COVER PAGE

Official Study Title: Phase I/II, Historical Controlled, Open-label, Non-randomised, Single-centre Trial to Assess the Safety and Efficacy of EF1αS-ADA Lentiviral Vector Mediated Gene Modification of Autologous CD34 + Cells From ADA-deficient Individuals

10-MI-29 Protocol v17.0. dated 28-Jun-2017

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UCL INSTITUTE OF CHILD HEALTH

Great Ormond Street Hospital for Children NHS Foundation Trust

Protocol Version 17.0, dated 28 June 2017

Full title of trial	Phase I/II, historical controlled, open-label, non-randomised, single-centre trial to assess the safety and efficacy of EF1αS-ADA lentiviral vector mediated gene modification of autologous CD34+ cells from ADA-deficient individuals
Short title	LV Gene therapy for ADA deficiency
Version and date of protocol	Version 17.0, 28 June 2017
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Sponsor protocol number	10-MI-29
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Active IMP(s):	EF1αS-ADA lentiviral vector transduced patient CD34+ cells
Placebo IMP(s):	N/A
Phase of trial	Phase I/II
Trial sites(s)	Great Ormond Street Hospital for Children NHS Foundation Trust (single-site)

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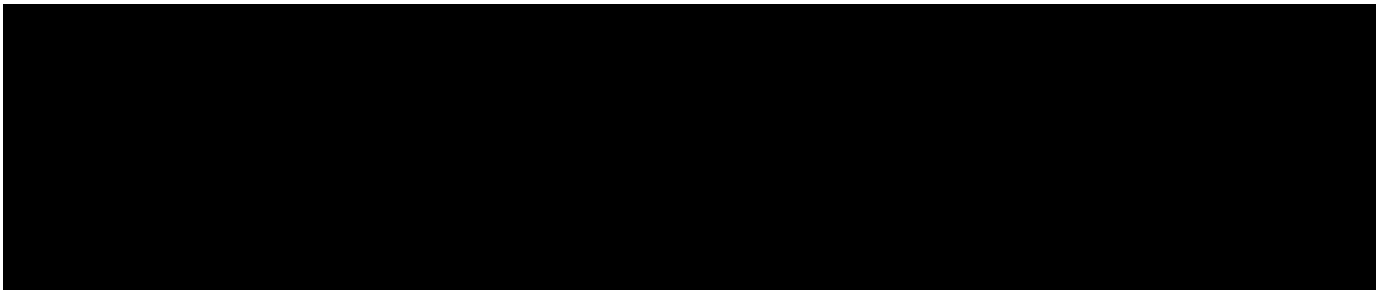

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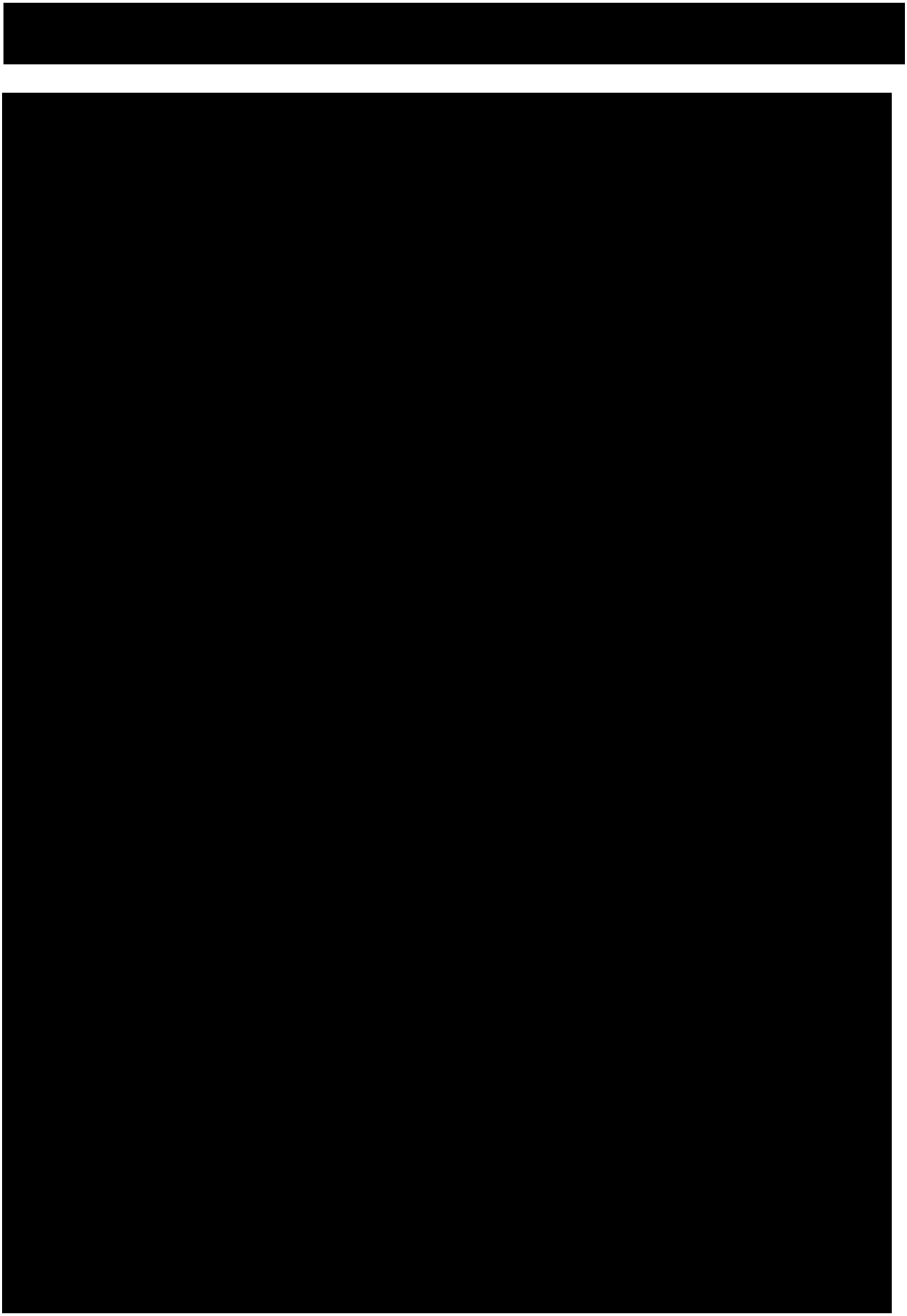
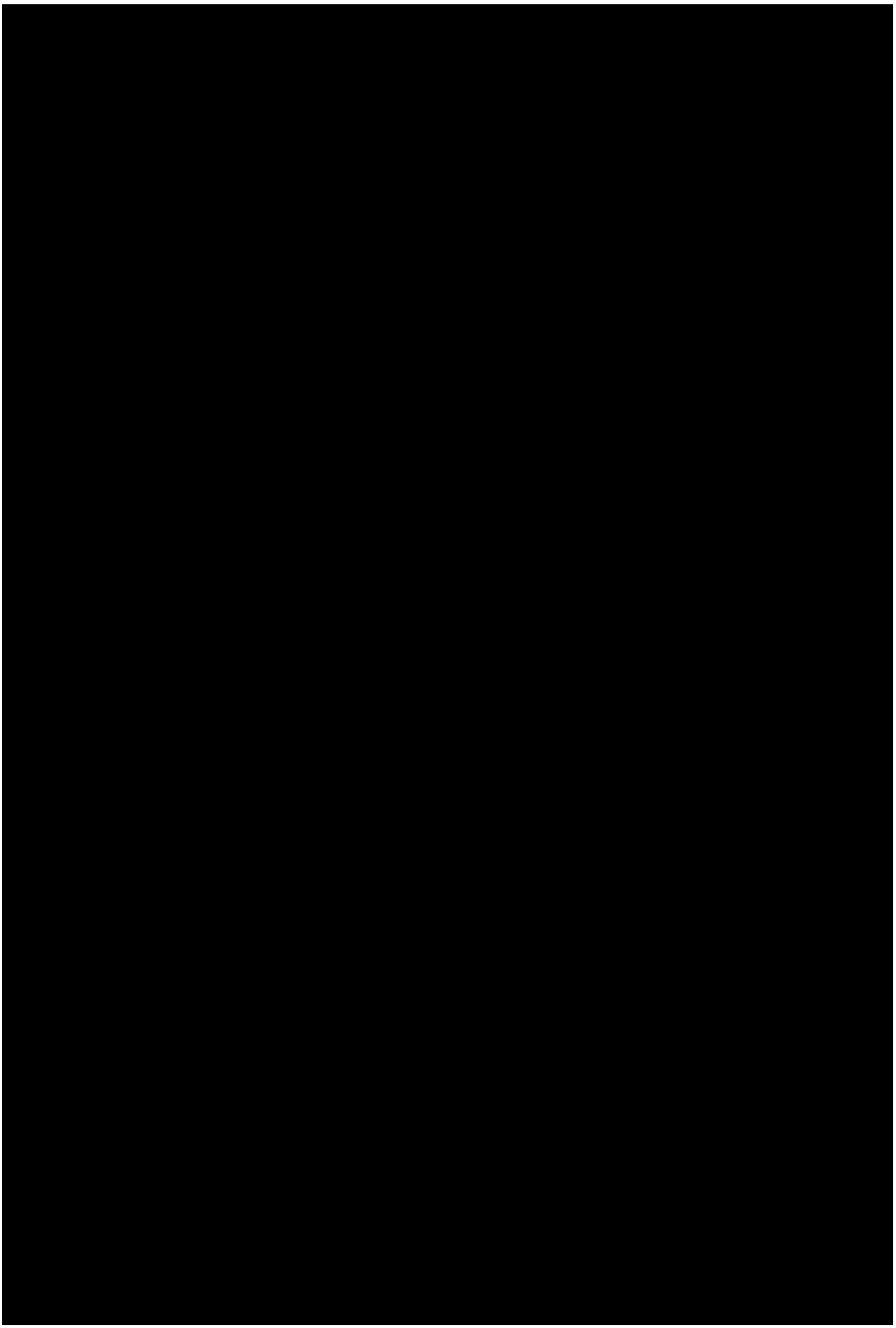
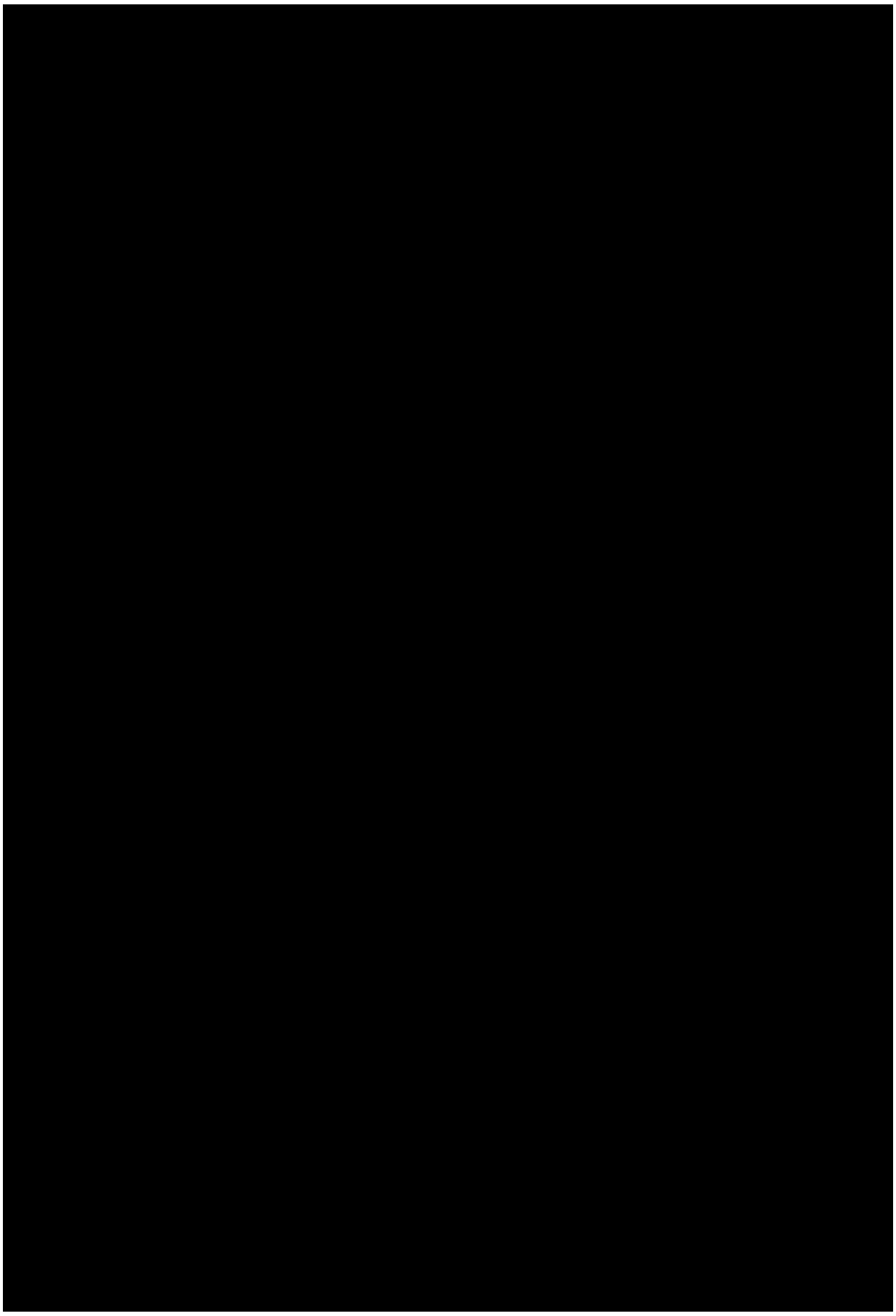
List of abbreviations

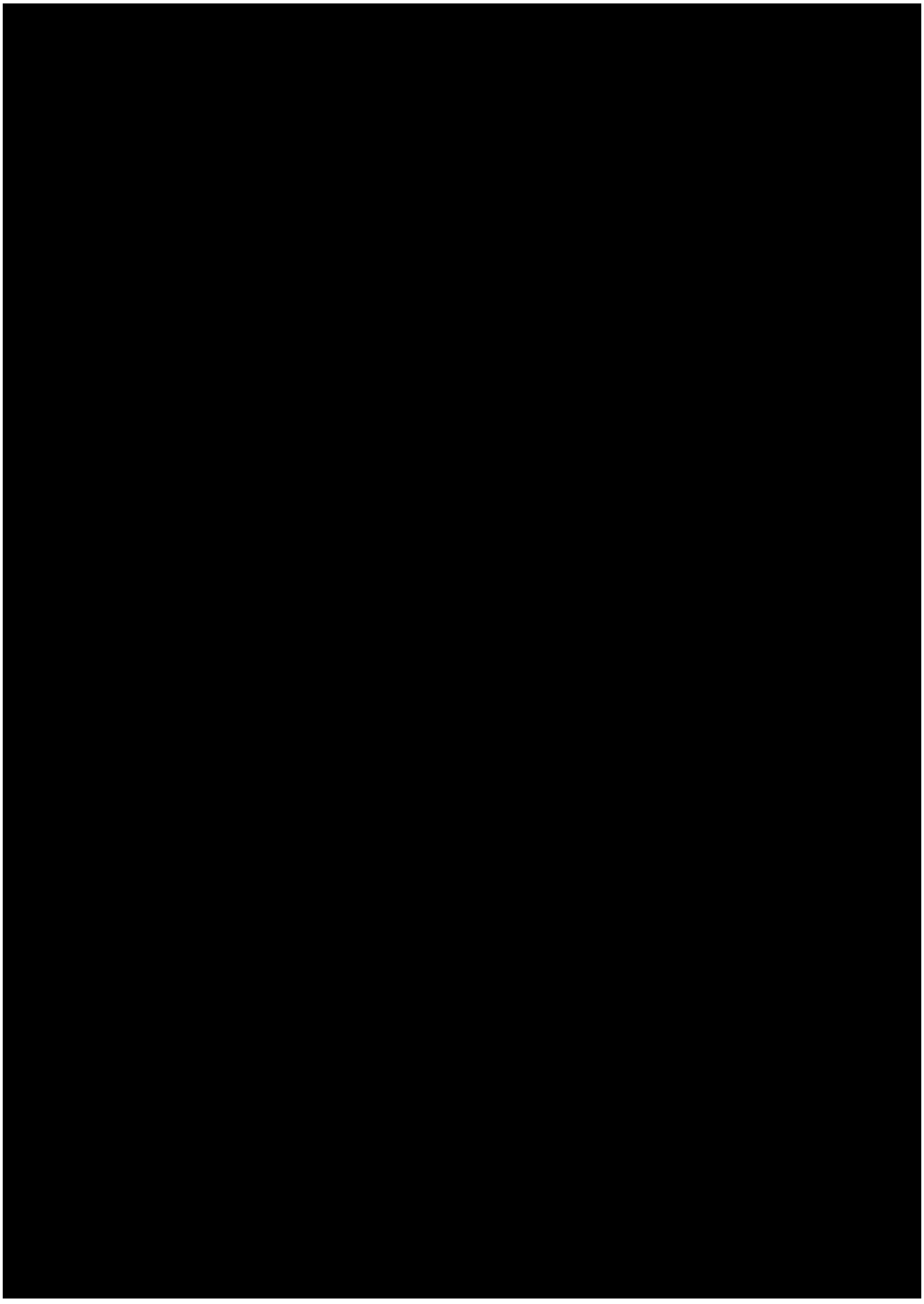
ADA	Adenosine deaminase
AE	Adverse Event
AR	Adverse Reaction
BCG	Bacillus Calmette–Guérin
cDNA	complementary Deoxyribonucleic Acid
CI	Chief Investigator
CTA	Clinical trial authorisation
CUP	Compassionate Use Program
dAdo	Deoxyadenosine
dATP	DeoxyATP
dCydK	Deoxycytidine kinase
EBMT	European Society for Blood and Marrow Transplantation
eCRF	electronic Case Report Form
EFS	Event-Free Survival
ERT	Enzyme Replacement Therapy
GCP	Good Clinical Practice
GMP	Good Manufacturing Practice
GOSH	Great Ormond Street Hospital
GTAC	Gene Therapy Advisory Committee
GvHD	Graft versus host disease
HAPLO	Haploidentical
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HSC	Haematopoietic stem cells
HSCT	Haematopoietic stem cell transplantation
ICH	Institute of Child Health
IgRT	Immunoglobulin Replacement Therapy
IMP	Investigational Medicinal Product
LSS	Lymphocytes subset
LTR	Long Terminal Repeats
LTR	Long Terminal Repeats
LV	Lentiviral vector
MFD	Matched Family Donor
MHRA	Medicines and Healthcare products Regulatory Agency
MMUD	Mismatched Unrelated Donor
MSD	Matched Sibling Donor

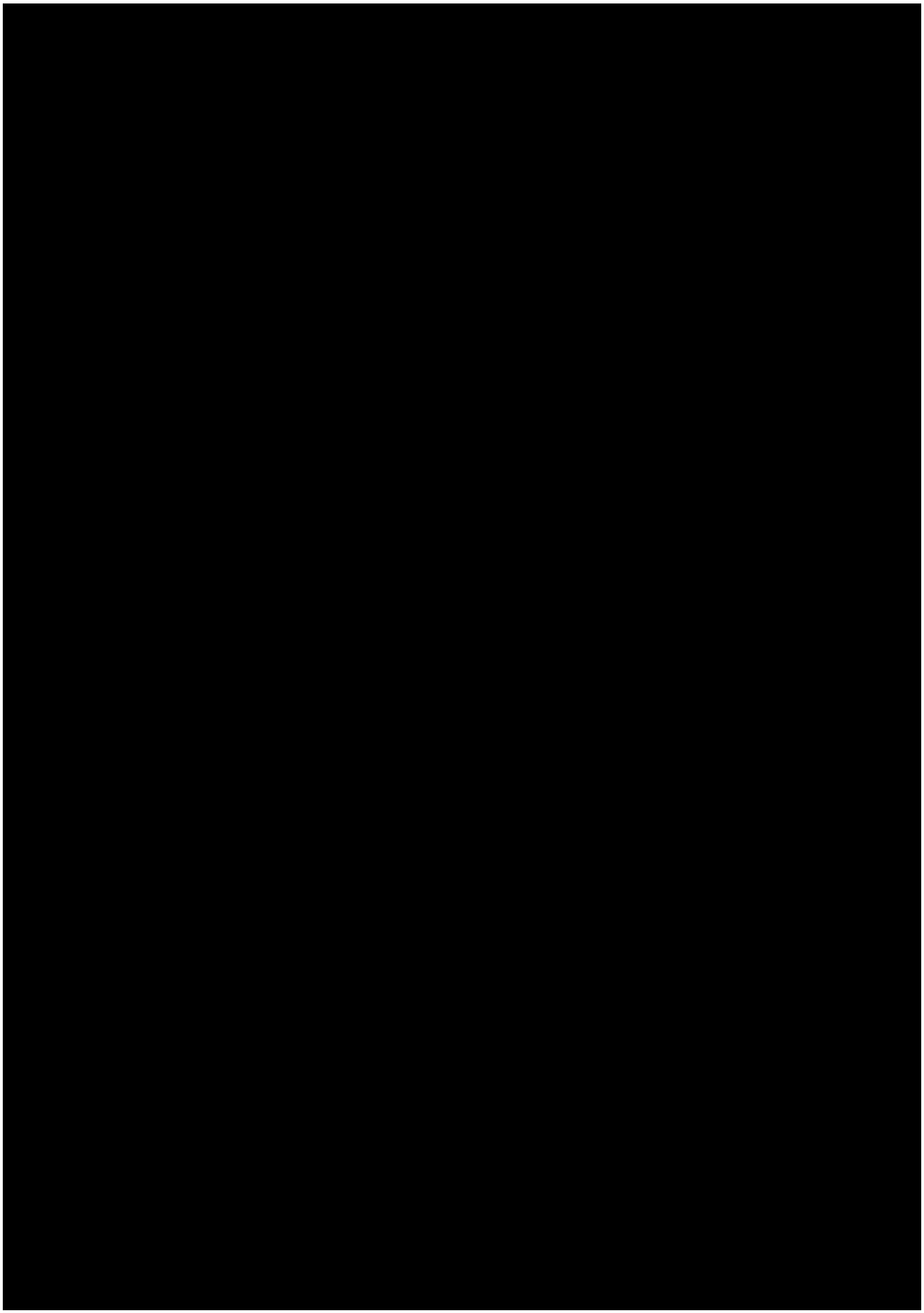
MUD	Matched unrelated donor
OKT3	anti-CD3 complex antibody
OS	Overall Survival
PBSC	Peripheral Blood Stem Cells
PCR	Polymerase Chain Reaction
PI	Principal Investigator
PID	Primary Immune deficiency
PIL	Patient Information Leaflet
R&D	Research and development
RCL	Replication-competent lentivirus
rIL-2	recombinant interleukin 2
RRE	Rev responsive element
SAE	Serious Adverse Event
SAHH	S-adenosylhomocysteinehydrolase
SCID	Severe combined immune deficiency
SFFV	Spleen Focus Forming Virus
SIN	Self INactivating
SRC	SCID-repopulating cells
SSAR	Suspected Serious Adverse Reaction
SUSAR	Suspected Unexpected Serious Adverse Reaction
TCR	T-cell Receptor
TRECs	T Cell receptor excision circles
UCL	University College London
UCLA	Children’s Hospital Los Angeles
VSV	Vesicular Stomatitis Virus
WPRE	Woodchuck Post-transcriptional Regulatory Element
γc	Common cytokine receptor gamma chain











2 Summary

Title: Phase I/II, historical controlled, open-label, non-randomised, single-centre trial to assess the safety and efficacy of EF1αS-ADA lentiviral vector mediated gene modification of autologous CD34+ cells from ADA-deficient individuals

Short Title: LV gene therapy for ADA deficiency

Investigational Medicinal Product (IMP): EF1αS-ADA lentiviral vector transduced patient CD34+ cells

Phase of trial: Phase I/II

Objectives:

The primary objective is to assess the safety and efficacy of EFS-ADA LV mediated gene therapy for treatment of ADA-SCID patients. This will be achieved via the following specific objectives:

Safety

1. To assess clinical, haematological and immunological progress of patients.
2. To assess vector integration sites and clonal proliferation.

Efficacy

1. To assess overall survival (OS) at 1 year for patients treated with IMP
2. To assess event free survival (EFS) at 1 year for patients treated with IMP

   overall and event free survival at 1 year between patients treated with IMP and patients treated with allogeneic HSCT
3. To assess at each visit engraftment success and resulting immunological and metabolic effects in IMP treated patients using:
   a. Vector copy numbers in peripheral blood leukocytes
   b. Cellular and humoral immune system recovery
   c. ADA activity and reduction in dATP in peripheral blood cells

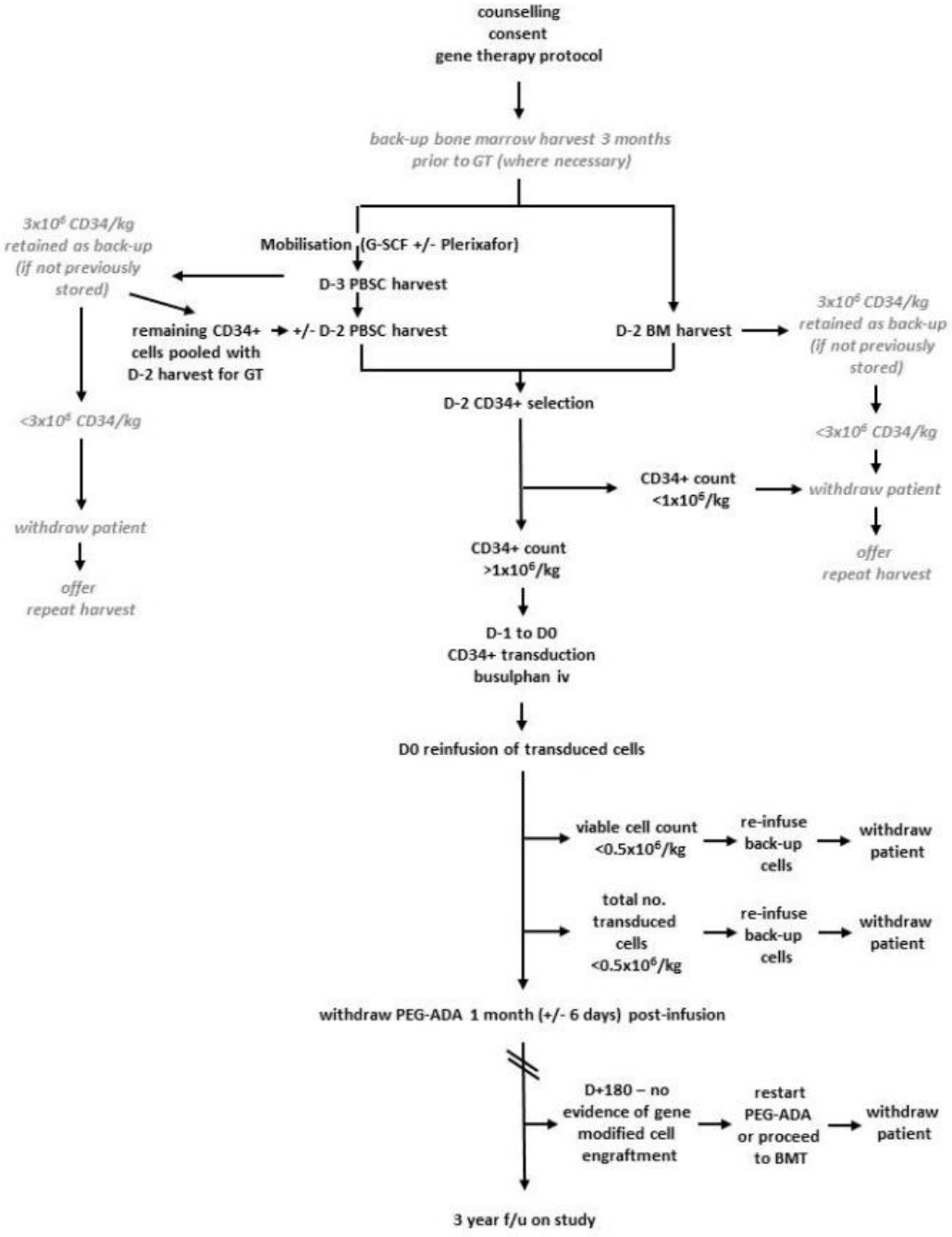
The secondary objectives are to:

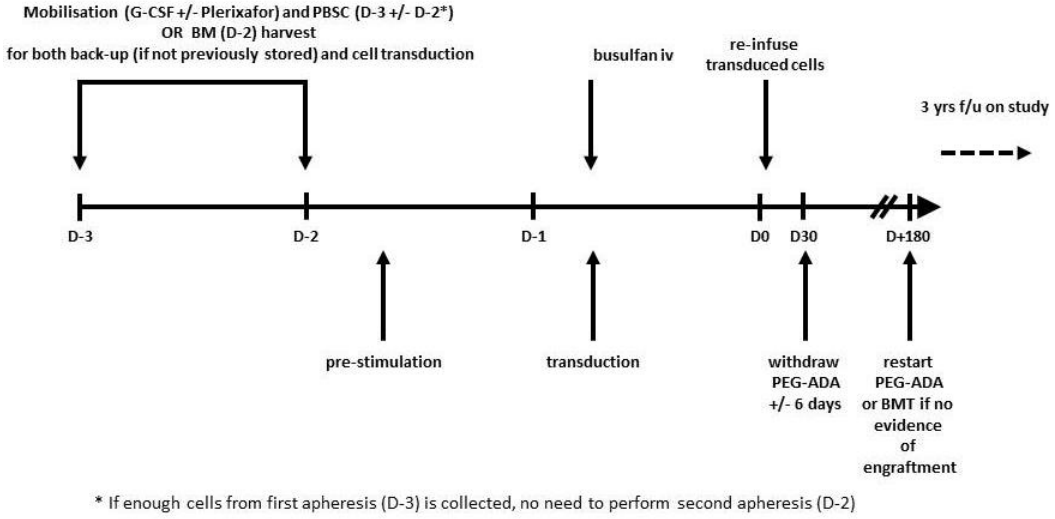
- Compare survival and event free survival outcomes at 2 and 3 years between patients treated with IMP and patients treated with allogeneic Hematopoietic Stem Cell Transplantation (HSCT)

- Determine the percentage of IMP treated patients requiring immunoglobulin replacement therapy at all evaluation points from 18 months onwards
- Compare frequency of infections, and growth of pathogenic microorganisms over 3 years
- Evaluate the longitudinal clinical effect in terms of improved immunity
- Evaluate tolerability of conditioning regimen
- Evaluate feasibility of the transduction procedure

Type of trial: Phase I/II, historical controlled, open-label, non-randomised, single-centre trial

Trial design and methods: Summary flowchart of the patients treated with gene therapy





Recruitment period: 5 years

Trial duration per participant: From consent until 3 years post-infusion of transduced CD34+ cells

Estimated total trial duration: 8 years

Planned trial sites: Great Ormond Street Hospital for Children NHS Foundation Trust (single-site trial)

Total number of participants planned:

Patients treated with gene therapy on trial**:** Up to 10 treated patients

The trial will include two other sub-groups: patients treated with gene therapy off trial under GOSH Special License constituting the Compassionate Use Program (CUP) and patients treated with Haematopoietic Stem Cell Transplantation (HSCT) constituting the historical control group.

