TITLE PAGE

Information Type: Clinical Protocol

Clinical Trial Protocol Title: A phase I/II clinical trial of hematopoietic stem cell gene therapy for the Wiskott-Aldrich Syndrome (TIGET-WAS)

Study Number: TIGET-WAS

Compound Number: OTL-103 (formerly known as GSK2696275 and Telethon003)

Development Phase: I/II

EudraCT number: 2009-017346-32

Protocol Version: 12, 19 Sep 2023

Rev.	Description of the revisions	Date
1	First issue	15-10-2009
2	As requested by ISS: action to be taken in case of microbiological contamination	22-01-2010
3	Editing and integration of amendment 1 related to Informed Consent Form: reporting a case of leukemia occurred during a study conducted in Germany with the retroviral vector encoding WASP	14-10-2011
4	Editing, integration of new endpoints, better definition of time line for endpoints, and integration of amendment 4 related to Informed Consent Form: reporting 4 cases of leukemia	10-05-2012
	Cytokine (SCF) production changed from Amgen to mobile Genix due to the interruption of the manufacturer production of cytokine (SCF) by Amgen. [ref: Amendment 5]	28-02-2013
5	Follow up extension (from 3 years to 8 years); Promoter change from Fondazione Centro San Raffaele del Monte Tabor to Ospedale San Raffaele. [ref: CTA Amendment 6]	10-04-2013
6	Change sample size (from 6 to 8), additional option for administration of MPB only, elimination of exclusion criterion 5 (revertant cell population) and change Financial Sponsor (from Fondazione Telethon to GSK R & D Ltd) [ref: CTA Amendment 7];	10-03-2014
	Communication of change in analytical method for testing WAS LVV QC [ref: Amendment 8];	25-09-2014
7	Discretional use of ATG in patients with clinical autoimmunity prior to gene therapy, addition of Plerixafor use for PBSC mobilization and change in Medicinal Product primary package (bag). Change of PI. [ref: CTA Amendment 9]	09-01-2015
	Communication related to minor changes in Certificate of Analysis for SCF and Flt-3L [ref: Amendment 10]	09-06-2015
8	Change regulatory sponsor (promoter) from Ospedale San Raffaele to GSK Research & Development Ltd) [ref: CTA Amendment 11]	29-Jan-2016
9	Extended Long-Term Follow up (post-8 years); new assessment on platelet activation and morphology [ref: CTA Amendment 12]	31-01-2018
10	Change of Sponsorship to Orchard Therapeutics	27-06-2018
11	Entry to a separate observational long-term follow-up (OLTFU) study; simplified strategy for RCL testing; possibility to perform remote follow-up visits.	22-05-2020
	Communication related to change in PV and medical monitor contact details; deletion of the mention of special interest for disease-related events	14-07-2021

	Communication related to Medical Monitor and Sponsor	16-02-2023
	Communication related to Medical Monitor and Sponsor Signatory	04-08-2023
12	Change of Sponsorship (from Orchard Therapeutics to Fondazione Telethon ETS)	19-09-2023

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PROTOCOL SYNOPSIS

Title (Acronym)	A phase I/II clinical trial of hematopoietic stem cell gene therapy for the Wiskott-Aldrich Syndrome (TIGET-WAS)
Objectives **Primary Objective** **Secondary Objectives**	**Primary objectives:** 1) To evaluate the safety of the administration of autologous CD34+ cells transduced with a lentiviral vector containing the WASP gene in patients with WAS, after a reduced intensity conditioning regimen; 2) To evaluate the long-term engraftment of WASP-expressing transduced cells; 3) To evaluate the efficacy of gene therapy assessed as: 3.1) Improvement of the patient's immune function, specifically of T cell function and antigen-specific responses to vaccinations; 3.2) Improvement of thrombocytopenia. **Secondary objectives:** To evaluate the efficacy of gene therapy in improving the patient's clinical conditions assessed by a reduction in frequency of severe infections, and bleeding episodes and reduction of autoimmunity phenomena and eczema.
Endpoints **Primary Endpoints** **Secondary Endpoints**	**Safety endpoints:** Primary safety endpoints: 1) Conditioning regimen-related safety, consisting in the absence of engraftment failure or delayed hematological reconstitution (prolonged aplasia) and surveillance of non hematological regimen related toxicity (for clinical features CTCAE ≥2, for metabolic/laboratory CTCAE ≥3); 2) Safety of lentiviral gene transfer into HSC: - Short-term safety and tolerability of lentiviral-transduced cell infusion; - Long-term safety of lentiviral-transduced cell infusion (absence of Replication competent lentivirus (RCL) and abnormal clonal proliferation). Secondary safety endpoints 1) Lack of immune response to transgene 2) Overall safety of the treatment. **Efficacy endpoints:** Primary efficacy endpoints: 1) Overall survival; 2) Sustained engraftment of genetically corrected hematopoietic stem cells in peripheral blood and/or in bone marrow; 3) Expression of vector-derived WASP; 4) Improved T-cell functions;

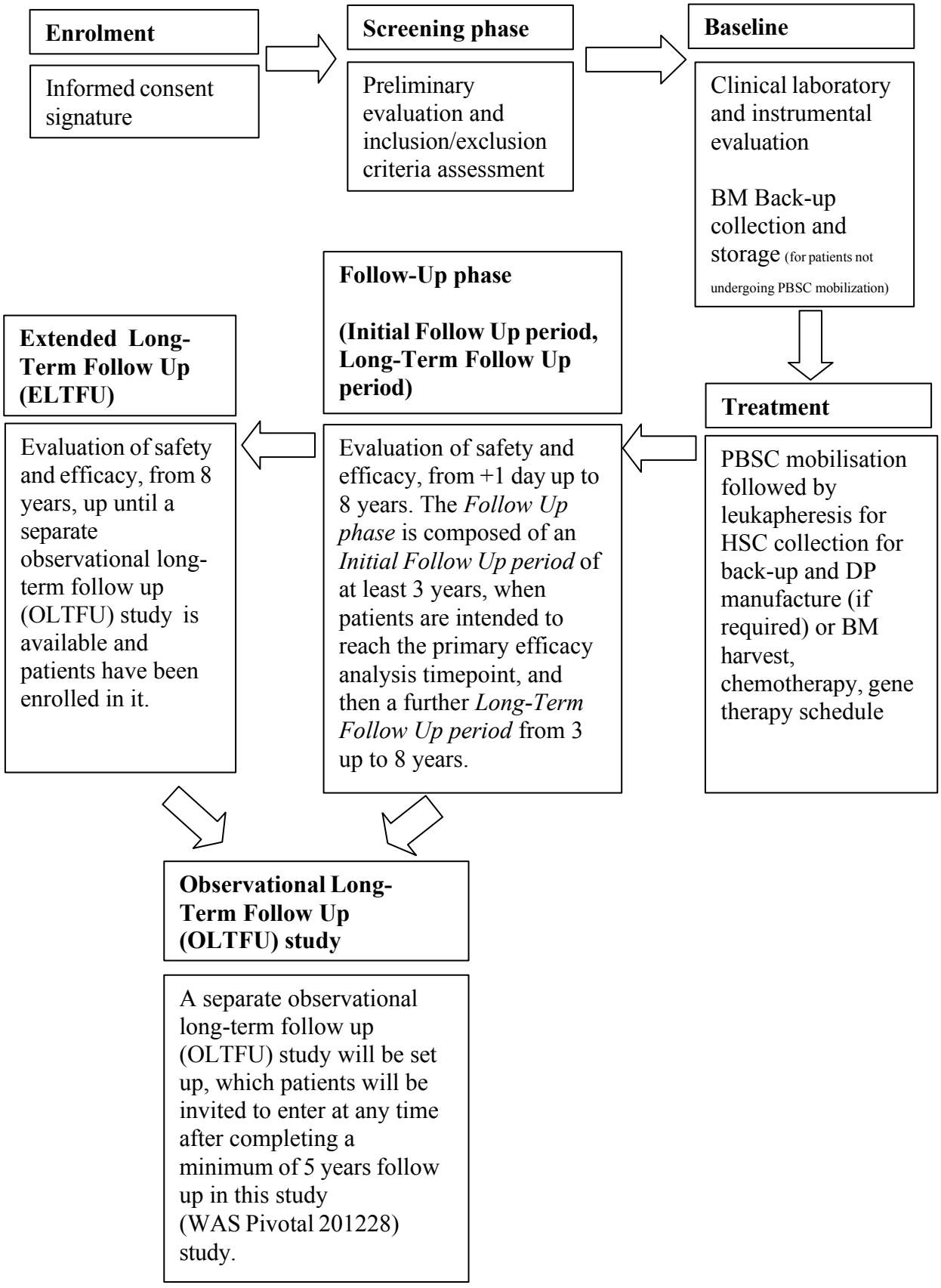
	5) Antigen-specific responses to vaccination; 6) Improved platelet count and MPV normalization. Secondary efficacy endpoints: 1) Multiline age engraftment of genetically corrected cells; 2) Reduced frequency of severe infections; 3) Reduced bleeding episodes; 4) Reduced autoimmunity phenomena; 5) Improvement in eczema; 6) Improved quality of life. Exploratory efficacy endpoint: 1) Normalisation of platelet activation profile and morphology
Criteria of assessment for: **Safety** **Efficacy**	**Safety assessment**: 1) For safety of LV gene transfer a monitoring plan and analyses of integrations will be performed. 2) For immunogenicity, antibody response to WASP protein will be evaluated. 3) For overall safety a descriptive analysis will be performed on AE, AR, SAE/SAR, UAR, SUSAR, and events will be classified for frequency, severity and body system involved. **Efficacy assessment**: 1) Overall Survival; 2) Engraftment of genetically corrected hematopoietic progenitors and/or differentiated cells in peripheral blood and/or in bone marrow; 3) Expression of vector-derived WASP; 4) Improved in vitro T cell functions; 5) Improved platelet count and normalization of mean platelet volume (MPV); 6) Reduced bleeding episodes; 7) Antigen-specific responses to vaccinations; 8) Reduced frequency of severe infections; 9) Modification in eczema and autoimmunity phenomena; 10) Improved quality of life; 11) Platelet morphology and activation after stimulation with agonists.
Protocol design	Non randomized, open label, monocentric, phase I/II, prospective, involving a single infusion of autologous CD34 positive cells transduced with a 3rd generation VSV-G pseudo typed lentiviral vector in 8 patients affected by WAS.

	Description of the Protocol Five protocol phases are foreseen, after signature of the informed consent: 1) *Screening phase*, during which the conditions required by the clinical protocol for patients' enrollment will be assessed and fulfillment of the inclusion/exclusion criteria of the study will be evaluated; 2) *Baseline phase,* carried from the end of the screening phase to the day before the harvest of peripheral blood stem cells, if performed, or the day before Rituximab administration (day -22); 3) *Treatment phase*, from the end of the Baseline to day 0; 4) *Follow Up phase*: the *Follow Up phase* is composed of *an Initial Follow-Up period* of at least 3 years, when patients are intended to reach the primary efficacy analysis timepoint, and then a further Long-Term Follow-Up period from 3 up to 8 years; 5) *Extended Long-Term Follow Up (ELTFU) phase*: patients who complete the *Follow up phase* will then be contacted annually, in order to collect long-term safety and selected efficacy data, until a separate observational long-term follow-up (OLTFU) study is initiated and patients have enrolled in it. Once the OLTFU study is set up, patients will be invited to enter the OLTFU study at any time after they have completed a minimum of 5 years follow up in this study (WAS Pivotal, 201228). As part of the OLTFU study, patients will continue to be followed up for a total of 15 years from the date of treatment with gene therapy.
Experimental drug description (dose/regimen/etc.)	**Medicinal Product** The Medicinal Product consists of autologous CD34+ cells collected from the bone marrow and/or peripheral blood and transduced with a lentiviral vector encoding WASP controlled by *WAS* promoter sequences (w1.6W). **Dosage indications** A minimum dose of 2 x 10^6 CD34+ cells/Kg (maximum 20 x 10^6 CD34+ cells/Kg) and an optimal dose of 5-10 x 10^6 CD34+ cells/Kg will be infused i.v. **Conditioning Regimen** A reduced conditioning regimen will be administered before the autologous engineered stem cells infusion: - *I.v. Busulfan:* body weight-based and AUC targeted dose (range: 0,8-1,2 mg/kg/dose), 8 (+ 1) doses administered every 6 hours from day -3 to day -1; - *I.v. Fludarabine*: 30 mg/sqm/day, on days -3 and -2; - *I.v. Rituximab*: 375 mg/sqm/day, on day -22.

	I.v. ATG Thymoglobuline may be used at PI discretion in patients with clinical autoimmunity before day-18.
Inclusion criteria **- Inclusion** **- Exclusion**	**Inclusion Criteria** 1) Diagnosis of WAS defined by genetic mutation and at least one of the following criteria: - Severe WAS mutation; - Absent WASP expression; - Severe clinical score (Zhu clinical score ≥ 3). 2) No HLA-identical sibling donor AND 3.1) Negative search for a matched unrelated donor (10/10) or an adequate unrelated cord blood donor (6/6) within 4-6 months; OR 3.2) Patients of > 5 years of age who are not candidate to unrelated allogeneic transplant based on clinical conditions. AND 4) Parental/guardian/patient signed informed consent. **Exclusion Criteria** Patients who meet any of the following criteria will be excluded from study admission: 1) Patients positive for HIV-infection; 2) Patients affected by neoplasia; 3) Patients with cytogenetic alterations typical of MDS/AML; 4) Patients with end-organ functions or any other severe disease which, in the judgment of the investigator, would make the patient inappropriate for entry into this study; 5) Patients who underwent an allogeneic hematopoietic stem cell transplantation in the previous 6 months; 6) Patients who underwent an allogeneic hematopoietic stem cell transplantation with evidence of residual cells of donor origin.
Sample size	Eight patients will be treated in this study
Accrual/duration timeline	Patients will undergo regular follow up after gene therapy to assess the efficacy and safety of the treatment. 1) For each subject the main study phase will be completed once the Initial Follow Up period of 3 years following treatment with OTL-103 is finished. 2) A Long- Term Follow up period will then be conducted from 3 years up to 8 years after the reinfusion of transduced cells.

	3) For subjects who complete the Long-Term Follow Up phase, an ELTFU phase will then continue from 8 years after the reinfusion of transduced cells until a separate OLTFU is initiated and subjects have enrolled in it. As part of the OLTFU study, patients will continue to be followed up for a total of 15 years from the date of treatment with gene therapy. The end of the study is when all subjects have completed a minimum of 5 years follow up and transitioned to a separate OLTFU study. Should a patient decide not to consent to participate in the ELTFU nor transition to the separate OLTFU study, then the subject will be considered to have completed this study and will be transitioned to standard of care according to local practice.
Statistical design	A descriptive analysis will be performed for all the endpoints (safety and efficacy). Percentage of subjects with sustained engraftment of genetically corrected hematopoietic stem cells at 1 year and percentage of those with detectable vector-derived WASP expression will be calculated, with 95% Confidence Intervals. The mean change between the pre-gene therapy and the 1-year values of vitro T cell proliferation will be calculated, with 95% CI. The percentage of subjects who improved the level of platelet count at one, two and three years after gene therapy compared to baseline, will be estimated, with 95% CI. When all the treated patients complete the 3-year regular follow up visit after gene therapy (study phase), a formal statistical analysis will be performed to assess the efficacy and safety of the investigational product (study phase analysis). A dedicated clinical study report will be generated based on all the data available at this time point. At the end of the study, a final statistical analysis will be conducted with all data available and the clinical study report will be updated accordingly. **Rules for**: **Protocol modification**: - 2 out of the first 3 patients suffering of prolonged aplasia; - 2 or more failures of engraftment of transduced cells. **Early Stopping**: - 2 transplant-related deaths; - 1 malignant proliferation related to gene therapy. In the interest of the patients' safety, the third patient will be treated when: - at least 1 of the first 2 patients will achieve hematopoietic recovery; - at least 60 days after the first treatment. For the subsequent patients the above rules will be applied.

TRIAL FLOW-CHART



SPONSOR and PRINCIPAL INVESTIGATOR INFORMATION PAGE

SPONSOR legal registered address	Fondazione Telethon ETS [redacted]
Principal Investigator	[redacted]

SPONSOR SIGNATORIES

Fondazione Telethon ETS

[redacted]

Fondazione Telethon ETS

LABORATORY INFORMATION PAGE

Routine haematology **Clinical chemistry** **Immunophenotyping** **Genetic analysis** **Microbiology**	[redacted]
PK analysis	[redacted]
Serum antibody levels to vaccinal antigens	[redacted]
RCL detection	[redacted]

INVESTIGATOR PROTOCOL AGREEMENT PAGE

- I confirm agreement to conduct the study in compliance with the protocol.
- I acknowledge that I am responsible for overall study conduct. I agree to personally conduct or supervise the described study.
- I agree to ensure that all associates, colleagues and employees assisting in the conduct of the study are informed about their obligations. Mechanisms are in place to ensure that site staff receives the appropriate information throughout the study.

Investigator Name:

Investigator Signature	Date

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ABBREVIATIONS AND DEFINITIONS

ADA-SCID	Adenosine deaminase severe combined immunodeficiency
AE	Adverse event
AIEOP	Italian Association of Paediatric Hematology and Oncology
AIHA	Autoimmune haemolytic anaemia
ALD	Adrenoleukodystrophy
ANC	Absolute neutrophil count
ASGT	American Society of Gene & Cell Therapy
ATG	Anti-thymocyte globulin
AUC	Area under the curve
AR	Adverse reaction
BMT	Bone marrow transplantation
CGD	Chronic granulomatous disease
CIS	Common insertion sites
CMV	Cytomegalovirus
CRF	Case reports forms
CTCAE	Common Terminology Criteria for Adverse Events
DC	Dendritic cells
DMSO	Dimethyl sulfoxide
DP	Drug Product
DS	Drug Substance
EBMT	European Society for Blood and Marrow Transplantation
eCRF	Electronic case report form
EBV	Epstein Barr virus
EMA	European Medicines Agency
FACS	Fluorescence-activated cell sorting
FFS	Failure Free Survival
GCP	Good clinical practice
G-CSF	Granulocyte-colony stimulating factor
GITMO	Italian Bone Marrow Transplantation Group
GMP	Good manufacturing practice
GTMPs	Gene Therapy Medicinal Products
GVHD	Graft versus host disease
ELTFU	Extended long-term follow up
HIV	Human immunodeficiency virus
HCP	Healthcare Professional
HSC	Hematopoietic stem cells
HSCT	Hematopoietic stem cell transplantation
HSPC	Hematopoietic stem or progenitor cell
HPC	Hematopoietic progenitor cell
SR-TIGET	The San Raffaele Telethon Institute for Gene Therapy
IB	Investigators brochure
IC	Informed consent
IMPD	Investigational Medicinal Product Dossier

ISCT	International Society for Cellular Therapy
IV	Intravenous
IVIG	Intravenous immunoglobulin
ITP	Idiopathic thrombocytopenic purpura
JACIE	Joint Accreditation Committee ISCT-Europe & EBMT
LAM-PCR	Linear-amplification mediated polymerase chain reaction
LM-PCR	Ligation- mediated polymerase chain reaction
LTR	Long terminal repeats
LV	Lentiviral vectors
mAb	monoclonal Antibody
MCB	Master Cell Bank
MLD	Metachromatic leukodystrophy
MLV	Moloney leukemia virus
MMFD	Mismatched family donor
MMUD	Mismatched unrelated donor
MNC	Mononuclear cell
MND	Myeloproliferative sarcoma virus enhancer, Negative control region, Deleted, dl587rev primer-binding site substituted
MOI	Multiplicity of Infection
MPB	Mobilized peripheral blood
MPV	Mean platelet volume
MSD	Matched sibling donor
MUD	Matched unrelated donor
NCI	National Cancer Institute
NK	Natural killer
OLTFU	Observational long-term follow up
OOS	Out Of Specification
OS	Overall Survival
PB	Peripheral blood
PBSC	Peripheral blood stem cells
PID	Primary immune deficiency
PLT	Platelet
PPD	Pharmaceutical Pharma Development
qPCR	Quantitative polymerase chain reaction
RCL	Replication-competent lentivirus
SAE	Severe adverse event
SAR	Serious adverse reaction
SCID	Severe combined immune deficiency
SCID-X1	X-linked severe combined immune deficiency
SUSAR	Suspected unexpected serious adverse reaction
TRECs	T cell-receptor excision circles
TCR	T cell-receptor
TPO	Thrombopoietin
TU	Transduction Unit
UAR	Unexpected adverse reaction
UCB	Umbilical Cord Blood

VCN	Vector copy number
VSV-G	Vesicular stomatitis virus glycoprotein
WAS	Wiskott-Aldrich syndrome
WASP	Wiskott-Aldrich syndrome protein
WIP	WASP-interacting protein
XLN	X-linked neutropenia
XLT	X-linked thrombocytopenia

1. BACKGROUND AND RATIONALE

1.1. Scientific Background and Rationale

1.1.1. WAS – Description of the condition

Wiskott-Aldrich Syndrome (WAS) is a rare X-linked primary immunodeficiency disease with an estimated incidence of 1:250.000 males and characterized by micro-thrombocytopenia, recurrent infections and eczema; this syndrome is associated with a high incidence of autoimmunity phenomena and lymphoid malignancies (Burns et al. 2004).

The disease is due to mutations in the WAS gene which encodes the cytoskeletal regulator Wiskott-Aldrich Syndrome protein (WASP) (Derry et al. 1994). WASP is expressed exclusively in hematopoietic cells where it functions as a regulator of actin reorganization by linking various signals received at the cell surface to Arp2/3-mediated actin nucleation.

WASP is an effector of cdc42 and controls actin nucleation for the formation of filopodia and membrane protrusions at the leading edge of hematopoietic cells. Consequently, WASP plays a pivotal role in cell migration and establishment of cell-to-cell contacts such as immunological synapses (Notarangelo and Ochs 2003). Defective WASP expression has been reported to cause functional impairments in hematopoietic precursors, platelets, T cells, B cells, Natural Killer (NK) cells, dendritic cells (DC), monocytes/macrophages, neutrophils and mast cells.

In T cells, WASP is an essential component of the signal transduction cascade initiated by T cell-receptor (TCR) engagement contributing to the establishment of an immunological synapse. WASP is recruited to lipid rafts and involved in lipid raft clustering to the site of the immunological synapse following TCR and CD28 triggering (Dupre et al. 2002). WASP deficiency in T cells is associated to reduced activation and to downstream defects of cytokine production and proliferation (Trifari et al. 2006).

In highly motile cells such as immature DC, WASP is essential for motility and for the formation of podosomes, which are actin-rich, highly dynamic cytoskeletal structures. In phagocytic cells such as macrophages and DC, clearance of apoptotic cells is accompanied by the recruitment of WASP to the phagocytic cup. A failure in apoptotic cell clearance due to WASP deficiency may perpetuate tissue inflammation and facilitate autoimmunity (Leverrier et al. 2001).

Thrombocytopenia is the most frequent clinical manifestation of WAS: platelet number can be drastically reduced in the blood of WAS patients, and the residual circulating platelets are morphologically abnormal (reduced in size). Patients with a milder form of the disease (X-linked thrombocytopenia, XLT) are characterized mainly by thrombocytopenia. Peripheral destruction of the platelets in the spleen contributes to the thrombocytopenia, since a correction of platelet count and size can be reached after splenectomy. Accelerated destruction of platelets could be the consequence of either intrinsic platelet morphological abnormalities or of an autoimmune reaction.

B cells defects include abnormalities of migration, homing, and also deficient production of antibodies, particularly against polysaccharide antigens. This may reflect a marked depletion of marginal zone splenic B cells (Meyer-Bahlburg et al. 2008;Westerberg et al. 2008). Microvilli expressed on WAS deficient B cells appear to be shorter and less dense in cell contacts than in wild type cells.

The absence of WASP has been associated with numerous hematopoietic cell defects including motility, antigen uptake by endocytosis, adhesion, and activation (Burns, Cory et al. 2004), (Ochs and Notarangelo, 2005). The numerous and distinct cellular defects in hematopoietic cells from WAS patients are related to a high degree of diversity of clinical manifestations of the disease. It is reasonable to think that specific clinical manifestations arise from specific intrinsic cellular defects. However, the complexity of the physiopathology of WAS suggests that mechanisms requiring the interplay of different hematopoietic cell subsets are defective.

1.1.2. Molecular aspects of the disease

WAS gene is composed of 12 exons, encoding a 502 amino acids (a.a.) protein (WASP) with an expression restricted to hematopoietic cells. WASP is located in the cytoplasmic compartment with the highest density along the cell membrane. WASP is a member of a family of cytoskeletal regulator proteins (WASP/SCAR) involved in cytoskeleton reorganization by activating Arp 2/3 mediated actin nucleation.

The C-terminal domain of WASP activates the actin nucleation activity of Arp 2/3 complex by bringing Arp 2 in the proximity of Arp 3. The N-terminal region constitutively interacts with WIP (WASP-interacting protein), which plays the role of a chaperon controlling the localization and level of WASP within the cell. WASP interacts with the small Rho GTPase Cdc42, which has the capacity to induce a conformational activation resulting in interaction with Arp 2/3. Indeed, in the absence of stimuli, WASP exists in a folded auto-inhibited conformation. WASP also interacts with signalling proteins and tyrosine kinases (Nck, Fyn, Btk, Grb2) that regulate its activity. Additional modifications of WASP play a role in modulating whether the protein is in its auto-inhibitory or activatory state. Tyr 291 phosphorylation by different tyrosine kinases (Lyn, Btk, Hck) directly activates WASP, independently of Cdc42, and stabilizes WASP active conformation. On the contrary, constitutive binding of WIP stabilizes the inactive conformation.

Overall, WASP is involved in receptor-mediated signalling cascades that activate Arp 2/3 and actin remodelling. Receptors with a link to WASP include TCR, BCR, co-stimulatory receptors and chemokine receptors. Actin remodelling plays an important role in cellular response allowing change in shape or motility, endocytosis, vesicular trafficking, and membrane reorganization.

1.1.3. Clinical manifestations

1.1.3.1. Thrombocytopenia and bleeding episodes

Platelets are the cells that display the most drastic numeric reduction in the blood of WAS patients and the most profound morphological abnormalities (reduced mean platelet volume). However, it is still unclear how mutations in the WAS gene (independently of the severity of the mutation) alter platelet numbers and size. There are contrasting reports on the capacity of megakaryocytes from WAS/XLT patients to produce pro-platelets to normal levels. WASP-defective platelets show defects of filopodia formation and F-actin reorganization upon adhesion. In addition, platelets from XLT/WAS patients express high level of phosphatidylserine, which is a signal for macrophage mediated platelet destruction. Thrombocytopenia has also been proposed to result from accelerated destruction via an autoimmune mechanism, as observed in the murine model of the disease (Prislovsky et al. 2008). Indeed, WAS/XLT patients consistently present high levels of antibodies adherent to the surface of platelets. As a consequence of the

platelet defect, bleeding episodes are a common manifestation in WAS (>80% incidence). The number of life-threatening episodes of bleeding (intracranial haemorrhage, intestinal bleeding) appears to be higher in WAS patients as compared to the XLT patients. This covers up to 20% of the death cases and severity of these episodes appears to be related to the number of platelets (severe episodes mainly in patients with platelets <10.000/µl, less severe episodes in patients with platelets = 50.000 - 100.000/µl) (Imai et al. 2004).

1.1.3.2. Recurrent infections

WAS patients have a high susceptibility to develop infections from numerous micro-organisms including bacteria, viruses and fungi (Imai, Morio et al. 2004). Bacterial infections due to encapsulated bacteria are the most frequent cause of morbidity, since WAS patients produce IgG2 against polysaccharide antigens at reduced levels. Respiratory infections, together with systemic infections (meningitis and sepsis), are commonly observed. Among viral infections, the most commonly reported are due to Herpes simplex viruses (type 1 and 2), which can be severe and disseminated. Infections due to cytomegalovirus (CMV) can cause encephalitis and hepatitis in WAS patients. These patients also develop *Molluscum contagiosum* infections and warts due to Papilloma viruses, at a relatively high frequency. Fungal infections are also common and mainly due to Candida and Aspergillus, while opportunistic pulmonary infections due to *Pneumocystis jirovecii* are also frequent.

1.1.3.3. Eczema

Eczema affects 80% of WAS patients in the course of the disease and the typical skin lesions resemble acute or chronic eczema in appearance and distribution (Imai, Morio et al. 2004). XLT patients also frequently develop eczema, although at a minor grade and for reduced periods, as compared to WAS patients. It is not known what the link is between WASP deficiency and eczema. In WAS patients, eczema is often associated to high IgE levels and could be of an allergic nature or due to an imbalance in Th2 cytokine production. It is also possible that other genetic or environmental factors contribute to this clinical manifestation.

