and provide direct access to source data documents and other records relative to study conduct.

Monitors will be appointed by the sponsor. Monitors will be appropriately trained and will have the scientific and/or clinical knowledge needed to monitor the trial adequately. They will be familiar with the investigational product(s), the protocol, written informed consent form and any other written information to be provided to patients, the Sponsor’s or designee’s SOPs, GCP, and the applicable regulatory requirement(s).

Deviation from study protocol

Every deviation from the study protocol will be specified and documented. The investigator must consult with the monitor and discuss the type and extent of deviation.

Audits and inspections

To ensure compliance with GCP and all applicable regulatory requirements, authorised representatives of Fondazione Telethon ETS (or delegate), regulatory authority, or Independent Ethics Committee (IEC) may visit the site to perform audits or inspections, including source data verification.

This study will be part of the sponsors risk-based audit program. The purpose of a sponsor audit or inspection is to systematically and independently examine all study-related activities and documents to determine whether these activities were conducted, and data were recorded, analysed, and accurately reported according to the protocol, GCP guidelines of the International Conference on Harmonization (ICH), and any applicable regulatory requirements.

The investigator should contact Fondazione Telethon ETS immediately if contacted by a regulatory agency about an inspection

The Sponsor and the Investigators guarantee their availability for audits and/or inspections made by the regulatory agencies.

Record Retention

Records and documents, including signed informed consent forms and insertion site analysis records, pertaining to the conduct of this study must be retained by the investigator and central laboratory for 30 years from the issue of the final Clinical Study Report / equivalent summary unless local regulations or institutional policies require a longer retention period. No records may be destroyed during the retention period without the written approval of the sponsor. No records may be transferred to another location or party without written notification to the sponsor. If it becomes necessary for Fondazione Telethon ETS or delegate, or the Regulatory Authority to review any documentation relating to the study, the Investigator must permit access to such records.

9.9. Limitations of the research methods

The retrospective nature of the study has benefit in terms of not requiring additional samples from subjects, but as the samples are limited to those that are already in storage, there are limitations related to the number of subjects with samples, the number of samples per subject and the timing of the samples relative to gRV-GT.

Eligible samples are not available for all subjects that have received gRV-GT. It is expected that samples from approximately 15 subjects will be tested. This reflects a significant proportion of the more of 40 subjects treated to date and so should provide a reasonable overview of insertion site profiles.

The number of samples per subject will be variable. For subjects with only one sample it will only be possible to describe the insertion site profile at a single time-point and these subjects will not be included in the analysis of clonal dynamics over time.

The timing of samples relative to gRV-GT will be variable, so comparison of insertion site profiles at specific timepoints post-treatment will be limited.

It is known that insertion site profiles for individual subjects during the first year following gene therapy can be highly fluctuant due to the variability of the engraftment of gene-corrected cells and in the timing of the expansion of individual clones. Samples taken less than 6 months after gene therapy will not be included in this study.

The study design does not allow for the prospective collection of samples. Any additional follow-up that requires further sampling will not be possible under this protocol.

9.10. Other aspects

None.

10. Protection of human subjects

10.1. Ethical approval and subject consent

10.1.1. Ethical considerations

This study will be conducted in accordance with the protocol and with:

- Consensus ethical principles derived from international guidelines including the Declaration of Helsinki and Council for International Organizations of Medical Sciences (CIOMS) International Ethical Guidelines
- Applicable ICH-GCP Guidelines
- Applicable laws and regulations

Ethical approval for this study will be obtained. The investigator must submit written approval to Fondazione Telethon ETS or delegate. Any amendments to the protocol will require IEC approval before implementation of changes made to the study design.

The investigator will be responsible for the following:

- Providing written summaries of the status of the study to the IEC annually or more frequently in accordance with the requirements, policies, and procedures established by the IEC.
- Providing oversight of the conduct of the study at the site and adherence to requirements of ICH guidelines, the IEC, European regulation 536/2014 for clinical studies (if applicable), and all other applicable local regulations protection of human subjects.

