

## Supplementary Online Content

### **Safety and early efficacy outcomes for lentiviral fibroblast gene therapy in recessive dystrophic epidermolysis bullosa.**

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## Supplemental Methods

**Supplemental Table 1. Trial protocol synopsis**

<b>Title of clinical trial</b>	<b>Phase 1 study of lentiviral-mediated <i>COL7A1</i> gene-modified autologous fibroblasts in adults with recessive dystrophic epidermolysis bullosa (RDEB).</b>
<b>Protocol Short Title/Acronym</b>	<b>LENTICOL-F</b>
<b>Study Phase</b>	1
<b>Sponsors</b>	<b>Lead Sponsor:</b> King's College London (King's Health Partners) <b>Co-Sponsor:</b> Guy's and St Thomas' NHS Foundation Trust
<b>Chief Investigator</b>	Professor John A McGrath
<b>Medical condition or disease under investigation</b>	Recessive Dystrophic Epidermolysis Bullosa (RDEB)
<b>Purpose of clinical trial</b>	To assess whether intradermal injections of self-inactivating (SIN) lentiviral vector (LV)-mediated <i>ex vivo</i> transduced autologous fibroblasts expressing codon-optimised <i>COL7A1</i> (investigational medicinal product – IMP), are safe and have potential efficacy in adults with RDEB.
<b>Primary objective</b>	To evaluate the safety of intradermal injections of SIN LV-mediated <i>COL7A1</i> gene-modified autologous fibroblasts in adults with RDEB.
<b>Secondary objectives</b>	<ol style="list-style-type: none"> <li>1. To evaluate the potential efficacy of intradermal injections of SIN LV-mediated <i>COL7A1</i> gene-modified autologous fibroblasts in adults with RDEB at week (W) 2, month (M) 3 and M12 after the IMP injections.</li> <li>2. To evaluate the immune response against the recombinant type VII collagen (C7) at W2, W4, M3, M6 and M12 after the IMP injections compared to baseline.</li> </ol>
<b>Trial Design</b>	Phase 1, non-randomized, open-label, single-center, proof-of-concept study.
<b>Endpoints</b>	<p><b><u>Primary Endpoints</u></b></p> <ul style="list-style-type: none"> <li>• Adverse events (AEs), Serious Adverse Events (SAEs), Adverse Reactions (ARs) and Serious Adverse Reactions (SARs) at each visit over a 12-month follow-up period.</li> </ul>

	<p><b><u>Secondary Endpoints</u></b></p> <ul style="list-style-type: none"> <li>• Skin biopsy analysis of treated skin at W2, M3 and M12 compared to untreated skin: <ol style="list-style-type: none"> <li>I. C7 protein expression by immunofluorescence microscopy (IF)</li> <li>II. Morphology of anchoring fibrils at the dermal-epidermal junction (DEJ) by transmission electron microscopy (TEM)</li> <li>III. Vector copy number by quantitative polymerase chain reaction (qPCR)</li> </ol> </li> <li>• Serum analysis for: <ol style="list-style-type: none"> <li>IV. Detection of anti-C7 antibodies by enzyme-linked immunosorbent assay (ELISA) (against NC1 and NC2 domains of C7) and indirect immunofluorescence (IIF) at W2, W4, M1, M3, M6 and M12 post-injections</li> <li>V. Detection of T-cell responses to the full length C7 by enzyme-linked immunosorbent spot (ELISPOT) assay at W4, M6 and M12 post-injections.</li> </ol> </li> </ul>
<b>Sample Size</b>	5–10 patients
<b>Summary of eligibility criteria</b>	<p><b><u>Inclusion criteria</u></b></p> <ol style="list-style-type: none"> <li>1. Clinical and genetic diagnosis of RDEB with confirmed bi-allelic <i>COL7A1</i> mutations.</li> <li>2. A reduced number or morphologically abnormal anchoring fibrils confirmed by TEM.</li> <li>3. At least 5x3cm of intact skin on the trunk and/or extremities that is suitable for cell injections.</li> <li>4. Able to undergo local anesthesia.</li> <li>5. Subjects aged ≥ 17years and able to give informed consent prior to the first study intervention.</li> </ol> <p><b><u>Exclusion criteria</u></b></p> <ol style="list-style-type: none"> <li>1. Subjects who received other investigational medicinal products within 6 months prior to enrolment into this study.</li> <li>2. Past medical history of biopsy proven skin malignancy.</li> <li>3. Subjects who have received immunotherapy including oral corticosteroids (Prednisolone &gt;1mg/kg) for more than one week (intranasal and topical preparations are permitted) or chemotherapy within 60 days of enrolment into this study.</li> <li>4. Known allergy to any of the constituents of the investigational medicinal product (IMP).</li> <li>5. Subjects with <b>BOTH</b>: <ul style="list-style-type: none"> <li>• positive serum antibodies to C7 confirmed by ELISA <b>and</b></li> <li>• positive IIF with binding to the base of salt split skin.</li> </ul> </li> <li>6. Subjects with positive results for HIV, Hepatitis B, Hepatitis C, HTLV or Syphilis.</li> <li>7. Subjects who are pregnant or of child-bearing potential who are neither abstinent nor practising an acceptable means of contraception when this is in line with the usual and preferred lifestyle of the subject, as determined by the Investigator, for 12 months after the cell injections.</li> </ol>
<b>IMP, dosage and route of administration</b>	<b>IMP:</b> SIN LV-mediated <i>ex vivo</i> transduced autologous fibroblasts expressing codon-optimised <i>COL7A1</i> .