Inclusion and Exclusion Criteria

1. Patients treated with gene therapy(on trial)

Inclusion criteria

1. Diagnosis of ADA-SCID confirmed by DNA sequencing OR by confirmed absence of <3% of ADA enzymatic activity in peripheral blood or (for neonates) in umbilical cord blood erythrocytes and/or leucocytes or in cultured foetal cells derived from either

chorionic villus biopsy or amniocentesis, prior to institution of PEG-ADA replacement therapy

2. Patients who lack a fully Human leukocyte antigen (HLA)-matched family donor

3. Patients (male or female) <5 years of age OR

   Patients (male or female) ≥ 5 years to 15 years of age who have preserved thymic function as evidenced by presence of >10 % naïve T cells (CD4+45RA+27+ cells)

4. Parental/guardian signed informed consent

Exclusion criteria

1. Cytogenetic abnormalities on peripheral blood
2. Evidence of active malignant disease
3. Known sensitivity to busulfan
4. If applicable, confirmed pregnancy (to be tested in patients above 12 years old)

2. Patients treated with HSCT, historical control group

Inclusion criteria

1. Diagnosis of ADA-SCID confirmed by DNA sequencing OR by confirmed absence of <3% of ADA enzymatic activity in peripheral blood or (for neonates) in umbilical cord blood erythrocytes and/or leucocytes or in cultured fetal cells derived from either chorionic villus biopsy or amniocentesis, prior to institution of PEG-ADA replacement therapy
2. Patients (male or female) between 0-18 years at time of treatment
3. Patient treated with an allogeneic haematopoietic stem cell transplantation since 2000

3. Compassionate Use Patients (CUP)

Another sub-group of patients have been also treated off-trial with the IMP under a compassionate basis under GOSH Special License. They were treated following the same protocol schedule and procedures as the on-trial patients and they will be included in the analysis (details in the SAP)

3 Introduction and Background

3.1 Adenosine deaminase deficient – severe combined immunodeficiency (ADA-SCID)

Severe combined immunodeficiencies (SCID) are a heterogeneous group of inherited disorders characterised by a profound reduction or absence of T lymphocyte function. As a result, children with SCID are susceptible to recurrent and severe infection with pneumonia, diarrhoea and failure to thrive being the most common clinical manifestations. Without treatment most children die in the first year of life from overwhelming infection.

SCID arises from a variety of molecular defects which affect lymphocyte development and function (Fischer et al., 1997). Adenosine Deaminase (ADA) deficient SCID accounts for approximately 10-20% of all cases of SCID and was the first form of SCID in which the underlying defect was identified (Giblett et al., 1972). Over 18 different genetic defects have now been shown to give rise to a clinical and immunological phenotype of SCID and these include 1) defects in the lymphocyte specific signalling molecules, common γ chain (Noguchi et al., 1993), JAK-3 (Macchi et al., 1995), and IL-7 receptor α (Puel et al., 1998), 2) in molecules that control immunoglobulin gene rearrangement, RAG-1/2 (Schwarz et al., 1996), Artemis (Moshous et al., 2001) and Cernunnos (] and 3) in subunits of the CD3 receptor complex (Rieux-Laucat et al., 2006; Soudais et al., 1993) amongst others. The incidence of all forms of SCID is variously reported to be between 1: 50,000 and 1: 75,000 and the incidence of ADA-SCID to be in the order of 1: 200,000 and 1: 1,000,000 live births (Hershfield and Mitchell 1995) although the prevalence may be higher within specific ethnic populations (Sanchez et al., 2007).

3.1.1 Adenosine deaminase deficiency

Adenosine deaminase is an enzyme that is expressed in all tissues of the body. During DNA breakdown, ADA catalyses the deamination of deoxydenosine (dAdo) and adenosine to deoxyinosine and inosine respectively (reviewed in (Hirschhorn, 1993). The lack of ADA results in the accumulation of dAdo in both intracellular and extracellular compartments. Intracellularly, dAdo is then converted by deoxycytidine kinase (dCydK) to deoxyadenosinetrisphosphate (dATP) which accumulates within the cell. The buildup of these two metabolites has profound effects on lymphocyte development and function and is the most likely cause of the immunological defects. dATP inhibits the enzyme ribonucleotide reductase which is necessary for DNA replication and repair (Takeda et al., 1991; Lee et al., 1984) and also induces apoptosis in immature thymocytes. dAdo inactivates the enzyme S-adenosylhomocysteinehydrolase (SAHH) (Hershfield et al., 1979) (Benveniste et al., 1995): SAHH accumulation inhibits transmethylation reactions and the lack of SAHH activity as a

consequence of ADA deficiency may also contribute to the immunodeficiency. It has also been speculated that the effects of adenosine acting through G protein receptors on the surface of thymocytes may play a role in the pathogenesis of the disease (Benveniste and Cohen, 1995) (Apasov et al., 2001).

Although ADA is expressed ubiquitously, the most profound effects are manifest in the immune system (non-immunological consequences are present however and are described in section 3.1.4.2.). This may be explained by the expression pattern of ADA which is highest in the thymus as a result of high lymphocyte turnover (Adams and Harkness, 1976) (Van der Weyden and Kelley, 1976) and also by increased expression of dCydK in lymphocytes which serves to increase dATP accumulation in immune cells more than in other tissues (Carson et al., 1977).

3.1.2 ADA gene: structure and function

The ADA gene locus has been mapped to 20q13.11 (Tischfield et al., 1974) on the basis of evidence from studies of somatic cell hybrids containing translocations involving chromosome 20 ((Mohandas et al., 1980), ADA activity in patients with chromosome 20 structural abnormalities (Philip et al., 1980) (Petersen et al., 1987) and *in situ* hybridisation with an ADA cDNA probe (Jhanwar et al., 1989). The gene spans 32kb and is organised into 12 exons separated by 11 introns (Valerio et al., 1983) (Wiginton et al., 1983). Promoter activity was localised to a 135bp region immediately 5' of the major transcription start site (-95 relative to the AUG translation start site). The promoter consists of 82% G + C residues, lacks the TATA and CAAT sequences found in the promoter regions of many developmentally regulated genes and possesses six GC boxes homologous to the consensus binding site for Spl, a eukaryotic 'zinc finger' transcriptional activator (Kadonaga et al., 1987) (Pugh and Tjian, 1990). The murine ADA promoter has similar characteristics (Ingolia et al., 1986).

cDNA sequences of normal human DNA were first characterized in 1983 by a number of different laboratories (Valerio et al., 1983) (Wiginton et al., 1983). The 1.5kb ADA mRNA consists of a 1089 nucleotide open reading frame together with 5' and 3' untranslated sequences. The resulting protein consists of 363 amino acids with a deduced molecular weight of 40.7Kd but in western blot studies the actual protein species varies between 36-44Kd. The ADA enzyme is expressed in all tissues of the body but levels vary over a wide range (Adams and Harkness, 1976) (Van der Weyden and Kelley, 1976; Hirschhorn et al., 1978). In humans, the highest activity is in the thymus and in other lymphoid tissues with lowest expression in erythrocytes. Among non-lymphoid tissues, high levels are found in the villi of the duodenal epithelium and in other areas of the gastrointestinal tract (GIT) and also in the cerebrum. There

is a different pattern of expression in other species with mice having higher levels in the GIT than in the thymus (Lee, 1973) (Chinsky et al., 1990).

ADA in human tissues exists in several different physical forms, which can be distinguished electrophoretically and by size. ADA is largely an intracellular enzyme although a low level of surface associated enzyme is detectable on a fraction of blood cells (SenGupta et al., 1985; Aran et al., 1991). The various forms of ADA represent a combination of genetic polymorphisms and isoenzymes generated by post-translational modifications and in some tissues, binding of the monomeric catalytic ADA gene product (Schrader et al., 1976) (Daddona and Kelley, 1977; Schrader and Stacy, 1977) to a non-catalytic (200kD) homodimeric glycoprotein termed ‘conversion factor’, ‘binding protein’ or ‘complexing protein’ (ADA-CP) (Daddona and Kelley, 1978; Schrader et al., 1976). It has been speculated that ADA-CP may regulate the activity of ADA or contribute to extracellular Ado catabolism (Trotta, 1982) or that it may mediate the renal clearance of monomeric ADA (Schrader and Bryer, 1982; Schrader et al., 1990; Schrader et al., 1984). However, interaction with ADA-CP does not affect the activity of ADA and in some tissues the two proteins do not co-localise (Schrader et al., 1987; Dinjens et al., 1989).

ADA-CP has been identified as a protein known both as CD26 and dipeptidyl peptidase IV (DPPIV) (Morrison et al., 1993; Kameoka et al., 1993). CD26 was first defined as an antigen on activated human T lymphocytes (Fox et al., 1984), and DPPIV as a widely distributed ectoenzyme that cleaves peptides including several hormones, neuropeptides, and cytokines (Hegen et al., 1990) (Ulmer et al., 1990). CD26 and DPPIV cDNAs from human T cells and intestine predict the same 766-residue, 88-kD polypeptide. This type II membrane protein has a large extracellular domain consisting of a glycosylated "stalk", a cysteine-rich segment (residues 290–552), and a COOH-terminal region that bears the serine protease (DPPIV) active site (residues 628–632). Residues 294 and 340–343 of the cysteine-rich segment are essential for binding ADA (Dong et al., 1997). Although it was initially suggested that binding of ADA to CD26 was important for protection of lymphocytes from the extracellular effects of Ado, recent studies on a healthy adult with defective ADA–CD26 binding suggests that interaction of these proteins is not essential for the development or maintenance of immune function in humans (Richard et al., 2000).

3.1.3 Mutations in ADA-SCID patients

Direct analysis of mutant ADA alleles from ADA deficient patients has revealed a wide variety of molecular defects. Most of the patients analysed are compound heterozygotes and several deletions and splicing defects have been found as have a larger number of point mutations in the coding region of the structural gene. Two large deletion mutations, one 3250bp deletion of

the promoter and exon 1 (Berkvens et al., 1987; Markert et al., 1988) and the other deleting exons 1-5 (Hirschhorn et al., 1992), led as might be expected to no mRNA production. A number of splice site mutations have been identified that result in skipping of specific exons or in activation of cryptic splice sites. Most of these mutations generate premature translation stop codons and result in a decreased level of mRNA. ADA missense mutations have been found throughout the coding region of the gene with no particular mutation 'hotspots' (reviewed in Hershfield and Mitchell 1995). Determination of the three-dimensional structure of the murine ADA has permitted modeling of the possible effects of the amino acid substitutions on human ADA function. Two mutations are directly involved in binding ADA substrates Glu_{217} (→Lys) hydrogen bonds to the N-1 atom of substrate through the side chain carboxyl and His_{15} (→Asp) coordinates with the zinc co-factor (Arrendondo-Vega et al., 1998). Several other mutated residues are close to the active site or to peptide segments that deploy active site residues.

In vitro analysis of the residual ADA activity of mutant alleles has been determined by expression of the mutant in the ADA-deleted *E.coli* strain SO3834 (Arredondo-Vega et al., 1998). Alleles associated with severe ADA SCID expressed 0.001%-0.6% of wild type activity whereas 3 alleles from partial ADA deficient patients expressed 5%-28% of normal. There was also a strong inverse correlation between mutant ADA activity and dATP level at time of patient diagnosis. These data suggest that the severity of the mutation and level of residual ADA activity may influence metabolic and clinical outcome.

Although analysis of the effects of missense mutations by modeling of ADA structure or by in vitro assays may be informative, in most cases of ADA-SCID, mutant ADA protein is present at only very low levels or is undetectable by western blotting. Thus the primary effect of some point mutations *in vivo* may be to increase the rate of enzyme degradation by interfering with protein folding or by destabilizing the mature protein and predisposing it to proteolysis.

3.1.4 The pattern of clinical disease

3.1.4.1 Classical presentations

ADA-SCID is estimated to affect between 1 in 200,000 and 1 in 1,000,000 births although the frequency may be greater in certain geographical areas (Hershfield and Mitchell 1995)(Sanchez et al., 2007). Clinically it is characterised by severe and recurrent infections and a high frequency of opportunistic infections. The clinical presentation in ADA-SCID is similar to patients with autosomal forms of SCID and it is difficult to distinguish between the different forms of SCID on the basis of clinical presentation alone. The mean age at diagnosis for all types of SCID is 6.6 months (Buckley et al., 1997) and this most likely reflects the time when the protective effect of placentally transferred maternal immunoglobulin has diminished

and children have been exposed to a range of microorganisms. The most common infective problems are oral candidiasis, respiratory infection due to Pneumocystis jiroveci, respiratory syncitial virus and parainfluenza 3, adenoviral infection, persistent diarrhoea and failure to thrive. In countries, which administer anti-tuberculous vaccination to infants with bacillus Calmette-Guerin (BCG), disseminated infection with BCG has occurred. Live polio vaccine has also caused poliomyelitis and carditis but only rarely and this is probably due to the continued presence of maternal immunoglobulin at the time of initial vaccination. Physical findings are unremarkable except for evidence of infection and the absence of lymph nodes and pharyngeal lymphoid tissue.

3.1.4.2 Non-immunological manifestations in ADA-SCID

Unlike other forms of SCID, ADA deficient patients show a number of non-immunological abnormalities which may reflect the importance of ADA expression in other systems. Costochondral abnormalities and skeletal dysplasias are well documented (Cederbaum et al., 1976) and ADA-SCID children have been noted to have a ‘rachitic rosary’ appearance. Other systemic abnormalities include neurological abnormalities involving motor function (Hirschhorn et al., 1980), bilateral sensorineuronal deafness (Tanaka et al., 1996), hepatic dysfunction (Bollinger et al., 1996) and renal mesangial sclerosis (Ratech et al., 1985). Non-immunological manifestations are also found in ADA deficient mice, which die perinatally from hepatocyte degeneration but also show pulmonary and intestinal defects (Migchielson et al., 1996; Mitani et al., 1993). More recently studies on ADA SCID patients post bone marrow transplantation show defects in cognitive and behavioural function despite correction of immunological abnormalities (Rogers et al., 2001; Titman et al., 2008) and again highlights the systemic nature of the disease.

3.1.4.3 Delayed/late onset disease

Approximately 10-15% of all cases of ADA deficient patients have a milder phenotype with less severe immunological abnormalities and clinical course. In these patients there is residual ADA activity as a result of the specific gene defect and consequently less profound metabolic derangement (Morgan et al., 1987). In these patients, recurrent infections may start to occur after 2-3 years of age and patients are eventually diagnosed following investigation for a combined immunodeficiency. At the far end of this spectrum are a handful of adults who have been diagnosed with ADA deficiency. Two sisters with a long-standing history of pulmonary insufficiency and warts were identified following investigation of their CD4 lymphopaenia (Shovlin et al., 1994). Other individuals with lymphopaenia and diagnosis in adulthood have been described (Ozsahin et al., 1997). In these patients there was only a mild metabolic abnormality in comparison with the levels of dATP and dAdo seen in patients with full blown ADA-SCID.

3.1.4.4 Partial ADA deficiency

A number of individuals have now been described with partial ADA deficiency. In these individuals there is differential expression of ADA in different cell lineages with very low or undetectable levels in erythrocytes (<2%) but ~4-70% of normal activity in fibroblasts (Hirschhorn et al., 1979; Borkowsky et al., 1980). As a result of significant activity in nucleated cells, there is very little metabolic derangement in erythrocytes and there is normal immune function. In vitro expression studies of the mutant alleles from such individuals demonstrates residual activity and suggests that there is a genotype-phenotype correlation (Daddona et al., 1983).

3.1.4.5 Reversion mutations in ADA-SCID

Patients have now been described in whom an in vivo reversion mutation to wild type sequence has resulted in restoration of wild type ADA activity to patient T cells. Detailed analysis of these patients showed that although B cells and other lineages showed the presence of two mutated alleles, T cell lines contained one mutant and one wild type allele. The wild type allele expressed a functional ADA protein resulting in half normal ADA activity in the cell lines. In one patient in vivo reversion resulted in progressive clinical improvement and unexpectedly mild biochemical and immunological abnormalities suggesting that T cells modified to wild type may have a powerful survival and growth advantage over ADA deficient cells (Hirschhorn et al., 1996). A similar phenomenon has been described in a patient with X-SCID and was used as an important for model for subsequently successful clinical gene therapy trials (Stephan et al., 1996). In a second individual the use of PEG-ADA resulted in a decrease in ADA activity in peripheral blood mononuclear cells which could have resulted from PEG-ADA abolishing the selective advantage of revertant cells in vivo (Ariga et al., 2001).

3.1.5 Diagnosis of ADA deficiency

ADA deficiency unlike many of the other immunodeficiencies can be reliably diagnosed by enzymatic assays. Intracellular ADA activity can be measured by the ability of cells to convert the substrate adenosine to inosine and hypoxanthine using high performance liquid chromatography (HPLC). Further, dATP build-up in erythrocytes, accumulation of intracellular and extracellular dAdo and lack of SAHH activity are specific characteristics of the disease and can all be measured to give an accurate and unambiguous diagnosis. In carriers of the condition, intermediate levels of ADA activity have been found leading to reliable identification of carrier status. The availability of such precise enzymatic assays has to a certain extent obviated the need for genetic diagnosis, although this has been carried out in specialist laboratories.

Enzymatic assays have also been used in prenatal diagnosis of ADA deficiency. In the first trimester of pregnancy, cultured cells from chorionic villus sampling (CVS) can be assayed for ADA enzymatic activity (Dooley et al., 1987). This is a more reliable source than fresh material from the CVS. In the second trimester diagnosis has been performed on cultured amniotic cells. Direct analysis of enzyme and metabolite levels in amniotic fluid has not proved useful.

Genetic diagnosis is also available in specialist laboratories and is useful for confirming the findings observed by metabolic analysis. In certain cases, the use of genetic analysis and mutant allele expression has been able to shed light on the variability in severity of presentation.

3.1.6 Prognosis

Classic ADA-SCID has an extremely poor prognosis without treatment. Death occurs in the first year of life from infectious complications. Although treatments based on physical isolation into a sterile environment can provide protection from infection and prolong life (for example, the case of the 'bubble baby'), such cases are extremely rare. Upon diagnosis, patients are commenced on bacterial, viral and pneumocystis prophylaxis and immunoglobulin substitution therapy. In some cases fungal prophylaxis is also commenced. In a few atypical cases, patients have been maintained on this regime for a number of years. However, it is generally accepted that in the vast majority of cases, prophylactic therapy is only a means of protecting the child until more definitive treatment with either stem cell transplantation or PEG-ADA can be performed.

3.2 Management options for ADA-SCID

3.2.1 Haematopoietic stem cell transplantation for ADA-SCID

Haematopoietic stem cell transplantation (HCST) is the treatment choice that is most widely available to most physicians and transplant centres. Until recently, data on the outcome of ADA-SCID transplants has been limited as most SCID transplant papers have presented data on the outcome of all SCID types rather than by specific molecular defect (Antoine et al., 2003). For these reasons, a multicenter retrospective study gathering data on outcome of haematopoietic stem cell transplantation for ADA-SCID has been conducted. In this study, we analysed outcome of HSCT in 106 patients with ADA-SCID who received a total of 119 transplants.(Hassan, 2012)

Donor source had a major impact on the outcome of HSCT (fig 1). Overall survival was higher in Matched Sibling Donor (MSD) and Matched Family Donor(MFD) HSCTs (86% (42 patients) and 81% (12 patients) respectively). Comparison of survival outcomes in MisMatched Unrelated Donor (MMUD) and haploidentical transplants [29% (7 patients) and 43% (30

patients) respectively]to those of MSD HSCT showed a highly significant difference ($p<0.0001$). Comparison of overall survival in MSD to MUD HSCT also showed a significant difference ($p<0.05$). In haploidentical transplants, even though overall survival was 43%, stable engraftment was only noted in 11 (37%) patients. The majority of deaths in the Matched Unrelated Donor (MUD), MMUD and haploidentical transplants (HAPLO) were in the first 100 days after HSCT, with relatively few late deaths. The causes of death were mainly related to pneumonitis/respiratory failure and sepsis which formed over 50% of all deaths. Other major causes included graft versus host disease (15%) and fungal infection (11%).

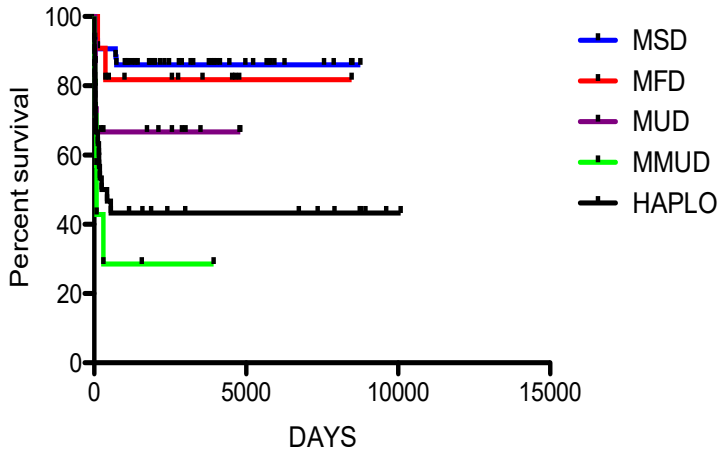


Figure 1: *Kaplan-Meier curve showing overall survival in relation to donor source. MSD–matched sibling donor; MFD–matched family donor; MUD-matched unrelated donor; MMUD-mismatched unrelated donor; HAPLO-haploidentical donor (data from manuscript in preparation).*

The majority of MSD/MFD transplants were undertaken without any chemotherapy as were a smaller number of MUD and HAPLO transplants and this may have had a major effect on outcome. When conditioning itself was studied in a univariate analysis, there was a significantly improved overall survival in unconditioned transplants in comparison to myeloablative intensity conditioning transplants (79 % vs 55%; $p=0.003$). Reduced intensity conditioning transplants had an overall survival of 64% and this was not statistically different from outcomes following unconditioned procedures.

The poor outcome in mismatched donor transplants may be attributable to the vulnerability of patients to the toxicities associated with cytoreductive chemotherapy and it has been argued that an alternative approach would be to infuse T cell depleted stem cells without any chemotherapy. Data available from a single North American centre suggests however, that this may not necessarily improve Event Free Survival (EFS) outcome. Of 19 patients undergoing such procedures, 14 survived (73%) but of these only 7 were successfully engrafted (DFS 37%) with the remainder rejecting the unconditioned transplant (Booth et al.,

2007). Similarly in the HSCT data collected recently, of 4 fully matched unrelated donor transplants performed without conditioning, 2 patients did not engraft effectively suggesting that conditioning is necessary in all scenarios other than a fully matched sibling or family donor.

The above data on HSCT outcome may help in guiding decision making with respect to the use of gene therapy. Certainly a fully matched sibling or family donor transplant is associated with a considerable degree of success both in terms of survival and sustained immune recovery and would be the definitive treatment of choice. By contrast, the current evidence suggests that mismatched transplants, both from unrelated and haploidentical donors, whether undertaken with or without conditioning have a poor chance of success and in the conditioned setting are associated with high mortality rate. The data from matched unrelated donor transplants (66% survival in 15 patients (fig 1)) also suggests that there is a significant risk of mortality associated with the procedure.

3.2.2 ADA enzyme replacement therapy

The toxic compounds that accumulate in ADA deficiency (dATP) cross the cell membrane poorly but are metabolised to ADA substrates (dAdo) that rapidly equilibrate with plasma via the nucleoside transporter. Thus maintaining sufficient levels of circulating 'ectopic' ADA either in the plasma or in a population of cells can normalise metabolite levels in enzyme deficient cells. This was initially demonstrated by the use of repeated red cell transfusions for the treatment of ADA deficiency (Polmar, 1978). Metabolic and immunological correction was seen but the effects were transient and outweighed by the risk of viral transmission and iron overload.

PEG-ADA is a bovine form of ADA conjugated to polyethylene glycol. The covalently bound PEG is intended to prevent proteolysis and uptake by cells and to prolong circulating life and reduce immunogenicity. PEG-ADA has been used since 1987 for the treatment of ADA-SCID patients who lacked a genotypically identical donor (reviewed in (Hershfield, 1995a; Hershfield, 1995b). PEG-ADA avoids the risks of red cell transfusion and the amount of enzyme activity provided is equivalent on a ml to ml basis to ~1800 times the ADA activity of packed erythrocytes. Three mls of PEG-ADA contains the ADA equivalent of ~10^{12} T lymphocytes. The preparation is given as an intramuscular injection at ~30 u/kg on a weekly basis.

Data on long term follow up of immunologic function and outcome in PEG-ADA treated patients has been limited since there has been no central point for data collection. However a recent review (Gaspar et al., 2009) has highlighted outcomes in over 185 patients treated with

PEG-ADA until September 2008 (about 90% of those ever treated). PEG-ADA has been used as initial therapy for patients who lacked a related HLA-identical donor, when assessment of risk and benefit by physicians and parents favoured Enzyme Replacement Therapy (ERT) over other options (Hershfield, 2004; Booth et al., 2007).

Overall, 70% of patients treated with PEG-ADA began ERT at <1 year of age (50% were <6 months old). Half of the remaining patients started treatment at 1-3 years of age, and half at 3-34 years of age. Many of these latter “delayed” or “late’ onset patients had pulmonary disease or other consequences of chronic immune deficiency, which made them poor candidates for partially mismatched HSCT with conditioning.

Up to September 2008, 98 patients were receiving PEG-ADA approximately half of the number that had begun ERT. About 20% of patients had died while on therapy; the remainder had discontinued ERT to undergo a potentially curative procedure. More than two thirds of the transplants were performed within a year of starting PEG-ADA, as soon as clinical condition was stable and a suitable donor had been identified. During the first decade after PEG-ADA received FDA approval in 1990, survival following these “elective” transplants was about 50% (Hershfield, 2004), similar to that for partially mismatched transplants in ADA-deficient SCID patients who had not received prior ERT.

Figure 2 plots the estimated probability of survival vs. length of treatment with PEG-ADA. Half of the deaths on ERT occurred within the first 6 months (40% in the first month), due to conditions present at diagnosis. The overall probability of surviving 20 years on ERT is estimated to be 78%. A patient alive 6 months after starting ERT had about 90% probability of surviving the next 12 years. Conditions contributing significantly (3-5 patients each) to mortality beyond 6 months include refractory hemolytic anaemia at 1-3 years; chronic pulmonary insufficiency after 5 to 15 years; and lymphoproliferative disorders after 5 to 15 years of ERT (Kaufman et al., 2005; Husain et al., 2007). Hepatocellular carcinoma developed in two patients, one just starting ERT after failing an unconditioned haploidentical HSCT, and a second after 10 years of ERT. Another patient died of hepatoblastoma discovered after 2 years of ERT, but thought to be present at diagnosis of ADA deficiency. Late deaths due to acute infection appear to be uncommon, but a patient recently died of measles after 10 years of treatment.