10.1.2. Subject consent

Before OSR can send samples from subjects treated with Strimvelis gene therapy to the laboratory, OSR will be responsible for ensuring that informed consent for using retained available samples had been provided in previous study participation, allowing for optional exploratory research.

If such consent had not been obtained or the consent form cannot be made available for the study monitor review, the Principal Investigator will ensure that the subject and/or parents/legal representative are given full and adequate information about the nature, purpose, possible risk and benefit of the study. Subjects must also be notified that participation in the study is voluntary and that they are free to discontinue from the study at any time, thus sample results will not be processed further.

Subjects should be given the opportunity to ask questions and allowed time to consider the information provided. The signed and dated informed consent must be obtained before samples are shipped to the central laboratory. The Principal Investigator(s) must maintain the original, signed Informed Consent Form. Another original, signed Informed Consent Form must be given to the subject.

The medical record must include a statement that written informed consent was obtained or available before the subject was enrolled in the study and the date the written consent had been obtained. The authorised person obtaining the informed consent must also sign the informed consent form.

10.2. Subject confidentiality

Each participant's identity will be known only to OSR. The samples sent to the central laboratory and the dataset used in the analysis will contain coded subject identifiers only.

All parties will ensure protection of subject personal data and will not include subject names on any study forms, reports, publications, or in any other disclosures, except where required by law. In accordance with Italian law, subjects (and their parent/carer) will be informed about data handling procedures and asked for their consent. The study will be conducted in accordance with all applicable privacy and data storage requirements.

Regulatory authorities or Fondazione Telethon ETS-approved auditors may inspect the study files, which may include personally identifiable information of participants. In all cases, personally identifiable information will not be transmitted to Fondazione Telethon ETS.

11. Management and reporting of adverse events/adverse reactions

Not applicable for this retrospective methodology study. The role of the Medical Monitor for this study will include maintain general oversight of data, discussion with the Principal Investigator and input regarding the need for exploratory analyses.

12. Plans for disseminating and communicating study results

Where required by applicable regulatory requirements, the principal investigator signatory will be identified for the approval of the clinical study report. The principal investigator will be provided reasonable access to statistical tables, figures, and relevant reports and will have the opportunity to review the complete study results. Fondazione Telethon ETS will also provide the investigator with the full summary of the study results.

The procedures and timing for public disclosure of the results summary and for development of a manuscript for publication will be in accordance with standard editorial and ethical practice.

12.1. Target Audience

The information generated by this study will be transmitted to Regulatory Authorities as required, primarily the EMA. The results may be disseminated externally via manuscripts or presentations.

12.2. Study reporting and publications

Fondazione Telethon ETS plans to include the report in the Periodic Benefit-Risk Evaluation Report (PBRER) according the legal requirements established in Regulation (EC) No 726/2004, and a final report will be submitted to the EMA within the timelines reported at Par.6.

In accordance with the 2010 EU pharmacovigilance legislation, information about this Post-Authorisation Safety Study has been entered into the publicly available European Network of Centers for Pharmacoepidemiology and Pharmacovigilance (ENCePP) Register of Studies and then transitioned to the HMA-EMA Catalogue of Real-World Data (RWD) sources, that replaced ENCePP since the beginning of 2024. The study protocol was also entered into the register before the start of data collection and will also be entered into clinicaltrials.gov. Updates to the study protocol in case of substantial amendments, progress reports where applicable, and the final study report will also be entered in the register.

Any publication of the results from this study will be consistent with standard editorial and ethical practices and guided by the Uniform Requirements for Manuscripts Submitted to Biomedical Journals: Writing and Editing for Biomedical Publication of the International Committee of Medical Journal Editors, updated April 2010 (ICMJE, 2010). The rights of the Investigator and of the Sponsor with regard to publication of the results of this study are described in the Investigator agreement. To comply with the requirements of the EU Pharmacovigilance Regulation, the final manuscript of the article will be submitted to the Agency and competent authorities, within two weeks of first acceptance for publication.

