TECHNICAL REPORT (PART B)

COVER PAGE

PROJECT	
Project number:	101056712
Project name:	**H**LA-homozygous iPSC-cardiomyocyt**E** **A**ggregate manufacturing techno**L**ogies for allogenic cell therapy to the heart
Project acronym:	HEAL

REPORTING PERIOD ⚠ *Please note that you must report on the entire reporting period.*	
RP number:	REP-101056712-2
Duration:	from 01/03/2024 to 31/08/2025

#@PER-REP-HE@#
#@PRO-GRE-PG@#

1. EXPLANATION OF THE WORK CARRIED OUT AND OVERVIEW OF THE PROGRESS

Following a successful first reporting period, the second reporting period has been equally productive, particularly benefiting from the 12-month non-monetary extension granted to the project. While this extension could not accelerate existing delays, it has allowed the project to continue progressing in a structured and stepwise manner, ensuring both research quality and thoroughness.

The project is now well-positioned to advance rapidly toward achieving its overall objectives:

- Develop scaled up GMP compliant processes for the production, quality assessment, storage and distribution of HLAh iPSC derived cardiomyocytes (iPS-CMs) aggregates (iPS-CMAs).
- Establish the immunogenicity aspect of HLAh iPSC and iPS-CMAs derived thereof
- Assessment and improvement of safety of iPS-CMAs for
    - Genetic integrity
    - Implementation and function of suicide gene
    - Toxicology / tumorigenicity
    - Arrhythmogenicity
- Complete regulatory preparations for first in man study of HLAh iPS-CMAs for heart repair.
- Establish IP strategy, cost effectiveness, distribution logistics for heart therapy and other diseases

Significance of the Extension

The 12-month extension entails no additional funding but is critical for the successful completion of key project objectives delayed due to unforeseen challenges such as logistical constraints, regulatory approvals, and adjustments to experimental timelines, as detailed in the granted amendment.

This extension enables the consortium to:

- Successfully complete all planned research activities.
- Maximize the scientific output.
- Fully utilize the existing budget.
- Maintain the quality and integrity of the project outcomes.

The consortium remains fully committed to delivering high-quality scientific results while addressing regulatory, clinical, and commercial requirements.

Consortium Collaborations and Progress

Communication within the consortium remains exceptional and highly collaborative throughout the project.

Partner Transition:

During the second reporting period, the consortium underwent a partner transition from Paracelsus Medizinische Privatuniversitat Salzburg (PMU) to BSZ Serologis GmbH (SERO). This transition was seamless as the PI remained unchanged, allowing ongoing work to proceed without interruption. Additionally, SERO fullfils all requirements previously met by PMU. The partner change was finalized during this reporting period.

Partner Engagement:

Although Biological Industries (BI) concluded most of their work in the first reporting period, they continue to actively participate in all consortium meetings. BI PIs join online for annual meetings, maintaining valuable engagement and contributions.

Ongoing Communication Activities:

- Regular laboratory exchanges conducted through personalized workshops
- Frequent monthly and weekly meetings within focused working groups
- Quarterly all-consortium TC meetings to updated on project progress
- Annual in-person consortium meeting (February 2025 at UMCU, Utrecht, Netherlands)

Research Progress: The WP-defined research program is advancing efficiently toward the project's objectives. Due to the extension of the project deadlines of deliverables and milestones are adjusted due to the unforeseen changes in the project progress.

Key Achievements to Date:

- Sucessfull continuation of HLA iPSC lines culture across partner laboratories, including establishment working cell bank creation in recipient labs, characterization, optimization of cardiac differentiation, and upscaling in various suspension culture platforms utilizing newly developed differentiation media; improvements in cryopreservation protocols have also been achieved.
- Entirely matrix-free, automation-friendly hiPSC expansion strategy was demonstrated, facilitating the development of good manufacturing practice (GMP)-compliant closed-system manufacturing and iPSC-CMA production in 2 liter scale enabledComprehensive assessment of immunogenicity profiles for HLAh iPSCs and iPS-CMAs.
- Continuous monitoring of genetic integrity in cells and advancement in the targeted introduction of suicide genes via gene editing.
- Substantial progress in establishing advanced methods for assessing the genetic integrity / safety of volume produced CMsAdvancement and innovation in iPS-CMA transportation logistic for cell delivery from CATD and MHH to UMCU established
- Improved strategies for iPS-CMA cryopreservation established
- iPC-CMA transplantation into the large animal myocardial infarct model in pigs initiated
- Dissemination: HEAL-symposium in frame of the German Stem Cell Network (GSCN) annual event 2024 performed; HEAL progress delivered to a leading global audience of translational stem cell scientist, clinicians and companies.
- Request for PEI consultutation meeting on the Drug Product (DP) characterization initiated

Table 1a: Deliverables (M19-M36)

Del. No.	Del.	Deliverable Name	Partner	Due Date	Updated Due Date	Status
WP 01 - UPSCALING of iPS-CM production						
1.2	**2**	HLA class I and II KO iPSC lines banked for iPS-CMA production and assessment in WP2/3	CATD	20, 30.04.2024		submitted
1.3	**3**	ACF GMP media effective in STBRs in 51 scale	BI	24, 30.08.2024		submitted
1.5	**5**	Set of validated SOPs; report on QA/QC assays for GMP iPS-CMA production/characterisation	CATD	30, 28.02.2025		submitted
WP 02 - IMMUNOGENICITY of HLA homozgygous iPS-CM						
2.1	**8**	Immunophenotyping of HLAh iPS-CMs	PMU	24, 30.08.2024		submitted
WP 03 - SAFETY genetic integrity and suicide gene						
3.1	**11**	Validated Nude Rat Assay for Tumorgenicity	INNO	12, 30.08.2023	25, 30.09.24	rejected 1PR/ resubmitted
3.3	**13**	Genetic integrity of iPSCs after mass production identifying genomic aberrations to enable cell line selection	HUJI	18, 28.02.2024	25, 30.09.24	rejected 1PR/ resubmitted
3.4	**14**	Generic pipeline for the genomic analysis of iPSC-based therapy	HUJI	24, 30.08.2024		rejected 1PR/ resubmitted
WP 04 - REGULATION EMA						
4.1	**19**	Report on the Development Plan completion stage	EATRIS	24, 30.08.2024		submitted
WP 05 - COST EFFECTIVENESS HTA, DISSEMINATION AND EXPLOITATION						
5.3	**24**	HTA results for iPS-CMA heart therapies	EATRIS, UoX	24, 30.08.2024	27, 30.11.24	submitted

Table 1b: Milestones (M19-M36)

No.	Milestone Name	WP	Partner	Due Date
M15	Decision on optimum protocol for genomic analysis of iPSCs based on completed analysis of genetics integrities by different methodologies	3	HUJI	20, 30.04.2024
M16	Immune function assays operative	2	PMU	24, 31.08.2024

1.1 Objectives

#@WRK-PLA-WP@#

Summary of progress towards the achievement of each of the project objectives within the reporting period M19-M36

Medizinische Hochschule Hannover (MHH):

Upscaling iPSC expansion and iPS-CM production by closed system manufacturing

Advanced closed system manufacturing and upscaling the iPSC-CM production process in stirred tank bioreactors (STBRs) has been achived and resulted into high impact publications with reference to the HEAL project funding.

This includes:

An entirely matrix-free, highly efficient, flexible and automation-friendly hPSC expansion strategy is demonstrated, facilitating the development of good manufacturing practice-compliant closed-system manufacturing in large scale.

Bioprocess upscaling for iPSC-CM aggregates production in 2 liter scale enabling the production of > 1 billion iPSC-CM in a single batch process

Promoting progression towards GMP-compliant iPSC-CM aggregates production in stirred bioreactors, a more controlled strategy has been developed by us and published in an SOP-like protocol. Modifying this strategy in collaboration with CATD has led to an advanced production and iPSC-CMA tranportation protocol used for the successful production and delivery of iPSC-CMA from MHH to the administration into the heart in the pig model directed by UMCU.**Development of iPSC lines that carry an iCASP9 suicide gene**

The results of the research show that none of the examined samples showed any acquired genetic changes and that neither the longer cultivation time nor the gene editing provide a negative impact on the functionality of the cells

Development of SOPs for GMP-compliant generation of iPSC lines carrying an iCASP9 suicide gene

Development of SOP conducted.

Catalent Düsseldorf (CATD):

Establish GMP workflow and conduct pilot runs for GMP-compliant HLAh iPS-CMA production

CATD has successfully established scalable and GMP-compatible workflows for the expansion of iPSCs, their differentiation into cardiomyocytes (iPSC-CMs), and the optimization of gene editing strategies in iPSCs. The cardiac differentiation protocol was progressively scaled from 20 mL cultures up to 500 mL suspension cultures, yielding up to 1.3×10^9 cardiomyocytes with consistently high purity (>90% $cTnT^+$). Compatibility studies in PBS Vertical Wheel bioreactors confirmed that the process is robust and transferable to GMP-relevant culture systems. A dissociation and re-aggregation step implemented at the cardiac progenitor stage further improved aggregate uniformity, enhanced cardiomyocyte purity, and ensured the generation of injection-compatible aggregates, while maintaining genomic stability. Feasibility for transport and administration was demonstrated, as iPSC-CM aggregates retained high viability after 24 hours of cold storage in saline and remained structurally intact after injection through clinical-grade needles.

In parallel, a standardized GMP-oriented workflow was developed, covering all steps from iPSC thawing, expansion, and differentiation through dissociation/re-aggregation and final preparation for transport and injection. This workflow was complemented by the implementation of in-process controls and quality control assays, including a highly sensitive residual iPSC assay (miRNA302/367 qPCR) and a potency assay based on contractility measurements in 3D constructs, ensuring product safety and functionality.

In addition, an optimized gene editing workflow was established, enabling precise gene editing of R26 iPSCs under GMP-compatible conditions. This workflow includes improved single-cell cloning, expansion, and QC strategies, facilitating the generation of clonally derived, genetically edited iPSCs.

To support long-term production needs, a new iPSC banking process was validated, enabling the generation of up to 360 cryovials per bank using single-cell passaging. This approach is harmonized with existing R&D protocols used for iPSC maintenance prior to cardiac differentiation, resulting in improved culture homogeneity and enhanced consistency for downstream applications.

In summary, CATD has advanced iPSC technologies by integrating upscaling, differentiation, banking, and gene editing into a coherent, GMP-oriented platform that establishes a solid foundation for the upcoming pilot runs for the iPS-CMs.

Sartorius Biological Industries (BI):

Objective 1– GMP-grade iPSC ACF Expansion Medium

An ACF expansion medium (Nutri3D hPSC ACF medium) for monolayer and aggregate suspension culture was developed and validated under cGMP and USP/Ph. Eur. standards. A 100 L GMP (Pilot) batch was produced with full supporting documentation, including IFU, SOPs, and QC protocols. After passing QC testing, the medium was shipped to partners (MHH, Catalent) for evaluation in Erlenmeyer flasks and 150 mL STBR. The medium was successfully tested with the consortium iPSC line R26-6 (Catalent) and subsequently applied in cardiac differentiation using CDM3 and internal formulations.

Objective 2 – GMP-grade iPSC-CM Differentiation Medium (CDM3) & Prototype Development

A small-scale 3D differentiation platform was established based on the MHH protocol using WNT-pathway modulators (CHIR99021, IWP2), enabling QC testing of differentiation yield and purity by cTNT, SA, and pan-MHC. GMP-grade CDM3 medium was produced, QC-tested, and shipped to MHH. Two formulation formats were developed (suitable to follow expansion using Nutri3D ACF medium) : a basal medium stored at +4 °C combined with a supplement mix at –20 °C, and a single supplement mix (–20 °C) for use with DMEM/F12. QC-SOPs were established, and validation was performed through Wnt/β-catenin modulation using iPSC lines R26-6 hHLA and RCRP005N. The medium achieved performance targets with cell yields of ≥1M cells/mL and purity of ≥80% (cTNT, SA). Stability testing confirmed six months for both basal and supplement mixes, 14-28 days for the complete medium at 2–8 °C.

University of Düsseldorf (UDUS):

Develop scaled up GMP compliant processes for the production, quality assessment, storage and distribution of HLAh iPSC derived cardiomyocytes (iPS-CMs) aggregates (iPS-CMAs).

UDUS is actively involved in culturing the project specific HLA homozygous iPS cell line under GMP conditions in small-scale (well plates and flasks) for testing of different culture and freezing conditions. The quality of the product is analyzed by flow cytometry applying cardio specific markers.

The freezing and storage of cardiomyocytes aggregates is currently under evaluation applying different freezing protocols for optimization of viability and recovery. In parallel cryopreservation of

single cardiomyocytes is performed, as well as the testing of a re-aggregation procedure prior to freezing.

The Hebrew University of Jerusalem (HUJI):

WP3 aims at the analysis and interpretation of genetic integrity of human pluripotent stem cells (hPSCs). Within this WP we have previously conducted a survey of cancer-related mutations in human pluripotent stem cells and their derivatives, we analyzed >2,200 transcriptomes from 146 independent lines in NCBI's Sequence Read Archive. 22% of the samples had at least one cancer-related mutation. Of these samples, 64% had TP53 mutations, which conferred a pronounced selective advantage, perturbed target-gene expression and altered cellular differentiation. These findings underscore the need for robust surveillance of cancer-related mutations in pluripotent cells, especially in clinical applications.

We have recently expanded our analysis in two different directions:

1. Analysis of chromosomal aberrations in hPSCs, defining that 12% of male hPSC samples have lost their chromosome Y and identified loss of chromosome Y in differentiated cells from HPSCs to all three germ layers. The broad analysis sheds light on the effects of loss of chromosome Y in hPSCs, suggesting a novel dosage-sensitive mechanism regulating transcription of ribosomal protein genes.

2. We have generated an in-depth analysis of adult stem cells in addition to PSCs to identify cancer-related mutations. We have thus identified cancer-related mutations in 18% of mesenchymal stem cell samples, and in 41% of neural stem cell samples. We have shown a lineage-specific profile of cancer-related genes, demonstrating that TP53 is a central mutated gene in human PSCs but not in mesenchymal or neural stem cells. We thus propose a categorization of these mutated samples for further appreciation of their severity, emphasizing the importance of genetic screening in pluripotent and adult stem cell lines.

Universitair Medisch Centrum Utrecht (UMCU):

Asses arrhythmogenicity potential of iPS-CMAs and optimize delivery & retention

Though we have experienced delays, UMCU has made substantial progress towards the specific task (3.6): Asses arrhythmogenicity potential of iPS-CMAs and optimize delivery & retention. Arrhythmogenicity is a potential specific safety issue related to transplantation of iPSC-CMAs. Myocardial infarct induced ventricular scaring may lead to tachyarrhythmias in affected patients. In addition, it has been reported in experimental studies, that the transplantation of iPS-CMs may induced donor CM-derived undesired action potentials, promoting the risk of arrhythmogenic events.

To assess the arrhytmogenic risk after iPSC-CMA transplantation, a total of 7 pigs with cardiac ischemia reperfusion damage were transplanted with saline or iPSC-CMAs. The occurenence of cardiac arrhytmias was monitored by implantable cardiac monitors and cardiac function was assessed with cardiac MRI. Four weeks after transplantation animals were sacrificed and tissue harvested for further processing. Follow-up analysis, including assessing the presence, frequency and duration of cardiac arrhytmias and assessing the retention of the iPSC-CMA in cardiac tissue are curently being performed. A detailed description on the progress is provided in section 1.2.

InnoSer (INNO):

Toxicology / tumorigenicity- validated nude rat assay for tumorigenicity

iPSC-derived cellular products are not subject to conventional pharmacokinetic safety testing and require biodistribution tumorigenicity studies. InnoSer will perform toxicology, biodistribution and tumorigenicity studies of the CM aggregates for the project. InnoSer has validated a qPCR assay with respect to detection sensitivity and specificity for human Alu repeat DNA detection to allow

biodistribution analysis. An NSG tumorigenicity mouse model assay has been validated based on previous regulatory input from the Paul-Ehrlich Institut (PEI). The model has been validated with the iPSC cell line from Catalent and a Hela cell line as positive control. Differentiated CMA have been spike with undifferentiated IPSCs to confirm the sensitivity of the model to show tumor formation in case of contaminating undifferentiated iPSCs in the transplanted products. qPCR and histopathology were used to confirm the presence of human tumor cells. The assay and model are now ready to test the final CMA cells product. Suicide gene experiments and tumorigenicity assays are scheduled to start in Q4 2025.

The European Advanced Translational Research Infrastructure in Medicine (EATRIS):

Development Plan to ensure alignment with regulatory requirements (TPP)

During this reporting period the first task was to complete Deliverable D4.1 which aimed to establish a Development Plan to address project weaknesses and ensure alignment with regulatory requirements. Central to this plan was the drafting of a Target Product Profile (TPP), created collaboratively within the first year of the project and refined through an iterative process across all partners. The TPP acted as both a roadmap and a monitoring tool, enabling progress assessment, gap identification, and corrective actions where necessary. Complementing the TPP, a review and feedback process had been initiated, including the development of SOPs for toxicity and carcinogenicity studies, which will inform the preclinical sections of the IMPD and IB whose development and drafting was a core part of this reporting period. Where consortium expertise was insufficient, the process of acquiring regulatory guidance at a Scientific Advice from PEI was initiated, ensuring that development parameters, efficacy tests, and clinical trial designs remained compliant moving forward. Early project outcomes defined in this period included the definition of product identity, based on myocardiocyte precursors, supported by ongoing antigen characterization studies and dosage range determination by MHH and Catalent. The manufacturing process, considered a key component of identity for ATMPs, continued to be standardized and compared between partners to establish robust parameters. Importantly, a cryopreservation step was integrated into the production workflow, addressing both clinical availability and logistical challenges associated with the short shelf-life of live cell products. Together, these efforts provided the foundation for the finalization of a complete IMPD which was proactively developed during tis reporting period with the aim to be completed by project conclusion.

- To Assess the current regulatory background of the HEAL project outputs and guidance for the IMP class. Devise a TPP
- Preparation and completion of the request for Scientific Advice to the national competent authority (PEI) and to the EMA
- Completion of CTA document (including IMPD and IB) ready for submission to the CTA process to be clinical trial ready by the end of the project lifespan

Paracelsus Medizinische Privatuniversität (PMU) & Serologis (SERO):

During the reporting period beneficiary PMU terminated its participation within HEAL. The beneficiary was changed to Serologis (SERO) with most of the people involved remaining the same since the team changed to SERO as well.

PMU:

Task 2.2 Comprehensive immunophenotyping of HLA-homozygous iPS-CMs [M01-M24]

5 Establish a comprehensive 40-parameter immunophenotype panel for iPS-CM at single-cell level.

6 Deliverable D2.1: Immunophenotyping of HLAh iPS-CMs

Task 2.3 Innate and adaptive immune response profiling applying HLA- matched iPS-CMAs and HLAh matched vs. haploidentical surrogate responder lymphocytes [M06-M32]

7 Validate immunogenicity of HLAh iPS-CM in HLA-matched mixed leucocyte and cytotox responses (in absence / presence of representative immunosuppressants).
8 Deliverable D2.2: Immune response data predicting immune-suppression requirements of HLA-matched iPS-CMAs

In project month 19-28 we extended the surface immune phenotyping of iPS-CM using spectral flow cytometry (sFCM) from 16 markers (reported in Milestone No. 10) to a total of 54 markers for comprehensive immunophenotyping. For testing the immunogenicity of HLAh iPS-CM we successfully adapted protocols to assay allorecognition by T cells. Further, we modified protocols testing T and NK cell-mediated cytotoxicity for use with iPS-CM. Preliminary results show expansion and differentiation of T cells in response to co-culture with iPS-CM indicating active allorecognition. Cytotoxicity against iPS-CM was observed for pooled unmatched effector (T and NK) cells but not for single donor haplo- or full-matched effectors.

A detailed description of the work carried out and the respective results is given below and in the submitted deliverable (D2.1).

SERO:

At SERO we used the whole 54 marker combination on cardiomyocytes generated independently at MHH and CATD which were kept under various conditions relevant to the transplantation scenario and potentially inflammatory microenvironmental cues. In addition, we benchmarked the combined marker expression pattern against PBMCs and Jurkat cells.

Associated partner- University of Oxford (UOX):

Legal and Regulatory Analysis

This task is completed with Deliverable 5.4a submitted on 31/07/2025. It provides a comprehensive review of data protection and privacy requirements under European law relevant to allogeneic HLA-homozygous iPSC-derived cardiomyocytes used in ATMPs. The report covers privacy considerations, protection of donor personal and genetic data, information security, and cybersecurity aspects during cell therapy development, alongside an assessment of the data generated at each technical step and its potential to identify donors.

Institutional Readiness Assessment

A full draft of Deliverable 5.4b was completed and uploaded to the consortium intranet on 31/07/2025. This report examines the readiness of two European university hospitals to adopt HEAL's novel iPSC-CMA therapy. Using the Institutional Readiness framework, the study drew on interviews with clinical and pharmacy experts, evaluating infrastructure, clinical and procedural preparedness, and organisational capacity. Key findings are due to be presented at the 8th HEAL teleconference on 06/10/2025 and a final version of the report will be submitted as Deliverable 5.4b following the incorporation of any final feedback from consortium members.

1.2 Explanation of the work carried out per WP

Overview of ongoing work per WP in M19-M36:

Table 2 shows the overview of reached Milestones and Deliverables throughout the second reporting period. Also shown are deviations in Milestones and Deliverables. More detailed explanations are given under 5. DEVIATIONS FROM ANNEX 1 AND ANNEX 2 of this report.

Table 2

P No	Lead partner	TASK	1st Reporting Period																2nd Reporting Period																		3rd Reporting Period																	
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54
1	MHH	**WP title: UPSCALING of iPS-CM production**																																																						
		ACF cGMP-compliant media						M4										M12								D3												D4/ M14				M14				D4										
		Upscaling iPSC expansion and iPS-CM production						D1																																														
		HLAh iPSC cell banks and HLA gene knockout			M1								M9								D2																																			
		GMP workflow and pilot runs for iPS-CMs																														D5												M17										D6
		iPC-CMA crypopreservation																																																			D7			
2	PMU	**WP title: IMMUNOGENICITY of HLA homozygous iPS-CM**																																																				
		HLAh iPSC/iPS-CM differentiation efficiency			M2																																																	
		Immunophenotyoing of HLAh iPS-CMs												M10												D8																													
		Immune response/ immunosuppression																								M16																															
		Machine learning correaltion algorithm																																																			D9			
3	HUJI	**WP title: SAFETY genetic integrity and suicide gene**																																																				
		Genetic integrity of HLAh iPSC																							D13																														
		Genetic integrity of mass produced iPS-CMs																																																	D18					
		Generic pipeline for iPSC genetic integrity																				M15				D14																													
		Integration of suicide gene in HLA-Hz iPSC															M11																																			D15&D16				
		Tumorgenicity assays and studies																									D11																											D17		
		Arrythmogenicity potential of iCMAs																																				D22																	D24
4	EATRIS	**WP title: REGULATION EMA**																																																			
		State of art review and FTO						M5																																														
		Regulation and guidance for the IMP																																																				
		Preliminary documentation assessment																																																			
		Preparation of the request for Scientific Advice											M8																											M8								M18	D20					
		Revision of documents for CTA																M13								D19																												D21
5	CATD	**WP title: COST EFECTIVENESS HTA, DISSEMINATION EXPLOITATION**																																																			
		FTO and IP Monitoring			D22																																																		
		Freedom-to- operate strategy						M6																																														
		Examination of social aspects of iPSC and iCMA										M7																										D25																
		Optimisation of methodology																																				D23																	
		Distribution Logistics																																								M20													
		HTA and business models																												D24																									D26
6	MHH	**WP title: MANAGEMENT and DISSEMINATION**																																																			
		Consortium communications and governance			D29																																																	
		Dissemination and communication			D28	M3		M20																																											M19				
		Progress monitoring and risk management						D30																																														
		Reporting and administration						D31																																														
		Financial management												D32																																								

Milestone | Deliverable | Deviation (Milestone & Deliverable) | Old Due Date

1.2.1 Work Package 1

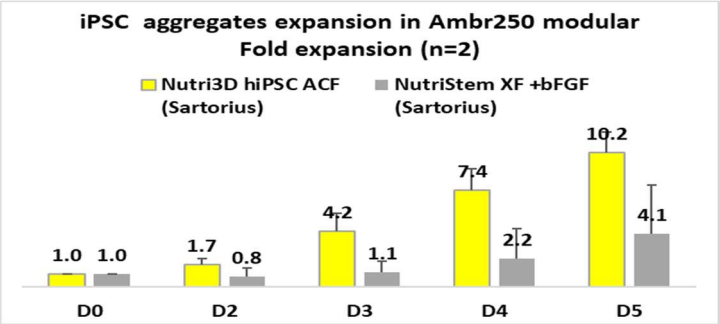
WP 1: Upscaling of iPS-CM production: **Lead Beneficiary MHH**

The overall aim of WP1 is to establish upscaling and cGMP adaption of an iPSC-CM production process in suspension culture to provide the controlled, regulatory-compliant cell product for clinical testing.

BI:

Task 1.1 Establish ACF cGNP-compliant culture and differentiation media [M1-M24]

The project is ready for technology transfer to OPS. Stability data shows 15 months for two PD batches and 9 months for an additional batch, while the 50L OPS pilot batch successfully passed 6 months of stability testing. The medium is designed to perform equal to or better than NutriStem XF in 2D culture and to outperform competitor media in suspension. Complete medium stability is confirmed for 30 days at 2–8 °C, with a final shelf-life target of 18 months. Next steps include industrialization and continuation of the small-scale bioreactor run, which is currently ongoing.

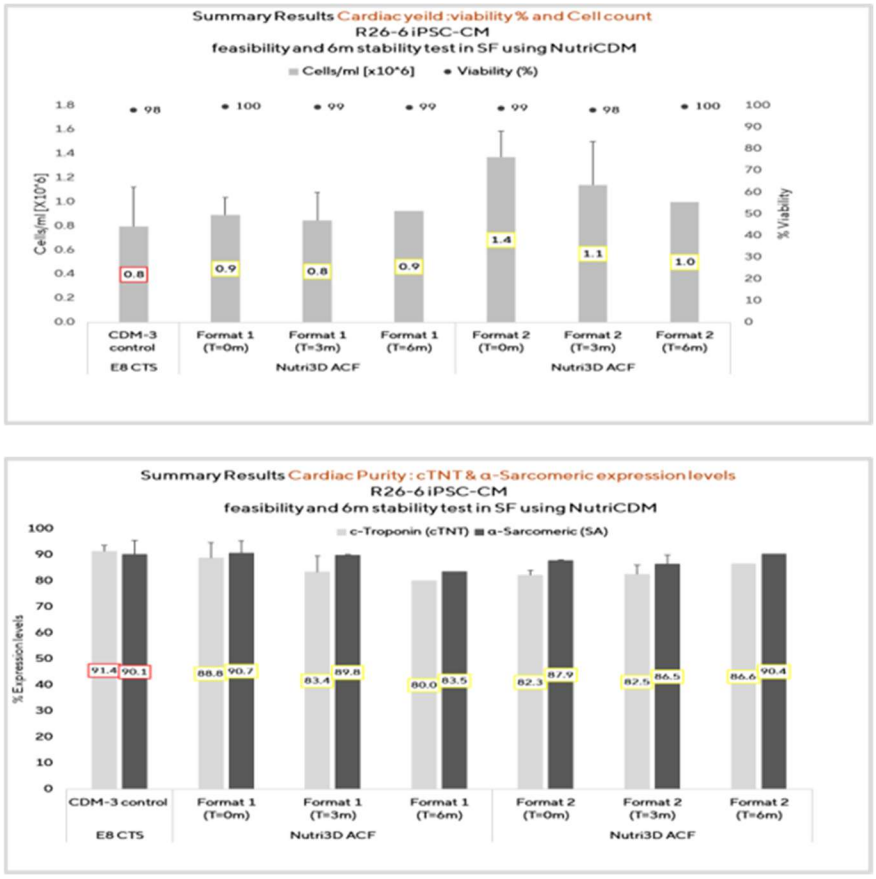


Graph 1. Average fold expansion hiPSC (A18945 Themo) expanded as aggregates in Ambr250 modular using Nutri3D hPSC ACF and a variety of other ACF and XF commercial media all supplemented with Poloxamer.

Task 1.2 Upscaling iPSC expansion and iPS-CM production by closed system manufacturing

Development of a specialized ACF medium was achieved to support the 3D differentiation of hiPSC-derived aggregates, previously expanded in Nutri3D hPSC ACF medium, into cardiomyocytes for clinical cell therapy applications. The medium is available in two formats, either as a basal solution stored at +4 °C with a supplement mix at –20 °C, or as a single supplement mix for DMEM/F12 stored at –20 °C. Validation was performed under QC-SOP conditions, with the Wnt/β-catenin pathway confirmed and testing conducted on the R26-6 hHLA and RCRP005N iPSC lines. The system demonstrated a yield of at least 1 million cells per ml with a cardiomyocyte purity of ≥80% (cTNT, SA). Stability studies verified 6 months for both the basal medium and supplement mix, while the complete medium maintained stability for 30 days at 2–8 °C, with an overall shelf-life target of 12 months. Performance was benchmarked to meet or exceed competitor media in suspension differentiation.