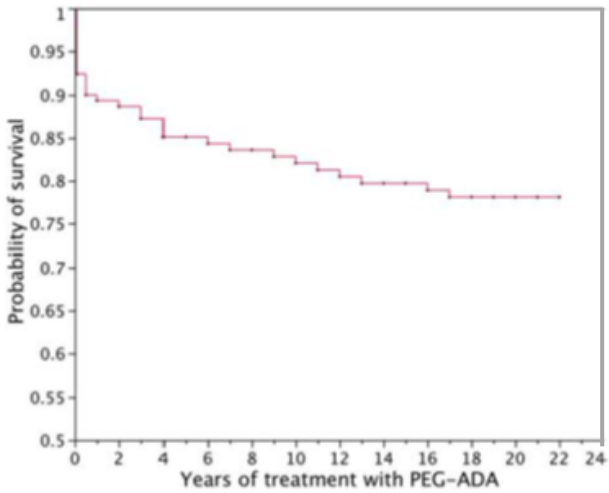


Figure 2: *Estimated probability of survival while receiving PEG-ADA (from Gaspar et al., 2009).*

Immune dysregulation has been seen in a few patients following commencement of PEG-ADA. Thrombocytosis has been described although there were no adverse consequences with resolution after a few weeks of treatment (Marwaha et al., 2000). Two patients developed refractory immune haemolytic anaemia (Hershfield, 1995b), one of whom required prolonged immune suppression and died of candida sepsis and the other discontinued PEG-ADA and died of complications of a mismatched HSCT. In a significant number of patients (~50%), recovery of immune function also leads to development of antibodies against bovine epitopes of PEG-ADA (Chaffee et al., 1992). In a minority of these individuals an inhibitory antibody directed at the ADA active site develops and results in enhanced ADA clearance. In one case tolerance was induced and in another increased clearance was overcome by increasing the PEG-ADA dose.

The other major limitation to PEG-ADA therapy is cost. As an orphan drug, the cost is high and treatment of a small child may cost between £150,000–£300,000 per year. With increase in age and size, these costs will only increase and since ADA therapy is palliative and not curative, treatment must be continued throughout the life of the patient.

In summary, PEG-ADA provides an often life-saving therapy at the time of diagnosis, when other options may be unavailable or less predictably effective. If ERT is continued beyond 6 months, there is a high probability that clinical benefit can be sustained for at least a decade. However, PEG-ADA may not be easily available in some countries and its high cost is a barrier to long-term ERT, and more uncertainty exists about how long term immunological and clinical benefit can be maintained beyond 8-10 years. An additional concern with ERT beyond 5yrs is the emergence of serious complications, described above, including lymphoid and possibly hepatic malignancies, and progression of chronic pulmonary insufficiency.

3.2.2.1 PEG-ADA treatment and long-term outcome

In the absence of a fully matched related or unrelated donor, a number of centres have chosen to maintain patients on long term PEG-ADA replacement therapy. Until very recently no formal data has been published on the immunological and clinical outcome in these patients but a number of reports now suggest that maintaining patients on long-term PEG-ADA may result in prolonged immunodeficiency and increased susceptibility to infection.

Paper 1: (Chan et al., 2005)

This paper evaluated the immune reconstitution of nine ADA SCID patients who have received PEG-ADA for 5-12 years (median 9 years). The T lymphocyte counts in these patients increased initially but as seen also in patients treated at GOSH, decreased thereafter to low levels. The graph below (Fig 3) shows that levels range between 12-500 T cells/mm^3 after 5-12 years and in all patients the absolute level is well below the lower limit of normal. Further impairment of cellular immunity was demonstrated by low levels of CD4+ cells and impaired T cell responses to both mitogens and specific antigens.

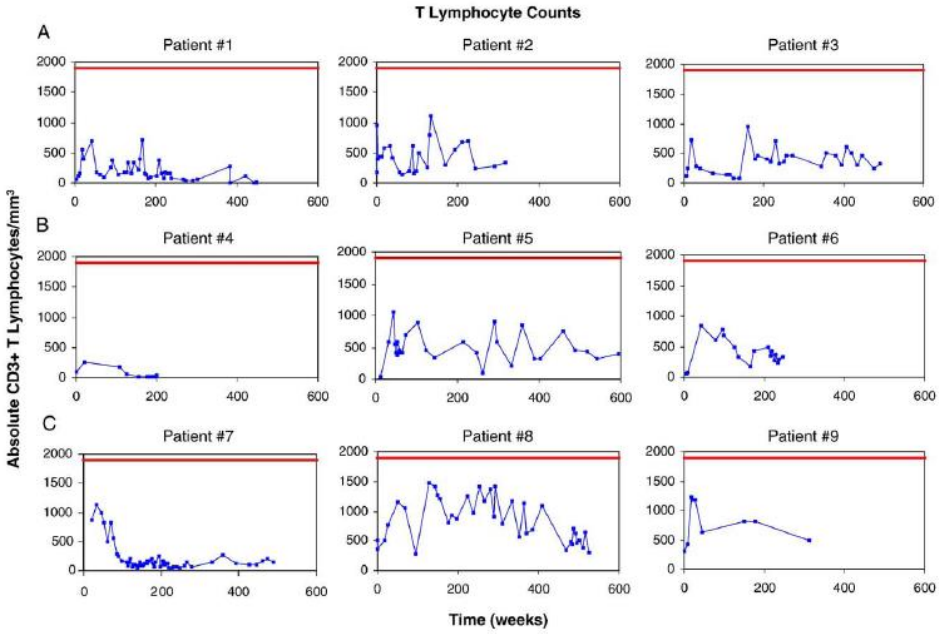


Fig. 1. Absolute T lymphocytes counts over time for nine ADA-deficient SCID patients receiving PEG-ADA enzyme replacement therapy. The lower limit of normal (10th percentile) is shown as a thick horizontal bar.

Figure 3: *Analysis of thymic output and TCR diversity. (A) Number of TREC and CD3+ lymphocytes in samples obtained from five PEG-ADA-treated patients (#1–#5) at 4.1 and 6.1 years after PEG-ADA therapy initiation. The mean (*) and standard deviation (vertical line) of values obtained in age-matched controls are shown for comparison. Figure from Chan B et al., 2005.*

Paper 2: (Malacarne et al., 2005)

In this study, five ADA-SCID patients who received PEG-ADA for 5-8 years (mean 6.7yrs) were assessed. These patients were also T lymphopaenic and importantly also showed decreased levels of TRECs (T cell receptor excision circles – an indicator of thymic activity) in comparison to age matched controls (Fig 4). These patients also showed a decreased response to mitogens and an increased tendency of T cells to apoptosis.

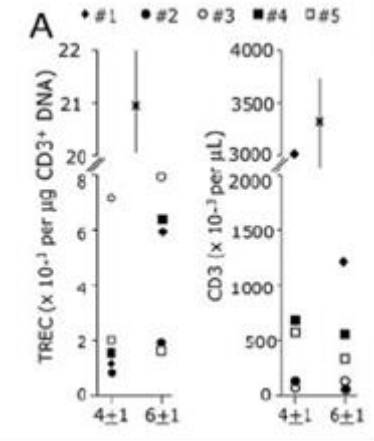


Figure 4: *Analysis of thymic output and TCR diversity. (A) Number of TREC and CD3+ lymphocytes in samples obtained from five PEG-ADA-treated patients (#1–#5) at 4.1 and 6.1 years after PEG-ADA therapy initiation. The mean (*) and standard deviation (vertical line) of values obtained in age-matched controls are shown for comparison. Figure from Malacarne F et al., 2005.*

Paper 3:(Serana et al., 2010)

In a follow up to the Malacarne study above, a comparative analysis of patients receiving HSCT (n=5) and PEG-ADA (n=8) was undertaken. This study demonstrates that patients on ERT show decreased numbers of total lymphocytes/CD3+/CD19+ cells over time, all of which are below the normal range for age. Patients on PEG-ADA in comparison to HSCT recipients also show decreased thymic output and restricted T cell receptor repertoires (Fig 5).

Papers 4 and 5: ***(Kaufman et al., 2005; Husain et al., 2007)***

These clinical reports document 2 children both of whom after 10 years of PEG-ADA therapy developed an EBV positive cerebral lymphoma and Burkitt's lymphoma respectively. Despite chemotherapy the first patient died 5 months after diagnosis of the tumour and the second patient responded to chemotherapy. In both patients there was poor recovery of cellular immunity on PEG-ADA which may have contributed to development of lymphoma. Other patients have also been reported who have developed malignancies after long term PEG-ADA administration.

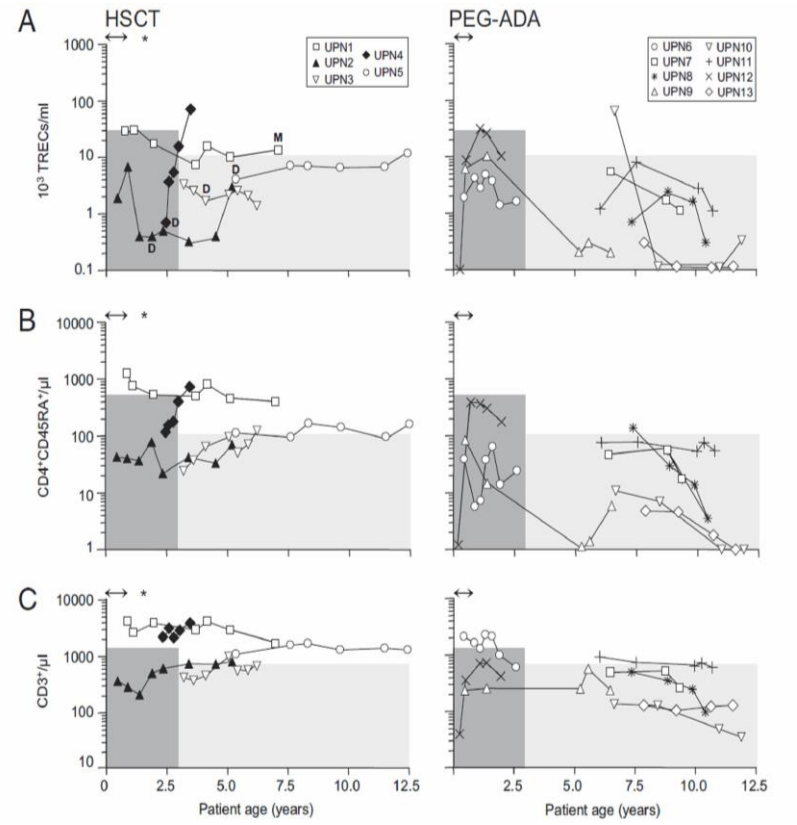


Figure 5: *Analysis of thymic output and T cell numbers in 5 HSCT treated patients and 8 PEG-ADA-treated ADA patients over time. The shaded areas represent the age related normal values in healthy children. A) Number of TRECs B) Naïve T cell numbers as characterized by CD4+CD45RA+ expression C) Total T cell numbers. Figure from Serana et al., 2010.*

These reports suggest that continued PEG-ADA treatment leads to demonstrable defects of T cell function and impaired thymic activity that may lead eventually to significant clinical infectious complications. It is also very probable that attempts to treat patients by either HSCT or stem cell gene therapy at this late stage will be unsuccessful or of limited efficacy due to reduced thymic function and increased infective burden (Thrasher et al., 2005).

Thus although PEG-ADA maybe an excellent short term measure to stabilise children with ADA-SCID, its prolonged use may be counter-productive.

3.2.2.2 PEG-ADA and neurological outcome

PEG-ADA results in more systemic delivery of ADA enzyme. As a result it may be thought that PEG-ADA therapy may have a more beneficial effect on the non-immunological consequences of ADA deficiency. However, at present there is no conclusive data to suggest that PEG-ADA treatment improves neurocognitive outcome. In a preliminary study, we have compared patients treated with PEG-ADA alone with those treated by HSCT and although the numbers are small, there is no significant difference between the two groups. Anecdotal evidence from other physicians also suggests that PEG-ADA treated children continue to have behavioural problems.

3.2.3 Alternative therapy for ADA-SCID based on somatic gene transfer

Allogeneic bone marrow transplantation for ADA-SCID has a number of limitations especially when a genotypically matched donor is unavailable. PEG-ADA can be very beneficial but also has certain side-effects, poor long term immune recovery and is extremely expensive. An alternative therapy based on somatic gene transfer of the ADA gene into haematopoietic stem cells has been proposed by several investigators and clinical trials have been undertaken with variable results. For the reasons stated below, ADA-SCID been considered an attractive condition for treatment by somatic gene therapy.

1. It is a monogenic disease well characterised at the molecular level.

2. ADA is widely expressed in haematopoietic and non-haematopoietic cells and is not under complex regulatory control.

3. Over-expression of the gene has not been associated with significant complications.

4. Suboptimal levels of gene expression result in immunological correction.

5. Introduction of the ADA gene into T cell precursors confers a survival and developmental advantage to their progeny.

6. Introduction of the ADA gene into haematopoietic stem cells may restore functionality of B lymphoid and T lymphoid cells.

Three recent clinical trials of somatic gene therapy for ADA-SCID have shown complete correction of the immunological and metabolic defects and thus verifies the feasibility and efficacy of this approach in human subjects. In all three studies, no adverse effects have been seen.

3.2.4 Clinical trials of gene therapy for ADA-SCID

3.2.4.1 T lymphocyte gene therapy

In 1990 the first clinical trial of gene therapy for ADA deficiency was started on two girls in the United States (Blaese et al., 1995). Both patients had had commenced PEG-ADA therapy but in each case there had been an initial good response to PEG-ADA with a subsequent deterioration in total lymphocyte numbers and *in vitro* and *in vivo* specific lymphocyte responses. The treatment protocol involved LASN retroviral vector supernatant transduction of peripheral T cells apheresed from each patient and stimulated with a combination of recombinant interleukin 2 (rIL-2) and OKT3 (an anti-CD3 complex antibody). The transduced expanded T cells were returned to the patients 9-12 days later by peripheral infusion. The procedure was repeated 11-12 times at regular intervals for each patient over a period of 18-24 months. The efficiency of retroviral gene transfer varied considerably from one round of transduction to another with a range of 0.1%-10% transduced cells. The level of peripheral blood lymphocyte ADA was in keeping with the PCR findings with a significant rise in Patient 1 but no change from pre-treatment levels in Patient 2. In both patients there was an initial rapid rise in the T cell count before stabilising in the normal range for Patient 1 and a slight increase from the pre-treatment levels for Patient 2. Cell mediated immunity was shown to improve in both patients as assessed by the development of DTH skin test reactivity to a variety of antigens and also by improvement in *in vitro* T cell immune responses. Humoral immune function improved with an increased antibody response to Hib and tetanus immunisation. One interesting observation arising from this study is the prolonged survival of the transduced T cells. Vector positive cells are detectable for over 10 years after the last infusion, a far longer survival time than originally predicted (Muul et al., 2003).

3.2.4.2 Peripheral blood lymphocyte and T cell depleted bone marrow gene therapy

A slightly different approach to gene therapy was taken by Bordignon and colleagues in 1992 (Bordignon et al., 1995). Two structurally identical vectors expressing the human ADA cDNA and distinguishable by the presence of alternative restriction sites in a non-functional region of the viral LTR, were used to transduce peripheral blood lymphocytes (PBLs) and T cell depleted bone marrow independently. Thus using restriction digest analysis, it was possible to identify the origin of transduced cells. Efficiency of transduction varied from 2% to 40% of PBLs and from 30%-40% of bone marrow cells.

Two patients were initially treated by this protocol (4 further patients have been treated following the initial report). Both had followed a similar clinical course with diagnosis at the age of two years and treatment with PEG-ADA. Both patients received infusions of gene modified PBLs and T cell depleted bone marrow regularly over a 1-2 year period. Analysis of vector transduced cells from peripheral blood indicated that initially all vector-positive cells were

derived from transduced PBLs. However, 3 years after initiation, and 1 year after discontinuation of gene therapy, peripheral lymphocytes showed the alternative restriction digest pattern indicating conversion of the circulating transduced lymphocytes from a PBL-derived to a bone marrow derived population. Further analysis of T lymphocyte clones, granulocytes and erythrocytes taken at this time point also showed a predominantly bone marrow origin. These findings suggested that there had been stable transduction of early haemopoietic progenitors capable of generating multilineage progenies. Moreover, the continued presence of transduced bone marrow cells and their differentiation into mature circulating cells appears to confirm the assumption that a small number of ADA+ cells have a survival advantage in an ADA -ve environment.

The frequency of transduced cells varied with 2-4.7% of T cells being vector positive and 17-25% of clonable bone marrow progenitors. Unsurprisingly, given the low frequency of transduced cells in the circulation the level of total ADA activity in total circulating nucleated cells has remained low (5-18% of normal values). There was an increase in absolute lymphocyte and T cell numbers in both children into the normal range and there was an improvement in T cell proliferative responses to non-specific and specific stimuli. The T cell repertoire was analysed by Vβ usage and demonstrated the development of a normal T cell repertoire.

Although these results appeared promising, both children remained on PEG-ADA therapy, albeit with decreasing doses. With greater follow-up, reports from this study did not show any significant improvement in immunological or metabolic parameters and patients remained on PEG-ADA.

3.2.4.3 CD34+ stem cell gene therapy

Hoogerbrugge and colleagues performed retroviral mediated gene transfer into CD34+ bone marrow cells of 3 children with ADA deficiency in an attempt to effect a cure with a once only procedure (Hoogerbrugge et al., 1996). Two of the subjects were on enzyme replacement therapy at the start of the trial, and in the third child PEG-ADA was only started 3 months after the gene therapy procedure. Gene transfer resulted in a 5-12% transduction frequency in *in vitro* colony forming cells. Transduced granulocytes and mononuclear cells persisted in the circulation for 3 months as shown by PCR analysis. In one patient the transduced gene was detected in the bone marrow at 6 months after gene transfer. Subsequently, no evidence of the transduced gene was detectable in either the bone marrow or peripheral blood. In addition, ADA gene expression was not detected at any time point.

A further study also targeted the CD34+ population but on this occasion from cord blood derived cells (Kohn et al., 1995). Three prenatally diagnosed infants were treated by transplantation of transduced autologous CD34+ selected umbilical cord blood cells. All 3 patients were started on PEG-ADA in the first few days of life. By progenitor colony assay, it was shown that 12-21% of CD34+ cells had been transduced in the three patients. The frequency of peripheral lymphocytes containing the LASN vector as shown by semiquantative PCR, was 1/3000 to 1/100000 18 months after transplantation. At this time point, ADA activity in unselected T cells was measured and found to be barely above the levels found in ADA SCID patients. The results suggested that retroviral mediated stable transduction of early progenitors in cord blood was possible and that engraftment of these cells without prior cytoablation was possible. However, the small numbers of transduced peripheral blood lymphocytes and the very low level of ADA expression in unselected cells suggested that significant immune reconstitution was unlikely and PEG-ADA administration was continued.

3.2.4.4 PEG-ADA withdrawal leads to proliferation of gene transduced cells

The use of PEG-ADA has always clouded the issue of whether these early trials were successful in correcting immune function and it has always been felt that only complete withdrawal of replacement therapy would answer this question. To date two patients from two different studies have undergone elective withdrawal of PEG-ADA. Kohn et al. at Children's Hospital Los Angeles (UCLA) attempted withdrawal in one patient from their cord blood gene transfer program. Over two months of observation, there was a 30-100 fold increase in the proportion of gene transduced T cells. However, there was an overall 25% decrease in the total numbers of T cells and a decrease in the B and NK cell numbers. This prompted investigators to restart PEG-ADA and the patients remains on enzyme replacement to this day (Kohn et al., 1998).

A patient from the Milan study also stopped PEG-ADA on the grounds that there was a plateau in immune function and the investigators reasoned that stopping PEG-ADA may provide an advantage to transduced cells (Aiuti et al., 2002b). The discontinuation of PEG-ADA led to a selective growth advantage of gene transduced T lymphocytes as assessed by PCR quantification, fluorescence in situ hybridisation and T cell clonal analysis. Transduced cells expressing the ADA vector increased during PEG-ADA withdrawal eventually replacing the non-transduced T cell population, reaching nearly 100% of circulating lymphocytes. Absolute T cell counts increased and stabilised to levels significantly higher than those observed during PEG-ADA treatment. Functional immunological parameters also improved with demonstration of vaccine specific responses and responses to the ΦX174 neoantigen. Intracellular PBL ADA activity also increased by 1 log and persisted at approximately 50% of normal individuals (which is in the range for immunologically normal heterozygote parents) but there was a rise

in the red blood cell dATP levels suggesting that the total mass of gene corrected lymphocytes producing ADA was not sufficient for systemic detoxification. Nevertheless this patient has remained off PEG-ADA and is clinically well. It has not been reported whether prophylactic medication has been discontinued.

There may be several reasons for the difference in outcome between the two patients described. However the most important factor is likely to be the total number of gene transduced cells at the time of PEG-ADA withdrawal. In the UCLA study this level was only 0.01% whereas 1-3% of T cells in the Milan study were ADA gene positive prior to PEG-ADA discontinuation. Therefore the pool of cells capable of selective proliferation and immune system repopulation was initially much greater. However, both studies demonstrate that gene transduced cells do possess a proliferative advantage and this may have been blunted by the use of PEG-ADA.

3.2.5 ADA-SCID gene therapy without PEG-ADA therapy and with the use of a mild conditioning regime

A major progress in gene therapy for ADA-SCID was accomplished following the adoption of an improved gene transfer protocol into bone marrow CD34+ cells combined with the administration of a nonmyeloablative dose of busulfan chemotherapy prior cell reinfusion (Aiuti et al., 2002a). Since year 2000, 15 children lacking an HLA-identical sibling donor were enrolled in this experimental protocol (Aiuti et al., 2009)(and unpublished data). Most patients displayed an inadequate response to enzyme replacement therapy or had failed mismatched related transplant. To exploit the selective advantage for ADA-transduced cells in a toxic environment, no PEG-ADA was given after gene therapy. The reduced dose of busulfan induced a transient myelosuppression without organ toxicity, which was sufficient to achieve long-term engraftment of gene corrected hematopoietic stem cells (Aiuti et al., 2002a; Aiuti et al., 2009). The dose of $CD34^+$ cells infused and the efficiency of gene transfer were shown to be critical factors in determining a higher proportion of gene corrected myeloid cells engrafting in patients. The large majority of lymphocytes were ADA-transduced, confirming that PEG-ADA withdrawal favours the selective survival of gene corrected cells (Aiuti et al., 2002b; Kohn et al., 1998; Aiuti et al., 2009) (and unpublished data). Clonal analysis of long-term repopulating cells revealed the presence of shared vector integrations among multiple hematopoietic lineages, thus proving the engraftment of multipotent hematopoietic stem cells (Aiuti et al., 2007). All children are alive and the outcome of the first ten treated patients has been recently reported. Eight of the 10 patients remain currently off enzyme replacement therapy, with persisting ADA expression and systemic detoxification up to 8 years after treatment. Nine patients displayed recovery of polyclonal thymopoiesis, significant increase in T-cell counts (although 4 patients did not reach normal levels) and full correction of T-cell

function, including susceptibility to apoptosis and proliferative responses to mitogens and antigens (Cassani et al., 2008). Intravenous immunoglobulin treatment was discontinued in 5 children with evidence of antigen-specific antibodies to vaccinal antigens and pathogens. The progressive restoration of immune and metabolic functions led to significant improvement of patients' physical development and protection from severe infections, without adverse events related to gene therapy.

In a similar trial performed in London at our centre (Great Ormond Street Hospital for Children NHS Trust), using an alternative retroviral construct and a single dose melphalan conditioning regime (and latterly busulfan iv 4mg/kg), 7 patients have been treated (Gaspar et al., 2006) (and unpublished data, manuscript submitted). Three patients have shown very good immune recovery and in a fourth, gene transduced cells contributed to immune recovery although PEG-ADA was initially re-started and then stopped. Failure of gene therapy was seen in three patients, in one case as a result of a poor stem cell harvest and in two others due to low level stem cell transduction efficiency. In the two most successfully reconstituted patients, ADA expression was observed in different haematopoietic lineages, including red blood cells, leading to effective metabolic detoxification. The large majority of T cells and NK cells were gene corrected, while significant gene marking was also observed in granulocytes and monocytes. In these two individuals re-initiation of thymopoiesis after gene therapy was also noted.

The clinical trials conducted at the National Institute of Health and Children's Hospital of Los Angeles have provided important information on the role of myelosuppression in favouring engraftment of gene corrected HSC. In the first study, four patients undertook gene therapy without receiving conditioning, resulting in low levels of marking in two patients and no sustained immunological improvement (Sokolic et al., 2008). The second clinical trial included low-dose myelosuppression with busulfan and withdrawal of PEG-ADA and has enrolled six patients with a follow-up ranging between two months and two and a half years. This version of the trial led to superior immunological and metabolic outcome with two out of three patients with a follow-up longer than 1 year having derived clinical benefit as shown by normalisation of in vitro T function and improvement of immunoglobulin production that in one case allowed for normal responses to vaccinations. In all but one case, production of ADA by PBMCs is at levels between 30-100% of normal leading to sustained metabolic control similar to that observed after HSCT (K. Shaw, personal communication, ASGT 2009, San Diego, CA). One patient experienced a prolonged cytopenia following busulfan conditioning, as the consequence of a pre-existing cytogenetic abnormality, pointing out at a potential limitation for patients subject to autologous gene transfer (Engel et al., 2007).

Preliminary reports from a Japanese gene therapy trial under which two ADA-SCID patients were also treated with retroviral gene transfer into CD34+ bone marrow cells after withdrawal of PEG-ADA treatment, but in the absence of any myeloablative conditioning, have suggested that some immunological reconstitution can be observed in such a setting. Pending the availability of further details, the extent of the improvement in comparison with the results obtained in patients subjected to prior conditioning remains unclear (Otsu et al., 2006).

ADA-deficiency represents a paradigmatic approach for gene therapy approaches for inherited blood borne disorders. The results of clinical trials from 2000 onwards indicate that gene therapy with non-myeloablative conditioning is associated with significant clinical benefit and is now an option to be considered for all ADA-SCID patients lacking an HLA-identical sibling donor. The results from the trials in Milan, London and the US in which at least 30 patients have been followed for longer than 1yr are impressive both in terms of toxicity and efficacy. All patients have survived therapy which compares favourably with HSCT data and ~70% of patients have near full or partial recovery of immune function allowing complete cessation of PEG-ADA therapy (See Table 1). With respect to non-immunological manifestations, it remains to be determined whether gene therapy will be superior to allogeneic transplant in preventing the neuro-cognitive complications frequently observed in these children after transplant (Honig et al., 2007; Titman et al., 2008).

Center	No. Patients	F/U (yrs)[1]	Off Enzyme	Survival	DFS[2]
Milan	17	0.9 – 10.5	14/17	100%	82.4%
London	8	0.5 – 7.5	4/8	100%	50%
CHLA-NHGRI UCLA-NHGRI	6 5	2– 5 0.1-2	3/6 4/5	100% 100%	50% 80%
TOTAL	**36**	**0.1 – 8.0**	**25/36**	**100%**	**69.4%**

Table 1: *Results from 3 different trials of gene therapy for ADA-SCID (gathered from published data (Aiuti et al., NEJM 2009), GOSH experience in London and personal communication (D.B. Kohn/F. Candotti).*

3.2.6 Safety of ADA-SCID gene therapy using a gammaretroviral vector

The use of gammaretroviral vector mediated gene therapy has been associated with insertional mutagenesis in 3 different gene therapy trials. This is includes 5 patients treated by gene therapy for SCID-X1 (Hacein-Bey-Abina et al., 2008; Howe et al., 2008), 2 patients

treated for X-CGD (Ott et al., 2006; Stein et al., 2010) and 1 patient treated for Wiskott-Aldrich syndrome (Christoph Klein, unpublished data). In both the X-CGD and the WAS trials, the vector backbone and promoter (SFFV) are very similar to the vector construct and promoter used in our current GTAC 073 gammaretroviral study.

For ADA-SCID, analysis of gammaretroviral vector integrants has been analysed in a similar way to the studies for the other immunodeficiencies and shows a very similar insertional profile. Integrations are found predominantly in and around the transcriptional start site of genes and insertions have been found in genes associated with cell cycle control, cell signalling and near known oncogenes such as LMO2 (Aiuti et al., 2007). In our study (GTAC 073) in which ADA transgene transcription is under the control of the SFFV LTR, we have seen insertions into LMO2 and particularly into the MDS-EVI1 locus which is a favoured insertion site for SFFV based vectors and was the insertional event that led to leukaemia formation in the X-CGD trial (Ott et al., 2006, Stein et al., 2010).

Despite these worrying integration events, none of the patients treated (now over 30 patients) on the 3 trials have shown evidence of clonal proliferation or adverse events related to gene transfer. This may indicate that gene therapy for ADA-SCID has a favourable risk-benefit profile, as opposed to gene therapy for SCID-X1 (Hacein-Bey-Abina et al., 2003; Howe et al., 2008) although further long term follow up is necessary. Nevertheless, safety monitoring should be continued to be strictly implemented long-term in all patients, according to guidelines of regulatory agencies.