13. References

Baum C, Hegewisch-Becker S, Eckert H et al. Novel retroviral vectors for efficient expression of the multidrug resistance (mdr-1) gene in early hematopoietic cells. J Virol, 1995;69(12):7541–7547.

Braun CJ, Boztug K, Paruzynski A, Witzel M, Schwarzer A, Rothe M. Gene therapy for Wiskott-Aldrich Syndrome - long term efficacy and genotoxicity. Science Translational Medicine, 2014;6:227-233.

Brugman M, Suerth JD, Rothe M, Suerbaum S, Schambach A, Modlich U et. al. Evaluating a ligation-mediated PCR and pyrosequencing method for the detection of clonal contribution in polyclonal retrovirally transduced samples. Human Gene Therapy Methods, 2013;24 (2):68-79.

Cavazzana M, Bushman FD, Miccio A, Andree-Schmutz I, Six E. Gene therapy targeting haematopoietic stem cells for inherited diseases: progress and challenges. Nat Rev Drug Discov, 2019 Jun;18(6):447-462.

Candotti F, Shaw KL, Muul L, Carbonaro D, Sokolic, R, Choi C, Schurman SH. Gene therapy for adenosine deaminase–deficient severe combined immune deficiency: clinical comparison of retroviral vectors and treatment plans. Blood. 2012 Nov 1;120(18):3635-46.

Chaffee S, Mary A, Stiehm ER, Girault D, Fischer A, Hershfield MS. IgG antibody response to polyethylene glycol-modified adenosine deaminase in patients with adenosine deaminase deficiency. J Clin Invest, 1992;89(5):1643-1651.

Chapel H, Geha R, Rosen F. Primary Immunodeficiency diseases: an update. Clin Exp Immunol, 2003;132 (1):9-15.

Chun JD, Lee N, Kobayashi RH, Chaffee S, Hershfield MS, Stiehm ER. Suppression of an antibody to adenosine-deaminase (ADA) in an ADA-deficient patient receiving polyethylene glycol modified adenosine deaminase. Ann Allergy, 1993;70(6):462-466.

Gabriel R, Eckenberg R, Paruzynski A, Bartholomae C, Nowrouzi A, Arens A et al. Comprehensive genomic access to vector integration in clinical gene therapy. Nature Medicine, 2009;15:1431-1436.

Gaspar HB, Bjorkegren E, Parsley K et al. Successful Reconstitution of Immunity in ADA-SCID by Stem Cell Gene Therapy Following Cessation of PEG-ADA and Use of Mild Preconditioning. Mol Ther, 2006 Oct;14(4):505-13.Gaspar HB. Bone marrow transplantation and alternatives for adenosine deaminase deficiency. Immunol Allergy Clin North Am, 2010; 30(2):221-236.

Gaspar HB, Cooray S, Gilmour KC, 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, 2011;Aug 24;3(97):97ra80.

Gillet NA, Malani N, Melamed A, Gormley N, Carter R, Bentley D, et al. The host genomic environment of the provirus determines the abundance of HTLV-1-infected T-cell clones. Blood, 2011;117(11):3113-22.

Gini C. Sulla misura della concentrazione e della variabilita dei caratteri. Transactions of the Real Istituto Veneto di Scienze. 1914;LIII:1203.6.

Hassan A, Booth C, Brightwell A, Allwood Z, Veys P, Rao K et al. Inborn Errors Working party of the European group for Blood and Marrow Transplantation and European Society for Immunodeficiency. Outcome of haematopoietic stem cell transplantation for adenosine deaminase-deficient severe combined immunodeficiency. Blood, 2012;120 (17):3615-3624.

Hirschorn R, Grunebaum E, Roifman C, Candotti F. Immunodeficiency due to defects of purine metabolism. In: Ochs HD, Smith CIE, Puck JM., editor. Primary immunodeficiency diseases: a molecular and genetic approach [3rd edition]. New York:Oxford University Press, 2014.