Graphs 2+3. Stability data of NutriCDM



Graph 2+3: Data represent mean ± SD of *n* = 3 independent R&D batches run in parallel. Upper graph: cell density (cells/mL) and viability for two formats over time. Lower graph: cardiac purity for the two formats.

MHH:

Task 1.2 Upscaling iPSC expansion and iPS-CM production by closed system manufacturing [M1-M24]

In frame of this collaborative project, MHH has focussed on both advancing closed system manufacturing and upscaling the iPSC-CM production process in stirred tank bioreactors (STBRs). Notably, this work has led into high impact publications with reference to the HEAL project funding

In brief, it was enabled to perform STBR-controlled, chemical aggregate dissociation and optimized passage duration of 3 or 4 days, thereby promoting exponential hPSC proliferation, process efficiency and upscaling by a seed train approach. Intermediate high-density cryopreservation of suspension-derived hPSCs followed by direct STBR inoculation importantly enabled complete omission of matrix-dependent 2D (two-dimensional) culture. Optimized 3D cultivation over 8 passages (32 days) cumulatively yielded ≈4.7× 10^{15} cells, while maintaining hPSCs' pluripotency, differentiation potential and karyotype stability. Gene expression profiling reveals novel insights into the adaption of hPSCs to continuous 3D culture compared to conventional 2D controls. Together, an entirely matrix-free, highly efficient, flexible and automation-friendly hPSC expansion strategy is demonstrated, facilitating the development of good manufacturing practice-compliant closed-system manufacturing in large scale.

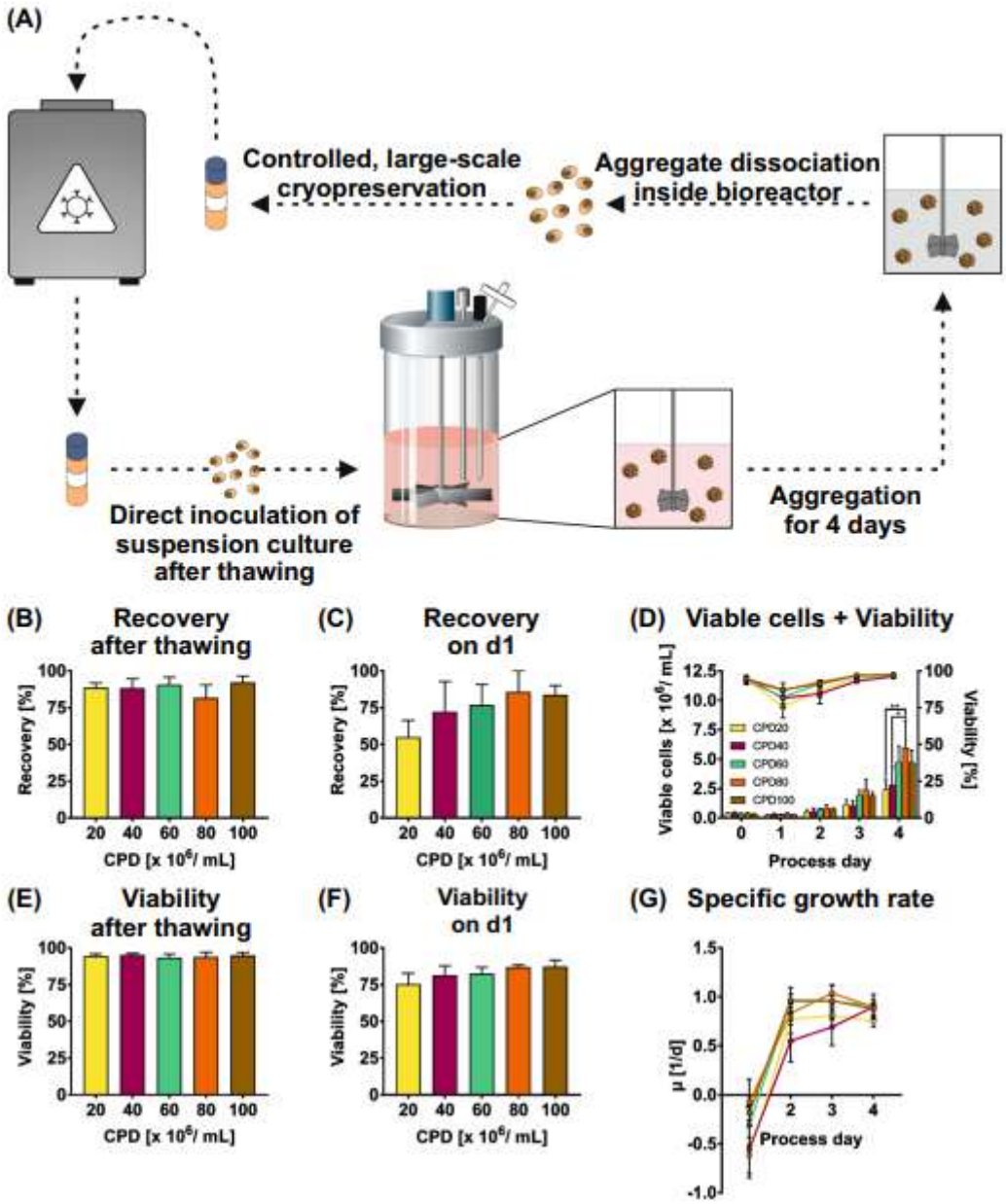


Figure 1: Cryopreservation of suspension-derived hPSCs for direct inoculation in suspension culture overcoming the need for 2D preculture thus promoting closed system manufacturing.

Regarding process upscaling of the iPSC-CM aggregates production, we have recently achieved production in 2 liter scale, enabling the production of > 1 billion iPSC-CM in a single batch process .

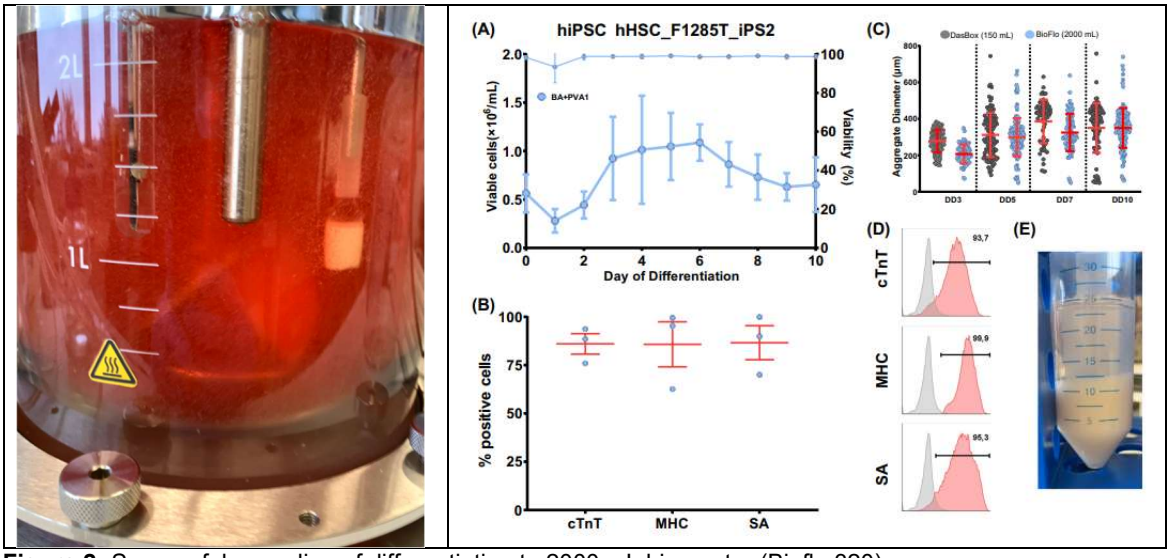
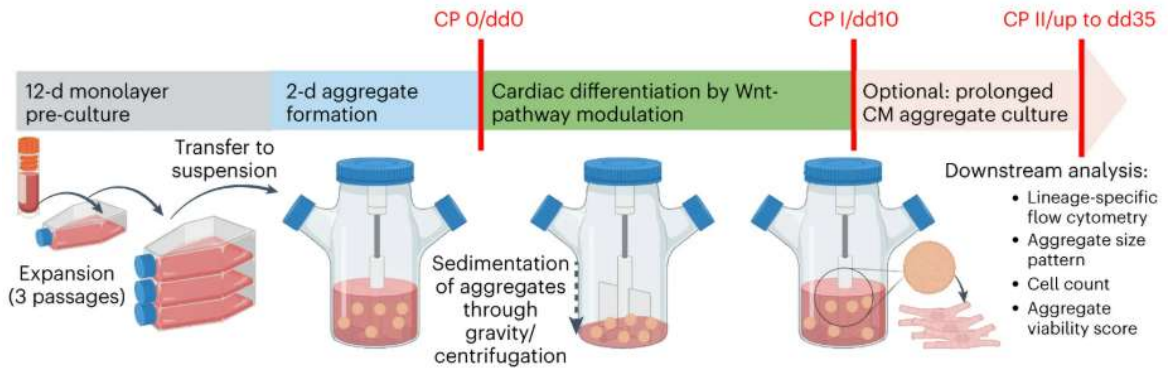


Figure 2: Successful upscaling of differentiation to 2000 mL bioreactor (Bioflo 320)

This work represents an important step toward the envisioned iPSC-CM aggregates production in 5 liter scale (MS14) which is currently in progress.

Promoting progression towards GMP-compliant iPSC-CM aggregates production in stirred bioreactors, a more controlled strategy has been developed by us and published in an SOP-like protocol.



The timeline is shown in the upper row, and the applied culture strategy and vessels are shown in the lower row. hPSCs are thawed and expanded in monolayer (Steps 1–28). Single cells are then used to inoculate stirred single-use spinner flasks, where cell-only aggregates form (Steps 29–42). Through WNT-pathway modulation, differentiation into CMs is induced (Steps 43–85). After 10 d, CMs can be analyzed for yield and purity and applied for downstream applications. Alternatively, CM aggregates can be kept in spinner flasks for ≤35 d without major cell loss or changes in CM purity (Steps 86–117). dd, day of differentiation. Figure created with BioRender.com.

Figure 3: Schematic display of key steps and timelines of the protocol and the applied culture strategy and vessels

Further modifying this strategy in collaboration with CATD (see next pagraph) has led to an advanced production and iPSC-CMA tranportation protocol (in saline at 4°C for up to 24h; see next paragraph by CATD) which has been used for the successful production and delivery of iPSC-CMA from MHH to the initiated administration into the heart in the pig model (see description below; UMCU:Task 3.6 Asses arrhythmogenicity potential of iPS-CMAs and optimize delivery & retention).

References:

Kriedemann N, Manstein F, Hernandez-Bautista CA, Ullmann K, Triebert W, Franke A, Mertens M, Stein ICAP, Leffler A, Witte M, Askurava T, Fricke V, Gruh I, Piep B, Kowalski K, Kraft T, Zweigerdt R. Protein-free media for cardiac differentiation of hPSCs in 2000 mL suspension culture. Stem Cell Res Ther. 2024 Jul 18;15(1):213. doi: 10.1186/s13287-024-03826-w.

Kriedemann N, Triebert W, Teske J, Mertens M, Franke A, Ullmann K, Manstein F, Drakhlis L, Haase A, Halloin C, Martin U, Zweigerdt R. Standardized production of hPSC-derived cardiomyocyte aggregates in stirred spinner flasks. Nat Protoc. 2024 Jul;19(7):1911-1939. doi: 10.1038/s41596-024-00976-2. Epub 2024 Mar 28.PMID: 38548938

Ullmann K, Manstein F, Triebert W, Kriedemann N, Franke A, Teske J, Mertens M, Lupanow V, Göhring G, Haase A, Martin U, Zweigerdt R. Matrix-free human pluripotent stem cell manufacturing by seed train approach and intermediate cryopreservation. Stem Cell Res Ther. 2024 Mar 25;15(1):89. doi: 10.1186/s13287-024-03699-z.PMID: 38528578

CATD:

Task 1.2 Upscaling iPSC expansion and iPS-CM production by closed system manufacturing [M1-M24]

As part of the ongoing collaborative project, CATD contributed to the optimization and upscaling of iPSC-derived cardiomyocyte (iPSC-CM) production focused on the technologies available within their GMP facility. The aim was to facilitate a robust and reproducible differentiation process that could be transitioned from small-scale research formats to larger-scale production.
To achieve this, CATD adapted the cardiac differentiation protocol to progressively larger culture volumes using Erlenmeyer Flasks (20 mL, 50 mL, 125 mL, 250 mL, and 500 mL) while fine-tuning agitation parameters to maintain optimal aggregate morphology and differentiation efficiency. At each scale, cardiomyocyte purity was assessed via flow cytometry for cTnT expression, consistently demonstrating high differentiation efficiency (>90%) (**Figure 4A**). The 500 mL scale yielded approximately 1.3 × 10^9 cardiomyocytes per culture, confirming scalability without compromising quality (**Figure 4B**).
In addition, we evaluated process compatibility with equipment present in our GMP area, including the PBS Vertical Wheel bioreactor. This setup maintained both high $cTnT^+$ purity (98%) and yields comparable to the Erlenmeyer flask approach, further validating the potential for integration into a GMP production pipeline (**Figure 4C**).
CATD contribution ensured that the upscaling workflow was aligned with available GMP technologies, creating the foundation for the GMP workflow establishment for the iPSC-CM manufacturing.

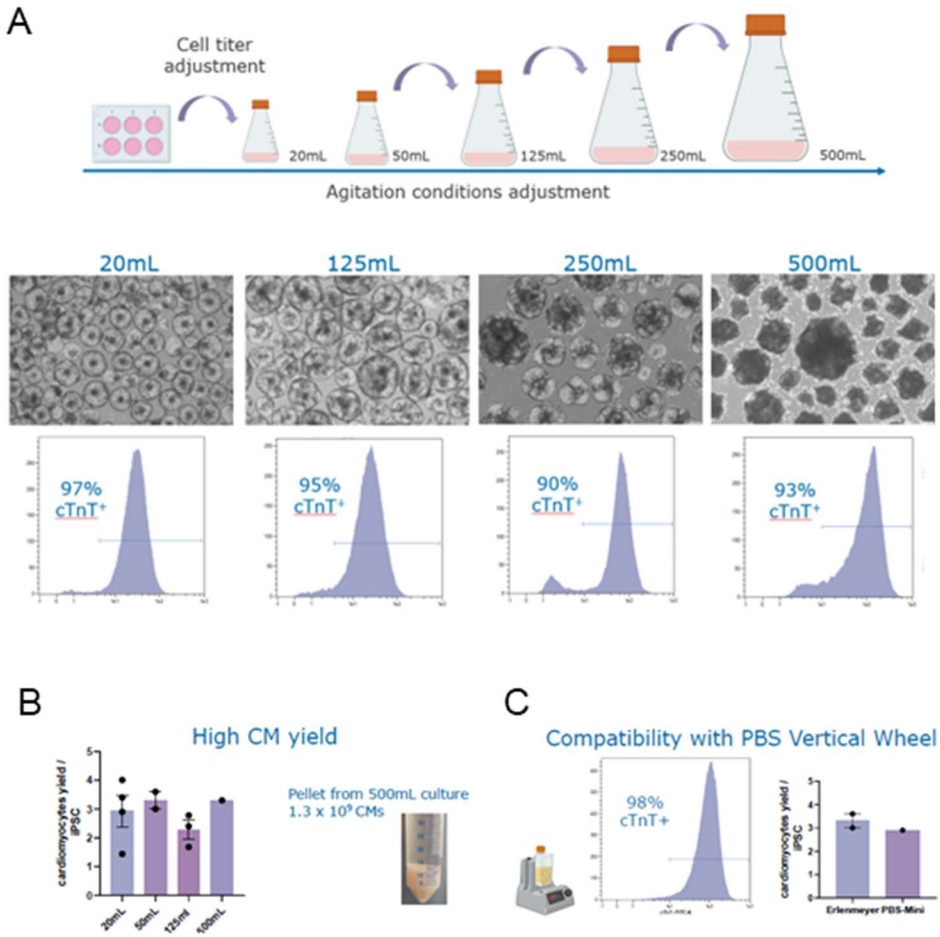


Figure 4: A. *Upscaling of iPSC-CM differentiation using GMP-compatible equipment. Cardiac differentiation was progressively scaled from 20 mL to 500 mL cultures with optimized agitation conditions. Representative brightfield images show aggregate morphology at each stage, with flow cytometry confirming high cardiomyocyte purity (cTnT⁺ >90% across all scales).* ***B.*** *The 500 mL process yielded ~1.3 × 10⁹ cardiomyocytes.* ***C.*** *Compatibility testing with the PBS Vertical Wheel bioreactor maintained high differentiation efficiency (98% cTnT⁺) and comparable yields to Erlenmeyer flask cultures, supporting its suitability for GMP-compliant upscaling.*

To optimize the final conditions of the cardiac differentiation process, a dissociation and re-aggregation step was introduced at day 8, during the cardiac progenitor stage. This modification aimed to generate uniformly sized and mechanically stable aggregates that could be delivered through a fine-gauge needle without structural disruption—an essential requirement for the downstream applications such as intramyocardial injection in the pig models. In this approach, cardiac progenitor aggregates were enzymatically dissociated into single cells, followed by controlled re-aggregation under defined conditions (**Figure 5A**). Brightfield images show that prior to re-aggregation, aggregates displayed heterogeneous morphology and larger sizes, whereas post re-aggregation, cultures consisted of compact, uniform aggregates with significantly reduced diameter variability (**Figure 5B**). Gene expression analysis revealed that the reaggregation process was also having a positive impact in the purity of the cardiomyocytes, by decreasing the expression of the pluripotent markets and off-target genes and increasing the expression of cTnT (**Figure 5C**). Furthermore, e-SNP karyotyping performed by the partner HUJI demonstrated normal genomic integrity, confirming that the additional dissociation step did not compromise chromosomal stability (**Figure 5D**). This process refinement enables the generation of high-quality, size-controlled cardiac aggregates suitable for injection while preserving differentiation efficiency and genetic stability.

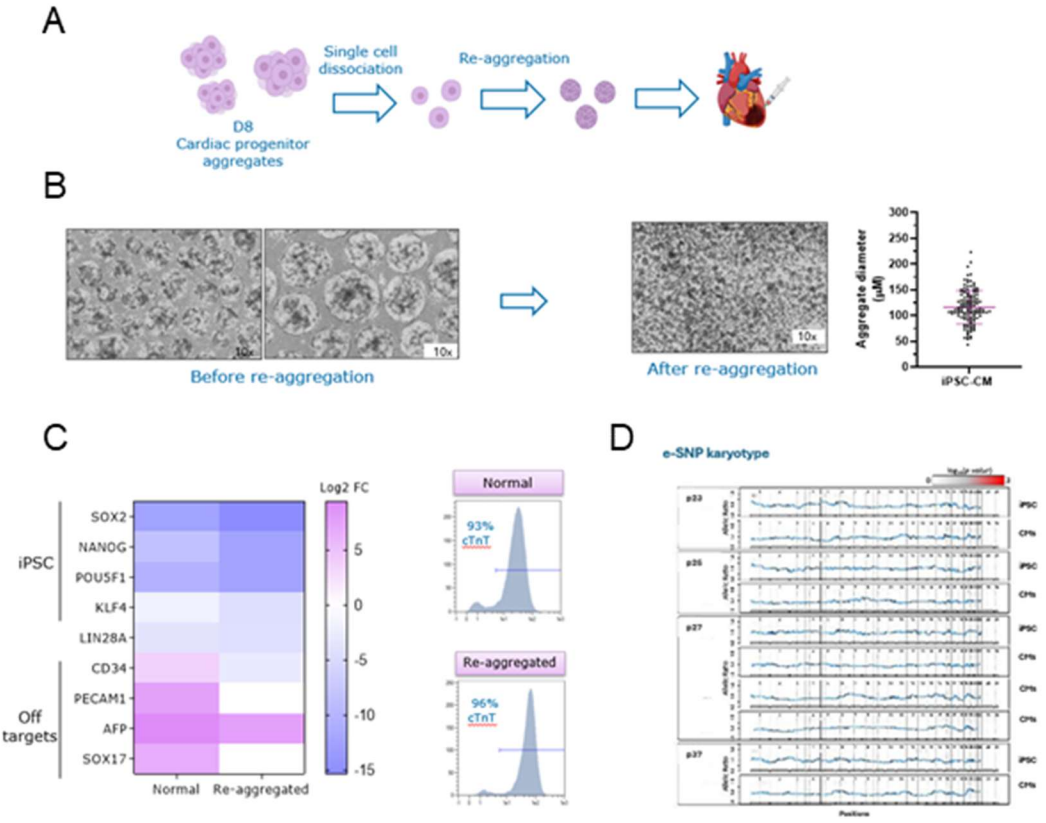


Figure 5:. *Optimization of cardiac aggregate size through dissociation and re-aggregation.* ***A****. A day 8 dissociation/re-aggregation step was implemented to produce uniformly sized, mechanically stable aggregates suitable for needle injection.* ***B.*** *Brightfield imaging and size quantification show reduced diameter variability after re-aggregation.* ***C****. Gene expression profiles and flow cytometry confirmed increased purity after re-aggregation.* ***D.*** *e-SNP karyotyping verified genomic stability.*

Transport Stability and Needles Compatibility of iPSC-CMs

CATD first evaluated the transport stability of iPSC-derived cardiomyocyte (iPSC-CM) aggregates. Aggregates stored in 0.7% NaCl at 4 °C for 24 h maintained high viability and exhibited a high recovery rate (**Figure 6A**). However, viability slightly declined after extended storage for 42 h and 48 h (**Figure 6B**), indicating that short-term cold storage preserves cell integrity but prolonged storage might slightly compromise cell survival. Cells were passed through needles of different gauges to assess potential mechanical stress during syringe ejection. All needle sizes tested performed equally well in maintaining cell viability and aggregate integrity; however, for intramuscular cardiac injections, the 27G needle is preferred, and cells appeared unaffected by passage through this needle. Next, the impact of needle design on aggregate integrity was assessed. A custom-bent needle, developed to facilitate surgical intramuscular injections in the pig's heart, was tested (**Figure 6C**). Brightfield imaging demonstrated that iPSC-CM aggregates remained intact following passage through both 27G and 25G custom needles (**Figure 6D**), suggesting that the needle modifications do not adversely affect aggregate structure. Based on this results, a 27G needle was selected for further applications.
Overall, these results indicate that iPSC-CM aggregates can be transported in 0.7% NaCl at 4 °C for at least 24 h without compromising viability, supporting the feasibility of short-term cell transport for the animal studies.

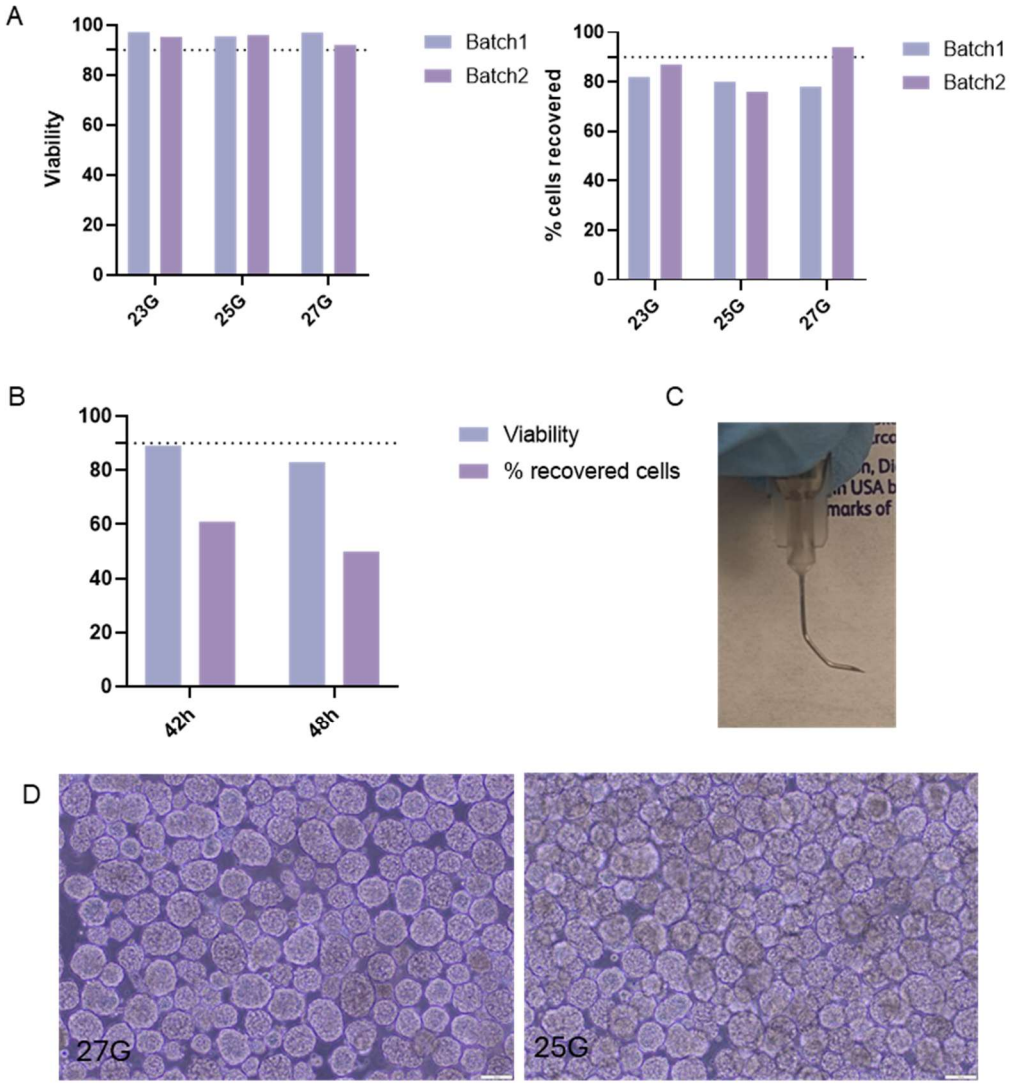


Figure 6: *Evaluation of transport stability and needle parameters for iPSC-CMs.* ***A****. iPS-derived CM aggregates were stored in 0.7% NaCl at 4 °C for 24 h show high viability and high recovery rate.* ***B****. Viability decreases after 42 h and 48 h.* ***C****. Custom-bent needle, designed to assist surgeons during injection was tested.* ***D****. Brightfield imaging confirmed that aggregates remained intact after passage through the 27G and 25G custom-made needle.*

Task 1.3 Establish HLAh iPSC working cell bank and HLA class I and II gene knockout [M1-M20]

Task accomplished as reported in first reporting period.

Task 1.4 GMP workflow establishment and conduction of pilot runs for iPS-CMs production [M24-M42]

Based on the process development described above, a final Standard Operating Procedure (SOP) describing the production and characterization of iPS-CMs was established and shared with the partner MHH to use and document the generation of iPS-CMs for the animal studies.
While at this stage the GMP-specific SOP and QA/QC assay validation are not yet available, we provide the foundational step toward full GMP compliance. Furthermore, the development of a potency assay to access the contractility of the generated iPS-CMs and a residual iPSC assay were developed and will be further described below.

Based on process development in Task 1.2, iPS-CMA manufacturing and QC processes will be implemented in CATD's QM system to comply with cGMP.

Generation of IPSC-derived cardiomyocytes in 3D SOP

The SOP describes the standardized process for generating iPSC-derived cardiomyocytes in suspension as aggregates using the PBS-MINI MagDrive Vertical Wheel Bioreactor with single-use vessels (PBS-MINI 0.1 or PBS-MINI 0.5). The procedure ensures consistent and reproducible differentiation of iPSCs into cardiomyocytes under controlled conditions, supporting the production of iPSC-derived cardiomyocytes for research purposes and preclinical animal safety studies.

The process consists of five key steps:

3. Thawing – Retrieval and thawing of cryopreserved iPSC stock for culture initiation.
4. Expansion of iPSC – Cultivation of iPSCs, including medium changes and passaging, to ensure optimal cell growth.
5. Cardiac differentiation – Induction of iPSC differentiation into cardiomyocytes in suspension using the PBS-MINI 0.1 or PBS-MINI 0.5.
6. Single-cell dissociation and re-aggregation – Enzymatic dissociation of iPSC-derived cardiomyocytes into single cells, followed by controlled 3D re-aggregation.
7. Cell collection and transport preparation – Harvesting of cardiomyocyte aggregates and preparation for shipment or further processing.

Appropriate conduction of these steps according to the respective documentation (SOPs and the respective attachments) is expected to result in production of high-quality iPSC-derived cardiomyocytes ready for transport and subsequent injection. Figure 4 shows an exemplary overview of this process.

