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Full-length article

A comprehensive report of long-term stability data for a range ATMPs: A need to develop guidelines for safe and harmonized stability studies

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ABSTRACT

Background aims: Advanced therapy medicinal products (ATMPs) are novel drugs based on genes, cells or tissues developed to treat many different diseases. Stability studies of each new ATMP need to be performed to define its shelf life and guarantee efficacy and safety upon infusion, and these are presently based on guidelines originally drafted for standard pharmaceutical drugs, which have properties and are stored in conditions quite different from cell products. The aim of this report is to provide evidence-based information for stability studies on ATMPs that will facilitate the interlaboratory harmonization of practices in this area.
Methods: We have collected and analyzed the results of stability studies on 19 different cell-based experimental ATMPs, produced by five authorized cell factories forming the Lombardy "Plagencell network" for use in 36 approved phase I/II clinical trials; most were cryopreserved and stored in liquid nitrogen vapors for 1 to 13 years.
Results: The cell attributes collected in stability studies included cell viability, immunophenotype and potency assays, in particular immunosuppression, cytotoxicity, cytokine release and proliferation/differentiation capacity. Microbiological attributes including sterility, endotoxin levels and mycoplasma contamination were also analyzed. All drug products (DPs), cryopreserved in various excipients containing 10% DMSO and in different primary containers, were very stable long term at <-150°C and did not show any tendency for diminished viability or efficacy for up to 13.5 years.
Conclusions: Our data indicate that new guidelines for stability studies, specific for ATMPs and based on risk analyses, should be drafted to harmonize practices, significantly reduce the costs of stability studies without diminishing safety. Some specific suggestions are presented in the discussion.
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Introduction

Extensively manipulated cells for therapeutic use are called advanced therapy medicinal products (ATMPs) and can be genetically engineered or not (somatic) [1,2]. Most ATMPs are produced by quite extensive culture methods and are generally cryopreserved at <-150°C in liquid nitrogen vapors to allow time for the execution of all quality controls on the finished product before administration to patients and to allow multiple dosing [3–6]. By law, they need to be produced according to specific guidelines and regulations (*e.g.*, the EU Good Manufacturing Practices [GMP] guidelines, published on the Eudralex website, and the U.S. Food and Drug Administration [FDA] current GMP regulations [cGMP], published on the FDA website) [7]. In the European Union, the same GMP regulations already established for standard drugs were initially applied to ATMPs [8,9]. In 2017, a specific GMP guideline dedicated to ATMPs was approved by the

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European Commission (EC) [10], since it is understood that cells and tissues are quite different from standard pharmaceutical drugs [4].

Stability studies determine a drug product (DP) "shelf life," *i.e.*, they guarantee that the final packaged DP maintains its identity, potency and sterility according to the approved manufacturer's specification throughout its valid period, in defined storage conditions [11–13]. Present guidelines and regulations prescribe that preliminary stability data should be available before an experimental product is used in a clinical trial, a minimum shelf life should be assigned, and further stability data should be planned and built up with real-life data subsequent to initial clinical use, eventually extending shelf life [6,14]. Unfortunately, the current guidance documents do not offer much support to establish the best methods and modalities of testing ATMP stability. This lack of formal advice can lead to uncertainty and different practices in different cell factories and different countries. Furthermore, the many assays to control DPs often are not standardized or harmonized between laboratories.

An official network of the five academic cell factories, called "Plagencell," all of which are authorized by Italy's national authorities (Agenzia Italiana del Farmaco, AIFA, Rome, Italy), has recently been established in the Lombardy region. As a first investigation, the Plagencell network has set out to collect and analyze the stability studies performed in the last 13 years on the experimental ATMPs internally produced for approved phase I/II clinical studies. The aim was to try to pinpoint the major bottlenecks and propose specific elements useful for the future establishment of guidelines more specific for this type of DPs, based on scientific evidence and real-life data.

Methods

The Plagencell network of cell factories

The Plagencell Cell Factories are numbered in the text and figures as follows: CF1, Center of Cellular Therapy "G. Lanzani", ASST Papa Giovanni XXIII, Bergamo; CF2, Laboratory of Cellular and Gene Therapy "S. Verri", ASST San Gerardo, Monza; CF3, Laboratorio di Medicina Rigenerativa - Cell Factory, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan; CF4, Cell Therapy Production Unit UPTC, IRCCS Istituto Neurologico Carlo Besta, Milan; and CF5, UOSD Cell Factory, Fondazione IRCCS Policlinico San Matteo, Pavia.

ATMP manufacturing, cryopreservation and thawing

Different types of experimental ATMPs were produced in GMP conditions following validated standard operating procedures (SOPs). These were either stored at 2° to 8°C until use or cryopreserved in different solutions (human Plasma AB or saline solution supplemented with human serum albumin (HSA) containing 10% dimethyl sulfoxide (DMSO, GMP grade; WAK-Chemie Medica, Steinbach, Germany; or Cryoserv; Mylan Institutional, Canonsburg, PA, or Alchimia, Ponte San Nicolò, Italy) or Cryostor CS10 (BioLife Solutions, Bothell, WA) [15–18]. Cells were frozen in double clinical bags (CryoMACS Freezing bag; Miltenyi Biotec, Bergisch Gladbach, Germany; or SAFE2 Cell Cryobag; Paolo Gobbi Frattini, Tovo di S. Agata, Italy; or HemoFreeze bag; Fresenius Kabi, Bad Homburg, Germany) or cryovials (Nalgene, Thermo Scientific, Waltham, MA) using controlled-rate freezing instruments and stored in vapor-phase liquid nitrogen storage tanks. The liquid nitrogen freezers were connected to an automated filling system and equipped with remote alarm systems; temperature and nitrogen levels were monitored and registered continuously.

Tubes or bags were thawed in a thermostatic bath at 37°C or dry thawing device; products were in some cases diluted in NaCl 0.9% supplemented with HSA to evaluate cellular viability and identity. Diluted or undiluted DPs were inoculated in hemoculture bottles to assess sterility.

Quality controls: viability and immunophenotype

Quality controls were performed on the final DPs and their intermediate products in GMP conditions, using different methods, instruments and reagents according to the SOPs of each laboratory, and are therefore only briefly summarized below. All methods were validated and applied the same specifications as those for ATMP batch release, as established by each cell factory [19]. The methods and specifications are indicated in Tables 2, 3, and 4.

Viability was measured either by Trypan Blue (Sigma Aldrich-Merck, Darmstadt, Germany) dye exclusion and counting in a hemocytometer, or by staining with propidium iodide (PI; Sigma Aldrich-Merck or BD Biosciences, San Jose, CA) or 7-aminoactinomycin D (7-AAD; BD Biosciences), followed by standard flow cytometry evaluation. Immunophenotypes were identified by staining with specific fluorescently labeled antibodies and flow cytometry analysis.

Quality controls: potency assays

Potency assays were those known for the type of cell product: immunosuppression and proliferation/differentiation for mesenchymal stromal cells (MSCs), cytotoxicity for T cell–derived products and T cell activation potential for dendritic cells (DCs). Cytotoxicity assays were performed using different methods according to each cell factory's internal protocols: (1) calcein-AM (Sigma Aldrich-Merck) loading of target cells and calcein release measured in an automated plate reader after 4-h coincubation of targets with effector cells (FLUOstar Optima, BMG LabTech), (2) carboxy fluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR) labeling of target cells followed by analysis of target cell death (apoptosis and necrosis) by PI/annexin V staining, (3) labeling of target cells overnight with ^{51}chromium (^{51}Cr, $Na_2{}^{51}CrO_4$ solution; PerkinElmer, Boston, MA), followed by 5-h ^{51}Cr release assay and gamma counter readout, or (4) 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay (Promega, Medison, WI), as previously described [20].

Immunosuppression assays were performed by coincubation of ATMPs with peripheral blood mononuclear cells (PBMCs) stimulated with phytohemagglutinin (PHA; Sigma Aldrich-Merck). Proliferation was measured with either CFSE and flow cytometry or ^{3}H-thymidine [21.22]. Proliferation was measured either by cell counting or capacity to form colonies in vitro [23,24].

To measure the capacity of DCs to activate lymphocytes, mixed lymphocyte reactions (MLRs) were set up. Briefly, PBMCs (responder cells) were co-cultured with irradiated or mitomycin-C–treated stimulating (S) cells at different ratios. S cells were DCs, autologous PBMCs (for auto-MLR, negative control), or allogeneic PBMCs (for allo-MLR, positive control). After 5 days of culture, proliferation was assessed using MTS reagent (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; Promega, Milano, Italy) and measured by absorbance at 540 nm [25]. Alternatively, proliferation was measured using an 18-h ^{3}H-thymidine (PerkinElmer) incorporation assay [25].

IFNγ production was measured by ELISPOT procedures. Briefly ATMPs were seeded on 96-well multiscreen filter plates coated with IFNγ antibody (Mabtech, Nacka, Sweden), in the absence or presence of relevant peptide mixes. After incubation for 24 h, plates were processed according to standard procedures, and spot-forming units (SFU) were counted using an ELISPOT reader (Bioline, Torino, Italy) [22]. The osteogenic differentiation potential of bone marrow (BM)-derived MSCs in 2D monolayers or 3D scaffolds in vitro, as well as in vivo ectopic bone formation, was assessed as described previously [26].

Karyotyping

Karyotyping was performed on an aliquot of cells by Q- or G-banding according to SOPs of the local Clinical Genetics Laboratories [27].