	<p><b>DOSAGE:</b> 0.8–1.2 million cells suspended in 0.25ml of 0.9% saline per injection over 1 cm<sup>2</sup> of intact skin (3 injections of IMP at a single timepoint).</p> <p><b>ROUTE OF ADMINISTRATION:</b> Intradermal injections.</p>
<b>Active comparator product (Control)</b>	None.
<b>Maximum duration of study participation</b>	32 months (each patient will be followed up for a total of 12 months post-IMP injections and screening will take place up to 4 months prior to the intervention).
<b>Version and date of protocol</b>	Version 4.0, 01/MAR/2016

*Production of third-generation SIN LV encoding codon-optimized full-length COL7A1 cDNA.* This was manufactured as described in Georgiadis *et al.* (2016).<sup>1</sup>

*Skin procurement and biopsies.* Alongside the baseline biopsy from the left upper arm, a 6mm punch biopsy was obtained for the skin procurement from an area of intact, unblistered skin under local anesthesia (1% xylocaine) using aseptic technique. The skin procurement was then loosely wrapped in sterile gauze soaked in 0.9% sodium chloride solution, placed in a sterile universal container with a sealed lid and transported at 2–8°C with a temperature log (TempMate®) in a medical cooler bag from the Clinical Research Facility (CRF) at Guy’s Hospital, London, U.K to Cellular Therapies, GOSH, London, UK, for cell culture and manufacture of gene-modified autologous fibroblasts. During follow-up visits at 2 weeks, 3 months and 12 months after treatment, two ~6 mm shave skin biopsies were obtained from the left upper arm, one from the injected skin and one from the non-injected skin (as control) ~2 cm away from the injected skin. The non-injected skin biopsy was obtained before the injected skin biopsy using a separate biopsy kit in order to avoid cross-

contamination. Both biopsies were further sectioned for IF, TEM, qPCR and snap-frozen and archived for integration site analysis in the event of serious adverse reactions.

*Isolation and propagation of fibroblasts.* The bottle containing the skin sample was cleaned by wiping with 70% ethanol and then transferred to a clean production room. The skin biopsy was washed with PBS and immersed in a tube containing 5mL of neutral protease NB (1 unit /ml); and incubated at 37°C for 3 hrs. Three hours later, the tissue was transferred into a petri-dish and the epidermis was peeled off from the dermis using sterile forceps. The remaining dermis was cut into small pieces (about 1 mm<sup>2</sup>) and transferred into a fresh tube containing 5mL of collagenase NB6 (0.45 units/ml), and incubated at 37°C for 2.5 hrs. The tissue/cells were re-suspended in PBS and pipetted up and down several times to obtain a homogeneous single cell suspension. An equal volume of culture media was then added into the tube to neutralize the digestion solution. The cell suspension was centrifuged to pellet the cells, re-suspended in 3ml culture media and seeded in a T25 cell culture flask. **Note:** the newly seeded cells were incubated for at least 3 days without disturbing. The media was changed 3 to 4 days after seeding. After this point, the media was changed twice a week.

*Gene Engineering.* Once fibroblasts reached confluence, cells were trypsinized and seeded in a 24-well cell culture plate 24 hrs prior to the transduction with *LV-PGK-coCOL7A1* viral vector. In total 10 to 11 wells were seeded with the cells (1x10<sup>5</sup> cells/well) for virus infection. Remaining cells were re-seeded back into the T25 flask as negative control. **Note:** At this stage, cells were cultured in the culture medium without antibiotics as per Medicines and Healthcare products Regulatory Agency (MHRA) for the Advanced Therapy Investigational Medicinal Products (ATIMP)

guidelines 24 hours post-seeding. Cells were then transfected by directly adding thawed virus stock solution into the wells containing fibroblasts at the MOI 5.