Hirschorn R. Immunodeficiency disease due to deficiency of adenosine deaminase. In: H Ochs, C Smith, J Puck, editor. Primary Immunodeficiency Diseases. Oxford: Oxford University Press, 1999:121-139.

Knight S, Collins M and Takeuchi Y. Insertional mutagenesis by retroviral vectors: current concepts and methods of analysis. Curr Gene Ther, 2013;13(3):211-227.

Lainka E, Hershfield MS, Santisteban I, Bali P, Seibt A, Neubert J, et. al. Polyethylene glycol-conjugated adenosine deaminase (ADA) therapy provides temporary immune reconstitution to a child with delayed-onset ADA deficiency. Clin Diagn Immunol, 2005;12 (7):861-866.

Otsu M, Yamada M, Nakajima S et al. Outcomes in two Japanese adenosine deaminase-deficiency patients treated by stem cell gene therapy with no cytoreductive conditioning. J Clin Immunol, 2015;May 35(4):384-98.

Sauer AV, Aiuti A. New insights into the pathogenesis of adenosine deaminase-severe combined immunodeficiency and progress in gene therapy. Curr Opin Allergy Clin Immunol, 2009;9 (6):496-502.

Schmidt M, Hoffmann G, Wissler M, et al. Detection and direct genomic sequencing of multiple rare unknown flanking DNA in highly complex samples. Hum Gene Ther, 2001 May 1;12(7):743-9.

Schmidt M, Carbonaro DA, Speckmann C et al. Clonality analysis after retroviral-mediated gene transfer to CD34+ cells from the cord blood of ADA-deficient SCID neonates. Nat Med, 2003;Apr;9(4):463-8.

Shannon C. 1948. A mathematical theory of communication. Bell Syst Tech J, 1948;27:379-423, 623-656.

Shaw KL, Garabedian E, Mishra S et al. Clinical efficacy of gene-modified stem cells in adenosine deaminase–deficient immunodeficiency. J. Clin. Invest, 2017;127,1689–99.

Six E, Gandemer V, Magnani A, Nobles C, Everett J, Male F et al. LMO2 associated clonal T cell proliferation 15 years after gamma-retrovirus mediated gene therapy for SCIDX1. ASGCT 20th Annual Meeting. 2017;Session 413:Abstract 753.

Williams DA. Curing genetic disease with gene therapy. Trans Am Clin Climatol Assoc. 2014;125:122-8; discussion 128-129.

Scala, Nat. Comm. 2023 - PMID: 37244942

Wagner, Nat. Comm. 2022 - PMID: 35764632

Olgasi, Mol Ther Methods Clin Dev. 2021 - PMID: 34853801

Gentner, N Engl J Med. 2021 Nov - PMID: 34788506

Cesana, Nat Med. 2021 - PMID: 34140705

Milano, Sci Transl Med. 2019 - PMID: 31118293

Petrillo, Hum Gene Ther. 2019 - PMID: 31037976

Marktel, Nat Med. 2019 - PMID: 30664781

Sessa, Lancet 2016 - PMID: 27289174

Jofra Hernández, Mol Ther. 2021 - PMID: 33010230

Cesana, Nat Med. 2021 - PMID: 34140705

ASGCT 2023 Abstract 215 Oral communication – Cesana et al. First Case of Leukaemia in Retroviral Gene Therapy for ADA-SCID - Molecular Therapy Vol 31 No 4S1, April 2023, Cesana Cicalese et al - Nat Comm. Submitted

ASGCT 2023 Abstract 7 Oral communication – Migliavacca et al. Gene Therapy for Adenosine Deaminase Deficiency: Long-Term Outcome and Post-Marketing Experience - Molecular Therapy Vol 31 No 4S1, April 2023 □ Paper Clinico: Migliavacca et al - Nat Med. Accepted.

ESGCT 2023 - Migliavacca et al. Gene Therapy for Adenosine Deaminase Deficiency: Long-Term Outcome and Post-Marketing Experience

14. Annex 1. List of stand-alone documents

None.