Table 1
ATMPs manufactured by Plagencell Network cell factories and major clinical trials

ATMP cell type (abbreviation)	ATMP (abbreviation)	Manufacturer[a]	Study Identification	Use	Phase	No. of batches produced for trials (including compassionate use)	Reference
Mesenchymal stromal cells (MSCs)	Bone marrow–derived (BM-MSC)	CF1 + CF2	NCT01764100	Allogeneic	I/II	73	[33]
		CF1	NCT02012153	Autologous	I	6	[31,37]
		CF1	NCT01854957	Autologous	I/II	35	[31]
		CF1	NCT02260375	Allogeneic	I/II	4	[30]
		CF1	NCT02565459	Allogeneic	I/II	5	
		CF1	EudraCT No. 2016-004804-77	Autologous	I	7	
		CF3	EudraCT No. 2019-002749-40	Autologous	IIb	1	
		CF3	NCT04759105	Autologous	IIb	9	
		CF3	NCT01824121	Autologous	I	5	
		CF3	NCT02065167	Autologous	II	3	
		CF5	EudraCT No. 2021-004755-17	Allogeneic	I/II	44	
		CF5	NA	Allogeneic	NA	18	
	Umbilical cord–derived (UCM)	CF1	NCT02032446	Allogeneic	I/II	18	Submitted
	$CD362^+$ selected BM stromal cells (ORBCEL-M)	CF1	NCT02585622	Autologous	I	3	
	bone marrow–derived (BM-MSC) + biomaterial	CF3	NCT03325504	Autologous	III	5	
		CF3	NCT01842477	Autologous	II	7	[45]
	Cord blood–derived (CB-MSC)	CF3	NCT04034316	Allogeneic	II	11	
		CF3	EudraCT No. 2011-001387-21	Allogeneic	I	11	
		CF3	Eudra CT 2020-001577-70	Allogeneic	I/IIa	3	
	Adipose tissue–derived (AD-MSC)	CF3	EudraCT No. 2020-005336-29	Autologous	II	Clinical trial under submission to competent authorities	
	Adipose tissue–derived (AD-MSC)	CF4	Documents in preparation	Allogeneic	I	/	
	AD-MSC loaded with Paclitaxel (PacliMES)	CF4	EudraCT No. 2020 -005928-11	Allogeneic	I	/	
Polyclonal T cells	Blinatumomab-expanded T cells (BET)	CF1	NCT03823365	Autologous	I	15	
	Cytokine-induced killer (CIK) cells	CF1 + CF2	NCT01186809	Allogeneic	II/A	110	[32]
		CF1	ISS 64499-PRE21-848	Allogeneic	I	11	[42]
		CF1	NCT03821519	Allogeneic	I/II	8	
	Anti-leukemia cytotoxic T cells (CTL)	CF5	EudraCT No. 2019-003362-41	Allogeneic	I/II	24	
	Adenovirus-specific T lymphocytes (LTC-ADV)	CF5	EudraCT No. 2008-000523-25	Allogeneic or autologous	I/II	5	
	Epstein–Barr virus–specific CTL (CTL-EBV)	CF5	CE n. 15/97	Allogeneic or autologous	I/II	43	[35,39]
	Cytomegalovirus-specific T lymphocytes (LTC-CMV)	CF5	NA	Allogeneic or autologous	NA	9	
Gene modified polyclonal T cells	CAR-CD19 gene-modified CIK (CAR-CIK-CD19)	CF1 + CF2	NCT03389035	Allogeneic	I/II	35	[38]
		CF1 + CF2	EudraCT No. 2020-005025-85	Allogeneic or Autologous	II	/	
Dendritic cells (DCs)	Autologous GSC a-loaded mature DCs (DENDR STEM)	CF4	EudraCT No. 2013-002100-13	Autologous	I	2	
	Tumor lysate–loaded mature DCs (DENDR-1)	CF4	EudraCT No. 2008-005035-15	Autologous	I/II	65	
	Tumor lysate–loaded mature DCs (DENDR-2)	CF4	EudraCT No. 2008-005038-62	Autologous	I	40	[36]
Stem cells	Human fetal neural stem precursor cells (hNPC-DP)	CF2	EudraCT No. 2016-002020-86	Allogeneic	I	15	
	BM-derived $CD133^+$ stem cells (BM-CD133-SC)	CF2	EudraCT No. 2012-005267-27	Autologous	I	18	

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Microbiological contamination

Endotoxin levels were measured by gel clot assay or the kinetic chromogenic method performed in-house (LAL-PTS system; Charles River, Wilmington, MA) or by Eurofins Biolab (Vimodrone, MI, Italy) according to Ph.Eur.2.6.14. Possible contamination with aerobic and anaerobic microbes was measured by hemoculture methods, using BactAlert (Biomerieux) or BACTEC (BD Diagnostics) according to the manufacturers' instructions and Ph.Eur.2.6.27. Mycoplasma contamination was measured using the culture method or by polymerase

Table 2
Stability studies on cryopreserved final DPs and data at longest time point

ATMP (manufacturer)	Excipients	Primary container	Freezing method	Storage	Shelf life assigned (no. batches tested)	QC test performed to assign shelf life	Test method	Test specifications	QC test result before freezing	QC test result at latest time point tested
BM-MSC +UCM (CF1)	Human plasma AB + 10% DMSO	CryoMACS Miltenyi infusion bag	Controlled-rate freezer	≤−150°C	8 years (n=3)	Viability	Trypan blue	≥80% viability	94.5% ± 3.2%	89.3% ± 3.3%
						Immunophenotype	Cytofluorimetry	$CD73^+$ ≥90%	97.4% ± 1.2%	99.6% ± 0.2%
								$CD90^+$ ≥90%	99.3% ± 0.5%	99.8% ± 0.3%
								$CD105^+$ ≥90%	97.1% ± 0.6%	98.9% ± 1.4%
						Immunosuppression	Cytofluorimetry	≥30% PBMC proliferation inhibition at E:T ratio 10:1	ND	64.8% ± 3.4%
						Karyotype	QFQ-banding	No metaphases or no chromosomic aberration or non-clonal chromosomic aberrations in ≤10% of metaphases [27]	Compliant	Compliant
						Sterility	Bact/Alert	Sterile	Sterile	Sterile
						Mycoplasma	Culture	Absence	Absent	Absent
						Endotoxin Level	Chromokinetic	<7 EU/mL	< 3.5 EU/mL	< 3.5 EU/mL
BM-MSC (CF2)	HSA 20% + 10% ACD + 10% DMSO	Nalgene cryovial	Controlled-rate freezer	≤−150°C	49 months (n=1)	Viability	Cytofluorimetry	>70% viability	87.7%	82.3%
						Immunophenotype	Cytofluorimetry	$CD73^+$ ≥70%	99.3%	99.5%
								$CD90^+$ ≥70%	99.7%	99.6%
								$CD105^+$ ≥70%	99.9%	99.7%
BM-MSC (CF5)	NaCl 0.9% + 5% HSA+ 10% DMSO	Nalgene cryovial	Nalgene controlled-rate manual freezing system	≤−150°C	6 years (n=3)	Viability	Trypan blue	≥90% viability	96.0% ± 1.5%	93.0% ± 3.0%
						Immunophenotype	Cytofluorimetry	$CD73^+$ ≥85%	98.0% ± 0.7%	94.0% ± 6.0%
								$CD90^+$ ≥85%	99.0% ± 0.5%	96.0% ± 5.0%
								$CD105^+$ ≥85%	96.0% ± 0.9%	94.0% ± 8.0%
						Differentiation potential	Cell culture and specific staining	Differentiation into adipocytes and osteocyte	Yes	Yes
						Immunosuppression	^{3}H TdR	≥30% inhibition at E:T ratio 1:2	65.0% ± 14.0%	71.0% ± 9.0%
						Sterility	Bactec	Sterile	Sterile	Sterile
ORBCEL-M (CF1)	Cryostor CS10	CryoMACS Miltenyi infusion bag	Controlled-rate freezer	≤−150°C	45 months (n=3)	Viability	Trypan Blue	≥70% viability	85.3% ± 3.5%	90.1% ± 2.9%
						Immunophenotype	Cytofluorimetry	$CD73^+$ ≥95%	99.9% ± 0.1%	99.8% ± 0.1%
								$CD90^+$ ≥95%	99.9% ± 0.1%	99.9% ± 0.1%
								$CD105^+$ ≥95%	99.6% ± 0.5%	98.3% ± 2.3%
						Sterility	Bact/Alert	Sterile	Sterile	Sterile
						Mycoplasma	Culture	Absence	Absent	Absent
						Endotoxin Level	Chromokinetic	< 10 EU/mL	≤5 EU/mL	≤5 EU/mL
CB-MSC (CF3)	NaCl 0.9% + 10% HSA + 10% DMSO	CryoMACS Miltenyi freezing bag	Controlled-rate freezer	≤−150°C	57 months (n=3)	Viability	Cytofluorimetry	≥80% viability	88.5% ± 6.2%	87.6% ± 3.7
						Immunophenotype	Cytofluorimetry	$CD45^-CD90^+CD105^+$ ≥90%	92.8% ± 1.4%	98.3% ± 1.2%
						Proliferation Potential	Gentian Violet	Colony formation	52.0 ± 22.4	19.0 ± 15.8
							Fold expansion	≥2 fold	24.4 ± 7.1	6.8 ± 4.3
						Karyotype	G-banding	46,XX or 46,XY	Compliant	Compliant
						Sterility	Bact/Alert	Sterile	Sterile	Sterile
AD-MSC (CF4)	NaCl 0.9% + 5% HSA + 10% DMSO	Nalgene cryovial	Controlled-rate freezer	≤−150°C	1 years (n=1)	Viability	Trypan blue	≥80% viability	96.1%	92.9%
						Immunophenotype	Cytofluorimetry	$CD73^+$ ≥80%	98.6%	97.6%
								$CD90^+$ ≥80%	94.5%	94.9%
								$CD105^+$ ≥80%	90.5%	91.0%
						Sterility	Bact/Alert	Sterile	Sterile	Sterile
						Mycoplasma	PCR	Absence	Absent	Absent
						Endotoxin Level	Gel Clot	<117 EU/mL	≤0.5 EU/mL	≤0.5 EU/mL
PacliMES (CF4)		Nalgene cryovial		≤−150°C	1 years (n=3)	Viability	Trypan blue	≥80% viability	91.0% ± 0.5%	90.5% ± 2.2%