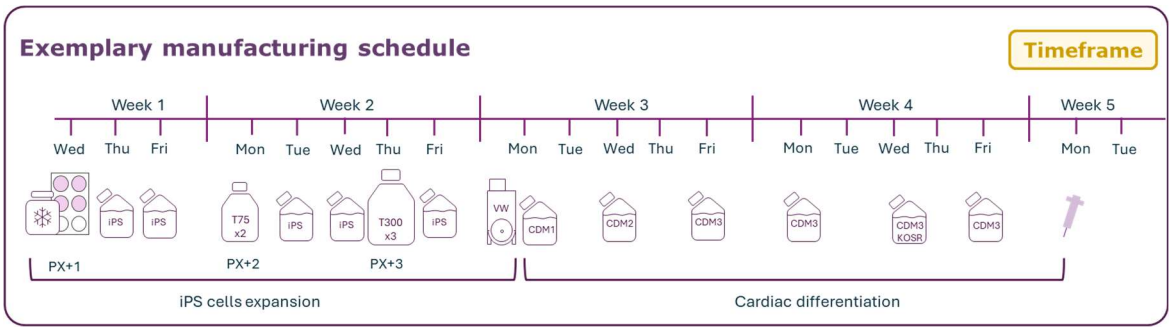


Figure 7: Overview of the possible timeframe to perform a cardiac differentiation in a PBS-MINI 0.5 vessel with 300mL differentiation culture enough to produce 300M of iPSC-derived CMs as aggregates.

The process spans 5 weeks and involves cell thawing, expansion, differentiation, re-aggregation, and final preparation for injection. It begins with the thawing of hiPS R26 cells, which are expanded through multiple passages in StemMACS iPS-Brew medium until at least 300 million cells are obtained. On Day 0, cells are harvested and seeded into a PBS-MINI MagDrive Vertical Wheel Bioreactor with CDM1 medium at 0.75 million cells per mL to initiate cardiac differentiation, which progresses through sequential media changes (CDM1 → CDM2 → CDM3) over 9 days. On Day 9, cardiomyocyte aggregates are dissociated using TrypLE and re-seeded at 1 million cells per mL in re-aggregation medium containing CDM3, Knockout Serum Replacement, and ROCKi, followed by continued maintenance in the bioreactor. By Day 14, a final cell titer assessment is performed, and 250 million cardiomyocytes are harvested, washed, and resuspended in 0.9% NaCl at 100M cells/mL before being loaded into syringes for transport at 4°C. The prepared suspension remains stable for up to 24 hours before injection, ensuring high-quality cardiomyocytes for preclinical studies and potential therapeutic applications.

In-process controls and QC tests

In-process controls (IPCs) and additional checks during production are conducted to ensure a high product quality and to recognize possible shortcomings in a timely manner. At the end of the differentiation, QC tests will be performed to ensure the quality of the iPSC-derived cardiomyocytes and to access if the cells meet the criteria to be released.

An overview of the IPCs and QC tests is shown in the following list and in the **Figure 8**.

- IPC1: FACS (flow cytometrical analysis) to confirm the identity of iPSC-derived cardiomyocyte at day 9 before single cell dissociation and re-aggregation, using Cardiac troponin T (cTnT) antibody (the cardiomyocyte population should exceed than 85%).
- QC1: Measurement of the cardiac aggregates size by taking representative pictures under the microscope of a 4mL sample (at least 5 different areas) and measure the diameter using ImageJ.
- QC2: Viability of the aggregates using the NC200 Nucleocount.
- QC3: FACS (flow cytometrical analysis) to confirm the identity of of iPSC-derived cardiomyocyte at day 14, using Cardiac troponin T (cTnT) antibody (the cardiomyocyte population should exceed than 85%).
- QC4: Mycoplasma testing on the culture supernatant before transport.
- QC5: Residual iPSC assay using RT-qPCR to assess the presence of undifferentiated iPSCs within the aggregates by detecting the expression of the miRNA302-367 cluster.
- QC6: Sterility testing performed on the backup cells after 24h at 4C in NaCl.
- QC7: Viability of the aggregates using the NC200 Nucleocount after after 24h at 4C in 0.9% NaCl.

Figure 8: Overview of in-process controls (IPCs) and QC tests.

Sampling of IPC1 is documented in the single cell dissociation protocol (CATD RD_SOP_09_A07). Sampling of QC1-QC7 are documented in the cell collection and transport preparation protocol (CATD RD_SOP_09_A08). Residual iPSC assay is conducted according to CATD RD-17_DRAFT_SOP_qRT-PCR. FACS analysis is conducted according to CATD RD-19_DRAFT_SOP. Mycoplasma testing is conducted according to CATD RD-10_SOP and Sterility test is conducted by Quade according to Ph. Eur. 2.6.27.

Residual iPSC assay- Method qualification

As part of the final process for iPSC-derived cardiomyocyte (iPSC-CM) production, a residual iPSC assay was implemented to ensure the absence—or presence below an acceptable threshold—of undifferentiated pluripotent stem cells in the final product. Residual iPSCs pose a potential safety risk due to their tumorigenic capacity, and therefore their detection is a critical quality attribute for clinical translation.

The assay detects residual iPSCs based on the expression of miRNA302/367, a pluripotency-associated microRNA, using a highly sensitive TaqMan-based qPCR method. This target was selected for its specificity to undifferentiated iPSCs and absence in terminally differentiated cells.
The residual iPSC assay was integrated into the manufacturing workflow SOP as a quality control (QC) release test. A method qualification study, performed under R&D conditions, was planned and executed to establish the acceptance criteria for subsequent GMP method validation. In accordance with ICH Q2(R2) Validation of Analytical Procedures guidelines (**Figure 9A**), the qualification plan included experimental assessment of key analytical performance characteristics: accuracy, repeatability, intermediate precision, specificity, limit of detection (LOD), limit of quantification (LOQ), linearity, and range. **Figure 9B** summarizes qualification results for accuracy, repeatability, and LOQ. The method demonstrated an accuracy of 92.7%, defined as the closeness of agreement between the measured value and the theoretical reference value (**Figure 9B**). Repeatability was confirmed across technical replicates, with consistent results even when cDNA samples were diluted up to 1:100,000 (**Figure 9C**). LOQ was determined by spiking defined proportions of undifferentiated iPSCs into terminally differentiated cells. The assay was able to reliably detect as low as 0.001% iPSCs (**Figure 9D**) with both accuracy and precision. These data confirm that the residual iPSC assay meets the defined criteria, supporting its suitability as a future GMP release test and providing a critical safeguard for product safety in the final clinical workflow.

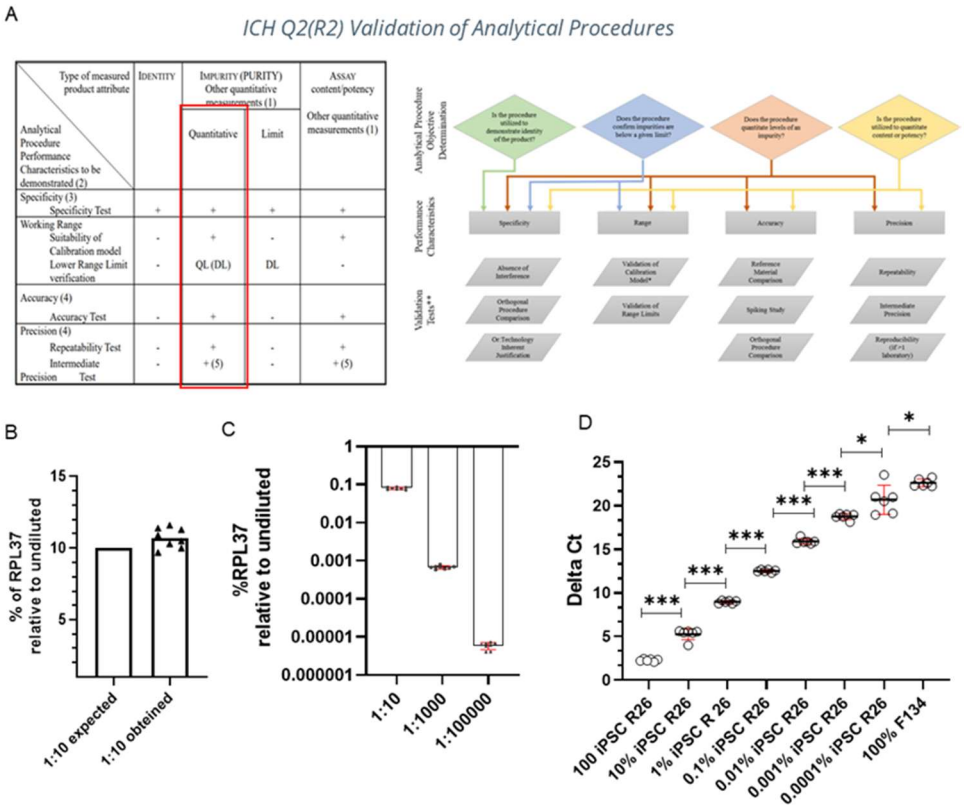


Figure 9: Qualification of the residual iPSC assay. **A**. ICH Q2(R2) Validation of Analytical Procedures guidelines **B**. The method showed 92.7% accuracy, **C** high repeatability even at 1:100,000 cDNA dilution **D**. lower limit of quantification of 0.001% spiked iPSCs.

Development of a potency assay

Robust potency assays are critical in GMP settings to ensure consistent quality and functional performance of the product. Among the key functional attributes, contractility provides a direct measure of cardiomyocyte performance and is a critical parameter for batch release and quality control (QC).
To quantitatively evaluate contractility, CATD developed a potency assay using the recently aquired myrImager (Myriamed GmbH), a high-throughput video-optical imaging system specifically designed for functional readouts of iPSC-CM contractility. The platform was applied in combination with myrPlates, which feature two flexible poles (stretchers) embedded in each well. For all comparative experiments, TM5 stretchers with an E-modulus of 4.73 Nm/mm² were used (**Figure 10A**). Each

stretcher is coated with a fluorescent dye that is excited by UV light emitted by the myrImager; the resulting fluorescence is captured by the imaging system and enables precise tracking of pole displacement over time. These displacements directly reflect the forces generated by iPSC-CM tissue contractions, providing a quantitative and reproducible measure of contractility (**Figure 10B**).

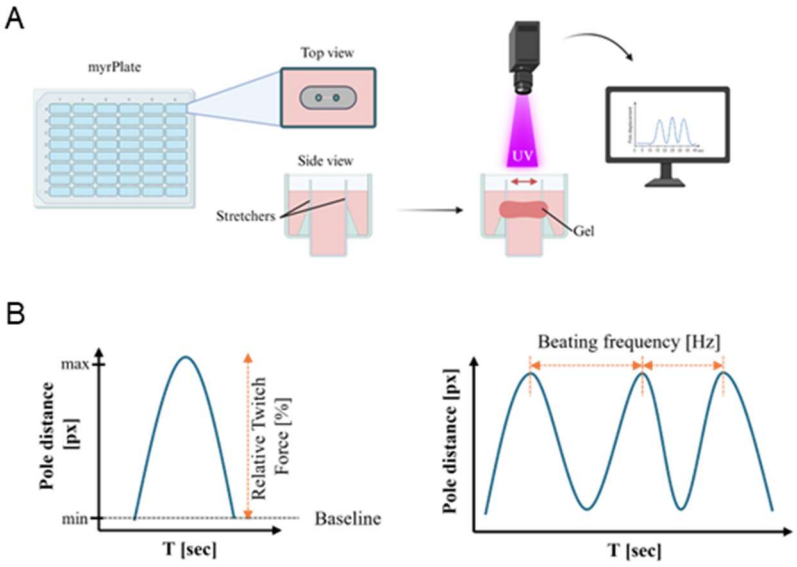


Figure 10:. Schematic overview of the contractility assay using the myrImager platform. **A**. iPSC-derived cardiomyocyte tissues are cultured on myrPlates, which contain two flexible poles (stretchers) embedded in each well. Upon tissue contraction, the poles are displaced. Each pole is coated with a UV-excitable fluorescent dye, and displacement is recorded at 37 °C using the myrImager high-throughput imaging system. **B**. The fluorescence signal is tracked over time to quantify contractile behavior, including contraction force (left) and beat frequency (right).

Assay optimization involved systematic evaluation of matrix composition, cell type selection, seeding density, cell ratios, and culture media formulation. Standardizing these parameters was critical to reduce inter-assay variability and to establish a robust, GMP-aligned potency assay suitable for QC applications.

1. Matrix selection

To identify the most suitable hydrogel matrix for forming gels within the predefined myrPlate molds, fibrin- and collagen-based gels were compared. The results revealed distinct differences between the two matrices. Fibrin-based gels partially supported iPSC-CM contractility but failed to form rings around the poles, resulting in no pole displacement. In contrast, collagen-based gels provided a more robust matrix that facilitated rapid and proper ring formation around the poles, leading to increased pole displacement, as shown in **Figure 11A-B**. Based on these findings, collagen was selected as the matrix for gel formation.

2. Cell types used

The inclusion of non-myocytes can improve the contractility force of the iPSC-CM. In this regards, different cell types were tested: human foreskin fibroblasts (derived from neonatal male tissue), foetal hepatocyte-derived fibroblasts F134 (originating from foetal liver tissue), and MSCs differentiated in-house from iPSC R26 (iMSCs). By day 8, clear differences in contractile force between conditions became apparent (**Figure 11C**). Gels containing HFFs displayed weaker pole displacement, indicating lower twitch force compared to constructs containing F134 fibroblasts or iMSCs. Notably, tissues with F134 or iMSCs showed comparable levels of contractile force and beating frequency, suggesting that both cell types can be used for the current assay. However. using MSCs derived from the same iPSC line as the cardiomyocytes ensures genetic consistency, reduced contamination and immunogenic risk, and streamlined GMP documentation, making the potency assay more reproducible and regulatory-compliant than using primary fibroblasts.

3. Cell titer

To optimize the potency assay, we compared gel constructs containing either 0.5 M or 1 M total cells, regardless of the CM-to-non-myocyte ratio, to see how cell number affects ring formation and contractility. Constructs with 1 M cells formed tight rings around the poles within three days, while 0.5 M constructs took up to ten days depending on the cell ratio. Higher cell numbers also led to earlier detectable contractility, stronger pole displacement, and higher beating frequency (**Figure 11D-E**). These results indicate that 1 M cells per gel promotes faster and more reliable ring formation and contractile measurements, so subsequent assays were performed with 1 M cells in collagen-based constructs of CMs and MSCs.

4. Media composition

Three maintenance media were tested —KO-DMEM, IMDM, and RPMI—on cardiac constructs. Only KO-DMEM, especially when supplemented with ITS, supported consistent tight ring formation within three days and generated measurable contractile forces capable of displacing the myrPlate poles, while IMDM and RPMI led to incomplete rings and undetectable contractility (**Figure 11G**).

In summary, collagen-based gels were the most suitable matrix for supporting contractile 3D constructs in the myrPlate format. Among the non-myocyte populations tested, iPSC-derived MSCs effectively supported functional tissue formation when co-cultured with CMs. BCM + ITS was the only maintenance medium that consistently sustained contractions. The optimized model—1 M cells per gel at a 60:40 CM:MSC ratio—produced the tightest ring formation and allowed reliable, consistent quantification of contractility by iPSC-derived CMs, as showed in **Figure 11H**.

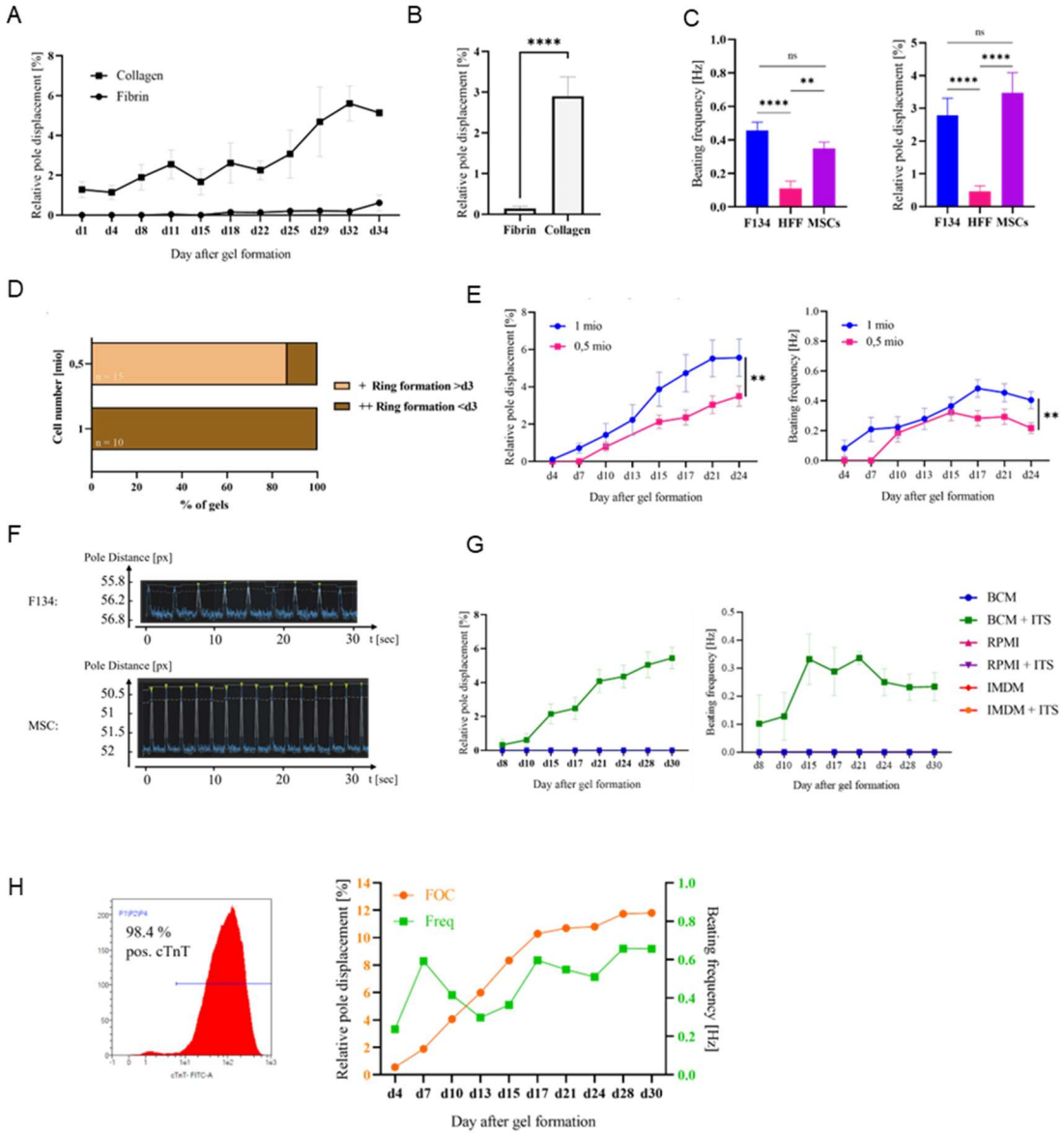


Figure 11: Characterization and functional assessment of iPSC-CM/iMSC collagen-based 3D constructs. **A-B**. Quantitative analysis reveals collagen matrix to be more suitable for the myrPlates. **C**. Comparison of beating frequency and contractility force in constructs with non-myocyes **D**. Distribution of constructs per ring formation quality at different total cell numbers. **E**. Longitudinal analysis of contractile force and beating frequency in 0.5 M versus 1 M cell constructs. **F**. Representative contraction traces from F134 (top) and iMSC-containing gels(bottom). **G**. Beating frequency over 30 days in constructs cultured under different medium conditions; BCM + ITS supports consistent spontaneous contraction (green). **H**. Flow cytometry analysis confirms high purity of cTnT-positive CMs (left); correlation of force of contraction (FOC) and beating frequency (Freq) over time in optimized constructs (right).

UDUS:

Task 1.1 Establish ACF cGMP-compliant culture and differentiation media [M1-M24]

In cooperation with both CATD and MHH we have established the workflow of iPSC culture handling, of the project specific HLA-homozygous iPS cell line in a GMP compliant environment. The optimized cardiomyocyte differentiation protocol based on rational and titrated signalling pathway perturbations was successfully implemented in two different systems (6-well plates and Erlenmeyer flasks) (**Figure 12**).

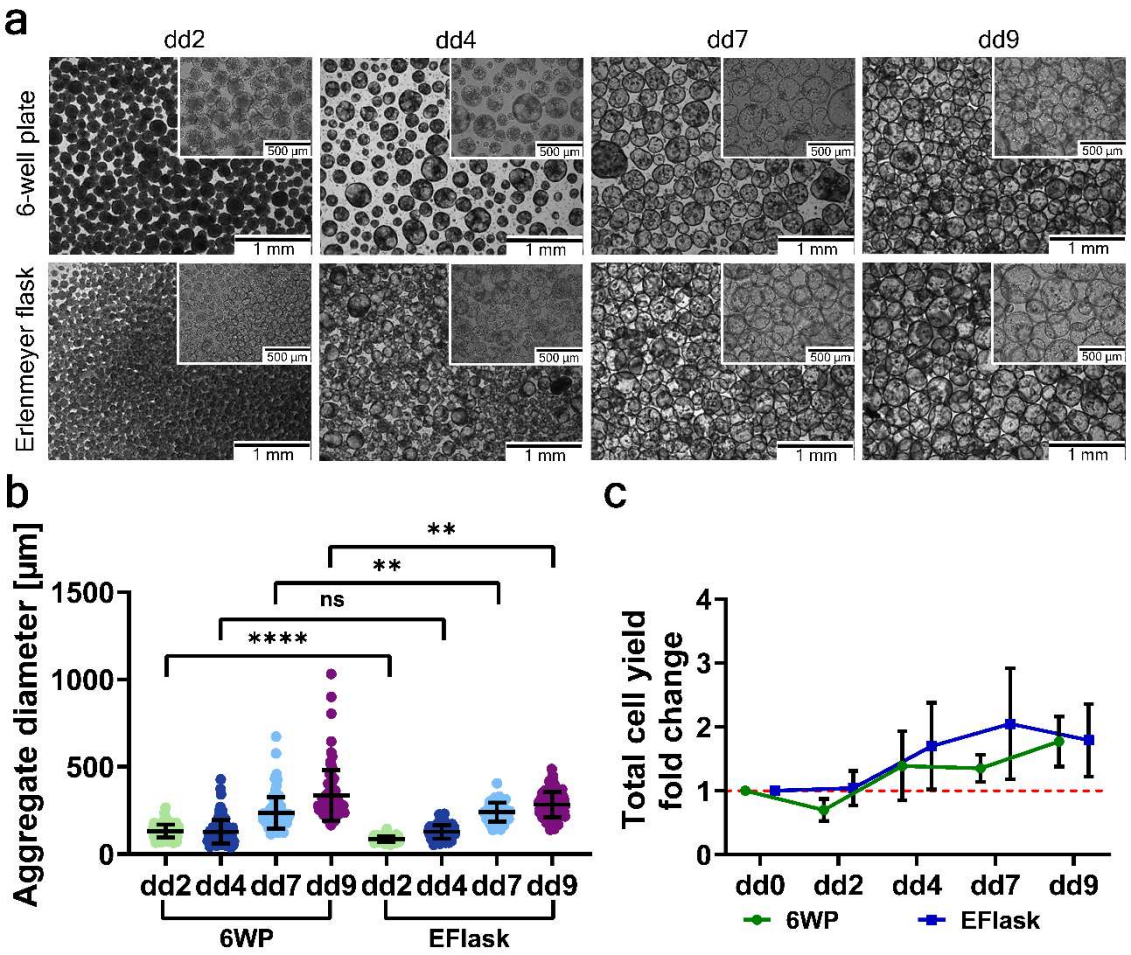


Figure 12: 3D-Suspension Culture for Cardiomyocyte Differentiation. (a) Representative bright field images for morphological assessment of the differentiation at different time-points. Scale bar 1mm and 500 µM. (b) Aggregate diameter distribution during differentiation; shown are individual values of aggregates from n=3 experiments and mean values ± SD (for each experiment >40 aggregates were measured); Data were analyzed for statistical significance using an unpaired t-test (**p ≤ 0.01, ****p ≤ 0.0001). (c) Cell yields depicted as fold changes at different time-points during differentiation (mean values ± SD, n=3).

The differentiation in either platform yielded morphologically similar cardiomyocyte aggregates (CMAs) (**Figure 12A**). Depending on the platform utilized for the differentiation (6WP or EFlask), the CMA size differs significantly (**Figure 12B**). CMAs generated in 6WP were more heterogeneous and reached an average size of about 337µm±144µm. CMAs generated in EFlasks reached significantly smaller and more homogeneous average aggregate sizes of about 283µm±72µm. Both platforms showed a ~1.8-fold increase in cell yield compared to the seeded number of iPS cells (**Figure 12C**).

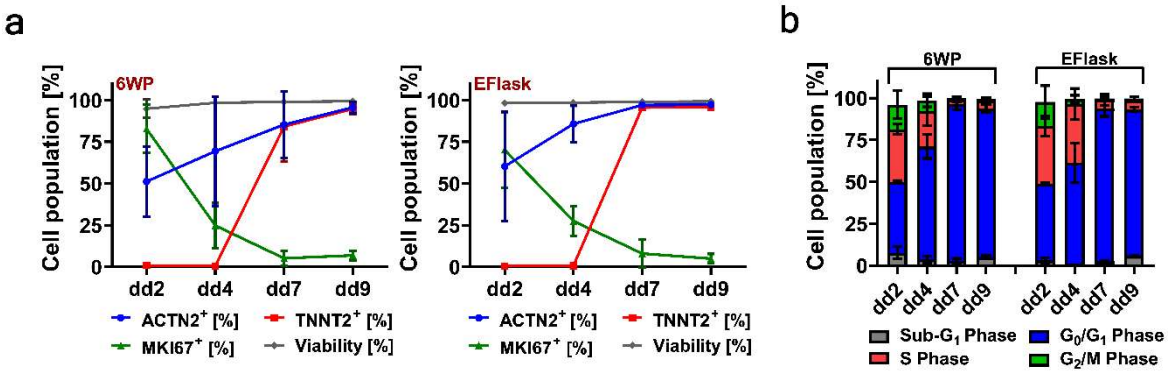


Figure 13: Flow Cytometry Analyzes for the Characterization of Cardiomyocyte Aggregates during the Differentiation. (a) Investigation of cardiac marker expression ACTN2 and TNNT2, proliferation marker MKI67,

and the viability of cells at different time points (mean values ± SD, n=3). (b) Cell cycle phase distribution during differentiation (mean values ± SD, n=3).

For the characterization of the CMAs flow cytometry of cardiac markers was deployed focussing on ACTN2 and TNNT2 expression during the differentiation (**Figure 13**). Independent of the utilized platform, both cardiac marker expressions exceeded ≥95% by differentiation day 9. As cardiomyocytes are characteristically withdrawn from active cell cycle, both the proliferation marker MKI67 and a cell cycle analysis were performed. The level of MKI67 expression drastically decreased during the differentiation, reaching a level of ~7% by dd9 (Figure 10A). Concurrently, cell cycle analysis revealed that from dd7 onward most of the cells resided in the G_0/G_1 phase (≥87%) and only a low percentage of cells in either S (≤5%) or G2/M (≤2%) phase (**Figure 13B**).

Additionally, to exclude the potential presence of residual iPSC contaminations, both the pluripotency marker POU5F1 was analyzed by flow cytometry as well as gene expression level of miR-02/367 HT was analyzed by qPCR (**Figure 14**).