*Expansion, characterization and cryopreservation of gene engineered cells.* 1 to 3 days post-transduction, when cells reached confluence, gene engineered fibroblasts were trypsinized, pooled from all 10 or 11 wells and washed with culture media once. Washed cells were re-suspended and seeded in a fresh T75 flask with 20 mL culture medium. Untransduced fibroblasts were also propagated in parallel and were used as negative control. Culture medium was changed twice a week for both cultures. Gene engineered fibroblasts were cultured until the cell numbers reached ~ 9 – 10 million cells, which took approximately 3 - 4 weeks post-transduction, with variation expected between individuals. Once the desired cell number was achieved, cells were trypsinized, washed and re-suspended in culture medium. Both transfected and untransfected cells were counted and divided into several portions as following:  $0.5 \times 10^6$  cells for pro-viral copy number assessment;  $0.5 \times 10^6$  cells for *in situ* cyto-immunofluorescence staining for C7;  $0.5 \times 10^6$  cells for flow cytometry staining for C7 and  $2 \times 10^6$  cells for immunoblotting for C7; The remaining cells were pelleted again. The cell pellets were reconstituted in cryopreservation medium with the concentration of  $2 \times 10^6$  cells / mL and aliquoted 1 mL per cryo-vial for freezing. Initially, the cryopreserved cells were kept in a Mr Frosty™ freezing container and stored at  $-80^{\circ}\text{C}$  for 24 to 48 hrs and then the cells were transferred to a vapor phase liquid nitrogen vessel for long term storage. The temperature of the liquid nitrogen vessel was monitored daily to ensure the temperature was maintained between  $-150$  to  $190^{\circ}\text{C}$ .

*Release of the IMP.* The release was done in two stages, pre-release and final release. *Pre-release.* Pre-release required quality assurance (QA) and a qualified person (QP) checking all relevant documents including sterility tests, the IMP

specificity and any deviations and file notes generated during the production period meet GMP requirements and have been completed and authorized.

*Final release.* This procedure was performed on the day, when the IMP was delivered to the hospital for the patient. The QP checked all documents for QA and Batch Manufacturing Record (BMR), in particular the gram staining result, liquid nitrogen temperature monitor recordings and release criteria based on the product specification (Supplemental Table 2). Once the QP was satisfied the production results meet QA, the following certificates were issued: a batch release certificate, a certificate of analysis, a certificate for material of origin free from transmissible spongiform encephalopathy (TSE) / bovine spongiform encephalopathy (BSE). The issuing of the certificates then allowed IMP dispatch to the trial site at the Guy's Hospital Clinical Research Facility.

*Investigational Medicinal Product (IMP) Packaging and delivery.* A total of three 1 mL syringes were packed with viable gene engineered autologous fibroblasts (2.4 - 3.6 x 10<sup>6</sup> cells per syringes) for the clinical trial. Syringes were placed into a 150mm petri dish and sealed with Tegaderm clinical tape. Each syringe and the petri dish were clearly labeled with the patient ID,IMP information and IMP use information. The product was finally placed into a medical cooler bag at ambient temperature, along with a TempMate® temperature monitor and delivered to the trial site at Guy's Hospital Clinical Research Facility.



**Supplemental Table 2. Specification for releasing RDEB autologous fibroblasts.**

\*depending on the mutation, some subjects may have residual protein expression.

<b>Test</b>	<b>Day/week during manufacturing</b>	<b>Specification for release of gene engineered RDEB cells (IMP)</b>
<b>BACTalert</b>	Weekly	No organisms detected
Mycoplasma test (Mycoplasma genus DNA)	Before release	Not detected
<b>Gram staining</b>	Before release	No organisms detected
<b>Cell number</b>	Before release	2.4-3.6X10 <sup>6</sup> viable gene engineered cells. 0.25ml cell suspensions containing 0.8-1.2 x10 <sup>6</sup> live cells in saline/1mL syringe capped with luer-screw, total 3 syringes
<b>Stable lentiviral transduction</b>	Specific qPCR	Copy number = 0.01-1.0 copy/cell
<b>C7 protein expression*</b>	Flow cytometry, Immunostaining and Immunoblot	Restoration of C7 expression compared to non-modified control (for information)
<b>Cell storage temperature</b>	Daily record	Less or equal to -70°C