(*continued on next page*)

Table 2 (*Continued*)

ATMP (manufacturer)	Excipients	Primary container	Freezing method	Storage	Shelf life assigned (no. batches tested)	QC test performed to assign shelf life	Test method	Test specifications	QC test result before freezing	QC test result at latest time point tested
	NaCl 0.9% + 5% HSA + 10% DMSO		Controlled-rate freezer			Immunophenotype	Cytofluorimetry	$CD73^+$ ≥80%	98.4% ± 0.4%	99.3% ± 0.9%
								$CD90^+$ ≥80%	93.9% ± 3.9%	94.1% ± 4.0%
								$CD105^+$ ≥80%	91.5% ± 7.7%	83.4% ± 1.8%
						Paclitaxel content	HPLC/Mass spectrometry	≥0.05 pg/cell	0.5 ± 0.2	0.5 ± 0.2
						Cytotoxicity	MTT	≥13.5 ng/mL (Paclitaxel equivalent concentration)	13.7 ± 0.0	13 ± 0.0
						Sterility	Bact/Alert	Sterile	Sterile	Sterile
						Mycoplasma	PCR	Absence	Absent	Absent
						Endotoxin Level	Gel Clot	<117 EU/mL	≤0.5 EU/mL	≤0.5 EU/mL
BET (CF1)	Human plasma AB + 10% DMSO	CryoMACS Miltenyi infusion bag	Controlled-rate freezer	≤−150°C	2 years (n=1)	Viability	Cytofluorimetry	≥80% viability	80.7%	94.5%
						Immunophenotype	Cytofluorimetry	$CD3^+$ ≥90%	84.5%	85.7%
CIK (CF1)	Human plasma AB + 10% DMSO	Hemofreeze bag Fresenius	Controlled-rate freezer	≤−150°C	7 years (n=1)	Viability	Cytofluorimetry	≥80% viability	96.9%	98%
						Immunophenotype	Cytofluorimetry	$CD3^+CD56^+$ ≥40%	51.3%	48.8%
						Cytotoxicity	Calcein-AM release	≥10% lysis at E:T ratio 30:1	ND	45.1%
CIK (CF2)	HSA 20% + 10% DMSO + 10% ACD	SAFE2 Cell cryobag Gobbi Frattini	Controlled-rate freezer	≤−150°C	2 years (n=3)	Viability	Cytofluorimetry	≥70% viability	79.2% ± 5.8%	91.9% ± 3.6%
						Immunophenotype	Cytofluorimetry	$CD3^+CD56^+$ ≥40%	45% ± 7.1%	44.4% ±2.4%
CTL (CF5)	NaCl 0.9% + 5% HSA+ 10% DMSO	Nalgene cryovial	Nalgene controlled-rate manual freezing system	≤−150°C	8 years (n=3)	Viability	Trypan blue	≥80% viability	94% ± 5%	88% ± 3%
						Immunophenotype	Cytofluorimetry	$CD3^+/CD3^-CD56^+$ value at freezing ± 10%	98% ± 2%	98% ± 2%
						Cytotoxicity	^{51}Cr release	Cytotoxicity value at freezing ± 10%	25% ± 15%	27% ± 7%
						Sterility	Bactec	Sterile	Sterile	Sterile
LTC-ADV (CF5)	NaCl 0.9% + 5% HSA + 10% DMSO	Nalgene cryovial	Nalgene controlled-rate manual freezing system	≤−150°C	13.5 years (n=5)	Viability	Trypan blue	≥80% viability	100% ± 0%	96% ± 0.5%
						Immunophenotype	Cytofluorimetry	$CD3^+/CD3^-CD56^+$ value at freezing ± 10%	98% ± 2%	99% ± 1%
						Cytotoxicity	Cr^{51} release	Cytotoxicity >25% at E:T ratio 20:1	62% ± 20%	45% ± 18%
						Sterility	Bactec	Sterile	Sterile	Sterile
CTL-EBV (CF5)	NaCl 0.9% + 5% HSA + 10% DMSO	Nalgene cryovial	Nalgene controlled-rate manual freezing system	≤−150°C	4 years (n=3)	Viability	Trypan blue	≥80% viability	100% ± 0%	96% ± 0.5%
						Immunophenotype	Cytofluorimetry	$CD3^+/CD3^-CD56^+$ value at freezing ± 10%	98% ± 2%	99% ± 1%
						Cytotoxicity	Cr^{51} release	Lysis >25% at E:T ratio 20:1	62% ± 20%	45% ± 18%
						Sterility	Bactec	Sterile	Sterile	Sterile
LTC-CMV (CF5)	NaCl 0.9% + 5% HSA + 10% DMSO	Nalgene cryovial	Nalgene controlled-rate manual freezing system	≤−150°C	2 years (n=3)	Viability	Trypan blue	≥80% viability	100% ± 0%	85% ± 5%
						Immunophenotype	Cytofluorimetry	$CD3^+/CD3^-CD56^+$ value at freezing ± 10%	95% ± 1.5%	97% ± 2%
						IFN-γ production	ELISPOT assay	Spot forming unit/10^5 cells > 25	473 ± 236	290 ± 122
						Sterility	Bactec	Sterile	Sterile	Sterile
CARCIK-CD19 (CF1)	0.9% NaCl + 11.2% HSA + 10% DMSO + 10% ACD	CryoMACS Miltenyi double bags	Controlled-rate freezer	≤−150°C	1 year (n=1)	Viability	Trypan blue	≥70% viability	94%	82.8%
						Immunophenotype	Cytofluorimetry	$CD3^+$ ≥90%	98.5%	98.6%
								$CD3^+56^+$ ≥30%	57.7%	57.7%
								CAR^+ ≥20%	35.9%	39.9%
						Cytotoxicity	Cytofluorimetry	≥25% at E:T ratio 5:1	69.3%	44.3%
						Sterility	Bact/Alert	Sterile	Sterile	Sterile
CARCIK-CD19 (CF2)	0.9% NaCl + 11.2% HSA + 10% DMSO + 10% ACD	SAFE2 Cell cryobag Gobbi Frattini	Controlled-rate freezer	≤−150°C	1 year (n=2)	Viability	Trypan blue	≥70% viability	97.2% ± 1.8%	84.0% ± 10.9%
						Immunophenotype	Cytofluorimetry	$CD3^+$ ≥90%	99.2% ± 0.2%	99.5% ± 0.3%
								$CD3^+56^+$ ≥30%	57.8% ± 7.5%	52.3% ± 1.3%
								CAR ≥20%	56.5% ± 13.6%	44.8% ± 14.0%

(*continued on next page*)

Table 2 (*Continued*)

ATMP (manufacturer)	Excipients	Primary container	Freezing method	Storage	Shelf life assigned (no. batches tested)	QC test performed to assign shelf life	Test method	Test specifications	QC test result before freezing	QC test result at latest time point tested
DENDR STEM (pre-final formulation) (CF4)	NaCl 0.9% + 0.5% HSA + 10% DMSO	Nalgene cryovial	Controlled-rate freezer	≤−150°C	1 year (n=1)	Viability	Trypan blue	≥75% viability	97%	96%
						Immunophenotype	Cytofluorimetry	$CD80^+$ ≥ 73%	76.7%	83.8%
								$CD83^+$ ≥ 35%	69.9%	58.3%
								$CD86^+$ ≥ 92%	97.9%	95.8%
								HLA-DR ≥ 88%	99.7%	99.3%
						MLR	MTS	>18%	92.2%	94.5%
						Sterility	BactAlert	Sterile	Sterile	Sterile
						Mycoplasma	PCR	Absence	Absent	Absent
						Endotoxin Level	Gel-Clot	<2.86 EU/mL	≤0.5 EU/mL	≤0.5 EU/mL
DENDR-1 DENDR-2 (CF4)	NaCl 0.9% + HSA 0.5% + 10% DMSO	Nalgene cryovial	Controlled-rate freezer	≤−150°C	1 year (n=3)	Viability	Trypan blue	≥75% viability	93.0% ± 3.8%	93.9% ± 0.0%
						Immunophenotype	Cytofluorimetry	$CD80^+$ ≥ 73%	75% ± 0.8%	75.2% ± 0.0%
								$CD83^+$ ≥ 35%	62.7% ± 10%	62.3% ± 0.1%
								$CD86^+$ ≥ 92%	94.3% ± 3.1%	95.7% ± 0.0%
								HLA-DR ≥ 88%	97.1% ± 3.7%	99.7% ± 0.0%
						MLR	^{3}H TdR	Stimulation index (SI) >3	14.9±4.7	16.8±0.6
						Sterility	BactAlert	Sterile	Sterile	Sterile
						Mycoplasma	PCR	Absence	Absent	Absent
						Endotoxin Level	Gel-Clot	<2.86 EU/mL	≤0.5 EU/mL	≤0.5 EU/mL

ND, not done; QC, quality control

chain reaction (PCR), in all cases according to European Pharmacopeia (Ph.Eur.2.6.7; Ph Eur.2.6.21). Tests were carried out either in a GMP-qualified external laboratory (Eurofins Biolab) or in-house.

Results

The cell factory network and ATMPs produced for clinical use

The Plagencell cell factory network was created to optimize the development and production of innovative ATMPs for clinical use and is part of a wider collaboration between research institutes and hospitals in the Lombardy region (see Methods for details), which collaborate for the development of treatments for several devastating human diseases. The five cell factories are academic and have been approved for production and testing of experimental ATMPs for phase I/II clinical trials [28].