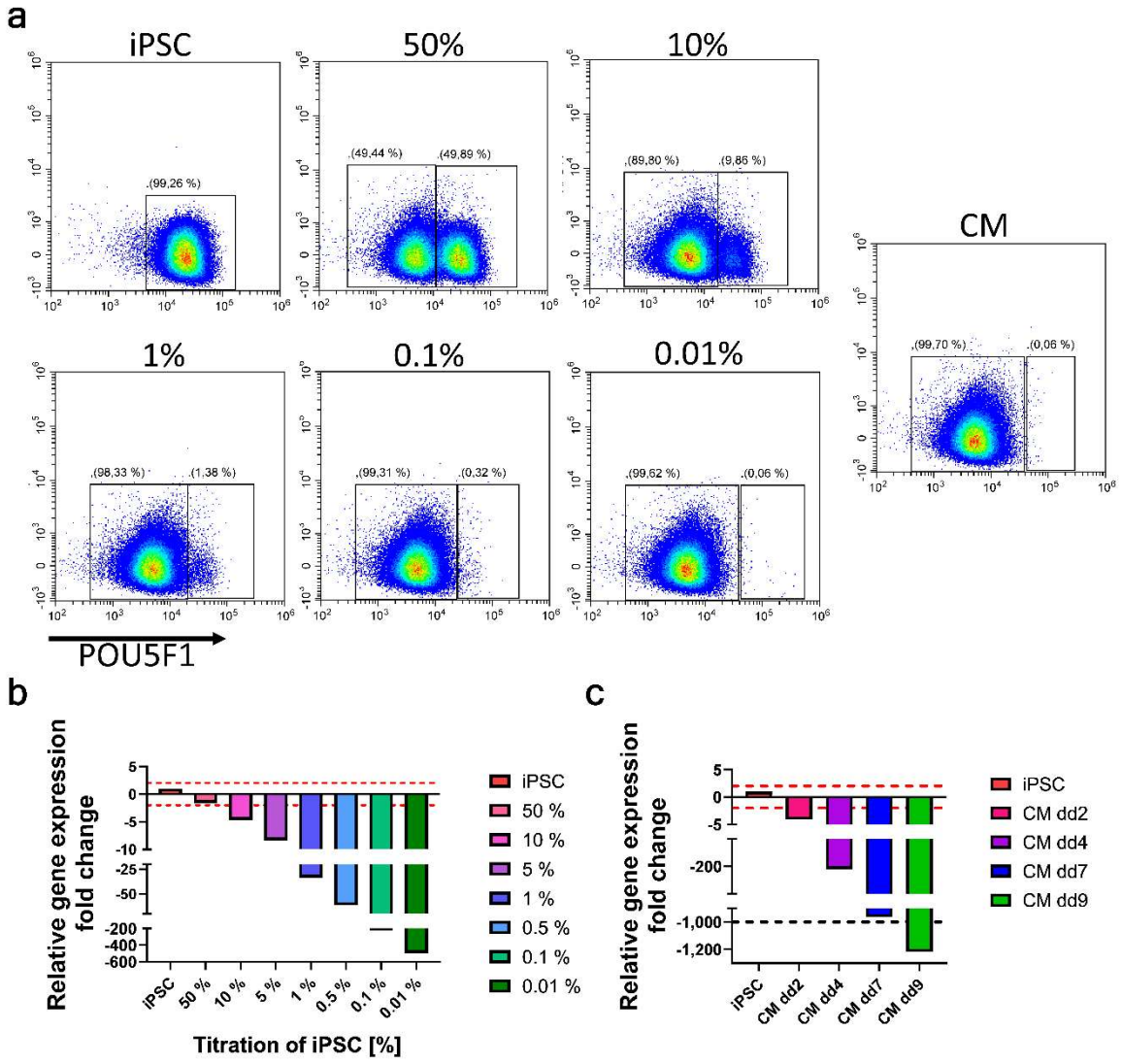


Figure 14: Quality control assessment for residual contaminating iPS cells. (a) Flow cytometry detection of POU5F1 with different concentrations of iPSC (50 % to 0.01 %); (b) Detection limit determination for the expression of miR302/367 HT with different concentrations of iPSC (50 % to 0.01 %) via qPCR; (c) Determination of residual iPSC during the 9-day differentiation of cardiomyocytes, the threshold of -1000-fold was determined as the value for no residual iPSC present in the cell sample; the red dotted lines at 2-fold and -2-fold mark significant up- or down-regulation of the gene expression.

iSPC-spiking of various concentrations (50% to 0.01%) indicated the detection of ~0.1% residual iPSC in a sample via flow cytometry (**Figure 14A**) and the sensitive detection of as low as 0.01%

residual iPSC in a sample via real-time qPCR (**Figure 14B**). Analyzing CMAs during the differentiation process revealed no residual iPSC contamination by dd9 (<0.1% POU5F1 expression measured via flow cytometry and < -1000-fold down regulated gene expression of miR-302/367 HT measured via qPCR).

Task 1.5 Establish iPS-CM aggregate (iPS-CMA) cryopreservation [M6-M36]

Cryopreservation experiments with CMAs differentiated from the specific HLA-homozygous iPS cell line are ongoing. The focus here is a GMP compliant controlled freezing process.

Due to the complex structure of cardiac aggregates, cryopreservation effects were assessed to determine the influence of freezing on structure, viability, phenotype and function. CMAs from dd9 onward (dd9 to dd11) expressing >85% ACTN2 and TNNT2 were further utilized for cryopreservation purposes. Both commercially available freezing media (Stemdiff-CMFM, CS-10, Nutrifreeze-D10, and Stem Cellbanker) and basic freezing media with different additives (CMFreeze, 10% HSA, 10% KOSR, 90% KOSR) were tested for their cryoprotective capabilities for the cryopreservation of CMAs.

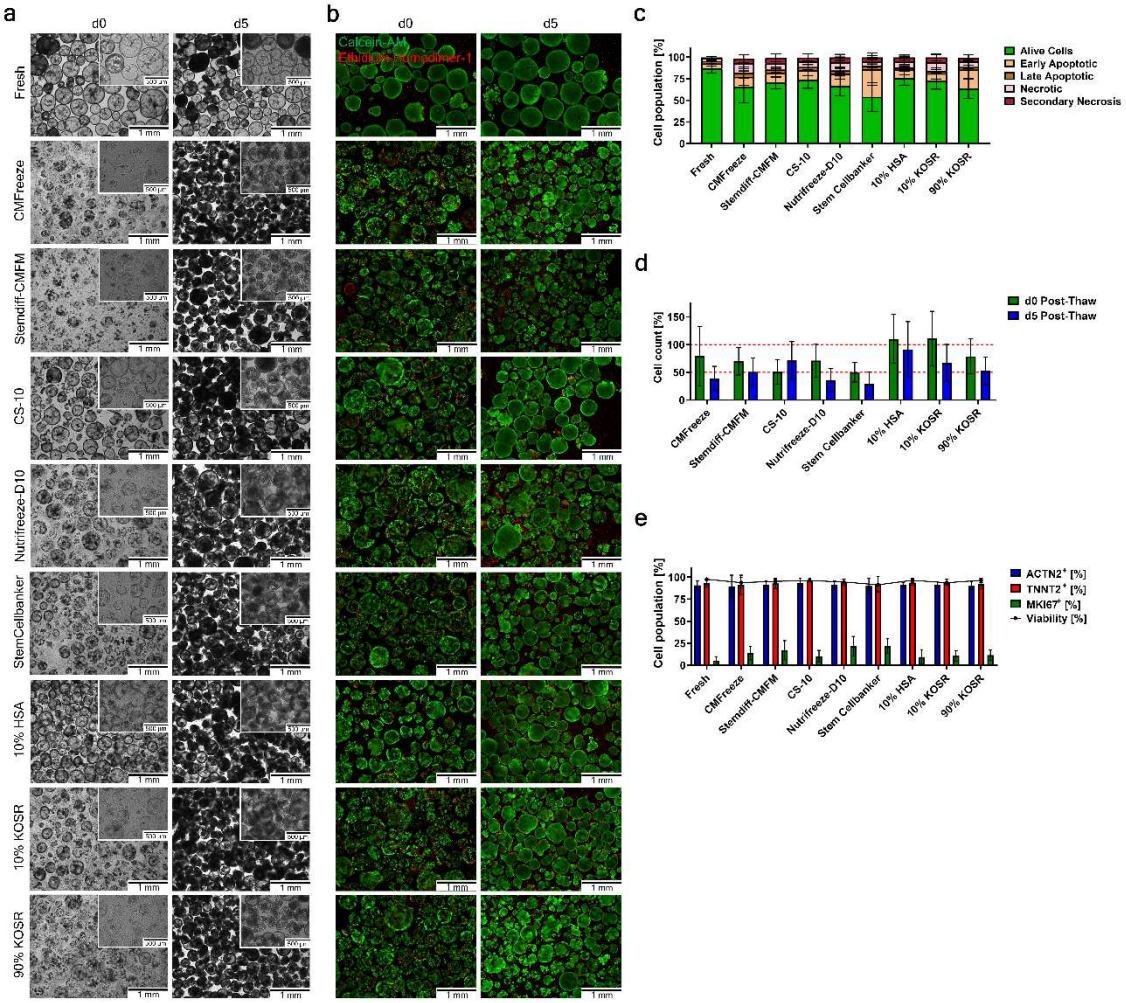


Figure 15

On d0 post-thaw, morphology assessment of cryopreserved CMAs revealed a heavily impaired aggregate structure with most aggregates displaying a disrupted outer membrane, independent of the freezing medium. Freezing media CS-10 and 10% HSA were performing best in regard to protection of the aggregate structure, as the two conditions had the least visual disruption of CMA

integrity. On the other hand, the freezing media CMFreeze, Stemdiff-CMFM, and 90% KOSR had the most drastic CMA structure disruption on d0 post-thaw. However, after a 5-day post-thaw culture, all conditions were able to recover their aggregate integrity of most CMAs. Compared to the fresh sample, CMAs that underwent freezing appeared to have a denser structure compared to the more cystic appearance of the fresh cells.

Even though aggregate structure was compromised immediately after cryopreservation, whole aggregate viability staining revealed that most of the cells in the aggregates were alive and only a few cells in the outer regions of the aggregates were dead as well as single cells that had detached from the aggregates (**Figure 15B**). After the post-thaw culture (d5), the CMAs were completely viable and the only dead cells in the culture were the remaining single cells that had detached from the aggregates. Further assessment of the viability of CMAs directly post-thaw through flow cytometry analysis with an apoptosis assay (Annexin V/PI) revealed first signs of ongoing apoptosis of cells in all conditions (**Figure 15C**). After cryopreservation, all conditions had a lower number of viable cells (≤75%, ~87% for fresh cells) and a higher number of cells of both early stages of apoptosis (9%-32%, ~5.4% for fresh cells) and necrosis (5%-11%, ~3% for fresh cells). Both late stage apoptotic and secondary necrotic cell levels were similar to those of fresh cells (3%-4% and 1%-7% respectively). CMAs frozen with Stem Cellbanker had the lowest number of alive cells (53.9%±16.7%), while CMAs cryopreserved with either Stemdiff-CMFM, CS-10, 10% HSA, or 10% KOSR had the highest living cell numbers (70.4%±7.2%, 73.9%±10.1%, 75.6%±7.9%, and 72.4%±9.6% respectively).

To highlight the importance of the consideration of onsetting post-thaw cryoinjury, the recovery of cells was analyzed both immediately post-thaw (d0) and after a short post-thaw culture (d5) (**Figure 15D**). Immediately post-thaw (d0), most conditions had high recoveries of ≥50%, with both the conditions 10% HSA and 10% KOSR performing best out of the tested freezing media with recoveries of ~110%. By d5, most conditions had a severe drop in the recovery. The commercially available freezing medium Stem Cellbanker had the most profound cell loss with a recovery of only 29.20%±20.84%, followed by Nutrifreeze-D10 with 35.45%±21.86%, and CMFreeze with 38.77%±21.93%. Moderate recoveries were achieved with either Stemdiff-CMFM (50.71%±25.56%), or 90% KOSR (52.68%±25.08%). The best results were accomplished with either 10% KOSR (67.25%±33.15%), CS-10 (71.17%±34.16%), or 10% HSA (91.23%±50.10%). Notably, all conditions had a high batch-to-batch variability as evidenced by the high SD values.

The recovered CMAs were characterized via flow cytometry for their cardiac marker (ACTN2 and TNNT2) and proliferation marker (MKI67) expression and their viability on d5 post-thaw (Figure 11E). No phenotypic drift could be analyzed with all conditions displaying comparable levels of both ACTN2 and TNNT2 populations (~90% and ~93% respectively). The proliferation capacity of the CMAs was slightly elevated in all conditions as evidenced by higher MKi67 expression (≥10%) as compared to the fresh sample (5.46%±4.39%). Similar to the observed high viability of the whole aggregates (**Figure 15B**), flow cytometry analysis confirmed that by d5 post-thaw the CMAs were highly viable (≥91%) independent of the freezing media.

To overcome the high batch-to-batch variability for the CMA cryopreservation, different approaches are being investigated.

1. Pre-treatment of the cells prior to cryopreservation with additives known to inhibit apoptosis, e.g. Y-27632 or small molecule cocktails like CEPT
2. Single cell freezing with a re-aggregation process post-thaw
3. Re-aggregation process (3 to 5 days) prior to cryopreservation and freezing the re-aggregated CMAs

1.2.2 Work Package 2

WP2: Immunogenicity of HLA homozygous iPS-CM; Lead Beneficiary PMU & SERO

The overall objective of WP2 is to create a comprehensive set of immune phenotype and functional data obtained in vitro by partially matching iPSC-CMs with responder lymphocytes to train a predictive AI-based algorithm correlating immunophenotype and immune response.

Task 2.1 Production of HLA iPSC as semi-universal cell source [M1-M3]

Task accomplished as reported in first reporting period.

PMU & SERO:

Table 3 and 4 show Milestones and Deliverables through the reporting period (M19-M28). Also shown are deviations of Milestones and Deliverables. More detailed explanations are given below and under 5. DEVIATIONS FROM ANNEX 1 AND ANNEX 2 (IF APPLICABLE) of this report.

Table 3: Milestones Partner PMU M19-28

No.	Milestone name	WP	Partner	Due Date	Means of verification	Status
M16	Immune function assays operative	2	PMU	31.08.2024	Preliminary data	Submitted 07.09.2024

Table 4: Deliverables Partner PMU M19-28

No.	Deliverable name	Partner	Due Date	Status
D2.1	Immunophenotyping of HLAh iPS-CMs	PMU	31.08.2024	Submitted 07.09.2024
D2.2	Immune response data predicting immune-suppression requirements of HLA-matched iPS-CMAs	PMU	31.04.2025	Delayed

SERO:

Deliverables D2.2 and D2.3 received new due dates (31.04.2026 and 31.08.2026) due to the granted cost-neutral extension. They were therefore transferred to SERO and are not part of this reporting period.

Task 2.2 Comprehensive immunophenotyping of HLA-homozygous iPS-CMs [M1-M24]

PMU:

We established a 21-parameter panel for spectral flow cytometry based on the screening results reported in Milestone No.10. The panel consists of key markers relevant for immune recognition and modulation of the immune response (activation / suppression; **Table 5**). In addition it contains a viability stain to exclude dead cells and anti-cTNT antibody for identification of cardiomyocytes. The panel was tested for sensitivity to detect different cellular states by interrogating iPSCs versus iPS-CMs. In addition, IFNγ-stimulated iPS-CM were included since inflammatory conditions can change the immunological properties of iPS-derived cell products (Petrus-Reurer S, Romano M, Howlett S, Jones JL, Lombardi G, Saeb-Parsy K. Immunological considerations and challenges for regenerative cellular therapies. Commun Biol. 2021 Jun 25;4(1):798. doi: 10.1038/s42003-021-02237-4. PMID: 34172826; PMCID: PMC8233383.) Clustering by t-stochastic neighborhood

embedding (tSNE) readily resolved d0 iPSCs from differentiated iPS-CM based on marker expression patterns. Likewise, IFNγ-stimulated iPS-CMs were clearly separated from the latter two (**Table 5**). The tSNE plot in the lower left shows cTNT expression of the different cell types with iPS-CMs being positive for cTNT while iPSCs are negative. To avoid confounding effects of cTNT expression on the clustering this marker was excluded as a parameter for clustering. We examined iPS-CMs from HLAhet PMUi001-B (001B), HLAho R26_6, and R26_6 DKO to assess differences in the immune profile between strains. Samples from five independent differentiations per strain were included. As shown in **Figure 16B**, tSNE plots do not reveal dominant strain-specific separation of samples. Rather, the different samples appear interspersed indicating a high degree of similarity between each other.

Table 5: Antibodies included in the 21-parameter panel with corresponding information

TARGET	FLUOROCHROME	CLONE	VENDOR	DILUTION
VIABILITY	Zombie NIR		Biolegend	1:2000
cTNT	Brilliant Violet 421	13-11	BD	1:166
HLA-ABC	Pacific Blue	W6/32	Biolegend	1:25
HLA-DR	eFluor 506	LN3	eBioscience	1:100
CD276	Brilliant Violet 605	7-517	BD	1:75
HLA-E	Brilliant Violet 650	3D12	BD	1:25
ICOSL	Brilliant Violet 711	2D3/B7-H2	BD	1:33
CD137L	Brilliant Violet 786	C65-485	BD	1:50
galectin-3	Alexa Fluor 488	B2C10	BD	1:33
CD164	RB545	N6B6	BD	1:66
CD273 (PD-L2)	PE	MIH18	Biolegend	1:25
HLA-F	PE-Dazzle 594	3D11	Biolegend	1:20
CD80	PerCP	MEM-233	eBioscience	1:20
CD112	PerCP-Cy5.5	TX31	Biolegend	1:100
MICA/B	PE-Cy7	6D4	Biolegend	1:20
HLA-G	APC	87G	eBioscience	1:25
CD86	NovaFluor Yellow 690	IT2.2	eBioscience	1:25
CD47	Alexa Fluor 700	CC2C6	Biolegend	1:33
CD155	BUV737	SKII.4	BD	1:25
CD30	APC-Fire 750	BY88	Biolegend	1:25
CD274 (PD-L1)	APC-Fire 810	MIH3	Biolegend	1:33

Analysis of individual markers confirmed similar expression patterns across strains. CD276, CD164, CD112, and CD155 showed uniform expression in all samples. Galectin-3 and CD47 were expressed in all samples with some variation between individual differentiations (**Figure 16A**).

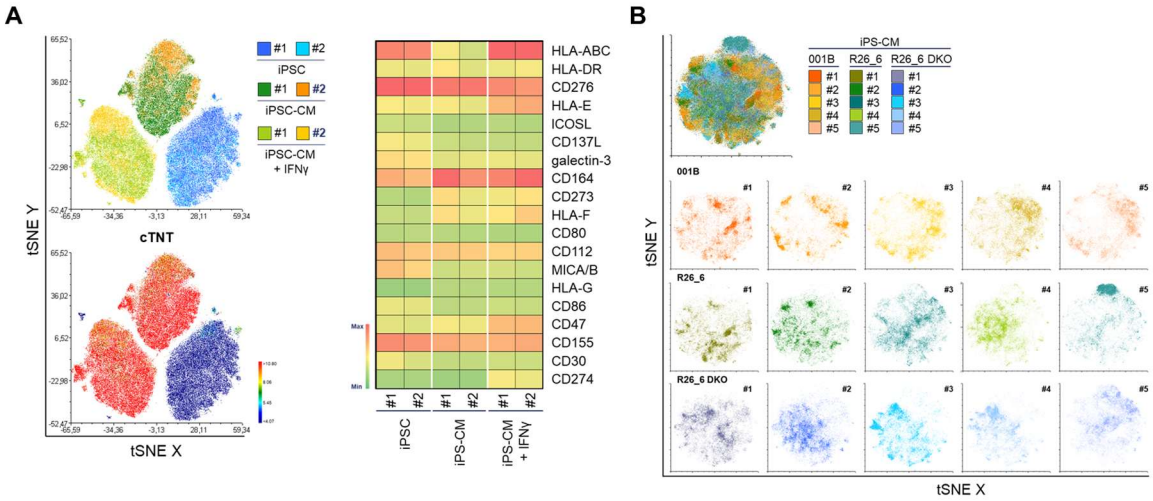


Figure 16: Deep immune-phenotyping of iPSCs and iPS-CMs. (**A**) Left: Dimensionality reduction (tSNE) of d0 iPSCs and iPS-CM, in the absence or presence of IFNγ, based on 21-parameter expression profile. Right: Corresponding heatmap based on scaled mean fluorescence intensity (MFI) marker expression. (**B**) Day 10 phenotype distribution of 5 independent differentiations per strain (001B, R26_6, R26_6 DKO).

By contrast, HLA-ABC was consistently downregulated in HLAho R26_6 compared to HLAhet 001B (**Figure 17A, B**). For comparison R26_6 DKO lacking HLA class I and class II expression was analysed as well. Statistical analysis confirmed significant downregulation of HLA-ABC in R26_6 with levels being only slightly above the HLA class I deficient control (**Figure 17C**).

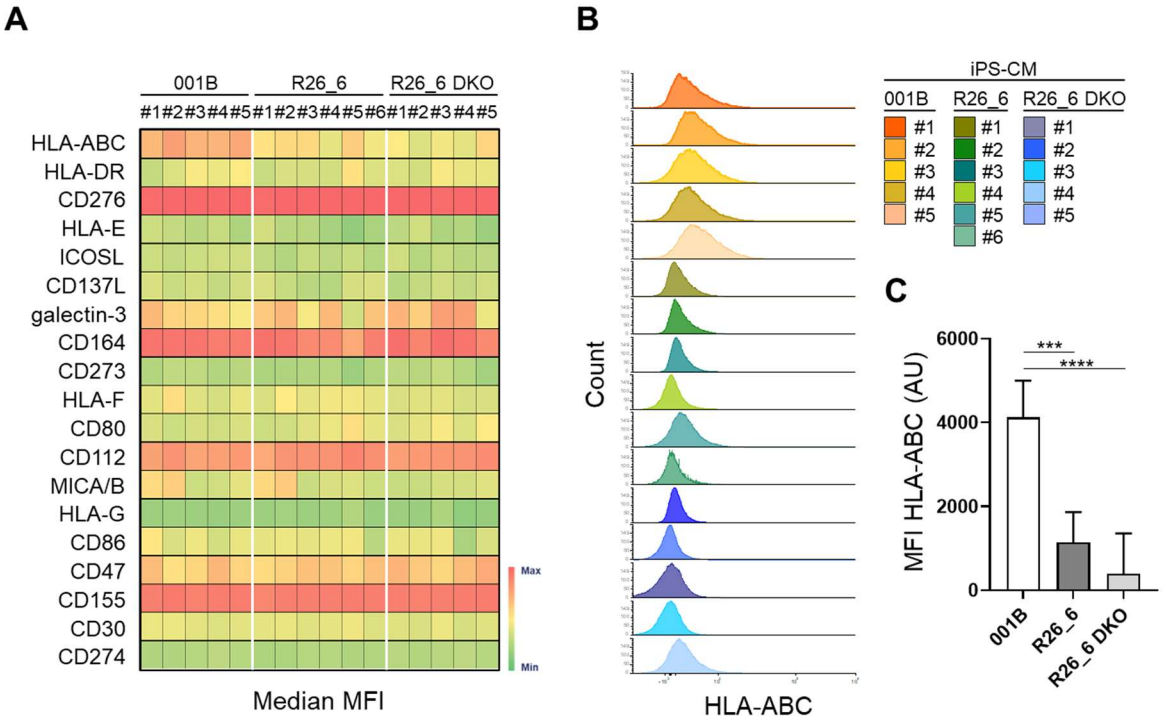


Figure 17: Marker expression in iPS-CMs from different strains and runs. (**A**) Heatmap of day 10 iPS-CM marker expression in five independent differentiations of PMU001B, R26_6, R26_6 DKO, as indicated. (**B**) Corresponding HLA-ABC expression histograms. (**C**) Statistical analysis of differences in MFI for HLA-ABC.

To investigate the effect of inflammatory conditions on marker expression HLAh R26_6 iPS-CM were treated with IFNγ. Cells clustered together depending on whether they were stimulated or not (**Figure 18A**). Of note, stimulated cells from sample 1 were slightly set apart from stimulated cells from sample 2 and 3. Treatment with IFNγ induced pronounced and uniform upregulation of HLA-ABC and HLA-E and slight to moderate upregulation of PD-L1 (CD274) and CD47 (**Figure 18B**). In

addition, stimulated cells from sample 2 showed slight upregulation of PD-L2 (CD273), HLA-F, and HLA-DR. This indicates some variability among iPS-CM from different runs in their stress response with potential consequences for immune recognition. We further extended the immune phenotyping by a 18-parameter panel for purity determination. The panel consists of markers for identification of contaminating iPSCs and a variety of non-cardiomyocytes such as CD34 (hematopoietic stem cells) or CD31 (endothelial cells). The panel was tested on iPSCs and three independent cardiomyocyte differentiations including on subpar run (**Figure 19A**). Dimensionality reduction analysis faithfully separated iPSCs from iPS-CM. In addition, non-cardiomyocytes from the subpar run (iPS-CM 1, cl. cTNT neg. 2) as well as cTNT negative cells from the other two differentiations (cl. cTNT neg. 1) were clearly separated from cardiomyocytes as well as iPSCs based on the marker expression profiles of the individual populations (**Figure 19B, C**). The expression of CD140b, CD201, and CD43 of the non-cardiomyocyte population cl. cTNT neg. 2 could indicate very early hemangiogenic development while absence of all markers investigated here precludes identification of cells in cl. cTNT neg. 1.

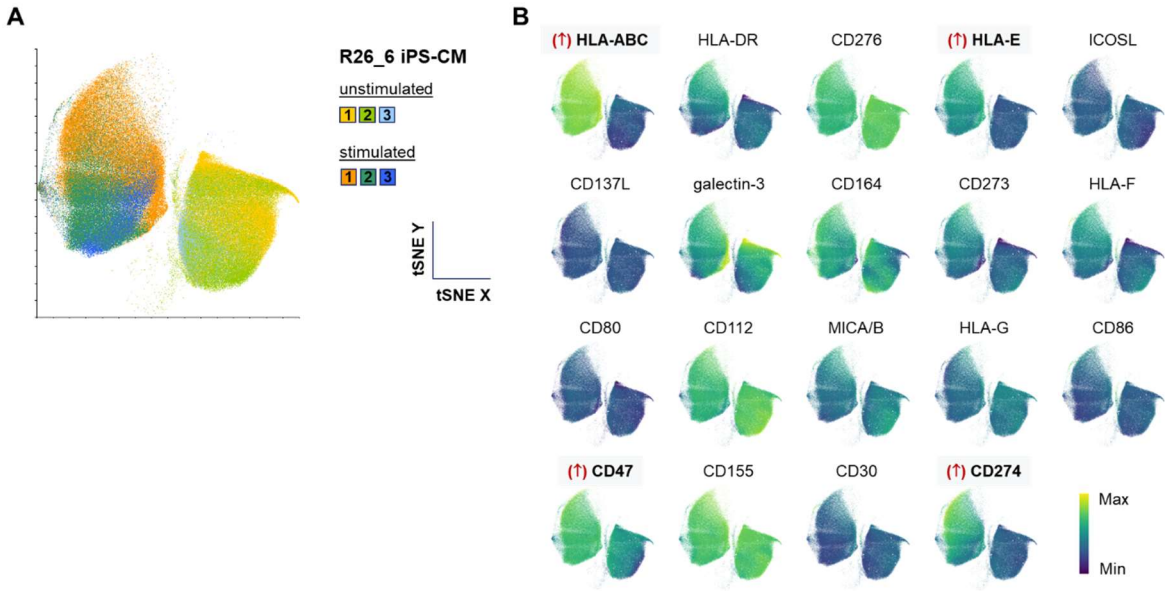


Figure 18: Deep immune-phenotyping of R26_6 iPS-CMs. (**A**) Dimensionality reduction (tSNE) of iPS-CM from 3 independent differentiations, in the absence or presence of IFNγ, based on 21-parameter expression profile and resolved by sample identity. Unstimulated and stimulated samples with the same number represent iPS-CMs from the same run. (**B**) Expression level of each individual marker in the samples analyzed in (A). Expression is represented as color heat ranging from no expression (Min, dark blue) to high expression levels (Max, yellow).

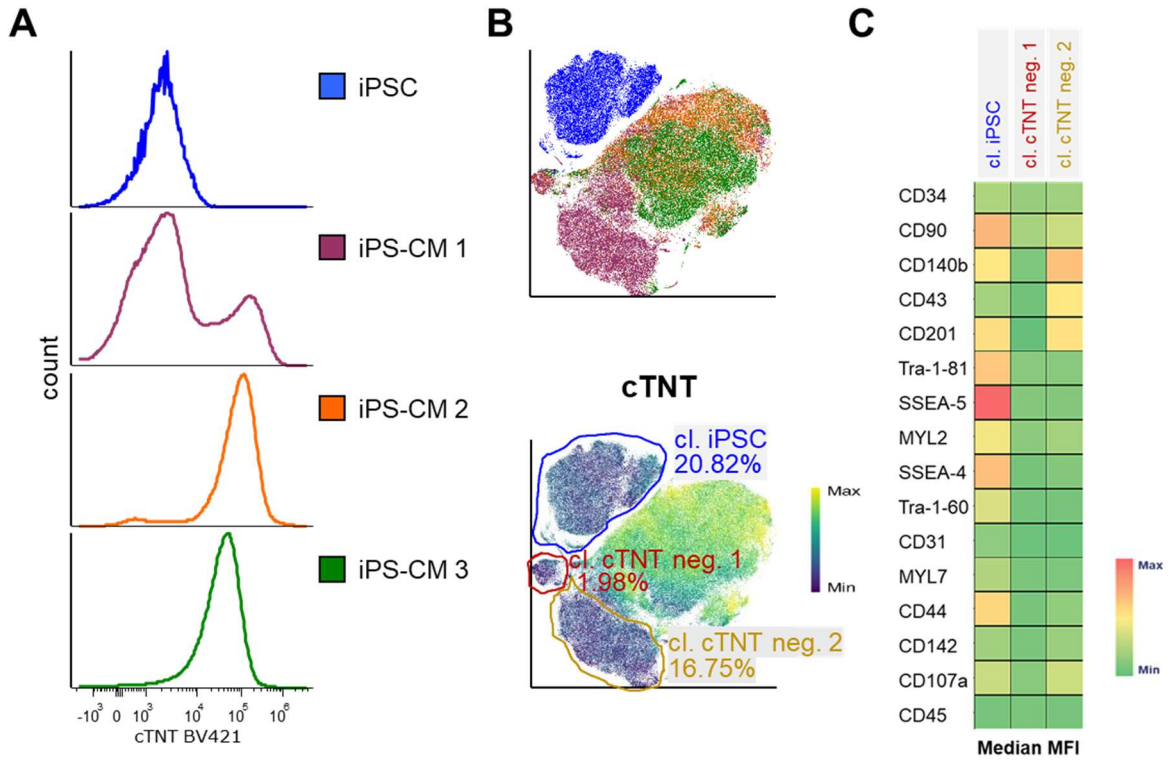


Figure 19: Quality control (QC) panel for identification of contaminating cells in cardiomyocyte differentiations. (**A**) Expression of cTNT in cells used for analysis, including iPSCs and cells from three independent differentiations. (**B**) Dimensionality reduction (tSNE) of samples shown in (A). Upper: Color coding resolved by sample ID. Lower: cTNT expression depicted as color heat from no expression (Min, dark blue) to high expression levels (Max, yellow). (**C**) Heatmap depicting marker expression patterns of iPSCs (cl. iPSC) and cTNT negative populations contaminating differentiations iPS-CM 1-3 (cl. cTNT neg. 1, cl. cTNT neg. 2).