*DNA extraction and Quantitative PCR.* Genomic DNA was extracted from cells (0.5x10<sup>6</sup> cells) using QIAGEN genomic DNA extraction kit in accordance with the manufactures instructions. The titer of concentrated LV-coCOL7A1 virus was determined by infecting 293T cells with serial dilutions of concentrated LV-coCOL7A1. Three days after transduction, cells were harvested and copies of HIV Psi packaging element (Psi) encoded by the vector were determined by quantitative polymerase chain reaction (qPCR). Qualified plasmid standards encoding Psi and human albumin sequences were used in qPCR (Supplemental Table 3). Transduction efficiency was determined at week-4 post-transduction.

**Supplemental Table 3. Details of primers used for vector copy number (VCN) RT-qPCR.**

<b>qPCR for detection of the psi (<math>\psi</math>) packaging signal in the pCCL lentiviral backbone</b>	
HIV-psi Forward Primer 5' CAG GAC TCG GCT TGC TGA AG 3'	Invitrogen
HIV-psi Reverse Primer 5' TCC CCC GCT TAA TAC TGA CG 3'	Invitrogen
HIV-psi Probe 5' <b>FAM</b> -CGC ACG GCA AGA GGC GAG G <b>TAMRA</b> -3'	Applied Biosystems
Albumin Forward Primer 5' GCT GCT ATC TCT TGT GGG CTG T 3'	Invitrogen
Albumin Reverse Primer 5' ACT CAT GGG AGC TGC TGG TTC 3'	Invitrogen
Albumin Probe 5' <b>VIC</b> -CCT GTC ATG CCC ACA CAA ATC TCT CC- <b>TAMRA</b> 3'	Applied Biosystems
<b>STANDARD CURVE</b> Human fibrosarcoma HT1080 cell line with 1 copy CAR19 per cell  DNA extracted from Clone-C3 are serial diluted from 10 <sup>5</sup> to 10	In house

*C7 expression using flow cytometry.* Cells (~0.5x10<sup>6</sup> cells) were fixed in Fix & Perm<sup>®</sup> Medium A (FIX & PERM<sup>®</sup> Cell Permeabilization Kit; Cat no. GAS004, Life technologies, UK) for 20 mins at RT in the dark. Then the cells were washed with 2-3 % FBS/PBS, in 2ml, twice; spun down at 1600 rpm for 4 mins at RT. Cell pellet was re-suspended in Fix & Perm<sup>®</sup> Medium B with C7 antibody (COL7A1 Antibody (LH7.2): sc-53226; Santa Cruz Biotechnologies, UK; 1:25 dilution), and incubated at 4°C overnight in dark. The cells were washed with 2 %FBS/ PBS, twice and pelleted at 16,000 rpm for 4 mins. The cell pellet was re-suspended in the Fix & Perm<sup>®</sup> Medium B with FITC-goat anti-mouse 2<sup>ry</sup> antibody (1:200 dilution) (Life technologies, UK), and

incubated at RT for 1hr 30 mins in dark. The cells were washed with 2% FBS/PBS twice, and re-suspended in 0.5 mL 2-3% FBS/PBS and analyzed using flow cytometry immediately.

*Western blot.* Cells ( $2 \times 10^6$ ) cells were suspended in cell lysis buffer composed of 50 mmol/L Tris-HCl pH8.0, 150 mmol/L NaCl, 5 mmol/L EDTA, cocktail protease inhibitors and 1 mmol/L phenylmethanesulfonylfluoride. Samples were lysed by pipetting the cells up and down repeatedly until the cell pellet dissolved completely, and then incubated for 20 mins on ice. The samples were then centrifuged at 12,000 rpm for 10 mins to pellet the insoluble cell debris at 4°C. The total protein concentration in the supernatant was determined using Bio-Rad protein assay kit (Hertfordshire, UK). Samples from cell lysate were further diluted in 5x sample buffer containing 100 mmol/L dithiothreitol, 10% sodium dodecyl sulphate, 30% glycerol, 0.001% bromophenol blue, and 0.5 mmol/L Tris-HCl pH6.8. Equal quantities of total protein were loaded in 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis. After electrophoresis, proteins were transferred to polyvinylidene fluoride membranes and incubated with C7 antibody overnight at RT with shaking. Next day membranes were washed and further incubated with mouse anti-rabbit secondary antibody conjugated with HRP (Sigma-Aldrich, UK) and signals were detected using the ECLplus system (GE Healthcare, UK).