Table 1 lists the experimental ATMPs, clinical trials and numbers of DP batches that are the object of this report. The ATMPs belong to five different cell types as follows: (1) MSCs isolated from BM, umbilical cord (UC), adipose tissue (AD) and cord blood (CB). In one case, AD-derived MSCs were loaded with an anti-neoplastic agent (Paclitaxel). (2) Polyclonal T cells expanded from peripheral blood (PB) or CB, including cytokine induced killer (CIK) cells, blinatumomab expanded T cells (BETs), cytotoxic T lymphocytes (CTLs) directed against Epstein–Barr virus (EBV) or leukemic cells, and T cell lines (LTCs) specific for cytomegalovirus (CMV) or adenovirus (ADV). (3) One gene-modified ATMP, produced by two cell factories, consists of CIK cells transduced with an anti-CD19 chimeric antigen receptor (CAR), using a nonviral transposon-based transfection method (CAR-CIK-CD19). (4) DCs starting from monocytes isolated from leukapheretic material and loaded with tumor cells or cell lysates. (5) Stem cells, including BM-derived $CD133^+$ cells and neural precursor stem cells (NPCs), derived from fetal human brain (Table 1). Some ATMP types were made by more than one cell factory and entered stability studies in each. They were produced for 36 approved clinical trials, and overall 668 batches of cells have been produced in the context of these trials (Table 1).

Stability studies performed on cryopreserved DPs

Nineteen DPs produced (listed in Table 2) were cryopreserved to <−150°C and stored in continuously monitored nitrogen vapor freezers. Because of the required cell volumes, treatment modalities and different practices, DPs were frozen in different types of primary containers (freezing bags or cryovials) and with different excipients, including DMSO added to human plasma or physiological solution containing different percentages of HSA and anticoagulant citrate dextrose (ACD), or in preformulated freezing medium (Cryostor CS10) (Table 2). All ATMPs produced entered stability studies to assign an initial shelf life. The latter was extended once the results of the longer stability studies became available, so some ATMPs currently have a 7- to 13.5-year stability at <−150°C. Only the longest validated shelf lives are reported in Table 2. The ATMPs more recently introduced have a shorter assigned stability due to the shorter period of testing or lack of clinical need to extend shelf life beyond 1 year.

The quality control methods performed for stability studies differed according to the ATMP and the cell factory (Table 2) but remained constant throughout each individual stability study. Usually, at least three batches of DPs produced in GMP and cryopreserved in the same concentrations, excipients and containers as those of the DPs for clinical use were tested in these studies at each time point, although in some cases, single batches were tested at different time points, following a matrixing approach.

The tests performed in stability studies included in all cases viability (by flow cytometry or Trypan Blue), identity and purity (immunophenotype). Acceptable viability was between ≥70% and ≥80% for 18 of 19

Table 3
Stability studies performed on freshly manufactured, non cryopreserved DPs

ATMP (manufacturer)	Excipients	Primary container	Storage	Shelf life assigned (n ° batches tested)	QC test performed to assign shelf life	Test method	Test specifications	QC test result before freezing	QC test result at latest time point tested
BM-MSC+ biomaterial (CF3)	NaCl 0.9% + 5% HSA	Syringe with a screwed luer lock cap	4–8°C	24 h (n=10 before shipping, n=5 at latest time)	Viability	Cytofluorimetry	>80% viability	98.7% ± 0.6%	93.9% ± 0.7%
					Immunophenotype	Cytofluorimetry	$CD73^+$ ≥90%	95.5% ± 2.9%	99.8% ± 0.0%
							$CD90^+$ ≥90%	97.7% ± 2.2%	99.8% ± 0.2%
							$CD105^+$ ≥90%	97.1% ± 2.7%	98.8% ± 1.3%
							HLA Cl II ≤ 20%	2.1% ± 3.9%	1.3% ± 1.0%
					Proliferation potential	Crystal violet staining	Cell adhesion to a 3D scaffold	[26]	
						Cell count	Fold Increase ≥2 at day 6 in vitro		
					Differentiation potential	Cell culture and specific staining [26]	Ex vivo osteogenic differentiation compared to controls		
						In vivo bone-formation assay	Repair of induced calvaria defects after 4 weeks		
BM-MSC (CF3)	NaCl 0.9% + 5% HSA	Syringe with a screwed luer lock cap	4–8°C	24 h (n=3)	Viability	Cytofluorimetry	≥80% viability	97.1% ± 0.8%	85.3% ± 5.0
					Immunophenotype	Cytofluorimetry	$CD73^+$ ≥90%	99.9% ± 0.0%	99.8% ± 0.3%
							$CD90^+$ ≥90%	99.6% ± 0.4%	99.4% ± 0.6%
							$CD105^+$ ≥90%	99.8% ± 0.2%	99.7% ± 0.4%
					Sterility	Bact/Alert	Sterile	Sterile	Sterile
AD-MSC (CF3)	Ringer lactate	Syringe with a screwed luer lock cap	2–8°C	24 h (n=3)	Viability	Cytofluorimetry	≥80% viability	84.1% ± 0.3%	86.4% ± 2.9%
					Immunophenotype	Cytofluorimetry	$CD73^+$ ≥80%	98.9% ± 0.8%	99.7% ± 0.3%
							$CD90^+$ ≥80%	99.9% ± 0.1%	100.0% ± 0.1%
							$CD105^+$ ≥80%	99.4% ± 0.5%	98.5% ± 1.7%
							$CD166^+$ ≥80%	99.0% ± 0.7%	99.0% ± 1.1%
					Sterility	Bact/Alert	Sterile	Sterile	Sterile
hNPC −DP (CF2)	NaCl 0.9% + 0.028% HSA	MACS GMP cell expansion bag; Miltenyi	2–8°C	4 h (n=2)	Viability	Trypan Blue	≥45% viability	60.5% ± 13.5%	55.7% ± 4.1%
BM-CD133-SC (CF2)	NaCl 0.9% + 5% HSA	50-mL polypropylene tube	4–8°C	12 h (n=3)	Viability	Trypan Blue	≥80% viability	92.1% ± 4.3%	92.4% ± 1.2%
					Immunophenotype	Cytofluorimetry	$CD133^+$ ≥80%	88.9% ± 4.04%	88.1% ± 2.3%

Table 4
DP holding time after thawing

ATMP (manufacturer)	Thawing method	Post-thaw processing	Shelf life assigned upon thawing (n° batches tested)	QC test performed to assign shelf life	Test method	Test specifications	QC test result before freezing	QC test result at latest time point tested after thawing
BM-MSC (CF2)	37°C water bath	Administered immediately	15 min (n=3)	Viability	Trypan Blue	≥70% viability	95.8% ± 1.1%	91.1% ± 4.5%
BM-MSC (CF5)	37°C water bath	Infused after dilution in NaCl 0.9% + 4% HSA	4 h (n=3)	Viability	Trypan Blue	≥80% viability	100% ± 0.0% (96.0% ± 7.0%) *	85.0% ± 9.0%
CB-MSC (CF3)	37°C water bath or dry thawing device	Dilution 1:1 in NaCl 0.9% + 10% HSA + 12% ACD-A	30 min (n=3)	Viability	Cytofluorimetry	≥80% viability	95.3% ± 0.6%	82.3% ± 1.5%
				Cytofluorimetry				Immunophenotype $CD45^{-}CD90^{+}CD105^{+}$ ≥90%
				94.8% ± 3.6%	94.5% ± 3.0%			
Proliferation Potential	Gentian Violet staining	colony formation	16% ± 6.6%	16% ± 6.1%				
	Cell count	Fold Increase >2	22.0% ± 12.1%	25.0% ± 19.3%				
CTL (CF5)	37°C water bath	Infused after dilution in NaCl 0.9% + 4% HSA	1 h (n=3)	Viability	Trypan Blue	≥70% viability	94.0% ± 1.7% (83.0% ± 5.0%)*	81.0% ± 7.0%
LTC-ADV (CF5)	37°C water bath	Infused after dilution in NaCl 0.9% + 4% HSA	2 h (n=3)	Viability	Trypan Blue	≥70% viability	100% ± 0.0% (91.0% ± 1.7%)*	79.0% ± 2.5%
DENDR-1 DENDR-2 (CF4)	37°C water bath	Administered immediately	20 min (n=3)	Viability	Trypan Blue	≥75% viability	98.2% ± 0.2%	96.3% ± 1.0%

*At thawing.

cryopreserved DPs and a very stringent ≥90% in 1 case. These specifications were demonstrated to be maintained for all ATMPs at the longest storage time point tested (Table 2). Results of the time course of viability assays, performed on several ATMPs representative of different cell types, are shown in Figure 1. In the case of CIK (Figure 1D), the data derive from analyses from two different cell factories (CF1 and CF2). In