SERO:

In the present reporting period, we finalized an extended immune recognition panel consisting of 21 parameters. In combination with the two panels established at by PMU we are now able to perform a 54 marker immune phenotyping on iPS-CM using only three tubes per sample. The respective panels are **p1: immune recognition**, **p2: extended immune recognition & QC**, and **p3: QC**. Each panel includes a viability dye and cTNT for identification of viable cardiomyocytes. These panels were used on iPS-CM generated at either MHH or CATD. The iPS-CM were used in their native state, after dissociation and re-aggregation for 72 hours, after re-aggregation followed by a 24 hour incubation period in saline on 4°C, or after stimulation of the native aggregates with IFNγ (**Figure 20, left panel**). The first panel - p1: immune recognition – distinguished IFNγ treated iPS-CM from all other samples similar to what was detected previously in other samples (**Figure 16A, Figure 18**). In addition, the 4°C sample clustered separately from all other conditions. The second panel, p2: extended immune recognition, distinguished the 4°C sample from all others. The IFNγ stimulated samples did not cluster separately indicating a marker expression profile which is not affected by proinflammatory cues. This is supported by the corresponding heatmap representation of the marker expression (**Figure 20, middle panel**). The last panel resulted in clustering of all iPS-CM together while cells representation small populations of cTNT- cells clustered away from these. Of note, the 4°C sample appeared to harbour slight differences to the other iPS-CM since parts of it where set apart in the tSNE. The heatmap representation of the marker expression profiles confirms highly similar expression profiles. However, deviations were noted for the 4°C sample (CD34), and the native and the IFNγ treated samples from MHH (CD43) (**Figure 20, right panel**).

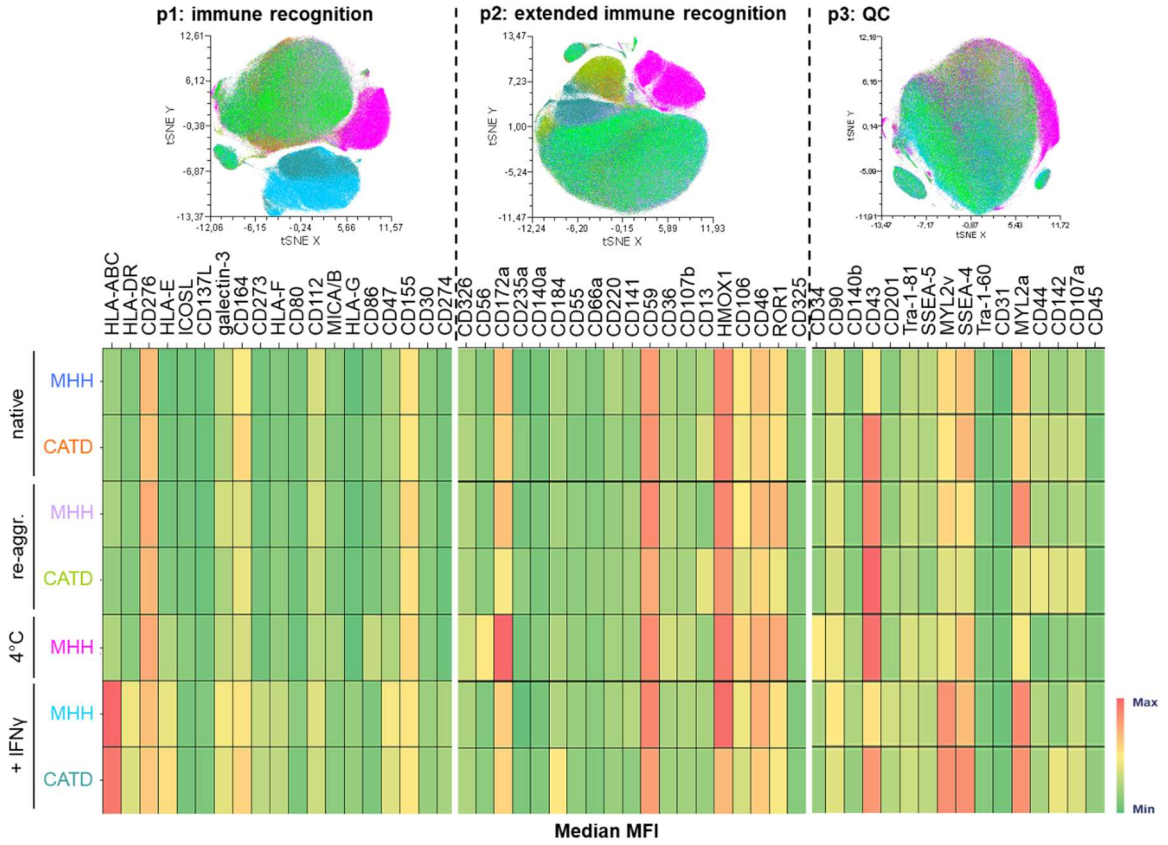


Figure 20: Clustering with tSNE and heatmap representation of samples measured with all three immune phenotyping panels. Upper: tSNE of samples fromm two different production sites (MHH, CATD) under different conditions stained with the full 3-tube immune phenotyping panel. Lower: Corresponding heatmap representation of MFI values for all included markers.

For further comparison we examined the corresponding histogram overlays of the individual markers for each of the panels. The IFNγ treated samples showed strong upregulation of HLA-ABC and moderate to weak upregulation of HLA-DR, -E, -F and -G, galectin-3, CD273, CD47, and CD274 (**Figure 21A**). For all other markers no difference was detected (**Figure 21B-C**). The 4°C sample displayed moderate increase of HLA-E and CD86 expression and a weak but detectable upregulation of MICA/B (**Figure 21A**). In addition, CD56 and CD172a (SIRPα) showed increased expression (**Figure 21B**). While no differences were detected between samples generated at MHH or CATD in panel 1 and 2, panel 3 revealed altered expression of CD43 in non-reaggregated iPS-CM from MHH. Specifically, these samples showed a negative and a positive population (**Figure 21C**). This pattern disappeared in the reaggregated samples indicating either a dynamic regulation of this marker due to the reaggregation procedure or loss of the CD43 negative population.
To perform a hierarchical clustering of the samples we exported the scaled expression values of all markers and analysed them in R using the ComplexHeatmap package. In addition, we included data from iPS-CM generated at our site (R26.6 SERO and 001B SERO). We as well included Jurkat cells and PBMCs with or without IFNγ stimulation. This was done in order to have a reference signature from cells known to evoke an alloreponse when cultured with responder PBMCs. PBMCs and Jurkat cells showed distinct marker profiles with pronounced expression of HMOX1, CD46, CD43, HLA-ABC, CD47, CD184, and CD45 (**Figure 22**). In addition, PBMCs expressed CD55 while moderate levels of CD59, CD155, CD164, and CD31 were detected in Jurkat cells. Human iPS-CM showed a clearly different yet characteristic marker profile with strong to moderate expression of HMOX1, cTNT, CD276, CD59, CD155, and CD164. CD164 showed some variability among samples with lower expression in reaggregated samples from MHH. Of note, samples from the same production site (MHH, CATD or SERO) clustered together indicating subtle differences in the marker expression profile. An exception to this is samples stimulated with IFNγ. If and how these differences are relevant on a functional level regarding the immune response will be further investigated.

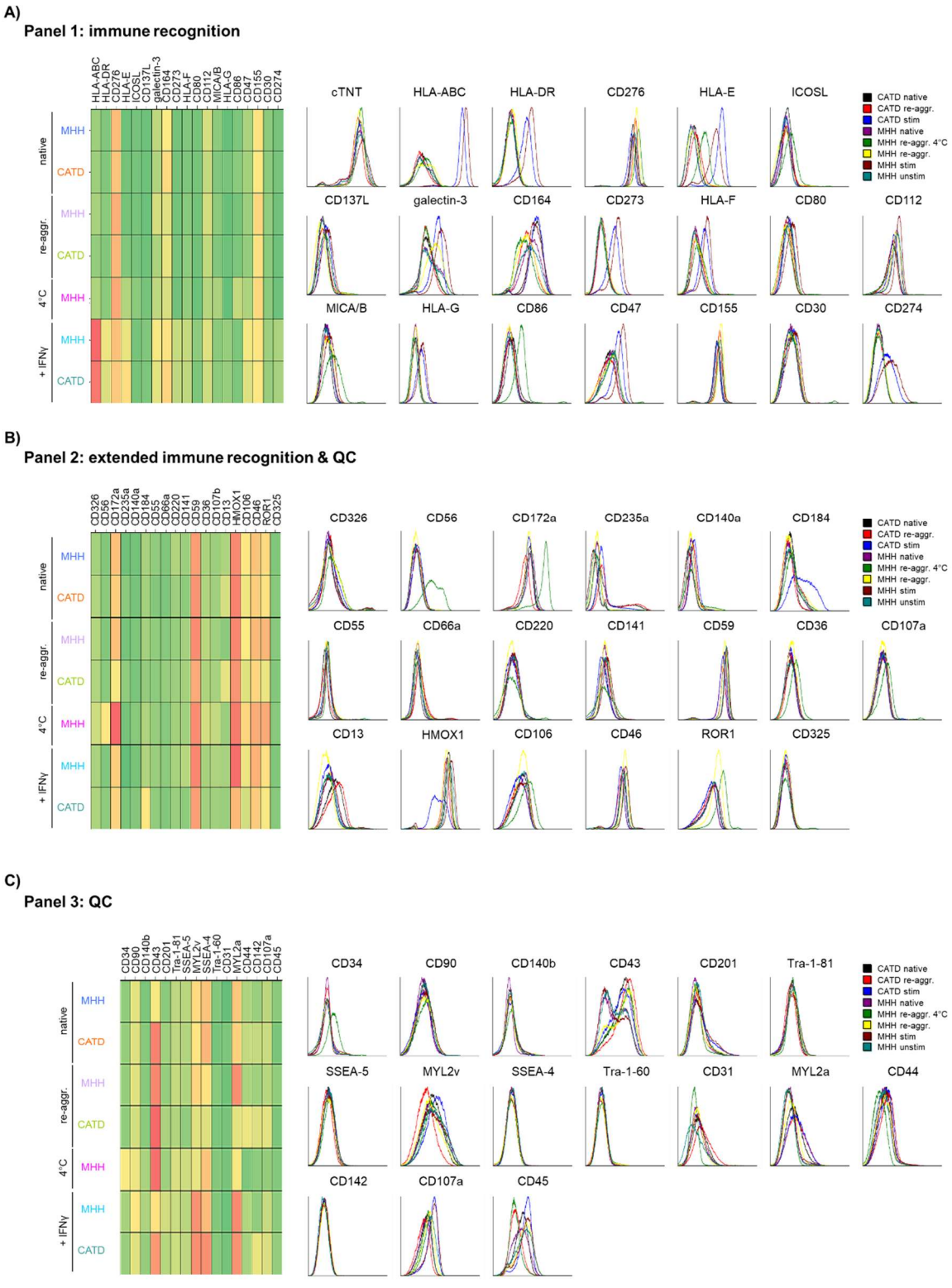


Figure 21: Histogram overlays of all 54 individual markers measured on samples from MHH and CATD. **(A)** Histogram overlays of markers included in panel 1: immune recognition. **(B)** Histogram overlays of markers included in panel 2: extended immune recognition & QC. **(C)** Histogram overlays of markers included in panel 3: QC.

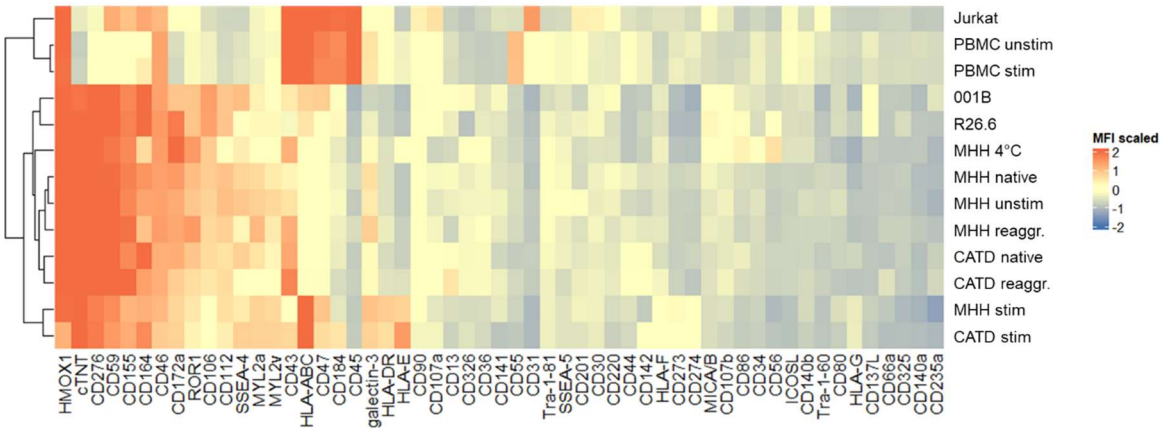


Figure 22: Unsupervised hierarchical clustering of samples based on the combined marker expression profile from the three immune profiling panels. Scaled values for all markers were exported to R and subjected to unsupervised clustering using the ComplexHeatmaps package in R.

Task 2.3 Innate and adaptive immune response profiling applying HLA- matched iPS-CMAs and HLAh matched vs. haploidentical surrogate responder lymphocytes [M6-M32]

PMU:

We adapted established co-culture protocols to assess T cell responses (Ketterl et al., 2015; Chimienti et al., 2022) to the specific needs of iPS-CMs as target cells. Surrogate recipient PBMCs were co-cultured with these cells to investigate allorecognition by T cells. Alloresponse induction was measured as change in the proportions of i) viable CD4+ and CD8+ T cells after 7, 14, 21, and 28 days and ii) naive, central memory, effector memory, and effector T cells (T_{naive}, T_{CM}, T_{EM}, T_{EFF}) after 7 and 14 days of co-culture using flow cytometry (**Figure 23A**). Results are expressed relative to PBMCs only containing IL-2 necessary for the culture (IL-2 control). CD4+ T cells show a tendency to decrease by d14 compared to d7 in both, PMUi001-B (HLAhet) and R26_6 (HLAh). However, R26_6 DKO show an increase by d21 which is lost by d28. Conversely, CD8+ cytotoxic T cells (CTL) show a trend towards increased levels on from d14 onwards compared to d7 while there is no change in R26_6 DKO cultures (**Figure 23B**). Regarding their activation status CD4+ as well as CD8+ show an increase of T_{naive} and T_{CM} at the expense of T_{EM} and T_{EFF} by day 7. After 14 days this shifts to predominant increase of T_{CM} (CD4+ T cells) and T_{EM} (CD8+ T cells), likely as a result of differentiation of the previously expanding naive T cells (**Figure 23C**). These results indicate allorecognition of the target cells by T cells and induction of an allo-immune response which is more pronounced in CTLs.

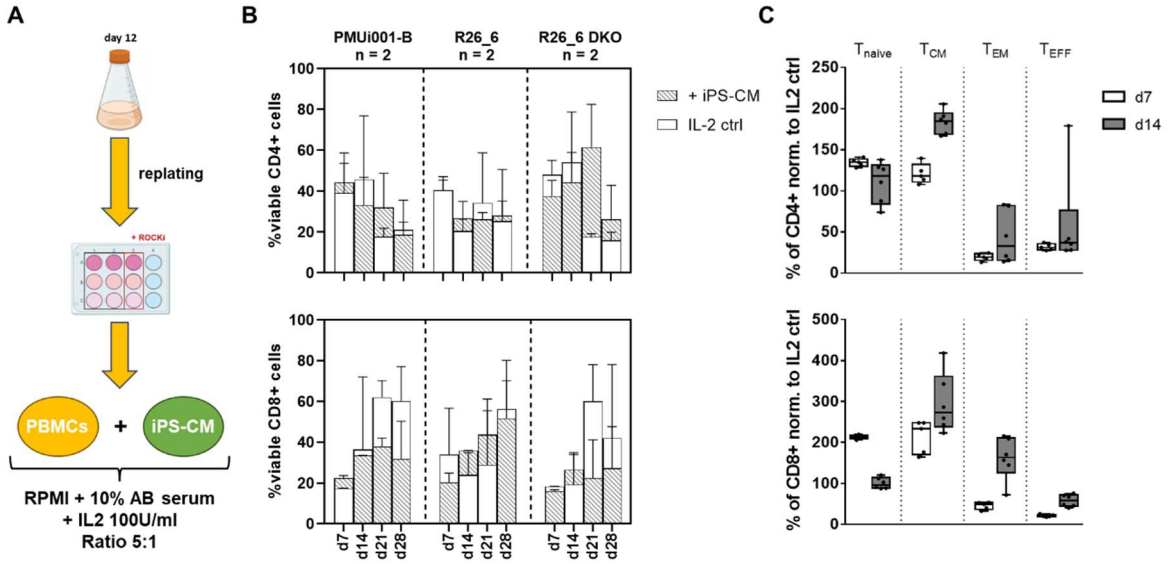


Figure 23: Flow cytometric analysis of T cell responses in unmatched iPS-CM / PBMC co-cultures. (**A**) Experimental outline of co-cultures for allorecognition analysis. (**B**) Frequencies of viable CD4+ (upper) and CD8+ (lower) T cells after 7, 14, 21, and 28 days of co-culture with indicated iPS-CMs. Hatched bars: cultures containing PBMCs and iPS-CMs (+ IL-2), open bars: PBMCs only (+IL-2). (**C**) Frequencies of T_{naive}, T_{CM}, T_{EM}, T_{EFF} among CD4+ and CD8+ T cells after 7 and 14 days of co-culture. Percentages were normalized against IL-2 control; n = 2, technical replicate = 3.

To monitor cytotoxicity of T and NK cells we optimized our standard protocol to iPS-CM with special emphasis on optimal probe concentration and signal-to-noise ratio as these vary between culture conditions and target cell types. Assay setup was carried out using unmatched PBMC pools of 10 donors each and effector-to-target ratios were 1:1, 5:1, 10:1, and 20:1. Specific lysis of HLAhet PMUi001-B iPS-CM by pooled PBMCs was 7.52 % at 1:1 and increased nearly linear to 90.87 % at 20:1. Specific lysis of HLAh R26_6 iPS-CM was 20.59 % at 1:1 and increased only moderately to 42.82 % at 20:1 (**Figure 24A**). These data suggest efficient recognition and lysis of iPS-CM by NK and/or T cells with a decreased impact on HLAh iPS-CM at higher effector-to-target ratios. Strangely, when using single donors PBMCs either haplo- or full-matched to R26_6 we only detected low levels of specific lysis between 4.65 % and 15.80 % with no differences between strains or donors/matching (**Figure 24B**). Since these results are rather contradictory, we are currently in the process of validating the results from both experimental setups.

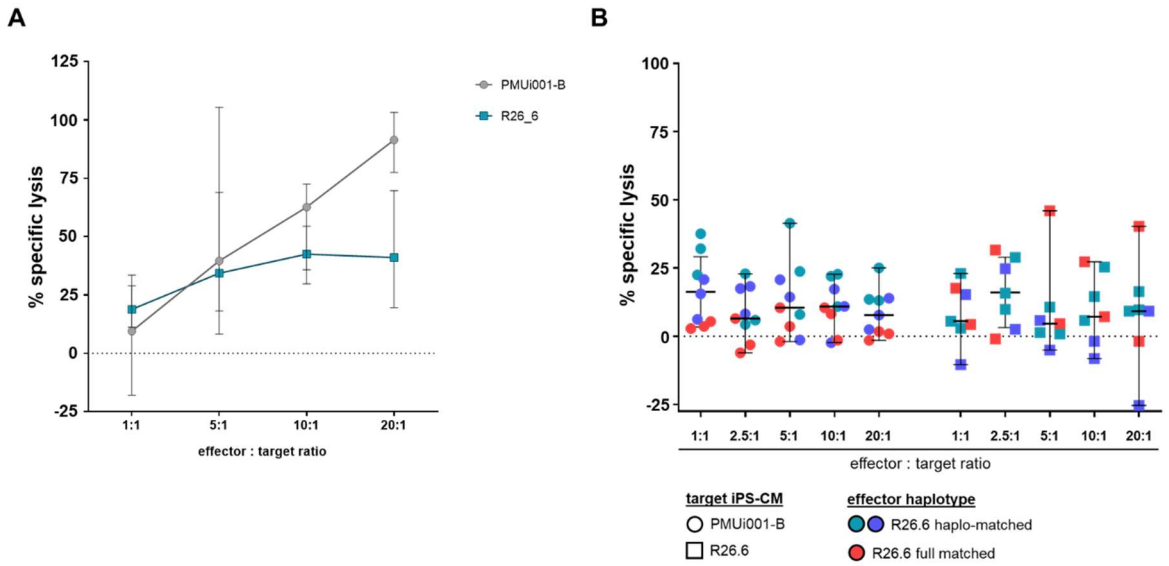


Figure 24: Cytotoxic effect of PBMCs against iPS-CMs. (**A**) Specific lysis mediated by unmatched pooled PBMCs against HLAh and HLAhet iPS-CMs; n = 4. (**B**) Lysis of HLAh and HLAhet iPS-CM by single donor PBMCs haplo- or full-matched to HLAh R26_6; technical replicates = 2-3.

Task 2.4 Machine learning correlation algorithm to predict immunogenicity [M18-M42]

PMU:

No work done yet since this task depends on the input of task 2.3. Please refer to section **5. Deviations from Annex 1 and Annex 2** for further information.

CATD:

Task 2.2. Comprehensive immunophenotyping of HLA-homozygous iPS-CMs [M1-M24]

A staff scientist from CATD travelled to MHH with iPSC-derived cardiomyocytes (both original aggregates and re-aggregates), with and without interferon-gamma stimulation, to learn and perform immunophenotypic staining together with the PMU/ SERO staff scientist.

1.2.3 Work Package 3

WP 3: Safety genetic integrity and suicide gene; Lead Beneficiary HUJI

The overall aim of WP3 is to generate new knowledge and tools to support progress in the safety aspect of iPS-CMAs and iPSC-based therapies in general.

HUJI:

Task 3.1 Assessing the genetic integrity/ safety of HLAh iPSC [M01-M18]

Task accomplished in first reporting period.

Task 3.2 Assessing the genetic integrity / safety of volume produced CMs [M24-M42]

Aneuploidy is a condition in which a cell has an abnormal number of chromosomes. In humans, there are a few viable trisomies, but the only full monosomy is in chromosome X. In accordance, there are several recurrent trisomies in human pluripotent stem cells (hPSCs), but the only recurrent full monosomy is X0, resulting from the loss of one of the X chromosomes. Conversely, X0 monosomy can also be the result of chromosome Y loss. In humans, the mechanism behind loss of chromosome Y (LOY) is still not fully understood. However, LOY occurs in aging and cancers, but its extent and implications in hPSCs have not been studied. Here, we analyzed over 2,650 RNA sequencing (RNA-seq) samples from hESCs and their differentiated derivatives to detect LOY. We found that 12% of hPSC samples have lost their chromosome Y and identified LOY in differentiated cells from all three germ layers. Differential expression analysis revealed that LOY samples showed a decrease in expression of pluripotency markers and in ribosomal protein (RP) genes. Strikingly, significant RP transcription downregulation was observed in most RP genes, although there is only one expressed Y-linked RP gene. We further analyzed RP expression in Turner syndrome and Diamond-Blackfan anemia samples and observed overall downregulation of RP transcription. This

broad analysis sheds light on the scope and effects of LOY in hPSCs, suggesting a novel dosage-sensitive mechanism regulating RP gene transcription in LOY and autosomal ribosomopathies.

This study has recently been published:

Sarel-Gallily, R., Gunapala. K.M., Benvenisty, N.: Large-scale analysis of loss of chromosome Y in human pluripotent stem cells: Implications for Turner syndrome and ribosomopathies. Stem Cell Reports 20:102471 (2025).

Task 3.3 Generic pipeline for assessment of genetic integrity of iPSCs therapeutic derivations [M12-M24]

Human pluripotent stem cells (PSCs) are commonly used in developmental research, disease modeling, and regenerative medicine due to their ability to differentiate into all cell types and seemingly endless proliferative capabilities. Likewise, tissue-derived adult stem cells (ASCs) play a major role in biomedical research, and some of them can readily propagate in culture due to their self-renewal capacity. Specifically, mesenchymal stem cells (MSCs) isolated from a broad range of sources (bone marrow, adipose tissue, cord blood, and more) can differentiate into muscle, bone, cartilage, and adipocytes, and are used in multiple clinical trials. Neural stem cells (NSCs), which are mainly isolated from fetal central nervous system and differentiate into neuronal and glial cells, are central in understanding brain development and disease, and are also used in clinical trials. The self-renewal capacity of PSCs and ASCs implies that they are constantly engaged in DNA replication, increasing their susceptibility to DNA damage and genomic defect accumulation.

Human pluripotent stem cells are known to harbor mutations in tumor-associated genes, and here we aimed to examine the status of adult stem cells. We have thus identified cancer-related mutations in 18% of about 600 mesenchymal stem cell samples, and in 41% of about 200 neural stem cell samples. We have shown a lineage-specific profile of cancer-related genes, demonstrating that TP53 is a central mutated gene in human pluripotent stem cells but not in mesenchymal or neural stem cells. We suggest that the lineage-specificity of tumor-associated genes correlates with their expression levels and with tumor-specific mutations in patients. We have also demonstrated the consequences of mutations in oncogenes and tumor suppressor genes on the transcriptome of each specific stem cell lineage. We therefore propose a categorization of these mutated samples for further appreciation of their severity and emphasize the importance of genetic screening in pluripotent and adult stem cell lines (see Table 1).

Table 6 | Categorization of mutated samples

Ranking of mutated samples by presumed severity of identified mutations. In category B, loss-of-heterozygosity (LOH) is perceived as mutations with allelic frequencies of more than 0.8. In category D, genes are known to promote tumor formation in heterozygous individuals, and in category E, genes have no increased tumorigenicity in heterozygous individuals.

Category of mutated sample	**Example**	**Number of samples**	**Percentage [%]**
A - 2/3 mutated cancer-related genes	*TP53* + *NF1*	26	8.6
B - Tumor-suppressor genes + LOH	*TP53*	24	7.9
C - Oncogenes	*HRAS*	78	25.7
D - Tumor-suppressor genes (tumor-prone carriers)	*APC*	164	54.1
E - Tumor-suppressor genes (normal carriers)	*ERCC5*	14	4.6

This study has recently been accepted for published:

Jung, J., Benvenisty, N.: Drivers and implications of lineage-specific cancer-related mutations in human pluripotent and adult stem cells Stem Cell Reports (in press) (2025).

MHH:

Task 3.4 GMP-compliant genetic engineering of a suicide gene into iPSC lines to advance transplant safety [M1-M36]

In this part of the project, MHH has addressed and successfully achieved several goals:

1. Generation of R26_6 suicide lines with biallelic inducible iCASP_dTomato suicide switch in the proliferation-specific CK1 locus under defined conditions
2. Functionality and specificity of suicide system in vitro was shown by:
    - Sensitivity of undifferentiated suicide clones even after induction with low AP1903 dimerizer concentrations
    - Survival of undirected differentiated cells after suicide induction with AP1903 dimerizer
    - Unaffected beating rates of undirected differentiated cardiomyocytes
    - Confirmation of downregulated suicide and reporter gene expression during undirected differentiation by qPCR
    - Survival of different directed differentiated cell types iCM, iEC and iSMC
    - High expression of CDK1 in undiff R26_6 and suicide clones and downregulation after differentiation into iEC, iSMC, iCM, tested by qPCR
    - Genomic integrity of R26_6 and suicide clones 4, 6 and 27

Since the detailed PCR testing of the iCASP9_dTomato knock-in lines in the last report period revealed an unwanted and unexpected random off-target additional integration the gene targeting was repeated and alternative clones without additional off-target integrations were generated.