*Immunocytochemistry (ICC).* Cells ( $\sim 0.5 \times 10^6$  cells/per coverslip in 24 well plate) were fixed in 4% neutral buffered formalin, washed once with 1xPBS and permeabilised with 0.1% Triton X-100 in PBS. Cells were incubated with blocking solution (1% BSA in 0.1% PBS) for 20mins at RT, and then incubated overnight at 4°C with C7 primary antibody (LH7.2; sc-53226; Santa Cruz Biotechnologies, UK; 1:25 dilution). After washing, cells were incubated with Alexa-488 goat-anti-mouse 2<sup>nd</sup>

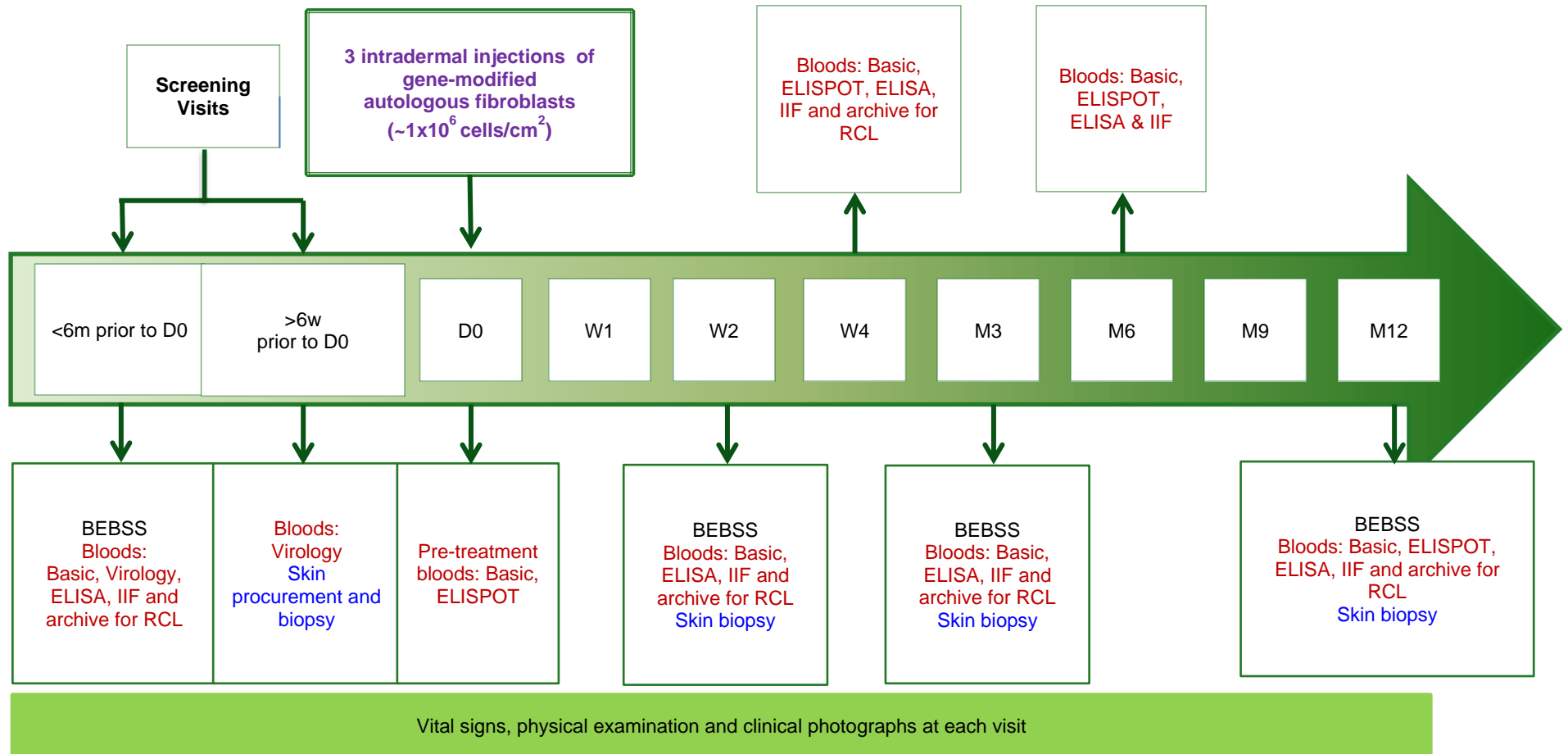
antibody (1:200 dilution, Life technologies UK) and with DAPI (4',6-Diamidino-2-Phenylindole) (Life technologies, UK) Rhodamine phalloidin stain (1:1000) (Sigma-Aldrich, UK) for 20 mins at RT. The slides were mounted with Prolong gold (Life technologies, UK). Micrographs were taken using an upright fluorescence microscope (BX51, Olympus, UK).