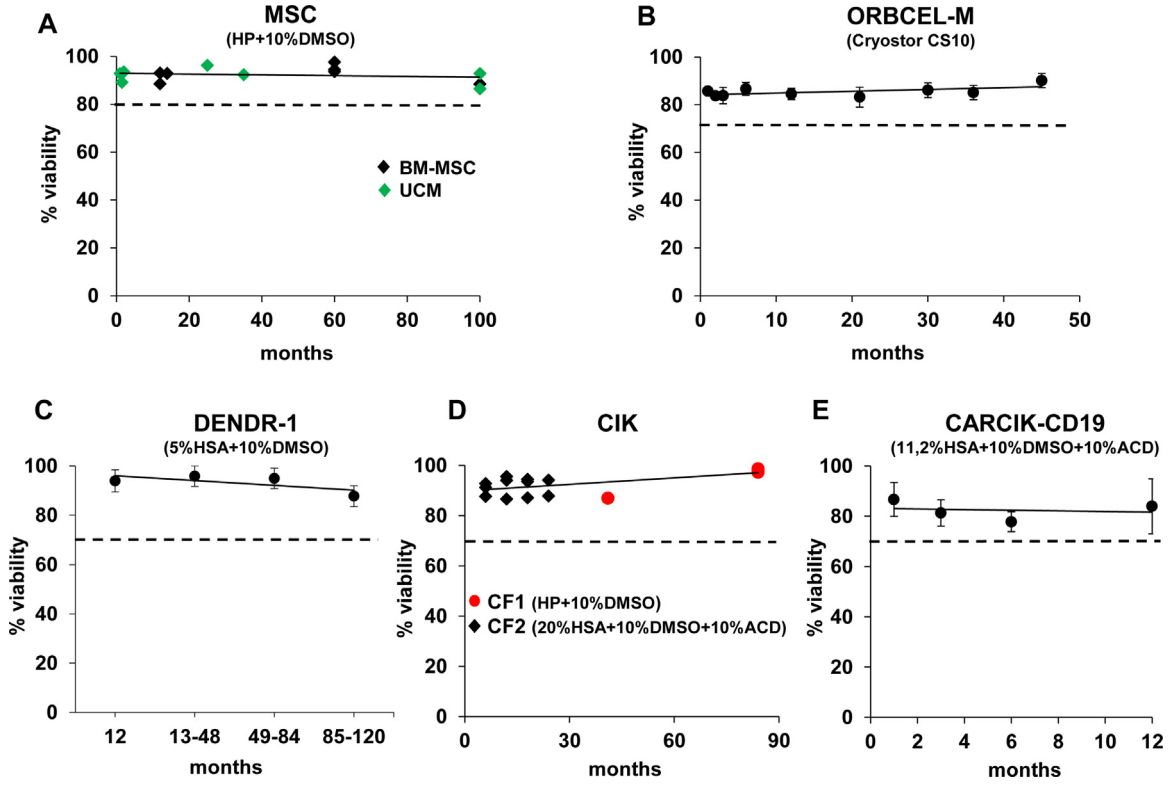

Figure 1. Viability of different ATMPs during storage in nitrogen vapor. The viability of ATMPs produced in GMP and stored in their final containers at <−150°C for extended periods of time was tested at different time points. Representative data are shown for different subtypes of mesenchymal stromal cells: BM-MSC and UCM (A) and ORBCEL-M (B), DCs (C) and unmodified or genetically modified CIK cells (D and E respectively). In D, the excipients used for CIK cryopreservation in the two different cell factories (CF1 and CF2) are shown. The dotted lines indicate the minimum percentage viability required for each ATMP according to the specifications set by each cell factory. The excipients used are indicated. At least three batches of each ATMP were used in the viability studies, all time points included.

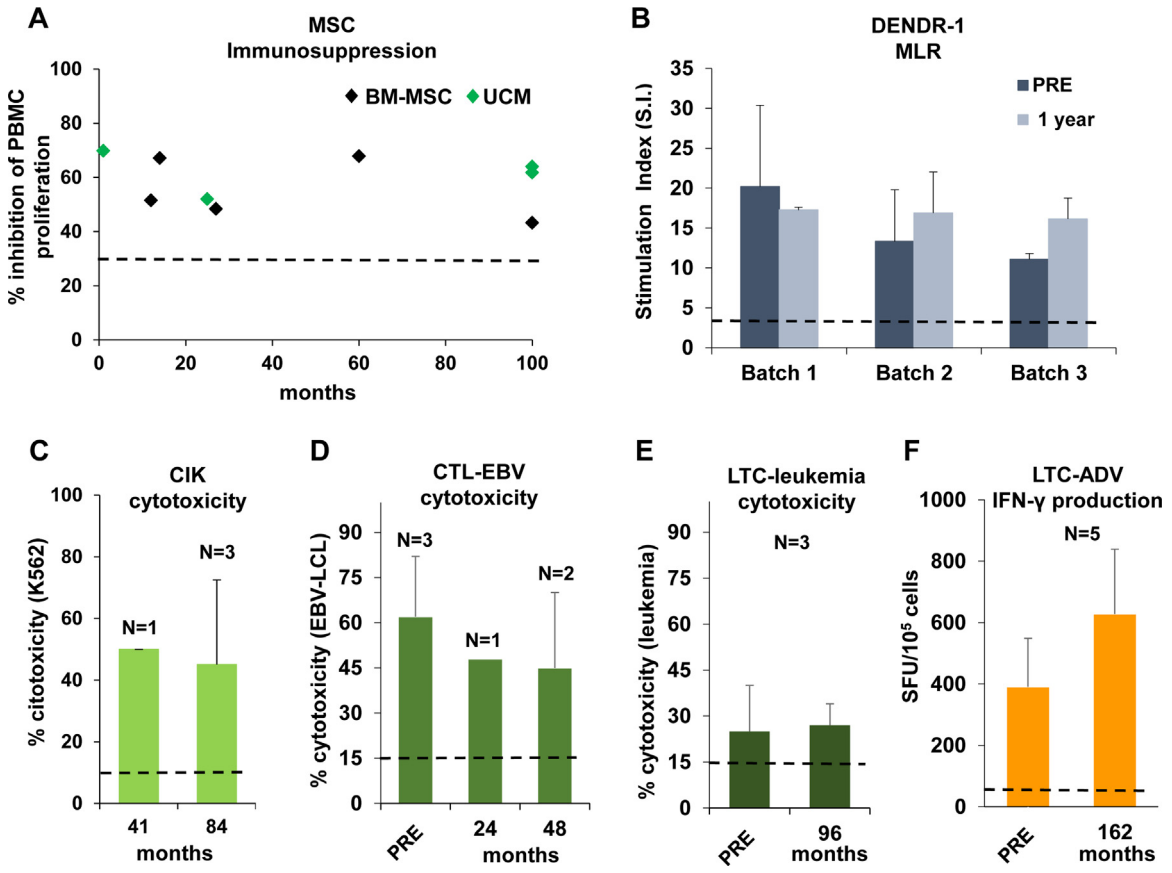

Figure 2. Potency assays of DPs cryopreserved for extended times. Potency assays were performed on ATMPs produced in GMP and stored in their final containers at <−150°C for extended periods of time. Exemplificative potency results obtained with different types of ATMPs are shown: immunosuppressive activity of BM-MSC and UCM on T cell proliferation induced by PHA (A), MLR potential of PBMCs in presence of DCs (B), cytotoxic activity of CIK against the K562 leukemic target cell line (C), specific cytotoxicity of CTL-EBV against autologous EBV-lymphoblastoid cell line (D), specific cytotoxicity of anti-leukemia CTL against patient leukemic blasts (E) and IFNγ production by LTC-ADV in response to adenovirus peptides (F). At least three batches of each ATMP were used overall in the potency studies.

this case, however, the same testing method was used (cytofluorimetry), and the DP was produced according to the same procedure and used in a multicenter clinical trial involving both cell factories (NCT01186809, Table 1). Of note is that the trend lines that fit the viability data are almost flat and show a very limited downward trend. In addition, they remain always well above the thresholds even for extended periods, suggesting that the viability of the DPs should be maintained above the established limit for at least 10 to 13 years, and this was observed for all products tested in long-term studies (Figure 1 and Table 2).

Like viability, in all cases tested, ATMPs stored for extended periods at <−150°C maintained their initial identity and purity (immunophenotype), without any loss or tendency for diminished expression of specific markers over time, even after the longest tested period of up to 13.5 years (Supplementary Figure S1 and Table 2).

Potency assays were performed in vitro for 13 out of 19 cryopreserved ATMPs. These included the capacity to mediate immunosuppression, T cell activation, cytotoxicity, cytokine production in presence of leukemic or cell targets expressing viral antigens, proliferation and differentiation potential (Table 2 and Material and Methods). The results show that, as for viability, identity and purity, there was no significant loss of potency, nor any tendency for a significant reduction over time, even over extended periods of up to 13.5 years (Figure 2 and Table 2). Aspects of ATMP safety were not always included in stability studies, owing to lack of clear guidelines. For three of 11 MSC-based ATMPs, karyotype analysis was performed after cryopreservation [27]. These results were found to be according to specifications.

More frequently, microbiologic contamination during storage was investigated, an unlikely but possible event that may occur in case of defective containers or their deterioration over time [29]. Thus, for 14 of 19 cryopreserved ATMPs, bacterial sterility was tested and, for seven, also mycoplasma and endotoxin contamination, the latter a marker of some kinds of contamination. In all cases analyzed, the maintenance of sterility could be validated for freezing bags or cryovials for 1 to 13,5 years (Table 2).

Stability studies of fresh DPs

Some ATMPs cannot be cryopreserved at the end of production. Stability of five of these DPs was analyzed, with the aim to establish whether the cells were still viable after a storage at 2° to 8°C, for the length of time necessary before infusion into patients, considering worst-case situations. The data obtained show that stability of non-cryopreserved DPs, washed and resuspended in infusion medium, can be assigned a 4- to 24-h shelf life, based on results of viability (five of five), immunophenotyping (four of five) and in one case potency assay (proliferation potential in vitro and cell differentiation in vivo) (Table 3). In two cases, sterility after 24-h storage was also verified, since containers were in this case syringes and therefore potentially less protected from the environment.

Stability studies on cryopreserved intermediates

Past guidelines have suggested the need to control the stability of cryopreserved intermediates that are generated during expansion of some ATMPs, such as MSCs, and stored in cryovials. We collected the stability studies performed in the five cell factories on cell intermediates, and the details of the most complete data are available as Supplementary Table S1. The tests performed include in all cases (three

of three) viability, immunophenotype and bacterial sterility. Cell recovery (one of three) and proliferation capacity (two of three) were also investigated. Shelf lives of 1 to 9 years could be assigned to different cryopreserved intermediates, confirming the long-term stability of cryopreserved intermediates.

Holding time of DPs after thawing

The cryopreserved cells need to be thawed before infusion into patients. Although the procedures plan for immediate infusion, stability testing has been introduced to define a maximal holding time for the thawed DP that takes into account the need to perform quality controls before infusion or transport to the clinical unit. Viability of the cells is again the most frequently tested attribute verified in stability studies of thawed DPs, but in some cases immunophenotype and capacity to expand in culture or form colonies has also been investigated (Table 4). The data generated on five DPs allow us to define a maximal holding time upon thawing ranging from 15 min to 4 h, specific for each ATMP in its excipient storage temperature and container (Table 4).