The safety of gene therapy for ADA-SCID is also highlighted by the long term follow-up (>10yrs) of patients treated in clinical trials in the 1990's. Though these patients derived little benefit from the gene therapy procedure, a number of patients continue to have significant numbers of gene transduced cells (1-10%) without any evidence of clonal proliferation or insertional mutagenesis (Muul et al., 2003; Schmidt et al., 2003).

From these studies it is clear that gene therapy is a very promising treatment strategy for the management of ADA-SCID. There is strong evidence for immunological and metabolic correction in the majority of patients treated. There is nevertheless, a potential risk associated with the continued use of conventional gammaretroviral vectors in which transcription of the ADA gene is under the control of the viral LTR. The use of alternative vector systems that can promote similar or improved immune recovery but with an enhanced safety profile would be of significant advantage in the furthering the use of gene therapy for ADA-SCID.

3.3 Lentiviral vectors for gene therapy of ADA SCID

3.3.1 Lentiviral vectors

Lentiviral vectors were derived from components of the HIV-1 lentivirus and have been found to have several attributes that make them potentially more effective and safe than retroviral vectors (Naldini et al., 1996; Zufferey et al., 1998; Zufferey et al., 1997). Lentiviral vectors offer several potential advantages over conventional gammaretroviral vectors including higher efficiency, lack of dependence on cell division, and possibly a more favourable integration profile. This means that cell culture periods can be minimized, and that HSC activity can be more easily preserved. This may also mean that the risk of harmful mutagenesis during *ex vivo* cell culture is minimized. Recently, lentiviral vectors have been utilized in a clinical trial of HIV gene therapy in the US, and are currently in trial for adrenoleukodystrophy (Cartier et al., 2009), β-thalassemia in France (Cavazzana-Calvo et al., 2010) and Wiskott-Aldrich syndrome (GTAC 146) (where patients additionally receive full myeloablative alkylating agent conditioning). The lentiviral (LV) vector proposed for this clinical trial is a 3rd generation replication-defective hybrid viral particle made by core proteins derived from HIV and the envelope of the unrelated Vesicular Stomatitis Virus (VSV). The system is based on 4 non-overlapping expression constructs in order to maximize the segregation of *cis* and *trans* acting functions. The lentivector is packaged using three separate plasmids, two expressing HIV proteins (gag/pol and rev), and the other expressing glycoprotein envelope protein of the VSV (VSV-G) combined with a self-inactivating (SIN) transfer vector carrying a major deletion in the 3' long terminal repeat (LTR) region. The system is engineered in such a way that minimal homology regions are present between packaging and transfer vectors, thus minimizing the likelihood of homologous recombination events and the generation of replication competent lentiviruses (RCLs). The packaging construct is deleted of all HIV accessory proteins (vpu, vpr, nef, vif) and Tat. The packaging system segregates gag/pol and rev genes in two separate plasmids. The Rev responsive element (RRE) maintained in the gag/pol plasmid makes the gag/pol gene expression Rev dependent. As the transcripts of gag and pol genes contain cis acting repressor sequences, they are expressed only in the presence of Rev, expressed in trans on a separate plasmid, which promotes their nuclear export and expression by binding to RRE. The transfer vector encodes a codon optimised human ADA gene cDNA and sequences necessary for expression, incapsidation, reverse transcription and integration of the viral genome. In order to minimize the risk of RCL generation, the 3' LTR of the transfer vector has been deleted in the U3 region. A mutated woodchuck post-transcriptional regulatory element (WPRE) sequence is incorporated in the vector to enhance transgene expression.

3.3.2 Rationale for using a lentiviral vector for gene delivery

Lentiviral vectors were developed in the mid 1990's with the potential advantage over retroviral vectors of being capable of gene transduction of non-dividing cells (Naldini et al. Science, 1996). Ideally, this will lead to more effective transduction of quiescent HSC with preservation of stem cell function. Lentiviral vectors are more effective than retroviral vectors when compared using multiple surrogate assays for human HSC, including in vivo growth in NOD/SCID mice and LTCIC assays of quiescent CD34+/CD38- cells (Miyoshi et al., 1999; Bai et al., 2003; Case et al., 1999). Lentiviral vectors can perform effective transduction during just 1-2 days of culture; this shorter time of culture has been clearly proven to increase the survival of pluripotent stem cells (Mazurier et al., 2004). More effective gene transfer to long-term reconstituting pluripotent hematopoietic stem cells may lead to more robust and rapid immune reconstitution than with using retroviral vectors. Additionally, lentiviral vectors configured to lack strong the strong enhancer elements of retroviral long-terminal repeats, present in typical retroviral vectors, have been shown to have lower risks for causing insertional oncogenesis in a number of *in vitro* and *in vivo* models (Modlich et al., 2006; Modlich et al., 2009; Zychlinski et al., 2008; Montini et al., 2006). This may translate into a lower risk for causing lymphoproliferative disorders in gene therapy subjects.

3.3.3 The EF1αS-ADA lentiviral vector

Our laboratory has constructed an ADA cDNA lentiviral vector containing the enhancer/promoter from the human EF1-α gene (Figure 6). The EF1-α enhancer/promoter used is "shortened" by deletion of the first intron of EF-1α, which possesses strong enhancer activity and because of its potential to *trans*-activate adjacent cellular genes upon insertion. The "EF1αS" promoter has been shown to direct high level transcription of reporter genes in murine hematopoietic cells and to have significantly reduced *trans*-activation potential compared to retroviral LTR (Zychlinski et al., 2008).

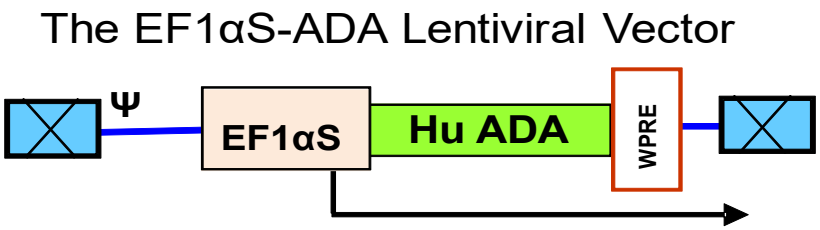


Figure 6: *huADA cDNA = human ADA cDNA; EF1αS = elongation factor 1α short (see text for description); WPRE = woodchuck hepatitis post-transcriptional regulatory element.*

There have been no clinical trials to date utilising lentiviral vectors for gene-correction of ADA-SCID. We propose to use the EF1αS-ADA lentiviral vector with the human ADA cDNA to transduce autologous CD34+ cells from the bone marrow of ADA-deficient SCID patients. We will evaluate transduction efficiency, gene marking, ADA levels, and immune function in ten (n=10) subjects. We hypothesise that the EF1αS-ADA lentiviral vector will result in increased transduction efficiency of the CD34+ cells. We anticipate that this increased transduction will result in better engraftment of gene-corrected stem cells, which will in turn result in increased gene marking, more rapid and robust production of gene-corrected lymphocytes that express ADA, and a reconstituted immune system.

3.4 Pre-clinical data

3.4.1 Transduction and expression of ADA in cell lines

In initial experiments, we compared the ability of the EF1αS-ADA lentiviral vector to express ADA in comparison to the current clinical grade gammaretroviral vector (Sfada/W) used in the present clinical trial GTAC 073. Following transduction of 3 different cell lines, ADA expression following transduction of cells with the EF1αS-ADA lentiviral vector (normalised for vector copy number) was either equivalent to or superior than the expression seen in cells transduced with the Sfada/W vector. This included an EBV transformed LCL from a patient with ADA deficiency (Fig 7). Total ADA activity is shown in Fig 7a and activity normalised for vector copy number is shown in Fig 7b.

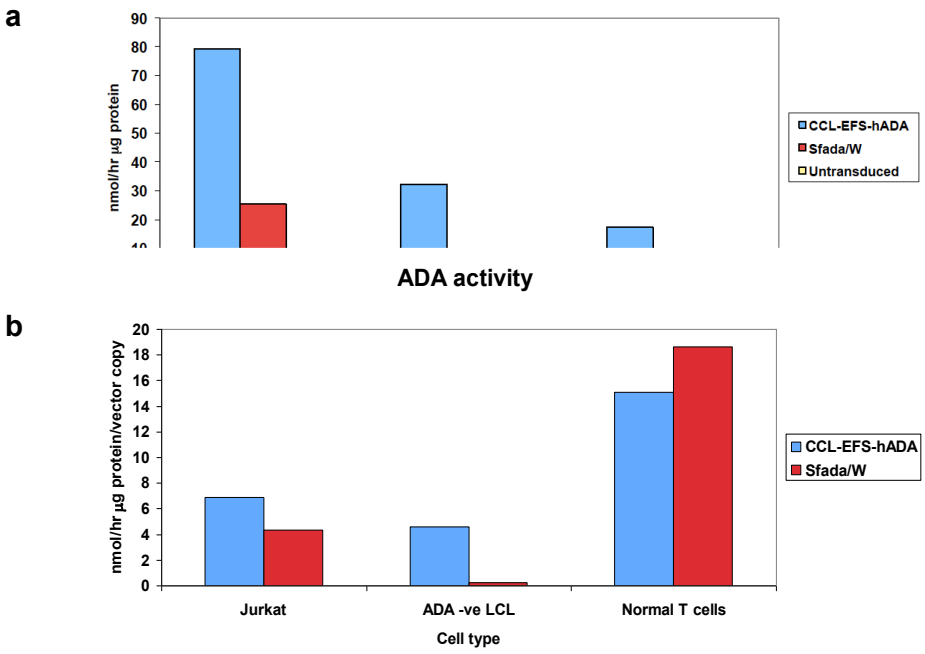
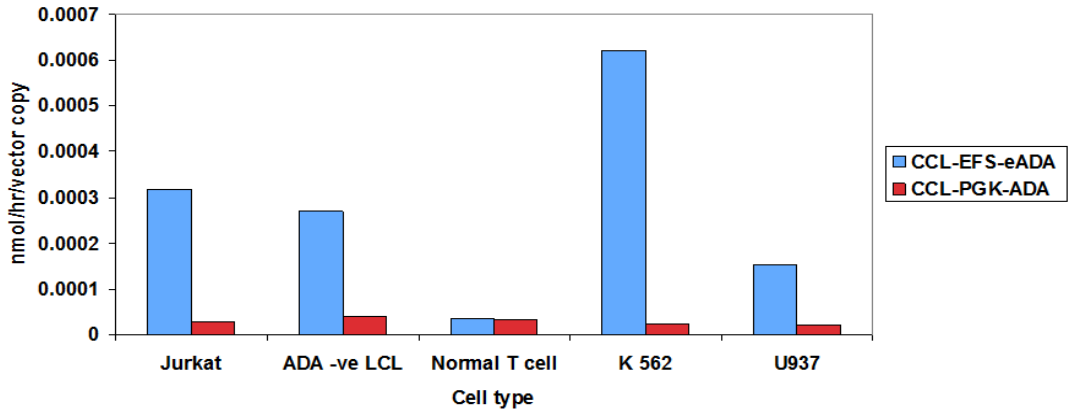


Figure 7: *ADA expression/vector copy number following transduction of Jurkat cells, ADA deficient EBV-LCLs and normal T cells using a EF1αS-ADA lentiviral vector or the Sfada/W gammaretroviral vector - a) total ADA activity, b) activity normalised for vector copy number.*

In further studies in our laboratory we compared the EF1αS-ADA lentiviral vector with a similar lentiviral vector in which the ADA gene was transcribed by an internal PGK promoter. A number of different cell lines were transduced including ADA-ve LCLs, primary T cells from a normal individual, Jurkat T cells, U937 cells and K562 cells. Viral supernatant of a similar titre and similar MOI were used for transduction purposes and in all cell lines other than the U937 line, the EF1αS-ADA lentiviral vector showed a higher transduction efficiency when transduced cells were analysed by intracellular ADA analysis. Cells were then analysed for ADA activity above baseline levels (as a low level of ADA activity is found in all human cell lines) and activity normalised for vector copy number which was determined by vector specific qPCR. In all cell lines other than normal primary T cells, the EF1αS-ADA lentiviral vector showed markedly higher ADA activity/copy number than the PGK-ADA lentiviral vector (Figure 8). Total ADA activity is shown in Fig 8a and activity normalised for vector copy number is shown in Fig 8b.

a



b

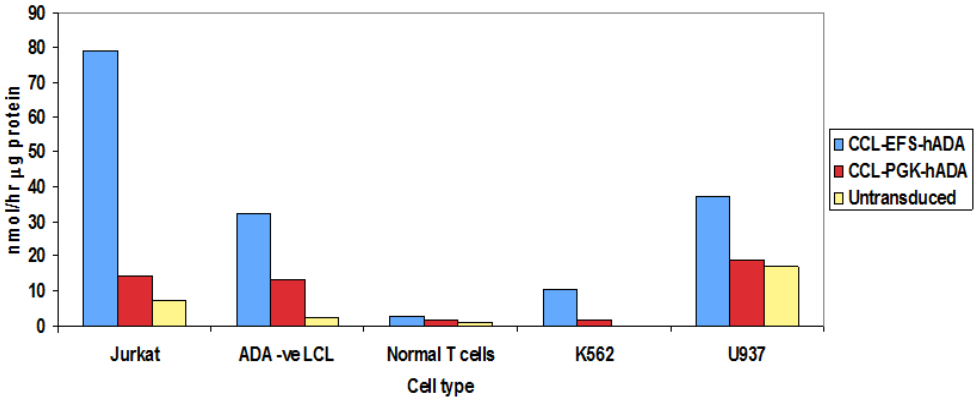


Figure 8: *ADA activity measured by HPLC and corrected for copy number in a variety of cell lines and in normal T cells - a) total ADA activity, b) activity normalised for vector copy number.*

In similar experiments performed by collaborators at UCLA, transduction of HT29 (colon carcinoma), C.E.M. (T cell leukemia), and an HTLV-1-transformed T cell line from a patient with ADA-deficient SCID again showed increased ADA activity/vector copy in cells transduced with the EF1αS-ADA lentiviral vector.

3.4.2 Transduction and expression of ADA in CD34+ cells

To determine the ability of the EF1αS-ADA lentiviral vector to express ADA in human haematopoietic stem cells, CD34+ cells from cord blood or bone marrow of normal or ADA deficient patients respectively was obtained and transduced with the EF1αS-ADA lentiviral vector using the same cytokine cocktail to be used in the clinical transduction protocol. Following culture to allow cell number expansion, cells were stained and assessed for intracellular ADA expression by flow cytometry. Significant levels of ADA expression are seen in transduced cells from both ADA deficient and wild type CD34+ cells (Fig 9).

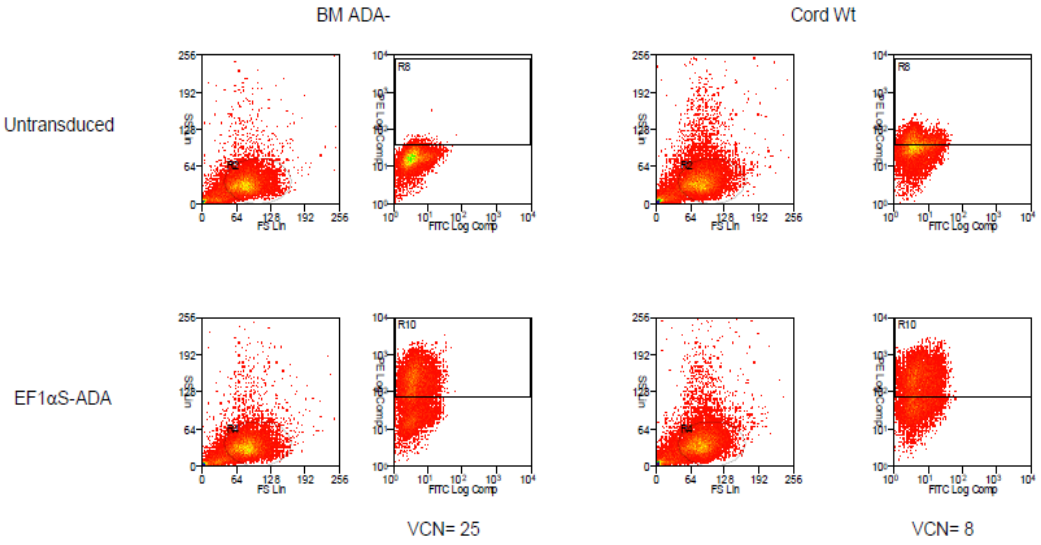


Figure 9: *Intracellular ADA expression following EF1αS-ADA lentiviral vector transduction of CD34+ cells from wild type and ADA deficient individuals (VCN=vector copy number).*

In further experiments, CD34+ cells bone marrow from an ADA deficient patient were transduced using either the EF1αS-ADA lentiviral vector, the PGK-ADA lentiviral vector or the gammaretroviral vector currently used in the GTAC 073 trial. Transduced cells were then grown on stromal cells in conditions conducive for development into T or NK cells (Schmitt and Zuniga-Pflucker, 2002). Viability and the extent of differentiation into the different lineages were similar for all 3 vectors. Following differentiation, the percentage of cells showing expression of ADA transgene was highest in those transduced with the gammaretroviral vector

and at slightly lower levels for those transduced with either the EF1αS-ADA lentiviral vector or the PGK-ADA lentiviral vector (Fig 10).

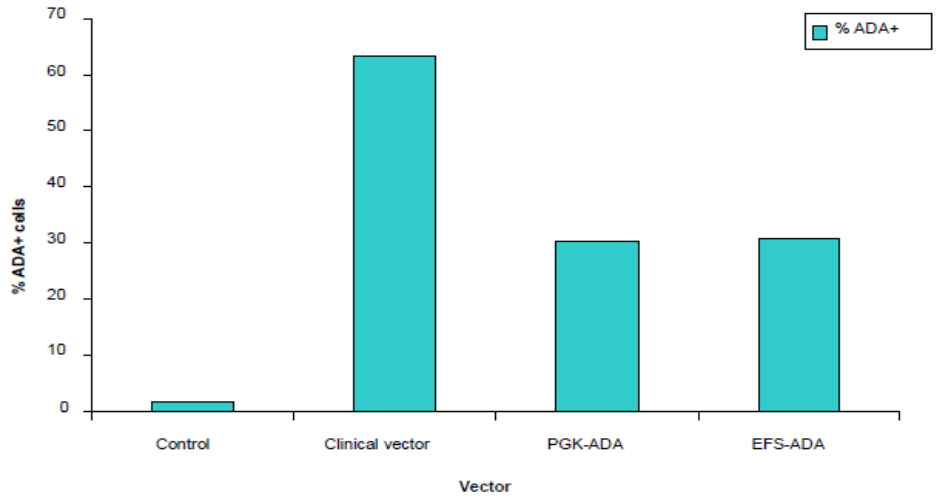


Figure 10: *CD34-ve cells from an ADA deficient patient were taken and transduced with the clinical grade gammaretroviral vector, pCCL EF1aS-ADA LLV vector or a pCCL PGK ADA LV vector and grown on a murine stromal layer expressing Notch-delta 1ligand. Following T cell differentiation the percentage of cells expressing ADA is shown.*

Studies of these lentiviral vectors were also performed using human CD34+ cells isolated from normal umbilical cord blood by collaborators at UCLA. The CD34+ cells were transduced using the culture conditions to be used for the proposed clinical trial, testing a range of vector concentrations (10^6, 10^7 and 10^8 TU/ml). The transduced CD34+ cells were grown in culture for two weeks after transduction, and then cell samples assayed to quantify the vector copies/cell, using qPCR and the ADA enzymatic activity, using a colorimetric biochemical assay. Expressed ADA enzymatic activity per vector copy was then calculated. These studies showed that transduction, as measured by vector copy per cell, is directly related to the concentration of vector during transduction, with 10^7 TU/ml leading to approximately 2 copies/cell. Expression of the transferred ADA cDNA was at a similar level by a lentiviral vector with an internal viral LTR (pCSO-re-MCU3-hADA), the EF1αS-ADA lentiviral vector to be used in this clinical trial and a gammaretroviral vector.

These in vitro studies demonstrate that in both cell lines and in primary progenitor cells, the EF1αS-ADA lentiviral vector is able to transduce cells at efficiencies comparable to gammaretroviral vectors used successfully in current gene therapy studies and is also able to express the ADA cDNA leading to ADA activity at similar levels to that shown by gammaretroviral constructs.

3.4.3 Transduction of CD34+ cells and expression of ADA *in vivo*

In further experiments, we have transduced CD34+ cells selected from ADA-SCID patient bone marrow with either the EF1αS-ADA lentiviral vector (LV.SIN.EFS.hADA), the clinical SFFV LTR based clinical vector used in the current GTAC 073 trial (SFada) or a vector expressing GFP only (LV.SIN.EFS.eGFP) and engrafted transduced cells into 8-10 week old irradiated immunodeficient mice (NSG (NOD/SCID/gamma $c^{-/-}$) mice) (Fig 11). Six mice were used in each group although one of the SFada cohort died. In both bone marrow (Fig 11a) and the spleen (Fig 11b) of recipient NSG mice, cells transduced with either the LV.SIN.EFS.hADA or SFada construct engrafted at similar levels whereas no evidence of CD45+ cells was seen in mice receiving cells transduced with a GFP containing vector. Development of CD19 B cells or CD3 T cells in the spleen was similar between the two constructs. B cell differentiation was the most abundant cell type seen in the bone marrow or spleen and formed the vast majority of engrafted human CD45+ cells.

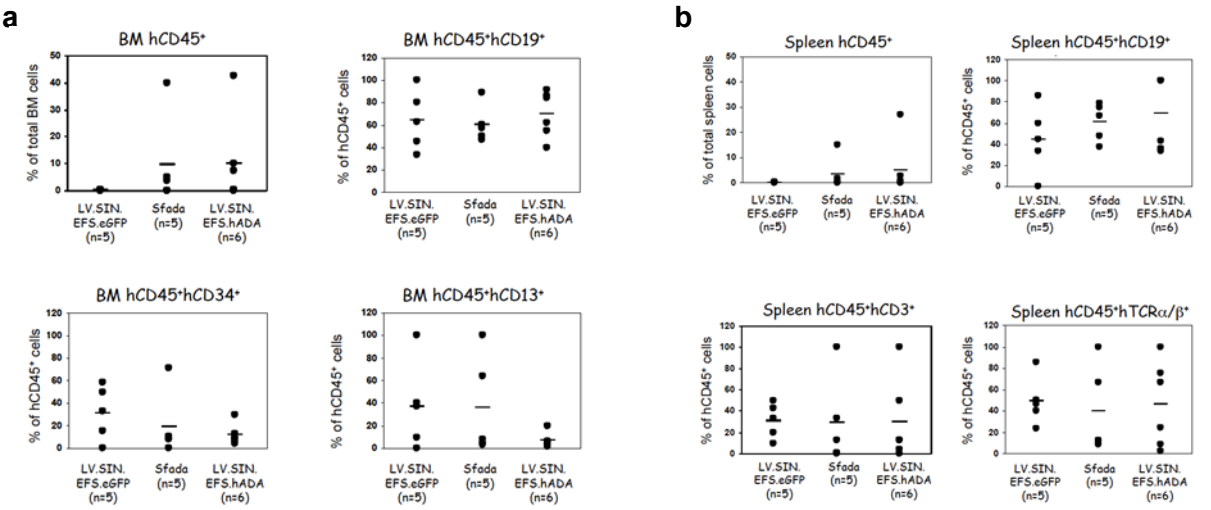


Figure 11: *Transduction of ADA deficient CD34+ bone marrow with the LV.SIN.EFS.eGFP, SFada and LV.SIN.EFS.hADA vectors and engraftment into immunodeficient NOD/SCID/γc$^{-/-}$ murine recipients. a) levels of bone marrow engraftment of hCD45+ cells, CD34+ cells, CD19+ B and CD13+ myeloid cells, b) levels of splenic engraftment of hCD45+ cells, CD19+ B cells and CD3+ T cells and TCRα/β.*

These studies show that following transduction with the EF1αS-ADA lentiviral vector, ADA deficient CD34+ cells are able to engraft and differentiate in a human xenograft model at levels that are similar to that seen following transduction with the current SFada vector.

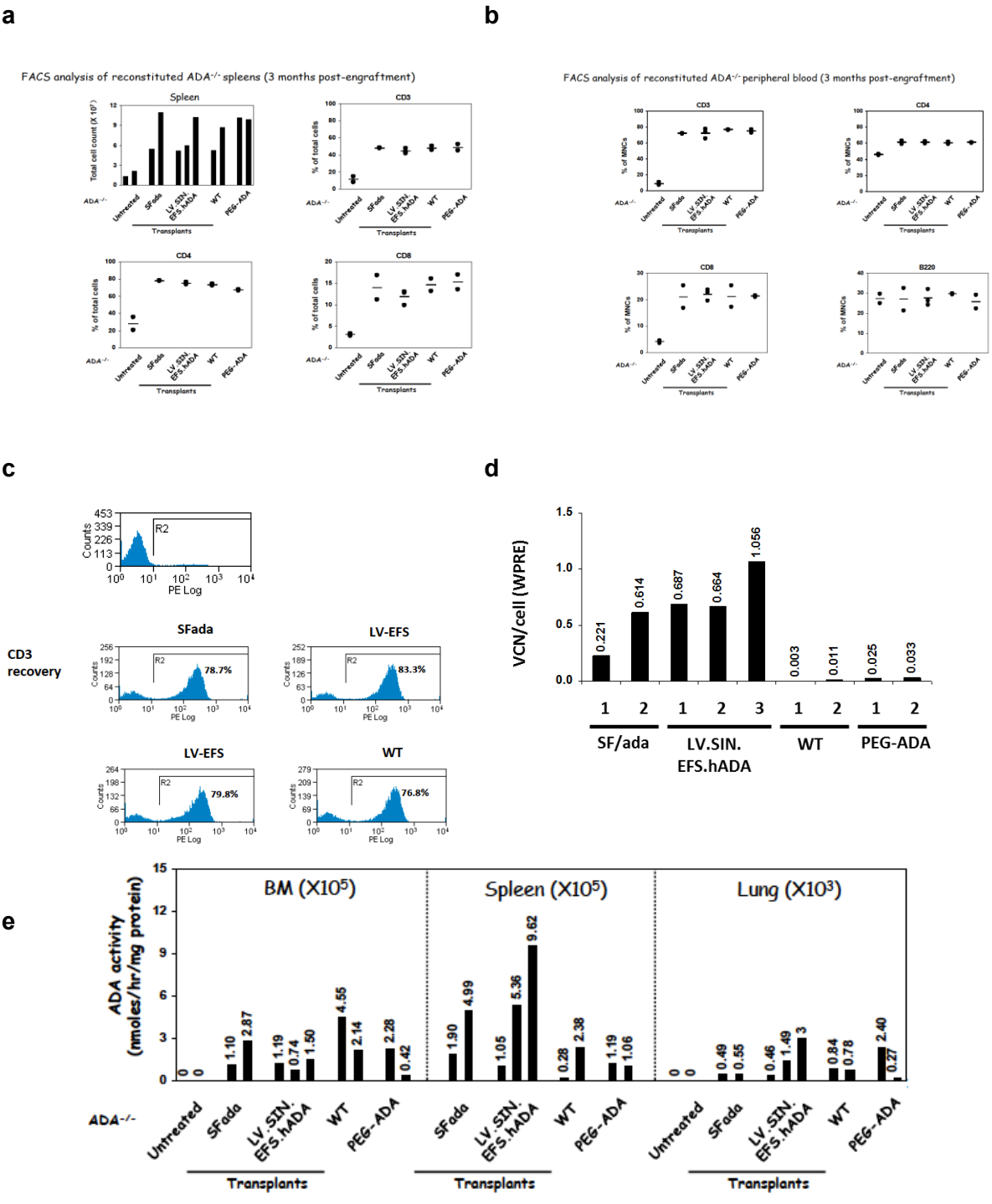
3.4.4 SIN lentiviral vector promotes immune recovery in a murine model of ADA deficiency

Early models of ADA deficient mice were limited by embryonic or peri-natal lethality as a result of severe hepatocellular and pulmonary damage. A two stage transgenic strategy in which ADA expression is restricted to trophoblasts rescues ADA null mice from pre- and peri-natal lethality and allows post natal study of mice lacking ADA expression. In this model, many features of the human disease are accurately represented. Mice show abnormalities of thymic architecture with a significant block in thymocyte development at the CD4-CD8- double negative stage. In vitro analysis of thymocytes from these mice also demonstrates defects in later stages of thymocyte development due to the toxic effects of dATP and deoxyadenosine accumulation. In the periphery, there is a profound lymphopaenia with a severe T, B and NK cell deficiency that is seen in humans together with abnormalities in splenic lymphoid architecture and a paucity of splenic T cell populations. Murine ADA deficiency also manifests a number of non-immunological abnormalities including lung, renal, costochondral and neurological changes. Pulmonary problems are present in mice from ~postnatal day 12 and are associated with significant lung inflammatory changes, eventually leading to the death of mice by postnatal day 22 from pulmonary insufficiency, unless early treatment is initiated. Mice die by three weeks of age but can survive in response to enzyme replacement therapy with PEG-ADA (see below). The immunological and systemic defects found in this murine model resemble closely many features of the human disease and this model is therefore an ideal system in which to test the ability of new ADA vectors to correct the disease phenotype.