1.1.3.4. Autoimmunity

The incidence of autoimmune diseases associated with classical WAS is high (40% and 72% in 2 independent patient cohorts) and is not necessarily correlated with the overall disease severity or complete absence of WASP expression (Dupuis-Girod et al. 2003; Imai, Morio et al. 2004). Indeed, this complication can also develop in patients with otherwise milder disease. In addition, WAS patients often suffer from more than one autoimmune manifestation at the same time (25 to 36%). The most common manifestations (80%) are autoimmune haemolytic anaemia (first autoimmune manifestation to appear in the course of the disease), cutaneous vasculitis, IgA nephropathy (also common in XLT patients) and arthritis.

Less common autoimmune manifestations in WAS patients are: chronic inflammatory intestinal disease and idiopathic thrombocytopenic purpura (as a consequence of splenectomy). WAS patients with autoimmune manifestations have a 25% risk to develop tumors (as compared to 5% for WAS patients without autoimmunity).

In addition, 75% of patients developing tumors had signs of autoimmunity in earlier follow up. Thus, autoimmunity is associated with an increased risk of mortality and this is particularly true for autoimmune haemolytic anaemia and idiopathic thrombocytopenic purpura.

Despite being an important cause of morbidity and mortality, the mechanisms of WAS-associated autoimmunity are still poorly understood. WASP may be required for normal thymic maturation and theoretically, self-reactive T cells may escape negative selection because of defective TCR-induced apoptosis, as described for WASP-deficient murine lymphocytes.

Impaired function of WASP-deficient T cells, B cells, macrophages, or DC could all destabilize important mechanisms participating in the maintenance of normal tolerance. In addition, recent studies have demonstrated defects in homeostasis and suppressor functions of natural regulatory T cells (Marangoni et al. 2007), which may at least partly account for the autoimmune disorders associated with WAS.

1.1.3.5. Cancer

Together with severe hemorrhages and autoimmune manifestations, tumors are the major complication of WAS. They occur in 13-22% of the patients with a median age of onset of 9.5 years (Imai, Morio et al. 2004). This reported frequency most probably underestimates the current situation since the median survival of WAS patients has been improving over the last decades, thereby increasing the risk to develop tumors. WAS associated-tumors are mainly lymphoreticular malignancies, since leukemia, myelodysplasia and lymphoma cover 90% of the cases. This is most probably linked to the restricted expression and function of WASP in hematopoietic cells. Immunodeficiency is likely to contribute as a proportion of malignancies are associated with Epstein-Barr virus (EBV) and there may be specific immune-surveillance defects (such as impaired NK or cytotoxic T cell cytolysis). However, this is unlikely to be the full explanation, as the highest lymphoma risk (44%) may be conferred by a single splice site mutation that is otherwise associated with a mild clinical phenotype. Interestingly, there also appears to be a significant incidence of myelodysplasia in severe WAS patients. Although the mechanisms of malignancy remain undetermined, there may be a role for WASP in human cell division and maintenance of genomic stability (Moulding et al. 2007) as has been reported for the WASP homolog in fission yeast.

1.1.4. Classification of WAS patients

Historically, the clinical classification of WAS patients arose from a large cohort of patients classified into 5 severity scores (Zhu et al. 1997). This was based on the following clinical findings: microthrombocytopenia, eczema, immunodeficiency, autoimmune disorders/cancer (Table 9).

Approximately 300 unique mutations have been reported in the WAS gene (WASPbase: http://pidj.rcai.riken.jp/WASPbase/). Studies in large cohorts of patients have recently shown that the disease severity correlates with the severity of WASP expression defect, which in turn correlates with the type of mutation in the WAS gene. Therefore, direct measurement of WASP residual expression by Western blot or by flow cytometric analysis is useful for predicting the phenotypic outcome of WAS (Imai, Morio et al. 2004; Jin et al. 2004; Lutskiy et al. 2005). Missense mutations usually result in residual expression of a mutated protein and are associated with a mild clinical form of the disease (XLT), in which only platelet defects are present (Villa et

al. 1995). In contrast, nonsense mutations usually totally impair protein expression and are associated with the WAS severe clinical phenotype consisting in thrombocytopenia and recurrent infections and eventually autoimmunity and cancer.

Therefore, the expression of WASP has been proposed as a main prognostic parameter by the Italian Association of Paediatric Haematology and Oncology (AIEOP), which has adopted guidelines, which characterize WAS patients based on WASP expression in combination with clinical signs.

1.1.5. Current methods of treatment and limitations

Clinical management of patients with WAS represents significant challenges, particularly in attenuated phenotypes where the natural history of disease progression is unclear. Splenectomy, by decreasing excessive sequestration and elimination of platelets, is an effective treatment for thrombocytopenia, but carries a significant long-term risk of sepsis and is therefore probably indicated only in severe disease where there is no prospect for curative intervention. Moreover, it may not be effective in the setting of autoimmune thrombocytopenia in addition to intrinsic platelet defects. At present, hematopoietic stem cell transplantation (HSCT) from an HLA-identical sibling is the treatment of choice for WAS since it has been reported to lead to 82 to 88% long-term survival in different European and American centers (Filipovich et al. 2001; Kobayashi et al. 2006; Ozsahin et al. 2008), with an average of 87% in the American study (Table 1). However, the majority of the patients do not have such a donor available. As an alternative, WAS patients have been receiving HSCT from HLA-matched unrelated donors (MUD). Following this procedure, the survival rate had been previously estimated to be 71% (Filipovich, Stone et al. 2001), with a higher success rate when performed before the age of 5 (Table 2). Recent studies have reported improved survival rates (80-81%) after MUD HSCT, possibly due to the availability of a detailed HLA-matching system for unrelated donors, with further indication that prompt diagnosis and intervention is crucial for successful outcome (Kobayashi et al. 2006; Pai et al. 2006) (Table 3). The success of MUD transplantation is however hampered by a risk to develop autoimmune complications when complete chimerism is not obtained (Ozsahin et al. 2008). HSCT from alternative donors (partially HLA-matched relatives) has led to disappointing results, with 5-year overall survival of only 55%, which dropped to 25% when patients over 2 years of age were analyzed (Table 4). When successful, HSCT can lead to restoration of platelet counts and immune functions. In case of stable mixed chimerism, clinical conditions can also be improved, although a number of these patients show recurrence of clinical symptoms later on. In contrast to SCID, in which severe impairment of hematopoietic precursor differentiation conveys space and a selective advantage for engrafted progenitors with the potential for normal differentiation, the differentiation of hematopoietic cells in WAS is not blocked since all mature subsets are present in the blood of patients, although at reduced levels (platelets, B cells and T cells). Therefore, successful HSCT in WAS patients requires myeloablative conditioning before transplantation, usually performed with a combination of alkylating agents (Busulfan + Cyclophosphamide + ATG, Fludarabine + Melphalan + ATG/Alemtuzumab).

Table 1 5-year survival after allogeneic HSCT in WAS patients according to donor type

Parameter	HLA-Identical Siblings	Mismatched related donor	MUD
Overall Survival			
	87%	52%	71%
Overall survival according to age at the time of HSCT			
<5 Years	90%	53%	84%
>5 Years	79%	38%	<30%
Overall survival according to performance score at the time of HSCT			
< 90%	94%	41%	70%
≥ 90%	96%	61%	71%
Overall survival according to pre-HCST infection at the time of transplant			
No	84%	59%	71%
Yes	100%	35%	55%

from (Filipovich, et al. 2001)

Table 2 Efficacy of HSCT in a large cohort of WAS survivors

Disease Status	HLA-Identical Siblings	Mismatched related donor	MUD
Cured	73%	57%	73%
Improved	20%	24%	27%
Unchanged	7%	5%	0%

from (Filipovich, Stone et al. 2001)

Table 3 Overall survival (OS) and failure-free survival (FFS) 5 years after stem cell transplantation in WAS patients, according to donor type and age at the time of transplant

	OS	FFS
HLA-Identical Siblings (n= 67)	81.8%	64.3%
Mismatched related donor	37.5%	37.5%
MUD BMT	80%	75.2%
MUD CORD	80%	71.4%
<5 Years	79.8%	72%
>5 Years	53.8%	46.2%

From (Kobayashi, Ariga et al. 2006)

Table 4 Overall survival (OS) 7 years after stem cell transplantation in WAS patients, according to donor type and age at the time of transplant

	OS
HLA-Identical Siblings (n=45)	88%
MUD BMT (n=32)	71%
≤2 Years	82%
>2 Years	55%
Other relative (n= 19)	55%
≤2 Years	75%
>2 Years	25%

From (Ozsahin, Cavazzana-Calvo et al. 2008)

In recent years, survival rates after HSCT have improved, in particular in the MUD setting.

In a recent retrospective collaborative study (Moratto et al., 2011), long-term outcome and donor cell engraftment were analysed in 194 patients with WAS, who have been treated by HSCT in the past 30 years. Donor type included matched sibling donor (MSD), mismatched family donors (MMFD), MUD, and umbilical cord blood (UCB). Overall survival was 84.0%, resulting higher for those treated after year 2000 (89.1% 5-year survival). This is probably a result of recent improvement of outcome after transplantation from mismatched family donors and for patients who received HSCT from an unrelated donor at an older age (> 5 years). Younger age was associated to best 5-year survival in patients treated with MUD HSCT (91.9% for pts < 2 years old at time of HSCT; 73.3% for pts > 5 years of age), but it did not significantly affect survival in patients treated by MSD or MMFD. An increase in survival after HSCT from MMFD was also observed in most recent transplant, but the number of patients is relatively small to draw conclusions. Mixed chimerism was associated with an increased risk of incomplete lymphocyte count reconstitution and development of autoimmunity after HSCT. Moreover, persistent thrombocytopenia after HSCT was strongly associated with low or null myeloid chimerism, suggesting that robust and stable engraftment of donor-derived myeloid cells is required to correct this defect, because of the lack of selective advantage for WASp-positive cells in the myeloid compartment.

In another recent paper by Shin et al. (Shin at al., 2012), the outcome of HSCT in 47 WAS patients treated at a single center since year 1990 has been reported. Donor type included MSD, MUD, mismatched unrelated donors (MMUD) and UCB. Outcome improvement after year 2000 was observed also in this study, from a 5-year OS of 62.5% for patients transplanted during years 1990-2000 to 90.8% and 2001-2009, respectively. In multivariate analysis, age was only marginally significant, and the extent of HLA-mismatch did not significantly affect the incidence of acute GVHD, chronic GVHD or survival. Post-transplant autoimmune cytopenias were frequently diagnosed (55% patients), but their occurrence was associated with donor type, acute or chronic GVHD or mixed chimerism.

1.1.6. Rationale for GT in WAS

Allogeneic HSCT from an HLA-identical donor can cure all the pathological aspects related to WAS. However, in the absence of an HLA-matched bone marrow donor, currently available

alternative strategies are to be considered unsatisfying. In fact it has been clearly demonstrated that results with HLA-mismatched, especially T-cell depleted, parental grafts are discouraging, with an unacceptable incidence of transplant related mortality (Filipovich, Filipovich, Stone et al. 2001; Kobayashi, Ariga et al. 2006). Consequently, for patients affected by WAS who lack a suitable stem cell donor, *ex vivo* gene therapy represents an alternative approach, since it is in principle applicable to all patients. In principle, gene therapy has several advantages over allogeneic transplant, including the lack of graft-versus-host disease and reduced toxicity.

In recent years gene therapy via autologous hematopoietic stem cells (HSC) has been used as an alternative therapeutic option in several immunodeficiencies. The successful use of oncoretroviral vectors derived from murine leukemia virus (MLV) to treat patients affected with X-linked severe combined immunodeficiency (Hacein-Bey-Abina et al. 2002) and Adenosine deaminase deficiency (Aiuti et al. 2002; Aiuti et al., 2009) has opened the perspective for application of gene therapy to other immunodeficiencies, including WAS. Indeed, primary immunodeficiencies are the best candidates for gene correction because of the possibility to use HSC as targets, the potential *in vivo* selective advantage of the corrected cells and the possibility to correct the immune defect even when partial engraftment of engineered cells occurs. There is a consensual agreement that also in the case of WAS, the optimal cell target to deliver the healthy copy of the WAS gene is the hematopoietic stem cell. Indeed, the multilineage progeny of HSC engineered to express the normal WAS gene should be functional and able to expand in the treated patients, and to correct the phenotype of WAS.

Preclinical studies suggest that lentiviral vectors (LV) are to be considered superior to the retroviral vectors both for efficacy and safety reasons (Kay et al. 2001). In general, LV derived from HIV provide stable long-term gene expression in vitro and *in vivo* and they present a number of advantages over gamma-retroviral vectors for the transduction of haematopoietic stem cells. From the safety point of view, LV are based on a self-inactivating configuration in order to minimize the risk of producing replication-competent lentivirus (RCL), they can be adapted to incorporate the use of a physiological gene promoter, and are likely to possess a safer integration profile as compared to retroviral vectors. Lentiviral vectors have recently entered the clinic with wide-ranging applications, as several trials are ongoing or are beginning in Europe and US to treat HIV infection, neurodegenerative syndromes or genetic diseases such as thalassemia.

1.1.6.1. Exploiting the selective advantage for WAS-expressing cells

It is commonly accepted that a selective advantage for the transduced cells is a key factor for the success of gene therapy and various lines of evidence suggest that restoration of WASP gene expression by lentiviral vectors in WASP-deficient cells may also confer a selective advantage.

In heterozygous females with a nonsense WASP mutation, the pattern of X chromosome inactivation occurs in a non-random manner during the migration of early haematopoietic progenitor cells from fetal liver to bone marrow, suggesting a chemotactic (or survival) advantage for haematopoietic progenitors and stem cells with a normal WASP expression (Lacout et al. 2003). In addition, recently, it has been described that WASP expression confers a selective advantage to the development of hematopoiesis dependent on the receptor c-Kit (Mani et al., 2009). This finding suggests that WASP transduced progenitor cells, which are exposed during gene transfer to stem cell factor (c-Kit ligand), may preferentially survive and/or expand in vitro and in vivo.

Furthermore, somatic mosaicism has been reported to spontaneously occur in T lymphocytes (including regulatory T cells) and more unusually in B and NK cells of WAS patients, leading to a milder clinical phenotype (Wada et al. 2001; Konno et al. 2004; Lutskiy, Rosen et al. 2005; Humblet-Baron et al. 2007). A selective advantage of gene-corrected cells over the null cells has also been observed in T lymphocytes both *in vitro* and in bone marrow reconstitution experiments in mice (Klein et al. 2003; Dupre et al. 2004). Thus, a proliferative advantage is expected to be conferred by WASP expression in the lymphoid compartment following gene therapy in WAS patients. Further indirect evidence for a strong survival or proliferative advantage of WASP-expressing lymphocytes comes from studies of mixed chimera status in WAS patients after HSCT (Yamaguchi et al. 2002).

A recent analysis of the possible advantage for WASP-expressing cells over WASP-negative cells in a competitive setting of WASP +/- mice has been documented by Westerberg et al (Westerberg, de la Fuente et al. 2008) providing insight into which lineages are more likely to be corrected by gene therapy.

The authors demonstrate that WASP is important for the development and maturation of T-cell and B-cell lineage cells, but the selection for WASP-expressing myeloid cells appears less robust than in other lineages, although in humans expressions of WASP appears to confer a significant advantage already in hematopoietic progenitor cells (HPCs), as previously shown in carrier females.

Although the lack of donor myeloid engraftment is associated with increased risk of persistent thrombocytopenia after allogeneic HSCT (Yamaguchi, Ariga et al. 2002; Pai, DeMartiis et al. 2006), data in heterozygous WASP +/- mice have shown that a moderate excess of WASP+ platelets was present in the circulation. While it remains to be seen whether such increase reflects advantage in the number and/or differentiation of WASP-expressing megakaryocytes or an increased peripheral consumption of WASP-negative platelets, these data represent an encouraging result in view of the potential use of gene therapy in WAS.

1.1.6.2. Choice of conditioning regimen and cell dose

We envisage to administer a reduced intensity conditioning regimen assuming that a stable mixed chimerism (with gene corrected and uncorrected cells) in bone marrow and peripheral blood will be sufficient to provide clinical benefit with a low regimen-related toxicity. We estimate that partial engraftment of corrected cells in the bone marrow, resulting from a reduced intensity conditioning, will lead to an engraftment of corrected cells in the peripheral compartment sufficient to provide improvement of immune and hematological functions.

Based on our experience in combining *ex vivo* gene therapy with low doses of intravenous Busulfan in children affected by ADA-SCID, we will adopt iv Busulfan as myelotoxic drug (Aiuti et al., 2002).

The total dose of Busulfan will be increased as compared to the ADA-SCID trial in which lower levels of corrected myeloid cells are sufficient to lead to clinical benefit. In order to achieve correction of the lymphoid compartment, and especially T cells, we will complete the conditioning regimen with Fludarabine to target lymphoid progenitors and mature lymphocytes. Indeed, the mechanisms of selective advantage in WAS are different than the ones in ADA-

SCID. In ADA-SCID patients treated by gene therapy, the selective advantage takes place during differentiation of HSC into mature B cells and T cells.

In contrast, the studies on WAS patients with mixed chimerism following HSCT or with revertant mutation in the T cell compartment indicate that the selective advantage for WASP+ cells might take place mainly through peripheral expansion upon antigen triggering rather than during thymic differentiation (Wada, Schurman et al. 2001; Yamaguchi, Ariga et al. 2002).

An additional contribution to the selective advantage of corrected T cells might be the previously reported susceptibility to apoptosis of WASP-deficient cells (Rawlings et al. 1999).

Based on these considerations, a low dose of Fludarabine will be optimal to break the homeostasis in the compartment of early lymphoid progenitors in order to boost, at an early stage of differentiation, the expansion of genetically corrected T cell precursors.

In addition, through its activity on the naïve T cell compartment, Fludarabine will favor the establishment of a pool of corrected naïve T cells in the periphery. The selective advantage described above should favor the expansion of genetically corrected memory T cells, during encounter with antigens.

We will also introduce in the conditioning anti-CD20 monoclonal antibody (Rituximab), a biological agent that depletes peripheral B cells and has been shown to provide a clinical benefit in several autoimmune disorders. Anti-CD20 will serve as depleting agent for B cells and particularly of autoreactive cells, thereby facilitating the engraftment and expansion of gene corrected B cells expressing WASP. In addition, anti-CD20 will act as pre-emptive treatment for lymphoproliferative disorder due to EBV, which represents a high risk factor for the development of lymphoma in WAS patients.

Another important consideration is the presence of self-reactive T cells responsible for autoimmune complications in WAS patients. For patients with autoimmune manifestations, the use of ATG Thymoglobuline as an additional lympho-depleting agent may be considered to eliminate auto-reactive memory T cells.

Based on these considerations, we plan to use iv Busulfan (a total dose of 6.4-9.6 mg/kg adjusted for patient's weight and AUC targeted) and Fludarabine (at total dose of 60 mg/sqm) as preparative regimen prior to gene therapy, associated to a single dose of Rituximab (at the dose of 375 mg/sqm).

A detailed description of the conditioning regimen is presented in Section 10.5 ,Table 7 .

In case the conditioning regimen described above will not produce mixed chimerism associated with clinical benefit, the protocol will be amended to include fully myeloablative conditioning. This may include re-treatment of the patients originally conditioned with the reduced intensity protocol one year after the first gene therapy treatment. The choice of the regimen will depend also on the results of the parallel trials which will be adopted different full conditioning regimens: the UK trial for WAS gene therapy and the TIGET trial for gene therapy in metachromatic leukodystrophy (MLD).

A direct correlation between cell dose and engraftment is a well-known phenomenon in hematopoietic transplant medicine and has been established also for ADA-SCID gene therapy trial (Aiuti et al., 2009). A minimum of 2 x 10^6 CD34+ cells/kg is usually required to safely perform an autologous hematopoietic stem cell transplant. Therefore, a dose of 2 x 10^6

transduced CD34+ cells/kg is the minimum target dose at infusion in our study, and we will aim at infusing 5-10 x 10^6 CD34+ cells/Kg. The goal is to collect a slightly higher number of cells than usually recommended for a conventional un-manipulated transplantation because of the subsequent procedures (mononuclear cell (MNC) purification, positive selection, transduction and QC). Therefore, we will harvest approximately 20-30 bone marrow (BM) ml/kg (max 35 ml/kg) patient body weight with a minimum target $CD34^+$ cell count (before any manipulation) of 5 x 10^6/kg. In the case of use of mobilized peripheral blood (MPB), leukapheresis will be performed to achieve a minimum target $CD34^+$ cell count (before any manipulation) of 5 x 10^6/kg.

1.1.6.3. Choice of lentiviral vector

Initial studies using oncoretroviral MLV-based vectors for *exvivo* gene therapy of WAS have demonstrated the feasibility of gene transfer in WAS patient cells (Candotti et al. 1999; Dupre, Aiuti et al. 2002; Strom et al. 2003) or in hematopoietic progenitor cells of WAS null mice (Klein, Nguyen et al. 2003; Strom, Turner et al. 2003). However, the requirement for high-frequency HSC transduction positions lentiviral vectors as prime candidates for HSC gene transfer in gene therapy for WAS. Indeed, LV offer a unique combination of advantages for the proposed application: i) they integrate efficiently into a variety of cell types *ex vivo* and *in vivo*; ii) they allow stable and robust transgene expression; iii) they significantly alleviate the safety concerns associated with RV integrations. Because of these features, LV enable efficient gene marking of mouse and human HSC (the latter ones being assayed in mouse xenografts as non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice with minimal in vitro manipulation and cell perturbation, allowing for full maintenance of stem cell properties and multiclonal repopulation of chimeric hosts (Miyoshi et al. 1999; Guenechea et al. 2000; Guenechea et al. 2001; Ailles et al. 2002). As detailed below, LV have recently been approved for clinical testing.

The vector selected for the WAS gene therapy trial contains as internal promoter the 1.6 kb-long sequence of the WAS gene endogenous promoter. In the context of a LV, the WAS promoter appears weaker in driving WAS mRNA expression than other internal promoters, such as EF1-alfa. Nevertheless, it leads to optimal expression of the protein (Charrier et al. 2007), thereby suggesting an optimal regulation of expression by the WAS promoter. This regulatory element restricts transgene expression mainly to hematopoietic cells (Dupre, Trifari et al. 2004), thus limiting the possibility of transgene toxicity to this system.

In summary, the lack of satisfactory therapeutic options for many patients affected by WAS with no HLA identical donor, together with experimental proofs of concept and a relatively good understanding of the molecular basis of this disease, provide a strong rationale for designing a gene therapy approach to WAS based on lentiviral vector technology.

1.1.7. Telethon-Généthon common efforts in the development of lentiviral vector gene therapy for WAS

The Medicinal Product was developed to the clinical application under a collaborative agreement between the non-profit funding agencies Telethon and Généthon. Généthon is an Institute located in Evry (Paris) sponsored by the non-profit organization AFM. Généthon has obtained Orphan Drug Designation from EMA (EU/3/05/345, 24/01/2006 “lentiviral vector containing the human

WASP gene"). The manufacturing, purification process and the specifications of the vector developed at MolMed (for Telethon) are very similar to the ones developed at Généthon. We acknowledge the contribution of preclinical data from investigators at Généthon (Anne Galy) and Great Ormond Street Hospital (Adrian Thrasher) for this application. A different clinical trial sponsored by Généthon is being conducted at Necker Hospital, Paris (PI: Marina Cavazzana Calvo), UCL Institute of Child Health, London (PI: Bobby Gaspar) and Children's Hospital Boston (PI: David Williams) with the same lentiviral vector. A myeloablative conditioning will be used to prepare the patient to the infusion of transduced CD34+ cells. The results of these clinical trials will be useful to compare the effects of the different conditioning protocols and obtain crucial information that could be used to modify the clinical trial in case of toxicity or lack of engraftment.

Following the submission of this protocol, Fondazione Telethon has received its own orphan drug designation (ODD) for Telethon003 (Autologous CD34+ cells transfected with lentiviral vector containing WAS cDNA ODD#10-3043) in the US on April 30th 2010 for the treatment of WAS. An ODD for the same medical product was also obtained in Europe on 06/06/2012 by EMA (European Medicines Agency).

1.1.8. Ethical considerations

The rare nature of the condition, the pediatric target population and the specificity the gene therapy treatment pose critical ethical questions that were addressed following the recent guidelines issued by the ad hoc group of pediatric clinical trials for the implementation of the EMA Directive 2001/20/EC (2008).

Based on the above considerations, the clinical course and therapeutic options in WAS indicate that the objective of developing alternative therapies for WAS appears not only to be justified, but desirable in ethical terms. Although the transplantation of allogeneic HSC is a curative therapy, this option is limited to those patients who have access to a compatible donor. In addition, allogeneic HSC transplant is associated to severe risks for the patient and, therefore, is usually limited to those patients with a severe clinical course of the disease.

Moreover, there is general consensus that gene therapy is an ethically justified approach for the treatment of severe diseases, considering the previous successful attempts in primary immunodeficiencies.

The results of the preclinical studies with the medicinal product, as well as the preliminary results of a clinical trial with similar products (same vector for a different condition or different vector for the same condition) provide evidence of the biological activity and efficacy of genetically corrected autologous HSC transduced with a lentiviral vector, therefore supporting a reasonable expectation of a benefit for the individual participating in the study.

The risks associated with the procedures have also been carefully analyzed. The risks associated with the procedure (bone marrow harvest, conditioning, cell reinfusion) are not superior to those expected from a standard conditioning regimen for transplant of allogeneic HSC, and actually are expected to be lower, considering the reduced conditioning regimen. The specific risks related to the use of lentiviral vector transduced cells are discussed extensively below in a dedicated session. The vector technology and vector design employed in this study is believed to be the most updated, reliable and safe currently available method for stable vector integration in the

human genome. The extensive preclinical studies conducted in vitro and in mice for long-term period of observations by our group and our collaborators have not shown any adverse event, including clonal proliferation. Nevertheless, the patient and/or patient's proxy will be extensively informed about the potential risks of gene transfer, which are inherent to the use of integrating vector.

In summary, the experimental treatment proposed can be classified under the category of "Greater than minor increase over minimal risk with benefit for the individual that is especially favorable in relation to available alternative approaches for the individual", according to the ad hoc group guidelines for the implementation of pediatric clinical trials of the EMA. In the case of the proposed WAS gene transfer protocol, the risk-benefit evaluation is in favor of the enrollment and treatment of patients with a severe condition, in the absence of a suitable donor for allogeneic HSC.

2. PRODUCT MANUFACTURING AND CHARACTERIZATION

2.1. Medicinal product definition

Drug product (DP) is defined as autologous CD34+ cells genetically modified with a lentiviral vector (LV) encoding for the human WAS cDNA resuspended in their final formulation medium.

Medicinal product manufacturing and release and LV supernatant production will be performed at MolMed S.p.A. (Molecular Medicine S.p.A., Via Olgettina 58, 20132 Milano), a GMP (Good Manufacturing Practice) certified facility authorized by Italian Ministry of Health according to D.L.vo 166/2003 for the production of sterile medicinal products, the production of specific medicinal products obtained with particular technologies (i.e. gene therapy), and for primary packaging operations.

2.2. Lentiviral vector encoding for WASP

According to the draft revision of Annex I Part IV of 2001/83 Directive, the drug substance (DS) is defined as autologous CD34+ cell enriched population that contains haematopoietic stem and progenitor cells (HSPC) transduced *ex vivo* using a lentiviral vector encoding the human (WAS) gene.

The LV proposed for this application is a pseudo-type vector made by a core of HIV-1 structural proteins and enzymes, the envelope of the VSV and a genome containing HIV-1 cis-acting sequences, no viral genes and one expression cassette for the WAS transgene under the control of the WAS 1.6Kb promoter. The 3 vector components (core, envelope and genome) are transiently expressed in vector producer cells by 4 different constructs: 2 core packaging constructs, the envelope construct and the transfer vector construct. Only the transfer vector transcript is transferred and integrated into the target cells. The latter has been optimized for maximal transduction efficiency and stable constitutive transgene expression in target cells.

The proposed LV is replication-defective by design. No viral genes are transferred to target cells. Because the vector is pseudotyped by the envelope of an unrelated virus, wild-type HIV cannot be generated by recombination among the constructs used to make vectors. Moreover, the lack of homology between the envelope and the core packaging sequences makes recombination highly unlikely between these constructs (Vigna and Naldini 2000). The main safety concerns associated with *ex vivo* administration of LV are: i) contamination by replication competent lentiviral recombinant, ii) mobilization and recombination of the vector with wild-type virus in infected hosts, iii) insertional mutagenesis.