This second round of gene targeting resulted in seven clones with correct biallelic (homozygous) knock-in of the suicide/reporter gene into the CDK1 Locus without additional integrations. Three clones were selected for further analysis and use in the project (**Figure 25**)

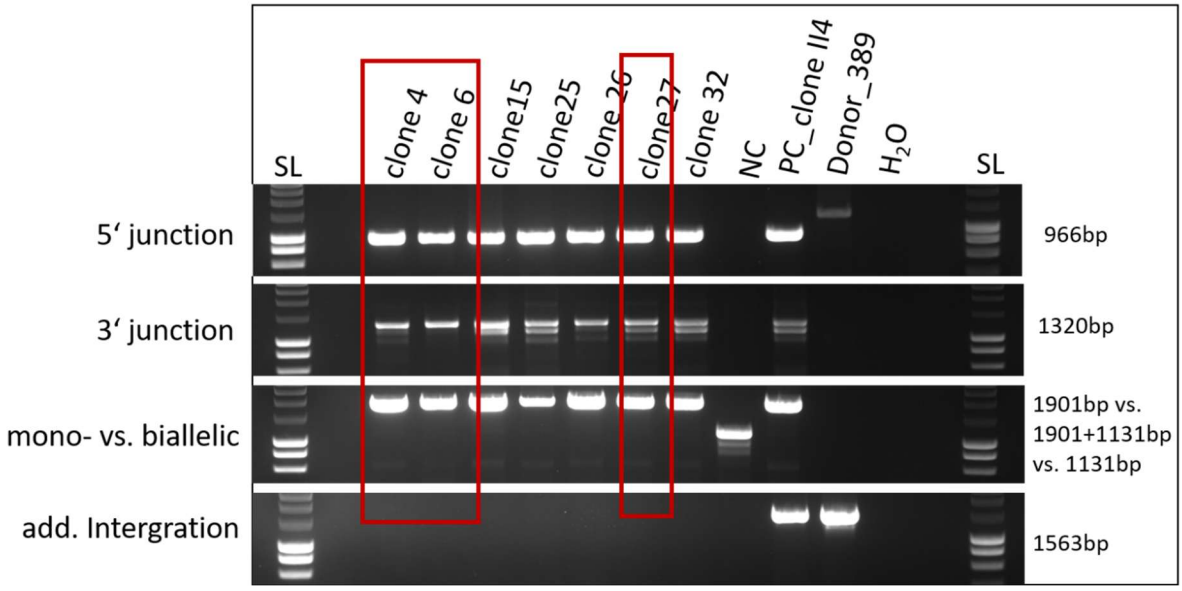


Figure 25: Detailed PCR targeting analysis showed successful biallelic integration of the iCASP_dTomato transgene into the CDK1 Locus without additional integrations. Tree clones (red rectangles) were selected for futher use.

A first proof of concept test for functionality of the suicide gene containing clones 4, 6 and 27 was successful. The generated R26_6 suicide clones showed, as expected, strong sensitivity to treatment even with low concentrations of the dimerizer/inducer of apoptosis AP1903 even after short treatment times (**Figure 26**).

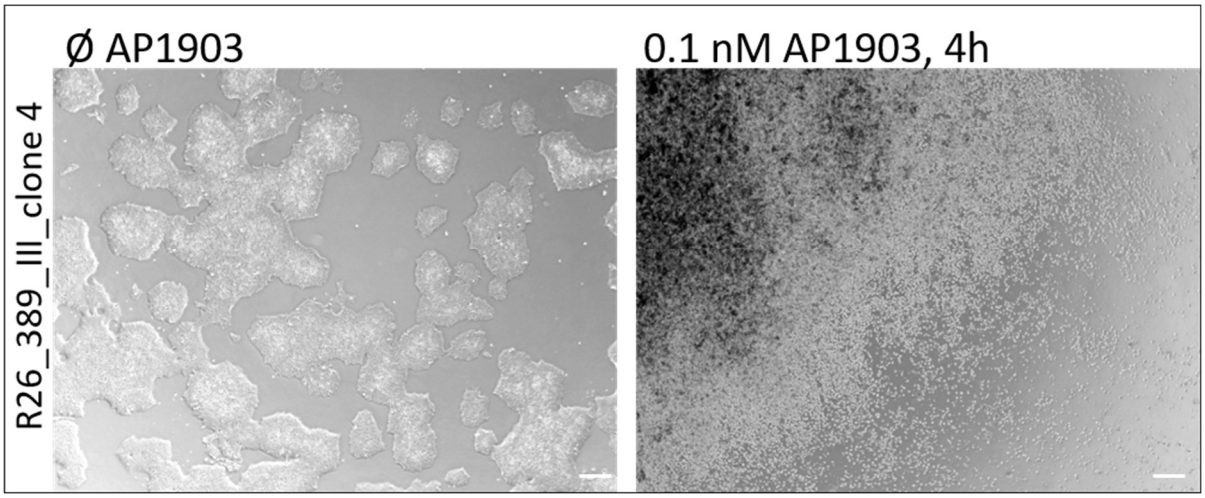


Figure 26: Exemplarily selected R26_6 suicide clone 4 showed sensitivity to treatment with low concentrations (0.1nM). Scale bar 100 µm

In a second proof of concept testing, cultures of undirected differentiated cells (via embryoid bodies, EB's) were tested for their sensitivity to the dimerizer and the induction of apoptosis. Due to the integration of the suicide gene into the CDK1 locus, it was proposed that differentiated, slowly dividing cell have a decreased expression of this cell-cycle-specific gene and therefore also of the iCASP suicide gene and the accompanying increased resistance to AP1903. As expected, the differentiated cells of the suicide clones exhibit resistance against AP1903 (**Figure 27**), indicating that the knock-in of the iCASP suicide cassette into the CDK1 locus mediates the required specificity for expression exclusively in differentiated cells and a selective elimination of only undifferentiated cells is possible.

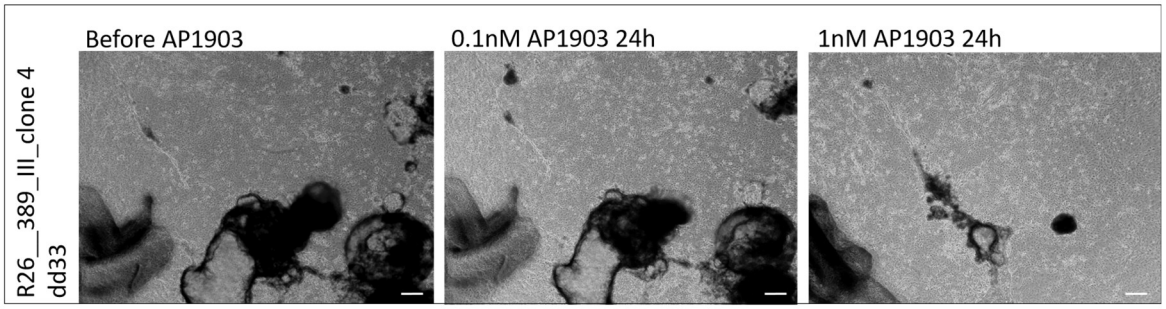


Figure 27: Differentiation cultures (dd3, differentiation day 3) exhibit resistance against 0.1 and 1 nM AP1903 after 24h and 48 h (data not shown). Scale bar 100 µm

To verify the decreasing transgene expression in the time course of differentiation we subsequently tested the expression of the transgenes iCASP and dTomato, integrated into the CDK1 locus of the transgenic suicide clones and in R26_6 parental cells via quantitative PCR (qPCR). As expected, the expression of the integrated transgenes was not detectable in non-transfected parental cells. qPCR revealed decreasing expression of the iCASP and dTomato transgenes in cells of the suicide clones during the course of differentiation, indicating again the linked expression of the transgenes within the CDK1 locus (**Figure 28**).

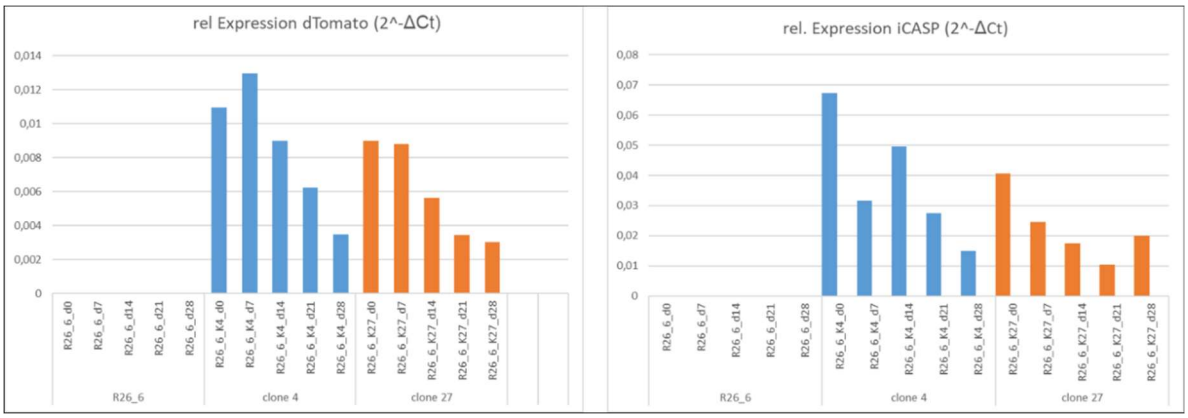


***Figure 28**: Expression of CDK1 driven transgenes dTomato and iCASP is not present in R26_6 parental cells and downregulated during random differentiation of R26_6 suicide clones 4 and 27.*

Since the treatment of R26_6 suicide clones seem not induce apoptosis in the differentiated cultures, we were interested in determining whether the chemical inducer affects the functionality of the differentiated cells by influencing the beating rate of iPSC-based cardiomyocytes. The beating rate is a highly sensitive parameter of the functionality of cardiac myocytes. Given that cardiomyocytes are the cells of primary interest in the HEAL project, we therefore aimed to investigate this parameter.

Cells from R26_6 suicide clone 27 and the parental line were differentiated undirected and treated with various concentrations of AP1903. For each condition, three contracting, cardiomyocyte-containing areas (EBs) were examined in the differentiation cultures. The beating rates were determined before and after treatment with different AP1903 concentrations at multiple time points (**Table 7 and 8**).

Table 7: Treatment conditions of contracting embryoid bodies to determine the influence of AP1903 on the beating rate

concentrations dimerizer	*timepoints*
Ø AP1903	pre treatment
0.01nM AP1903	4h
0.1nM AP1903	24h
1nM AP1903	48h

Table 8: Beating rates in undirected differentiated contracting aggregates after treatment with AP1903

	pre			4h			24h			48h		
Klon27	EB1 dd22	EB 2 (rot) dd28	EB3 (grün) dd28	EB1 dd22	EB 2 (rot) dd28	EB3 (grün) dd28	EB1 dd22	EB 2 (rot)	EB3 (grün)	EB1 d22	EB 2 (rot)	EB3 (grün)
Kontrolle (9)	14	20	28	20	18	34	32	38	34	/	36	30
0,01 nM (11)	34	14	26	36	16	34	42	16	50	44	14	32
0,1nM (8)	52	30	50	54	32	52	52	38	58	52	20	36
1nM (1)	30	46	50	44	72	68	52	76	96	52	40	32
	pre			**4h**			**24h**			**48h**		
R26_6	EB1	EB 2 (rot) dd28	EB3 (grün) dd28	EB1 dd22	EB 2 (rot) dd28	EB3 (grün) dd28	EB1 dd22	EB 2 (rot)	EB3 (grün)	EB1 dd22	EB 2 (rot)	EB3 (grün)
Kontrolle (4)	42	14	30	42	28	52	66	30	40	/	24	34
0,01nM (3)	30	26	68	24	0	100	22	0	86	16	34	70
0,1nM (2)	68	32	46	78	60	90	64	48	68	66	32	54
1nM (1)	40	44	15	48	78	36	44	42	26	42	32	20

Our results show that the cardiomyocyte beating rate in undirected, differentiated, contracting embryoid bodies (EBs) is not negatively affected by AP1903 treatment (**Figure 29**). The differentiated cells survive and the cardiomyocytes continue to beat without significant variations in beating rate, indicating that the suicide gene integrated in the CDK1 locus appears to be specific and the system performs as intended.

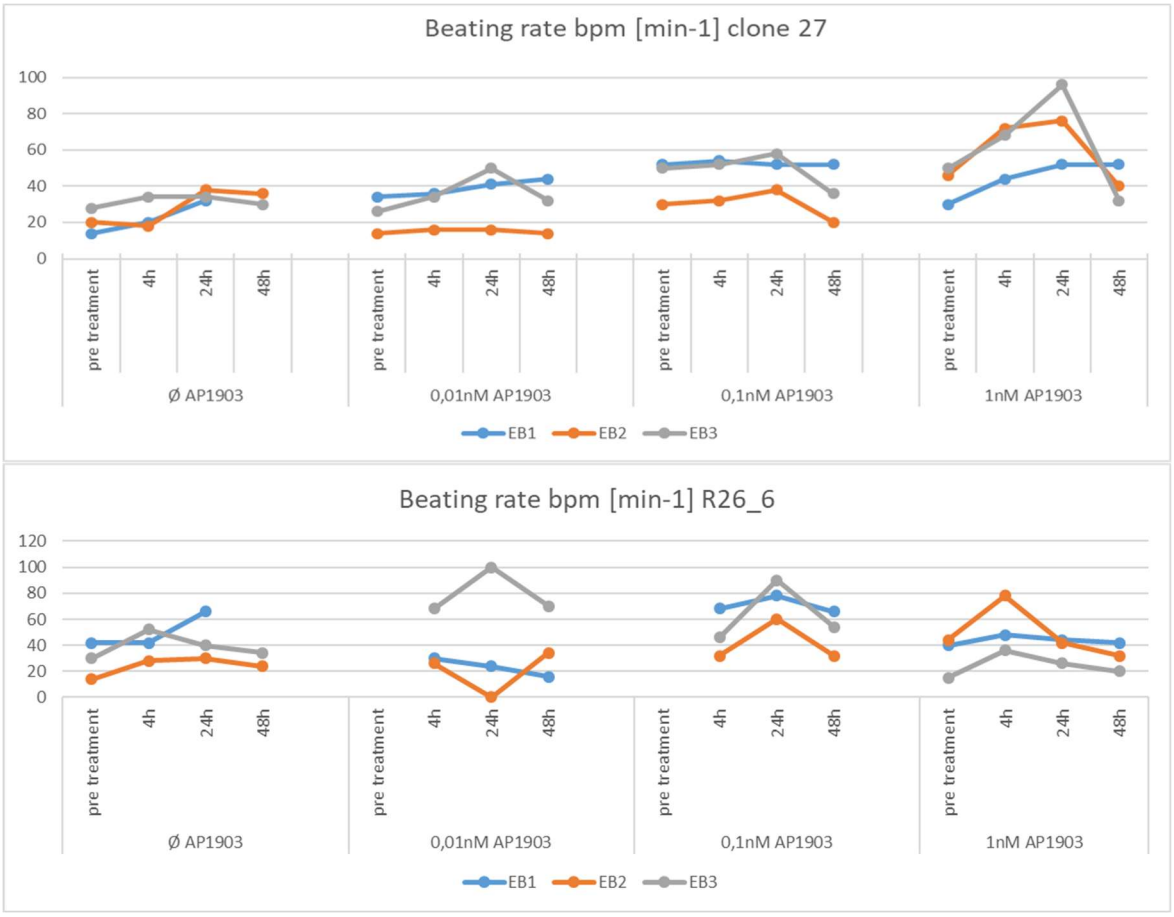


Figure 29: *Cardiomyocyte beating rate in undirected differentiated Embryoid Bodies in R26_& parental cell line and suicide clone 27 is not negatively affected by AP1903 treatment. EB= Embryoid body; bpm = beats per minute*

Since these experiments was conducted with cardiomyocytes that were generated by random undirected differentiation of parental and suicide clone iPSC's, in the next step we wanted to analyze whether the suicide gene selectivity is also present in directed cardiac-like differentiated cells. Therefore, cells were subjected to directed differentiation by applying chemical Wnt pathway modulators (*CHIR* (GSK3ß-inhibitor) = wnt-activation and *Wnt* = wnt-inhibition). The resulting contracting induced cardiomyocytes (iCM) containing aggregates (**Figure 30**) were treated with different AP1903 concentrations and contraction behavior was analyzed.

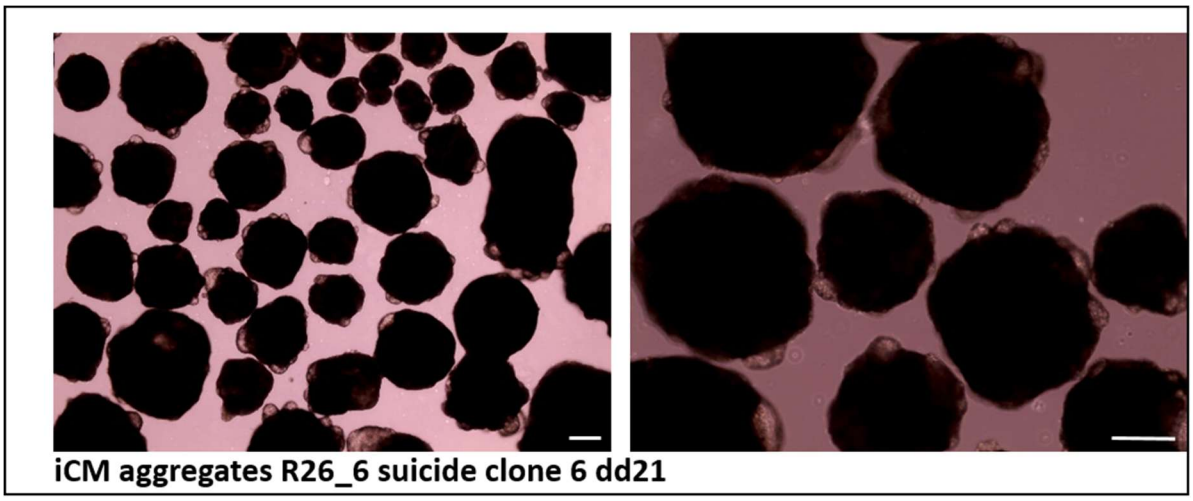


Figure 30:iPSC-based cardiac aggregates at differentiation day 21 (dd21). Scale bar 100µm

The directed differentiated cardiac aggregates were also not affected by the inducer of the suicide gene and kept on beating (**Table 9**) under AP1903 treatment, what again might suggest that non-cycling or slowly dividing differentiated cardiomyocytes survive and the specificity of the suicide system is indicated.

***Table 9**: Contractions in directed differentiated cardiac aggregates after treatment with different AP1903 concentrations*

iCM aggregates d21, 24h after AP1903 treatment				
	AP1903	**1nM AP1903**	**0.1 nM AP1903**	**0.01 nM AP1903**
R26_6	+	+	+	+
clone4	+	+	+	+
clone6	+	+	+	+
+ = still contracting aggregates				

In the further course, we tested the specificity of the suicide system in in the R26_6 transgenic clones in other iPSC-based differentiated derivatives. We applied our established protocols to derive cardiomyocyte-like cells (iCM), endothelial cells (iEC) and smooth muscle cells (iSMC). To perform cell counting differentiated cells were subsequently dissociated and seeded in specific cell densities in 12-well plates. Furthermore, we treated the cells with different concentrations of AP1903 and counted them again after 24 hours to assess their response to the suicide gen-inducing agent, as schematically shown in **Figure 31**.

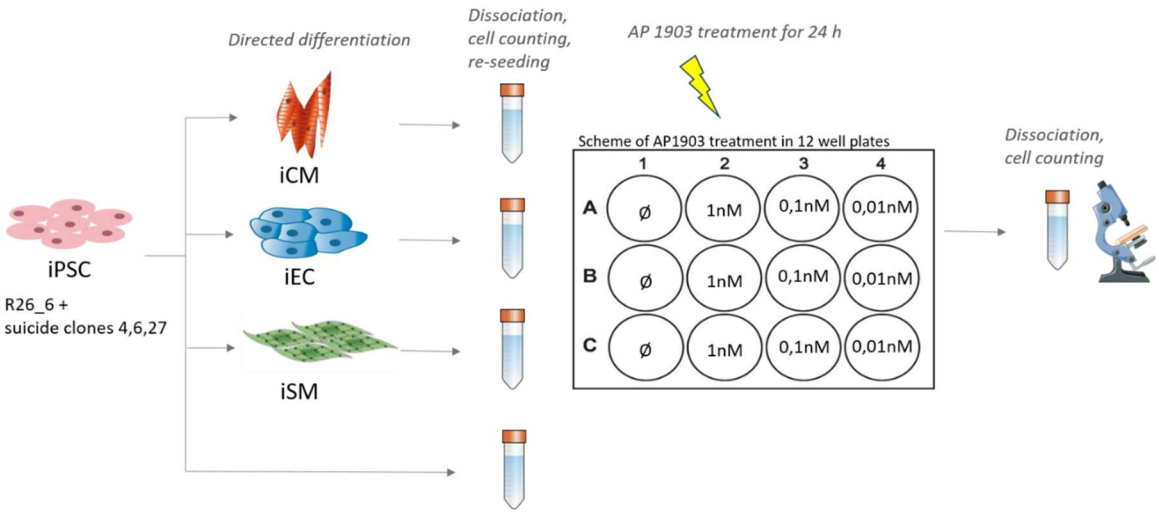


Figure 31: Experimental setup to analyse the suicide system- specificity in varying iPS-based derivatives. ICM= iPSC-based cardiomyocytes, iEC=iPSC-based endothelial cells, iSMC= iPS-based smooth muscle cells.

The results, depicted in **Figure 32** revealed that differentiated cell types were not affected by the AP1903 dimerizer treatment, regardless of their proliferation capacity (Mean PD/day depicted in **Figure 32**, bottom). In contrast, undifferentiated cells of the suicide clones underwent apoptosis, as expected. These results indicate that CDK1 expression is already downregulated in all tested differentiated cell types, and that the suicide system is effective for removing undifferentiated cells, but may selectively protect differentiated cells from apoptosis in our in vitro system.

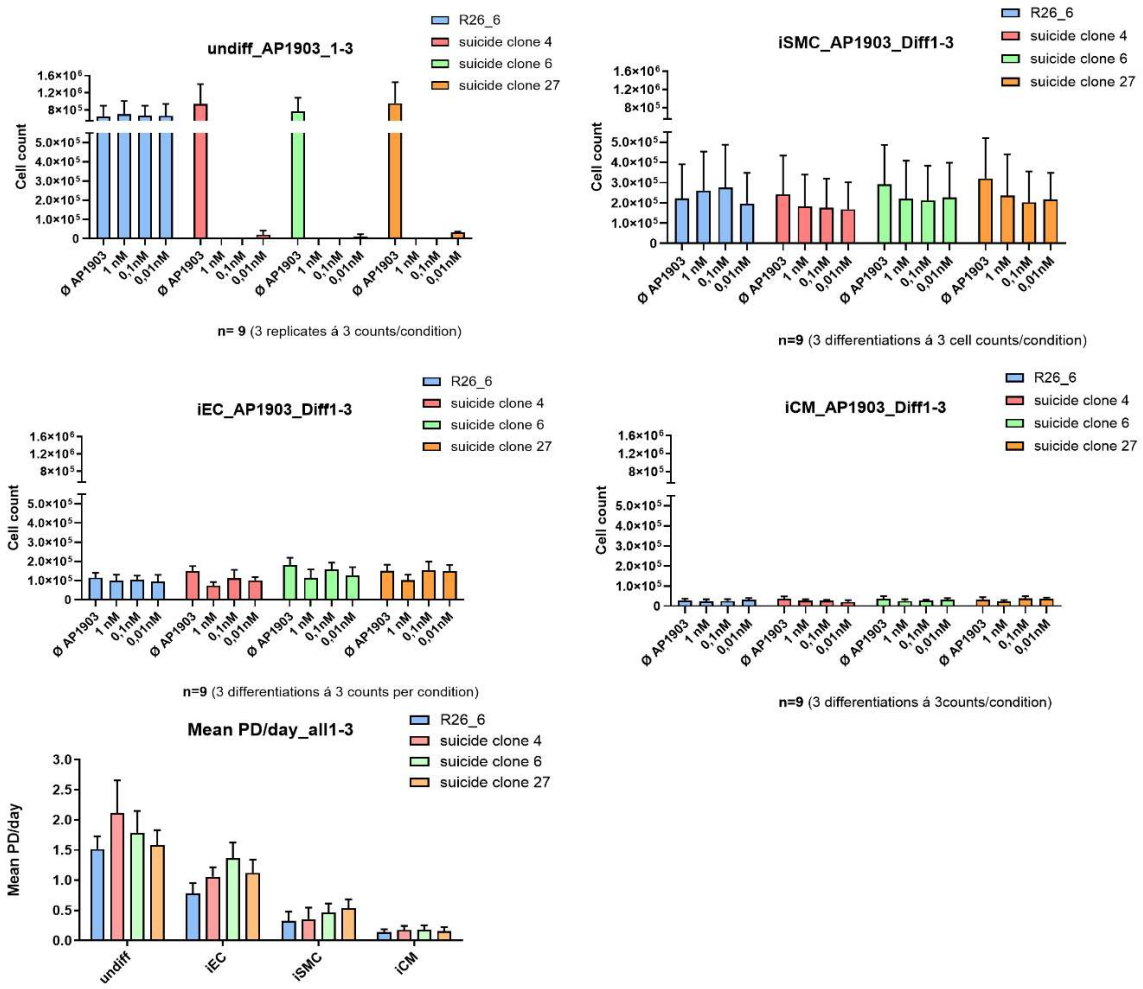


Figure 32: Cell counts and population doublings (PD) of AP1903 treated undifferentiated iPSC, iECs, iCMs and iSMCs. Undifferentiated iPSCs of suicide clones revealed sensitivity to inducing dimerizer AP1903. Differentiated derivatives of suicide clones showed resistance to all AP1903 concentrations, regardless of their proliferation capacity.

To further confirm a decreased CDK1-driven suicide gene expression and thereby specificity of our system, we performed RT-qPCR-based expression analysis for CDK1 in all differentiated cell types and compared to undifferentiated control cells of each suicide clone.

This analysis indeed suggests that CDK1 expression is high, in the undifferentiated cells, as proposed, while it is strongly reduced in the differentiated iEC, iSMC and iCM (**Figure 33).** Notably, the expression in the non-genetically modified differentiated cells (R26-6) is higher than in the suicide clones (clones 4, 6 and 27). This could indicate that the CDK1 expression in the transgenic clones might be negatively influenced by the homozygous knock-in of the transgene in both alleles of the CDK1 gene.

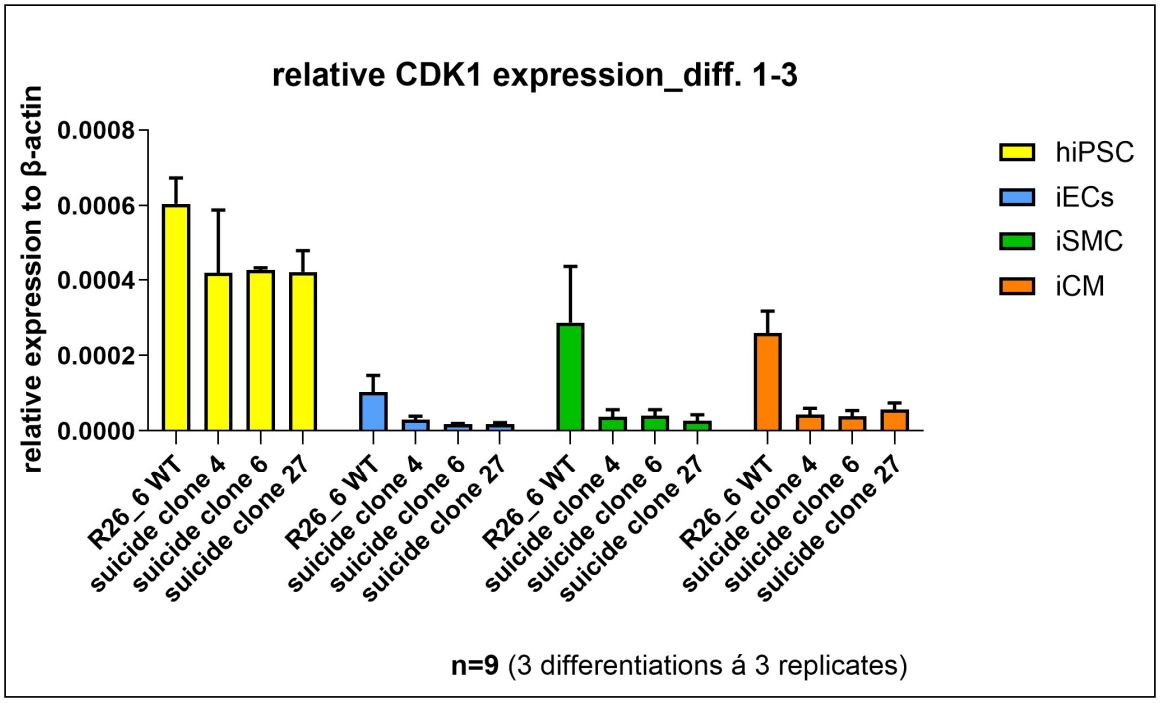


Figure 33: qPCR Analysis of CDK1 expression in undifferentiated and differentiated derivatives (iEC, iSMC, iCM) of R26_6 parental cell line and transgenic suicide clones 4, 6 and 27. CDK1 expression was fpund to be high in undifferentiated cells, whereas it was strongly reduced in differentiated derivatives.