The ADA-/- mouse colony was purchased and established at the ICH mouse facility. Murine lin-ve cells were either transduced using standard protocols with 1) the LV.SIN.EFS.hADA (n=3) or 2) SFada (n=2). Cells were returned to lethally irradiated recipients. Mice were analysed after 12 weeks and compared with ADA-/- mice, mice that had undergone wild type transplant experiments (WT) or mice that had been treated with enzyme replacement therapy alone. Mice reconstituted with the LV.SIN.EFS.hADA vector have increased numbers of total cells in the spleen in comparison to ADA-/- mice (Fig 12a). The recovery of total cell numbers and of specific lymphocyte subpopulations including CD3, CD4 and CD8 T cells, are similar to that seen in mice reconstituted with SFada vector transduced cells and mice treated with wild type cells or PEG-ADA replacement therapy. Similarly analysis of peripheral blood (Fig 12b) shows very low % of T cells in the mononuclear fraction of ADA-/- mice whereas reconstituted mice with both vectors, WT cells or PEG-ADA treatment all show a marked increase in T cell recovery. B cell reconstitution is again similar between the different treatment groups. A representation of the equivalence in T cell reconstitution is shown (Fig 12c). Importantly, the vector copy number in the peripheral blood of mice transduced with either the

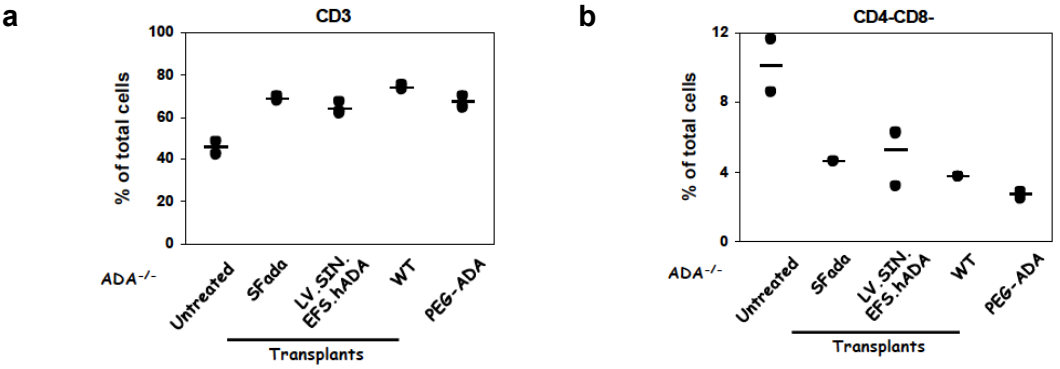
LV-EFS or SFada vector were similar suggesting that even with similar levels of virus transduction, equivalent levels of T cell reconstitution can be achieved (Fig 12d). ADA enzymatic activity was similar in both the marrow and spleen of mice transduced with LV-EFS vector in comparison to mice treated with SFada vector, WT transplants or PEG-ADA and were greatly increased from levels seen in ADA-/- mice. Similar results were also seen in a non-immune organ such as the lung (Fig 12e).

Figure 12



In the thymus, the major abnormalities in the ADA-/- mice are a block in thymocyte development with an accumulation of CD4-CD8- thymocytes. Reconstituted mice with either vector or with WT or PEG-ADA treatment show similar levels of reduction of this population (fig 13).

Figure 13



Importantly, all the reconstitution experiments in ADA-/- mice were undertaken with clinical grade LV.SIN.EFS.hADA vector demonstrating that the clinical grade vector has equivalent immune reconstitution potential to the current SFada clinical vector that has been used successfully in clinical trials.

3.4.5 Genotoxicity of the EF1αS-ADA lentiviral vector

It is clear from pre-clinical and clinical trial data that there is a need to move away from the use of gammaretroviral vectors which have the potential for insertional mutagenesis to the use of vector designs which have reduced transformation potential. The EF1αS-ADA lentiviral vector design is likely to be significantly less mutagenic than current gammaretroviral vectors for two major reasons:

- The use of the self-inactivating (SIN) lentiviral vector backbone
- The use of the internal EF1αS promoter for transgene transcription rather than the viral LTR

3.4.5.1 SIN Lentiviral vector constructs are safer than LTR intact gammaretroviral constructs

The risk of insertional mutagenesis from a SIN lentiviral vector design in general is low as determined in both *in vitro* and *in vivo* systems. One *in vitro* assay has now been developed and tested by a number of groups and is gaining recognition as the most reliable test of the transformation potential of different viral constructs. The *in vitro* immortalisation assay (IVIM)

(Modlich et al., 2006; Modlich et al., 2009) detects clonal dominance of insertional mutants which expand in initially polyclonal cultures of primary murine haematopoietic cells within two weeks after gene transfer and acquire serial replating ability, the extent of which provides a measure of clonal fitness. When different vector designs were tested in this assay the SIN lentiviral vector design shows significantly less transformation potential than current LTR intact gammaretroviral designs (Modlich et al., 2006; Modlich et al., 2009).

To test the oncogenic capacity of SIN-lentiviral vectors *in vivo*, Montini et al., utilized the tumor-prone Cdkn2a-/- mouse model (Montini et al., 2006). Haematopoietic stem cells from these mice were isolated and transduced in similar conditions with SIN LV or LTR intact gammaretroviral constructs expressing reporter genes. A vector with a similar backbone as the proposed ADA vector, but expressing a neutral eGFP reporter gene, was tested in this system and no genotoxicity was found even upon integration of ~6 copies of vector per cell. In contrast, LTR-active gammaretroviral vectors were measurably genotoxic, suggesting that the intrinsic genotoxicity of the pRRL LV (the backbone used in this study) is low.

3.4.5.2 The EF1αS promoter is safer than the viral LTR

Using the IVIM assay, the EF1αS promoter was compared under equivalent conditions to the SFFV LTR in the context of a SIN gammaretroviral vector (Zychlinski et al., 2008). In this assay a SIN vector with the internal EF1αS promoter was significantly (P<0.01) less mutagenic than a SIN vector with the internal SFFV promoter. Under conditions that resulted in death of mock-transduced cells, the SIN vector containing an internal EF1αS promoter was unable to induce sustained growth of mutants, even when the average copy number was >40. In contrast, transformed clones were always observed with the SIN vector containing the internal SFFV promoter, and with this vector, an average copy number of ~2 was sufficient to induce transformation. This indicates that the transactivating potential of the SIN vector with the EF1αS promoter is at least substantially diminished. Of note, SIN vectors containing 'insulator' elements flanking the internal SFFV promoter-driven cassette showed a significantly greater degree of transforming activity that the SIN vector with the EF1αS promoter (4/4 experiments; P< 0.01). Together these studies indicate that the transactivating potential of gammaretroviral vectors are substantially reduced by SIN configurations (Modlich et al, 2006), and further reduced by the utilization of EF1αS internal regulatory sequences.

Further *in vivo* studies have also established the low transformation capacity of SIN vectors containing the EF1αS promoter. These experiments were conducted as safety studies for a trial of gene therapy for SCID-X1 (GTAC 116) and are relevant here because of the use of the same EF1αS promoter. SIN gammaretroviral constructs containing the EF1αS promoter and driving expression of the IL2RG or GFP reporter transgene were evaluated in murine

transplant experiments in wild type C57/BL6 experiments. Briefly lin-ve cells from donor mice were isolated and transduced under standard conditions with vectors containing EF1αs-IL2RG or EF1αS-GFP or media alone (mock controls). Twenty mice were transplanted for each experimental group and were observed for a period of 10-12 months. There were 17 evaluable mice in the IL2RG group, 17 evaluable mice in the EGFP group and 18 evaluable mice in the mock group. On average, there was 60-80% engraftment in each group. The average vector copy number in peripheral blood mononuclear cells, determined by real-time PCR at 2 months post-transplant was 1.25 copies/cell for the *IL2RG* test group and 2.2 copies/cell for the EGFP control group. Two host cell derived malignancies were noted in the test *IL2RG* group. One vector-negative thymoma and an ovarian tumor were noted in the GFP vector group. Host cell derived malignancies are not unexpected in this mouse model, and the frequency is not higher than previously reported values by our group. There were no cases of vector positive donor cell derived leukemias in the *IL2RG* test group. There was one vector positive, donor cell derived T cell lymphoma noted in a C57BL/6J recipient mouse which received the EGFP control vector. These data suggest that in the context of a SIN configuration and coupled with the IL2RG gene, the EF1αS promoter has not been associated with demonstrable tumorigenesis in wild type murine transplant experiments.

3.4.5.3 Data from other similar LV constructs

Some surrogate information can also be gained from analysis of another SIN LV vector. The EF1αS-ADA lentiviral vector backbone for this study is the same as that approved in a trial of lentiviral gene therapy for Wiskott-Aldrich (WAS) syndrome (GTAC 146). The promoter and transgene for the WAS vector have been removed and replaced with the EF1αS-ADA cassette but the other structural elements of the virus remain the same. The WAS vector has been tested *in vivo* in was^{null} recipients with a follow up of 12 months. was^{null} mice transplanted with cells transduced with the WAS vector at high or low copy number had no difference in survival or tumour formation in comparison to was^{null} mice transplanted with untransduced cells from WT or was^{null} mice. Tumours seen were either of non-haematopoietic origin or were derived from host cells suggesting that tumour formation was not driven by vector integration. A similar frequency of tumours was seen in control cohorts (Marangoni et al., 2009). These data suggest that this WAS SIN LV vector construct has no significantly increased risk of tumour formation compared to standard transplant experiments. The EF1αS-ADA lentiviral vector is therefore likely to show similar safety to the above construct.

3.4.5.4 IVIM assay to test genotoxic potential of the EF1αS-ADA lentiviral vector

We have set up the IVIM assay at our laboratory and tested the transformation ability of SFFV LTR driven vectors expressing GFP (SF91.GFP) or dsRED (SF91,dsRED) reporter genes, the current SFFV LTR clinical gammaretroviral vector used in GTAC 073 (SFada/W) and the

EF1αS-ADA lentiviral vector. In consecutive experiments, cells transduced with the LTR driven gammaretroviral vectors give rise to transformed clones with replating ability whereas no transformed clones above background (or mock transduced) levels were detectable in cells transduced with the EF1αS-ADA lentiviral vector (Table 2) despite equivalent or higher vector copy numbers. In this assay, the genotoxic potential of the EF1αS-ADA lentiviral vector is considered to be low and significantly lower than the transformation potential of the vector currently used in clinical trials.

Table 2: IVIM data

Vector	Replating clone number (100 cells/well)	Normalized Replating clone number (100 cells/well)	VCN per cell	Replating frequency	Replating index
Mock	9 11 5	0 0 0	0.007 0.008 0.000	0 0 0	0 0 0
SFada/W	**17** **17** **17**	**8** **6** **7**	**4.9** **4.7** **0.9**	**0.00087** **0.00065** **0.00076**	**0.00018** **0.00014** **0.00084**
SF91. **dsRED**	**27** **33** **16**	**18** **22** **4**	**5.4** **4.8** **1.4**	**0.00208** **0.00260** **0.00043**	**0.00039** **0.00054** **0.00031**
SF91.GFP	**65** **24** **34**	**56** **11** **18**	**1.3** **1.4** **0.2**	**0.00875** **0.00122** **0.00208**	**0.00694** **0.00089** **0.01226**
LV.SIN. **EFS.hADA**	**3** **6** **3**	**0** **0** **0**	**5.3** **4.2** **2.7**	**0** **0** **0**	**0** **0** **0**

In order to test objectively whether clones derived following transduction were viable and had significant cytokine independent proliferative capacity, clones were analysed using the WST-1 assay. The assay is based on the reduction of WST-1 by viable cells which produces a soluble formazan salt. The procedure involves initial incubation of cells with the WST-1 reagent, followed by spectrophotometric assay of coloured product.

In two experiments independent experiments, any clones derived from cells transduced with the SFada/W, SF91.GFP, EF1αS-ADA LV vectors or mock transduced cells were studied further in the WST-1 assay. As seen in Fig 12, in both experiments, clones derived following SFada/W or SF91.GFP transduction showed levels of proliferation that were far higher than the baseline proliferation seen in mock transduced clones. In contrast any clones derived following EF1αS-ADA LV transduction showed negligible proliferative capacity and were no greater than levels of proliferation seen in mock transduced cells.

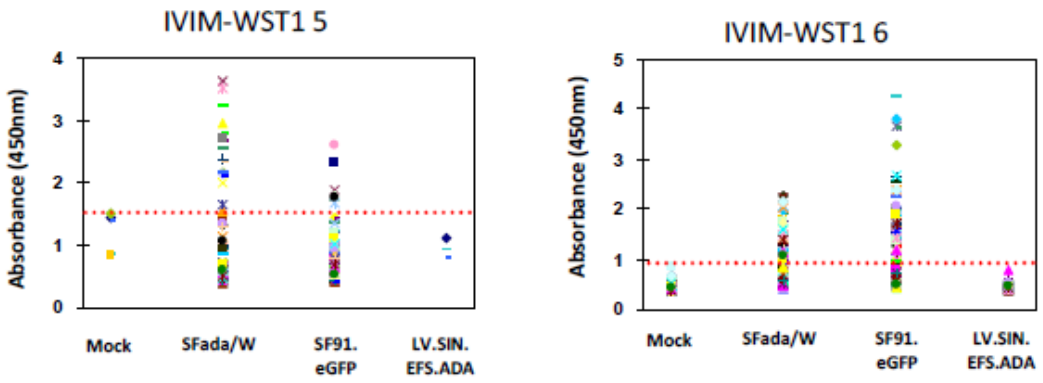


Figure 12: *Proliferative capacity of clones generated following transduction were tested in a WST-1 assay. Clones from SFada/W and SF91.GFP transduced cells show high levels of proliferation whereas clones from EF1αS-ADA LV transduced cells have levels of proliferation no higher than that seen in mock transduced cell clones.*

3.4.6 Clinical grade vector is able to effectively transduce human ADA-/- CD34+ stem cells

Clinical grade LV.SIN.EFS.hADA vector was characterised for its ability to transduce CD34+ cells from ADA-/- patients. Using clinical grade reagents and a clinical protocol (see section 7.6.4) involving 18 hrs prestimulation and followed by 1 round of lentiviral transduction for 24hrs, either 1 x 10^8 (~MOI 100) or 5 x 10^7 (~MOI 50) viral genomes were added to ~1 x 10^6 CD34+ cells. As shown in table 3a, these clinically applicable transduction conditions resulted in effective CD34+ cell transduction with viral copy numbers of ~1–1.3 copies/cell. In addition there was preservation of CD34+ integrity with little change in the percentage of stem cells through the culture period form D0–D3 (table 3b).

Table 3

a

Test	Result	Specification
Total cell dose	1x10^8: 1.78 x10^6 5x10^7: 1.78 x10^6	N/A
Gram stain	N.D. scale up in R&D lab	No organisms seen
Culture D1	N.D. scale up in R&D lab	No organisms seen
Transduction efficiency (mean copies/cell)	1x10^8: 1.34 copy/cell 5x10^7: 0.96 copy/cell	>0.3 copies/cell

b

Day	Culture Conditions	Total CD34+	True stem cells
D0	Untransduced	94%	55%
D3	Untransduced	89%	51%
D3	1 x 10*8 Ig/ml	92%	58%
D3	5 x 10*7 Ig/ml	91%	58%

These data show that using a clinically applicable protocol, the clinical grade vector generated for this study is able to show effective transduction of CD34+ cells from ADA deficient patients at levels that are acceptable for clinical use and maintain CD34+ cell integrity.

3.5 Continuation of PEG-ADA for 1 month after infusion of gene modified cells

There has been considerable debate as whether PEG-ADA either blunts or improves the survival of gene modified cells. In previous clinical trials, PEG-ADA has been withdrawn prior to the infusion of gene-modified cells.

Two lines of evidence now suggest that continuation of PEG-ADA may be of benefit:

a) In a recently published murine study (Carbonaro et al., 2012) demonstrated that the continued use of PEG-ADA for 1 month after gene modified cell infusion did not diminish immune reconstitution but in fact allowed better thymic development possibly due to its protective effect on thymic epithelium.
b) In two ADA-patients treated off trial on a compassionate basis, we also continued PEG-ADA for 1 month after gene modified cell infusion. In both cases PEG-ADA was continued because of the clinical state of the patients and the concern that removal of enzyme may have adverse clinical consequences. In both cases we have seen engraftment of gene modified cells and emergence of naïve T cell cells at 6 and 7 months post treatment.

From this we would suggest that 1) continued PEG-ADA usage is not detrimental to gene modified cell engraftment, 2) it may promote improved thymic T cell development and 3) its continued use is clinically safer for patients.

For these reasons we intend to continue PEG-ADA for 1 month after infusion of gene modified cells.

3.6 Busulfan dosage

The busulfan dosage to be used in this study differs from previous studies. In this study, weight adjusted iv busulfan dosing will be given as detailed below:

Body-weight	mg/kg/day
3 to 15kg	5.1
15 to 25kg	4.9
25 to 50kg	4.1
50 to 75kg	3.3
75 to 100kg	2.7

This allows us to use the maximum daily dosage of iv busulfan and is based on European Society for Blood and Marrow Transplantation (EBMT) guidelines for Busulfan dosing in children and a recent publication (Bartelink et al., 2012).

The reason for the change is to achieve the correct balance between level of conditioning and safety. A higher busulfan dose will allow a greater degree of myeloablation and therefore greater engraftment of gene modified cells. This dosing scheme has recently been used in two ADA-SCID patients treated off-trial on a compassionate basis and in both cases the dose (both received 5.1mg/kg) was well tolerated with no toxicities.

3.7 Summary and rationale for use of the EF1αS-ADA lentiviral vector for gene therapy

- Gammaretroviral vector mediated gene therapy for ADA has shown impressive efficacy and safety long term in 3 international studies thus far.
- Safety has been excellent with 100% survival and no serious adverse events but concerns regarding the insertional profile of gammaretroviral vectors need to be addressed.
- Lentiviral vectors encoding the EF1αS internal promoter driving human ADA show efficacy of transduction and expression that is comparable to gammaretroviral constructs *in vitro.*
- Lentiviral vectors have an insertional profile that differs from gammaretroviral constructs and show decreased potential for transformation *in vitro* and in *in vivo* tumour prone models.
- The LV.SIN.EFS.hADA vector shows decreased transformation potential in an *in vitro* model of stem cell transformation and in *in vivo* transplantation experiments the EF1αS-promoter in a SIN configuration does not show transformation potential.

- The LV.SIN.EFS.hADA vector shows effective reconstitution of T cell numbers and ADA activity in a murine model of ADA-/- deficiency and is comparable to the current clinical grade SFada gammaretroviral vector.
- The LV.SIN.EFS.hADA vector shows effective transduction of ADA-/- CD34+ cells and leads to successful engraftment in NSG xenograft experiments and is comparable to the current clinical grade SFada gammaretroviral vector.
- A clinically applicable protocol for transduction of ADA-/- CD34+ cells leads to levels of stem cell transduction that are applicable for clinical use.
- Lentiviral vector mediated gene therapy for ADA-SCID has potential for high levels of efficacy and improved safety.

4 Trial Design

This is a historical controlled, non-randomised phase I/II clinical trial to assess the safety and efficacy of autologous transplantation of CD34+ cells from ADA-deficient SCID infants following human ADA cDNA transfer by the EF1αS-ADA lentiviral vector with comparison of outcomes to historical control group. Subjects will receive the infusion of their autologous, transduced cells following marrow cytoreduction with busulfan.

4.1 Patients treated with gene therapy (on study)

- Patients will be selected on the basis of inclusion and exclusion criteria as detailed below
- A back-up harvest will be retained in case of failure of reconstitution following conditioning and re-infusion of transduced cells
- For the transduction protocol (see appendix 3 for schematics), CD34+ cells will be purified from G-CSF mobilised Peripheral Blood Stem Cell (PBSCs) recovered by leukapheresis (Plerixafor may be added to enhance mobilization), whenever possible or from bone marrow harvested under general anaesthetic. Back-up cells of $3x10^6$ $CD34^+$/kg will be stored from a single procedure if sufficient cell counts available (and if not previously stored). The remaining $CD34^+$ cells will be taken forward for transduction. If $<3x10^6$ $CD34^+$ cells/kg are harvested from the back-up, the patient will be withdrawn from the study

- Patients in whom of <1 x 10^6 CD34+ cells/kg are available for transduction will not proceed to conditioning, will be withdrawn from the study and will be offered a repeat harvest in approximately. 3 months
- Patient conditioning will be initiated immediately after bone marrow or PBSC collection (busulfan iv. weight adjusted dose)
- After purification, CD34+ cells will be immediately transduced with the lentiviral vector as detailed below, then evaluated for sterility, cell count, and viability, and immediately infused into patients
- Several other tests for safety including testing for RCLs will be performed on re-infused cells and results will be recorded retrospectively
- If less than 0.5 x 10^6 viable transduced CD34+ cells/kg are returned to the patient, then back-up bone marrow/PBSCs will be returned and patients will be withdrawn from the study
- PEG-ADA will be withdrawn one month (+/- 6 days) post-infusion
- If there is no bone marrow recovery by 6 weeks (ANC<0.5 x 10^9/L or platelets < 20 x 10^9/L) the back-up harvest will be re-infused
- Patients will be followed up on study at 1 month, 6 weeks, 3, 6, 9, 12, 18, 24, 30 and 36 months post gene therapy. Visit windows will be +/- 5 days for visits at 1 month, 6 weeks and +/- 2 weeks for visits at 3, 6, 9, 12, 18, 24, 30 and 36 months post gene therapy If after 180 days there is no evidence of the transgene in PBMCs by qPCR or there is no evidence of T cell recovery, then patients will re-start PEG-ADA and will be withdrawn from the study

PEG-ADA may be restarted at PIs discretion prior to this time point on clinical grounds e.g infective problems or delayed immune reconstitution and at this stage will be withdrawn from the study.

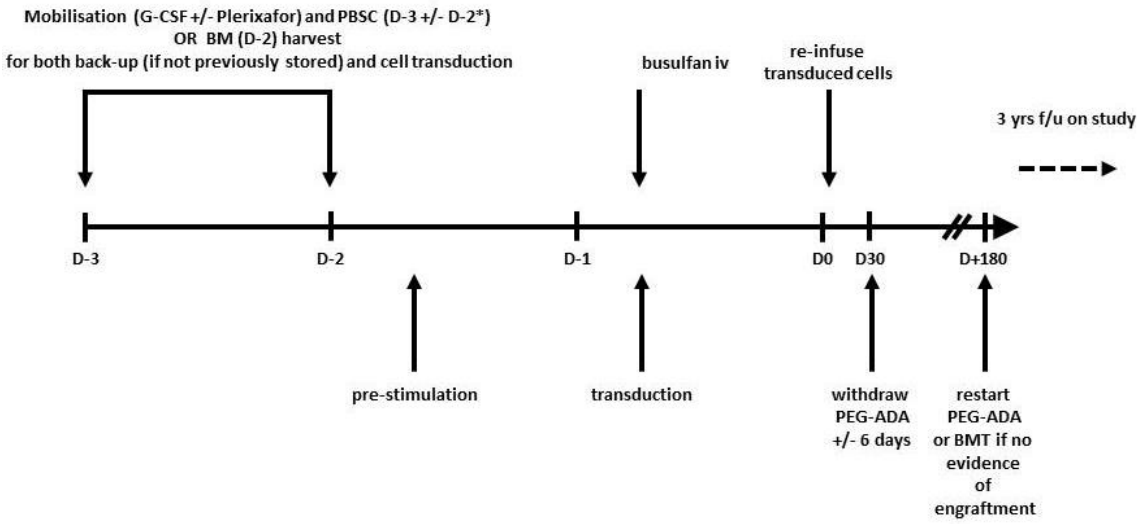
4.2 Patients treated with gene therapy (off study): CUP group

In addition to the patient treated on trial, another sub-group of patients treated on compassionate basis (CUP) with the same gene therapy under GOSH License, that followed the procedure of this protocol are included within this protocol and will be part of the analysis (see section 8.9). Details about this group of patient can be found in Appendix 9.

4.3 Historical control group: HSCT group

Historical HSCT data from a database of ADA-SCID patients treated with allogeneic HSCT from GOSH will be collected as comparator group and considered for the analyses (see section 8.9). Details about this group of patient can be found in Appendix 9.

Trial summary



* If enough cells from first apheresis (D-3) is collected, no need to perform second apheresis (D-2)

4.2 Trial objectives

4.2.1 Primary objectives

The primary objective is to assess the safety and efficacy of EFS-ADA LV mediated gene therapy, the Investigational Medicine Product (IMP) for treatment of ADA-SCID patients. This will be achieved via the following specific objectives:

Safety

1. To assess clinical, haematological and immunological progress of patients
2. To assess vector integration sites and clonal proliferation. This analysis will be performed at a specialised laboratory in Germany and the data will be reviewed and interpreted by the PI and the study team

Efficacy

1. To assess overall survival (OS) at 1 year for patients treated with IMP
2. To assess event free survival (EFS, defined below in Section 5.2.2) at 1 year for patients treated with IMP
    - To compare OS and EFS at 1 year between patients treated with IMP and patients treated with allogeneic HSCT
3. To assess at each visit engraftment success and resulting immunological and metabolic effects in IMP treated patients using:

a. Vector copy numbers in peripheral blood leukocytes

b. Cellular and humoral immune system recovery

c. ADA activity and reduction in dATP in peripheral blood cells

4.2.2 Secondary objectives

The secondary objectives are to:

- Compare survival and event free survival at 2 and 3 years between patients treated with IMP and patients treated with allogeneic HSCT
- Determine the percentage of IMP treated patients requiring immunoglobulin replacement therapy at all evaluation points from 18 months onwards
- Compare frequency of infections, and growth of pathogenic microorganisms over 3 years
- Evaluate the longitudinal clinical effect in terms of improved immunity
- Evaluate tolerability of conditioning regimen. These data will be reviewed and interpreted by the PI and the study team
- Evaluate feasibility of the transduction procedure. These data will be reviewed and interpreted by the PI and the study team

4.3 Study endpoints

4.3.1 Primary endpoints

- To assess survival at 1 year for patients treated with IMP.
- To assess event free survival at 1 year for patients treated with IMP.
    - To compare survival and event free survival at 1 year for patients treated with IMP compared to patients treated with allogeneic HSCT.
- Success of engraftment of transduced cells as assessed by vector copy number in the cells at each study visit.
- Reconstitution of cell mediated and humoral immunity as assessed by evidence of changes in T cell number, function and circulating immunoglobulin levels at each study visit.
- Correction of metabolic abnormalities as assessed by dATP and evidence of ADA activity in erythrocytes at each study visit compared to published data from HSCT treated patients

The following will be analysed at a specialist laboratory in Germany and the results reviewed and interpreted by the PI and the study team.

- Analysis of frequency of vector integration into known proto oncogenes
- Analysis of frequency of clonal expansion associated with vector integration near proto oncogene

4.3.2 Secondary endpoints

1) Survival and Event free survival at 2 years and 3 years

2) Reduction in frequency of infections (evaluated at 1, 2 and 3 years after treatment based on clinical history and examination)

3) Long term immune reconstitution as assessed by sustained improvement in thymic function assessed by TRECS after 2 years post-gene therapy

4) Serum immunoglobulin levels for IgA, IgG and IgM and patients requiring immunoglobulin replacement therapy (recorded dichotomously).

5) Feasibility of the transduction procedure assessed by availability of greater than 0.5 x 10^6 CD34+ cells/kg after transduction; undetectable RCL (determined retrospectively); and CD45+ cell viability after transduction equal to or greater than 50%, in accordance with the final product release criteria. These data will be reviewed and interpreted by the PI and the study team.

4.3.3 Additional Endpoints

1) T Cell V Beta Panel

2) Phytohaemagglutinin (PHA) stimulation Result

3) CD3 Stimulation

4) Integration Analysis Result

5) Bone Marrow Aspirate (if performed)

Morphology (Normal/ Abnormal).

Integration (Normal/ Abnormal).

Vector copy number in BM-MNC.

6) Tetanus Vaccination Response

5 Patient selection and recruitment

5.1 Patients treated with gene therapy (on trial)

Up to 10 treated patients will be recruited from the Immunology Unit at Great Ormond Street Hospital. Referrals for enrolment in the study will be accepted from centres worldwide. Individuals will be selected for inclusion on the basis of the following defined criteria:

5.1.1 Inclusion criteria

1. Diagnosis of ADA-SCID confirmed by DNA sequencing OR by confirmed absence of <3% of ADA enzymatic activity in peripheral blood or (for neonates) in umbilical cord blood erythrocytes and/or leucocytes or in cultured foetal cells derived from either chorionic villus biopsy or amniocentesis, prior to institution of PEG-ADA replacement therapy

2. Patients who lack a fully HLA-matched family donor

3. Patients (male or female) < 5years of age OR

   Patients (male or female) ≥ 5years to 15rs of age who have preserved thymic function as evidenced by presence of >10 % naïve T cells (CD4+45RA+27+ cells)

4. Parental/guardian signed informed consent

5.1.2 Exclusion criteria

1. Cytogenetic abnormalities on peripheral blood

2. Evidence of active malignant disease

3. Known sensitivity to busulfan

4. If applicable, confirmed pregnancy (to be tested in patients above 12 years old)

5.1.3 Justification for enrolment criteria

In this study gene therapy will be offered in preference to a matched unrelated donor HSCT. To date over 30 patients have been treated with a gene therapy protocol involving cessation of PEG-ADA and non-myeloablative conditioning. All patients have survived the treatment and ~70% have been able to stop PEG-ADA. Furthermore, patients who have failed gene therapy have been able to restart PEG-ADA without any compromise to clinical or immunological well-being. In contrast, MUD transplants have a 67% survival (n=15). For these reasons, gene therapy offers a safer initial alternative and carries a high rate of success. If gene therapy fails,

patients can restart PEG-ADA or proceed to a transplant without compromising the success of the latter procedure.