The combination of the third-generation packaging system with a SIN vector expressed in producer cells by a chimeric 5'LTR (long terminal repeat) provides an advanced multi-split design (four constructs are used to make vector) expressing only a fractional set of HIV-1 genes (three out of nine in the wild-type virus: *gag, pol* and *rev*) with two built-in safety mechanisms that would interfere with the generation of an unlikely RCL: expression of the *gag pol* gene conditional on trans-complementation by a separately expressed Rev protein, and self-inactivation of the vector LTR.

Concerning the risk of insertional mutagenesis, several factors suggest a lower risk for LV than for conventional onco- or gamma-retroviral vectors. Firstly, despite the extensive replication of

HIV-1 in chronically infected immuno-compromised hosts, there is no evidence of increased oncogenesis directly linked to viral integration.

Secondly, HIV-1 integration site selection does not show a bias for the promoter regions of transcriptionally active genes or for gene subsets involved in growth control and oncogenesis as observed for MLV-based vector (Schroder et al. 2002; Wu et al. 2003; Mitchell et al. 2004). These features suggest a lower chance for LV to activate oncogenes at the integration site (De Palma et al. 2005).

Thirdly, the lack of transcriptionally active LTR, combined with a moderately active internal promoter to drive transgene expression, is likely to be a major safety advantage of the proposed vector. As described below in the preclinical studies of this application, we have challenged the predicted safety of the proposed vector design by transducing HSPC from a tumor-prone mouse model in conditions representative of those proposed for the clinical trial with either the SIN-PGK LV or prototypical MLV-based RVs and provided direct experimental evidence that integration of a SIN LV (with an internal PGK promoter) has low to undetectable genotoxic potential.

2.3. Establishment of MCBs

Vector plasmids are produced by incorporation into *E. coli* and a Master Cell Bank (MCB) is generated for each plasmid after selection. Each *E. coli* MCB is tested for a number of parameters among which viability, microbial purity, identity, and plasmid retention. Each plasmid preparation is suitably characterized to confirm identity, sterility, purity and to assess the level of contaminants and the stability.

A subclone of the human kidney 293T cell line (from Stanford University) originally selected at the Salk Institute (La Jolla, CA, USA) for its high yield performance in production of LV by transient transfection is used for MCB establishment. 293T cells were chosen as the cell line for LV production because of the vastly superior performance (10-fold) as compared to 293 cells in pilot experiments performed at SR-TIGET, MolMed and Genethon laboratories.

The MCB was generated at Genethon; the entire cell banking procedure, including formulation of the culture media, equipment cleaning, and all cell manipulations, was performed in GMP conditions, according to Standard Operating Procedures (SOP). The MCB was tested for the absence of microbial and viral contaminants, and for purity according to international guidelines (PTC in the characterization of cell lines used to produce biological – 1993, ICH Topic Q5D, Note for guidance on the quality, preclinical and clinical aspects of gene transfer medicinal products – EMA/CPMP/BWP/3088/99).

2.4. Lentiviral Vector production

The LV supernatant is produced by transient quadri-transfection of the producer cells 293T with GMP plasmid preparations encoding for two core packaging constructs (gagpol and rev), the envelope construct (VSV-G) and the transfer vector construct, followed by a vector purification process involving DNase (Benzonase) treatment, anion exchange chromatography, concentration by ultra-filtration, gel filtration and final sterilizing filtration. Each batch of LV will be characterized for viral particles and titer, infectivity, viral proteins, vector integrity and identity, for the presence of selected impurities related to the producer cells or the manufacturing process

and for the absence of microbial contaminants (bacteria, fungi, mycoplasma and viruses) and RCL.

LV production is performed at Molmed S.p.A., as described in the investigational medicinal product dossier (IMPD).

2.5. Production of transduced CD34+ cells

The final medicinal product is made of LV-transduced CD34+ cells expressing human WASP resuspended in the final formulation medium. CD34+ cells will be purified from bone marrow aspirates and/or from mobilized peripheral blood.

Transduced cell production is performed at Molmed S.p.A., as described in the IMPD. The production of WASP transduced CD34+ cells will be divided into the following steps.

2.5.1. Cell isolation

2.5.1.1. Isolation of BM CD34+ cells

Bone marrow harvest (minimum 5, maximum 20 x10^6 CD34+/kg) will be diluted, stratified with lymphoprep and centrifuged in order to separate blood fractions. A cell suspension primarily made of mononuclear cells (MNC) will be collected. The cells will be tested for viability and cell concentration. MNC thus obtained will be labeled with magnetic beads by 30 min. incubation at 5°C mixing 200 µl CD34+ reagent (CD34 Microbeads, Milteny Biotec) with 100x10^6 MNC/ml. At the end of the incubation, the cells will be washed and MNC loaded on the magnetic column device. CD34+ cell positive and negative fractions will be then eluted from the column and assayed for sterility, viability and cell number, purity and clonogenic potential (see Investigator's Brochure).

2.5.1.2. Isolation of mobilized peripheral blood CD34+ cells

Autologous leukoapheresis product will be transferred in two or more 250 ml tubes and then centrifuged at 1000±50 rpm for 30±5 minutes at room temperature to remove plateles from mononuclear cells (MNC). The cell pellet, primarily made of mononuclear cells (MNC), will be re-suspended and tested for viability and cell concentration. MNC thus obtained will be labeled with magnetic beads by 30 min. incubation at room temperature mixing 200 µl CD34+ reagent (CD34 Microbeads, Milteny Biotec) with 100x10^6 MNC/ml. At the end of the incubation the cells will be washed and MNC loaded on the magnetic column device. CD34+ cell positive and negative fractions will be then eluted from the column and assayed for sterility, viability and cell number, purity and clonogenic potential.

2.5.2. Transduction of CD34+ cells

On day -3 of the production process**,** purified CD34+ cells will be seeded at final concentration of 1x10^6/ml in serum free medium (Cell Gro) supplemented with the cytokines SCF, TPO, FLT3-L and IL-3, in gas-permeable bags coated with retronectin, and incubated for 24 hours at 37°C in 5% CO_2 atmosphere. On day -2 and -1, the LV (1x10^8TU/ml, multiplicity of infection (MOI) 100) will be added to the cell suspension and incubated for 16 hours (over-night) (first and second round of transduction). On day 0, at the end of the transduction, cells will be washed

and re-suspended in the final formulation medium to obtain the final Drug Product. Aliquots will be retained for the Quality Control characterization (see Investigator's Brochure for detailed list of QC tests and specifications). Since the medicinal product is freshly infused, the interval time prior to infusion does not allow the execution of all Quality Control (QC) tests, therefore the results for some of them will be obtained only after patient treatment. In order to maximize the number of QC tests results available at the time of infusion, the manufacturing process is put on hold for few hours at the Drug Substance stage and aliquots for most of the QC tests are retained at this stage. Results for viability, immunophenotype, endotoxin and mycoplasma tests will be available before infusion (see the Investigator's Brochure).

The outlined QC strategy has been designed taking into consideration the peculiar nature of the manufacturing process, and the definitions of the Drug Substance and the Drug Product reported above. In this light, the manufacturing process can be considered as a single intervention starting from the collection of the cells from the patient up to the re-injection and, therefore, the DP can be considered equivalent to the DS as only a change in the formulation medium is performed.

If an Out Of Specification (OOS) occurs, responsible physicians will be immediately informed.

Before starting the production of clinical batches, the validation of the manufacturing process will be completed repeating three times the whole production process.

2.6. Preclinical studies

2.6.1. Introduction

A large body of safety and efficacy preclinical data has been generated in the past decade for the use of retroviral and lentiviral vectors encoding the Wiskott-Aldrich syndrome protein (WASP). A summary of the available preclinical safety and efficacy studies is provided below.

Studies conducted using onco-retroviral vectors encoding WASP under either an internal promoter or the LTR demonstrated *in vitro* correction of TCR-driven activation, immunological synapse assembly, restoration of WASP expression and actin cytoskeleton architecture in both T and B cells. *In vivo* studies conducted in was$^{-/-}$ mouse model resulted in the differentiation of WASP positive lymphocytes with restored TCR-driven proliferation. Furthermore, an *in vivo* selective advantage for gene-corrected lymphocytes was also observed (Klein, Nguyen et al. 2003).

More recently, a number of third generation lentiviral vectors encoding WASP under the control of constitutive or autologous promoters have been tested *in vitro* and *in vivo* in the was$^{-/-}$ mouse model.

Re-expression of WASP was demonstrated *in vitro* in different patient hematopoietic cell lineages (T, B and DC). T cell proliferation, IL-2 secretion, F-actin polarization and correction of DC cytoskeleton defects in DC, as well as improvement of colitis were observed *in vivo* along with a confined expression of WASP to the hematopoietic compartment, when expressed under the autologous promoter, and a selective advantage for corrected lymphocytes. Overall, the results obtained from these preliminary studies along with the absence of any short and long term adverse reactions in treated animals indicate that gene therapy treatment for WASP is a feasible, well tolerated approach and prompted the selection of the final WASP vector for preclinical studies.

2.6.2. Preclinical studies performed with the proposed therapeutic vector

2.6.2.1. In vitro studies

2.6.2.1.1. T cells

Over the past few years, we have tested the efficacy of *WAS* gene transfer with the w1.6W LV in T cells from numerous WAS patients. Transduction levels ranged from 20 to 80% depending on vector concentration used for transduction (MOI 10 = $1x10^7$ TU/ml to MOI 100 = $1x10^8$ TU/ml). Transduction levels were comparable for T cells of each patient. Importantly, the percentage of transduced T cells expressing WASP progressively increased during long-term (9-30 weeks) culture indicating that $WASP^+$ T cells have a selective growth advantage over $WASP^-$ T cells *in vitro*. This phenomenon was very reproducible since it was observed in 11 out of 11 transduced cell lines kept in long-term cultures. WASP expression in transduced cells reached normal levels and no over-expression was observed. Correction of functional defects including TCR-driven proliferation and IL-2 production was reproducibly achieved after transduction at the dose of $1x10^8$ TU/ml, which resulted in approximately 1-3 vector copy per cell. Clones of transduced $CD4^+$ T cells were generated to determine the relationship between number of integrated vectors, WASP expression levels and functional correction. One to two vector copies of the WASP-encoding vectors were sufficient to restore WASP expression and IL-2 production. Importantly, no evidence for toxicity (abnormal proliferation, IL-2 independent growth, abnormal morphology) could be observed in long-term cultures of transduced T cell clones or bulk cell lines (up to 30 weeks).

2.6.2.1.2. B cells

Our collaborator A. Galy (Généthon) tested *WAS* transgene expression in WASP-negative EBV-transformed B cell lines from 3 WAS patients following transduction with the w1.6W LV. Cells were analyzed 8 days post-transduction to evaluate WASP mRNA and protein expression using respectively a quantitative real-time RT-PCR assay, intracellular fluorescence-activated cell sorting (FACS) detection or Western blotting. Levels of *WAS* mRNA and protein expression correlated with the number of integrated copies per cell, leading to restoration of WASP expression in B cell lines.

2.6.2.1.3. Dendritic cells

Our collaborators at Généthon also tested *WAS* transgene expression in monocyte-derived DC. These cells were prepared from the blood of 2 WAS patients by culture of adherent blood cells in the presence of GM-CSF and IL-4 for 2 weeks. Cells were transduced with the w1.6W vector at the dose of $1x10^8$ TU/ml. Quantitative RT-PCR showed that w1.6W vector induced *WAS* mRNA expression in these preparations of cells compared to untransduced cells. Functional correction was documented by the restoration of podosome formation in transduced DC.

Collectively, these data demonstrate that transduction of T cells, B cells and DC from WAS patients with the w1.6W vector leads to WASP expression and to functional correction. Importantly no toxic effect or event of transformation was documented in any of the models tested.

2.6.2.1.4. CD34+ cells

As a prerequisite for the development of a gene therapy protocol for WAS, the safety and efficacy of WAS gene transfer into HSC isolated from WAS patients had to be validated. This has been reported recently using both a retroviral vector and a lentiviral vector (Dewey et al. 2006; Charrier et al. 2007) and confirmed with GMP-grade LV. Results with the w1.6W lentiviral vector indicate that WAS HSC can be normally purified from the bone marrow and can be transduced at high levels, while retaining *ex vivo* survival rates and ability to form colonies similar to those of control HSC. The restoration of WASP expression was demonstrated in the differentiated progeny of WAS HSC by Western Blot. To assess if WASP over-expression could be toxic in HSC, we tested the effect of vector-driven WASP expression in normal HSC in vitro. No perturbation of *in vitro* differentiation of hematopoietic stem cells from healthy donors was observed following transduction. In addition to hematopoietic cells, we found that WASP was expressed also in normal human endothelial cells (which can express the CD34 antigen), but transduction with w1.6W LV did not result in increased expression of the endogenous WASP. Collectively, these results demonstrate the feasibility of lentiviral vector-mediated *ex vivo* WAS gene transfer into hematopoietic cells.

2.6.2.2. *In vivo* studies

In vivo proof of principle data were obtained using 2 different WAS KO mouse models. Experiments were carried out in collaboration between TIGET and Généthon. Murine Lin– cells transduced at different viral MOI with 1 or 2 hit transduction procedure were re-infused in sex-mismatched animals. Mice were followed for up to 12 months post-transplantation and monitored for a number of different functional parameters.

2.6.2.2.1. Long-term engraftment and WASP expression

We have generated solid evidence of long-term engraftment and efficacy of the gene therapy approach based on the w1.6W lentiviral vector in $Was^{-/-}$ transplanted mice (Marangoni et al., 2009). Fifty transplanted mice were followed for up to 12 months. Engraftment and transduction efficiency, vector copy number, functional correction and blood counts were evaluated at different time points throughout the observation period. A full size transgenic WAS protein was produced in mice cells as assessed by Western Blot and FACS analyses. High, stable and multilineage donor cell engraftment and WASP expression were achieved up to 12 months after gene therapy. Noteworthy, a selective advantage for T and B lymphocytes expressing transgenic WASP was observed.

2.6.2.2.2. Safety in the mouse model

Autopsy and histopathology examination were performed at sacrifice. None of the analyzed parameters revealed side effects related to the treatment. Survival rate of the treated mice was within the normal range and the observed hematopoietic tumors (4) were of host origin and did not contain vector sequences. Such events were also seen in KO mice treated with untransduced Lin- cells and were therefore not attributable to a side effect of the gene therapy procedure.

In order to further evaluate if the WASP-encoding vector is associated with *in vivo* tumorigenesis, secondary transplantation experiments were designed and performed at our collaborating site (Généthon). A total of 32 recipient mice were irradiated and infused with bone

marrow cells obtained from recipients of primary transplants with transduced cells. Mice were observed up to 6 months. Multi-lineage and long-term persistence of the w1.6W vector could be measured, attesting that long-term hematopoietic stem cells were initially transduced with the vector and that the vector did not prevent them to function for extended periods of time. No tumors of donor/transduced cell origin were observed. In conclusion, we documented successful engraftment of transduced cells in primary and secondary transplantation protocols. The study design and analytical methods allowed the detection of tumors in the mice, and there was no evidence of clonal expansion or malignancy induced by the treatment with the WASP-encoding vector, no evidence of hematopoietic toxicity from the WASP-encoding vector, and no evidence of toxicity from the WAS transgene as expressed in this system.

2.6.2.2.3. Efficacy in the mouse model

Ex vivo gene therapy in the *was*$^{-/-}$ murine model allowed long-term correction the T cell defects, restoring anti-CD3 proliferation and production of various Th1 and Th2 cytokines (Marangoni et al., 2009). B cell-counts were improved in mice receiving Lin- cells transduced at high MOI. Moreover, *ex vivo* gene therapy resulted in correction of the defect in B cell-migration in response to CXCL13 and improvement of antibody response to polysaccharides (Pneumococcal vaccine) at 4-5 months after gene therapy as compared to untreated was-deficient mice. Importantly, mice treated with LV gene therapy showed restoration of cytoskeletal functions in myeloid cells, as assessed by the presence of podosome-forming cells in dendritic cells differentiated *in vitro*, at levels of 40-100% of normal. Although Was$^{-/-}$ mice show only a moderate defect in platelet count, we evaluated the effect of gene therapy on platelets. Transplant of Lin- cells transduced at high MOI, but not low MOI, resulted in significant improvement in platelet count to levels achieved with transplant of wild type Lin- cells.

Mice deficient in WASP in the 129/Sv background develop a TH2-mediated autoimmune colitis, with a severe colitis developing after irradiation and transplant of WAS-KO cells. Data generated at Gènethon showed that the colon of mice transplanted with cells transduced with the WASP-encoding vector was significantly ameliorated and only focal or mild defects persisted in some mice; in one set of experiments diarrhea was measured and a reduction was observed in the animals treated with gene therapy.

In summary, cumulative results from gene therapy in the murine model show that the proposed w1.6W vector is capable of significant biological correction of multiple disorders.

2.7. Studies and considerations on safety related to the medicinal product

2.7.1. Biodistribution and vector shedding

It is expected that the biodistribution of the autologous transduced CD34+ cells reflects that of untransduced HSPC, which migrate to the lympho-hematopoietic organs of conditioned transplant recipients. In addition, the hematopoietic progeny of the engrafted HSC will also infiltrate non-hematopoietic tissues to become resident or migrating extra-vascular hematopoietic lineage cells. Biodistribution studies were performed in RAG2-/-IL-2rγc-/- neonate mice, which represent a suitable model to study engraftment and differentiation of human HSPC (Traggiai Chicha et al. 2004). Human derived umbilical cord blood (UCB) CD34+ cells were transduced with clinical grade vector according to the manufacturing procedure established for the clinical

trial. The transduction efficiency was on average 72% as assessed in a CFU-C assay and the mean integrated VCN on cultured cells was 1.5. After transduction, $0.3x10^6$ human CD34+ UCB cells were transplanted into each RAG2-/-IL2r-γc-/- neonate mouse. This dose corresponds to $1x10^8$ CD34+ cells/kg, thus largely exceeds the $5x10^6$/kg average dose that will be administered to patients. Control animals were transplanted with untransduced (UT) or unmanipulated (UM) UCB cells, transplanted in equal doses. Mice were euthanized 2 months post-infusion and their lympho-hematopoietic organs (BM, liver, spleen, thymus) were collected for further studies. In addition, brains and gonads (as representative non-hematopoietic organs) were collected from male mice after perfusion with saline solution.

We found no differences in the biodistribution of the UT, UM and transduced human UCB cells, including CD34+ cells in the BM. A normal differentiation pattern into T cells was observed in the thymus and differentiated B cells were normally represented in the BM and spleen of mice treated with transduced human HSPC. qPCR (quantitative polymerase chain reaction) documented the presence of LV in hematopoietic organs of all mice transplanted with transduced CD34+ cells. The results obtained from both brain and gonads for LV and human genome sequences were below the sensitivity threshold of the assay (< 16 copies in 10^5 cells).

Upon transduction, the vector becomes stably integrated as a provirus within the chromatin of target cells. Because of the replication-defective nature of the vector, there is no possibility of spontaneous mobilization of the provirus from the transduced cells unless helper functions are also provided in the transduced cells either by recombination during vector production or by super-infection with wild-type HIV in an infected host. Both of these possibilities are extremely unlikely, because: 1) the multi-split design of the packaging system and the lack of homology between the envelope and transfer vector genome make extremely unlikely that a replication-competent recombinant be generated during production; 2) the self-inactivating feature of the vector LTR prevents transcription from the proviral LTR and thus prevents vector mobilization even in the case of recombination with the packaging constructs or superinfection of the transduced cell by wild-type HIV-1, as we have previously reported (Bukovsky et al. 1999). There is however the concern that some infectious vector particles remain associated with the transduced cells and that can be subsequently released to transduce bystander cells as shown with our laboratory-grade vector stocks in CD34+ cells cultured with stromal cells and other Authors using mouse HSPC (Pan et al. 2007). Whereas the amount of vector being released is conceivably limited and thus the chance of off-target transduction is low, the potential release of some infectious vector from the transduced cells after *in vivo* infusion may raise concerns on its biodistribution and the potential transduction of unwanted cell types which are typically associated with *in vivo* vector administration. We have set up experiments specifically aimed at assessing the extent of vector release from previously transduced human HPSC. Our results show that: 1) vector release and bystander transduction can occur but at substantially lower level when using vector stocks from the large-scale production and purification process used for GMP production, as compared to laboratory grade stocks. This difference may be due to the fact that the latter are concentrated by ultracentrifugation without further purification and may contain larger amount of impurity and aggregated particles; 2) exposure of the transduced HSPC to autologous human plasma significantly inhibits bystander transduction, so that it becomes virtually undetectable when using large scale LV stocks. These findings suggest that in the clinical setting, in which the transduced cells are inoculated into the bloodstream of human

patients, the potential occurrence of bystander cell transduction may be reduced to negligible levels.

Moreover, to directly assess the eventual occurrence of bystander cell transduction upon *in vivo* infusion of transduced cells, we have exploited the vector integration studies performed in the organs of *Rag2-/-, Il2r-gamma chain-/-* mice transplanted with transduced HSPC. DNA extracted from the organs of the transplanted mice was analyzed for the content of vector DNA and human genome by qPCR as described above. In the experiment performed we have observed corresponding estimates for: 1) the extent of human cell repopulation estimated by FACS analysis for cells expressing the human hematopoietic marker CD45 and the proportion of human genome over mouse genome in the total DNA extracted from the organ (as estimated by qPCR for human telomerase and mouse b-actin); 2) the vector content *per* human genome in the different organs (as estimated by qPCR for vector sequence versus human telomerase). The results obtained with this strategy, indicate that in the mouse organs tested the vector distributes together with the human cells and that at least the vast majority of the detectable vector genome remains associated with the human genome in stable proportions, thus without showing indication of mobilization or bystander transduction of mouse cells.

In parallel, another PCR able to discriminate whether LV DNA integrated in mouse genome was set up, in order to exclude that a mobilization from human to mouse cells had occurred. This PCR amplifies vector DNA integrated in the proximity of mouse repetitive and highly interspersed B2 SINE family elements (Follenzi et al. 2002). As these sequences constitute approximately 0.7% of total mouse genomic DNA (estimates ranging from 2500 to 100000) and are only found at low abundance in humans (< 50), it is likely that most integrants in the mouse genome are found at a PCR-amplifiable short distance from one such element. The results of these studies have allowed to establish that bystander transduction does not occur above a threshold detection level in several tissues of the mice transplanted with transduced HSPC.

2.7.2. Germline transmission

Because the risk of inadvertent germ line transmission associated with the administration of *ex vivo* transduced human cells is considered “low” or “minimal” according to EMA and ICH, respectively, and animal testing of human cells may be difficult or not meaningful, non-clinical germ line transmission studies of human genetically modified cells are not recommended (EMA Guideline on non-clinical testing for inadvertent germline transmission of gene transfer vectors, EMA/273974/2005 and CHMP/ICH/469991/2006).

Moreover, no evidence of germ line transmission nor of transgene expression in germ cells after *in vivo* delivery of RV and LV into experimental animals have been reported to date. In a study of testicular tubule injection of LV in mice, the sperm collected from recipient testes were able to generate normal pups and none of the offspring carried the transgene (Ikawa et al. 2002). Rat spermatogonial stem cells, however, can be effectively transduced *ex vivo* by LV and generate viable transgenic offspring upon re-implantation into rat testes. Moreover, LV can transduce in vitro fertilized eggs from a variety of species, including mouse, rat and pig. Indeed, LV are becoming common tools for the generation of transgenic animals by in vitro oocyte transduction followed by in utero implant (Park 2007).

Thus, given the concerns for residual infectious vector associated with the transduced HSPC, we have expanded the biodistribution study of vector and HSPC progeny in *Rag2-/-, Il2r-gamma*

chain-/- mice described above to test for the presence of LV genome in the gonads at the time of sacrifice, 2 months post-transplant. For this experiment, we extracted DNA from the gonads and performed: 1) qPCR for vector and human genome content, as described above; 2) nested B2-specific PCR for vector integrated within the mouse genome, as described above. The assays are designed in a way that if any vector DNA is detected in the gonads it should be associated with the human genome, and in corresponding ratio as observed for the other organs of the mouse. Our results show no detectable vector in the gonads of those mice by qPCR, which was also confirmed by nested B2-specific PCR.

Last but not least, we should remind that the concerns for germ line transmission of the genetic modification are unfortunately further mitigated by the severe disability and short life expectancy of the patients to be enrolled in the trial, and by the administration of conditioning regimen which is often associated to the induction of sterility.

2.7.3. Genotoxicity and carcinogenicity

Insertional oncogenesis is a possible consequence of vector integration into the human genome. Several decades of studies of gamma- or onco-retrovirus induced lymphomas/leukemias (Lund et al. 2002; Suzuki et al. 2002; Uren et al. 2005) in mice have shown that proviral insertions near or within proto-oncogenes can trigger their over-expression and/or aberrant transcription and lead to cell transformation. Both mechanisms of oncogene activation are dependent on the strong transcriptional enhancer/promoter elements contained in the viral Long Terminal Repeat (LTR) (Uren, Kool et al. 2005). Initially, the gamma retrovirus-derived vectors (gRV) proposed for gene therapy applications were considered to pose a much lower risk of insertional mutagenesis compared to gamma-retrovirus infection, given the lack of replication and the limited number of integrations occurring during the *ex vivo* transduction procedure. Unfortunately, however, gRV-induced activation of the *LMO2* oncogene and/or other oncogenes resulting in leukemogenesis has now been reported in 5 out of 20 X-linked SCID patients treated with HSPC gene therapy (Hacein-Bey-Abina et al. 2003; Pike-Overzet et al. 2007). Similarly, clonal expansions resulting in myelodysplastic syndrome were ascribed to gRV insertion into the *EVI1, PRDM16* or *SETBP1* (Ott et al. 2006; Ott et al. 2007) proto-oncogenes in 3 CGD patients treated with HSPC gene therapy.

In light of these serious adverse events, the oncogenic risk of vector integration in HSPC had to be re-evaluated (Baum and Fehse 2003; von Kalle et al. 2004) and a great effort went into defining the integration pattern of the different vectors and its impact on endogenous gene transcription (Schroder, Shinn et al. 2002; Wu, Li et al. 2003; Hematti et al. 2004; Lewinski et al. 2006). A better understanding of the risk associated with gRV and LV in HSPC gene therapy is emerging from a growing number of pre-clinical studies which have modeled genotoxicity in *in vitro* cell immortalization assays and in *in vivo* murine models of leukemia/lymphoma development (Williams 2006; Will et al. 2007) and from transcriptional studies of gene de-regulation at the vector integration sites in cells transduced *in vitro* or retrieved from transplanted mice and patients (Recchia et al. 2006; Ryu et al. 2008).

Direct comparison of gRV and LV in these assays demonstrates that prototypical LV have a substantially lower oncogenic potential than gRV (Montini et al. 2006; Modlich et al. 2008). Important features contributing to the lower genotoxicity of LV as compared to conventional gRV are likely to be: 1) the self-inactivating design, which prevents LTR-dependent effects on

the transcription of genes neighboring the integration site; 2) the use of a vector internal promoter to drive transcription of the therapeutic transgene, which is chosen among those with moderate transcriptional activity as compared to gRV LTR (these aspects are discussed in more details in paragraph 6.4.).

Moreover, it has been demonstrated that both gRV and LV display a non random genomic distribution pattern, which is characteristic for each vector and that may thus influence the safety profile of each vector differently. Indeed, both gRV and LV integrate more frequently within genes than expected of random insertion throughout the genome, gRV preferentially integrate near the promoters and CpG islands of actively transcribed genes (Wu et al. 2003; Bushman et al. 2005). This preference makes it more likely for the LTR to interfere with regulation of the genes near the integration site (Schroder et al. 2002; Wu, Li et al. 2003; Bushman, Lewinski et al. 2005; De Palma et al. 2005). Furthermore, when hot spots of vector integration were identified in the mouse and human HSPC genome, striking differences were observed between gRV and LV (Montini, Cesana et al. 2006; Aiuti et al. 2007; Bushman 2007; Cattoglio et al. 2007; Deichmann et al. 2007; Schwarzwaelder et al. 2007). gRV, but not LV, integrations occurred at proto-oncogenes and genes involved in the control of cell proliferation with a frequency significantly higher with respect to the expected random frequency. LV integrations appeared to occur more broadly across gene classes and hot spots were found at low frequency and in locations characterized by high density of expressed genes.

Indeed, the unique propensity of gRV to integrate near potentially dangerous genes may be an important factor determining genotoxicity, and the lack of such preference in the case of LV may well contribute to the improved safety profile demonstrated in preclinical gene therapy models.

Beside the intrinsic features of the vector, however, other factors may contribute significantly to the risk of oncogenesis in gene therapy treated patients (Woods et al. 2006), including disease-related conditions that may increase the susceptibility to develop tumors (such as the accumulation of immature progenitors or immune deficiency) and the biological activity of the therapeutic transgene itself.