*DNA purification from skin tissues.* DNA was extracted from snap-frozen skin biopsies of each subject using QIAamp DNA mini kit (Qiagen, Crawley, UK). Thawed skin biopsy was cut up into small pieces and placed in a 1.5 ml microcentrifuge tube, and 180  $\mu$ l of Buffer ATL was added. 20  $\mu$ l of proteinase K was added and mixed by vortexing at room temperature. The tissue with the mixture was incubated at 56°C until the tissue was completely lysed. The sample was occasionally vortexed during incubation and briefly centrifuged to remove drops from the inside of the lid. 200  $\mu$ l of Buffer AL was added to the sample and mixed by pulse-vortexing for 15 s, incubated at 70°C for 10 min and briefly centrifuged. 200  $\mu$ l of 100% ethanol was added to the sample, again mixed by pulse-vortexing for 15 s and then briefly centrifuged. This mixture including the precipitate was carefully applied to the QIAamp Mini spin column without wetting the rim. The cap was closed and centrifuged at 6000 x g (8000 rpm) for 1 min. The QIAamp Mini spin column was placed in a clean 2 ml collection tube. The tube containing the filtrate was discarded. 500  $\mu$ l of Buffer AW2 was carefully added to the QIAamp Mini spin column without wetting the rim. With the cap closed, the mixture was centrifuged at full speed: 20,000 x g (14,000 rpm) for 3 min. The QIAamp Mini spin column was placed in a new 2 ml collection tube and centrifuged at full speed for 1 min. The old collection tube with the filtrate was discarded. The QIAamp Mini spin column was placed in a clean 1.5 ml microcentrifuge tube and 200  $\mu$ l Buffer AE carefully added before incubation at room temperature for 1 min followed by

centrifugation at 6000 x g (8000 rpm) for 1 min. The last step was repeated and the purified DNA was eluted in Buffer AE and placed at -20°C.