Discussion

In this report, we collected the results of stability studies performed on 19 different experimental ATMPs, belonging to at least five cell lineages and produced over the last 13 years in GMP conditions in five authorized academic cell factories in the context of phase I/II clinical trials [30–48]. The stability studies were performed mostly on the final cryopreserved DPs in their primary containers.

The data presented show that all cryopreserved experimental ATMPs maintained, when stored at <-150°C in different freezing media containing 10% DMSO, a very high level of viability over very long periods of time. Viability, cell identity and purity were maintained above the specifications for $\leq$13.5 years, and the trend did not indicate any significant decrease in viability and identity over these longest tested periods. The results of functional potency assays, carried out on 68% of stored DPs, confirmed the viability data [49]. The data presented are not surprising, since many other cell products or tissues are known to maintain high viability and functionality for extended times when resuspended in iso-osmotic solutions containing 10% DMSO and cryopreserved in nitrogen vapor [50–53]. Successful long-term storage of cell-based medicines for therapeutic transplantation has been extensively demonstrated over many years, for example, hematopoietic stem cells used in the context of hematopoietic transplantation, which can be conserved in a cryopreserved state for $\leq$20 years [54,55]. Human embryos stored at <-150°C for $\leq$12 years have been shown to be fully viable and implant successfully *in utero*, giving rise to healthy babies [56,57]. Worth noting is that the storage temperature was monitored continuously, and DPs were maintained continuously in the same nitrogen tanks throughout their storage period and were likely subject only to limited temperature oscillations, except during their manual insertion and retrieval [58,59]. Clearly, it is important that remote monitoring and alarm systems should be in place for the continuous assessment of tank functionality in cell factories, and comprehensive quality management procedures on cryostorage of ATMPs should also be established and routinely followed to mitigate the possible negative consequences of out-of-specification events [60].

The data here are important because they have been performed on multiple ATMPs produced by five cell factories over an extended period of time, and similar studies on this type of DPs have not been published so far. The results suggest that stability studies for novel ATMPs may not need to have extensive analyses at short time points ($<$1 y), but rather should verify and validate longer periods of storage. This is important in the planning of these studies, in which many aliquots of several batches of ATMPs need to be generated and set aside for long-term storage and analysis. Although it is true that many ATMPs are dedicated to single patients (in particular autologous or HLA-matched ATMPs) and generally infused in patients within 1 y of production, others can be given regardless of donor's HLA and are off-the-shelf products, for which validated long-term stability may be a useful characteristic. Stability studies are quite demanding, requiring multiple aliquots of at least three batches of ATMPs and the execution of a number of biological tests, including complex potency assays.

The International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) has established stability testing guidelines that provide comprehensive guidance on registration stability requirements for new drugs in the ICH regions (Europe, United States and Japan) [6,61]. These guidelines suggest stability testing of DPs every 3 months during the first year of storage, every 6 months over the second year and once a year thereafter. The testing plan also has to consider possible variables such as different drug concentrations and the need to test at least three batches. Although a matrixing design can be implemented to reduce the numbers of aliquots and tests to be performed [62], these studies are in any case very time consuming and costly. They also need to be repeated every time an excipient or container is modified or any other significant change is introduced to the cryopreservation procedure. However, in the absence of specific guidelines for cryopreserved ATMPs, practices vary between laboratories, as also evidenced in this report, and regulatory authorities performing inspections often require a time plan that is in line with the rules set for standard drugs. Reduced testing of drugs during the first years of ATMP storage, as suggested by the data presented, as well as harmonized practices would therefore facilitate cell factories and the development of ATMPs [4].

Another aspect of stability testing is the need to guarantee the sterility of the ATMPs throughout their shelf life, and therefore their safety [63,64]. Microbiologic controls should be part of stability study plans, in consideration of the fact that containers may lose their seal during time [29,65,66]. We show here that different DPs cryopreserved in different excipients in freezing bags with overwrap bags, or in cryovials, always maintained sterility for up to 13,5 years. This result is not particularly surprising, at least for freezing bags, which have a double barrier to contamination, which should avoid problems due to possible bag failures [29,67]. Sterility of products may indeed depend on the type and quality of the primary containers and perhaps to some extent on the excipients or additive present, but are unlikely to depend on the specific ATMPs. We therefore suggest that a novel risk-based matrixing approach could be used for sterility testing in the context of stability studies: a cell factory may be able to validate specific excipients and containers in long-term studies, by including in their analyses all the different tests available, including sterility, mycoplasma and endotoxin. We propose that, once specific excipients, additives and container have been validated within a cell factory for absence of microbial contamination in long-term studies, such tests would not be required to be repeated extensively for each new ATMP. Such a risk-based approach would provide a high level of guarantee and would reduce costs by avoiding the necessity of repeating all the tests on each ATMP, unless significant changes in containers, excipients, additives or storage methods are introduced.

Finally, an important aspect emerging from the present report is that there is a need to harmonize the quality control test methods, in particular potency assays, as well as define acceptable specifications, in particular for viability parameters.

To conclude, we believe that the results presented here are the most extensive analysis of stability studies of ATMPs published so far. On the basis of the scientific evidence presented, we propose that specific guidelines regarding stability studies and shelf life assignments for this type of DP should be introduced, that should (1) better define the type of tests that need to be performed for ATMPs (at a

minimum viability and immunophenotype, but also potency studies, at least at some time points); (2) revise significantly the frequency of stability testing for ATMPs—we would suggest that testing of viability, identity and potency at an early time point (*e.g.*, 1 month after freezing) should be sufficient to control the effect of the freezing procedure on these parameters and to assign a 1-year shelf life, if the 1-month testing is within specifications; stability testing for the same parameters could then be repeated at 1 year and every 2 years thereafter, and shelf life could be extended at each test by 2 years in presence of results within the expected specifications; (3) test the microbiological attributes on stored DPs after a risk analysis, and thus tests need not be repeated on all ATMPs or for all attributes, provided the same containers and excipients/additives and freezing procedures are used and appropriate times have already been tested at least in triplicate; (4) investigate holding times with viability studies for non-cryopreserved DPs and upon thawing of cryopreserved products, with possible matrixing for DPs belonging to the same cell types and conserved in the same solutions. All these changes would need to be justified within the stability plan after a risk-based analysis. A longer shelf life assigned outright to all cryopreserved ATMPs is consistent with other practices, for example, shelf life assigned to cryopreserved heart valves [50].

We strongly believe that such specific guidelines would harmonize practices between different laboratories and guarantee full quality of the ATMPs during the assigned shelf lives but also simplify stability studies and significantly reduce costs. Similar studies should be analyzed for other cell-based intermediates used by the industry, in particular for hybridomas or recombinant eukaryotic cells used for therapeutic antibodies and cytokine production, because simplified procedures may be put in place to reduce costs without diminishing safety.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.jcyt.2021.12.004.

References

[1] Jiskoot W. Advanced Therapy Medicinal Products: What's in a Name? Journal of pharmaceutical sciences 2020;109(11):3282–4.
[2] Regulation (EC) No 1394/ 2007, in: E.p.a.t. Council (Ed.) https://eur-lex.europa.eu/legal-content/IT/TXT/PDF/?uri=CELEX:32007R1394&from=EN.
[3] Hubel A, Spindler R, Skubitz AP. Storage of human biospecimens: selection of the optimal storage temperature. Biopreservation and biobanking 2014;12(3):165–75.
[4] Iyer RK, Bowles PA, Kim H, Dulgar-Tulloch A. Industrializing Autologous Adoptive Immunotherapies: Manufacturing Advances and Challenges. Frontiers in medicine 2018;5:150.
[5] A. Hubel, Preservation of Cells: A Practical Manual, Wiley Blackwell 2018.
[6] International Council on Harmonisation. Stability testing for new drug substances and products. ICH Q1A(R2), 2003, https://database.ich.org/sites/default/files/Q1A%28R2%29%20Guideline.pdf.
[7] Galli MC. ATMPs for Cancer Immunotherapy: A Regulatory Overview. Methods in molecular biology 2016;1393:1–9.
[8] European Directive 2001 /83/CE, in: E. Commission (Ed.) https://ec.europa.eu/health.
[9] Directive 2009 /120/EC, https://ec.europa.eu/health/sites/default/files/files/eudralex/vol-1/dir_2009_120/dir_2009_120_en.pdf.
[10] Guidelines on good manufacturing Practice specific to advanced therapy medicinal products 2017, https://www.ema.europa.eu/en/news/new-guidelines-good-manufacturing-practices-advanced-therapies.
[11] FDA Guidance for Industry: Stability Testing of Drug Substances and Drug Products. 1998, https://www.fda.gov/media/71707/download.
[12] Aashigari S, Ramya Goud R, Sneha S, Vykuntam U, Potnuri NR. Stability studies of pharmaceutical products. World Journal of Pharmaceutical Research 2018;8(1):479–92.
[13] Haider K, Akash MSH, Faheem A, Rehman K. Guidelines for drug stability and stability testing. In: Akash MSH, Rehman K, eds. Drug Stability and Chemical Kinetics, Eds., Springer; 2020:19–29 https://www.springer.com/gp/book/9789811564253.
[14] Food and Drug Administration, 21 CFR 312.23(a)(7)(ii), https://www.govinfo.gov/app/details/CFR-2011-title21-vol5/CFR-2011-title21-vol5-sec312-23.
[15] Rajan R, Matsumura K. Development and Application of Cryoprotectants, 1081. Advances in experimental medicine and biology; 2018. p. 339–54.
[16] Clarke DM, Yadock DJ, Nicoud IB, Mathew AJ, Heimfeld S. Improved post-thaw recovery of peripheral blood stem/progenitor cells using a novel intracellular-like cryopreservation solution. Cytotherapy 2009;11(4):472–9.
[17] Ginis I, Grinblat B, Shirvan MH. Evaluation of bone marrow-derived mesenchymal stem cells after cryopreservation and hypothermic storage in clinically safe medium. Tissue engineering. Part C, Methods 2012;18(6):453–63.
[18] Stylianou J, Vowels M, Hadfield K. Novel cryoprotectant significantly improves the post-thaw recovery and quality of HSC from CB. Cytotherapy 2006;8(1):57–61.
[19] Vigano M, Budelli S, Lavazza C, Montemurro T, Montelatici E, de Cesare S, Lazzari L, Orlandi AR, Lunghi G, Giordano R. Tips and Tricks for Validation of Quality Control Analytical Methods in Good Manufacturing Practice Mesenchymal Stromal Cell Production. Stem cells international 2018;2018:3038565.
[20] Lisini D, Nava S, Frigerio S, Pogliani S, Maronati G, Marcianti A, Cocce V, Bondiolotti G, Cavicchini L, Paino F, Petrella F, Alessandri G, Parati EA, Pessina A. Automated Large-Scale Production of Paclitaxel Loaded Mesenchymal Stromal Cells for Cell Therapy Applications. Pharmaceutics 2020;12(5).
[21] Capelli C, Zaccara E, Cipriani P, Di Benedetto P, Maglione W, Andracco R, Di Luca G, Pignataro F, Giacomelli R, Introna M, Vitali C, Del Papa N. Phenotypical and Functional Characteristics of In Vitro-Expanded Adipose-Derived Mesenchymal Stromal Cells From Patients With Systematic Sclerosis. Cell transplantation 2017;26(5):841–54.
[22] Comoli P, Schilham MW, Basso S, van Vreeswijk T, Bernardo ME, Maccario R, van Tol MJ, Locatelli F, Veltrop-Duits LA. T-cell lines specific for peptides of adenovirus hexon protein and devoid of alloreactivity against recipient cells can be obtained from HLA-haploidentical donors. Journal of immunotherapy 2008;31(6):529–36.
[23] Brennan MA, Renaud A, Amiaud J, Rojewski MT, Schrezenmeier H, Heymann D, Trichet V, Layrolle P. Pre-clinical studies of bone regeneration with human bone marrow stromal cells and biphasic calcium phosphate. Stem cell research & therapy 2014;5(5):114.
[24] Barilani M, Lavazza C, Vigano M, Montemurro T, Boldrin V, Parazzi V, Montelatici E, Crosti M, Moro M, Giordano R, Lazzari L. Dissection of the cord blood stromal component reveals predictive parameters for culture outcome. Stem cells and development 2015;24(1):104–14.
[25] Nava S, Lisini D, Pogliani S, Dossena M, Bersano A, Pellegatta S, Parati E, Finocchiaro G, Frigerio S. Safe and Reproducible Preparation of Functional Dendritic