In order to detect possible genotoxic effect of the WASP LV, a recently developed assay was performed by Gènèthon. This test detects the presence of clonal dominance of insertional mutants which expand in initially polyclonal cultures of primary murine hematopoietic cells within two weeks after gene transfer and acquire serial replating ability, the extent of which provides a measure of clonal fitness (Modlich et al., 2006). The test has some limitations, since it detects essentially only genotoxicity related to EVI1 insertions and is based on a mouse *in vitro* transformation after *in vitro* cytokine expansion. In none of the three assays conducted with WASP LV, induction of replating activity was observed. As expected, a positive control represented by gRV containing a strong myeloid viral promoter (SFFV) showed a high replating activity. Thus, the WAS vector did not exhibit any genotoxic potential, confirming its safety also in this context.

To efficiently analyze high numbers of lentiviral insertion sites in the DNA of transduced cells, Généthon has developed an improved high-throughput method called vector integration tag analysis (VITA), described in (Mantovani et al. 2006). VITA is based on the identification of genomic tags associated to the insertion sites, which are used as signatures of the integration events. We analyzed the integration sites following WASP-encoding w1.6W LV transduction of murine WKO cells and compared the pattern of integration sites obtained in a population of

hematopoietic progenitor cells cultured *in vitro* with that of cells capable of engraftment *in vivo* (Mantovani et al., 2009). Lineage-negative hematopoietic progenitor cells were prepared from WKO mice and were transduced *ex vivo* with the w1.6W LV and transplanted into lethally irradiated WKO mice. Extracts from the Lin- cells (pre-infusion), as well as blood and BM of mice after gene therapy were used to prepare genomic DNA and were compared by VITA technique to analyze the vector insertion sites.

A high number of unique junctions was found in the cultured cells (more than 90 unique junctions in a first approach without concatenation, more than 300 in a second approach with concatenation). In blood and BM samples, the numbers of unique junctions were significantly lower with respectively 17 to 28 unique sites in blood samples and 8 to 13 unique sites in BM samples, depending on the animal analyzed. In contrast to what observed with the cultured cell sample, the number of unique sites found in the blood/BM samples reached a plateau as number of sequences increased (80-120 junctions sequenced for each point). As previously reported by others, we found a bias toward insertion of the vector into genes, but no obvious bias for the distribution of insertions into specific categories of genes. No common integration sites between the *in vitro* and *in vivo* samples and no evidence of integrations shared by different mice were detected.

Altogether, the analysis of the w1.6W LV integration sites in murine cells revealed insertions into a polyclonal population, no hotspot and no insertion of the vector into previously identified CIS.

2.7.3.1. Integration analyses in other trials

LV-based HSPC gene therapy was recently tested in ALD patients. The vector tested was a third-generation LV similar to the one proposed for this trial, except for the internal promoter driving the therapeutic cDNA, which was derived from the strong MND LTR. A clear clinical benefit has been reported in the two treated patients together with stable polyclonal hematopoietic repopulation by the transduced HSPC and no evidence for skewing in genomic distribution or specific gene classes by comparing vector integration sites in pre- and post-transplant samples (Cartier N. et al., 2009). These data provide compelling evidence that LV provide for efficient, safe and long-term marking of human hematopoiesis as predicted by a large amount of pre-clinical studies.

In another recent trial of LV-based HSC gene therapy for beta-thalassemia, a self-limiting expansion of a clone bearing a LV insertion putatively leading to a gain-of-function in growth control was recently reported in a beta-thalassemia patient (Cavazzana-Calvo M. et al. Nature 2010). Further investigations are needed to clarify the potential contribution of this insertion to the observed clonal expansion and its benign or pre-malignant nature. Moreover, several additional factors may have contributed to the findings in the thalassemia trial, including a complex vector design and the use of non-purified low titer vector that resulted in the transplantation of very few transduced HSPC and a long-delayed *in vivo* marking rate.

In summary, our studies and recent preliminary findings from clinical trials indicate that integration of prototypical SIN LV has low genotoxic potential and, together with the high gene transfer efficiency and the therapeutic efficacy demonstrated in pre-clinical models, provide a major scientific rationale for advancing WAS LV to clinical experimentation.

2.7.4. Immunogenicity

Even though immunogenicity is considered in international guidelines for GTMPs, it is unlikely that the proposed therapeutic approach may elicit an immune response either against the therapeutic gene product or against the viral vector.

The infusion of genetically modified cells occurs, in the proposed clinical protocol, following the administration of a non-myeloablative conditioning regimen known to establish a certain degree of tolerance to donor encoded antigens in the context of allogeneic transplant.

Further, the drug product consists of CD34+ genetically modified cells exposed to the viral vector *ex-vivo*. It is recognized that this procedure has a lower anti-vector immunogenic risk when compared to *in vivo* administration of viral vectors. In addition, WASP is an intracellular protein, its expression in the vector is regulated by the endogenous, physiological promoter, and WAS protein levels are regulated by the WIP molecule.

For these reasons, no *in vivo* pre-clinical studies for immunogenicity have been performed. Moreover, there was no evidence of immune mediated elimination of WASP transduced cells in the murine model of gene therapy, since transduced cells persisted long term with evidence of selective advantage.

Nonetheless, a careful assessment of any immune response will be performed by monitoring anti-WASP and anti-HIV proteins (anti-p24) antibodies every 6 months for the first year, then once a year during the clinical trial follow up period.

2.7.5. Environmental risks

The gene treatment planned in this clinical trial does not involve particular risks in terms of public health. The cell infusion will occur in a dedicated room of the authorized area (Impianto Area for MOGM Class -MI/IC/IMP.2/01-002 Rev.2) in the Hematology and Bone Marrow Transplantation Unit, notified for the use of the specific GMOs.

The procedures and programs for the cleaning, disinfection and decontamination of the ward, the operating theater (if it is used) and the equipment do not differ from the standard ones in force for the management of patients who undergo bone marrow transplantation, according to Institutional SOPs.

All the solid and liquid waste contaminated by biological and/or potentially infected material are disposed of in compliance with the hospital regulations in force. All members of the staff, wearing gloves and a protection mask, are adequately educated and trained. In particular, the staff is shown how to apply the regulations in force to patients who receive multiple transfusions, are immunosuppressed and at high risk of infections.

In a recent review of the literature, no RCR was detected in 103 patients treated with *ex vivo* retrovirally transduced cells, nor in 342 patients treated *in vivo* with retroviral vectors (Schenk-Braat et al. 2007). Overall, in more than 18 years of clinical trials, there has been no report of cultural or molecular RCR test positivity, in more than 300 clinical trials conducted worldwide with retroviral vectors and a very high number of people considering healthcare staff, family members and other people. In addition, no RCL has been detected in the clinical trials currently conducted with SIN lentiviral vector (Cartier N et al., Science 2009). Even in the unlikely event of RCL, the risk of transmission is expected to be typical of a lipid membrane enveloped virus.

Therefore, risks for healthcare staff and others should be mostly limited to accidental venipuncture and accidental mucosal or parenteral exposure to body fluids. An emergency plan is active in the Hospital for this circumstance.

The patients will be followed to exclude the possible development of RCL infection (see above). Samples will be collected before treatment (at baseline) and after 1, 3, 6, 12 and 24 months, and then yearly after treatment, as suggested by FDA guidelines (Guidance for Industry, November 2006). In case the samples collected during the first two years result always negative for the screening tests HIV p24, VSV-G DNA and HIV-pol RNA, the subsequent yearly samples will be stored, but not analyzed. If one of the above- mentioned tests will result positive, the screening evaluations will be repeated at the next planned follow up visit; in the case two of the three screening test will result positive, a confirmatory RCL culture tests will be performed. Of note, HIV-derived RCL would conceivably be sensitive to – and treatable by – conventional anti-HIV therapy targeting all *pol*-derived enzymes (RT, protease and integrase). We can estimate that the risk of acquiring an infection by wild type HIV for the patients enrolled in the clinical study is superimposable to that of the population of the same age not under study.

2.8. Clinical trials performed in other countries with similar Drug Substance

2.8.1. Gene Therapy for Wiskott-Aldrich-Syndrome using a retroviral vector

Since 2006, a gene therapy clinical trial has been conducted in Hannover Medical School by Dr. C. Klein. The preliminary results have been presented at the 2008 American Society of Gene & Cell Therapy (ASGT) meeting and an update was given at the 2009 European Society for Blood and Marrow Transplantation (EBMT) Inborn Error meeting in Cambridge (UK). Ten patients were treated with infusion of autologous mobilized peripheral blood CD34+ cells transduced with a gammaretroviral vector encoding WASP, under the control of a viral promoter. All patients are alive, one patient underwent a bone marrow transplant due to failure of engraftment, associated with low dose of cells infused. The first two patients have reached >24 months of follow up and showed evidence of long-term gene marking in myeloid and lymphoid cells. Gene marking in myeloid cells is about 10-30%, in NK cells 50-80%, in T cells 80-95%. In addition, the majority of peripheral thrombocytes showed evidence of WASP expression and platelet counts have substantially improved. Functional reconstitution was documented in dendritic cells (podosome formation), T cells (proliferation in response to CD3-signaling), and NK cells (formation of immunological synapse). Of note, they did not develop any severe infections or bleeding after gene therapy. In the first patient eczema and autoimmune haemolytic anaemia have resolved, while anti-neutrophil and anti-PLT antibodies have disappeared. In the second patient eczema has resolved. Clinically, patients are in good conditions more than two years after gene therapy (Boztug K et al, 2010). These results are very encouraging and confirm our hypothesis that the mixed chimerism associated with reduced conditioning, also thanks to the occurrence of selective advantage, is sufficient to allow biological efficacy and clinical benefit. However, the majority of WAS patients treated with the retroviral vector encoding WASP have developed leukaemia during the study conducted in Germany (M. Schmidt, K Boztug personal communication). As of January 2014, 7 out of the 10 treated patients developed acute leukaemia (one acute myeloid leukemia (AML), four T cell acute lymphoblastic leukemia (T-ALL), and two primary T-ALL with secondary AML, with all six cases of T-ALL associated with a

dominant clone with vector integration at the LMO2) (Braun et al, 2014). It should be noted that the lentiviral vector used in the present study has a different origin and it does not contain the part of the retroviral vector that is potentially responsible of the leukaemia. While more extensive studies and longer follow up will be needed to determine the overall safety profile of gene therapy with gamma-retroviral vector, this finding further reinforces the choice of a potentially safer platform, based on HIV vector for gene transfer in WAS.

2.8.2. Gene Therapy for Adrenoleukodystrophy using a lentiviral vector

As mentioned above, a clinical trial of gene therapy based on HSC and LV for the treatment of cerebral Adrenoleukodystrophy (ALD) was started at the Hôpital Saint-Vincent de Paul and Hôpital Necker in Paris (Cartier N et al, Science 2009). X-linked adrenoleukodystrophy (X-ALD), a genetic disorder secondary to alterations in the ABCD1 (ATP-binding cassette, sub-family D [ALD], member 1) gene, results in defective peroxisomal oxidation and the accumulation of very long chain fatty acids (VLCFA) in all tissues. The disorder primarily affects the adrenal cortex and the nervous system.

Up to now, three patients suffering from cerebral ALD have been included. Autologous $CD34^+$ progenitor cells were collected from MPB and transduced *ex vivo* for 18 hours with a 3rd generation LV encoding the ALD protein cDNA under the control of the MND promoter. The LV used in this trial differs from the Drug Substance described in the present protocol for some aspects, including the presence in the transfer construct of a different promoter driving therapeutic gene expression, and the protocol of large scale GMP production. The gene transfer procedure of $CD34^+$ cells was based on a similar cytokine combination to the one used for the preparation of the Medicinal Product described in the present protocol. Transduced cells were frozen after transduction to perform all tests planned before infusion. After thawing and prior to re-injection, 20-40% of the transduced $CD34^+$ cells expressed the ALD protein with a mean of 0.7 copies of integrated provirus per cell. Transduced cells were re-infused into pharmacologically conditioned patients (receiving a full myeloablative regimen consisting of Busulfan and Cyclophosphamide). The first results of this study have shown that genetically modified $CD34^+$ progenitor cells transduced with the ALD protein-encoding LV are able to differentiate in all hematopoietic lineages (granulocytes, monocytes, B and T lymphocytes) i*n vivo*. The percentage of corrected lymphocytes and monocytes in the peripheral blood of treated patients remained stable from day 30 to the longest follow up (30 and 24 months for Pt 1 and Pt2, respectively). From 10% to 15% of $CD14^+$, $CD3^+$, $CD19^+$ and CD3-$CD56^+$ cells expressed the ALD protein. Test for vector-derived RCL and vector mobilization were negative up to the last follow up. Clinical and instrumental evaluations revealed stability of the disease and a course comparable to that of patients undergoing allogeneic HSC transplantation.

These results indicate that HSC gene therapy using HIV-1-derived LV is not associated with the emergence of RCL and vector mobilization, and that LV allow safe and efficient transduction of human HSC and maintenance of gene expression in vivo.

3. OBJECTIVES

The objective of the present gene therapy clinical trial is to evaluate the safety of the transduced stem cells infusion in patients affected by WAS after a reduced-intensity conditioning regimen and its efficacy in improving the patient's immune-function and thrombocytopenia.

3.1. Primary Objectives

1) To evaluate the safety of the administration of autologous CD34+ cells transduced with a lentiviral vector containing the WASP gene in patients with WAS, after a reduced intensity conditioning regimen.

The short-term and long-term safety of LV gene transfer into HSC will be evaluated on the basis of adverse event reporting and monitoring for the theoretical risk of systemic reactions to cell infusion, abnormal clonal proliferation and immune response to the transgene. Hematological reconstitution after non-fully myeloablative conditioning regimen will be closely monitored.

2) To evaluate the long-term engraftment of WASP-expressing transduced cells.

The proportion of multilineage WASP-expressing cells, indicating engraftment of repopulating transduced stem cells, will be evaluated at different time points after transplant.

3) To evaluate the efficacy of gene therapy assessed as:

3.1) Improvement of the patient's immune function, specifically of T cell function and antigen-specific responses to vaccinations

Improvement of *in vitro* T-cell functions and *in vivo* antibody production will be analyzed as part of the post-gene therapy follow up.

3.2) Improvement of thrombocytopenia

Increase in platelet count compared to baseline and normalization of mean platelet volume (MPV) at 1-year post treatment will be assessed according to the severity of thrombocytopenia before gene therapy.

3.2. Secondary objectives

A) To evaluate the efficacy of gene therapy in improving the patient's clinical conditions, assessed by reduction in frequency of severe infections, and bleeding episodes and reduction of autoimmunity phenomena and eczema.

A complete clinical and laboratory assessment of the patient after the proposed treatment is scheduled at different time points, to provide an overall evaluation of the clinical benefit.

4. INVESTIGATIONAL PLAN

This clinical study is based on *ex vivo* gene therapy for Wiskott-Aldrich syndrome using autologous hematopoietic stem/progenitor cells (purified CD34+ cells). Genetically modified cells will be re-infused to patients following a reduced intensity conditioning regimen (see chapter 10 for details), dispensed to eliminate the defective cells and favor the engraftment of gene corrected cells expressing a normal WASP. The eligibility of the patients will be evaluated according to enrolment criteria, which include patients suffering from a severe clinical condition or severe WAS phenotype, without a suitable matched donor for allogeneic transplant or ineligible for HSCT because of age > 5 years or for clinical features.

In this study, we plan to treat a total of **8** patients in a period of 5 years, which will be followed for the safety and efficacy endpoint measures. There is a *Follow Up phase* composed of an *Initial Follow Up period* of at least 3 years, when subjects are intended to reach the primary efficacy timepoint, and then a further *Long-Term Follow Up period* from 3 years up to 8 years. For patients who complete the *Follow Up phase*, from year 8 onwards there will be an additional extended long-term follow up (ELTFU) phase. During the ELTFU phase, patients will be contacted annually, in order to collect long-term safety and selected efficacy data, until a separate observational long-term follow-up (OLTFU) study is initiated, and patients have enrolled in it. Once the OLTFU study is set up, patients will be invited to enter the OLTFU study at any time after they have completed a minimum of 5 years follow up in this study (WAS Pivotal 201228).

The treatment will be administered at the Pediatric Immunohematology and Bone Marrow Transplantation Unit at the San Raffaele Scientific Institute in Milan, Italy. The investigators and the manufacturer of the product have a specialized expertise in hematopoietic stem cells manipulation, LV production and gene transfer procedure according to GMP standards. Clinical trials using the same lentiviral vectors have been implemented at the Institute for Child Health in London, Necker Hospital in Paris and Children's Hospital in Boston (see above). These clinical trials have adopted similar criteria for the enrolment of patients and similar safety and efficacy endpoints, but the conditioning regimen is different. The Genethon sponsored trial have adopted a myeloablative conditioning regimen based on the use of Busulfan 12 mg/kg, associated to Fludarabine (120 mg/sqm), possibly associated to anti-CD20 mAb and/or anti-Thymocyte globulin in the setting of autoimmunity. Information deriving from the preliminary results of the two trials will be exchanged and discussed in order to allow to define an optimal safe and efficacious conditioning for gene therapy in WAS patients.

4.1. Study Design

4.1.1. Type of study

This is a phase I/II, open label, non-randomized, prospective, single centre study.

4.1.2. Study duration

Enrolment phase: 5 years.

Starting date: April 2010

Follow Up phase: the *Follow Up phase* is composed of an *Initial Follow Up period* of at least 3 years after the reinfusion of transduced cells, when subjects are intended to reach the primary efficacy analysis endpoint, and then a further *Long-Term Follow Up period* from 3 up to 8 years after the reinfusion of transduced cells. In the *Initial Follow Up period*, patients will undergo regular follow up assessments to assess the efficacy and safety of the treatment.

Extended Long-Term Follow Up phase (ELTFU): patients who complete the *Follow Up phase* will then be contacted annually from year 8 onwards in order to collect long-term safety and selected efficacy data until a separate OLTFU study is initiated and patients have enrolled in it.

Once the OLTFU study is set up, patients will be invited to enter the OLTFU study at any time after they have completed a minimum of 5 years follow up in this study (WAS Pivotal 201228).

4.1.3. Study population

Patients of pediatric or adult age affected by WAS, with severe clinical phenotype and/or severe genetic mutation, who meet the inclusion/exclusion criteria described below.

4.2. Safety Endpoints

4.2.1. Primary safety endpoints

4.2.1.1. Safety of reduced conditioning regimen

a) *Hematological reconstitution*.

The absence of engraftment failure and prolonged aplasia (defined as ANC <500/µl at day +60, with no evidence of bone marrow recovery and requiring back-up administration) is assumed as proving the safety of reduced intensity conditioning regimen associated with the infusion of *ex vivo* transduced $CD34^+$ cells.

In case the first two patients present prolonged aplasia, modification of the conditioning regimen is introduced in the third patient and the rule is again applied for this patient and the following two.

b) *Regimen related non-hematopoietic toxicity*.

A surveillance of clinical and laboratory parameters (for clinical features CTCAE ≥2, for metabolic/laboratory CTCAE ≥3) of the treated patients (CTCAE will be applied during the first 100 days after transplant in order to assess the degree of morbidity associated to the reduced conditioning regimen).

Stopping rule: in case of 2 deaths occurring within 180 days from transplant (not superior to mortality reported from mismatched related donors, see Table 1, Table 2, Table 3 and Table 4).

For detailed follow up and timing of clinical and laboratory surveillance see Table 8a, Table 8b and Table 10.

4.2.1.2. Safety of LV gene transfer into HSC

a) *Short-term safety and tolerability of lentiviral-transduced cell infusion*

This will be evaluated on the basis of adverse events reporting and monitoring of the systemic reactions to cell infusion (fever, tachycardia, nausea and vomiting, joint pain, skin rash).

The short-term safety of lentiviral-transduced cells infusion consists in the absence of serious adverse reactions within 48 hours from infusion.

b) Long-term safety of lentiviral-transduced cell infusion

This will consist in the absence of:

- Replication competent lentivirus (RCL).
- Molecular monitoring of RCL will be assessed via ELISA for HIV p24 antigen in serum. A positive HIV p24 test result will be subject to confirmation. If confirmed, second level testing will include a) DNA PCR for VSV-G envelope (cells) and b) RT-PCR for HIV-pol RNA (serum). Should one of these second level tests also provide a positive result, an appropriate confirmatory test may be conducted.

  The tests will be performed at baseline and after 1, 3, 6, 12 months and 24 months. Samples will be stored every year thereafter. If one of the above mentioned tests has a positive result, the screening evaluations will be repeated at the next planned follow up visit; if two of the three screening test have a positive result, a confirmatory RCL culture tests will be performed.
- Abnormal clonal proliferation. Abnormal hematopoietic clonal expansion will be monitored by clinical and laboratory surveillance, repertoire study, and bone marrow examination.

Stopping rule: 1 event of malignant proliferation related to gene transfer.

4.2.2. Secondary safety endpoints

a) *Lack of immune response to transgene*

The lack of immune response towards the transgene will be measured by antibodies to WASP (immunoblot analyses).

b) Overall safety surveillance

This will be evaluated on the basis of clinical and laboratory parameters recorded and monitored during the follow up of the treated patients, according to the assessment plan. A descriptive analysis will be performed on AE, AR, SAE/SAR, UAR, SUSAR, and events will be classified for frequency, severity and body system involved.

4.3. Efficacy Endpoints

4.3.1. Primary efficacy endpoints

a) *Overall survival*

From day +1 to 3 years after gene therapy, and then monitored as part of the long-term follow up.

b) Sustained engraftment of genetically corrected hematopoietic stem cells in peripheral blood and/or in bone marrow

Presence of gene corrected cells assessed by quantitative PCR on peripheral blood and bone marrow cells at 1 year after gene therapy. Adequate engraftment is defined as ≥0.04 VCN/cell in BM CD34+ (equivalent to 4% assuming a VCN of 1) or ≥0.1 VCN/cell in peripheral blood T lymphocytes (equivalent to 10% assuming a VCN of 1).

c) Expression of vector-derived WASP

Presence of detectable vector-derived WASP expression at 1 year after gene therapy by FACS analyses and/or Western Blot.

In case of failure of engraftment of transduced cells in two or more patients, intensification of conditioning dose and increase in the minimal cell dose will be considered after an amendment to the study.

d) Improved T-cell functions

Improvement in in vitro T-cell proliferation upon stimulation with anti-CD3i mAbs at 1, 2, and 3 years after gene therapy as compared to pre-gene therapy values.

e) Antigen-specific responses to vaccinations

- Ability to mount a protective humoral response to nominal antigens, including antibodies to at least 4 out of 5 T cell dependent antigens tested (Tetanus Toxoid, Diphtheria, Hepatitis B, Pertussis, Haemophilus B) measured after the end of the vaccination schedule (foreseen >1 year after gene therapy). In case results are available on n<5 antigens, the rule of at least n-1 will be applied for defining success.

- Ability to mount a protective humoral response to unconjugated polysaccharide antigens (Pneumococcus), measured after the end of the vaccination schedule (foreseen >1 year after gene therapy).

- Positive cellular response to Tetanus Toxoid after vaccination, measured by *in vitro* proliferative response >1 year after gene therapy.

f) Improved platelet count and normalization of MPV

Sustained increase in platelet count compared to baseline during the 3 year follow up, analyzing the individual longitudinal profile, according to the planned follow up visit and including platelet counts collected for clinical reasons during follow up (in transfusion independent patients).

Patients will be also stratified according to the severity of thrombocytopenia before gene therapy in 5 groups (A, B, C, D and E, Table 5) and the patient will be defined as improved if there is a shift from one group to the subsequent.

Table 5 Stratification of platelet count according to severity of thrombocytopenia (count x10^9/L)

A	B	C	D	E
< 20	20- 49	50 - 99	100 – 149	≥150

In addition, normalization of MPV at 1-year post treatment compared to baseline will be evaluated.

The need for a platelet infusion will be evaluated by the Investigators based on several factors (see Section 6.11).

4.3.2. Secondary efficacy endpoints

a) *Longitudinal analyses of sustained engraftment of genetically corrected hematopoietic stem cells in peripheral blood and/or in bone marrow and expression of vector derived WASP*

Presence of gene corrected cells and WASP expression will be assessed at the time points shown in Table 8a and Table 8b, as described above.

b) Analyses of sustained multilineage engraftment of genetically corrected cells in peripheral blood and/or in bone marrow

Presence of gene corrected cells assessed by quantitative PCR on all the available peripheral blood and/or bone marrow cell subpopulations (BM subpopulations: GlyA+, CD15+, CD3+, CD19+, CD56+; PB subpopulations: CD15+, CD19+, CD56+) at 1, 2 and 3 years after gene therapy. The availability of all the subpopulations depends on the amount of samples and on the technical procedures.

Adequate engraftment is defined as ≥0.04 VCN/cell (equivalent to 4% assuming a VCN of 1).

c) Reduced frequency of severe infections

Decrease in number of severe infections as evaluated starting from the first year follow up visit and thereafter until the 3-year visit after the treatment, by , complete physical examinations, hematological and microbiological tests compared to clinical history from the 12 months prior to GT. Infections will be assigned a CTCAE grading (as described in Section 4.3.2.1) by the Investigators, identical to how severity grading was used in clinical history.

d) Reduced bleeding episodes

Reduction in bleeding manifestations when present, as assessed starting from the first year follow up visit and thereafter until the 3-year visit, by clinical monitoring, compared to clinical history from 12 months prior to GT. Information about bleeding will be recorded through follow up by collecting data from parents, clinical reports periodically sent by local doctors following the patients at home and by clinical evaluations performed at each follow up visit at our center. Data will be recorded in the patients' clinical charts. Bleeding events will be given CTCAE grading (as described in Section 4.3.2.1) by the Investigators, identical to how severity grading was used in clinical history.

e) Reduced autoimmunity phenomena and eczema

Reduction in laboratory markers (number and titer of antibody when available) and/or clinical manifestations of autoimmunity, as evaluated starting from the first year follow up visit and thereafter until the 3-year visit, by organ-specific and systemic autoantibodies, imaging and clinical follow up, compared to clinical history

Reduction in eczema, as evaluated by clinical score according to Imai et al. 2004. Specifically, eczema will be scored by the Investigators based on the following scale:

1. None

2. Transient (mild): Asymptomatic or mild symptoms; additional medical intervention over baseline not indicated (consistent with CTCAE grade 1).

3. Moderate: topical or oral intervention indicated; additional medical intervention over baseline indicated (consistent with CTCAE grade 2).

4. Severe: medically significant but not immediately life threatening; intravenous intervention indicated (consistent with CTCAE grade 3).

Eczema scores over time will be presented in a shift table relative to the baseline score.

f) Improved quality of life

Improved quality of life, measured after the first year of treatment by reduced hospitalization, reduced requirement of drugs, school attendance and social activities. The evaluation will be performed according to patients' age (in particular, social ability with peers will not be evaluated before 2 years of age and sport practice will not be considered before 4 years of age).

4.3.2.1. CTCAE grading scale

The severity of the infection and bleeding events will be assigned by the Investigators using the National Cancer Institute (NCI) Common Terminology Criteria for Adverse Events (CTCAE) definitions. The CTCAE displays Grades 1 through 5 with unique clinical descriptions of severity for each event. Grade 1 and Grade 2 events are respectively considered as mild and moderate. An event graded 3 or above will be considered for analysis.

If an infection or bleeding event is not listed as a unique clinical description in the CTCAE, clinical judgement will be applied to grade the event.

For infections, a Grade 3 event is one that requires intravenous antibiotics; and/or antifungal intervention; and/or antiviral intervention and/or invasive intervention is indicated. A Grade 4 event is an event that has life-threatening consequences, and/or urgent intervention indicated. A Grade 5 event is an event that results in death.

For bleeding events, a Grade 1 event is one that presents with mild symptoms; intervention not indicated. A Grade 2 event is one that presents with moderate symptoms (limiting age-appropriate instrumental activities of daily living); intervention indicated. A Grade 3 event is one that requires platelets or red blood cells transfusions; and/or invasive intervention; and/or hospitalization. A Grade 4 event is an event that has life-threatening consequences, and/or urgent intervention indicated A Grade 5 event is an event that results in death.

4.3.3. Exploratory efficacy endpoint

a) *Platelet activation profile and morphology*

Platelet activation after stimulation with agonists will be assessed after ≥3y post gene therapy by measuring the expression of activation markers on the cell surface by FACS analysis. Platelet morphology will be assessed at the same time using transmission electron microscopy.

5. PATIENT SELECTION AND RECRUITMENT

Our Centre is a reference at national and international level for diagnostic referral of WAS patients. The chance of recruitment will be supported by our activity of molecular screening, clinical follow up and co-operation within national (AIEOP) and international (EBMT) networks. In particular, our centre participates to the CSS-ID network of the Italian Paediatrics Oncology-Haematology Association (AIEOP), a national committee on primary immunodeficiencies.