Older patients have been shown to have a poorer response to gene therapy for ADA-SCID and also for SCID-X1 (Thrasher et al., 2005; Aiuti et al., 2009). This most probably relates to the low level of residual thymic function seen in older SCID patients as a result of diminished population of thymic tissue and also in the case of ADA-SCID ongoing thymic toxicity.

This also suggests that in the case of older patients (>5yrs of age), gene therapy will be of greatest benefit in those who have preserved thymic function as evidenced by the presence of > 10 % naïve t cells (CD4+45RA+27+ cells) and TRECs.

5.2 Patients treated with gene therapy (off trial): CUP group

A group of patients were treated under the special compassionate group program (GOSH special license) either because the study was not yet open and patients needed urgent treatment, or because they were outside of the inclusion/exclusion criteria or received followed a different process (ie, received the Imp in two infusions). Patients followed however the same protocol steps and study visits. For this reason, CUP patients are included in the analysis, as described in the Statistical Analysis Plan (SAP)

5.3 Historical Control group: HSCT group

A group of historical retrospective data has been collected from GOSH ADA-SCID patients treated with HSCT from year 2000. This group will be use as comparator data vs patients treated with gene therapy, to evaluate survival and event free survival, as described in the SAP. Below are the inclusion criteria for this population

Inclusion criteria

1. Diagnosis of ADA-SCID confirmed by DNA sequencing OR by confirmed absence of <3% of ADA enzymatic activity in peripheral blood or (for neonates) in umbilical cord blood erythrocytes and/or leucocytes or in cultured foetal cells derived from either chorionic villus biopsy or amniocentesis, prior to institution of PEG-ADA replacement therapy

2. Patients (male or female) between 0-18 years at time of treatment

3. Patient treated with allogeneic haematopoietic stem cell transplantation since 2000

5.4 Concomitant medication

Entry into this study will not affect concomitant treatments for ADA-deficiency, such as prophylactic antibiotics or intravenous gamma globulin therapy. However, the patient will be

withdrawn from cotrimoxazole prophylaxis at least day -1 prior to IMP infusion. Cotrimoxazole will be restarted when ANC > 1 x 10^9/l on 3 consecutive days after reinfusion of transduced cells. Aerosolised or intravenous pentamidine will be used as prophylaxis for *Pneumocystis jirovecii* in the intervening time period.

PEG-ADA will be continued until one month (+/- 6 days) post-infusion of gene-modified cells. PEG-ADA will be restarted if i) the child is withdrawn from the study ii) if after 180 days there is no evidence of gene modified cell engraftment and/or failure of T cell recovery.

PEG-ADA may be restarted at PIs discretion prior to this time point on clinical grounds e.g infective problems or delayed T cell reconstitution.

6 Enrolment of subjects

Patients will be enrolled following diagnosis and referral to GOSH. ADA is an inherited disease and infants normally present in the first year of life. Patients less than 5 years of age will be enrolled and also those >5 years who show evidence of thymic function will also be eligible for enrolment. Informed consent will be obtained from parents/guardians and assent where appropriate.

The Chief/Principal investigator will discuss the study at length with the parent/guardian of a potential new subject. A printed information leaflet will be supplied and the parent/guardian given ample time to consider his/her decision. The parent/guardian will be encouraged to ask further questions about the study to the Investigator or the clinical nurse specialist. Interpreters will be used if English is not the native tongue. Patient Information Leaflet (PILs) will be translated into native language.

Should a parent/guardian decide that the patient will participate they will be invited to sign the study consent form.

Consent will take place prior to any study specific procedures.

Once a patient has been enrolled onto the study, the ADA-SCID pre-gene therapy patient protocol (see Appendix 4 for details) will be completed, reviewed, signed off by the investigator and distributed to all concerned parties.

7 Treatment protocol

7.1 Informed consent procedure

ADA deficiency is an inherited disease and therefore most patients with this inherited disease are diagnosed while they are still infants and are therefore incapable of giving informed consent. Therefore, it is expected that the majority of the informed consent will be obtained from parents/guardians by the Chief investigator or co-investigator.

The Chief investigator/co-investigator will discuss the study at length with the parent/guardian of a potential new subject, including an explanation of the aims, methods, anticipated benefits and potential risks of the study. The Chief investigator/co-investigator will explain that the patients are under no obligation to enter the trial and that they can withdraw at any time during the trial, without having to give a reason.

A patient information leaflet will be supplied and the parent/guardian will be given ample time to consider his/her decision. The parent/guardian will be encouraged to ask further questions about the study to the Investigator or the clinical nurse specialist.

Should a parent/guardian decide that the patient will participate they will be invited to sign the study consent form. A copy of the patient information leaflet and signed consent form will be provided to the patient to take home. A further copy will be retained in the patient's medical notes and the original will be filed in the Trial Master File.

Informed consent will be obtained before any trial-related procedures are performed.

7.2 Randomisation procedures

This section is not applicable as the trial is non-randomised.

7.3 Emergency unblinding

This section is not applicable as this is an open-label trial.

7.4 Screening assessments

The first part of the study will involve performing laboratory tests to determine whether the subject meets the inclusion criteria and does not have a contraindication which would exclude them from the trial.

7.5 Treatment procedures

7.5.1 Harvest of a back-up autologous graft

If a back-up has not previously been stored, haematopoietic stem cells will be collected from the patient as a back-up at the time of leukapheresis (on day -3) or bone marrow harvest (on day -2) (see section 7.5.2). A back-up of 3 x 10^6 CD34+/kg will be stored.

The total cell count required for both back-up (3 x 10^6 CD34+ cells/kg) and for transduction (1 x 10^6 CD34+ cells/kg) is 4 x 10^6 CD34+ cells/kg.

If insufficient cells are collected then the total collection will be stored as back-up, the patient will be withdrawn from the trial and a further collection will be attempted 2-3 months later in order to meet the criteria specified above.

The back-up cells serve as a salvage procedure, should there be a lack of haematopoietic recovery observed following the injection of genetically-manipulated cells (back-up graft).

The bone marrow or leukapheresis cells will be frozen and stored un-manipulated in liquid nitrogen vapours (-162^{0}C and -180^{0}C) to constitute the back-up graft. The back-up will be used in case the final cell dose is very low as specified or if no haematopoietic recovery is observed after 6 weeks, following the gene therapy treatment.

7.5.2 CD34+ cells for gene therapy

CD34+ cells for the gene therapy will be obtained preferably by leukapheresis (possibly augmented with Plerixafor) of G-CSF mobilised peripheral blood stem cells (PBSCs). If PBSC leukapheresis is not clinically appropriate or is contraindicated or where mobilisation fails, a bone marrow harvest will be performed. PBSCs are the preferred choice since we will obtain far higher numbers of CD34+ cells which in turn will allow gene modification of larger stem cell numbers prior to reinfusion into the patient. Autologous cells will be stored if cell count is >4 x 10^6 CD34+ cells/kg (back-up: 3 x 10^6 CD34+ cells/kg and cells for transduction: 1x10^6 CD34+ cells/kg).

For PBSC collection, recombinant human granulocyte colony stimulating factor (rhu G-CSF) will be administered by subcutaneous injection (usually in the abdominal region) for 5 to 6 successive days (dose 5-16 μg/kg per day) (possibly augmented with Plerixafor) prior to harvest. CD34+ cell counts will be monitored from day 5, and leukapheresis performed on days 5 and/or 6 providing that the CD34+ cell count is greater than 1 x 10^4/ml. If a bone marrow harvest is to be used as the source of CD34+ cells, bone marrow will be harvested from the patient under general anaesthesia from the posterior iliac crests on both sites by multiple punctures. The amount of marrow collected will be equivalent to 20 ml/kg of body weight. This

will give a total nucleated cell count of greater than 10^8cells/kg. This in turn should yield a CD34+ cell dose of greater than 10^6cells/kg after CD34+ cell selection.

Patients from whom <1 x 10^6 CD34+cells/kg are available, will be withdrawn from the study prior to conditioning. A repeat harvest will be considered after a period of 2-3 months and the patient will be re-enrolled onto the study.

Patients withdrawn from the study prior to administration of transduced CD34+ cells will resume normal clinical care. Efficacy and safety assessments will not be carried out from the point of withdrawal and data will not be recorded in the Case Report Forms (CRFs).

7.5.3 CD34+ cell purification

CD34+ cells will be separated by standard CliniMACs cell purification protocols which are in routine use as part of the bone marrow transplantation programme. Operating procedures are available on request. Purified cells will be immediately transferred to the gene therapy laboratory for transduction.

7.5.4 CD34+ cell culture and transduction

CD34+ cells will be purified, cultured and transduced in a dedicated clinical gene therapy laboratory at Great Ormond Street Hospital. CD34+ cells will be purified using standard protocols for stem cell transplantation (CliniMACs), and seeded into fresh closed cell culture bags or flasks in serum free medium (X-VIVO-20, Lonza) supplemented with 1% human serum albumin (HSA, Baxter), and cytokines IL-3 (20ng/ml), SCF (300ng/ml), Flt-3 ligand (300ng/ml), TPO (100ng/ml), at a density of 0.5 - 1 x 10^6/ml. All cytokines (Peprotech) and culture reagents are approved for ex vivo clinical use. A small culture sample will be removed for sterility testing. After ~18 hours total pre-stimulation, cells will be cultured with EF1αS-ADA lentiviral vector supernatant for 24 hours in the same complete media at a cell concentration of 0.5 - 1 x 10^6/ml. Following transduction, a portion of cells will be removed for quality control (analysis of transduction efficiency, viability, sterility, RCL). The remaining cells will be resuspended in Human Serum Albumin (HSA) 2% in order to be administered to patient.

7.5.5 Testing prior to patient re-infusion

Samples are collected during and at the end of the procedure for cell viability (trypan blue stain, 7AAD staining) and sterility (for bacteria, fungi and mycoplasma). Transduction efficiency (transgene PCR), and RCL testing will be performed after infusion of cells and recorded retrospectively.

7.5.6 Patient conditioning regimen

During transduction of CD34+ cells, patient will commence conditioning. Patients will receive non-myeloablative conditioning with intravenous busulfan. Busulfan dose will be weight-dependent, in accordance with the recommendation below (table taken from (Bartelink et al., 2012)).

Body-weight	mg/kg/day
3 to 15kg	5.1
15 to 25kg	4.9
25 to 50kg	4.1
50 to 75kg	3.3
75 to 100kg	2.7

A wash-out period of at least 24 hours will be maintained prior to reinfusion of the transduced cells. Pharmacokinetic monitoring of busulfan levels will be performed after administration of the first dose.

7.5.7 Infusion of transduced cells

Cells will be washed and infused in a volume up to 20mls/kg intravenously over 30-45 minutes A baseline set of observations will be carried out before the infusion begins (temperature, pulse, respirations, oxygen saturations). These will then be repeated 15 minutes into the infusion and then again at the end of infusion. Regular monitoring (frequency and duration dependent on reaction/occurrence) will be commenced if anything abnormal (e.g. allergic reaction) occurs during the infusion or if antihistamines are administered.

7.5.8 Discontinuation of PEG-ADA one month after infusion of gene modified cells

PEG-ADA will be continued until one month (+/- 6 days) post-infusion of gene-modified cells. PEG-ADA will be restarted if i) the child is withdrawn from the study ii) if after 180 days there is no evidence of gene modified cell engraftment and/or failure of T cell recovery.

PEG-ADA may be restarted at PI's discretion prior to this time point on clinical grounds e.g. infective problems or delayed T cell reconstitution. In instances when PEG-ADA has been re-started, PEG-ADA may be withdrawn again, if the patient remains well and meets withdrawal criteria (i.e. has detectable PBSC ADA activity and/or detection of gene modified cells above baseline levels).

8 Evaluation and Follow-up

8.1 Assessment of safety and efficacy

8.1.1 Immunological reconstitution

Detailed analysis of immune recovery following gene therapy for primary immune deficiency is essential to evaluate the effectiveness of the procedure, and is largely beyond the scope of our routine clinical immunology resources. We have therefore established dedicated resources and methodologies that enable us to 1) measure the diversity and complexity of T and B reconstitution 2) analyse thymic education and output following gene modification of lymphocyte precursors and 3) measure cellular antigen specific responses.

The analyses will be carried out on pre- and post-treatment samples as shown in Appendix 5. The results will be interpreted as a change in value over time through a longitudinal analysis, for each patient.

- The lymphocyte subset counts (LSC) immunophenotyping panel will be carried out to show the distribution of cells and is used to detect an increase in naïve CD3+ T lymphocyte cell numbers and assess the development of normal distribution of cell populations. This will include the evaluation of CD3+, CD19+, CD16+, CD56+, CD3+, CD4+, CD8+ and naïve CD4+CD45RA+CD27+ cell numbers.

- B cell memory tests will be performed at months 12, 24, and 36. This parameter should be recorded as “not done” in the CRF for subjects that have already completed any of these three visits at the time of amendment (Version) implementation.

- TCR excision circles (TRECs) may be enumerated as a surrogate marker for new thymic emigrants following gene therapy.

- Whole blood lymphocyte proliferation assays will be carried out to test function of T cells and will include responses to mitogens (PHA stimulation and CD3 stimulation).

- Representation of TCR families by flow cytometric analysis (Vβ phenotyping), also forms an important part of monitoring for both physiological and potentially pathological clonal expansions.

- Restoration of antibody production (IgA, IgM, IgG), and serological responses to tetanus vaccinations will be assessed.

8.1.2 Metabolic correction

Metabolic assays will be carried out at the Purine Research Laboratory and will include analysis of red blood cell ADA activity, dATP and dAdo levels. These will indicate how effectively gene therapy has corrected the metabolic phenotype of ADA-SCID.

8.1.3 Molecular characterisation of gene transfer

Molecular characterisation of gene transfer in patient cells is also an important parameter for assessment of efficiency, and potentially for assessment of safety:

- Quantification of transgene copy numbers is determined on sorted cell populations by real-time PCR methodology. This will be performed on peripheral blood samples and on bone marrow samples (if available). Detailed integration analysis maybe used to investigate specific clonal expansions.

- Analysis of lentiviral vector integration sites will be performed by specialised PCR based techniques and by high throughput sequencing of lineage specific populations. This will be performed on peripheral blood samples and on bone marrow samples (if available). These assays will be performed at the times specified in appendix 5.

8.2 Investigations and monitoring schedule

See appendix 5 for tables of investigations.

8.2.1 Pre-treatment investigations

Pregene therapy visit will be carried out prior to gene therapy. This visit will include pre-treatment monitoring investigations, which should be carried out within 3 months of the start of gene therapy treatment according to Appendix 5

8.2.2 Post-infusion monitoring

Patients will be monitored on protocol at 1 month, 6 weeks, 3, 6, 9, 12, 18, 24, 30 and 36 months post-infusion. Window to carry out these visits is +/- 5 days for visits at 1 month, 6 weeks and +/- 2 weeks for visits at 3, 6, 9, 12, 18, 24, 30 and 36 months. Follow-up as part of the routine clinical care of post-transplant patients will be annual after this.

Monitoring, testing being performed and information to be collected on CRF are fully detailed in Appendix 5. Modifications to this protocol will be adopted as necessary to improve sample-processing capability, and a degree of flexibility regarding the actual dates of assessment will be maintained. Additional tests may be carried out in the event of a significant adverse effect to ensure optimal clinical care.

8.2.3 Safety monitoring

The safety of treatment will be summarised using AEs, laboratory assessments and clinical examination assessments. These will be analysed continuously from pre-gene therapy to study completion, depending on the data available. Further details are given in the SAP

8.2.4. Neurological and behavioural monitoring of patients

Cognitive and behavioural defects are well documented in ADA-SCID patients following HSCT (Rogers et al., 2001; Titman et al., 2008). It is thought that patients undergoing gene therapy will also suffer similar consequences.

We have recently set up a study protocol (funded by the Department of Health) to assess all immunodeficiency patients (both ADA-SCID and non ADA-SCID) for cognitive, educational and behavioural outcomes following HSCT. This assessment will therefore also be applied to all ADA-SCID patients undergoing gene therapy. In addition to this, we will administer a standard health related quality of life questionnaire to assess the general impact of the treatment on the child functions.

8.3 Treatment failure/lack of response

- Failure of immune reconstitution is determined by poor recovery of T lymphocyte numbers and function by day 180

OR

- by absence of gene corrected cell engraftment (<0.1 %) tested by quantitative PCR in peripheral blood and/or BM samples 6 months after the procedure.

- If there is no immune recovery/evidence of gene modified cell engraftment seen 180 days after gene therapy, PEG-ADA will be commenced OR the patient will undergo stem cell transplant from a matched unrelated donor depending on the clinical state of the child.

- If there is no matched unrelated donor available, the patient will be commenced on PEG-ADA OR if the referring health agency is unable to provide funding, the patient will undergo stem cell transplant from an unrelated or haploidentical parental donor.

- In the absence of a matched family or unrelated donor a second administration of the gene therapy treatment could be considered following specific approval from GTAC/MHRA on a case by case basis.

8.4 Withdrawal of individual subjects

A patient can withdraw or be withdrawn from protocol treatment in the study at any time from enrolment until the transduced CD34+ cells have been administered. Once a patient has been administered the transduced CD34+ cells study treatment is complete.

- Patients from whom $<3x10^6$ CD34+ cells/kg are harvested for the back-up, will be withdrawn from the study prior to conditioning and offered a repeat harvest
- Patients from whom $<1x10^6$ CD34+ cells/kg are harvested for transduction, will be withdrawn from the study prior to conditioning and offered a repeat harvest
- If less than 0.5 x 10^6 viable transduced CD34 + cells/kg are returned to the patient, then back-up bone marrow/PBSCs will be returned and patients will be withdrawn from the study
- If after 180 days there is no evidence of the transgene in PBMCs by qPCR or there is no evidence of T cell recovery, then patients will re-start PEG-ADA and will be withdrawn from the study

PEG-ADA may be restarted at PIs discretion prior to this time point on clinical grounds e.g infective problems or delayed immune reconstitution and at this stage will be withdrawn from the study. The decision for patients to remain off PEG-ADA is dependent on a combination of factors including:

1) metabolic detoxification as assessed by sustained red blood cell dATP levels <250 umol/l
2) T cell recovery as assessed by absolute T cell numbers ($CD3^+$ cells) >300cells/mm^3
3) engraftment of gene modified cells as assessed by vector copy number in $CD3^+$ cells >0.05 (>5% of total T cells

Subjects who wish to discontinue from the study at any time are free to do so. However the reasons for discontinuation should be documented by the investigator if possible.

Patients who withdraw from the study after administration of the transduced CD34+ cells for reasons other than those given above will be encouraged to have follow-up investigations so that the consequences of the administration can be documented and analysed.

Any patient withdrawn prior to administration of transduced CD34+ cells will be replaced in the study.

8.5 Off study criteria

A patient will be considered off study under the following circumstances:

1. The patient is withdrawn from the study prior to administration of transduced CD34+ cells
2. The patients/parent/guardian withdraws consent for study procedures and data collection
3. The patient is lost to follow-up

8.6 Follow-up of withdrawn subjects

Patients withdrawn from the study prior to administration of transduced CD34+ cells and patients for whom consent has been withdrawn for the study, will resume normal clinical care. Efficacy and safety assessments will not be carried out from the point of withdrawal and data will not be recorded in the Case Report Forms (CRFs).

8.7 Definition of end of trial

- The sponsor will notify GTAC and the MHRA of the end of the study within a period of 90 days.
- The end of the study is defined as the last patient's last scheduled visit according to the protocol, which will be the 3-year follow-up visit of the last patient entered into the trial.
- In case the study is ended prematurely, the sponsor will notify GTAC and the MHRA within 15 days, including the reasons for the premature termination.
- Within one year after the end of the study, the investigator/sponsor will submit a final study report with the results of the study, including any publications/abstracts of the study, to GTAC and the MHRA.

8.8 Trial stopping criteria

The trial will be stopped prematurely under the following circumstances:

1. Evidence of oncogenesis in any trial patient. The trial will only be re-started following agreement from both the MHRA and GTAC.

The events below will result in halting of the trial and will require a formal evaluation of the reasons for non-efficacy. The trial will only be re-started after discussion with and approval from the MHRA and GTAC:

1. Unsuccessful transduction of 3 consecutive patient $CD34^+$ cells to specified criteria
2. No evidence of vector integration in 3 consecutive patients at 6 months post-treatment
3. 3 consecutive patients needing to re-start enzyme replacement therapy prior to 1 year post-gene therapy

   (From our experience of these studies, 3 consecutive patients would be seen as a number whereby this could not have happened by chance and is most likely related to processes or procedures)
4. Emergence of new therapies for ADA-SCID, believed by the CI to be of greater benefit to this population of trial patients. The CI is in regular contact with other experts in the field of ADA-deficiency therefore is aware of new developments in the field. Regular reviews of the currently available treatment options for the subject population will be conducted by the PI throughout the trial.

Complete cessation of the trial would be made in consultation with the MHRA and GTAC.

8.9 Statistical analysis

Timing of analyses

One interim analysis, one primary analysis, one longer term follow-up and one end-of-study analysis are planned for this study.

An interim look will be done when all on-study patients (not including CUP) have reached at least 6 months' follow-up.

The primary analysis for the study will be done when all on-study patients have reached at least 12 months' follow-up. This interim analysis will be including CUP and HSCT groups.

The third analysis will be done when all IMP on-study patients have reached at least 24 months' follow-up. This interim analysis will be including CUP and HSCT groups.

The final analysis will be done at the end of the study. i.e. after on study patients, have completed the planned follow up of 3 years. This analysis will also include HSCT group.

8.9.1 Analysis populations

There will be 3 populations analysed in this study: The on-study patients treated with IMP (gene therapy), patients who were treated under compassionate use (CUP patients) and the historical comparator group of patients who received HSCT.

The safety analysis population will include all patients who received the IMP infusion, including both on-study patients and patients who were treated on a compassionate use basis. In addition, all the allogeneic HSCT controls patients (with any type of donor) will be included.

Further efficacy populations will be detailed in the statistical analysis plan (SAP).

8.9.2 Data analysis/description

Descriptive statistics will be employed for the socio-demographic characteristics of the study population.

The primary efficacy endpoints, overall survival (OS) and event-free survival (EFS) will be compared descriptively to a group of ADA-SCID patients treated by allogeneic HSCT (historical control group). The main secondary endpoint, will be summarised descriptively.

Data will be summarized in tables displaying the mean, standard deviation, median, minimum, maximum and number of subjects per group for continuous data (e.g. age, weight) or in tables displaying count and percentage for categorical data (e.g. gender, previous treatment). Data will be presented by visit, if applicable. Data listing by subject will be provided as appropriate.

All statistical analyses will be performed and data appendices will be created using SAS® version 9.4 or higher

Safety

Safety will be analysed descriptively by arm and study phase using the safety analysis population. Coding will be done using MedDRA version 18.1 or higher. The safety of treatment will be summarised using AEs, laboratory assessments and clinical examination assessments. These will be analysed continuously from pre-gene therapy to study completion, depending on the data available. Further details are given in the SAP.

Efficacy

Primary Endpoints

Statistical methodologies for comparing gene therapy to historical controls at all stages will be descriptive in nature and will utilize the following approaches:

1. OS and EFS will be described using Kaplan-Meier curves for patients treated with investigational gene therapy product and HSCT, respectively, and statistically compared by the log-rank test and proportional hazards models (where applicable).
2. OS and EFS rates post treatment will be described by group and their differences between groups presented along with associated exact binomial confidence intervals.

Main Secondary Endpoint

Percentage of subjects requiring immunoglobulin replacement therapy will be provided for each cohort (See Overview Section above) along with associated, two-sided, exact binomial, 95% confidence intervals.

The detailed statistical procedures will be provided in the statistical analysis plan (SAP).

9 Safety issues related to the gene therapy protocol

9.1 Risks

9.1.1 Venepuncture

Hazards associated with blood drawing are those associated with venepuncture and, those associated with the loss of red blood cells as a result of removal of blood from the procedures described in this protocol. Risks of venepuncture are pain, the potential for formation of a haematoma at the site of the needle stick and a small potential for infection at the site of needle stick. Occasionally, individuals having venepuncture may faint as a result of a vasovagal reaction to the procedure. These risks will be minimised by carefully preparing the site of needle puncture with alcohol swab, by using the smallest size needle required for the procedure, and by having the patient lie down or sit as appropriate for 5 or 10 minutes after venepuncture. The major risk of repeated blood drawing is anaemia from removal of red blood cells. To minimise this risk the amount of blood drawn for all purposes will be adjusted so as not to exceed the guidelines of 450 ml or 7.5 ml/kg (whichever is less) over any six-week period.

9.1.2 Infection

Withdrawal of PEG-ADA can have adverse effects on immunity, as well as on other organ systems. Once PEG-ADA is withdrawn, the numbers of circulating T, B, and NK lymphocytes will decline and may remain low for several months until the autologous transplant of gene-corrected CD34+ cells leads to immune reconstitution. It is possible that a severe infection may develop during this time and this could even be fatal if it does not respond to therapy. If immunity is severely decreased after discontinuation of enzyme replacement therapy and the subject experiences multiple or serious infections, then therapy may have to be resumed.

It is also possible that the cells for infusion could become infected with bacteria or fungus during the growth period in the laboratory. If there is any evidence of infection of the cells with unwanted agents and busulfan has already been administered, the cells will be returned to the subject with appropriate antibiotic cover. If the infection significantly affects viability (to a total cell dose of $<0.5 \times 10^6$/kg) then transduced cells will not be returned and the back-up

bone marrow will be infused. However, there is a remote chance that subjects could acquire a bacterial or fungal infection from the re-infused cells (this also applies to the back-up cells, if administered).

9.1.3 Busulfan toxicity

Busulfan has been widely used for myeloablation in bone marrow transplantation and has a well-described toxicity profile. When busulfan is used at high doses (16 mg/kg, which translates to approximately 40 mg/m^2 x 16 doses) along with cyclophosphamide, as administered prior to allogeneic bone marrow transplantation, significant toxicity including thrombocytopenia and neutropenia have occurred, busulfan-related seizures and emesis may occur and are largely preventable with prophylactic medication. Rarely, veno-occlusive disease and pulmonary fibrosis (the latter two toxicities seen with busulfan/cyclophosphamide combinations) have been described. However, the nature and extent of toxicity resulting from low-dose busulfan (4mg/kg iv. total dose) in a paediatric population are not known. A recent report in ten ADA-SCID children demonstrated evidence of neutropenia but suggested no limiting toxicity with 4 mg/kg (Aiuti, A et al., 2002, 2009). Our own experience (of 3 patients) has shown no significant toxicity. The complications of high dose busulfan in combination with other chemotherapy drugs have not been seen in any of the more than 25 ADA-deficient patients treated using low dose busulfan. Busulfan is mutagenic (a clastogen, inducing DNA damage) and another potential cause for cancer in this study group. Although busulfan is frequently used in combination with cyclophosphamide in the clinical setting, there is the risk that it independently can cause cancer, even with the low dose proposed in this study.

The dosage schedule in this study is based on the differential clearance of busulfan in children of different weights. There is no evidence for any greater toxicity as a result of this weight adjusted dosing schedule. In two patients treated off trial on a compassionate basis and who received 5.1mg/kg of iv busulfan, no toxicity was seen.

9.1.4 Bone marrow harvest

Bone marrow will be harvested from the patient under general anaesthesia from the posterior iliac crests on both sites by multiple punctures. Marrow will be collected into sterile bags containing ACD (acid citrate dextrose) (Baxter). The amount of marrow collected will be equivalent to 20 ml/kg of body weight. The risks of the procedure include the standard risks of general anaesthesia and complications associated with the bone marrow harvest procedure. The latter include pain at the site of puncture following the procedure, excessive blood loss resulting in anaemia and the potential for infection at the puncture site. The anaesthetic risks will be minimised by the anaesthetic being administered by a paediatric anaesthetist at a major paediatric tertiary referral centre. The patient will be given paracetamol pain relief after the

procedure and will be monitored carefully to check haemodynamic status. It is likely that the patient will need a blood transfusion after the bone marrow harvest. Infection risk will be minimised by careful cleansing of the site prior to needle insertion and strict adherence to sterile technique during the procedure.