ARTICLE IN PRESS

Cells for Immunotherapy in Glioblastoma Patients. Stem cells translational medicine 2015;4(10):1164–72.
[26] Veronesi E, Murgia A, Caselli A, Grisendi G, Piccinno MS, Rasini V, Giordano R, Montemurro T, Bourin P, Sensebe L, Rojewski MT, Schrezenmeier H, Layrolle P, Ginebra MP, Panaitescu CB, Gomez-Barrena E, Catani F, Paolucci P, Burns JS, Dominici M. Transportation conditions for prompt use of ex vivo expanded and freshly harvested clinical-grade bone marrow mesenchymal stromal/stem cells for bone regeneration. Tissue engineering. Part C, Methods 2014;20(3):239–51.
[27] Capelli C, Pedrini O, Cassina G, Spinelli O, Salmoiraghi S, Golay J, Rambaldi A, Giussani U, Introna M. Frequent occurrence of non-malignant genetic alterations in clinical grade mesenchymal stromal cells expanded for cell therapy protocols. Haematologica 2014;99(6):e94–7.
[28] Gaipa G, Introna M, Golay J, Nolli ML, Vallanti G, Parati E, Giordano R, Romagnoli L, Melazzini M, Biondi A, Biagi E. Development of advanced therapies in Italy: Management models and sustainability in six Italian cell factories. Cytotherapy 2016;18(4):481–6.
[29] Khuu HM, Cowley H, David-Ocampo V, Carter CS, Kasten-Sportes C, Wayne AS, Solomon SR, Bishop MR, Childs RM, Read EJ. Catastrophic failures of freezing bags for cellular therapy products: description, cause, and consequences. Cytotherapy 2002;4(6):539–49.
[30] Casiraghi F, Perico N, Podesta MA, Todeschini M, Zambelli M, Colledan M, Camagni S, Fagiuoli S, Pinna AD, Cescon M, Bertuzzo V, Maroni L, Introna M, Capelli C, Golay JT, Buzzi M, Mister M, Ordonez PYR, Breno M, Mele C, Villa A, Remuzzi G, Group M-LS. Third-party bone marrow-derived mesenchymal stromal cell infusion before liver transplantation: A randomized controlled trial. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons 2021;21(8):2795–809.
[31] Uccelli A, Laroni A, Brundin L, Clanet M, Fernandez O, Nabavi SM, Muraro PA, Oliveri RS, Radue EW, Sellner J, Soelberg Sorensen P, Sormani MP, Wuerfel JT, Battaglia MA, Freedman MS. M.s. group, MEsenchymal StEm cells for Multiple Sclerosis (MESEMS): a randomized, double blind, cross-over phase I/II clinical trial with autologous mesenchymal stem cells for the therapy of multiple sclerosis. Trials 2019;20(1):263.
[32] Introna M, Lussana F, Algarotti A, Gotti E, Valgardsdottir R, Mico C, Grassi A, Pavoni C, Ferrari ML, Delaini F, Todisco E, Cavattoni I, Deola S, Biagi E, Balduzzi A, Rovelli A, Parma M, Napolitano S, Sgroi G, Marrocco E, Perseghin P, Belotti D, Cabiati B, Gaipa G, Golay J, Biondi A, Rambaldi A. Phase II Study of Sequential Infusion of Donor Lymphocyte Infusion and Cytokine-Induced Killer Cells for Patients Relapsed after Allogeneic Hematopoietic Stem Cell Transplantation. Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation 2017;23(12):2070–8.
[33] Introna M, Lucchini G, Dander E, Galimberti S, Rovelli A, Balduzzi A, Longoni D, Pavan F, Masciocchi F, Algarotti A, Mico C, Grassi A, Deola S, Cavattoni I, Gaipa G, Belotti D, Perseghin P, Parma M, Pogliani E, Golay J, Pedrini O, Capelli C, Cortelazzo S, D'Amico G, Biondi A, Rambaldi A, Biagi E. Treatment of graft versus host disease with mesenchymal stromal cells: a phase I study on 40 adult and pediatric patients. Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation 2014;20(3):375–81.
[34] Bassetti B, Carbucicchio C, Catto V, Gambini E, Rurali E, Bestetti A, Gaipa G, Belotti D, Celeste F, Parma M, Righetti S, Biava L, Arosio M, Bonomi A, Agostoni P, Scacciatella P, Achilli F, Pompilio G. Linking cell function with perfusion: insights from the transcatheter delivery of bone marrow-derived CD133(+) cells in ischemic refractory cardiomyopathy trial (RECARDIO). Stem cell research & therapy 2018;9(1):235.
[35] Comoli P, Pedrazzoli P, Maccario R, Basso S, Carminati O, Labirio M, Schiavo R, Secondino S, Frasson C, Perotti C, Moroni M, Locatelli F, Siena S. Cell therapy of stage IV nasopharyngeal carcinoma with autologous Epstein-Barr virus-targeted cytotoxic T lymphocytes. Journal of clinical oncology : official journal of the American Society of Clinical Oncology 2005;23(35):8942–9.
[36] Eoli M, Corbetta C, Anghileri E, Di Ianni N, Milani M, Cuccarini V, Musio S, Paterra R, Frigerio S, Nava S, Lisini D, Pessina S, Maddaloni L, Lombardi R, Tardini M, Ferroli P, DiMeco F, Bruzzone MG, Antozzi C, Pollo B, Finocchiaro G, Pellegatta S. Expansion of effector and memory T cells is associated with increased survival in recurrent glioblastomas treated with dendritic cell immunotherapy. Neuro-oncology advances 2019;1(1). vdz022.
[37] Perico N, Casiraghi F, Todeschini M, Cortinovis M, Gotti E, Portalupi V, Mister M, Gaspari F, Villa A, Fiori S, Introna M, Longhi E, Remuzzi G. Long-Term Clinical and Immunological Profile of Kidney Transplant Patients Given Mesenchymal Stromal Cell Immunotherapy. Frontiers in immunology 2018;9:1359.
[38] Magnani CF, Gaipa G, Lussana F, Belotti D, Gritti G, Napolitano S, Matera G, Cabiati B, Buracchi C, Borleri G, Fazio G, Zaninelli S, Tettamanti S, Cesana S, Colombo V, Quaroni M, Cazzaniga G, Rovelli A, Biagi E, Galimberti S, Calabria A, Benedicenti F, Montini E, Ferrari S, Introna M, Balduzzi A, Valsecchi MG, Dastoli G, Rambaldi A, Biondi A. Sleeping Beauty-engineered CAR T cells achieve antileukemic activity without severe toxicities. The Journal of clinical investigation 2020;130(11):6021–33.
[39] Comoli P, Basso S, Zecca M, Pagliara D, Baldanti F, Bernardo ME, Barberi W, Moretta A, Labirio M, Paulli M, Furione M, Maccario R, Locatelli F. Preemptive therapy of EBV-related lymphoproliferative disease after pediatric haploidentical stem cell transplantation. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons 2007;7(6):1648–55.
[40] Perico N, Casiraghi F, Introna M, Gotti E, Todeschini M, Cavinato RA, Capelli C, Rambaldi A, Cassis P, Rizzo P, Cortinovis M, Marasa M, Golay J, Noris M, Remuzzi G. Autologous mesenchymal stromal cells and kidney transplantation: a pilot study of safety and clinical feasibility. Clinical journal of the American Society of Nephrology : CJASN 2011;6(2):412–22.
[41] Perico N, Casiraghi F, Gotti E, Introna M, Todeschini M, Cavinato RA, Capelli C, Rambaldi A, Cassis P, Rizzo P, Cortinovis M, Noris M, Remuzzi G. Mesenchymal stromal cells and kidney transplantation: pretransplant infusion protects from graft dysfunction while fostering immunoregulation, Transplant international : official journal of the European Society for. Organ Transplantation 2013;26(9):867–78.
[42] Introna M, Borleri G, Conti E, Franceschetti M, Barbui AM, Broady R, Dander E, Gaipa G, D'Amico G, Biagi E, Parma M, Pogliani EM, Spinelli O, Baronciani D, Grassi A, Golay J, Barbui T, Biondi A, Rambaldi A. Repeated infusions of donor-derived cytokine-induced killer cells in patients relapsing after allogeneic stem cell transplantation: a phase I study. Haematologica 2007;92(7):952–9.
[43] Belingheri M, Lazzari L, Parazzi V, Groppali E, Biagi E, Gaipa G, Giordano R, Rastaldi MP, Croci D, Biondi A, Rebulla P, Edefonti A, Ghio L. Allogeneic mesenchymal stem cell infusion for the stabilization of focal segmental glomerulosclerosis. Biologicals : journal of the International Association of Biological Standardization 2013;41(6):439–45.