Based on the incidence of the disease and the potential recruitment of candidate patients SR-TIGET expected enrolment rate is of about 2 patients per year for a total of eight patients.

5.1. Study of population

5.1.1. Inclusion criteria

1	Diagnosis of WAS defined by genetic mutation and at least one of the following criteria:	
		Severe WASP mutation
		Absent WASP expression
		Severe clinical score (Zhu clinical score ≥ 3)
2	No HLA-identical sibling donor	
AND		
3.1	Negative search for a matched unrelated donor (10/10) or an adequate unrelated cord blood donor (6/6) within 4-6 months	
OR		
3.2	Patients with age > 5 years who are not candidate to unrelated allogeneic transplant based on clinical conditions	
AND		
4	Parental/guardian/patient signed informed consent	

The severity of the genetic mutation will be defined by literature data (genotype-phenotype studies) and the level of WASP expression will be assessed by FACS. The identification of a MUD donor will be established based on high-resolution allele-level DNA matching for 10/10 alleles (HLA-A, -B, -C, -DRB, and -DQB1 loci). For cord blood transplantation, both cell dose (>2.5 x 10^7 nucleated cells/kg body weight) and HLA matching (low-resolution HLA-A, -B, and high-resolution DRB1) will be considered.

5.1.2. Exclusion criteria

Patients who meet any of the following criteria will be excluded from study admission:

1	Patients positive for HIV-infection
2	Patients affected by neoplasia
3	Patients with cytogenetic alterations typical of MDS/AML
4	Patients with end-organ functions or any other severe disease which, in the judgement of the investigator, would make the patient inappropriate for entry into this study
5	Patients who underwent an allogeneic haematopoietic stem cell transplantation in the previous 6 months
6	Patients who underwent an allogeneic haematopoietic stem cell transplantation with evidence of residual cells of donor origin

5.1.3. Sample size

Given the low frequency of disease and the degree of novelty of the proposed experimental approach, we plan to treat overall **8** patients, in an estimated 5-year period.

5.1.4. Patient withdrawal

The patients will leave the study in case of impossibility to perform the planned procedures and treatment before gene therapy.

In addition, the following circumstance is to be considered sufficient to withdraw from the study an enrolled patient:

- Withdrawal of consent by the parent/legal tutor or the patient.
- Any AE/SAE or clinical condition that prevents the long-term continuation of follow up.

5.1.4.1. Natural study termination for a single enrolled patient

The natural study termination, for a single enrolled patient, is when the patient has completed a minimum of 5 years follow up in this study (WAS Pivotal, 201228) and has transitioned and has transitioned to a separate OLTFU study. As part of the OLTFU study, patients will continue to be followed up for a total of 15 years from the date of treatment with gene therapy. Should a patient decide not to consent to participate in the ELTFU nor transition to the OLTFU study, then the subject will be considered to have completed this study and will be transitioned to standard of care according to local practice. This study will be considered to have ended once all subjects have transitioned into the separate OLTFU study or transitioned to standard of care according to local practice.

In addition, the following circumstances are to be considered as natural study termination:

a) Treatment of the patient with allogeneic hematopoietic stem cell transplantation
b) Loss to follow up
c) Death of patient

All patients will be included for evaluation until withdrawal.

5.2. Phases of study

Starting from the signature of the informed consent by patients' legal tutors, the subjects will be enrolled in the clinical trial.

Five protocol phases are foreseen after enrolment (see Figure 1, Study flow chart):

1) *Screening phase,* during which the conditions required by the clinical protocol for patient's enrolment will be assessed and fulfillment of the inclusion/exclusion criteria of the study will be evaluated.
2) *Baseline phase,* carried from the end of the screening phase to the day before PBSC mobilization, if performed, or the day before Rituximab administration (day -22).
3) *Treatment phase,* from the end of the Baseline to day 0.
4) *Follow Up phase*: the *Follow Up phase* is composed of an *Initial Follow Up* period of at least 3 years, when patients are intended to reach the primary efficacy timepoint, and then a further *Long Term Follow Up period* from 3 up to 8 years.
5) *ELTFU phase*: patients who complete the *Follow up phase* will then be contacted annually from year 8 onwards, in order to collect long-term safety and selected efficacy data, until a separate OLTFU study is initiated and patients have enrolled in it.

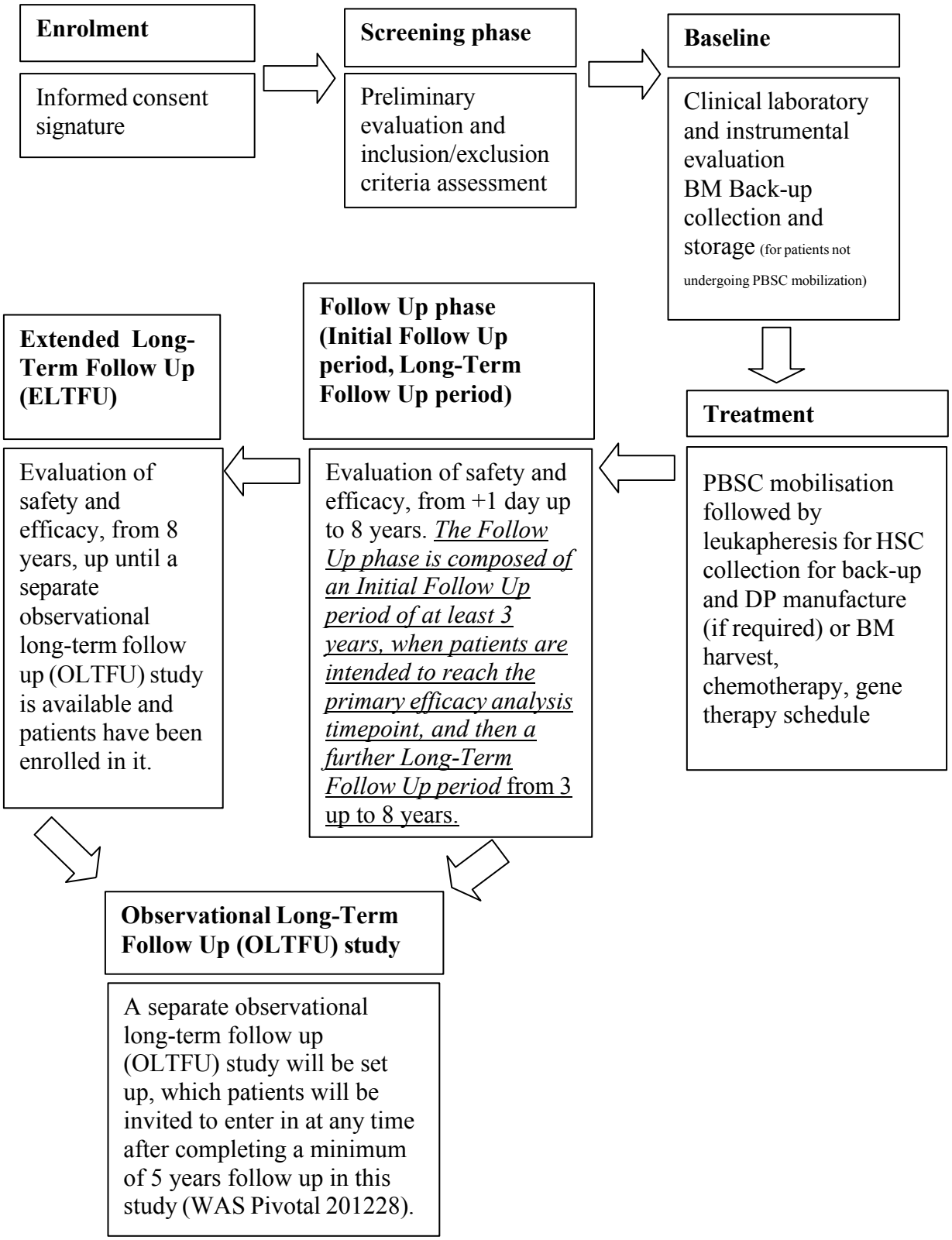
Once the OLTFU study is set up, patients will be invited to enter the OLTFU study at any time after they have completed a minimum of 5 years follow up in this study (WAS Pivotal, 201228).

If the subject and family are unable to attend clinic visits (due to, for instance, the health of the subject, or the inability to travel to the clinical site), but are willing to continue participation in the study, remote follow-up visits, i.e. visits conducted at the local healthcare professional (HCP) or healthcare facility close to where the subject resides may be considered as an alternative method of ensuring assessment of efficacy and safety while reducing the burden on the subject and subject's family. This alternative will be discussed with the PI, the medical monitor and the sponsor.

- Remote follow up visits should be avoided prior to the 3-year primary endpoints. Thereafter, remote follow-up visits may be conducted provided they are reviewed and agreed by the sponsor.
- The communication between the local HCP and the clinical site will be documented in a dedicated plan. This plan will also describe the arrangements of the remote follow-up visits and the responsibilities of the local HCP. The local HCP will have to record and report all observations pertinent to the subject's follow-up, including all safety events, and provide the source data to the subject's original treatment center. The Sponsor or the Principal Investigator may provide HCPs with tools to aid data collection. The Principal Investigator remains ultimately responsible for the conduct of the study and for the causality assessment of all potential safety events.
- Collection of biological samples might be performed at the local HCPs, in accordance with local regulations. If blood samples are collected during a remote follow-up visit, the volume of blood drawn should not exceed the volume required for scheduled

laboratory assessment. Local HCPs will not have access to the electronic case report form (eCRF) or any study database. Data entry will be performed by appropriately delegated personnel at the original treatment center.

Figure 1 Study Flow Chart



6. TREATMENT PROTOCOL

6.1. Treatment administration

The Medicinal product consists of autologous stem/progenitor cells from the bone marrow and/or mobilized peripheral blood transduced *ex vivo* with LV encoding the WAS cDNA to be administered intravenously.

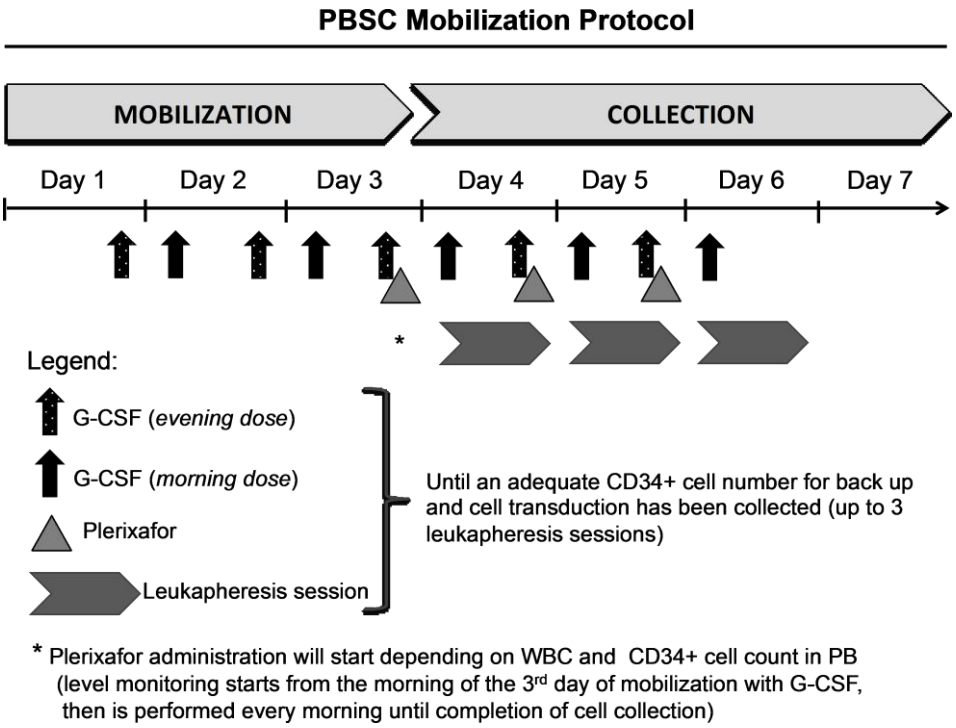
The procurement of BM/PBSC and treatment will be performed at Paediatric Immuno-haematology and Bone Marrow Transplantation Unit, which are part of the San Raffaele Stem Cell Programme (Director: Prof. F. Ciceri), Milan, Italy, accredited by the Italian Bone Marrow Transplantation Group (GITMO) and currently under JACIE (Joint Accreditation Committee ISCT (International Society for Cellular Therapy-Europe) & EBMT) accreditation.

Clinical Investigators and Clinical Staff will receive a training to provide education on the contained use of microorganism genetically modified (GMOs), according to Legislative Decree 206/01 (National Implementation of EU Directive 98/81/CE).

6.2. Stem cell back-up, collection and purification of mobilized CD34+ cells

A central venous catheter will be inserted to allow infusion of blood products, chemotherapy, parenteral nutrition and leukapheresis under general anesthesia. Before the procedure the patient may receive either intravenous immunoglobulin (IVIG) or platelets transfusion(s) in order to achieve a safe platelet count. About 5-10 weeks before the planned BM harvesting, a back-up of PBSC will be collected following G-CSF+Plerixafor stimulation (in case of foreseen use of MPB as only stem cell source, CD34+ cell will also be collected for future transduction). High doses of G-CSF administered twice daily will be used (10-12.5 mcg/kg in 2 divided doses) since they appear to mobilize more CD34+ cells. Moreover, starting from day 3 of G-CSF administration, an additional mobilizing agent, Plerixafor (AMD3100), will be administered in order to enhance PBSC mobilization (Vose et al, 2009; Yannaki et al. 2013; Gardellini et al. 2013; Aabiden et al. 2011; Vettenranta et al. 2012). The starting day of the Plerixafor administration could be shifted to day 4 or day 5 depending on WBC and CD34+ cell count/mcl in patient's PB. Patients will receive Plerixafor once daily at the dose of 0.24 mg/kg sub-cutaneously approximately 8-10 hours before standard leukapheresis is performed (see Figure 2– PBSC Mobilization Protocol) in agreement with internal procedure of the SR Stem Cells Program. G-CSF and Plerixafor administration will be repeated until sufficient mobilized peripheral blood (MPB) CD34+ cells are harvested (up to a maximum of 3 leukapheresis on 3 consecutive days or 7 days of G-CSF administration). According to clinical needs, an additional central or peripheral venous catheter can be inserted. During treatment with G-CSF + Plerixafor, complete blood cell count will be obtained daily and PB CD34+ cell count from the third day. Leukapheresis will be performed as soon as MPB CD34+ cell count reaches an adequate level and collections will be performed using a COBE Spectra blood under standard procedures. The optimum target number of CD34+ cells to collect for back-up is $5x10^6$/kg, although a minimum of $\geq 3x10^6$ autologous CD34+ cells/kg will be considered sufficient for safe transplantation. In case of concomitant collection of CD34+ cells for future transduction, an additional cell amount $\geq 5x10^6$ CD34+ cells/kg will be collected.

Figure 2 PBSC Mobilization Protocol



Failure of the procedure (*poor mobilization*) will be defined if PB CD34+ cell count remains <10 cells/µl in spite of mobilization, or an insufficient yield is reached after the third apheresis.

The procedure may be repeated to achieve an adequate cell dose for back-up and/or treatment.

An unmanipulated apheresis fraction containing at least $3x10^6$ CD34+ cells/kg with dimethyl sulfoxide (DMSO) at 10% final concentration will be stored in liquid nitrogen. Samples from each bag will be sent for detecting bacterial contamination.

The remaining fraction of the apheresis will be used to purify CD34+ cells by the Milteny ClinicaMACS immunomagnetic device. The purified stem cells, resuspended in 10% DMSO, will be stored in liquid nitrogen in at least two aliquots.

The MPB CD34+ cells may be used as cell source of genetically modified cells.

The stored back-up, together with the negative fraction following selection procedure, will be infused in case of engraftment failure or lack of reinfusion of the medicinal product, if conditioning regimen has been already started (see 10.12). In case the patients cannot undergo a collection of PBSC for clinical reasons (ex. patient's age, weight, unsuitability) or in case of poor mobilization, a back-up BM harvest targeting a minimum level of $\geq 1x10^8$ total nucleated cells per kg of body weight will be performed.

In the case of a dedicated collection of autologous umbilical cord blood performed at birth, this may be considered as a source of HSC for back-up, if adequate for cell number and quality.

6.3. Bone marrow harvest

BM will be collected in the operating room from the iliac crests under sterile conditions and using general anesthesia, on day –3. The bone marrow collection procedure is described in an internal SOP. The total volume of the required amount of bone marrow will be estimated according to the content of bone marrow CD34+ cells evaluated by previous bone marrow aspiration. On average approximately 20-30 BM ml/kg patient body weight (max 35 ml/kg) will be collected considering the subsequent need of manipulations (MNC selection, centrifugation, washing, positive selection, transduction).

The BM target $CD34^+$ cell count before any manipulation is $\geq 5x10^6$ CD34+ cells/kg to achieve a minimum dose of $2x10^6$ CD34+ transduced cells/kg. Hemoglobin values <7.0-7.5 g/dl will be corrected by irradiated and filtered red blood cells transfusion.

The harvested bone marrow, collected in a dedicated bag, sealed and identified, will be then transferred in a biohazard container to MolMed S.p.A, located near San Raffaele Hospital, by the research nurse or physician participating to the study.

BM harvest is not performed if MPB is used as the only stem cell source for transduction.

6.4. Cell manipulation and gene transfer with the lentiviral vector

6.4.1. BM CD34+ cell purification

Patients' HSC manipulation will be performed by MolMed S.p.A. in GMP conditions. The collected marrow will be diluted, stratified with lymphoprep and centrifuged in order to separate blood fractions. Final cell suspension primarily made of MNC will be collected. Positive selection on BM will be performed using immunomagnetic beads (CliniMACS, Miltenyi) and an immunomagnetic enrichment device. WBC counts will be performed in basal BM samples from each marrow bag, after buffy coat collection and both in the positive and negative cell fraction. Phenotypic expression of surface molecules (CD34) will be determined by direct labeling using standard methodology. After centrifugation to reduce volume, the negative fraction will be collected in divided bags with DMSO, frozen and stored in liquid nitrogen for possible infusion if engraftment failure will occur. Samples from each bag will be sent for detecting bacterial contamination.

6.4.2. Lentiviral vectors

Autologous bone marrow $CD34^+$ cells will be transduced with a clinical grade LV containing WAS gene. In this construct (w1.6W), the WAS gene is designed under the control of WAS autologous promoter. The w1.6W vector is further modified by introducing a mutation in the WPRE element. Viral supernatants will be produced in a GMP facility of the biotech company, MolMed S.p.A. (Milan, Italy).

6.4.3. Gene transfer of BM CD34+ cells

Purified CD34+ cells will be seeded in retronectin coated bags and further incubated for 24 hours at 37°C in CO_2 in serum free medium (Cell Grow) supplemented with the cytokines SCF, TPO, FLT3-L and IL-3. At the end of the incubation period, the cells will be transduced in the presence of cytokines with the LV-WASP vector. Two rounds of transduction are foreseen, each

lasting about 16 hours in the presence of $1x10^8$TU/ml of LV (corresponding to a Multiplicity of Infection – MOI - of 100), with a washout time of 12 hours, for a total of about 68 hours of *in vitro* culture. Since the cells will be freshly infused, only tests for endotoxin, mycoplasma, large T antigen DNA, and immunophenotype characterization will be completed and results will be available at the infusion time. The validated Hospital Sterility test (BacT/ALERT) is performed to allow a shorter response time and intermediate results will be available at the time of infusion. The detailed tests and specifications of the drug substance and drug product are reported in the investigator's brochure. At the end of the transduction procedure, the transduced $CD34^+$ cells will be harvested, washed with saline and resuspended in a minimum of 20 ml saline solution at the concentration of $10x10^6$/ml. The final drug product will consist of a cell suspension containing the transduced $CD34^+$ cells in a sterile syringe(s) or bag(s) of the suitable volume.

If the cells are detected to be contaminated, the medicinal product will not be returned to the patient and the patient will receive the unmanipulated back-up. If a positive result is obtained for the sterility test after the infusion, the patient will receive a specific antibiotic treatment and will be strictly monitored.

6.4.4. Gene transfer of MPB CD34+ cells

Mobilized peripheral blood (MPB) CD34+ cells may be used as cell source of genetically modified cells. Transduction will be performed as described above for BM CD34+ cells, starting from the previously stored CD34+ cells obtained from leukapheresis.

MPB may be used as single cell source:

1) in case of older patients (> 5 years old) to increase the dose of infused cells, if the expected collection from bone marrow is predicted not be sufficient to achieve the optimal target dose or for clinical reasons.

or

2) in case the patient has initiated conditioning and the transduced bone marrow CD34+ cells cannot be released for infusion. In this case the day of infusion will be postponed accordingly.

A mixture of MPB and BM CD34+ cells can be administered if required to obtain a sufficient cells dose.

6.5. Conditioning regimen

A reduced intensity-conditioning regimen will be administered before the autologous-engineered cells infusion based on the rationale described in chapter 5.

We will combine iv Busulfan (bodyweight-based and AUC targeted dose, see below, 8 +/- 1 doses administered consecutively from days –3 to –1), Fludarabine (total dose 60 mg/sqm, on days –3 and –2) and Rituximab (375 mg/sqm in single dose, on day -22).

Iv ATG Thymoglobuline may be used at PI discretion in patients with clinical autoimmunity before day-18 as an additional lympho-depleting agent to eliminate auto-reactive memory T cells.

Clinical data from the ADA-SCID trial indicate that the dose of administered CD34+ cell is one of the important factors in determining the engraftment of transduced progenitors, and thus the outcome of gene therapy. HSC freezing is associated to a significant reduction of the overall cell number, further increased by the mortality consequent to transduction. Thus, with the aim of preserving the maximum absolute number of $CD34^+$ cells, we will avoid freezing the transduced cells.

Since our protocol is based on the transduction and re-infusion of freshly isolated $CD34^+$ cells, the window of time between harvesting and transplantation will be limited to the minimum. The interval between the last infusion of drug and cell infusion will be sufficient to reduce drug concentration to safe levels for Busulfan (24-26 hours wash out) and Fludarabine (50-54 hours wash out).

6.5.1. Busulfan

Based on our experience in combining *ex vivo* gene therapy with reduced intensity in the 15 patients enrolled in the ADA-SCID gene therapy trial and previous pilot studies, we will adopt intravenous Busulfan (Busilvex) as myelotoxic drug. The alkylant will be administered at a higher dose than in ADA-SCID trial, because a significant level of myeloid engraftment can be a requirement for platelet correction in WAS patients.

Patients will receive one of five bodyweight-based doses of intravenous busulfan, according to the following scheme:

Table 6 Busulfan dosage according to patient's weight

< 9 kg	9 to < 16 kg	16-23 kg	> 23 – 34 kg	> 34 kg
1 mg/kg/dose	1.2 mg/kg/dose	1.1 mg/kg/dose	0.95 mg/kg/dose	0.8 mg/kg/dose

Patients will receive a total of 8 (+1) doses, given every 6 hours from day -3 to day -1. The dose of Busulfan and the number of doses may be adjusted to reach the target AUC (see below).

The mean half-life of i.v. Busulfan ranges between 2.83 to 3.90 hours.

We will monitor Busulfan plasmatic levels by temporized sampling following infusion after the first and the sixth dose.

The exposure to Busulfan, based on pharmacokinetic profiling, will be defined by the Pharmacological Institute of the University of Pavia. The dose of Busulfan will be adjusted to avoid excessive toxicity or insufficient exposure, aiming at a target AUC of 6000 ng/ml*h, equivalent to a cumulative target AUC of 48000 ng/ml*h (Malar R, et al. 2011). This value is equivalent to 53% of the myeloablative dose exposure (90000 ng/ml*h). Thus, in order to achieve this target cumulative dose of Busulfan, the fifth and the following doses will be adjusted according to the AUC obtained after the first dose. In particular, in case the estimated total target AUC ± 4000 ng/ml*h is achieved already after the 4^{th} dose, the administration of the following doses will be stopped. In case a lower AUC is detected at the first dose, the fifth and next doses should be increased both to reach the target AUC as well to replenish the reduced Busulfan exposure in the first four doses.

A dose adjustment will be performed if total predicted AUC shows more than 10% deviation from the target (total predicted AUC is < 43200 ng/ml*h or > 52800 ng/ml*h).

Dose adjustment calculation: D5 = D1*(target AUC – AUC1*4)/(AUC1*4)

D5 = 5th dose (mg)

D1 = 1st dose (mg)

AUC1 = AUC $_{0-\infty}$ after first dose

In case the total cumulative target is not reached after 8 doses, an additional dose may be administered according to the following calculation:

D9 = D5*[target AUC – (AUC1*4+AUC6*4)/AUC6

D9 = 9th dose (mg)

AUC6 = AUC $_{0-6h}$ after sixth dose

In case of administration of a ninth dose of Busulfan, the cell infusion will be scheduled to allow a wash-out time of 24 hours from the last dose.

6.5.2. Fludarabine

Fludarabine is a nucleoside analogue, currently included in non-myeloablative preparative regimens for HSCT as a drug inducing abrogation of lymphoid precursors in the bone marrow. Fludarabine is commonly used in conditioning regimen with a dose scheme ending at day -3 or -2 before reinfusion of donor HSC at a total dose of 60 mg/sqm. In the peripheral compartment, Fludarabine targets both resting and proliferating lymphocytes, producing a significant lymphopenia of B and T cells. It has been described that CD20^{+} B cells, CD4^{+} T cells (mainly naive (CD4^{+}CD45RA^{+}) T cells) and CD8^{+} T cells decrease profoundly upon Fludarabine treatment. Its half-life is 9.6 hours (3.1-26) measured in adult patients undergoing transplant (Jacobson 2005).

Table 7 Gene therapy schedule for WAS patients

DAY	*	-22	-20	-19	-18	-03	-02	-01	0
PBSC back-up ± CD34+ cell collection	■								
BM harvest ***						■			
iv RITUXIMAB 375 mg/sqm/day		■							
iv BUSULFAN Dose: see below							■	■	
iv FLUDARABINE 30 mg/sqm/day						■	■		
ATG THYMOGLOBULINE ** 2,5 mg/kg/day			■	■	■				
Infusion of transduced CD34+ cells									■

Busulfan bodyweight-based and AUC targeted dose, to be given every 6 hours for 8 doses (+/- 1) as described above (see Table 6).

*Usually performed about 5-10 weeks before treatment. BM collection is performed, if PBSC mobilization is not suitable.

**Iv ATG Thymoglobuline may be used at PI discretion in patients with clinical autoimmunity.

*** BM harvest is not performed if MPB is used as the only cell source for transduction

6.5.3. Rituximab

Rituximab, a monoclonal antibody directed against the CD20 molecule found on pre-B cells and mature B cells, was introduced in the late 1990s for the treatment of non-Hodgkin's lymphoma. Recently, this antibody has been used to treat autoimmune diseases, especially those associated with a prominent humoral component and with potentially pathogenic autoantibodies. Small cohort studies have indicated that rituximab could have an important role in the management of these disorders. Rituximab has also been utilized in the transplant setting, to diminish levels of alloreactive antibodies in highly sensitized patients, to manage ABO-incompatible transplants, and to treat rejection associated with B cells and antibodies. The exact mechanism by which rituximab exerts its effects in autoimmunity and transplantation remains unclear, as specific autoantibody or alloantibody levels often seem not to diminish in parallel with clinical improvement. A role for rituximab in depleting B cells and compromising their antigen-presenting function seems likely; rituximab might also inhibit T-cell activation. Rituximab has been successfully used to treat lymphoproliferative disorder following solid organ transplantation and is commonly employed as pre-emptive therapy for EBV-induced lymphoproliferation in the context of allogeneic HSCT. Rituximab will be used at 375 mg/sqm in a single dose on day -22.

6.6. Safety considerations on the procedure and conditioning regimen

Patients will undergo blood tests before and during treatment and in the follow up phase. In case blood removal will occur through venipuncture, the risks of these procedures are pain, formation of hematoma and minimal risk of infection at the puncture site. Blood tests will be planned on different days to minimize the risks of anemia due to blood loss.