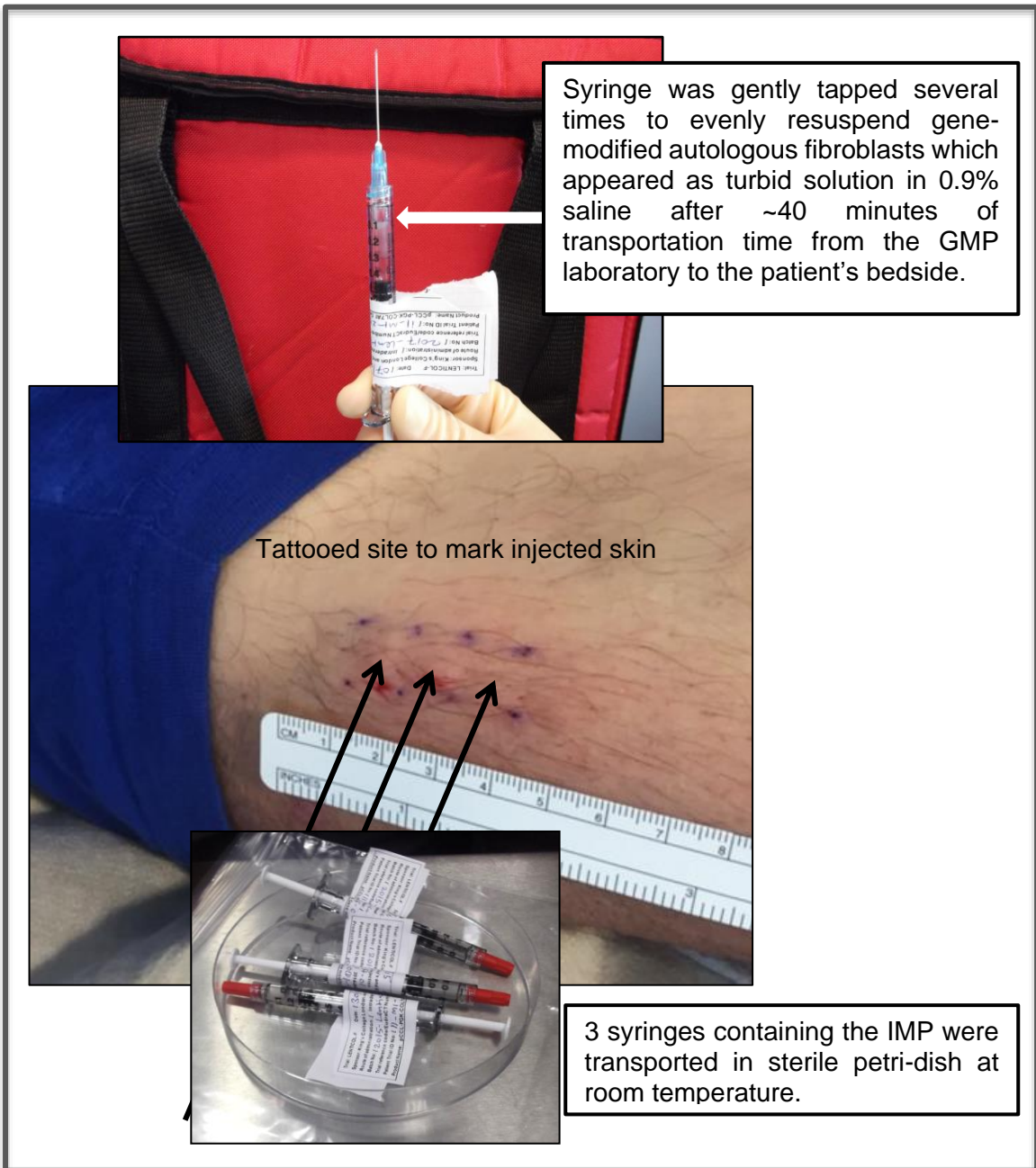
*Real-time quantification of vector copy number (VCN).* VCN was determined by real-time quantitative PCR using 100ng of DNA as a template for probe/primers specific to the PSI sequence of the lentivirus backbone and Human Albumin, used to quantify genomic DNA for normalization. A reference standard was obtained from serially diluted HT1080 fibrosarcoma cell line carrying one copy of integrated LV as detected and quantified by Southern blot. All reactions used Universal Master Mix (Applied Biosystems) and were run default conditions in the CFX96 Touch Real-Time PCR Detection System (Bio RAD). The data was analyzed using CFX Manager Software (Bio RAD).

## Supplemental Figures and Figure Legends



**Supplemental Figure 1. LENTICOL-F trial timeline.** An illustration of the schedule of visits for RDEB subjects on the trial. Each injection consists of 1.14–1.2 million cells suspended in 0.25ml of 0.9% saline. At the first screening visit, viral serology was tested for HIV, HepBsAg, HepBcAb, HepC1gG, HTLV 1&2 and Treponema pallidum. Basic blood tests included full blood count, urea and electrolytes, liver function tests, erythrocyte