9.1.5 Peripheral blood stem cell harvest

Treatment of patients or donors with granulocyte colony stimulating factor (G-CSF) and Plerixafor results in the mobilisation of bone marrow stem and progenitor cells into the peripheral blood, from where they can be harvested. The quality of cells collected in terms of engrafting potential has been such that many centres have virtually replaced transplantation of bone marrow with that of peripheral blood stem cells (PBSCs) particularly in the autologous setting. The advantages of this procedure (over bone marrow harvest) are that a larger number of stem cells can be harvested, and no anaesthetic is required. G-CSF is administered by subcutaneous injection for 6 days. On day 5, Plerixafor may also be administered by subcutaneous injection. The commonest side effect is of bone and muscle pain, which is easily controlled with mild analgesics. Rare reports (<1 in 1000 patients) exist of allergic-type reactions (including anaphylaxis, skin rash, urticaria, angioedema, dyspnoea and hypotension), usually when the drug has been injected intravenously. Temporary abnormalities of liver function and skin rashes have occurred in a very small number of patients, usually on long term therapy for neutropenia. Apart from extremely rare reports of spontaneous splenic rupture, the safety records of both G-CSF and Plerixafor are very good. If patients develop unanticipated inflammatory reactions, which in the opinion of the investigator are due to G-CSF and/or to Plerixafor toxicity, then administration will be terminated and the patient would then be proposed a bone marrow harvest.

After mobilisation, the cells are harvested by leukapheresis on two consecutive days from centrally placed cannulae. Each leukapheresis lasts from three to four hours and has been shown to be extremely safe both in adults and in children. Adverse reactions to apheresis procedures are rare, although vasovagal episodes related to needle insertions or transient volume loss can occur. The former reaction is prevented by lying down and if necessary, fluid administration. Paraesthesiae or tingling can also sometimes occur due to lowering of calcium levels by the citrate anticoagulant. This is readily relieved by slowing the rate or temporarily interrupting the anticoagulant infusion. The precautions taken to minimise pain, haematoma formation, risk of infection at needle site and vasovagal fainting are outlined above. Leukapheresis can be associated with some loss of red blood cells and plasma equivalent to 100 to 200 ml of whole blood though every effort will be made to keep this volume as low as possible. Insertion of the apheresis line can lead to occlusion of blood flow and therefore can result in thrombus formation. This is treated with anti-coagulant and regular screening to

monitor reduction of the size of the thrombus. Leukapheresis is a routine procedure with minimum side effects or complications. Potential complications include formation of haematoma at the site and a small risk of infection as with any intravenous catheter.

9.1.6 CD34+ cell infusion

Based on results from other gene transfer studies and our own experience, the infusion of cultured and gene altered autologous blood progenitors does not appear to be associated with any significant reactions. In this study, the medium used to culture the CD34+ cells is free of animal serum. However, infusion of any type of blood cell product can be associated with reactions resulting from clumping of these cells or other immediate reactions related to sticking of these cells to blood vessels in the lungs. Patients will be monitored as per national and hospital Blood product administration policy. Reactions are treated by stopping the infusion and providing oxygen, antihistamines, steroids and medications or fluids to increase blood pressure. Because infused cells in this study are autologous CD34+ cells, the possibility that this type of agglutination or vascular reaction will occur is very low. It is theoretically possible for the CD34+ cell cultures to become contaminated with microorganisms. One of the important safety features of this gene therapy protocol is that CD34+ cells will be cultured in a sealed bag culture system or in flasks in an isolator unit. Routine cultures will be performed on cell samples before infusion. Only interim sterility results will be available before infusion of cells. If significant growth of pathogenic microorganisms is detected, the cells will be returned with appropriate antibiotic cover, or if the infection significantly affects cell viability, the cells will not be returned to the patient.

9.2 Risks associated with the use of lentiviral vector

9.2.1 Insertional mutagenesis

Currently, there have been no reported cases of insertional mutagenesis in clinical trials using a lentiviral vector for gene therapy. A number of studies both in vitro and in in vivo (Modlich et al., 2009; Modlich et al., 2006; Montini et al., 2006) showed that the risk for insertional mutagenesis is less genotoxic with lentiviral vectors that lack strong retroviral LTR and may be further reduced with improved vector designs.

Risks from insertional mutagenesis have occurred in clinical trials of gene transfer using retroviral vectors. After immune reconstitution in 9 of 10 subjects participating in an X-SCID gene transfer study in Paris, France, four of these subjects developed leukaemia ≥ 2½ years after receiving the gene-transduced CD34+ cells (Hacein-Bey-Abina et al., 2003; Hacein-Bey-Abina et al., 2008). In a similar trial of gene therapy for X-SCID performed in London, England, one of 10 subjects also developed T cell leukaemia (Howe et al., 2008). One of these five patients who developed T cell leukaemia has died and the other four are in leukaemia

remission and continue to have restored immunity from the gene therapy. For three of the subjects with leukaemia, the proviral insertion sites have been mapped within or near (3 kb) of the LMO-2 gene, a human proto-oncogene associated with T-cell leukaemia. In a fourth case, two integrations sites were described: LMO-2 and BMI1, which is also a proto-oncogene. In a fifth subject, vector had integrated near a third proto-oncogene, CCND2 (Hacein-Bey-Abina S et al., 2008). These are the first reported cases of leukaemia developing in subjects participating in a clinical study of retroviral-mediated gene transfer to CD34+ cells. The probability of leukaemia developing in other participants of the French trial, or in participants of other trials, is not known. Similarly in a trial of gene therapy for X-CGD, 2 patients developed a myelodysplasia and an acute myeloid leukaemia as result of vector mediated integration events disrupting expression of the MDS-EVI1 oncogene ((Ott et al., 2006; Stein et al., 2010).

As of April 2011, 17 ADA-deficient SCID patients have undergone these protocols in Milan and 8 in London and 11 in the CHLA/NIH and UCLA/NIH trials (by personal communication from site PIs). No cases of leukaemia have occurred in any of the ADA subjects, in sharp contrast to the X-SCID or X-CGD results.

9.2.2 Replication-competent lentivirus (RCL) exposure

Generation of RCL is highly unlikely due to the multiply deleted nature of the vector and SIN configuration, and has not been noted in any preclinical study with similar vectors. Lentiviral supernatant and patient samples will however be tested for the presence of RCL by cell culture and PCR methodology. Patient samples will be tested at time points 0 (pre-treatment), 3 months, 6 months and one year after treatment. If all tests are negative during the first year, further samples will be taken annually through-out the patient's care at GOSH and archived for at least 15 years. If in the unlikely event that post-treatment samples are positive, further analysis will be undertaken to characterize the RCL.

9.2.3 Germ-line transmission of vector sequences

Issues of contraception and pregnancy are not applicable in infants and children before puberty. Because we will only be putting the EF1α-ADA vector into the CD34+ cells from bone marrow *ex vivo*, we do not anticipate that they can enter the germ cells.

9.2.4 Allergic/immunological responses to cell processing excipients

Stem Cell Factor (SCF) and thrombopoietin (TPO) are used to maintain stem cell viability and to promote gene transfer during the *ex vivo* transduction process. When these factors are administered parenterally, they may have untoward immunologic or allergic consequences such as thrombocytopenia or allergic reactions. The CD34+ cells cultured in these cytokines are thoroughly washed following *ex vivo* exposure. Since small amounts of residual factors

may remain, it is possible that the presence of these, and other cytokines used during the transduction process, may result in an adverse immunologic or allergic event. This hypothetical complication has not been reported from any of the clinical trials using these factors for *ex vivo* transduction.

9.2.5 Other adverse clinical consequences

Participation in this protocol may have deleterious effects on subsequent attempts at allogeneic HSC transplantation. These adverse effects could be due to "immunisation" to normal human ADA produced from the transgene. Most patients however, will have received bovine PEG-ADA, which is more likely to be immunogenic than human ADA; the development of significant inhibitory antibodies has been relatively rare in patients treated with PEG-ADA alone. It is possible that non-PEGylated human ADA could be more immunogenic or its expression in cells could induce more T cell responses than injected recombinant protein. Additionally, this protocol could lead to restoration of partial immunity, insufficient for complete protection from infections and yet sufficient to increase the risks of rejections of allogeneic cells. This partial immunity may also result from PEG-ADA alone, although it may be possible to eradicate this residual activity by complete PEG-ADA cessation. For patients who have not received PEG-ADA the gene transfer procedure would introduce these risks de novo, although it is difficult to know *a priori* the potential likelihood of these hypothetical risks.

As with any new form of therapy, there may be risks which are unknown or not anticipated.

10 Name description of the IMP and critical raw materials

10.1 Name and description of each IMP

- EF1αS-ADA lentiviral vector transduced patient CD34+ cells is designated as an IMP
- The IMP is patient-specific and corresponds to autologous CD34+ cells transduced ex vivo with the EF1αS-ADA lentiviral vector containing the human ADA gene in final formulation and container closure system, ready for intended medical use
- In this study EF1αS-ADA lentiviral vector is designated as a critical raw material
- The starting materials used for the production of the IMP consist of the viral vector and the patient’s CD34+ cells

10.2 Production, supply and release of the IMP

[redacted]

- Transduction of autologous CD34+ cells with the lentiviral vector will be carried out in the Gene Therapy Laboratory, Camelia Botnar laboratories, Great Ormond Street Hospital for Children NHS Trust.
- The transduced cells will be packaged in sealed transfer bags prior to being transported to the ward at Great Ormond Street Hospital (GOSH) for administration.
- An independent Qualified Person (QP) will be responsible for importation, sign off and release of the vector for use in the study

10.3 IMP accountability

IMP accountability is ultimately the responsibility of the Chief Investigator. This responsibility however will be delegated to the Clinical Scientist responsible for the transduction procedure.

Detailed records will be kept to allow for accurate accountability of the vector and transduced CD34+ cells. These records will include details of shipping, receipt, storage, use and destruction of the vector. Transfer of CD34+ cells from the gene therapy laboratory to the ward at GOSH and administration of the transduced CD34+ cells to patients will be recorded.

10.4 Description and justification of the trial treatment and dosage

The EF1αS-ADA lentiviral vector is used to transduce autologous CD34+ cells ex vivo. These transduced cells are then infused into the patient. The cell/product dose will consist of at least 0.5 x 10^6 CD34+ cells per kg of body weight transduced *ex vivo* with 1 x 10^8 i.g./ml of lentiviral vector to achieve an average of 0.3-2 integrated copies per cell. A higher dose would be expected but given that there is a survival advantage to gene corrected cells, this lower level has been designated as final release threshold in this study. This is supported by evidence gained from our first retroviral ADA trial here at GOSH, where two patients reconstituted effectively, having received less than 0.5x10^6 transduced $CD34^+$ cells/kg (Gaspar et al., 2011).

10.5 Dose modifications

A single dose of cells will be administered to the patient and the dose is individual to the patient since the gene-modified cells are the IMP.

10.6 Assessment of compliance

There is no compliance issue with IMP administration since gene modified cells are infused through a Hickman line and the infusion is monitored by a trained nurse.

10.7 Post-trial IMP arrangements

There is no provision to provide patients with IMP post-trial participation.

10.8 Name of each NIMP

- Cotrimoxazole
- Itraconazole
- Penicillin
- Aciclovir
- Immunoglobulin
- Calcium Folinate
- Busulfan

NIMP suspected Adverse Drug Reactions (ADR) or side effects will be reported through the yellow card system.

11 Recording and reporting of adverse events and reactions

11.1 Definitions

Adverse event

This is defined as any untoward medical occurrence in the patient administered an IMP that does not necessarily have a causal relationship with this treatment.

Adverse reaction

This is defined as any untoward and unintended response in a subject to an IMP, which is related to any dose administered to that subject.

Serious adverse event

A serious adverse event is defined as any untoward medical occurrence in the patient administered an IMP, which does not necessarily have a causal relationship with this treatment, and that at any dose:

- Results in death
- Is life threatening
- Results in persistent or significant or disability/incapacity
- Requires in-patient hospitalisation or prolongs existing hospitalisation
- Results in a congenital abnormality or birth defect

Medically significant (i.e. any event which the investigator considers significant but which is not covered by the above)

Suspected Serious Adverse Reaction (SSAR)

This is defined as an adverse reaction that is classed in nature as serious and which is consistent with the information about the IMP in question as set out in the Investigator's Brochure.

Suspected Unexpected Serious Adverse Reaction (SUSAR)

The definition of a SUSAR is a serious adverse drug reaction, the nature or severity of which is not consistent with the applicable product information (as set out in the Investigator's Brochure) or which adds significant information on the specificity or severity of an expected event.

11.2 Recording adverse events

All adverse events (related to the IMP, unrelated to the IMP, serious or not) will be recorded in the hospital notes and in the electronic Case Report Form (eCRF). An adverse events log may be adopted for the hospital notes.

If the investigator suspects that the disease has progressed faster due to the administration of the IMP, then he will report this as an unexpected adverse event to the sponsor.

Clinically significant abnormalities in the results of objective tests may also be recorded as adverse events.

The record of adverse events will include the following:

- **Clinical symptoms:** A simple, brief description
- **Severity:** we will categorise according to WHO classification (see appendix 7)
- **Causality assessment (relationship to treatment):** The assessment of relationship of adverse events to the administration of IMP is a clinical decision based on all available information at the time of the completion of the case report form. The following categories will be used:

Definitely: There is clear evidence to suggest a causal relationship, and other possible contributing factors can be ruled out.

Probably: There is evidence to suggest a causal relationship, and the influence of other factors is unlikely.

Possibly: There is some evidence to suggest a causal relationship (e.g. the event occurred within a reasonable time after administration of the trial medication). However, the influence of other factors may have contributed to the event (e.g. the patient's clinical condition, other concomitant events).

Unlikely: There is little evidence to suggest there is a causal relationship (e.g. the event did not occur within a reasonable time after administration of the trial medication). There is another reasonable explanation for the event (e.g. the patient's clinical condition, other concomitant treatments).

Not related: There is no evidence of any causal relationship.

Not Assessable

Note: events assessed as possibly, probably or definitely related to the IMP are considered adverse reactions (ARs)

- **Expectedness evaluation:** The following categories will be used:

Expected: An adverse event which is consistent with the information about the IMP listed in the Investigator Brochure or clearly defined in this protocol

Unexpected: An adverse event which is not consistent with the information about the IMP listed in the Investigator Brochure

- **Seriousness:** as defined for an SAE in section 11.2.1.

Collection, recording and reporting of adverse events (including serious and non-serious events and reactions) to the sponsor will be done according to the sponsor's SOP.

11.3 Expected disease adverse events

The following SAEs are considered disease related:

- Death from complications of the underlying disease process (SAE)
- Hospitalisation from complications of the disease (SAE)
- Prolonged hospitalisation from complications of the disease (SAE)
- Bone marrow aplasia for greater than 1 month (SAE/SSAR)

The following SAEs are considered related to the side effects of chemotherapy:

- Renal/liver/gastrointestinal/pulmonary/metabolic problems (SAE)
- Viral re-activation following chemotherapy and prior to immune recovery (SAE)
- Bone marrow aplasia for greater than 1 month (SAE/SSAR)

A full list of AEs considered related to the side effects of the chemotherapy are detailed in the undesirable effects section of the Summary of Product Characteristics (see Appendix 7).

11.3 IMP expected adverse events

The following ARs are considered expected reactions that could occur following administration of the IMP:

1. Lympho- or myelo-proliferation due to insertional mutagenesis (SSAR)
2. Chills/fevers/rigors at time of infusion (AR)
3. Rash (AR)
4. ADA overexpression leading to haemolysis (AR)
5. Immune response to transgene (AR)
6. Bone marrow aplasia for greater than 1 month (SAE/SAR)

11.4 Procedures for recording and reporting Serious Adverse Events

All SAEs will first be clearly documented in the patient's notes.

Events defined as serious will be reported immediately (within 24 hours of the any study team member observing or learning of the event), in as much detail as possible to the sponsor (Joint R&D office).

This event will be recorded in the sponsor's SAE form (F44) and emailed to [redacted] or [redacted] within the reporting timelines.

The Investigator may be asked by the sponsor to provide follow-up information, which should be reported to sponsor by completing SAE from (F44).

All SUSARs will be notified to the sponsor immediately (within 24 hours of any study team member observing or learning of the event) according to the sponsor's written SOP.

Elective and/or planned procedures do not require sponsor's SAE reporting, unless of course there is any complication arise that fulfils the definition as listed section 11.1 Definitions. In any case, these elective and/or planned procedures will be documented in the patient's notes and CRF.

11.4.1 Notification of deaths

Death will be reported to the sponsor irrespective of whether it is related to disease progression, the IMP, or an unrelated event.

11.4.2 Reporting SUSARs

SAE reported to the sponsor that is evaluated by the Investigator as related to the IMP and unexpected will be reported to the regulatory authorities (MHRA) and the ethics committee (GTAC) by the sponsor within the expedited reporting timelines:

Fatal or life threatening SUSARS: not later than 7 days after the sponsor has information that the case fulfils the criteria for a fatal or life threatening SUSAR, and any follow up information within a further 8 days.

All other SUSARs: not later than 15 days after the sponsor for pharmacovigilance has information that the case fulfils the criteria for a SUSAR.

11.4.3 Medically important events considered critical to the safety evaluation of the study

Abnormal transgene related lympho- or myelo-proliferation (i.e. in the absence of viral reactivation) is considered critical to the safety evaluation of the study and will be reported immediately (within 24-48hrs) to the sponsor using the sponsors reports of other safety issues

(F20) form. Such events will be reported to the MHRA and the GTAC by the sponsor within the expedited reporting timelines.

11.4.4 Long term follow-up

All patients are followed up lifelong and once these studies are officially closed the PI will continue to report SARs and SUSARS to the MHRA and GTAC in the same way as during the study.

11.4.5 Annual safety reports

Within 60 days of the anniversary of the clinical trial authorisation (CTA) date, an annual safety report will be sent to the MHRA and GTAC.

11.4.6 Annual progress reports

An annual progress report (APR) will be submitted to GTAC within 30 days of the anniversary date on which the favourable opinion was given, and annually until the trial is declared ended.

11.4.7 Pregnancy

Not applicable for this study.

11.4.8 Reporting urgent safety measures

The sponsor and the investigator may take appropriate urgent safety measures in order to protect the subjects of a clinical trial against any immediate hazard to their health or safety. If measures are taken, the sponsor shall immediately and in any event no later than 3 days from the date the measures are taken, give written notice, in the form of a substantial amendment, to the MHRA and GTAC of the measures taken and the circumstances giving rise to those measures.

In order to prevent any delays in the reporting timelines the sponsor has delegated this responsibility to the PI. Therefore the PI must report any urgent safety measures to the MHRA directly and in parallel to the sponsor.

11.4.9 Notification of serious breaches to GCP and/or the protocol

Any deviations, violations, potential serious breaches and urgent safety measures will be recorded.

The sponsor will be notified immediately of any case where the above definition applies during the trial conduct phase. The sponsor's SOP will be followed and all these events will be notified to GTAC and MHRA by sponsor.

12 Administrative aspects

12.1 Data handling, record keeping and sample storage

12.1.1 eCRF completion

All study data will be recorded on study specific eCRF. The eCRF will be completed by personnel authorised to enter or change data on the eCRF. The eCRF will track all modifications done by the relevant authorised person

12.1.2 Sample storage

A record of retained body fluids / tissue samples will be completed every time sample is stored. This includes the patient trial identification number, initials, date sample was stored, and storage location as well as the date the sample was moved or destroyed.

Samples (Cells, DNA & RNA) will be stored pre cell therapy and at regular intervals post cell therapy, and kept indefinitely at the bone marrow laboratory GOSH (or alternative approved location). Should it be required, samples will be disposed of in the appropriate manner according to local GOSH procedures, this will be detailed in the parent/guardian information sheet and consent obtained. Samples for RCL testing, integration analysis are sent outside GOSH for analysis only.

12.1.3 Record retention

Essential documents will be retained for a minimum of 25 years after completion of the trial. These documents will be retained for longer if required by the applicable regulatory requirements or the sponsor.

Records related to traceability of the IMP at site along with the patient identification code list will be retained at site for at least 30 years from the expiry date of the transduced cells.

The sponsor (or PI if delegated) will retain a copy of the final clinical trial protocol and Investigator Brochure along with the traceability records for the trial for at least 30 years from the expiry date of the transduced cells.

12.2 Patient confidentiality

In order to maintain patient privacy, all eCRFs, IMP accountability records, study reports and communications will identify the patient by initials, date of birth and the assigned unique patient trial number.

Direct access to the patient’s original medical records for verification of data gathered on the eCRFs and to audit the data collection process will be permitted for trial-related monitoring & audits by the sponsor, Research ethics committee review, and regulatory inspection(s).

12.3 Monitoring and audit

The trial has been initially monitored and audited by GOSH, this activity is now delegated by GOSH to Voisin Consulting Life Science.

12.4 Direct access to source data/documents

The investigator(s)/ institution(s) will permit trial-related monitoring, audits, GTAC review and regulatory inspection(s), providing direct access to source data/documents. Trial participants are informed of this during the informed consent discussion. Participants will consent to provide access to their medical notes.

13 Ethics and regulatory requirements

The sponsor will ensure that the trial protocol, patient information sheet, consent form, GP letter and submitted supporting documents have been approved by the MHRA and GTAC, prior to any patient recruitment. The protocol and all agreed substantial protocol amendments, will be documented and submitted for ethical and regulatory approval prior to implementation.

Before the site can enrol patients into the trial, the Chief Investigator or designee must apply for Site Specific Assessment from Trust Research & Development (R&D) and be granted written NHS R&D approval. It is the responsibility of the Chief Investigator or designee at each site to ensure that all subsequent amendments gain the necessary approval. This does not affect the individual clinician's responsibility to take immediate action if thought necessary to protect the health and interest of individual patients (see section 11.4.8 for reporting urgent safety measures).

Within 90 days after the end of the trial, the CI and sponsor will ensure that GTAC and the MHRA are notified that the trial has finished. If the trial is terminated prematurely, those reports will be made within 15 days after the end of the trial.

The CI will submit a summary report of the clinical trial to the MHRA and GTAC within 1 year after the end of the trial.

14 Finance

Finance for the study provided by

- Department of Health – vector costs
- Internal funds – to cover clinical costs

- Orchard Therapeutics

15 Insurance

Cover for negligent harm will be provided by the Great Ormond Street Hospital NHS Trust through the Clinical Negligent Scheme for Trusts (CNST). No-fault compensation insurance cover for any non-negligent harm will be provided by University College London (UCL).

16 Publication policy

Results of this study will be disseminated by publication, oral presentation at scientific meetings, and by direct communication with regulatory agencies.

17 Statement of compliance

The trial will be conducted in compliance with the protocol, GCP and the applicable regulatory requirement(s).

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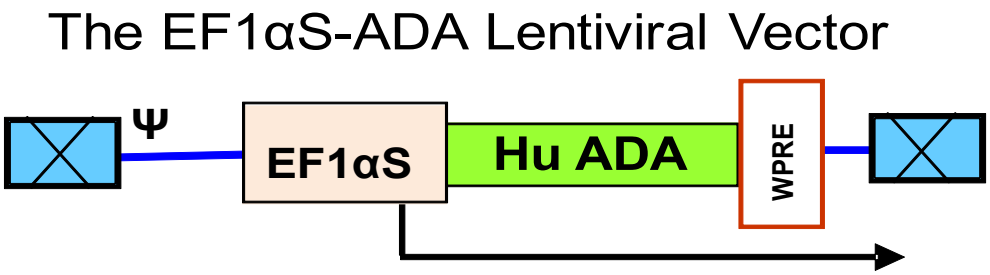
Appendix 1: Construction of EF1αS-ADA lentiviral vector

ADA Vector



EF1αS: short version of the EF1α promoter

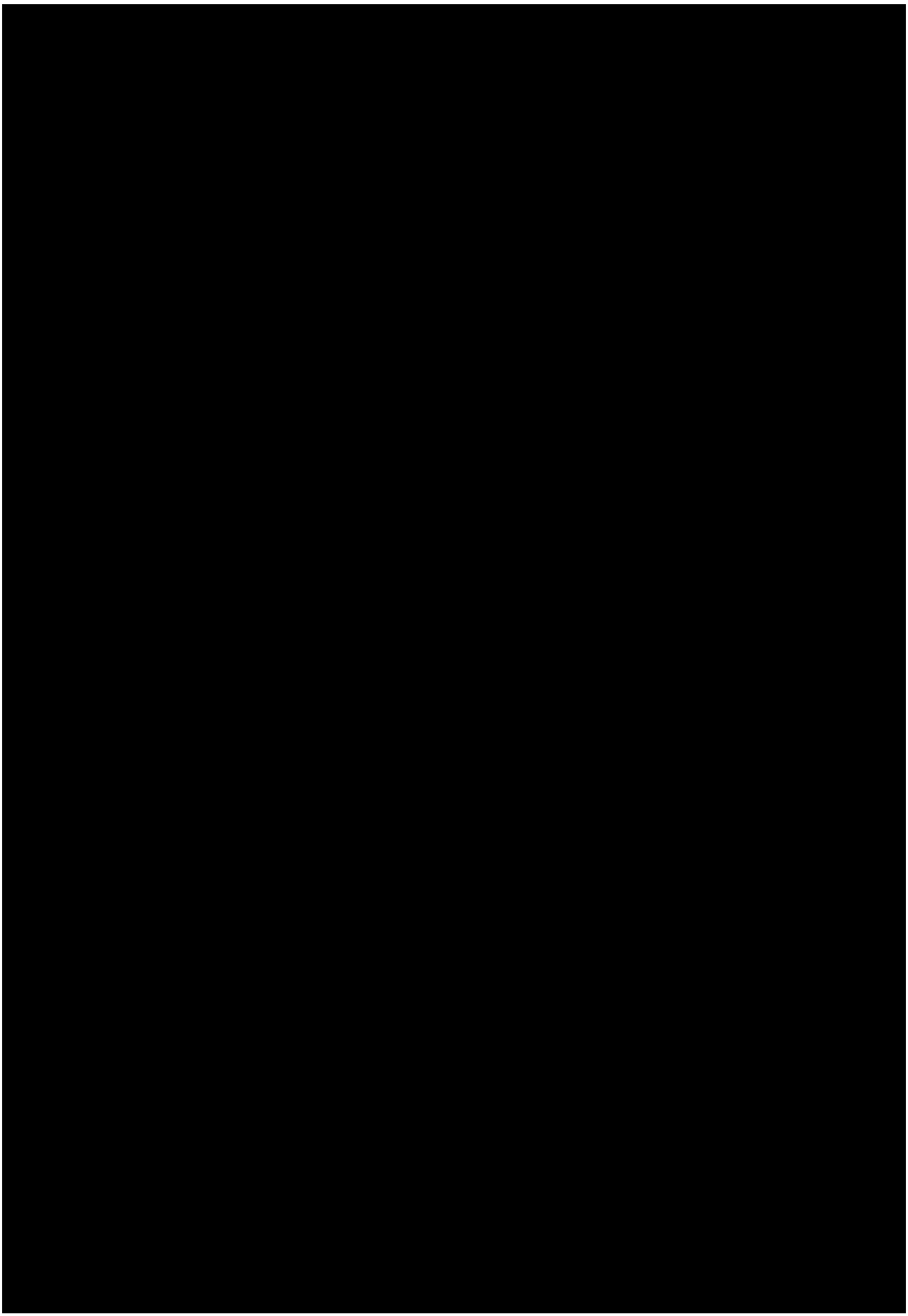
Hu ADA: human ADA cDNA (codon optimised)