Standard risks associated with implantation of a central venous catheter, collection of stem/progenitor cells by means of apheresis, and BM harvest under general anesthesia are to be taken into consideration (Moog et al. 2001; Nishimori et al. 2001; Mattioli et al. 2007). In particular, the risks of central catheter implantation include bleeding, formation of hematoma, pain, infections and rarely pneumothorax. The possible side effects of G-CSF administration for mobilization of CD34+ cells in the peripheral blood include fever, pain, and rarely allergic type reactions. Overall the safety record of G-CSF is very good, apart from extremely rare reports of spontaneous splenic ruptures. Plerixafor is registered for use in adults. The most commons adverse reactions reported in patients in conjunction with G-CSF were diarrhea, nausea, fatigue, injection site reactions, headache, arthralgia, dizziness and vomiting. The safety and efficacy of its use in children has not been studied in controlled clinical trials; several single centers studies reported no major adverse events with plerixafor administration in children (Pham HP et al. 2012; Son MH et al. 2013; Hong KT et al. 2012; Sevilla J et al. 2012).

Adverse reactions to leukapheresis can derive from transient volume loss, and lowering of calcium levels by the citrate anticoagulant. BM harvest can be associated with bleeding, formation of haematoma, pain, infections and those correlated with general anesthesia.

The risk of bleeding and bruising is expected to be increased in thrombocytopenic patients and will be minimized by the infusion of IVIG and/or platelets' transfusions before the procedure.

The risks associated with reduced intensity busulfan treatment are expected to be decreased compared to standard dose treatment. Busulfan toxicity includes myelosuppression, nausea,

vomiting, hair loss, mucositis, hepatotoxicity, seizures, hypo/hypertension, and lung fibrosis. Prolonged exposure to Busulfan may result in cataract formation, secondary tumors and sterility.

Fludarabine is a drug currently used in paediatric non-myeloablative preparative regimen in HSCT. Fludarabine toxicity includes bone marrow aplasia, nausea, vomiting, mucositis, and transient increase of liver enzymes. If given at high doses, Fludarabine can induce a progressive neurotoxicity due to demyelinating damage as main side effect. The patients enrolled in our clinical trial will receive lower doses of i.v. Fludarabine (standard dose in transplant: 100-150 mg/sqm). During a prolonged exposure to Fludarabine, transient visual disturbances and alterations of consciousness might occur: these scheduled events are described in literature as revertible with the drug's suspension.

ATG is an immunosuppressive drug approved in conventional conditioning as an agent inducing a selective decrease in T-cell count. Prior to and during ATG administration, adequate pre-medication will be dispensed to prevent anaphylaxis episodes or fever and chills, mainly described as occurring during initial infusions. Other common adverse effects include thrombocytopenia, leukopenia, headache, abdominal pain, nausea, diarrhoea, dyspnoea, and dizziness, reversible upon withdrawal. In addition, patients receiving ATG might develop severe infections resulting from over-immunosuppression. Particular caution will be adopted considering post-transplant lymphoproliferative disease. Thus, EBV-positive patients showing signs of viral reactivation after gene therapy, will receive appropriate pre-emptive therapy (Rituximab). Potentially increased risk of malignancy (non-Hodgkin's lymphoma, myeloma) has been reported as complication in long-term exposure of the drug.

The risks associated with Rituximab infusion include infusion reactions (that can be rarely fatal), nausea, vomiting, headache, wheezing, allergic reactions, severe muco-cutaneous reactions, LDH increase, hypo/hypertension. Cases of HBV reactivation and progressive multifocal leukoencephalopathy have been described after the use of this drug. Prior to Rituximab administration, adequate pre-medication will be dispensed to prevent anaphylaxis episodes or fever and chills, mainly described as occurring during initial infusions.

In case of microbiological contamination of bone marrow CD34+ cells, detected after the initiation of the conditioning regimen, culture will be stopped and the lot not released. A new lot will be prepared from mobilized CD34+ cells previously cryopreserved. Cells will be thawed, transduced and re-infused, as described in the IMPD, 3-7 days after the foreseen date.

Considering that the drug product consists of autologous cells, which are washed before cell infusion, no short-term toxicity is expected to occur. In this regard, it should be borne in mind that the infusion of autologous CD34+ cells from bone marrow or mobilized peripheral blood in children affected by haematological or oncological disorders usually does not cause side effects.

6.7. Packaging and labelling of the Medicinal Product

The final Medicinal Product will consist of a cell suspension containing the CD34+ cells transduced with the LV-WASP vector, resuspended in saline, in one or more sterile syringe(s) or bag(s) of the suitable volume.

The Medicinal Product will be labeled with a unique identification code of the patient and of the product, released by MolMed S.p.A, according to cGMP. The Medicinal Product will be released to an Investigator or another member of the investigating team designated by the Principal

Investigator and then transferred in a biohazard container at room temperature to the clinical ward for infusion, according to San Raffaele Stem Cell Program internal operating procedures.

6.8. Dose of the Medicinal Product

Each patient will receive a minimum dose of BM and/or MPB transduced $CD34^+$ cells of 2 x 10^6 / Kg, maximum dose of transduced $CD34^+$ cells of 20 x 10^6 / Kg, with a target of 5 - 10 x 10^6 / Kg, depending on the yield of cells available after transduction.

Transduced mobilized peripheral blood cells will be used in addition to BM in case of insufficient recovery of BM cells (< 2 x 10^6 CD34+ cells/Kg) or in place of BM in case transduced CD34+ cells are not released for infusion.

6.9. Infusion

At day 0, patients will be prepared for the infusion of the transduced cells. Before the infusion, all other i.v. drugs will be interrupted. After pre-medication (15-30 min before infusion) with chlorpheniramine (0.25 mg/kg, max dose 10 mg), transduced cells will be infused i.v. through the central venous catheter in about 20 minutes. At the end of infusion, saline solution will be used to wash the syringe(s)/bag(s) and the line. After the whole procedure, if needed, furosemide (0.2-1 mg/kg, max dose 20 mg) could be administered. Vital signs (BP, HR, SO_2) and symptoms will be monitored at baseline, then every ten minutes during infusion and every hour, for 3 hours after the infusion.

6.10. Hospitalization

The patients enrolled in the gene therapy trial will be followed at the Pediatric Clinical Research Unit/Pediatric Immuno-haematology and Bone Marrow Transplantation Unit, (within the San Raffaele Stem Cell Program), Milan, Italy, a certified AIEOP and GITMO center, currently under JACIE accreditation. At present, the Unit consists of: 3 single rooms for pediatric transplant in a dedicated and confined area; inpatients beds in the department of Pediatrics; day-hospital rooms; outpatient clinics.

During baseline, the patients will be hospitalized for implantation of a central venous catheter, for 3-5 days or according to clinical conditions. During treatment, the patient will be hospitalized for an average of 60 days during chemotherapy, gene therapy infusion and short-term follow up. Thereafter, patients will be followed as outpatients unless invasive procedures are required or complications will occur.

6.11. Supportive therapy

- During hospitalization, patients will be cared for in an isolation unit.
- Beginning before conditioning until recovery from severe neutropenia (ANC<500/µl) or discharge: oral decontamination and mouth care according to local standards.
- Pentamidine (nebulized) as first line *Pneumocystis jirovecii* prophylaxis from day -30 until complete immunological reconstitution.
- Antibacterial and antifungal prophylaxis according to local standards.

- Acyclovir as Herpes Simplex-prophylaxis until complete immunological reconstitution.
- Intravenous substitution to maintain immunoglobulin IgG >5g/l.
- Hydration of 3L/m^2 will be given during the conditioning phase (from day -4 to 24 hours after Busulfan).
- CMV (PCR), EBV (PCR) and adenovirus (PCR) viral load will be determined weekly till day +90. If repeatedly negative, after day +90, the interval of testing could be prolonged. Pre-emptive therapy with Ganciclovir or Foscarnet will be administered, according to local standards, if CMV positive.
- Pre-emptive therapy with Rituximab will be administered to EBV-positive patients after gene therapy in case of repeated increase of EBV viral load, according to local standards.
- Erythrocyte and thrombocyte concentrates will be substituted according to local standard. The decision to administer erythrocytes or platelets transfusions should incorporate individual clinical characteristics of the patients and not simply be a reflexive reaction to the platelet count. All blood products will be leukocyte depleted and irradiated.
- The need for a platelet infusion will be evaluated by the Investigators based on several factors, including:
    - platelets <20 x 10^9/L;
    - the presence of active bleeding;
    - stability of thrombocytopenia;
    - concurrent infection and febrile condition;
    - immune thrombocytopenic purpura;
    - use and response to thrombopoietin (TPO) receptor agonists;
    - presence of risk factors for bleeding (such as an upcoming invasive surgical procedure);
    - toxicity which may lead to an increased risk of bleeding;
    - subjects at risk of fall and trauma; and
    - overall clinical condition of the subject.
- Several factors are considered in deciding whether the interruption of IVIG can be stopped:
    - stable trough levels of IgG (immunoglobulin G) remaining at or above 5 g/L;
    - absence of symptoms of infections or positivity at laboratory tests for pathogens;
    - the presence of adequate levels of B cells in the peripheral blood;

- presence of other risk factors, for example, IVIG is rarely stopped during winter, as winter is considered a period of increased risk for developing upper respiratory tract infections such as influenza.

Based on these factors, the Investigators will consider first prolonging the interval of infusion of IVIG. If trough levels remain stable, IVIG will be stopped with the aim to start vaccinations after 3 months. During this period, IgG will be monitored to check the stability of their levels in serum.

- Antiemetic prophylaxis and analgesic according to local standards.
- Antiepileptic drugs will be dispensed for a short period to prevent seizures due to Busulfan administration.
- G-CSF in case of absolute neutrophil counts <500/microl, at day +45, following bone marrow evaluation.
- Parenteral nutrition will be introduced in case of severe loss of body weight or clinical decision.

The administration of other drugs will be allowed by the Investigators of the study depending on the clinical findings of the patient enrolled in this study.

6.12. Back-up reinfusion

In case of engraftment failure, the stored back-up, with or without the CD34 negative fraction following marrow manipulation, will be infused according to local standards. For the purposes of this study, engraftment failure is defined as failure to reach an absolute neutrophil count (ANC) > 500 neutrophils/µ1 with no evidence of BM recovery (i.e. hypocellular marrow) on day +60. The infusion of the stored back-up could be anticipated in case of lack of evidence of progression to engraftment and presence of a severe life-threatening complication.

6.13. Responsibilities

The Investigator, or other personnel allowed to store and dispense the treatment, will be responsible for ensuring that the medicinal product used in the study is securely maintained as specified in Investigator’s Brochure and in accordance with the applicable regulatory requirements.

All the treatment shall be dispensed in accordance with the Investigator's prescription and it is the Investigator's responsibility to ensure that an accurate record of medicinal product (issued and returned) is maintained.

7. STUDY ASSESSMENTS

7.1. Pre-treatment evaluation (screening and baseline)

After having obtained Informed Consent from parents or guardians, eligible patients will undergo a clinical, laboratory and instrumental pre-treatment work-up including also disease-specific evaluations (extensive immunological, microbiological and autoimmunity assessment). A BM aspirate will be obtained in order to evaluate morphology, cellularity, CD34+ cell content, and clonogenic activity.

Clinical evaluation, laboratory tests and imaging will be performed before treatment as scheduled in Table 8a and Table 8b:

a) **Clinical evaluation.** Medical history, physical examination with assessment of performance status (Lansky), including eczema assessment (according to Imai et al. 2004).

b) **Zhu score** assignment (Table 9).

c) **WAS mutation and WASP expression.** A search for WASP gene mutation will be performed if not available in the clinical records. The mutation will be classified as severe/non severe based on literature data (Imai et al., 2004) database information and prediction studies. The residual levels of WAS protein will be assessed by FACS analyses on peripheral blood and classified as either: a) present, b) reduced, c) absent. The presence of a population expressing normal levels of WASP and representing >5% of lymphocytes will be classified as: d) revertant.

d) **HLA-typing** (to exclude family donor or adequate unrelated donor at screening), if not previously performed; negative search for unrelated donor performed in the last two years, performed for at least for 4-6 months, will be considered a valid inclusion criteria.

e) **Specialist examination:** immunological, anesthesiological, ophthalmological, dental, cardiological evaluation.

f) **Diagnostic imaging and instrumental tests**: **Imaging n°1:** Chest X-ray, ECG, abdomen US scan, echocardiogram, Rx of left hand-wrist for evaluation of bone age, respiratory functional test (if children older than 5 years old). **Imaging n°2:** chest CT scan.

g) **Routine laboratory n°1:** blood count with differential (absolute count), including platelet count, MPV, CRP, LDH, AST, ALT, bilirubin (total, direct and indirect), blood glucose, BUN (Blood Urea Nitrogen), creatinine, creatine phosphokinase (CPK), ALP, GGT, electrolytes (Na, K, Ca, Mg), protein content, urine analysis. **Routine laboratory n°2:** ESR, protein electrophoresis, blood iron, transferrin, ferritin, reticulocytes, blood gases analyses, thyroid balance, coagulation (XDP, PT, APTT, FG).

h) **Bone marrow evaluation:** needle aspirate with immunophenotype: CD3+, CD3+/CD4+, CD3+/CD8+, CD19+, CD20+, CD22+, CD20+/CD22+, CD2+, CD16+/CD56+, CD19+/IgM+, CD19+/kappa, CD19+/lambda, CD71+/Glycophorin A+, CD15+, CD13+, CD38+/CD138+, CD61+, $CD34^+$ (ISHAGE), clonogenic progenitors (CFU-C), morphology, and karyotype. In case of previous allogeneic HSCT, a donor chimerism evaluation will be performed. Pre-therapy bone marrow evaluation will include $CD34^+$

cell purification and transduction of a small volume of BM aspirate (qPCR). Pre-treatment levels of genetically modified cells will be assessed in BM mononuclear cells. The BM aspirate will be planned on the occasion of other procedures requiring sedation, if possible.

i) **Microbiological evaluation: n°1**: Search for bacteria and fungi in nasal and pharyngeal swabs; CMV and EBV DNA search by molecular tests on PB plasma, stool culture, search for bacteria in urine. **n°2:** Search for Legionella antigen in urine. Search for Bacteria, Mycobacteria, fungi, *Pn. Jirovecii*, Legionella, Mycoplasma pn, Chlamydia pn in deep pharyngeal aspirates. Search for Respiratory Syncytial Virus (RSV) in nasal wash. Search for VZV, adenovirus and HSV1, HSV2, HHV6, HHV7 genomes in the PB plasma (molecular test); search for parasites and enteroviruses in the stools. **n°3:** Search for HCV, HBV, HIV genomes in PB plasma (molecular test), TPHA.

j) **Immunological evaluation n°1:** Immunophenotype with lymphocyte subpopulations (CD45+, CD14+, CD3+, CD3+/CD4+, CD3+/CD8+, CD19+, CD2+, CD16+/CD56+, percentage and absolute count. Serum immunoglobulins (IgG, IgA, IgM, total IgE). **n°2:** Immunophenotype with lymphocyte subpopulations (CD3+, CD4+, CD8+, CD16+, CD56+, CD14+, CD45+, TCR1+ (alfa/beta), TCR2+ (gamma/delta), CD4+/CD45RA+ &CD45RO+, CD8+/CD45RA+ & CD45RO+, CD4+/DR+, CD8+/DR+, CD4+/CD25+, CD8+/CD25+, CD3+/CD4+, CD3+/CD8+, CD19+, CD2+, CD16+/CD56+, kappa+, lambda+) percentage and absolute count. Serum immunoglobulins (IgG, IgA, IgM, total IgE). **n°3:** Proliferative response to mitogens (PHA, anti-CD3i, anti-CD3i+anti-CD28, IL-2, PWM). **n°4:** Proliferative response to Candida and alloantigens. Dose response of proliferation after anti-CD3i stimulation on PBMC and/or T-cell lines. Proliferative response to tetanus toxoid (if patient was vaccinated). TCR repertoire by FACS. **n°5** In patients who are not receiving routinely IVIG: serum antibody levels to vaccinal antigens (tetanus toxoid, diphtheria, hepatitis B, Pneumococcus, Pertussis, haemophilus B) and antibodies to pathogens (EBV VCA, measles, rubeola, CMV, HSV, VZV).

k) **Autoimmunity evaluation**, searching organ-specific and systemic antibodies in the serum: anti-platelet antibodies, anti-neutrophil cytoplasmic antibodies (p-ANCA. c-ANCA), anti-DNA antibodies (ADNA), anti-mitochondrial antibodies (AMA), anti-smooth muscle antibodies (ASMA), anti-Liver-Kidney-Muscle (LKM), Coombs tests (direct/indirect) and anti-nuclear antibodies (ANA). If ANA positive, anti-extractable nuclear antigens antibodies (ENA) should be evaluated. If ENA positive, the type should be specified (Anti-RNP, Anti SCL-70, Anti-Sm, Anti SSA, Anti SSB, Anti Jo1).

l) **Archiving of samples.** Samples from peripheral blood and bone marrow (serum, plasma, cell pellets, viable cells in DMSO, cell lines, DNA) will be archived stored at -80° at SR-TIGET, according to Institutional guidelines.

Repeat or unscheduled samples may be taken, or tests performed, for safety reasons or due to technical issues with the samples or tests.

For ethical reasons, under exceptional circumstances, tests or exams performed before patient's enrolment may be considered valuable for patient's pre-gene therapy evaluation.

7.2. Post-treatment evaluation

Patient's assessment will include complete medical examination, diagnostic imaging, microbiological tests, routine laboratory tests, immune profiling evaluation and specific immunological tests.

Clinical evaluation, laboratory tests and imaging will be performed after treatment as scheduled in Table 8a and Table 8b. For clinical evaluation and tests to be performed at time points after day +120, a timeframe of +/- 10% will be accepted, while for time points after 1 year, a timeframe of ± 30 days will be accepted.

a) **Clinical examination.** Anamnesis, physical examination with assessment of performance status (Lansky), including eczema assessment (Imai).

b) **Zhu score** will be re-assigned every year (Table 9).

c) **WASP expression**. Assessed by FACS analyses on peripheral blood.

d) **Specialist examinations**: cardiological, immunological, ophthalmological.

e) **Diagnostic imaging and instrumental tests**: **Imaging n°1**: Chest X-ray, ECG, abdomen US scan, echocardiogram, Rx of left hand-wrist for evaluation of bone age, respiratory functional test (if children older than 5 years old). **Imaging n°2**: chest CT scan. **Imaging n°3**: ECG, abdomen US scan, echocardiogram, Rx of left hand-wrist for evaluation of bone age, respiratory functional test (if children older than 5 years old). In addition, at 5th year: Chest X-ray or CT scan if clinical indication, and at 8th year: chest CT scan. **Imaging n°4**: ECG, abdomen US scan, echocardiogram, respiratory functional test (if children older than 5 years old).

f) **Routine laboratory n°1**: CBC with differential (absolute count), including platelet count, MPV (every year), CRP, LDH, AST, ALT, bilirubin, blood glucose, BUN, creatinine, creatine phosphokinase, ALP, GGT, electrolytes, protein content, urinalysis. **Routine laboratory n°2**: ESR, protein electrophoresis, blood iron, transferrin, ferritin, reticulocytes, blood gases analyses, thyroid balance, coagulation (XDP, PT, APTT, FG).

g) **Bone marrow evaluation**: needle aspirate with immunophenotype: CD3+, CD3+/CD4+, CD3+/CD8+, CD19+, CD20+, CD22+, CD20+/CD22+, CD2+, CD16+/CD56+, CD19+/IgM+, CD19+/kappa, CD19+/lambda, CD71+/Glycophorin A+, CD15+, CD13+, CD38+/CD138+, CD61+, CD34+(ISHAGE), clonogenic progenitors (CFU-C), morphology, and karyotype. PCR analysis on transduced cells will be performed separately, including the assessment of %LV+ colony-forming cells (CFC) from patient's BM-derived clonogenic progenitors.

h) **Microbiological evaluation**: **n°1**: Search for bacteria and fungi in nasal and pharyngeal swabs; CMV and EBV DNA search by molecular tests on PB plasma, stool culture, search for bacteria in the urine. **N°2**: Search for Legionella antigen in urine. Search for Bacteria, Mycobacteria, fungi, Pn. jirovecii, Legionella, Chlamydia pn. and Mycoplasma pn. in deep pharyngeal aspirates. Search for Respiratory Syncytial Virus (RSV) in nasal wash. Search for adenovirus in the blood (molecular test); search for parasites and enteroviruses in the stools. **N°3** Search for HSV1, HSV2, HHV6, HHV7, HCV, HBV, HIV genomes in the PB plasma (molecular test).

i) **Immunological evaluation n°1**: Immunophenotype with lymphocyte subpopulations (CD45+, CD14+, CD3+, CD3+/CD4+, CD3+/CD8+, CD19+, CD2+, CD16+/CD56+, percentage and absolute count. Serum immunoglobulins (IgG, IgA, IgM, total IgE). **n°2**: Immunophenotype with lymphocyte subpopulations (CD3+, CD4+, CD8+, CD16+, CD56+, CD14+, CD45+, TCR1+ (alfa/beta), TCR2+ (gamma/delta), CD4+/CD45RA+ & CD45RO+, CD8+/CD45RA+ & CD45RO+, CD4+/DR+, CD8+/DR+, CD4+/CD25+, CD8+/CD25+, CD3+/CD4+, CD3+/CD8+, CD19+, CD2+, CD16+/CD56+, kappa+, lambda+) percentage and absolute count. Serum immunoglobulins (IgG, IgA, IgM, total IgE). **n°3**: Proliferative response to mitogens (PHA, anti-CD3i, anti-CD3i+anti-CD28, IL-2, PWM). **n°4**: Proliferative response to Candida and alloantigens. Proliferative response to tetanus toxoid (if patient was vaccinated). Dose response of proliferation after anti-CD3i stimulation on PBMC and/or T-cell lines. TCR repertoire studies in the peripheral blood by FACS (Vbeta repertoire). **n°5** In patients who discontinued IVIG (as described in Section 6.11), a vaccination program will be started. Serum antibody levels to vaccinal antigens (tetanus toxoid, diphtheria, hepatitis B, Pneumococcus, Pertussis, Haemophilus B) or pathogens (EBV VCA, CMV, measles, rubeola, VZV, HSV) will be measured.

j) **Autoimmunity evaluation**, searching organ-specific and systemic antibodies in the serum: anti-platelet antibodies, anti-nuclear antibodies (ANA); anti-extractable nuclear antigens antibodies (ENA), if ANA positive; anti-neutrophil cytoplasmic antibodies (p-ANCA. c-ANCA); anti-DNA antibodies (ADNA); anti-mitochondrial antibodies (AMA); anti smooth muscle antibodies (ASMA), anti-Liver-Kidney-Muscle (LKM); Coombs' tests (direct/indirect).

k) **Evaluation of the presence of genetically modified cells**. Vector copy number (measured by quantitative PCR as VCN/cells) will be done after gene therapy in total nucleated cells (day +14), and afterwards in PB lymphocyte subpopulations (CD3+ and/or CD4+ and CD8+ T lymphocytes, B lymphocytes, NK cells) and granulocytes and in bone marrow cell subpopulations (CD15+, GlyA+, CD3+, CD19+, CD56+, CD34+).

l) **Archiving of samples**. Samples from peripheral blood and bone marrow (serum, plasma, cell pellets, viable cells in DMSO, cell lines, DNA) will be archived stored at -80° at SR-TIGET, according to Institutional guidelines.

m) **Platelet activation and morphology**: For platelet activation, the expression of activation markers on the surface of peripheral blood-derived platelets after stimulation with agonists will be assessed. For platelet morphology, the platelet perimeter, area and ultrastructure will be assessed by transmission electron microscopy.

Repeat or unscheduled samples may be taken, or tests performed, for safety reasons or due to technical issues with the samples or tests.

7.3. Extended Long-Term Follow Up

Both European and US regulatory agencies have expectations for the long-term follow up of patients who have been treated using gene therapy (EMA/CAT/GTWP/671639/2008 and revisions; EMEA/CHMP/GTWP/60436/2007; EMA/CAT/852602/2018; FDA LTFU guidance

January 2020; FDA, Considerations for the Design of Early-Phase Clinical Trials of Cellular and Gene Therapy Products, June 2015).

Patients who complete 8 years of follow up from the initial infusion of transduced stem cells (as part of the *Follow Up phase*) will then continue to be followed up as part of the ELTFU phase. During the ELTFU phase, patients will be contacted annually (+/- 3 months) by telephone, email or letter in an effort to collect long-term safety and selected efficacy data until a separate OLTFU study is available and patients have enrolled in it (see Table 8c). As part of the OLTFU study, patients will continue to be followed up for a total of 15 years from the date of treatment with gene therapy. Should a patient decide not to consent to participate in the ELTFU nor transition to the OLTFU study, then the subject will be considered to have completed this study (WAS Pivotal 201228) and will be transitioned to standard of care according to local practice.

Table 8a Pre and post-treatment evaluation (up to 36 months after gene therapy)

Parameter / **Time point**	Clinical Exam.***	Routine Lab. N° 1	Routine Lab. N° 2	Bone Marrow+ qPCR	Microbiology N° 1	Microbiology N° 2	Microbiology N° 3	Immunology N° 1	Immunology N° 2	Immunology N° 3	Immunology N° 4	Immunology N° 5	Zhu score	HLA typing and donor search	WASP Expression**	Autoimmunity	PCR Transduced cells (PB)	Archiving Blood/BM sample	Specialist Examination	Imaging N° 1	Imaging N° 2	AEs/SAEs recording	Platelet activation and morphology
Screening	X	X		X*			X		X				X	X	X			X					
Baseline	X	X	X		X	X			X	X	X	X	X		X	X		X	X	X	X		
Day -14	X	X						X															
Day 0	X	X						X										X				X	
+ 7 days	X	X			X													X				X	
+ 14 days	X	X			X			X									X	X				X	
+ 21 days	X	X			X													X				X	
+ 30 days	X	X		X	X	X			X						X		X	X				X	
+ 60 days	X	X			X				X						X		X	X				X	
+ 90 days	X	X	X	X	X	X			X	X					X		X	X				X	
+ 180 days	X	X	X		X				X	X	X				X	X	X	X				X	
+ 1 y	X	X	X	X	X	X	X		X	X	X	X	X		X	X	X	X	X	X		X	
+ 1,5 y	X	X	X						X						X		X	X				X	
+ 2 y	X	X	X	X	X	X	X		X	X	X	X	X		X	X	X	X	X	X		X	
+ 2,5 y	X	X	X						X						X		X	X				X	
+ 3 y	X	X	X	X	X	X	X		X	X	X	X	X		X	X	X	X	X	X	X	X	X[p]

* if the qPCR is not performed at this time point it may be performed on bone marrow collected for back-up or harvested for preparation of medical product.

** WAS mutation search carried out at screening if not available

*** Eczema will be evaluated as part of the clinical examination (according to Imai et al. 2004)

[P] Platelet activation and morphology will be assessed at at least one time point ≧ 3y post-GT

Repeat or unscheduled samples may be taken, or tests performed, for safety reasons or due to technical issues with the samples or tests.

Table 8b Long-term follow up evaluation

Parameter / Time point	Clinical Exam.***	Routine Lab. N° 1	Routine Lab. N° 2	Bone Marrow+ qPCR	Microbiology N° 1	Microbiology N° 2	Microbiology N° 3	Immunology N° 2	Immunology N° 3	Immunology N° 4	Immunology N° 5	Zhu score	WASP Expression	Autoimmunity	PCR Transduced cells (PB)	Archiving Blood/BM sample	Specialist Examination	Imaging N° 3	Imaging N° 4	AEs/SAEs recording	Platelet activation and morphology
+ 4 y	X	X	X		X		X	X	X	X	X	X	X	X	X	X	X		X	X	X[P]
+ 5 y	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X[P]
+ 6 y	X	X	X		X		X	X	X	X	X	X	X	X	X	X	X		X	X	X[P]
+ 7 y	X	X	X		X		X	X	X	X	X	X	X	X	X	X	X		X	X	X[P]
+ 8 y	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X[P]

*** Eczema will be evaluated as part of the clinical examination (according to Imai et al. 2004)

[P] Platelet activation and morphology will be assessed at at least one time point ≧ 3y post GT

Repeat or unscheduled samples may be taken, or tests performed, for safety reasons or due to technical issues with the samples or tests.

Table 8c Extended Long-Term Follow Up evaluation

Parameter	***Year 9+ p.a. (+/- 3 months)***
Social Life Is the patient living in a protected environment? Is the patient attending kindergarten/school or in employment (according to age)? - *Please comment.* How is the patient's social ability with peers (if applicable for age)? - *If abnormal please specify.* Does the patient practice any sport (if applicable for age)? - *Please comment.*	**X**
General Number of platelet infusions per year Complete Blood Count (CBC) including Platelet count/MPV	**X**

Table 8c Extended Long-Term Follow Up evaluation (Continued)

Parameter	*Year 9+ p.a. (+/- 3 months)*
Specialist laboratory assessments Integration Site Analysis (ISA)	**X**
Safety* SAEs AEs (related to bleeds, infections, autoimmunity, eczema, suspected malignancies)	**X**
Ig usage** Intravenous Ig Subcutaneous Ig	**X**

* SAE and AEs related to bleeds, infections, autoimmunity, eczema, suspected malignancies will be forwarded to TIGET as per current practice by the physician overseeing the patient in their home country. This includes the event, labs, and any other relevant data. In addition patients/family may also provide such information directly to TIGET. All such AEs should be entered into the eCRF within 30 days of awareness. SAEs, and any suspected malignancies, should be reported within 24 hours of awareness.