To further validate the usability of the suicide clones for the HEAL project, we collaborated with the group of Nissim Benvenisty in Israel to conduct a genome analysis. Our aim was to investigate whether the gene editing or the prolonged cultivation of the clones and parental cell line compromises the genome integrity. To this end, we collected RNA samples from the parental cell line and the suicide clones at low and high passages (see **Figure 34** for sample scheme).

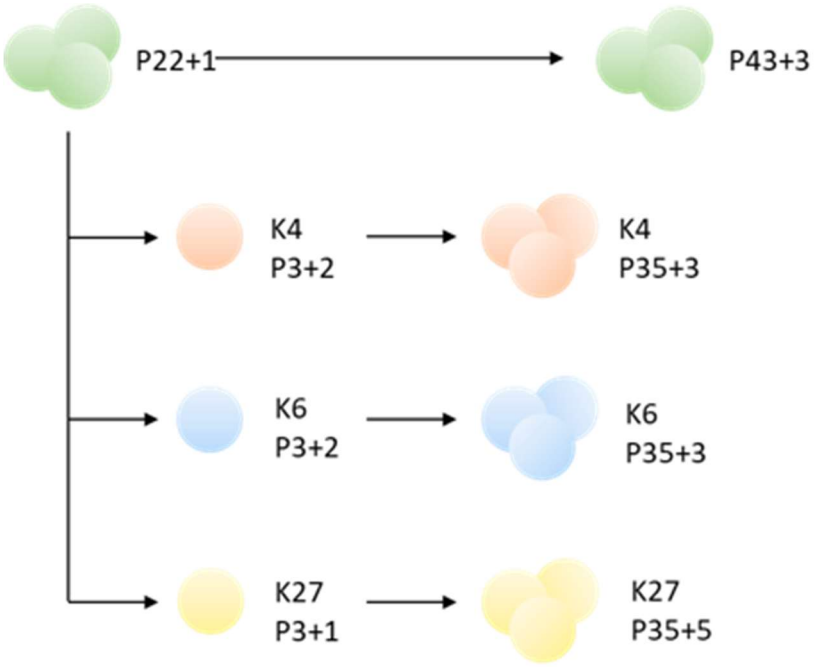


Figure 34: Sample scheme for RNA-based analysis of genomic integrity of R26_6 parental line and transgenic suicide clones 4, 6 and 27. K=clone, P= passage. Figure was kindly provided from Jonathan Jung of Benvenisty Group

Samples were analyzed by e-SNP-Karyotyping and a novel pipeline developed by the Benvenisty group for the identification of cancer-related mutations in hPSCs from RNA-seq data (RNA-seq to Cancer Mutation, RNA2CM).

The results provided no indication of acquired mutations or chromosomal aberrations. Examination by e-SNP-Karyotyping showed clean genomic integrity at the chromosomal level (**Figure 35**), with no significant abnormalities. RNA2CM analysis showed no evidence of acquired cancer-related mutations and identified a known single heterozygous mutation in the ATM gene in the parental cell line (**Figure 36**). In summary, these results show that none of the examined samples showed any acquired genetic changes and that neither the longer cultivation time nor the gene editing provide a negative impact on the functionality of the cells.

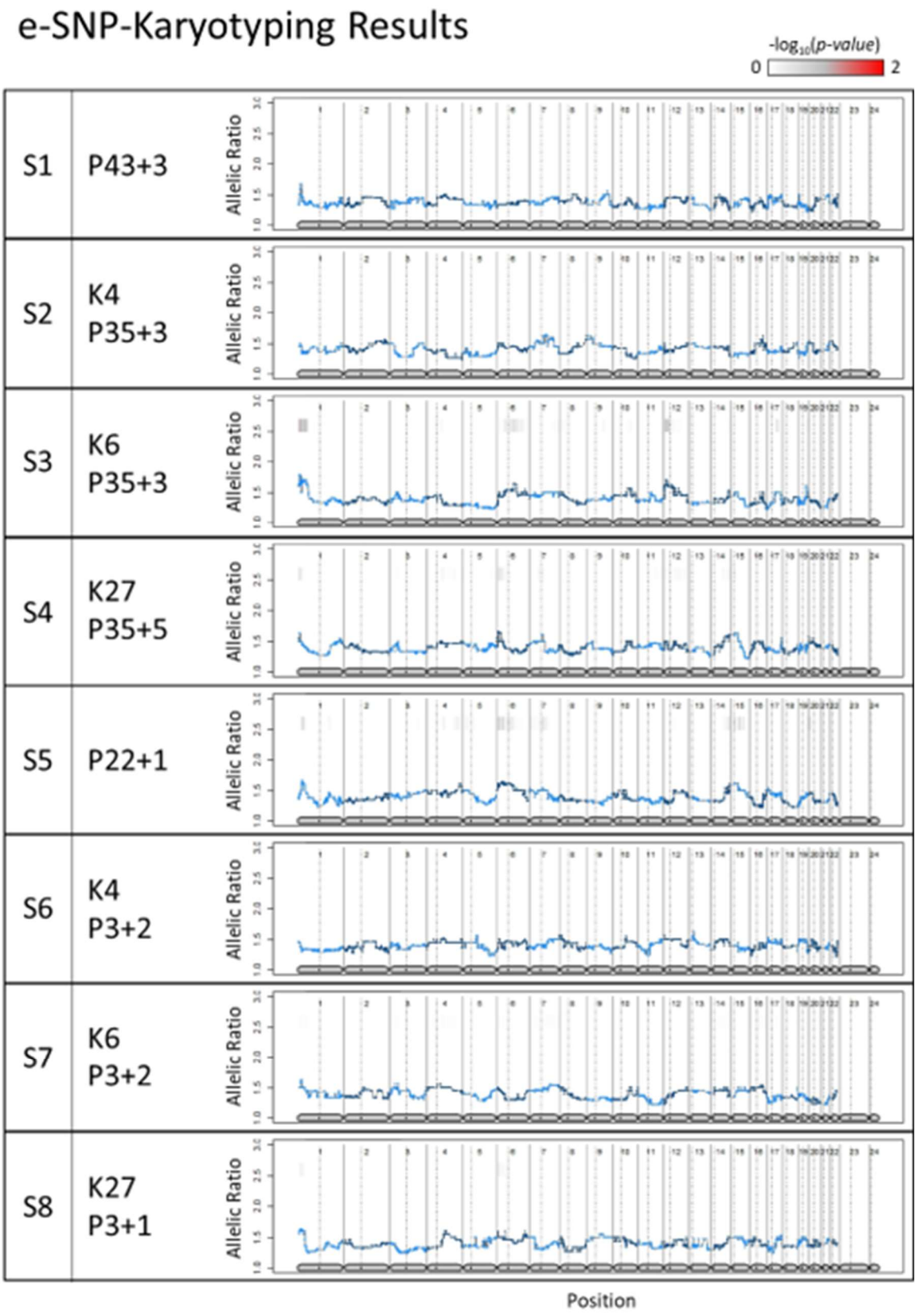
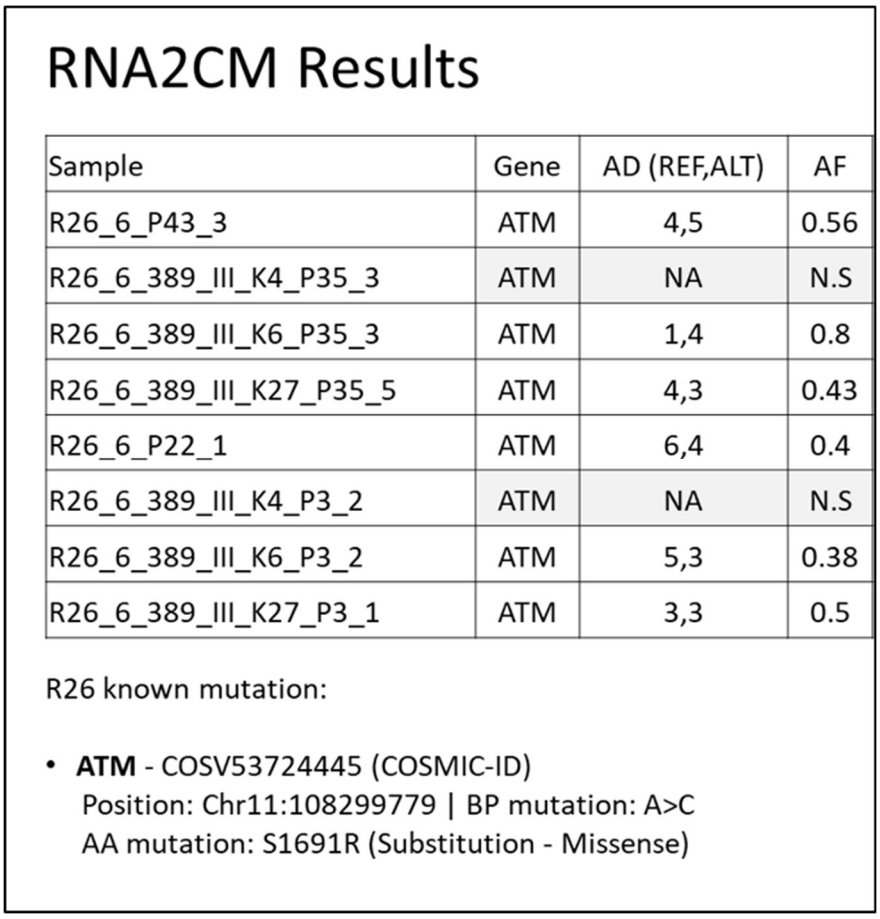


Figure 35: e-SNP-Karyotyping of R26_6 and suicide clones 4 (K4), 6 (K6) and 27 (K27) showed clean genomic integrity at the chromosomal level, with no significant abnormalities in low and higher passages (P). S= sample. Figure was kindly provided from Jonathan Jung of Benvenisty Group



RNA2CM Results

Sample	Gene	AD (REF,ALT)	AF
R26_6_P43_3	ATM	4,5	0.56
R26_6_389_III_K4_P35_3	ATM	NA	N.S
R26_6_389_III_K6_P35_3	ATM	1,4	0.8
R26_6_389_III_K27_P35_5	ATM	4,3	0.43
R26_6_P22_1	ATM	6,4	0.4
R26_6_389_III_K4_P3_2	ATM	NA	N.S
R26_6_389_III_K6_P3_2	ATM	5,3	0.38
R26_6_389_III_K27_P3_1	ATM	3,3	0.5

R26 known mutation:

- **ATM** - COSV53724445 (COSMIC-ID)
  Position: Chr11:108299779 | BP mutation: A>C
  AA mutation: S1691R (Substitution - Missense)

Figure 36: RNA2CM of R26_6 and suicide clones 4 (K4), 6 (K6) and 27 (K27) showed no acquired cancer-related mutations and identified a known single heterozygous mutation in the ATM gene at the original cell line.AD= Allelic Depth, REF= Reference, ALT= Alternative, AF= Allele Frequency, ATM= Ataxia-Telangietasia Mutated, K= clone, P= passage. Figure was kindly provided from Jonathan Jung of Benvenisty Group

Task 3.5: Development of SOPs for GMP-compliant generation of iPSC lines carrying an iCASP9 suicide gene [M32-M40]

The development of the SOPs was conducted. The documents will be made available to the consortium at the HEAL academic cloud:

https://sync.academiccloud.de/index.php/s/qa5XV3gk9Nh3ebc

CATD:

Task 3.4. GMP-compliant process for integrating 2 copies of a suicide gene into HLAh iPSC [M01 – M36]

Optimization of a gene editing GMP workflow to enable efficient knock-in in R26 iPSCs.

For the GMP-compliant generation of edited iPSCs carrying two copies of a desired transgene, it is essential to combine high knock-in (KI) efficiency with process robustness, reproducibility, and scalability. CATD goal was to establish a KI protocol that meets GMP requirements by ensuring

efficient genome integration, operational simplicity, and compatibility with clinical manufacturing — specifically avoiding complex cell sorting, enrichment, or selection steps.

Three parameters were identified as key to achieving GMP-relevant KI outcomes:

1. Efficient delivery of gRNA, nuclease, and donor DNA.
2. Promoting homology-directed repair (HDR) over non-homologous end joining (NHEJ).
3. Maximizing iPSC viability during and after genome editing.

For clinical translation, GMP-ready reagents and processses were selected. Ribonucleoprotein (RNP) complexes were based on Alt-R A.s. Cas12a Ultra or Alt-R A.s. Cpf1 (IDT), and donor templates used standard plasmid backbones compatible with GMP plasmid manufacturing. As a model system, a GFP KI into the AAVS1 locus was employed.
Optimization of nucleofection parameters using pMaxGFP plasmid identified the P4 Nucleofection Buffer with program CA167 as yielding the highest proportion of GFP^+ cells (**Figure 37A**). A 10-minute post-nucleofection recovery in RPMI medium significantly improved cell survival compared with standard iPSC medium, an essential improvement for maintaining viability in GMP-scale workflows (**Figure 37B**). Plasmid-only delivery was highly efficient, and RNP-only delivery generated high indel rates; however, co-delivery of RNP and donor plasmid in a single step yielded low KI rates and poor viability (**Figure 37C-D**).

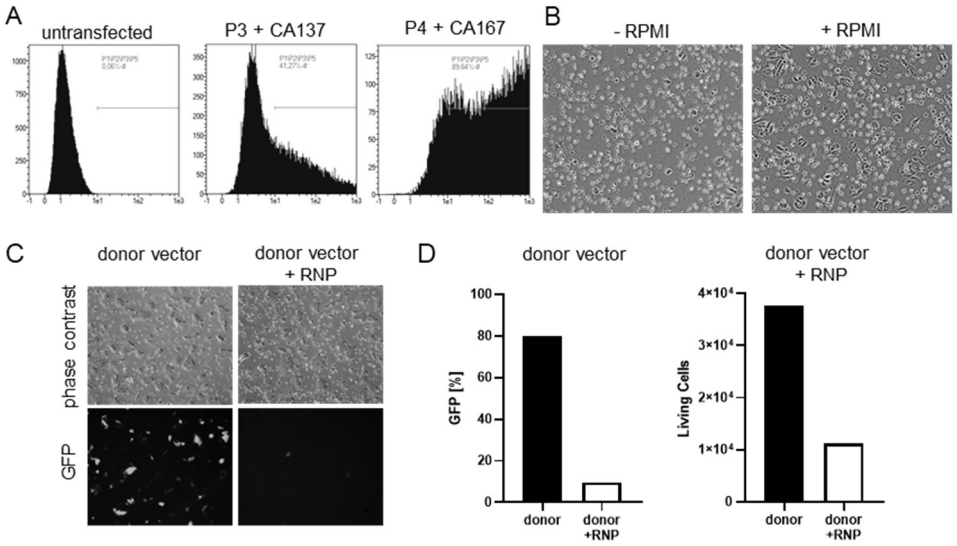


Figure 37: Optimization of Nucleofection. **A**. Flow cytometry results of cells 24 hrs after nucleofection with pMaxGFP plasmid using different nucleofection buffers and programs. **B**. Brightfield microscopy (4x magnification) comparing cell morphology and density 24 hrs after nucleofection with and without recovery step in RPMI medium. **C.** Fluorescence microscopy (4x magnification) images comparing nucleofection efficiency of a GFP donor only (left) or donor in combination with RNP (right) after 24 hrs. **D**. Flow cytometry quantification of transfection efficiency and cell numbers 24 hrs after nucleofection.

The most significant improvement came from a two-step sequential nucleofection — plasmid donor delivery on day 1 followed by RNP delivery on day 2 — which substantially increased KI efficiency (Figure 37A-B). The RPMI recovery step was indispensable for enabling two consecutive nucleofections without compromising viability.

Additional process refinements included (**Figure 38A-B**):

- Scaling up cell input per cuvette to 3 × 10^6 cells while maintaining the same plasmid-to-cell ratio, further improving KI efficiency and reducing variability.
- Post-RNP cold shock at 32 °C, enhancing HDR efficiency.
- Pre-conditioning in enriched culture medium for 48 hours before the first nucleofection, providing incremental gains in KI yield.

These combined optimizations increased KI efficiencies from ~3 % to up to 40 % (**Figure 38C-D**). The sequential donor–RNP delivery step proved critical; omitting it resulted in a complete collapse of KI efficiency (**Figure 38B**).
The protocol performed equivalently with Cas12a or Cas9 nucleases (**Figure 38E**), at multiple genomic loci including AAVS1 and B2M (**Figure 38F**) and in independent GMP iPSC lines (**Figure 38G**). Confirming its locus-, nuclease-, and line-independent applicability.
In summary, CATD have developed a scalable, reproducible, and GMP-compatible KI platform for iPSCs, eliminating the need for complex post-editing enrichment, supporting process standardization, and enabling manufacturing genome-edited iPSCs for therapeutic use.

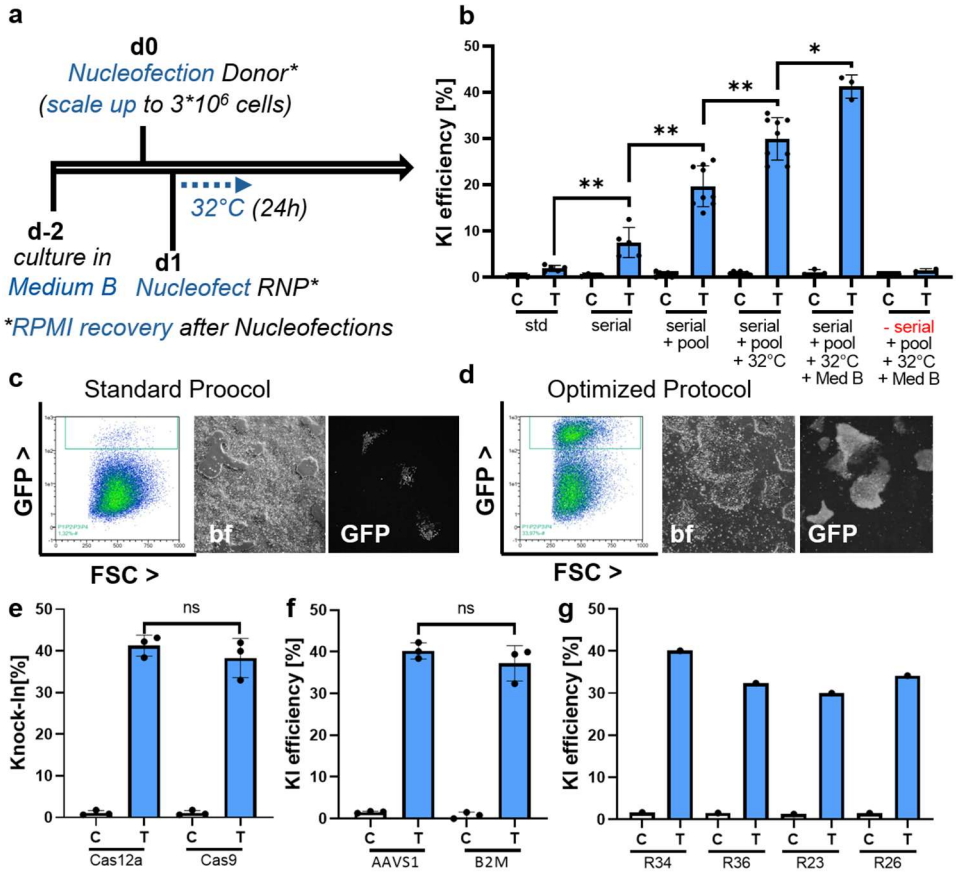


Figure 38: Optimized gene editing workflow enables efficient Knock-Ins in iPSCs. **A.** Overview of optimized gene editing protocol. Critical optimizations are highlighted in blue. **B**. Incremental increase of KI efficiency by implementation of each optimization indicated by flow cytometry quantification of GFP+ cells 10 days after nucleofection. C = control, T = target. Controls received donor and RNP, but an irrelevant gRNA was used. **C-D**. Example results of standard protocol **(C)** or fully optimized protocol **(D)** showing flow cytometry and fluorescence microscopy (with 4x objective) of GFP. **E-G** Flow cytometry quantification of GFP integration comparing Cas12a against Cas9 **(E),** different target loci **(F)** or different GMP iPS cell lines **(G).** Replicate numbers are indicated by dots overlaying bar charts. Error bars denote standard deviation.

INNO:

Task 3.5: Regulatory compliant tumorigenicity studies [M32-M40]

The toxicology, biodistribution and tumorigenicity study design was discussed with the project partners based on PEI requirements and final product specifications. It was decided to switch from nude rats to NSG mice as animal model since these are standardly used and since NSG mice do not pose a risk for NK cell-mediated rejection.

An MTA has been set in place for the transfer of the CATD iPSC line to be used as positive control for the validation of the NSG mouse tumorigenicity assay.

Hela cells are used as GLP compliant positive control cell line, a license for the use of these cells has been obtained and cells have been purchased.

A human DNA specific Alu repeat qPCR assay has been validated to allow determination of the biodistribution of human cells to different organ systems after transplantation.

Hela cells and iPSCs have been expanded and banked.

The in vivo tumorigenicity model validation experiments have been performed with confirmed tumorigenicity of the Hela positive control, undifferentiated IPSCs and CMAs spiked with undifferentiated iPSCs.

Human specific histopathology markers have been validated and used in the validation assay to identify human CMA and iPSC derived cells. Tumor formation and proliferation of these cells has been confirmed by ki67 staining and veterinarian pathologist assessment.

The tumorigenicity model and assays are now ready to test the final CMA cells product. Suicide gene experiments and tumorigenicity assays are scheduled to start in Q4 2025.

UMCU:

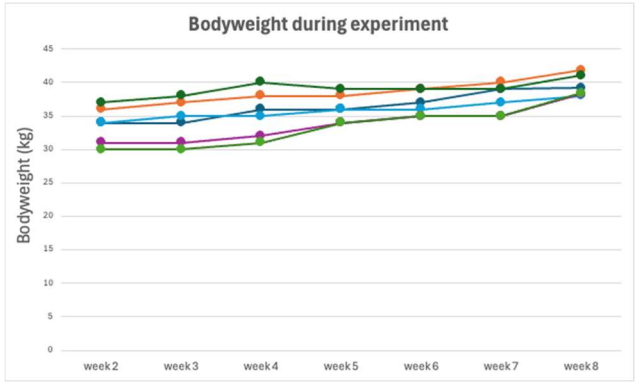
Task 3.6 Asses arrhythmogenicity potential of iPS-CMAs and optimize delivery & retention [M1-M14]

Both increased ethical considerations of authorities as a delayed progress for iPS aggregates production due to GMP compliant logistics of cell products delayed this objective. Due to unforeseen local safety restrictions at the animal facility that made it impossible to conduct the experiment, UMCU was forced to seek a subcontractor to collaboratively perform these experiments. With the consortium's approval, UMCU found a subcontractor for the planned pig experiments, leveraging their specialized expertise and state-of-the-art laboratory infrastructure.

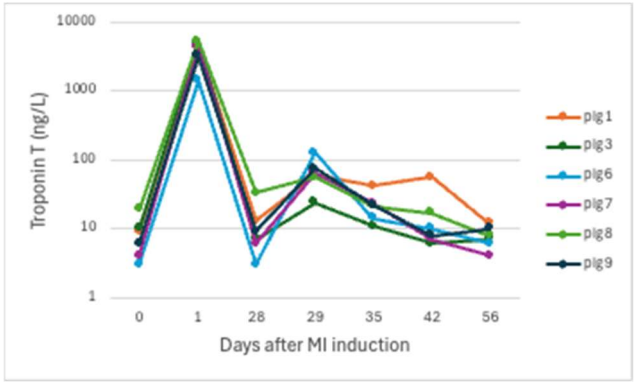
To assess the arrhytmogenic risk after iPSC-CMA transplantation, a total of 7 pigs with cardiac ischemia reperfusion damage were transplanted. Four weeks after induction of 90 minutes cardiac ischemia-reperfusion (I/R) injury, pigs have been injected with saline (n=3), iPSC-CMAs from MHH (n=2) and iPSC-CMAs from CATD (n=2). All these pigs succesfully reached the end of the experiment, except for one control animal that died at week 7 post-IR because of ventricular fibrillation. This indicates that no life-threathing arrhythmias occured in iPSC-CMA injected pigs. Implantable Cardiac monitors (Reveal LINQ, Medtronic) were implanted to closely monitor and record ECG changes upon I/R induction and transplantation of iPSC-CMAs. Cardiac function was assessed with cardiac MRI at one day prior to iPSC-CMA injection and at termination. Bodyweight was measured, which indicated no clear difference between saline and iPSC-CMA injected animals (**Figure 39A**). Troponin T was measured to monitor myocardial damage (**Figure 39B**), which showed an increase one day after I/R injury and iPSC-CMA injection and decreased gradually afterwards. In addition, plasma levels of AST, ALT, gamma-GT, creatinin, and ureum were determined to monitor for liver and kidney damage (**Figure 39C-F**). Four weeks after transplantation, animals were sacrificed and tissues were harvested for further processing. Follow-up analysis, including assessing the presence, frequency and duration of cardiac arrhythmias and assessing the retention of the iPSC-CMA in cardiac tissue are curently being performed. As the extensive data obtained from the Reveal devices requires more time to be analysed in detail than anticipated, UMCU’s milestone could not be reached on 31st of July 2025. However, we foresee not a problem in the completion of this objective before the end of 2025.

Figure 39: Bodyweight and biomarker characteristics

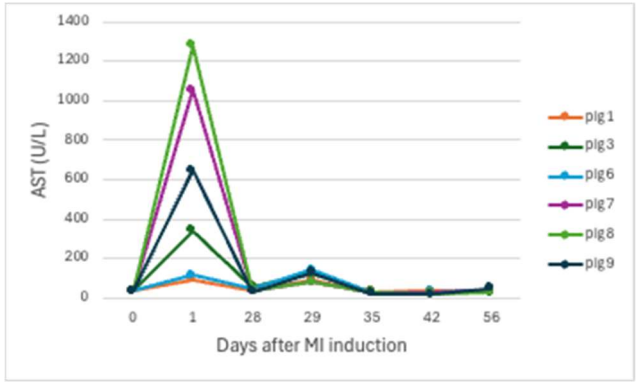
A: Bodyweight



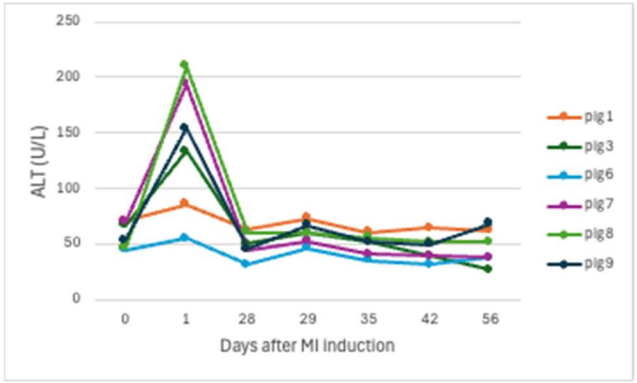
B: Circulating levels of Troponin T



C: Circulating levels of AST

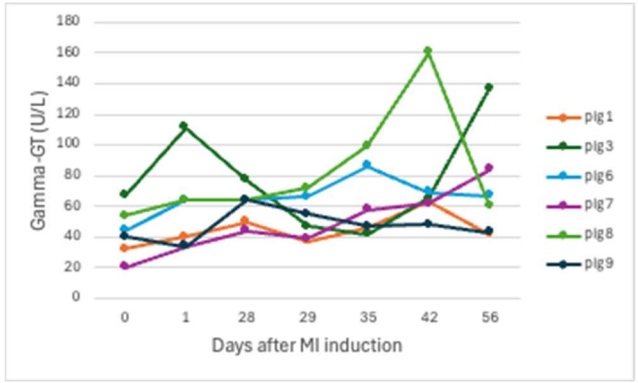


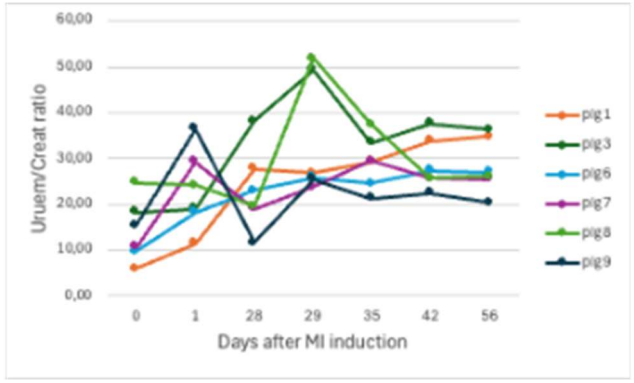
D: Circulating levels of ALT



E: Circulating Gamma-GT levels

F: Ratio of ureum and creatine (U/C ratio) in plasma





A. The bodyweight of all pigs stably increase during the course of the experiment. B. Cardiac troponin T release spikes after I/R induction and is normalized after 28 days. The transplantation procedure results in increased troponin release which gradually declines to normal levels in all pigs. AST, ALT, and Gamma-GT levels were determined as markers for liver damage . We observe spiked AST and ALT levels upon I/R injury, which return to physiological levels in all pigs (C, D). Gamma-GT seemed to slowely increase over time for all pigs with a slightly different trajectory for pig 3 and 8 (E). The ratio of ureum to creatinin in the plasma was determined as a proxy for kidney dysfunction. The levels vary between pigs but mostly show the same pattern. Pigs 3, 8, and 9 show a slightly altered U/C ratio upon the transplant procedure compared to the other pigs.