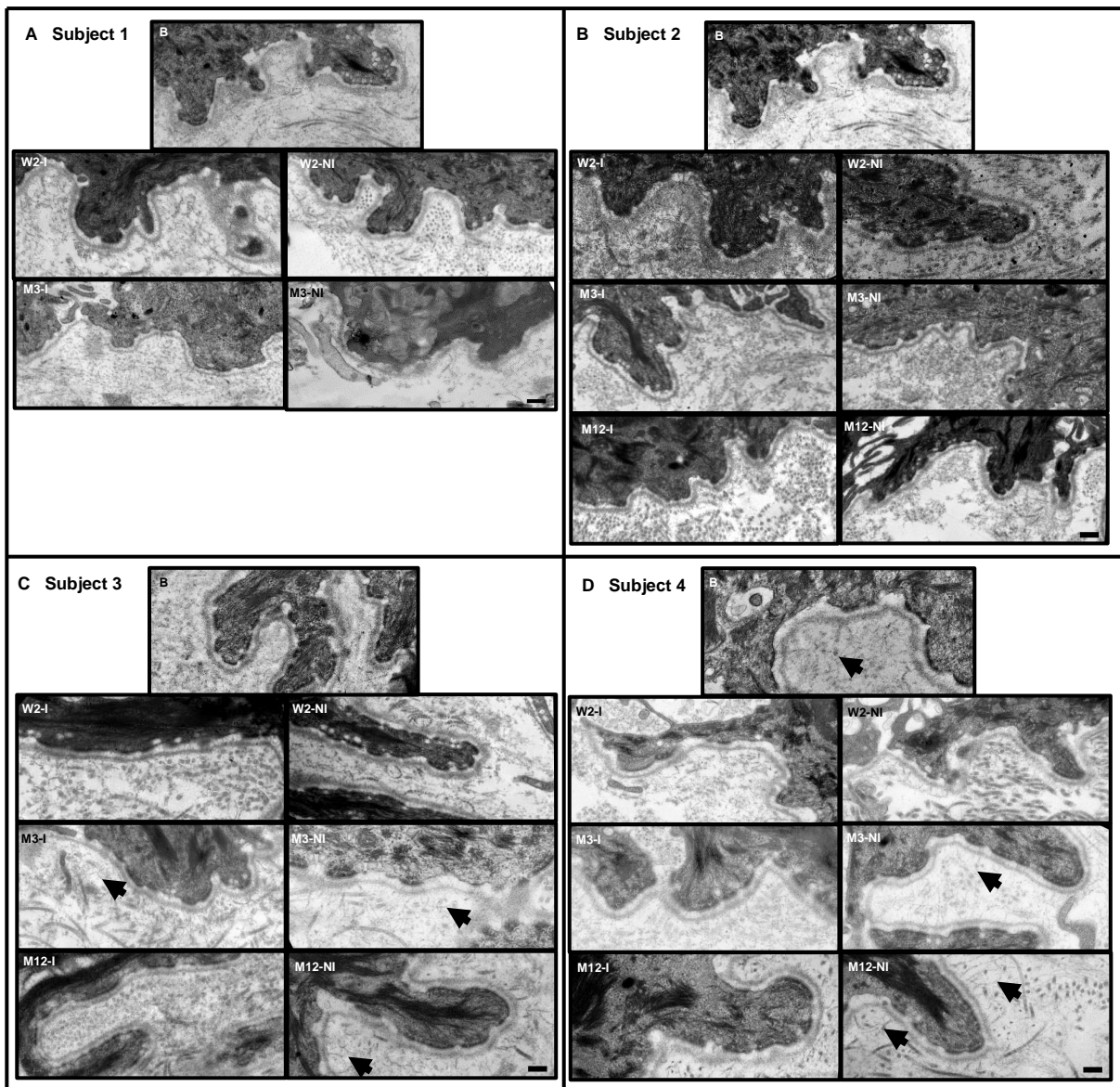
sedimentation rate and C-reactive protein. Skin procurement and biopsy at the second screening visit included 2 to 3 biopsies (one 6mm procurement for IMP production; and one to two 4mm for baseline C7 IF, TEM and qPCR). Skin biopsy at W2, M3 and M12 included two ~6mm biopsies for C7 IF, TEM, qPCR and for archive sample for integration site analysis in the event of serious adverse reactions (one from injected site and one from non-injected site). BEBSS, Birmingham Epidermolysis Bullosa Severity Score; D, Day; IIF, indirect immunofluorescence; M, Month; RCL, replication competent lentivirus; W, Week.



**Supplemental Figure 2. Intradermal injections of gene-modified autologous fibroblasts in Subject 3.** Three syringes, each containing  $\sim 1 \times 10^6$  gene-modified autologous fibroblasts suspended in 0.25ml of 0.9% sodium chloride were transported at room temperature from the GMP facility at Great Ormond Street Hospital to the patient's bedside at Guy's Hospital, London. The gene-modified fibroblasts appeared turbid within the clear saline solution, thus



gentle tapping of the syringe enabled even resuspension of the cells in saline prior to intradermal injection within the tattooed area of the patient's intact skin on the left upper arm.



**Supplemental Figure 3. Transmission electron microscopy (TEM) did not demonstrate mature AFs in Subjects 1–4 in treated skin. (A–D)** Thin sections of skin from injected and non-injected sites from the left upper arm were obtained from Subjects 1–4 at 2 weeks, 3 months and 12 months after treatment (only 2 weeks and 3 months in Subject 1 as he was withdrawn and re-enrolled as Subject 2). Only thin wisp-like rudimentary AFs were visualized in all subjects at these time-points. Occasional near-fan-shaped AF structures were seen in baseline, injected and non-injected skin in Subjects 3 and 4 (black arrows). B, baseline; I, injected skin; M, month; NI, non-injected skin; W, week. Scale bar: 0.25  $\mu$ m.

## Reference

1. Georgiadis C, Syed F, Petrova A, Abdul-Wahab A, Lwin SM, Farzaneh F, et al. Lentiviral Engineered Fibroblasts Expressing Codon-Optimized COL7A1 Restore Anchoring Fibrils in RDEB. *J Invest Dermatol.* 2016;136(1):284-92.