** Frequency to be specified if possible.

Table 9 WAS scoring system according to Zhu.

	XLT			WAS			
Clinical scores	0.5	1	2	3	4	5A	5M
Thrombocytopenia	+/-	+	+	+	+	+	+
Eczema	-	-	+/-	+	++	++/-	++/-
Immunodeficiency	-	-	+/-	+	++	++/-	++/-
Autoimmunity	-	-	-	-	-	+	-
Malignancy	-	-	-	-	-	-	+

The score is based on Zhu and colleagues (Zhu et al. 1997), with subsequent refinements (Bosticardo et al., 2009).

8. ASSESSMENT OF SAFETY

8.1. Adverse Events

8.1.1. Adverse Event (AE)

Any untoward medical occurrence in a patient or clinical trial subject administered a medicinal product and which does not necessarily have a causal relationship with this treatment.

8.1.2. Adverse Reaction (AR)

All untoward and unintended responses to an investigational medicinal product related to any dose administered.

8.1.3. Serious adverse event (SAE)

Any untoward medical occurrence or effect that at any dose results in death, is life-threatening, requires hospitalization or prolongation of existing hospitalization, results in persistent or significant disability or incapacity, or is a congenital anomaly or birth defect.

8.1.4. Unexpected adverse reaction (UAR)

An adverse reaction, the nature or severity of which is not consistent with the applicable product information (e.g. investigator's brochure for an unauthorized investigational product or summary of product characteristics for an authorized product).

8.1.5. Suspected unexpected serious adverse reaction (SUSAR)

An adverse event assessed as serious and unexpected and for which there is a reasonable suspected causal relationship with an investigational medical product.

8.2. Pharmacovigilance Procedure

8.2.1. Reporting of Serious Adverse Events (SAEs)

The Investigator shall report all SAEs to the Sponsor or its designee within 24 hours after becoming aware of the SAE.

SR-TIGET should inform the Sponsor of the following events within three days of gaining awareness:

- Positive result from replication competent lentivirus (RCL) testing
- Test results suggesting abnormal clonal proliferation

Events meeting the Hy's law criteria (ALT ≥ 3xULN and bilirubin ≥ 2xULN (>35% direct bilirubin) or ALT ≥ 3xULN and INR>1.5, if INR measured) will be reported as SAEs.

Patients with liver function abnormalities should be monitored until liver chemistries resolve, stabilise or return within baseline. Tests to define the aetiology of the abnormal liver function tests should be conducted as relevant.

SAEs should be reported using the paper SAE reporting form. After completion, the form should be scanned and emailed to the SAE contacts (Medical Monitor and Sponsor's pharmacovigilance designee) found at the beginning of the protocol on the Sponsor/Medical Monitor Contact Information page.

In rare circumstances if email communication is not available, notification by facsimile is acceptable.

8.2.2. Regulatory Reporting Requirements for SAEs

Prompt notification by the Investigator of SAEs related to study treatment is essential so that legal obligations and ethical responsibilities towards the safety of subjects and the safety of a product under clinical investigation are met.

The Sponsor has a legal responsibility to notify both the local regulatory authority and other regulatory agencies about the safety of a product under clinical investigation. The Sponsor will comply with country specific regulatory requirements relating to safety reporting to the regulatory authority, Institutional Review Board (IRB)/Independent Ethics Committee (IEC) and investigators.

Investigator safety reports are prepared for suspected unexpected serious adverse reactions according to local regulatory requirements and Sponsor policy and are forwarded to Investigators as necessary.

An Investigator who receives an investigator safety report describing a SAE(s) or other specific safety information (e.g., summary or listing of SAEs) from the Sponsor will file it with the IB and will notify the IRB/IEC, if appropriate according to local requirements.

Suspected Unexpected Serious Adverse Reaction (SUSAR)

In case of SAR that is unexpected, the Investigator/Sponsor must report the reaction as soon as possible (and in any case no later than 7 days for reactions that are fatal or life-threatening, and no later than 15 days for all other SUSARs) to the competent Regulatory Authorities and to the competent Ethics Committee (and that relevant follow up information is subsequently communicated per the applicable regulation/guideline).

8.2.3. Adverse event recording

All AE/AR, regardless of seriousness or relationship to Medicinal Product or expectedness, are to be recorded on the corresponding page(s) included in the Case Report Forms. Whenever possible, symptoms should be grouped as a single syndrome or diagnosis. The Investigator should specify the date of onset, maximal intensity, action taken with respect to Medicinal Product, corrective therapy given, outcome and his/her opinion as to whether there is a reasonable possibility that the event was caused by the study Medicinal Product.

The investigator shall keep detailed records of all adverse events, which are reported. Events occurring from enrolment to the administration of the autologous gene modified cells (Medicinal Product), including the preparatory phase of conditioning, will be recorded as Concomitant Diseases in the CRF. These may include events related to the procedures (e.g. CVC positioning, bone marrow harvest, mobilization and collection), chemotherapy, and other drugs. These reports must be provided to the competent Authorities if they so request.

8.2.4. Expected AE/AR

The following reactions are expected following G-CSF + Plerixafor administration, conditioning regimen, other concomitant drug administration, and blood products and will be recorded if ≥2 CTCAE grade for clinical manifestations, ≥ 3 CTCAE grade for laboratory parameters:

- Vomiting
- Nausea
- Stomatitis
- Mucositis
- Diarrhea
- Hair loss
- Fever of undetermined origin during neutropenia
- Fatigue
- Loss of appetite
- Cytopenia
- Bleeding
- Headache
- Hypotension
- Hypertension
- Vasomotor effects, including flushing
- Dizziness
- Arthralgias
- Injection site reactions
- Allergic reactions (to transfusions or drugs different from the medicinal product)
- Electrolytes disturbances*
- Liver and renal function alterations*

**Abnormal laboratory values or test results constitute adverse events only if they induce clinical signs or symptoms or require therapy.*

8.2.5. Follow up of AE / AR / SAE / SAR

The Investigator should take all appropriate measures to ensure the safety of the patients, notably he/she should follow up the outcome of any event (clinical signs, laboratory values or other, etc…) until the return to normal or until consolidation of patient conditions.

In the case of any **Serious Adverse Event or Serious Adverse Reaction**, the patient must be followed up until clinical recovery is complete and laboratory results have returned to normal, or until progression has been stabilized. When the clinical recovery lasts longer than thirty (30) days the Investigator will provide to the Ethics Committee an Additional Safety Report. This may imply that follow up will continue after the patient left the study and that additional investigations may be requested by the Ethical Committee.

8.2.6. Follow up procedure for withdrawn patients

All withdrawn patients should be recorded by the Investigator in the appropriate pages of the CRF when considered as confirmed. If possible, the patients should be assessed using the procedure planned for the end of study visit.

For the withdrawn patients, according to Italian regulations on gene therapy, the follow up will continue just for the safety aspects.

8.3. Assessment of safety endpoints

8.3.1. Safety of the conditioning regimen

Conditioning regimen-related safety consists in the absence of failure of hematological recovery and/or delayed hematopoietic reconstitution, which is defined as ANC<500 at +60 days after transplantation, with aplastic bone marrow and requirement for back-up infusion.

8.3.2. Safety of the transduced cell infusion

Short-term safety of LV-transduced cell infusion consists in the absence of severe adverse reactions after infusion.

Microbiological contamination of the drug product: although all manipulations will be performed under validated GMP procedures, collection of bone marrow cells and their manipulation *ex vivo* is potentially associated with contamination. Standard operating procedures aimed at reducing this risk will be implemented during collection. The final results of the sterility test will be available after patient infusion. If a contamination is detected, the patient will receive a specific antibiotic treatment and a strict monitoring.

Long-term safety of LV-transduced cell infusion consists in the absence of RCL, abnormal clonal proliferation and immune responses against the transgene.

The overall safety of the experimental treatment will be evaluated on the basis of clinical and laboratory parameters recorded and monitored during the follow up of the treated patients, based on the assessment plan. A descriptive analysis will be performed on AE, AR, SAE/SAR, UAR, SUSAR, and events will be classified for frequency, severity and body system involved.

8.4. Specific safety monitoring plan for gene therapy

As per gene therapy regulations, patients who received gene therapy with lentiviral vectors will be followed up for 15 years after treatment. The first three years of safety follow up (during the *Initial Follow Up* period) are described in Table 10 . After completion of this *Initial Follow Up period*, patients enrolled and treated in this study will continue to be followed from 3 years up to 8 years as part of the *Long-Term Follow Up period.*

Patients who complete the *Long-Term Follow Up period* in this study(i.e. 8 years of follow-up) will then be contacted annually from year 8 onwards (as part of the ELTFU phase) in order to collect long-term follow up safety and selected efficacy data, until a separate OLTFU study is initiated and patients have enrolled in it. Once the OLTFU study is set up, patients will be invited to enter the OLTFU study at any time after they have completed a minimum of 5 years of follow up in this study (WAS Pivotal 201228) . As part of the OLTFU study, patients will be followed up for a total of 15 years following treatment with gene therapy. In case no detectable engraftment of LV-transduced cells will be observed for >1 year, patients will be followed only for safety aspects.

8.4.1. Replication competent lentivirus (RCL)

Molecular monitoring of RCL will be assessed via ELISA for HIV p24 antigen in serum.

A positive HIV p24 test result will be subject to confirmation. If confirmed, second level testing will include a) DNA PCR for VSV-G envelope (cells) and b) RT-PCR for HIV-pol RNA (serum). Should one of these second level tests also provide a positive result, an appropriate confirmatory test may be conducted.

The tests will be performed at baseline, after 1, 3, 6, 12 months and 24 months. The samples will be stored every year thereafter. If one of the above mentioned tests are positive, the screening evaluations will be repeated at the next planned follow up visit; in the case two of the three screening tests are positive, a confirmatory RCL culture test will be performed.

8.4.2. Abnormal clonal proliferation (ACP)

For safety monitoring of abnormal clonal proliferation, the following plan will be implemented during the first 3 years of follow up:

A. First level screening *(every 6 months)*

1. Clinical evaluation

2. Blood count with differential to detect abnormal proliferation or cytopenia
3. Routine biochemical tests to detect abnormal organ functions
4. Serum immunoglobulins and protein serum electrophoresis, for the presence of altered or monoclonal immunoglobulin components
5. Flow cytometry on peripheral blood for lymphocyte subpopulations (CD2, CD3, CD4, CD8, CD56, CD16, TCR alfa/beta, TCR gamma/delta, CD19, Ig-kappa, Ig-lambda)
6. Flow cytometry with monoclonal antibodies for TCR Vbeta families
7. PCR analysis for measuring vector copy number (VCN).

B. Second level screening (every year):

1. Bone marrow aspirate, including morphological examination, cytogenetic analysis and detailed immunophenotyping
2. Cytogenetic analyses on lymphocytes (karyotype)

3. Archive pellets and viable cells of BM and/or PB.

C. Additional tests (if above tests suggest monoclonal expansion, to be performed on recent and archived samples):

1. BCR clonality (PCR)
2. Clonality analysis for TCR (PCR)
3. Establishment of cell lines
4. Molecular tests for identifying proviral integrants ((Linear-amplification mediated) LAM-PCR or (ligation-mediated) LM-PCR)
5. RT-PCR for expression of vector and genes next to the integration
6. All other appropriate imaging and laboratory tests.

Tests (1) to (5) are available as research grade at TIGET

Test of first and second level screening (I, II) are already included in the efficacy evaluation in Table 8a and Table 8b.

For the long-term follow up (from 3 years up to 8 years) the ACP safety-monitoring tests will be performed as follows:

1) First level screening will be performed every year from the 4th to 8th year of follow up.
2) Second level screening will be performed as follows:
   a. Bone marrow aspirate on *year 5 and 8*
   b. Cytogenetic analyses on lymphocytes (karyotype): every year from the 4th to 8th year follow up
   c. Archive pellets and viable cells of BM and/or PB: every year from the 4th to 8th year follow up

As part of the overall safety evaluation, a detailed analysis of LV integrations will be performed on PBMC and BM cells, to monitor the nature and distribution vector integration sites. These studies will help defining the clonal composition of the transduced cell graft and the eventual occurrence of clonal expansion.

8.4.3. Immune response to transgene

Patients will be monitored for antibodies anti-WASP for the lack of immune response to transgene every 6 months for the first year, then at year 2 and year 3. Moreover, anti-HIV antibodies (anti-p24) will be monitored at the same time points.

Table 10 Safety follow up (from 0 to 3 years)

Parameter			
Time point	RCL	ACP	Immune response to transgene
Baseline	**X**	**X**	**X**
+ 30 days	**X**		
+ 90 days	**X**		
+ 180 days	**X**	**X**	**X**
+ 1 y	**X**	**X**	**X**
+ 1,5 y		**X**	
+ 2 y	**X**	**X**	**X**
+ 2,5 y		**X**	
+ 3 y	**Archive**	**X**	**X**

9. ASSESSMENT OF EFFICACY

9.1. Sustained engraftment of genetically corrected cells in peripheral blood and/or in bone marrow

Positive engraftment will be assumed as detectable gene corrected cells on peripheral blood and bone marrow cells at 1 year after gene therapy. Adequate engraftment is defined as ≥ 4% CD34+ cells (equivalent to 0.04 VCN/cell) in the bone marrow or ≥ 10% peripheral blood T lymphocytes or peripheral blood mononuclear cells (when cells are not sufficient) (equivalent to 0.1 VCN/cell). This value is based on clinical experience in other gene therapy trials, which has demonstrated 4% average long-term engraftment of autologous hematopoietic stem/progenitor cells transduced with retroviral vectors in the bone marrow of paediatric patients with ADA-SCID and receiving reduced intensity, non-myeloablative conditioning (Aiuti et al., 2009; unpublished results).

To further confirm results, the PB T cell or PB MNC cells and the pre-GT sample will be performed at MolMed S.p.A.

In addition, the multilineage engraftment will be evaluated in BM and/or PB subpopulations (BM subpopulations: GlyA+, CD15+, CD3+, CD19+, CD56+; PB subpopulations: CD15+, CD19+, CD56+). If data on PB CD3+ are not available, the average of PB CD4+ and PB CD8 T cells will be calculated and reported as T-cell engraftment. Adequate multilineage engraftment is defined as ≥ 4% in all the available cells (equivalent to 0.04 VCN/cell).

Assessment parameter: PCR for transduced cells (specific for lentiviral vector sequences).

9.2. Expression of vector-derived WASP

Positive WASP expression will be assumed by presence of WASP protein on peripheral blood or bone marrow cells, as investigated by FACS analyses or Western Blot.

Assessment parameters: analyses of WASP expression (FACS or Western Blot with antibodies specific for WASP).

9.3. Improved T-cell functions

Improvement in T-cell proliferation upon stimulation with anti-CD3i mAbs ≥1 year after gene therapy (as compared to pre-gene therapy values) on PBMC and/or T-cell lines. The degree of correction will be evaluated with respect to normal controls.

Assessment parameters: proliferative responses in response to anti-CD3i mAb stimulation *in vitro*.

9.4. Antigen-specific responses to vaccinations

Ability to mount a humoral response to nominal antigens including antibodies to T-cell dependent antigens (Tetanus Toxoid, Diphtheria, Hepatitis B, Pertussis, Haemophilus B) and unconjugated polysaccharide antigens (Pneumococcus), measured after vaccination (foreseen >1 year after gene therapy). In case of failure of immunization with unconjugated polysaccharide antigens after vaccination, the patients will receive a conjugated polysaccharide vaccine. Positive

cellular response to Tetanus Toxoid after vaccination will be measured by *in vitro* proliferative response >1 year after gene therapy.

Assessment parameters: 4 out of 5 protective antibody titers after vaccination and antigen proliferative responses to tetanus toxoid. In case results are available on n<5 antigens, the rule of at least n-1 will be applied for defining success.

Protective antibody titer after unconjugated vaccination to Pneumococcus (the determination of this antibody titer will be performed by the laboratory of Dr. M. Eibl, Immunologische Tagesklinik, Wien, Austria).

9.5. Improved platelet count and normalization of MPV

Increase in platelet count compared to baseline, considered as the mean of the (at least three) determinations performed in the 3 months before beginning of treatment. Patients will also be evaluated according to a stratification of the severity of thrombocytopenia in 5 groups (see Section 8.3.1, Table 5).

The individual longitudinal profile will be analyzed according to the planned follow up visit and including platelet counts collected for clinical reasons during follow up (in transfusion independent patients).

The determination of platelet count following procedures that may alter platelet number (thrombocyte infusion, TPO agonists etc.) will not be considered for evaluation.

Normalization of MPV compared to baseline will be evaluated at 1 year after gene therapy.

Assessment parameters: Platelet count and MPV.

9.6. Reduced bleeding episodes

Reduction in severity and/or frequency of bleeding manifestations when present, starting from the first year follow up visit and thereafter until the 3-year visit compared to clinical history from the 12 months prior to GT. Information about bleeding will be recorded through follow up by collecting data from parents, clinical reports periodically sent by local doctors following the patients at home and by clinical evaluations performed at each follow up visit at our center. Data will be recorded in the patients' clinical charts. Bleeding events will be given CTCAE grading (as described in Section 4.3.2.1).

Assessment parameters: clinical evaluation.

9.7. Reduced frequency of severe infections

Decrease in number of severe infections (defined by CTCAE criteria , as described in Section 4.3.2.1) will be evaluated starting from the first year follow up visit and thereafter until the 3-year visit by clinical history, complete physical examinations, and microbiological tests, compared to clinical history from the 12 months prior to GT.

Assessment parameters: Clinical evaluation, laboratory tests, microbiology.

9.8. Modification of eczema and autoimmunity phenomena

Modification of eczema and of autoimmunity laboratory markers and/or clinical manifestations (if present before treatment), as evaluated starting from the first year follow up visit and thereafter until the 3-year visit by organ-specific and systemic autoantibodies, imaging and clinical follow up, as compared to clinical history.

Assessment parameters: clinical evaluation including eczema score (Imai, 2004) and autoimmunity laboratory tests.

9.9. Improved quality of life

Improved quality of life, measured after the first year of treatment by reduced hospitalization, reduced requirement of drugs, school attendance, social activities.

Assessment parameters: clinical evaluation, performed according to patients' age (ex. social ability with peers will not be evaluated before 2 years of age and sport practice will not be considered for evaluation before 4 years of age).

9.10. Platelet activation profile and morphology (exploratory endpoint)

Activation of platelets will be assessed after stimulation with different concentrations of the platelet activation agonists ADP and thrombin.

Assessment parameters: platelet activation will be assessed by cytofluorimetric measurement of surface activation markers P-selectin and GPIIbIIIa after stimulation with various concentrations of ADP and thrombin. The dose response curves will be compared with healthy controls.

Platelet morphology will be assessed by transmission electron microscopy.

Assessment parameters: platelet perimeter, area and ultrastructure. Morphology will be compared with healthy controls.

10. STUDY MONITORING

10.1. Responsibilities of the Investigator(s)

The Investigator(s) undertake(s) to perform the study in accordance with this Protocol, Good Clinical Practice and the applicable regulatory requirements.

The Investigator(s) should permit monitoring and auditing by the sponsor, and inspection by the appropriate regulatory Authority(ies).

The Investigator is required to ensure compliance with the investigational product schedule, visits schedule and procedures required by the protocol.

The Investigator agrees to provide all information requested in the Case Report Form in an accurate and legible manner.

10.2. Use and completion of Case Report Forms (CRFs)

It is the responsibility of the Investigator to maintain adequate and accurate CRFs to record all observations and other data pertinent to the clinical investigation.

Under SR-TIGET sponsorship paper CRFs were utilized; these should be completed in their entirety in a neat, legible manner to ensure accurate interpretation of data; a black ball point pen should be used to ensure the clarity of reproduced copy of all CRFs. Should a correction be made, the information to be modified must not be overwritten. The corrected information will be transcribed by the authorized person next to the previous value and dated.

An electronic CRF will be implemented; all data previously collected on paper CRF and all data collected following sponsorship transfer will be entered into defined CRFs. All these data will be transmitted electronically to the Sponsor or designee and combined with data provided from other sources in a validated data system.

Management of clinical data will be performed in accordance with applicable Sponsor standards and data cleaning procedures to ensure the integrity of the data, e.g., removing errors and inconsistencies in the data.

Adverse events and concomitant medications terms will be coded using MedDRA (Medical Dictionary for Regulatory Activities) CRFs (including queries and audit trails) will be retained by the Sponsor, and copies will be sent to the investigator to maintain as the investigator copy. Subject initials will not be collected or transmitted to the Sponsor according to the Sponsor policy.

10.3. Definition of Source Data

Source data include all the documents related to the patient during the trial. These include (but is not limited to) the medical records which support the SAE and SUSAR report forms, and may include patient's medical files, appointment book, original laboratory records, discharge summary, death certificate, autopsy report if available, as they are related to the trial and support the data in the CRFs.

All parameters asked for in the case report form should be documented in the source documents.

10.4. Trial Monitoring

The purposes of trial monitoring are to verify that:

- the rights and well-being of human subjects are protected;
- the reported trial data are accurate, complete, and verifiable from source documents;
- the trial will be conducted in compliance with the currently approved protocol/amendment(s), with GCP, and with the applicable regulatory requirement(s).

10.5. Qualifications of Monitors

Monitors should be appointed by the sponsor.

Monitors should be appropriately trained, and should have the scientific and/or clinical knowledge needed to monitor the trial adequately.

Monitors should be thoroughly familiar with the investigational product(s), the protocol, written informed consent form and any other written information to be provided to subjects, the sponsors SOPs, GCP, and the applicable regulatory requirement(s).

10.6. Monitoring Procedures

The monitor should follow the sponsor’s established written SOPs as well as those procedures that are specified by the sponsor for monitoring a specific trial.

10.7. Monitoring Report

The monitor should submit a written report to the sponsor after each trial-site visit or trial-related communication.

Reports should include the date, site, name of the monitor, and name of the investigator or other individual(s) contacted.

Reports should include a summary of what the monitor reviewed and the monitor's statements concerning the significant findings/facts, deviations and deficiencies, conclusions, actions taken or to be taken and/or actions recommended to secure compliance.

11. ETHICAL CONSIDERATION

This trial complies with the principles laid down by the Declaration of Helsinki (last version) and the ICH GCP guidelines.

This Protocol and its conduction comply with in use laws and guidelines of the Italian Ministry of Health.

11.1. Informed Consent

The Principal Investigator, according to applicable regulatory requirements, or a person designated by the Principal Investigator, should fully inform the patient and/or parents or legal representatives of all pertinent aspects of the clinical trial.

The Informed Consent Form and its updates used by the Investigator for obtaining the patient's informed consent must be reviewed and approved by Ethics Committee. Informed consent will be performed by the Principal Investigator, or a person designated by the Principal Investigator, at the original treatment center, and may take place remotely.

In case of the patient is minor (<18 years of age), the Informed Consent of the parents or legal representatives must be obtained; consent must represent the minor's presumed will and may be revoked at any time, without detriment to the minor.

The parents or legal representatives should have an interview with the investigator, or another member of the investigating team designated by the Principal Investigator, in which they are given the opportunity to understand the objectives, risks and inconveniences of the trial and the conditions under which it is to be conducted.

The minor should receive information according to its capacity of understanding, from staff with experience with minors, regarding the trial, the risks, and the benefits.

The person not able to give informed legal consent should receive information according to his/her capacity of understanding the trial, the risks, and the benefits.

Prior to a patient's participation in the clinical trial, the Informed Consent Form should be signed and dated by the patient and/or the parents or legal representatives, and by the person who conducted the informed consent discussion.

The patient and/or parents or legal representatives should receive an original copy of the signed and dated written informed consent form.

During the participation in the trial, the subject or legal representatives should receive a copy of the signed and dated consent form updates and an original copy of any amendments to the written information provided to subjects.

The patient and/or the parents or legal representatives may at any time, and without giving any explanation, withdraw from the trial. In any case the doctors will continue to follow the patient with proper attention and care.

11.2. Investigator's Brochure

The Investigator's Brochure (IB) should provide the investigators and others involved in the trial with the information to facilitate their understanding of the rationale for, and their compliance

with, many key features of the protocol, such as the dose, dose frequency/interval, methods of administration, and safety monitoring procedures.

The IB should contain the following sections, with appropriate references for each chapter (ICH Topic E6 GCP):

- Table of Contents
- Summary
- Introduction
- Physical, Chemical, and Pharmaceutical Properties and Formulation
- Nonclinical Studies
- Nonclinical Pharmacology
- Pharmacokinetics and Product Metabolism in Animals
- Toxicology
- Effects in Humans
- Pharmacokinetics and Product Metabolism in Humans
- Safety and Efficacy
- Marketing Experience
- Summary of Data and Guidance for the Investigator
- Reference on 1. Publications
  2. Reports

11.3. Minimizing distress

Repeated invasive procedures may be painful or frightening. This trial is designed to minimize the discomfort. The study is designed and conducted by investigators experienced in the treatment of paediatric/vulnerable subjects/patients.

The protocol and investigations have been designed specifically for the paediatric population and approved by an Ethical Committee expert in paediatric/vulnerable subjects/patients.

Practical considerations to ensure that participants' experiences in clinical studies are positive and to minimize discomfort and distress include the following:

- personnel skilled in dealing with the paediatric population and its age-appropriate needs, including skill in performing paediatric procedures;
- an environment with furniture, play equipment, activities, and food appropriate for age;
- the conduction of studies in a familiar environment such as the hospital or clinic where participants normally receive their care;
- approaches to minimize procedures related discomfort, such as:
  - local anaesthesia to place peripheral venous catheters;

- indwelling catheters (central or peripheral) rather than repeated venipunctures for blood sampling;
- collection of protocol-specific blood samples in occasion of routine blood sampling, performed for clinical reasons.

11.4. Minimizing Risk

Every effort has been made to anticipate and reduce known hazards. Investigators are fully aware and trained on all relevant preclinical and clinical toxicity of the medicinal product. To minimize risk in paediatric/vulnerable subjects clinical studies, investigators are experienced in studying the paediatric/vulnerable subjects population, including the evaluation and management of potential adverse events.

In designing studies, every attempt was made to minimize the number of procedures, consistent with good study design. Mechanisms are in place to ensure that the study can be put on hold or modified should an unexpected hazard be noted.

12. STATISTICAL DESIGN

12.1. Protocol modification and stopping rules

As already described in section 8.2 for relevant endpoints, rules for protocol modification and stopping serve as guidelines for discussing the continuation/modification of the study. The rules are defined below.

Protocol modification:

- 2 out of the first 3 patients suffering of prolonged aplasia;

- 2 or more failures of engraftment of transduced cells.

Early Stopping:

- 2 transplant-related deaths;

- 1 malignant proliferation related to gene therapy.

In the interest of the patients' safety, the third patient will be treated when:

- at least 1 of the first 2 patients will achieve hematopoietic recovery;

- at least 60 days after the first treatment.

For the subsequent patients the above rules will be applied.

12.2. Study Population and Timing of Analysis

All study patients will be included for evaluation until the earliest time point of the following: completion of a minimum of 5 years follow up in this study (WAS Pivotal, 201228) and transition to a separate OLTFU study, death, withdrawal or loss to follow up. Patients who, at 1 year after gene therapy, show absence of transduced cell engraftment in peripheral blood and bone marrow will be subsequently evaluated for safety aspects only.

At the end of the study, when all subjects have completed a minimum of 5 years follow up and transitioned to a separate OLTFU study, a final statistical analysis will be performed with all data available up to the transition to the OLTFU study, and a final clinical study report will be compiled.

12.3. Safety Endpoints

A descriptive analysis will be performed for all primary and secondary safety endpoints.

12.4. Efficacy endpoints

Primary efficacy endpoints

Overall survival

The Kaplan-Meier estimate of survival at 3 years from gene therapy will be obtained, with 95% Confidence Intervals, based on deaths by any causes related to disease. Survival will be also monitored as part of the long-term follow up.

Sustained engraftment of genetically corrected haematopoietic stem cells in peripheral blood and/or in bone marrow

The percentage of subjects who present at one year after gene therapy with adequate engraftment defined as ≥ 4% CD34+ in the bone marrow or ≥ 10% in peripheral blood T lymphocytes will be estimated and the 95% Confidence Intervals (CI) of this proportion will also be calculated. The description of the longitudinal profile over time of the percentage of gene corrected cells on both bone marrow and peripheral blood might be included as additional information.