[44] Gomez-Barrena E, Padilla-Eguiluz NG, Rosset P, Hernigou P, Baldini N, Ciapetti G, Gonzalo-Daganzo RM, Avendano-Sola C, Rouard H, Giordano R, Dominici M, Schrezenmeier H, Layrolle P, On Behalf Of The Reborne C. Osteonecrosis of the Femoral Head Safely Healed with Autologous, Expanded, Bone Marrow-Derived Mesenchymal Stromal Cells in a Multicentric Trial with Minimum 5 Years Follow-Up. Journal of clinical medicine 2021;10(3).
[45] Gomez-Barrena E, Padilla-Eguiluz N, Rosset P, Gebhard F, Hernigou P, Baldini N, Rouard H, Sensebe L, Gonzalo-Daganzo RM, Giordano R, Garcia-Rey E, Cordero-Ampuero J, Rubio-Suarez JC, Garcia-Simon MD, Stanovici J, Ehrnthaller C, Huber-Lang M, Flouzat-Lachaniette CH, Chevallier N, Donati DM, Spazzoli B, Ciapetti G, Fleury S, Fernandez MN, Cabrera JR, Avendano-Sola C, Montemurro T, Panaitescu C, Veronesi E, Rojewski MT, Lotfi R, Dominici M, Schrezenmeier H, Layrolle P. Early efficacy evaluation of mesenchymal stromal cells (MSC) combined to biomaterials to treat long bone non-unions. Injury 2020;51:S63–73. Suppl 1.
[46] Ball LM, Bernardo ME, Roelofs H, van Tol MJ, Contoli B, Zwaginga JJ, Avanzini MA, Conforti A, Bertaina A, Giorgiani G, Jol-van der Zijde CM, Zecca M, Blanc KLe, Frassoni F, Egeler RM, Fibbe WE, Lankester AC, Locatelli F. Multiple infusions of mesenchymal stromal cells induce sustained remission in children with steroid-refractory, grade III-IV acute graft-versus-host disease. British journal of haematology 2013;163(4):501–9.
[47] Ciccocioppo R, Bernardo ME, Sgarella A, Maccario R, Avanzini MA, Ubezio C, Minelli A, Alvisi C, Vanoli A, Calliada F, Dionigi P, Perotti C, Locatelli F, Corazza GR. Autologous bone marrow-derived mesenchymal stromal cells in the treatment of fistulising Crohn's disease. Gut 2011;60(6):788–98.
[48] Comoli P, Basso S, Riva G, Barozzi P, Guido I, Gurrado A, Quartuccio G, Rubert L, Lagreca I, Vallerini D, Forghieri F, Morselli M, Bresciani P, Cuoghi A, Paolini A, Colaci E, Marasca R, Cuneo A, Iughetti L, Trenti T, Narni F, Foa R, Zecca M, Luppi M, Potenza L. BCR-ABL-specific T-cell therapy in Ph+ ALL patients on tyrosine-kinase inhibitors. Blood 2017;129(5):582–6.
[49] International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products Q6B, 1999, https://database.ich.org/sites/default/files/Q6B%20Guideline.pdf.
[50] Burkert J, Kochova P, Tonar Z, Cimrman R, Blassova T, Jashari R, Fiala R, Spatenka J. The time has come to extend the expiration limit of cryopreserved allograft heart valves. Cell and tissue banking 2020.
[51] Underwood J, Rahim M, West C, Britton R, Skipworth E, Graves V, Sexton S, Harris H, Schwering D, Sinn A, Pollok KE, Robertson KA, Goebel WS, Hege KM. How old is too old? In vivo engraftment of human peripheral blood stem cells cryopreserved for up to 18 years - implications for clinical transplantation and stability programs. World journal of stem cells 2020;12(5):359–67.
[52] Mareschi K, Adamini A, Castiglia S, Rustichelli D, Castello L, Mandese A, Leone M, Pinnetta G, Mesiano G, Ferrero I, Fagioli F. Cytokine-Induced Killer (CIK) Cells, In Vitro Expanded under Good Manufacturing Process (GMP) Conditions, Remain Stable over Time after Cryopreservation. Pharmaceuticals 2020;13(5).
[53] Shafa M, Walsh T, Panchalingam KM, Richardson T, Menendez L, Tian X, Suresh Babu S, Dadgar S, Beller J, Yang F, Baghbaderani BA. Long-Term Stability and Differentiation Potential of Cryopreserved cGMP-Compliant Human Induced Pluripotent Stem Cells. International journal of molecular sciences 2019;21(1).
[54] Lisenko K, Pavel P, Kriegsmann M, Bruckner T, Hillengass J, Goldschmidt H, Witzens-Harig M, Ho AD, Wuchter P. Storage Duration of Autologous Stem Cell Preparations Has No Impact on Hematopoietic Recovery after Transplantation. Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation 2017;23(4):684–90.
[55] Kim GH, Kwak J, Kim SH, Kim HJ, Hong HK, Jin HJ, Choi SJ, Oh W, Um S. High Integrity and Fidelity of Long-Term Cryopreserved Umbilical Cord Blood for Transplantation. Journal of clinical medicine 2021;10(2).
[56] Revel A, Safran A, Laufer N, Lewin A, Reubinov BE, Simon A. Twin delivery following 12 years of human embryo cryopreservation: case report. Human reproduction 2004;19(2):328–9.
[57] Yuan Y, Mai Q, Ma J, Deng M, Xu Y, Zhuang G, Zhou C. What was the fate of human embryos following long-term cryopreservation (>/=12 years) and frozen embryo transfer? Human reproduction 2019;34(1):52–5.
[58] Angel S, von Briesen H, Oh YJ, Baller MK, Zimmermann H, Germann A. Toward Optimal Cryopreservation and Storage for Achievement of High Cell Recovery and Maintenance of Cell Viability and T Cell Functionality. Biopreservation and biobanking 2016;14(6):539–47.
[59] Pogozhykh D, Pogozhykh O, Prokopyuk V, Kuleshova L, Goltsev A, Blasczyk R, Mueller T. Influence of temperature fluctuations during cryopreservation on vital parameters, differentiation potential, and transgene expression of placental multipotent stromal cells. Stem cell research & therapy 2017;8(1):66.

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[60] Schiewe MC, Freeman M, Whitney JB, VerMilyea MD, Jones A, Aguirre M, Leisinger C, Adaniya G, Synder N, Chilton R, Behnke EJ. Comprehensive assessment of cryogenic storage risk and quality management concerns: best practice guidelines for ART labs. Journal of assisted reproduction and genetics 2019;36(1):5–14.
[61] International Council on Harmonisation. Stability testing of Biotechnological/Biological Products. ICH Q5C, 1995, https://database.ich.org/sites/default/files/Q5C%20Guideline.pdf.
[62] International Council on Harmonisation. Matrixing and Bracketing designs for stability Testing of New Drug Substances and Product. ICH Q1D, 2002, https://database.ich.org/sites/default/files/Q1D%20Guideline.pdf.
[63] Brown H, Mahler HC, Mellman J, Nieto A, Wagner D, Schaar M, Mathaes R, Kossinna J, Schmitting F, Dreher S, Roehl H, Hemminger M, Wuchner K. Container Closure Integrity Testing-Practical Aspects and Approaches in the Pharmaceutical Industry. PDA journal of pharmaceutical science and technology 2017;71(2):147–62.
[64] Golay J, Pedrini O, Capelli C, Gotti E, Borleri G, Magri M, Vailati F, Passera M, Farina C, Rambaldi A, Introna M. Utility of routine evaluation of sterility of cellular therapy products with or without extensive manipulation: Best practices and clinical significance. Cytotherapy 2018;20(2):262–70.
[65] Nieto A, Roehl H, Brown H, Nikoloff J, Adler M, Mahler HC. Evaluation of Container Closure System Integrity for Frozen Storage Drug Products. PDA journal of pharmaceutical science and technology 2016;70(2):120–33.
[66] Jere D, Sediq AS, Huwyler J, Vollrath I, Kardorff M, Mahler HC. Challenges for Cell-Based Medicinal Products From a Pharmaceutical Product Perspective. Journal of pharmaceutical sciences 2021;110(5):1900–8.
[67] Becherucci V, Bisin S, Ermini S, Piccini L, Gori V, Gentile F, Ceccantini R, De Rienzo E, Bindi B, Pavan P, Cunial V, Allegro E, Brugnolo F, Bambi F. Comparison of CryoMACS Freezing Bags with Maco Biotech Freezing-Ethinyl Vinyl Acetate Bags for Hematopoietic Progenitor Cells Cryopreservation Using a CD34(+)-Enriched Product. Biopreservation and biobanking 2020;18(5):454–61.