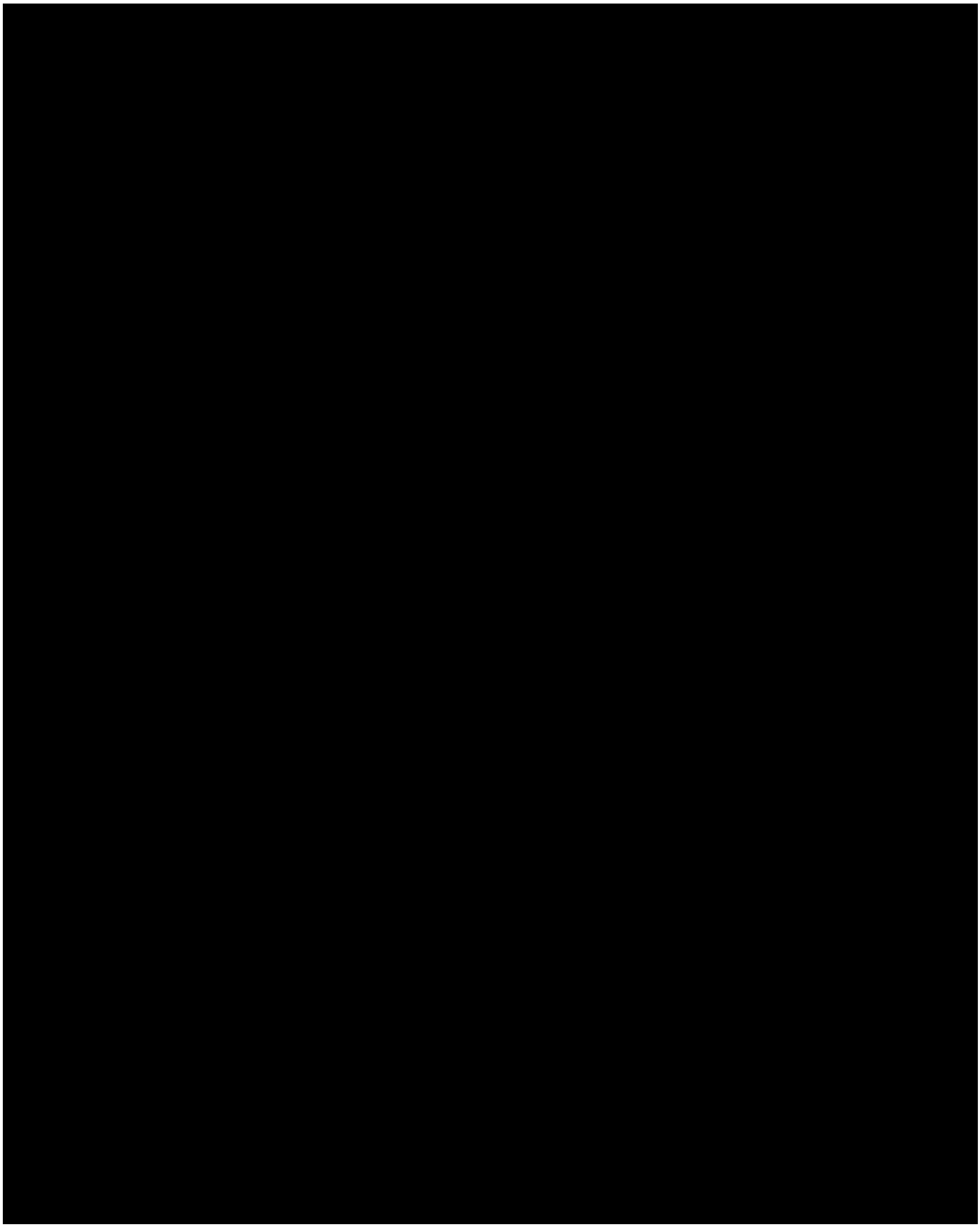
WPRE: mutated form of Woodchuck Hepatitis Virus post transcriptional regulatory element

[redacted]

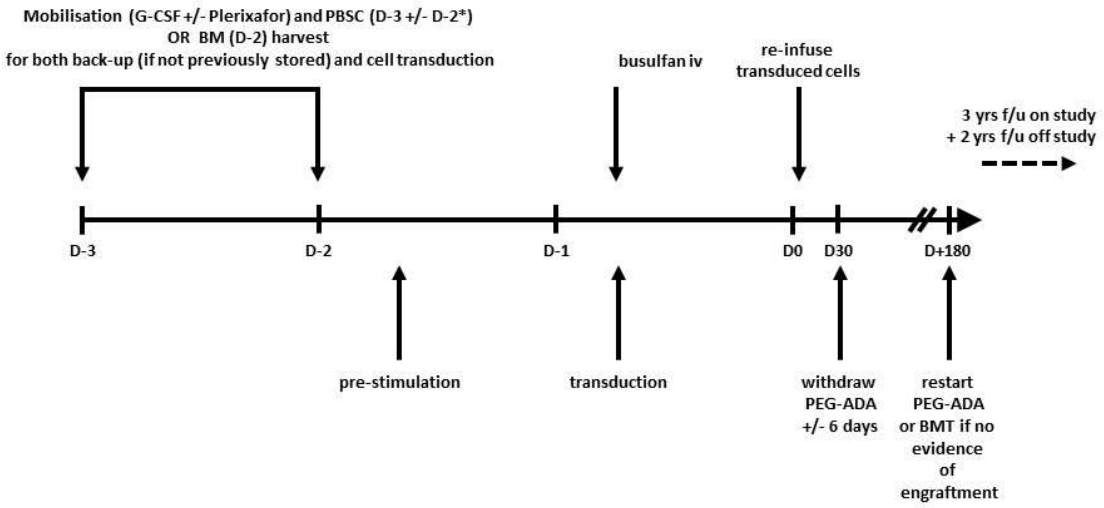
[redacted]

[redacted]





Appendix 3: Transduction protocol schematic



* If enough cells from first apheresis (D-3) is collected, no need to perform second apheresis (D-2)

Appendix 5: ADA-SCID patient monitoring protocol

		Timepoint (months) ^											
	Visit window			**+/- 5d**	**+/- 5d**	**+/- 2w**	**+/- 2w**	**+/- 2w**	**+/- 2w**	**+/- 2w**	**+/- 2w**	**+/- 2w**	**+/- 2w**
Assessment	**Parameter to be reported on the eCRF**	**PreGT**	**Treatment**	**1**	**1.5**	**3**	**6**	**9**	**12**	**18**	**24**	**30**	**36**
Inclusion / Exclusion criteria	-	**X**											
Pregnancy test for patient >12 years old	-	**X***											
Diagnosis	DNA sequencing (mutation, exon, polymorphism) OR Confirmed absence of <3% enzymatic activity	**X**											
Medical history	Infection/hospitalisation history Transplantation history	**X**											
Coagulation testing	PTT, APT, Thrombin Time, Fibrinogen	**X**											
Blood ABO testing	Determination, Rhesus	**X**											
Extended B cell memory	CD19 Naïve B cells CD19+IgD+CD27- Non Switched memory B cells CD19+IgD+CD27+ Class Switched memory B cells CD19+IgD-CD27+ Transitional B cells CD19+IgM++CD38++ Plasmablasts CD19+CD38++IgMwk CD21 low B cells CD19+CD21wkCD38wk	**X**							**X**		**X**		**X**
Virology	CMV, EBV, Adenovirus, HIV1, HIV2, VZV, Hep A, Hep B, HSV, Hep C, Syphilis, HTLV1, HTLV2, Toxoplasma	**X**											
Biochemistry	Sodium, Potassium, Urea, Creatinine, Calcium, Magnesium, Phosphate, Albumin, Alkaline Phosphatase (ALP), Alanine Transaminase(ALT), gamma-glutamyltranferase(GGT), Bilirubin	**X**		**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**
Glomerular Filtration	Weight, Creatinine,Distribution volume,Half-life Glomerular Filtration, corrected GFR	**X**											
Chest x-ray	Normal/Abnormal	**X**											
ECG	Normal/Abnormal	**X**											
Echocardiogram	Normal/Abnormal	**X**											
Lung function test if applicable\\	Normal/Abnormal	**X**											
Dental	Normal/Abnormal	**X**											
Audiology	Normal/Abnormal	**X**											
Maternal engraftment studies	Normal/Abnormal	**X**											

Assessment	Result to be reported on the eCRF	PreGT	Treatment	1	1.5	3	6	9	12	18	24	30	36
Back-up harvest	Harvest type BM or leukapheresis, CD34+cell count		X										
Harvest for IMP preparation	If leukapheresis : Mobilisation: G-CSF (date dose) +/- Plerixafor (date, dose), Leukapheresis harvest: patient's weight, date, CD34+cell dose for transduction If BM: patient's weight, date, CD34+cell dose for transduction)		X										
Conditioning	Patient weight, Busulfan dose, Busulfan AUC		X										
IMP administration	Infusion date, IMP expiry, IMP start date, IMP end date, IMP cell dose, IMP viability, IMP volume Observation before, +15min, after infusion		X										
Vital Signs	Patient's weight Temperature Heart rate Respiratory rate Oxygen saturation	X	X	X	X	X	X	X	X	X	X	X	X
Physical examination	Cardiovascular system Respiratory system Ear, Nose, Throat, Neck and Eyes (only at PreGT, 36 m) Neurological System (only at PreGT, 36 months) Abdominal Skin Other	X		X	X	X	X	X	X	X	X	X	X
Infection status	Evaluate any active infection at time of visit (respiratory tract, meningitis, sepsis, skin and mucosa, ear, nose and throat, abscess, persistent fever, other) Evaluate if there is any infection resulting in hospitalisation since last visit	X		X	X	X	X	X	X	X	X	X	X
PEG-ADA ERT status	Confirmation that if PEG-ADA has been administered or not since last visit	X		X	X	X	X	X	X	X	X	X	X
Blood Count	Haemoglobin, Platelet count White cell count Neutrophil count Lymphocyte count	X		X	X	X	X	X	X	X	X	X	X

Assessment	Result to be reported on the CRF	PreGT	Treatment	1	1.5	3	6	9	12	18	24	30	36
Lymphocyte subset counts	CD 3 CD19 CD16+ CD 56+ CD3+ CD4+ CD3+ CD8+ Naïve: CD4+CD45RA+CD27+	X		X	X	X	X	X	X	X	X	X	X
T cell function PHA stimulation**	Normal/Abnormal	X				X	X		X	X	X		
Lymphocyte function CD3 stimulation**	Normal/Abnormal								X			X	
GAM ***	Ig G level Ig A level Ig M level Immunoglobin status since last visit	X					X	X	X	X	X	X	X
TRECS	TRECS on T cells TRECS on MNCs	X					X		X		X		
Vβ Spectratyping	Reported as Normal/Abnormal						X		X	X	X	X	X
Vector copy number in cells (qPCR)	vector copy number in PBMC vector copy number in CD34 vector copy number in CD3 vector copy number in CD19 vector copy number in myeloid					X	X		X		X		X
Integration analysis	Normal/Abnormal					X****	X****		X		X****		X
Tetanus vaccination responses	Normal/Abnormal										X		X
ADA metabolite analysis (dATP) and ADA activity	Plasma deoxyadenosine level ADA (rbc) level dATP level	X		X	X	X	X	X	X	X	X	X	X
RCL (blood)	Reported as detectable / not detectable	X				X****	X****		X		X****		X

Assessment	Result to be reported on the CRF	PreGT	Treatment	1	1.5	3	6	9	12	18	24	30	36

Bone marrow aspirate for vector copy number qPCR and integration analysis (optional)	Morphology (Normal/ Abnormal) Vector copy number in BM-MNC Integration analysis (Normal / Abnormal)								x		x		x
Immunology save serum & SCLN (stored cells)	Serum collection date SCLN s collection date	x					x		x	x	x	x	x
Reporting of Adverse Event			x	x	x	x	x	x	x	x	x	x	x
Reporting of Concomitant Treatment^^			x	x	x	x	x	x	x	x	x	x	x

^Lab results to be entered into eCRF for these time points should be the ones closer to the consent date/ visit date
^^ Concomitant reporting: In case of any change in administering a concomitant medication i.e. dose, frequency etc. the previous medication should be stopped where the new medication has started
** Assay to be performed only if patient > 12 years of age*
*** Assay can only be performed if the lymphocyte count > 0.5 x 10^9/L*
 # Assay to be carried out until evidence of T cell recovery
**** Pre-gene therapy assay should be carried out only if not already done at diagnosis*
*****Sample are stored but analysed retrospectively only in case sample at 1 year visit or 3 year visit are positive*

PHA - phytohaemagglutinin stimulation, **Ig** – immunoglobulin, **TCR** - T cell receptor, **RCL** - replication competent lentivirus, **TREC** - TCR excision circles, **PCR** - polymerase chain reaction, **SCLN** - save cells in liquid nitrogen

Appendix 6: Undesirable effects of Busilvex (Busulfan)

(taken from the summary of product characteristics (Pierre Fabre Limited))

4.8 Undesirable effects

Undesirable effects in adults: Adverse events information is derived from two clinical trials (n=103) of Busilvex.

Serious toxicities involving the hematologic, hepatic and respiratory systems were considered as expected consequences of the conditioning regimen and transplant process. These include infection and Graft-versus host disease (GVHD) which although not directly related, were the major causes of morbidity and mortality, especially in allogeneic HPCT.

Blood and the lymphatic system disorders:

Myelo-suppression and immuno-suppression were the desired therapeutic effects of the conditioning regimen. Therefore all patients experienced profound cytopenia: leukpoenia 96%, thrombocytopenia 94% and anemia 88%. The median time to neutropenia was 4 days for both autologous and allogeneic patients. The median duration of neutropenia was 6 days and 9 days for autologous and allogeneic patients.

Immune system disorders:

The incidence of acute graft versus host disease (a-GVHD) data was collected in OMC-BUS-4 study (allogeneic) (n=61). A total of 11 patients (18%) experienced a-GVHD. The incidence of a-GVHD grades I-II was 13% (8/61), while the incidence of grade III-IV was 5% (3/61). Acute GVHD was rated as serious in 3 patients. Chronic GVHD (c-GVHD) was reported if serious or the cause of death, and was reported as the cause of death in 3 patients.

Infections and infestations:

39% of patients (40/103) experienced one or more episodes of infection, of which 83% (33/40) were rated as mild or moderate. Pneumonia was fatal in 1% (1/103) and life-threatening in 3% of patients. Other infections were considered severe in 3% of patients. Fever was reported in 87% of patients and graded as mild/moderate in 84% and severe in 3%. 47% of patients experienced chills which were mild/moderate in 46% and severe in 1%.

Hepato-biliary disorders:

15% of SAEs involved liver toxicity. HVOD is a recognised potential complication of conditioning therapy post-transplant. Six of 103 patients (6%) experienced HVOD. HVOD occurred in: 8.2% (5/61) allogeneic patients (fatal in 2 patients) and 2.5% (1/42) of autologous patients. Elevated bilirubin (n=3) and elevated AST (n=1) were also observed. Two of the above four patients with serious serum hepatotoxicity were among patients with diagnosed HVOD.

Respiratory, thoracic and mediastinal disorders:

One patient experienced a fatal case of acute respiratory distress syndrome with subsequent respiratory failure associated with interstitial pulmonary fibrosis in the Busilvex studies.

In addition, the literature review reports alterations of cornea and lens of the eye with oral busulfan.

Adverse events in newborn infants, children and adolescents:

Adverse events information are derived from the clinical study in paediatrics (n=55). Serious toxicities involving the hepatic and respiratory systems were considered as expected consequences of the conditioning regimen and transplant process.

Immune system disorders: The incidence of acute graft versus host disease (a-GVHD) data was collected in allogeneic patients (n=28). A total of 14 patients (50%) experienced a-GVHD. The incidence of a-GVHD grades I-II was 46.4% (13/28), while the incidence of grade III-IV was 3.6% (1/28). Chronic GVHD was reported only if it is the cause of death: one patient died 13 months post-transplant.

Infections and infestations:

Infections (documented and non-documented febrile neutropenia) were experienced in 89% of patients (49/55). Mild/moderate fever was reported in 76% of patients.

Hepato-biliary disorders:

Grade 3 elevated transaminases were reported in 24% of patients. VOD was reported in 15% (4/27) and 7% (2/28) of the autologous and allogenic transplant respectively. VOD observed were neither fatal nor severe and resolved in all cases.

Adverse reactions reported both in adults and paediatric patients as more than an isolated case are listed below, by system organ class and by frequency. Frequencies are defined as: very common (>1/10), common (>1/100, <1/10), uncommon (>1/1,000, <1/100).

System organ class	Very common	Common	Uncommon
Blood and lymphatic system disorders	Neutropenia Thrombocytopenia Anaemia Pancytopenia Febrile neutropenia		
Nervous system disorders	Insomnia Dizziness Depression	Confusion	Delirium Nervousness Hallucination Agitation Encephalopathy Cerebral haemorrhage Seizure
Metabolism And nutrition disorders	Anorexia Hyperglycaemia Hypomagnesaemia Hypokalaemia Hypocalcaemia Hypophosphatemia Oedema	Hyponatraemia	
Psychiatric disorders	Anxiety		
Cardiac disorders	Tachycardia Hypertension Hypotension Vasodilatation Thrombosis	Arrhythmia Atrial fibrillation Cardiomegaly Pericardial effusion Pericarditis Decrease ejection fraction	Femoral artery thrombosis Ventricular extrasystoles Bradycardia Capillary leak syndrome
Respiratory thoracic and mediastinal disorders	Dyspnoea Rhinitis Pharyngitis Cough Hiccup Epistaxis Abnormal breath sounds	Hyperventilation Respiratory failure Alveolar haemorrhages Asthma Atelectasis Pleural effusion	Hypoxia
Gastrointestinal disorders	Nausea Stomatitis Vomiting Diarrhoea Constipation Dyspepsia Anus discomfort Abdominal pain Ascites	Oesophagitis Ileus Haematemesis	Gastrointestinal haemorrhage
Hepato-biliary disorders	Hepatomegaly Jaundice		
Skin and subcutaneous tissue disorders	Rash Pruritis Alopecia	Erythema Pigmentation disorder Skin desquamation	
Musculoskeletal connective tissue disorders	Back pain Myalgia Arthralgia		
Renal and urinary disorders	Creatinine elevated Dysuria Oligurea	Bun increase Haematuria Moderate renal insufficiency	
General disorders and administration site conditions	Fever Headache Asthenia Chills Pain Allergic reaction Oedema general Pain or inflammation at injection site Chest pain Mucositis		

Appendix 7: WHO classification of acute/subacute toxic effects

ITEM	Grade 1 Toxicity	Grade 2 Toxicity	Grade 3 Toxicity	Grade 4 Toxicity
HEMATOLOGY				
Hemoglobin	9.5 - 10.5 gm/Dl	8.0 - 9.4 gm/Dl	6.5 - 7.9 gm/Dl	< 6.5 gm/Dl
Absolute Neutrophil Count	1000-1500/mm^3	750-999/mm^3	500-749/mm^3	<500/mm^3
Platelets	75000-99000/mm^3	50000-74999/mm^3	20000-49000/mm^3	<20000/mm^3
Prothrombin Time (PT)	1.01 - 1.25 x ULN	1.26-1.5 x ULN	1.51 -3.0 x ULN	>3 x ULN
Activated Partial Thromboplastin (APPT)	1.01 -1.66 x ULN	1.67 - 2.33 x ULN	2.34 - 3 x ULN	> 3 x ULN
Fibrinogen	0.75 - 0.99 X LLN	0.50 - 0.74 x LLN	0.25 - 0.49 x LLN	< 0.25 x LLN
Fibrin Split Product	20-40 mcg/ml	41-50 mcg/ml	51-60 mcg/ml	> 60 mcg/ml
Methemoglobin	5 - 9.9 %	10.0 - 14.9 %	15.0 - 19.9%	> 20 %
LIVER ENZYMES				
AST (SGOT)	1.25 - 2.5 x ULN	2.6 - 5 x ULN	5.1 - 10 x ULN	> 10 x ULN
ALT (SGPT)	1.25 - 2.5 x ULN	2.6 - 5 x ULN	5.1 - 10 x ULN	> 10 x ULN
GGT	1.25 -2.5 x ULN	1.6 - 5 x ULN	5.1 - 10 x ULN	> 10 x ULN
Alkaline Phosphatase	1.25 - 2. 5 x ULN	1.6 - 5 x ULN	5.1 - 10 x ULN	> 10 x ULN
Amylase	1.1 - 1.5 x ULN	1.6 - 2.0 x ULN	2.1 - 5.0 x ULN	> 5.1 x ULN
CHEMISTRIES				
Hyponatremia	130-135 mEq/L	123-129 mEq/L	116-122 mEq/L	< 116 or mental status changes or seizures
Hypernatremia	146-150 mEq/L	151-157 mEq/L	158-165 mEq/L	> 165 mEq/L or mental status changes or seizures
Hypokalemia	3.0 - 3.4 mEq/L	2.5 - 2.9 mEq/L	2.0 - 2.4 mEq/L or intensive replacement Rx required or hospitalization required.	< 2.0 mEq/L or paresis or ileus or life-threatening arrhythmia
Hyperkalemia	5.6 - 6.0 mEq/L	6.1 - 6.5 mEq/L	6.6 - 7.0 mEq/l	> 7.0 mEq/L or life-threatening arrhythmia
Hypoglycemia	55-64 mg/dL	40-54 mg/dL	30-39 mg/dL	<30 mg/dL or mental status changes or coma
Hyperglycemia (note if fasting)	116 - 160 mg/dL	161- 250 mg/dL	251 - 500 mg/dL	> 500 mg/dL or ketoacidosis or seizures
Hypocalcemia (corrected for albumin)	8.4 - 7.8 mg/dL	7.7 - 7.0 mg/dL	6.9 - 6.1 mg/dL	< 6.1 mg/dL or life threatening arrhythmia or tetany
Hypercalcemia (correct for albumin)	10.6 - 11.5 mg/dL	11.6 - 12.5 mg/dL	12.6 - 13.5 mg/dL	> 13.5 mg/dL life-threatening arrhythmia

Hypomagnesemia	1.4 - 1.2 mEq/L	1.1 - 0.9 mEq/L	0.8 - 0.6 mEq/L	< 0.6 mEq/L or life-threatening arrhythmia
Hypophosphatemia	2.0 - 2.4 mg/dL	1.5 -1.9 mg/dL or replacement Rx required	1.0 -1.4 mg/dL intensive Rx or hospitalization required	< 1.0 mg/dL or life-threatening arrhythmia
Hyperbilirubinemia	1.1 - 1.5 x ULN	1.6 - 2.5 x ULN	2.6 - 5 x ULN	> 5 x ULN
BUN	1.25 - 2.5 x ULN	2.6 - 5 x ULN	5.1 - 10 x ULN	> 10 x ULN
Creatinine	1.1 x 1.5 x ULN	1.6 - 3.0 x ULN	3.1 - 6 x ULN	> 6 x ULN or required dialysis
URINALYSIS				
Proteinuria	1+ or < 0.3% or <3g/L or 200 mg - 1 gm loss/day	2 -3 + or 0.3 - 1.0% or 3-10 g/L 1- 2 gm loss/day	4+ or > 1.0% or > 10 g/L 2-3.5 gm loss/day	nephrotic syndrome or > 3.5 gm loss/day
Hematuria	microscopic only	gross, no clots	gross+ clots	obstructive or required transfusion
CARDIAC DYSFUNCTION				
Cardiac Rhythm		asymptomatic, transient signs, no Rx required	recurrent/persistent; No Rx required	requires treatment
Hypertension	transient inc. > 20 mm; no Rx	recurrent, chronic, > 20 mm, Rx required	requires acute Rx; No hospitalization	requires hospitalization
Hypotension	transient orthostatic hypotension, No Rx	symptoms correctable with oral fluids Rx	requires IV fluids; no hospitalization required	requires hospitalization
Pericarditis	minimal effusion	mild/moderate asymptomatic effusion, no Rx	symptomatic effusion; pain; EKG changes	tamponade; pericardiocentesis or surgery required
Hemorrhage, Blood Loss	microscopic/occult	mild, no transfusion	gross blood loss; 1-2 units transfused	massive blood loss; > 3 units transfused
RESPIRATORY				
Cough	transient- no Rx	treatment associated cough local Rx	uncontrolled	
Bronchospasm, Acute	transient; no Rx < 80% - 70% FEV_1 (or peak flow)	requires Rx normalizes with bronchodilator; FEV_1 50% - 70% (or peak Flow)	no normalization with bronchodilator; FEV_1 25% - 50% (or peak flow retractions)	cyanosis: FEV_1 < 25% (or peak flow) or intubated
GASTROINTESTINAL				
Stomatitis	mild discomfort; no limits on activity	some limits on eating/drinking	eating/talking very limited	requires IV fluids
Nausea	mild discomfort; maintains reasonable intake	moderate discomfort; intake decreased significantly; some activity limited	severe discomfort; no significant intake; activities limited	minimal fluid intake
Vomiting	transient emesis	occasional/moderate vomiting	orthostatic hypotension or IV fluids required	hypotensive shock or hospitalization required for IV fluid therapy
Constipation	mild	moderate	severe	distensions w/vomiting

Diarrhea	transient 3-4 loose stools/day	5-7 loose stools/day	orthostatic hypotension or > 7 loose stools/day or required IV fluids	hypotensive shock or hospitalization for IV fluid therapy required
NEURO & NEUROMUSCULAR				
Neuro-Cerebellar	slight incoordination dysdiadochokinesis	intention tremor, dysmetria, slurred speech; nystagmus	locomotor ataxia	incapacitated
Mood	mild anxiety or depression	moderate anxiety or depression and therapy required	severe anxiety or depression or mania; needs assistance	acute psychosis; incapacitated, requires hospitalization
Neuro Control (ADL = activities of daily living)	mild difficulty concentrating; no Rx; mild confusion/agitation; ADL unaffected	moderate confusion/agitation some limitation of ADL; minimal Rx	severe confusion/agitation needs assistance for ADL; therapy required	toxic psychosis; hospitalization
Muscle Strength	subjective weakness no objective symptoms/ signs	mild objective signs/symptoms no decrease in function	objective weakness function limited	paralysis
OTHER PARAMETERS				
Fever: oral, > 12 hours	37.7 - 38.5 C or 100.0 - 101.5 F	38.6 - 39.5 C or 101.6 - 102.9 F	39.6 - 40.5 C or 103 - 105 F	> 40 C or > 105 F
Headache	mild, no Rx therapy	transient, moderate; Rx required	severe; responds to initial narcotic therapy	intractable; required repeated narcotic therapy
Fatigue	no decrease in ADL	normal activity decreased 25- 50%	normal activity decreased > 50% can't work	unable to care for self
Allergic Reaction	pruritus without rash	localized urticaria	generalized urticaria; angioedema	anaphylaxis
Local Reaction	tenderness or erythema	induration < 10 cm or phlebitis or inflammation	induration > 10 cm or ulceration	necrosis
Mucocutaneous	erythema; pruritus	diffuse, maculo papular rash, dry desquamation	vesiculation, moist desquamation, or ulceration	exfoliative dermatitis, mucous membrane involvement or erythema, multiforme or suspected Stevens-Johnson or necrosis requiring surgery

Appendix 8: Details of the historical HSCT control group

1. Objective

Historical data from ADA-SCID patients who were treated with Hematopoietic Stem Cell Transplantation (HSCT) at GOSH during the period of 2000-2016 will be collected. HSCT is currently the standard of care for ADA-SCID patients. These data will be used as historical control group in a current on-going gene therapy study "Phase I/II, historical controlled, open-label, non-randomised, single-centre trial to assess the safety and efficacy of EF1αS-ADA lentiviral vector mediated gene modification of autologous CD34+ cells from ADA-deficient individuals". The objective is to compare efficacy and safety of gene therapy versus HSCT to determine the optimal treatment.

2. Study population

The control group will be patients from 0-18 years of age with a confirmed diagnosis of ADA deficiency transplanted at Great Ormond Street Hospital (GOSH) since 2000. See inclusion criteria page 64.

3. Data to be collected

GOSH holds a registry / clinical database (European Society for Immunodeficiencies (ESID) and European Society for Blood and Marrow Transplant (EBMT) Registry, GOSH BMT clinical database) displaying information on these types of patients. Patients treated with HSCT have been identified who received a transplant at GOSH between 2000-2016. . ESID and EBMT will allow us to collect the below information:

- Demographics (gender, date of birth, date of death if any),
- Method of ADA-SCID diagnosis,
- HSCT treatment(s) including: date of treatment, transplant cells, donor type and outcome

However, to make this historical control group valid for marketing authorisation application, our discussions with regulatory agencies suggests that we need to collect further information valuable to compare the efficacy and safety of any new treatment in research vs. current HSCT standard of care as:

- Previous PEG-ADA ERT (and duration of treatment),
- Previous or current immunoglobulin therapy,
- Listing of relevant medical history, including infections (severe or opportunistic infections)
- Vaccination history and immunological response to vaccination

- Auto-immune disease
- Malignant disease / leukaemia
- Graft versus host disease
- ADA activity and ADA metabolites
- AEs and SAEs

This information is only available from the patient's records; we therefore plan to re-consent these patients to access their full medical records.

4. Data collection process

This is a retrospective, non-interventional data collection activity; the patients follow their routine medical practice. We will contact these patients or their authorised representative and seek their consent to access their medical notes and share the information with regulatory agencies and pharmaceutical companies involved with the licensing process. HSCT historical control group data team will work on the collection of data from GOSH registries and patients' records, data will be entered into an electronic Case Report Form (eCRF). This eCRF will be monitored as per standard process before it can be used for statistical analysis and submission to agencies for review and assessment.

5. Data Analysis

The comparison analysis between gene therapy and HSCT will be performed evaluating data related to:

Survival and event free survival, defined as need to return to PEG-ADA or second transplant at 1 and 2 years post treatment.

Use of immunoglobulin replacement therapy (IgRT) and infections post treatments. Infections are defined as infections requiring hospitalisation or prolonging hospitalization and/or documented infections by opportunistic pathogens (interstitial pneumonia, intractable diarrhoea)

Appendix 9: Details of the CUP group

Great Ormond Street Hospital holds a Specials License from the MHRA for the manufacture of gene modified products for compassionate use. This license allows a physician to treat a patient with an unlicensed product in case of a special need. These ADA-SCID patients treated under this Special License could not be enrolled into the clinical trial for various reasons: the trial was not yet opened and patients were in clinical need of treatment; the patient could not meet the inclusion/exclusion criteria; the final formulation of the product was different (cryopreserved instead of fresh transduced cells); the product could not meet the release specification, the trial recruitment was already completed and patients were in clinical need of treatment.

However, in the treatment of these patients we have applied similar standards to clinical trial i.e. the patient gives their consent to collect data, the same trial monitoring schedule/trial procedure is applied, and pharmacovigilance (SAE) is documented in the same way. These patients will be included part of the analysis for the safety and secondary efficacy objectives only as mentioned in section 8.9.