Expression of vector-derived WASP

The percentage of subjects who present at one year after gene therapy with detectable vector-derived WASP expression will be estimated, with 95% CI.

Improved T-cell functions

The mean change between the pre-gene therapy and the 1-year values of in vitro T-cell proliferation upon stimulation with anti-CD3i will be calculated, with 95% CI, if appropriate. The description of the longitudinal profile over time of the levels of the in vitro T-cell proliferation might be included as additional information. The degree of correction (reached at 1, 2 and 3 years from gene therapy) with respect to normal control levels will also be described.

Antigen-specific responses to vaccinations

The percentage of subjects who respond to vaccination (see Section 9.4) performed > 1 year after gene therapy will be estimated, with 95% CI.

Improved platelet count

The percentage of subjects who improved the level of platelet count at 1, 2 and 3 years after gene therapy compared to baseline, shifting from one category to the subsequent as defined in Section 9.5, will be estimated, with 95% CI. The relative mean change in platelet count with respect to pre-gene therapy value will also be given. The description of the longitudinal profile over time of the platelet count might be included as additional information.

Secondary efficacy endpoints

The percentage of subjects who present at one year after gene therapy with adequate multilineage engraftment defined as ≥ 4% in bone marrow and in peripheral blood cell subpopulations (BM subpopulations: GlyA+, CD15+, CD3+, CD19+, CD56+; PB subpopulations: CD15+, CD19+, CD56+) will be estimated and the 95% Confidence Intervals (CI) of this proportion will also be calculated.

A descriptive analysis of the other secondary efficacy endpoints will be provided.

12.5. Note on Confidence Interval calculation

The Confidence Intervals for proportions will be calculated according to the Wilson approach (Brown et al., 2001), while an exact Poisson procedure will be adopted for deriving CIs for incidence rates. Confidence Intervals for the means or mean changes in continuous variables will be derived with standard parametric methods, if appropriate.

12.6. Integration of results by other trials on GT

A meta-analysis with data coming from similar trials (the studies sponsored by Généthon to our current knowledge and others that may be implemented in the near future) is envisaged. This would be useful, based on a pooled estimate of safety and efficacy, to:

a) assess whether there is a meaningful heterogeneity in results between different trials;

b) compare outcome after gene-therapy with outcome after different types of conditioning regimen and cell preparation;

c) consider the opportunity for the clinical development of a phase II-III trial.

Any changes in the planned statistical methods will be documented in the study report.

12.7. Long-term follow up

Individual patient annual data, collected from the 3rd up to the 8th year after infusion of transduced stem cells (Table 8b), and at the end of the study, will be summarized by means of descriptive summary statistics or frequency tables as appropriate, as described above.

13. QUALITY ASSURANCE AND QUALITY CONTROL

13.1. Definitions

Audit: A systematic and independent examination of trial related activities and documents to determine whether the evaluated trial related activities were conducted, and the data were recorded, analysed and accurately reported according to the protocol, sponsor's standard operating procedures (SOPs), Good Clinical Practice (GCP), and the applicable regulatory requirement(s).

Monitoring: The act of overseeing the progress of a clinical trial, and of ensuring 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).

Quality Assurance (QA): All those planned and systematic actions that are established to ensure that the trial is performed and the data are generated, documented (recorded), and reported in compliance with Good Clinical Practice (GCP) and the applicable regulatory requirement(s).

Quality Control (QC): The operational techniques and activities undertaken within the quality assurance system to verify that the requirements for quality of the trial-related activities have been fulfilled.

13.2. Monitoring.

A comprehensive quality control can be ordered in the form of monitoring. It might include checking the whole course of the study, the management of documentation, the management of data, the management of subject enrolled, the management of experimental drug and the management of biological sample. The sponsor of the study will be in charge for monitoring.

The investigators will assure the monitoring of the clinical study to assure conformance to protocol as well as the completeness, correctness, and plausibility of the completed case report forms (CRF).

13.3. Deviation from study protocol.

Every deviation from the trial protocol must be specified and documented separately for each patient. The investigator must consult with the monitor and discuss the type and extent of deviation, as well as the possible consequences for further participation of the patient in the study. If the evaluability of a patient is questionable the coordinating investigator will be consulted.

13.4. Audits and inspections

If necessary, a comprehensive quality control could be ordered in the form of an audit. It might include checking the whole course of the study, the documentation, statistical analysis, and the investigators.

The sponsor guarantees the availability for the inspections from the regulatory agencies.

14. ADMINISTRATIVE RULES

14.1. Insurance

The conduct of the study is covered by an insurance policy.

The insurance policy is in accordance with local laws and requirements. A copy of the insurance certificate will be archived in the Trial Master File.

14.2. Financing

Up to January 2014 Telethon Foundation was the source of monetary support of this clinical trial, i.e.Telethon Foundation funded SR-TIGET to conduct the clinical trial at the Pediatric Clinical Research Unit and Stem Cell Program at Ospedale San Raffaele. An agreement (Convenzione) between Ospedale San Raffaele (via Olgettina, 60 – Milano) and Telethon Foundation (via G. Saliceto, 5/a – Roma) assigned a grant to Pediatric Clinical Research Unit TIGET. This grant covered the costs of the clinical trial.

As of January 2014, GlaxoSmithKline Research and Development Ltd, 980 Great West Road, Brentford London TW8 9GS, United Kingdom was the source of monetary support of the clinical trial.

As of April 2018, Orchard became the source of monetary support of the clinical trial.

As of December 2023, Fondazione Telethon became the source of monetary support of the clinical trial.

14.3. Final study report

The sponsor will be responsible for preparing formal interim clinical study reports and the final clinical study report.

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16. APPENDICES

16.1. Appendix 1 Summary of Significant Changes Introduced by Protocol Amendment Version 9

The following changes were made to the protocol:

1. Page 1. Version number, description of the revision and date added.
2. Page 3. Rationale for amendment updated as follows:

The purpose of the current amendment is to extend the follow up period post-gene therapy. This period is referred to as Extended Long-Term Follow Up. The overall objective of this change is to allow for the collection of long-term safety and selected efficacy data in support of a marketing authorisation application for the gene therapy treatment.

In addition, a new assessment on platelet activation and morphology has been added to the schedule of assessments. These assays have recently been developed and validated at SR-Tiget, hence they can now be used within this clinical trial. Abnormal platelet function and morphology are common features in WAS patients, hence it is important to understand whether platelets are able to return to a more normal phenotype after gene therapy.

In addition, bruising has been removed from the secondary objectives, CD61+ has been removed from the bone marrow analysis after gene therapy for the evaluation of the presence of genetically modified cells, and a small number of typographical errors have been corrected.

A list of all changes contained in this amendment is included in Appendix 1.

3. Page 3. Bruising removed from secondary objectives in protocol synopsis. Bruising is not recorded as a separate item in the CRF. Any severe bruising that is present will be recorded as an AE.

In addition, based upon observations within the programme, data collection about bruising occurrences prior to screening has been identified as being subject to recall bias, due to the high frequency of bruising occurring in WAS patients.

Wording has also been updated throughout the rest of the protocol synopsis and in Section 3.2 , Section 4.3.2 and Section 9.6 in order to be consistent with this update.

4. Page 4. Exploratory efficacy endpoint added to protocol synopsis as follows:

1) Normalisation of platelet activation profile and morphology

In order to be consistent with this update, wording has also been updated throughout the rest of the protocol synopsis and additional wording added in Section 4.3.3 as follows:

4.3.3. Exploratory efficacy endpoint

a) Platelet activation profile and morphology

Platelet activation after stimulation with agonists will be assessed after ≥3y post gene therapy by measuring the expression of activation markers *on the* cell surface *by FACS analysis. Platelet morphology will be assessed at the same time using transmission electron microscopy.*

In order to be consistent with this update, wording has also been added to Section Section 7.2 as follows:

m) Platelet activation and morphology: For platelet activation, the expression of activation markers on the surface of peripheral blood-derived platelets after stimulation with agonists will be assessed. For platelet morphology, the platelet perimeter, area and ultrastructure will be assessed by transmission electron microscopy.

Again, in order to be consistent with this update, wording has also been added in Section 9.10 as follows:

9.10. Platelet activation profile and morphology (exploratory endpoint)

Activation of platelets will be assessed after stimulation with different concentrations of the platelet activation agonists ADP and thrombin.

Assessment parameters: platelet activation will be assessed by cytofluorimetric measurement of surface activation markers P-selectin and GPIIbIIIa after stimulation with various concentrations of ADP and thrombin. The dose response curves will be compared with healthy controls.

Platelet morphology will be assessed by transmission electron microscopy

Assessment parameters: platelet perimeter, area and ultrastructure. Morphology will be compared with healthy controls.

In addition, Table 8a and Table 8b have been updated accordingly.

5. Page 6. The number of protocol phases has been updated from four to five and a new fifth phase has been added as follows:

5) Extended Long-Term Follow Up phase: after completion of 8 years of follow up after gene therapy, patients will be contacted annually in order to collect long-term safety and selected efficacy data, until a registry is available and patients have been enrolled in it.

In order to be consistent with this update, wording has also been updated throughout the rest of the protocol synopsis and in Section 4, Section 4.1.2, Section 5.2 and Section 8.4 .

In addition, wording has been added to Section Section 7.3 as follows:

7.3. Extended Long-Term Follow Up

Both European (EMEA/CHMP/GTWP/60436/2007) and US regulatory agencies (Food and Drug Administration (FDA)) have expectations for the long-term follow up of patients who have been treated using gene therapy.

After completion of a total of 8 years of follow up from the initial infusion of transduced stem cells, patients will be contacted annually (+/- 3 months) by telephone, email or letter in an effort to collect long-term safety and selected efficacy data, until a registry is available and patients have enrolled in it (see Table 8c). This part of the study is known as Extended Long-Term Follow Up (ELTFU). Should a patient decide not to consent to participate in the ELTFU nor transition to the registry, then the subject will be considered to have completed the protocol and will be transitioned to standard of care according to local practice.

In addition, Table 8c has been added to Section 7.3 as follows:

Table 8c – Extended Long-Term Follow Up evaluation

Parameter	***Year 9+ p.a. (+/- 3 months)***
Social Life Is the patient living in a protected environment? Is the patient attending kindergarten/school or in employment (according to age)? - *Please comment.* How is the patient's social ability with peers (if applicable for age)? - *If abnormal please specify.* Does the patient practice any sport (if applicable for age)? - *Please comment.*	**X**
General Number of platelet infusions per year Complete Blood Count (CBC) including Platelet count/MPV	**X**
Specialist laboratory assessments Integration Site Analysis (ISA)	**X**
Safety* SAEs AEs of Special Interest (such as bleeds, infections, autoimmunity, eczema, suspected malignancies)	**X**
Ig usage** Intravenous Ig Subcutaneous Ig	**X**

* SAE and AE's of special interest will be forwarded to TIGET as per current practice by the physician overseeing the patient in their home country. This includes the event, labs, and any other relevant data. In addition patients/family may also provide such information directly to TIGET. All SAEs, and any suspected malignancies, should be reported to Sponsor within 24 hours of awareness. Other AEs of Special Interest should be entered into the eCRF within 30 days of awareness.

** Frequency to be specified if possible.

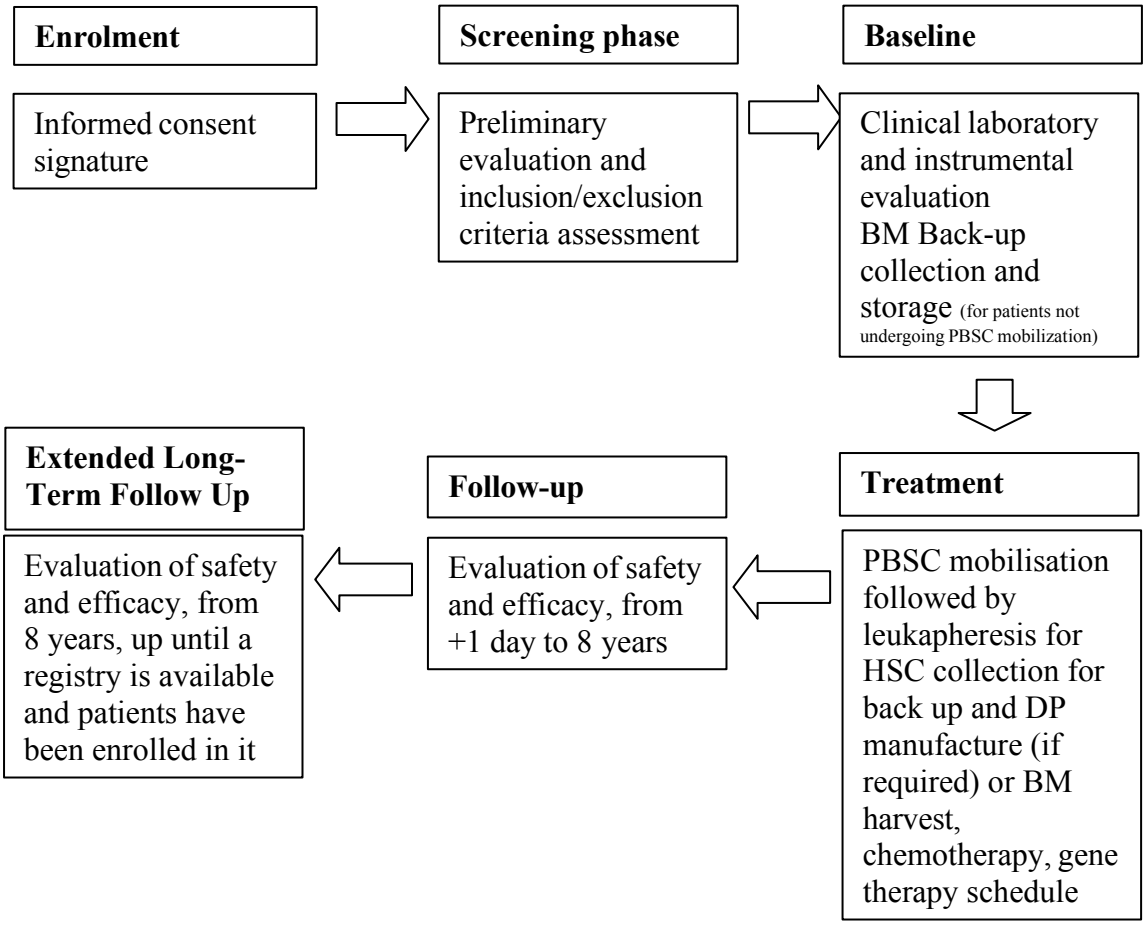
In addition, the trial flow chart has been updated (see 7 below).

6. Page 8. The end of the study has been updated as follows:

The end of the study is when all subjects have completed a minimum of 8 years follow up and transitioned to a registry. Should a patient decide not to consent to participate in the ELTFU nor transition to a registry, then the subject will be considered to have completed the protocol and will be transitioned to standard of care according to local practice.

In order to be consistent with this update, wording has also been updated in Section 5.1.4.1 , Section Section 12.2 and Section Section 12.7 .

7. Page 10. The trial flow chart has been updated as follows:



In order to be consistent with this update, this has also been updated in Figure 1 in Section 5.2 .

8. Page 52. Data and reference from a previous clinical trial has been updated in Section 2.8.1 , with the removal of previous out of date references, as follows:

As of January 2014, 7 out of the 10 treated patients developed acute leukemia (one acute myeloid leukemia (AML), four T cell acute lymphoblastic leukemia (T-ALL), and two primary T-ALL with secondary AML, with all six cases of T-ALL associated with a dominant clone with vector integration at the LMO2) (Braun et al, Sci Trans Med 2014).

The bibliography has been updated accordingly.

9. Page 77. Wording has been updated for the bone marrow evaluation after gene therapy in Section 7.2 as follows:

PCR analysis on transduced cells will be performed separately, including the assessment of %LV+ colony-forming cells (CFC) from patient's BM-derived clonogenic progenitors.

10. Page 78. CD61+ has been removed from the bone marrow analysis after gene therapy for the evaluation of the presence of genetically modified cells in Section 7.2 .

This has been removed due to the identification of technical issues related to the purification of the CD61+ cell fraction from patients' bone marrow.

In order to be consistent with this update, this has also been updated in Section 4.3.2 , Section 9.1 and Section 12.4 .

11. Page 82. Wording has been updated for reporting of SAEs to GSK in Section 8.2.1 as follows:

SR-TIGET should inform GSK of the following events within three days of gaining awareness:

- *Positive result from replication competent lentivirus (RCL) testing*
- *Test results suggesting abnormal clonal proliferation*

Events meeting the Hy's law criteria (ALT ≥ 3xULN and bilirubin ≥ 2xULN (>35% direct bilirubin) or ALT ≥ 3xULN and INR>1.5, if INR measured) will be reported as SAEs.

Patients with liver function abnormalities should be monitored until liver chemistries resolve, stabilise or return within baseline. Tests to define the aetiology of the abnormal liver function tests should be conducted as relevant.

12. Page 97. Wording has been updated for study population and timing of analysis in Section 12.2 as follows:

When all the treated patients complete the 8-year follow up visit after gene therapy, a statistical analysis will be performed with all data available and a clinical study report will be compiled accordingly.

At the end of the study, a final statistical analysis will be performed with all data available and a final clinical study report will be compiled.

13. Page 101. Wording has been updated for final study report in Section 14.3 as follows:

The sponsor will be responsible for preparing formal interim clinical study reports and the final clinical study report.

14. Minor typographical and table numbering errors have been corrected throughout.

16.2. Appendix 2 Summary of Significant changes introduced by Protocol Amendment Version 8

The following changes were made to the protocol:

1. Page 1. TIGET logo removed.
2. Page 1. GSK study number added
3. Page 1. GSK compound number added
4. Page 1. Version date amended and Protocol Amendment number added.
5. Page 2. Rationale for amendment added as follows:

Protocol Amendment Version 8: Rationale

In November 2013, GSK in-licenced GSK2696275 (previously described as Telethon 003) from Fondazione Telethon and SR-TIGET. While SR-TIGET currently remained the regulatory sponsor of the clinical study protocol at this time, GSK replaced Fondazione Telethon as the Financial Sponsor.

The purpose of the current protocol amendment is to formally register GSK as the new regulatory sponsor for the clinical trial and align data management and serious adverse event reporting methods with company processes. Where appropriate background information has been updated to reflect this change.

The overall objective of the changes described are to allow the existing all clinical data and to be collated by GlaxoSmithKline R&D in support of a marketing application for the gene therapy treatment.

A list of all changes contained in this amendment is included in Appendix 1 .

1. Protocol Synopsis moved into cover page section (pages 3-7)
2. Page 9: Medical monitor/Sponsor Information Page added.

Of note, Medical Monitor details remain unchanged from the prior amendment and version. A new GSK SAE contact has been added given GSK's role as regulatory sponsor.

3. Page 10: Sponsor Legal Registered Address has been added.
4. Page 11: GSK Sponsor signatory page has been added.
5. Page 12: Investigator Protocol Agreement Page added
6. Section 1. Background, the following text was removed:

The promoter of the study is Ospedale San Raffaele. The Ospedale San Raffaele is recognized by the Italian Ministry of Health as a Scientific Institute, carrying out biomedical research and clinical activities of relevant national interest (IRCCS).

Fondazione Telethon is a major italian non-profit foundation responsible for raising and distributing funds for biomedical research in Italy, primarily in universities, public and non-profit research institutes. Telethon's mission is to advance research toward the cure of muscular dystrophies and other rare genetic diseases.

The San Raffaele-Telethon Institute for Gene Therapy of Genetic Diseases (SR-TIGET) was established a joint venture between Fondazione San Raffaele and Fondazione Telethon for the implementation of basic and clinical research on gene therapy for genetic diseases. The mission of the Institute is to perform cutting edge science in the field of gene and cellular therapy and to promote the translation of basic discoveries into therapeutic advances.

7. The following text from Section 4.1.1 has been removed from the protocol.

Section 4.1.1. Type of Study

Principal investigators: Alessandro Aiuti, MD, PhD.

Treatment Center: Paediatric Clinical Research Unit, SR-TIGET/Paediatric Immunohematology and Bone Marrow Transplantation Unit-OSR.

Personnel: physicians, nurses and paramedical staff working in the Paediatric Immunohematology and Bone Marrow Transplantation Unit-OSR are fully qualified to assist patients affected by critical haematological diseases, such as WAS, and to conduct this clinical trial in accordance with applicable laws and regulations, as well as recognized good clinical practice.

8. Global Changes made to protocol include: Study promoter changed from 'Ospedale San Raffaele' to 'Sponsor' (which is GSK). Where 'HSR-TIGET'appeared in text, the abbreviation was changed to 'SR-TIGET.'
9. Page 75: 8.1.3 . ***or Serious Adverse Reaction (SAR)*** removed from heading: the definition for SAR is not included in the text section.

10. Page 76: Section 8.2 . Pharmacovigilance Procedure

The following text has been removed from the protocol:

8.2.1. Serious Adverse Events (SAEs): *SAEs will be reported according to the law and regulations in force.*

The following text replaces section 8.2.1:

8.2.1 Reporting of Serious Adverse Events (SAEs) to GSK

The Investigator shall report all SAEs to GSK within 24 hours after becoming aware of the SAE.

SAEs should be reported to GSK using the paper SAE reporting form. After completion, the form should be scanned and emailed to the SAE contacts (Medical Monitor and GSK Italy pharmacovigilance) found at the beginning of the protocol on the Sponsor/Medical Monitor Contact Information page.

In rare circumstances if email communication is not available, notification by telephone is acceptable, with a copy of the SAE form sent by overnight mail.

The following is added to the protocol:

8.2.2. Regulatory Reporting Requirements for SAEs

Prompt notification by the Investigator to GSK of SAEs related to study treatment (even for non-interventional post-marketing studies) is essential so that legal obligations and ethical responsibilities towards the safety of subjects and the safety of a product under clinical investigation are met.

GSK has a legal responsibility to notify both the local regulatory authority and other regulatory agencies about the safety of a product under clinical investigation. GSK will comply with country specific regulatory requirements relating to safety reporting to the regulatory authority, Institutional Review Board (IRB)/Independent Ethics Committee (IEC) and investigators.

Investigator safety reports are prepared for suspected unexpected serious adverse reactions according to local regulatory requirements and GSK policy and are forwarded to Investigators as necessary.

An Investigator who receives an investigator safety report describing a SAE(s) or other specific safety information (e.g., summary or listing of SAEs) from GSK will file it with the IB and will notify the IRB/IEC, if appropriate according to local requirements.

The following text has been removed from the protocol:

8.2.3. Suspected Unexpected Serious Adverse Reaction (SUSAR) *I n case of SAR that is unexpected, the Investigator/Sponsor must report the reaction as soon as possible (and in any case no later than 7 days for reactions that are fatal or life-threatening, and no later than 15 days for all other SUSARs) to the competent Regulatory Authorities and to the competent Ethics*

Committee (and that relevant follow up information is subsequently communicated within an additional eight days).

8.2.4. Periodic reporting *Once a year throughout the trial, the Investigator should provide to Competent Regulatory Authorities and the Ethics Committee an Annual Safety Report, that is a listing of all suspected SAR, which have occurred over this period and a report of the subjects' safety.*

11. Page 86: Section 10.2 . Documentation of data in the case report form (CRF)

The following wording has been removed from the protocol:

It is the responsibility of the Investigator to maintain adequate and accurate CRFs to record all observations and other data pertinent to the clinical investigation. All CRFs should be completed in their entirety in a neat, legible manner to ensure accurate interpretation of data; a black ball point pen should be used to ensure the clarity of reproduced copy of all CRFs. Should a correction be made, the information to be modified must not be overwritten. The corrected information will be transcribed by the authorized person next to the previous value and dated.

The following wording has been added to the protocol:

It is the responsibility of the Investigator to maintain adequate and accurate CRFs to record all observations and other data pertinent to the clinical investigation.

Under TIGET sponsorship paper CRFs will be utilized; these should be completed in their entirety in a neat, legible manner to ensure accurate interpretation of data; a black ball point pen should be used to ensure the clarity of reproduced copy of all CRFs. Should a correction be made, the information to be modified must not be overwritten. The corrected information will be transcribed by the authorized person next to the previous value and dated.

Under GSK sponsorship an electronic CRF will be implemented; all data previously collected on paper CRF and all data collected following sponsorship transfer will be entered into GSK defined CRFs. All these data will transmitted electronically to GSK or designee and combined with data provided from other sources in a validated data system.

Management of clinical data will be performed in accordance with applicable GSK standards and data cleaning procedures to ensure the integrity of the data, e.g., removing errors and inconsistencies in the data.

Adverse events and concomitant medications terms will be coded using MedDRA (Medical Dictionary for Regulatory Activities) and an internal validated medication dictionary, GSKDrug.

CRFs (including queries and audit trails) will be retained by GSK, and copies will be sent to the investigator to maintain as the investigator copy. Subject initials will not be collected or transmitted to GSK according to GSK policy.

16.3. Appendix 3: Summary of Significant Changes Introduced by Protocol Amendment Version 10

A list of all changes contained in this amendment are included below:

1. Date and protocol amendment number has been changed
2. Medical Monitor/Sponsor Information has been changed. An Orchard Therapeutics Medical Monitor and PPD SAE contact have been added given Orchard Therapeutics role as regulatory sponsor.
3. Sponsor Legal Registered Address has been added
4. Orchard Therapeutics Sponsor signatory page has been added
5. Orchard Therapeutics investigator protocol agreement page has been added
6. PPD has been added to the abbreviation/acronym list
7. Global changes made to protocol include:
    a. The acronym GSK has been changed to Orchard Therapeutics as regulatory sponsor.
    b. For the reporting of SAEs section, the acronym GSK has been changed to PPD.
    c. For the section on collection of pregnancy information, the acronym GSK has been changed to PPD.
    d. The protocol header number has been removed and name has been changed to Orchard Therapeutics.

16.4. Appendix 4: Summary of Significant Changes Introduced by Protocol Amendments Version 11 and 11.1

A list of significant changes contained in version 11 are listed below.

Throughout: text has been amended to allow all subjects to enter a separate observational long-term follow-up (OLTFU) study once it is set up and subjects been followed up for a minimum of 5 years, not just after subjects enter the (extended long-term follow-up) ELTFU phase. As part of the OLTFU study, patients will continue to be followed up for a total of 15 years from the date of treatment with gene therapy.

Throughout: text has been added to allow patients to attend remote visits (upon agreement by PI, medical monitor and sponsor,) and text has been added to describe these remote visits. Text has also been added to allow informed consent (performed by the PI or a person designated by the PI) to take place remotely.

Throughout: the severity assessments used for clinical events including bleeding, infections and eczema have been clarified upon FDA feedback.

8.4.1. Replication competent lentivirus (RCL): the strategy for RCL testing was simplified. In the previous version of the protocol, 3 assays (ELISA for HIV p24 antigen on serum, DNA PCR for VSV-G envelope on cells, and RT-PCR for HIV-pol RNA on serum) were used for first line detection of RCL. In the amended text, one test is performed in the first instance (HIV p24 antigen detection in the serum by ELISA), in line with EMA guideline CHMP/BWP/2458/03, which defines the best assays for the determination of RCL. Detection of HIV p24 antigen in serum by ELISA is identified as a validated and sensitive test, which is also routinely used in the clinic to determine HIV positivity in case of suspected viral infection by the wild type lentivirus. Should this test be positive, further analyses will be performed, including the two remaining tests mentioned above (DNA PCR for VSV-G envelope on cells, and RT-PCR for HIV-pol RNA on serum). If a positive result is obtained from at least one of the additional tests, an appropriate confirmatory test may be performed. This change is required since some of the commercially available tests to detect RNA-Pol HIV (e.g. Roche COBAS Ampliprep/COBAS Taqman HIV-1 v2.0 Test), are unable to differentiate between the DNA of gene corrected cells and HIV infection, potentially leading to false positive results.

Minor readability and typographical errors were corrected in the text

The following changes are contained in version 11.1:

Medical Monitor/ Sponsor information page: Contact details for the Medical Monitor and for pharmacovigilance SAE reporting have been updated to reflect changes in personnel and vendor.

Throughout: PPD (former pharmacovigilance vendor) was replaced by Sponsor/ Sponsor's pharmacovigilance designee.

Table 8c: The mention of 'special interest' was removed from the definition of events such as bleeds, infections, autoimmunity, eczema and suspected malignancies, which are disease related and do not meet the criteria for events of special interest in terms for PV safety analysis and reporting.

16.5. Appendix 5: Summary of Significant Changes Introduced by Protocol Amendments Version 12

A list of significant changes contained in version 12 are listed below.

a. the name of the previous Sponsor (Orchard Therapeutics) has been replaced with the name of the new Sponsor (Fondazione Telethon ETS) in the text.