CATD

Task 3.6 Assess arrhythmogenicity potential of iPS-CMAs and optimise delivery & retention [M1– M14]

Production and Documentation of iPSC-Derived Cardiomyocytes for Pig Injections

CATD generated two independent batches of iPSC-derived cardiomyocyte aggregates (CMAs) according to the established SOP. Both batches originated from the R26_6 iPSC line and were differentiated for 14 days before harvest. The final product was formulated in saline solution and filled into syringes at a concentration of 110 million cells in 1.1 ml, optimized for intramyocardial injection in pigs.
All production steps were documented, and a Certificate of Analysis (CoA) was issued for each batch to ensure compliance with predefined release criteria (**Figure 40**). Both batches fulfilled the specifications, with the exception of the first batch, which required clarification of sterility results. Nevertheless, cells were injected into the myocardium of the pigs.

Certificate of Analysis (CoA)

Product	iPSC-derived cardiomyocyte aggregates (CMAs)
iPSC line of origin	R26_6
Batch designation	Exp170
CMA age at filling	14 days after initiation of differentiation
Description	CMAs in saline solution filled into injection syringes at 110 M cells in 1.1 ml

Release criteria and batch-specific test results for R&D-grade iPSC-CMAs:

Test for	Category	Method	Specification	Result
Sterility	Safety	Ph.Eur. 2.6.27	Negative	Ambig.*
Mycoplasma	Safety	qPCR	Negative	Pass
cTnT expression	Identity / Purity	Flow cytometry	>85% positive	Pass
Aggregate size	Integrity	Microscopy	95% of CMAs with diameter of 50-200 µM	Pass
Viability before transport	Integrity	Viable cell count (NC-200)	>80% viable	Pass
Recovery after transport	Integrity	Viable cell count (NC-200)	>50 M viable cells per ml	Pass
Residual iPSC expression	Impurity	RT-qPCR on iPSC marker gene	>1,000-fold downregulated in CMAs vs. iPSCs	Pass
Contractility	Potency	EHT on myrimager	Spontaneous contractions / force generation	Pass

04/23/2025

Boris Greber, Ph.D.
Head of iPSC Research and Operations
Catalent Cell & Gene Therapy

Certificate of Analysis (CoA)

Product	iPSC-derived cardiomyocyte aggregates (CMAs)
iPSC line of origin	R26_6
Batch designation	Exp171
CMA age at filling	14 days after initiation of differentiation
Description	CMAs in saline solution filled into injection syringes at 110 M cells in 1.1 ml

Release criteria and batch-specific test results for R&D-grade iPSC-CMAs:

Test for	Category	Method	Specification	Result
Sterility	Safety	Ph.Eur. 2.6.27	Negative	Pending
Mycoplasma	Safety	qPCR	Negative	Pass
cTnT expression	Identity / Purity	Flow cytometry	>85% positive	Pass
Aggregate size	Integrity	Microscopy	95% of CMAs with diameter of 50-200 µM	Pass
Viability before transport	Integrity	Viable cell count (NC-200)	>80% viable	Pass
Recovery after transport	Integrity	Viable cell count (NC-200)	>50 M viable cells per ml	Pass
Residual iPSC expression	Impurity	RT-qPCR on iPSC marker gene	>1,000-fold downregulated in CMAs vs. iPSCs	Pass
Contractility	Potency	EHT on myrimager	Spontaneous contractions / force generation	Pending

04/23/2025

Boris Greber, Ph.D.
Head of iPSC Research and Operations
Catalent Cell & Gene Therapy

Figure 40: Certificate of analysis of the iPSC-derived CMs produced by CATD.

Detailed analysis of batch Exp171 confirmed high viability before transplant (97.4%) (**Figure 41A**), a high differentiation efficiency with 98% of cells expressing cTnT (**Figure 41B**), a reduced presence of residual iPSC (**Figure 41C**) and a proper uniform aggregate size (around 125µM) (Figure 37D).

Importantly the cells remained stable after 24h transport in 0.7% NaCl at 4C (94%) (**Figure 41E**) supporting their suitability for pig injection studies.

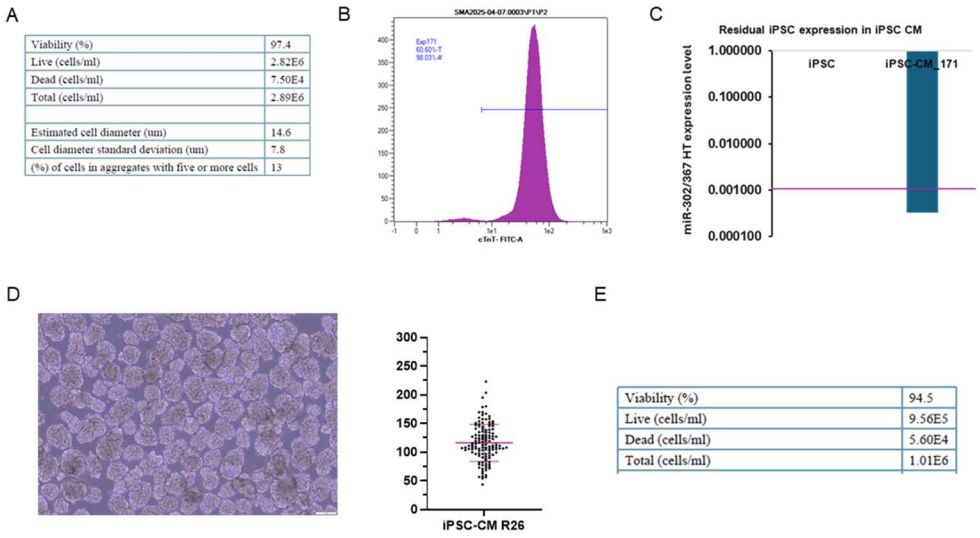


Figure 41: Characterization of iPSC-derived cardiomyocyte aggregates (Batch Exp171). Quality control analyses included cell viability before **(A)** and after transport **(E). B.** flow cytometry for cTnT expression. **C.** RT-qPCR for residual iPSC marker**. D.** microscopy of aggregates and size distribution.

1.2.3 Work Package 4

WP 4: REGULATION EMA; Lead Beneficiary EATRIS

The overall aim of WP4 is to investigate, compile and analyze regulatory requirements for a Clinical Trial Application (CTA), and to adapt the individual dossiers for a select country identified for a first-in-man trial based on the preclinical findings in HEAL

EATRIS

Task 4.1 Draft TTP document based on the SOTA and previous TECHNOBEAT results [M1-M6]

The deliverable established a Development Plan to address project weaknesses and ensure regulatory alignment. Central to this is the Target Product Profile (TPP), drafted within the first 12 months through an iterative consortium-wide process. The TPP functioned as a reference tool to assess project progress, identify gaps, and trigger corrective actions, reviewed annually.

Key activities of this deliverable included:

1 **TPP-driven monitoring and feedback process** covering SOPs for toxicity and carcinogenicity studies (e.g., qPCR assays, INNOSER). These SOPs inform the preclinical components of the IMPD and IB.

2 **Regulatory engagement**:

- Scientific Advice from PEI (D4.2) on clinical trial requirements and analytical tests (next objective below).

3 **Preliminary outcomes**:

1. **Product identity definition**: based on myocardiocyte precursors, antigen panel characterization, dosage range (MHH, Catalent), and manufacturing process parameters. **Manufacturing process updates**: cryopreservation step added for clinical and logistical reasons (ensuring product stability, on-demand availability, and process coordination).

Overall, D4.1 set the foundation for a complete Investigational Medicinal Product Dossier (IMPD) by project end, ensuring progress tracking, regulatory compliance, and structured feedback integration.

Task 4.2 Assessment of the current regulatory background and guidance for the IMP class [M2-M10]

Feedback on protocol discussions with PEI regarding the exploitation of induced pluripotent stem cells (hiPSCs) for clinically applicable heart repair has been reviewed by the consortium. This review included previous scientific advice consultations with PEI covering the reprogramming of human peripheral CD34+ cells into hiPSCs, their isolation and purification, the differentiation of hiPSCs into hiPSC-derived cardiomyocytes (hiPSC-CMs), and the purification and characterization of these differentiated cells.

The meeting minutes and protocols from these consultations have been integrated into the Target Product Profile (TPP) development where appropriate helping to complete a first fdraft of a TPP for this IMP after year 1. Additional gaps identified will now be subject to a follow-up scientific meeting with PEI in late 2025 as described in Task 4.4, including discussions on the specific cell type required for tumorigenicity studies.

A scientific advice process with PEI (D4.2) will be pursued to ensure regulatory compliance, focusing on requirements for clinical trials and the suitability of development plan parameters, especially regarding bioactivity and efficacy analytical testing. Furthermore, protocol assistance will be requested from PEI to discuss clinical development stages, endpoint selection criteria, and the relevance of efficacy parameters.

Task 4.3 Assessment of the preliminary documentation [M4-M10]

In collaboration with the project partners, a Development Plan has been initiated to address the weaknesses identified within the project. Central to this plan is the drafting of the TPP in the first 12 months, informed by the state of the art and prior TECHNOBEAT results. The TPP was populated through an iterative process with all relevant partners and is intended as both a planning and monitoring tool matching actual results with projected targets and triggering corrective actions when necessary (D4.1).

D4.1 also established a monitoring, review, and feedback process for preliminary documentation, including SOPs for toxicity and carcinogenicity studies. For example, a qPCR-based evaluation of carcinogenicity potential has been developed and initiated (INNOSER, Figure 2). These SOPs describe the preclinical studies feeding into the non-clinical and quality sections of the IMPD and IB, drafted on the basis of final product characteristics defined in the TPP and prior TECHNOBEAT data.

Regular team meetings have now defined key objectives to achieve a complete IMPD by the project's end:

1. **Product Identity:** (active principle as myocardiocyte precursors).

- Study of characterization are ongoing for antigen expression. The product identity is planned to be identified by a panel of antigen and the manufacturing process. Additionally, a range of dosage will be defined here by the MHH and Catalent Teams.

- The full manufacturing process is considered as an essential part of the identity for the ATMPs. Comparability between the product prepared at MHH and Catalent will further define the relevant parameters.

2. **Manufacturing Steps:**

   - A cryopreservation step before final formulation has been introduced to improve clinical feasibility (availability on request) and logistical handling (mitigating the limited shelf-life of live cell products requiring tight coordination between production and intervention).

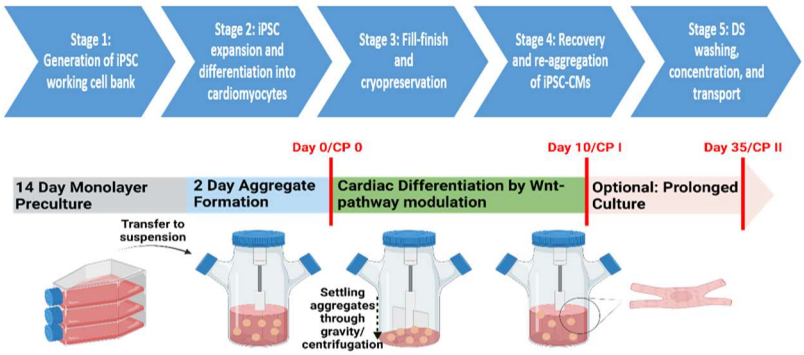


Figure 1- Defining a manufacturing process for an iPSC derived from cryopreserved intermediate.

Figure 42: Defining a manufacturing process for an iPSC derived from cryopreserved intermediate.

Gaps and challenges that cannot be resolved internally by the HEAL consortium will be addressed through additional scientific advice procedures with PEI.

Task 4.4 Preparation of Scientific Advice requests to the national competent authority & EMA [M10-M35]

The task was prioritised during this reporting period and involved finalizing the consortium's document for Scientific Advice with PEI, ensuring completeness of technical content and alignment with regulatory expectations. Final contributions from multiple discussion with the technology developers and the EATRIS regulatory team in this WP included flow cytometry data for product identity, in vitro assay results for PK, and updates on biodistribution plans from UMCU. The aim was to complete the document by early June 2025 which it now is, enabling MHH to apply for the Scientific Advice meeting in preparation for a potential session in early autumn which is being requested.

This objective and work to achieve it during this reporting period consolidates the project's progress in establishing a scalable, GMP-compatible workflow for differentiating iPSCs into cardiomyocytes and formulating cardiomyocyte aggregates (CMAs) for clinical application. Key regulatory questions were prepared on product viability, formulation handling, sterility testing, identity criteria, viral/genetic safety, residual iPSCs, proliferation, and biodistribution. These questions seek agency agreement on the consortium's proposed approaches to quality, safety, and regulatory compliance. The outcome of this task will guide the next stages of the IMPD process, which will now become a central focus of project activities.

Topics to be addressed at the PEI Scientiifc Advice:

Q: Viability at administration

Q: Drug Product formulation/manipulation at the site

Q: Sterility of the drug product

Q: Identity criteria

Q: Safety testing- viral safety and genetic mutations

Q: Safety testing - residual iPSCs

Q: Lack of PK data in vivo

Q: Biodistribution

Task 4.5 Revision of the documents prepared for the CTA [M10-M42]

The consortium has progressed towards the completion of a full Investigational Medicinal Product Dossier (IMPD) by the end of the project, with a structured focus on defining, characterizing, and validating the product while ensuring regulatory compliance. This work has been achieved by routine meetings between technology developers and the EATRIS regulatory team. A central task has been the precise definition of the drug substance, including whether cardiomyocytes should be considered as single cells or aggregates, and specifying critical parameters such as aggregate size, number of cells, and residual single cells. To meet regulatory expectations, practical methods such as imaging and machine-generated size distribution data are being applied. The aggregation and reaggregation procedures, including cryopreservation and recovery steps, are being described in detail within SOPs of the IMPD to guarantee reproducibility. Parallel to this, the product is being characterized post-reaggregation to confirm identity, potency, and purity through assays such as flow cytometry and antigen expression profiling. Validation of cryopreservation and reaggregation ensures consistent outcomes across production runs, safeguarding reliability of the final product.

Responsibilities for drafting sections of the IMPD have been distributed across partners to ensure efficient completion: CATD is documenting the manufacturing process, including facilities, authorizations, and critical steps; MHH is leading characterization data collection and analysis; and other partners are contributing to the biodistribution, PK, and safety testing sections. Preparation for the Scientific Advice meeting with PEI to complement IMPD development is underway as mentioned in point 2 above, focusing on key regulatory questions concerning drug product identity, viability, sterility, safety (including residual iPSCs and mutations), and formulation/manipulation at clinical sites. This feedback will be crucial to align the final IMPD with regulatory requirements. A clear timeline and division of responsibilities are now in place to project end, with MHH leading the formal advice request. These coordinated activities ensure that the consortium builds a comprehensive, regulator-ready IMPD, supporting the transition from development to clinical application. The recently approved no-cost extension to the project will allow the time required to finalize the IMPD more effectively and to completion.

1.2.3. Work Package 5

WP 5: Cost Effectiveness HTA, Dissemination and Exploitation; Lead Beneficiary CATD

The overall aim of WP5 is to adjust the methodology to meet requirements for future commercial exploitation, with an emphasis on the IP landscape and cost effectiveness of the approach.

EATRIS:

Task 5.1 Conducting of FTO analysis and ongoing monitoring of IP situation [M01-M42]

Building on previous IP analysis, EATRIS conducted an FTO investigation of the proposed methodology. During the project this process continuously monitor developments in the patent landscape (new applications, approvals, withdrawals, etc.) and provides regular reports to partners

HEAL consortium members have continuously identified IP which can potentially be used in the project. They have also flagged competing or overlapping IP held by others which should be evaluated to determine how to optimally proceed with technology development within the project. We have evaluated both of these categories, as seen later in the document, and added a third category: potentially competing IP as identified by novel search of patent databases. All IP deemed competing is identified by numbers preceded by symbol '➢', and each Patent Family is organized in catalog table which can provide easy navigation throughout the project, to be updated as we (and others) file new IP.

This is brief snapshot of the current IP landscape within the project:

1. IP intended as background within the project, should be free, clear, unencumbered, and remain active within the patent territories where it has been filed and established. There are several Patent Families which are claimed for use in the project, which are either under uncertain ownership, or where the patent has lapsed. These are noted in Patent Family details.

2. Presumably of particular project interest are the European granted patent positions. These were noted as lacking or at least currently incomplete in some of the applications. If there is a specific commercialization geography intended by the project consortium, specifically within or beyond Europe, the available IP coverage should support that as well as possible.

3. Some identified competing IP is in similar condition; patents which seemingly hinder the project's development, are incomplete from a geographic perspective, or have lapsed in some cases. It is therefore useful to examine the current legal status which was done to the extent possible.

4. For competing IP, granted patents pose more challenge because they permit the holder to prosecute infringement, which is ultimate benefit of patents. Granted patents which have lapsed, or pending applications do not present a direct infringement concern, but may still act as prior art if their claims overlap with the development methodology for the consortium.

The document which is continuously updated is intended to provide a baseline sampling of the IP in the field, and to provide a snapshot of their patent positions relative to that of the project participants in development and delivery of cell therapeutics. It attempts to convey the idea that IP is fundamental to therapeutic development, and cannot be separated from the science if the ultimate objective is to improve the standard of care through Advanced Therapies and Medicinal Products (ATMP).

This is a both a self-contained as well as a living document. It is intended to contain all the relevant IP information, with links to necessary details where identified. It can be updated as the project proceeds, on a frequency to be determined, and should keep a full inventory of all the relevant Patent Families as seen in the table. EATRIS advises having a periodic evaluation of the IP landscape in order to continuously renew the insight into how IP can shape the therapeutic development process. Our role within the project is to support these evaluations, so if there is anything further evaluation needed, please route these requests through EATRIS and we can deliver evaluations accordingly.

Structure of the document

1. Existing IP (prior art) from the patent numbers identified in the proposal:

    a) Proprietary background IP from inventors/institutes within the HEAL consortium, general information, status, claims, and assessment of claims and position

    b) Competing background IP as identified by the HEAL consortium, general information, status, claims, and assessment of claims and position

c) Competing background IP as identified by novel search, general information, status, claims, and assessment of claims and position

2. Competitive intelligence on developers of similar cell therapies, as it relates to IP (distributed throughout document)

3. Future IP directions (to be added later)

4. Appendices of related information

This document has been continuoulsy updated as results are generated, and with the changing FTO landscape.

High level overview of existing IP - a limited Freedom-to-Operate (FTO) scan

Patent applications - and ultimately granted patents - provide core IP that may be applicable to therapeutic development, by providing commercial exclusivity on the use of specific technologies to prohibit competitors from selling similar technologies. Importantly, this reduces competitive risk - and increases likelihood of obtaining the level of investment required to bring a cell therapy to market. But it is important to state upfront that it does not guarantee commercial success. Preventing others from infringing, and being commercially successful, and separate but equally important aspects.

A cell therapy is simultaneously a medical product which can be produced, evaluated, tested, and delivered, as well as a set of pre-defined manufacturing processes. Cell therapies grow in complexity with respect to IP requirements needed to establish a commercial position. The final product and process may be a collection of proprietary IP as well as IP which is in-licensed from other owners. For example, Novartis does not own the Ientiviral transfection IP used in the commercial version of Kymriah (their CAR-T product), but has negotiated a license on its use, or relies upon a manufacturing partner who has a license. Similarly in the field of allogeneic as well as autologous cell therapies, it is expected that each medicinal product, if eventually approved, will be a collection of proprietary and licensed IP.

IP may cover parameters of a finished, manufactured product - biological characteristics which describe the composition of the product - as well as processes and methodology to manufacture the product. For this reason, the complexity of cell therapy IP is expected to grow as firms specialize in developing certain portions of the finished product. Future cell therapies are likely to be integrated IP which is owned by the developer, as well as external technologies which are either in-licensed or subcontracted.

Patent search was conducted using methodology-based search terms. It is advisable that any patents identified are examined further, in order to understand the details. The Patent Lens website (lens.org) can be searched separately by novel terms, or by patent application numbers. It is important to note that patent applications are not public for 18 months after filing date. Therefore, the patent literature lags the scientific literature, by up to 1.5 years, assuming that patent applications are often filed just before publications on the underlying invention become publicly visible.

Index of related IP

Patent Families 1-6: Proprietary	Patent Family identified within proposal on p.30 ('The project will exploit the following patents held by the project partners')
Patent Families 7-13: FTO-Proposal	Patent Family identified within proposal on p.30 (' We are aware of some potentially overlapping patents...')
Patent Families 14-25: FTO-Novel Search (Dec 2022)	Identified via novel search at baseline

Patent Families 26-38 Identified via Boris Greber, Catalent (added June 2023)

Patent Families 29-36 Identified via novel search (June 2023)

Patent Families 37-XX Identified via future novel searches (September 2023)

Patent Family: category	**Patent number**	**Title**
Patent Family 1: Proprietary	9422521	Differentiation of pluripotent stem cells with a kinase inhibitor or PGI2
Patent Family 2: Proprietary	US9404085B2	Direct differentiation of cardiomyocytes from embryonic stem cells
Patent Family 3: Proprietary	EP18161619.4	Process for producing cardiac organoids
Patent Family 4: Proprietary	PCT/EP2018/061574	Stem-cell derived myeloid cells, generation and use thereof
Patent Family 5: Proprietary	US9,458,425	Differentiated Human Embryoid Cells and A Method for Producing them
Patent Family 6: Proprietary	PCT/IL2013/050441	Identification and characterisation of highly selective inhibitors of hESCs and IPSCs by high-throughput screening
Patent Family 7: FTO-Proposal	➢ US9663764B2	Generation of cardiomyocytes from human pluripotent stem cells
Patent Family 8: FTO-Proposal	➢ US9765299B2	Chemically defined albumin-free conditions for cardiomyocyte differentiation of human pluripotent stem cells
Patent Family 9: FTO-Proposal	➢ US9234176B2	Chemically defined production of cardiomyocytes from pluripotent stem cells
Patent Family 10: FTO-Proposal	➢ US9404085B2	Direct differentiation of cardiomyocytes from embryonic stem cells
Patent Family 11: FTO-Proposal	➢ US9422521B2	Differentiation of pluripotent stem cells with a kinase inhibitor or PGI2
Patent Family 12: FTO-Proposal	➢ US9458425B1	Differentiated human embryoid cells and a method for producing them
Patent Family 13: FTO-Proposal	➢ WO2013175474A2	Selective inhibitors of undifferentiated cells
Patent Family 14: FTO-Novel Search	➢ 20200263139	Methods for Directed Differentiation of Pluripotent Stem Cells to Hla Homozygous Immune Cells

Patent Family 15: FTO-Novel Search	➢ US10604739B2	Cardiomyocyte production
Patent Family 16: FTO-Novel Search	➢ 10260048	Generation of induced pluripotent stem cells from small volumes of peripheral blood
Patent Family 17: FTO-Novel Search	➢ US20210002615A1	Reagents and Methods With Wnt Agonists and Bioactive Lipids for Generating and Expanding Cardiomyocytes
Patent Family 18: FTO-Novel Search	➢ US20200297775A1	Enhanced Direct Cardiac Reprogramming
Patent Family 19: FTO-Novel Search	➢ WO 2013063305A2	Directed Cardiomyocyte Differentiation Of Stem Cells
Patent Family 20: FTO-Novel Search	➢ WO2021/173449 A1	Orthogonal Safety Switches to Eliminate Genetically Engineered Cells
Patent Family 21: FTO-Novel Search	➢ US2017/0044500 A1	Application Of Induced Pluripotent Stem Cells To Generate Adoptive Cell Therapy Products
Patent Family 22: FTO-Novel Search	➢ US 2022/0267801 A1	Elimination of Proliferating Cells From Stem Cell-Derived Grafts
Patent Family 23: FTO-Novel Search	➢ WO 2018/096343 A1	Controllable Transcription
Patent Family 24: FTO-Novel Search	➢ US 2021/0171903 A1	Universal Donor Stem Cells and Related Methods
Patent Family 25: FTO-Novel Search	➢ WO 2019/113169 A2	HLA Homozygous Induced Pluripotent Stem Cell (iPSC) Libraries
Patent Family 26: FTO-Novel Search, Catalent identified	➢ US9279103 B2	Simplified basic media for human pluripotent cell culture
Patent Family 27: FTO-Novel Search, Catalent identified	➢ EP3268480B1	Tools and Methods for Using Cell Division Loci to Control Proliferation of Cells
Patent Family 28: FTO-Novel Search, Catalent identified	➢ US8415155B2	Cardiomyocyte production

Patent Family 29: FTO-Novel Search	➢ WO 2023/104813 A1	Methods Of Cardiomyocyte Production
Patent Family 30: FTO-Novel Search	➢ WO 2023/286772 A1	Cardiomyocyte Production Method
Patent Family 31: FTO-Novel Search	➢ WO 2022/019768 A1	Closed Manufacturing Processes for Large Scale Manufacturing of Pluripotent Stem Cell Derived Cells
Patent Family 32: FTO-Novel Search	➢ US 2022/0088143 A1	Angiogenic Conditioning to Enhance Cardiac Cellular Reprogramming of Fibroblasts of the Infarcted Myocardium
Patent Family 33: FTO-Novel Search	➢ US 2022/0233600 A1	Cell Population Comprising Mesenchymal Cells, Pharmaceutical Composition Comprising the Same, and Method for Producing the Same
Patent Family 34: FTO-Novel Search	➢ EP 4071238 A1	Cardiomyocyte Aggregate Production Technique Improving Survival Rate Under Cryopreservation And Hypoxic Conditions
Patent Family 35: FTO-Novel Search	➢ EP 4123016 A1	Method For Purifying Cardiomyocytes
Patent Family 36: FTO-Novel Search	➢ WO 2022/221051 A1	Cardiomyocytes And Compositions And Methods For Producing The Same

A more comprehensive overview of each Patent family is available in the first version of the Report on IP/FTO situation (D5.1).

Task 5.2 Implementation of freedom-to-operate strategy [M04-M06]

The task was accomplished in the first reporting period.

UOX:

Task 5.3 Societal and ethical aspects of iPSC and iPSC-CMAs [M6-M42]

A: Conduct a legal and regulatory analysis of the data protection & privacy elements of the cell therapy production process

This task has been completed, resulting in deliverable 5.4a “D.5.4a “Regulatory analysis of data protection and privacy requirements” being completed and submitted to the consortium management on 31/07/2025

The Deliverable responds to Task 5.3.1 “Legal and regulatory analysis of the data protection elements of the cell therapy production process related to HLAh iPSC-derived CMs for cardiac cell therapy”. The submitted report comprises an extensive review of data protection and pri8vacy requirements relevant to allogeneic human induced pluripotent stem cells utilised as the basis for an ATMP and contains the following elements i) overview of how and why the HEAL cell therapy is subject to privacy and data protection considerations under European law, ii) review of those

requirements, outlining measures to ensure adequate and appropriate protection for any and all personal data pertaining to the original cell line donor, including genetic data, that is likely to be generated in the course of the development of the HEAL cell therapy. The Deliverable includes considerations relevant to European law on privacy, data protection and also includes discussion, where relevant, of information security and cybersecurity issues applicable to cell therapy research and development. The final part of the Deliverable reviews a list of technical processes likely to be utilized in the course of developing an iPSC-derived cardiomyocyte cell therapy product and assesses what data is collected or generated in each step and what potential each data type has with respect to the identifiability of the original cell line donor.

B: Qualitative assessment of Institutional Readiness

This task has been completed, resulting in a full draft of Deliverable 5.4b "'Regulatory analysis and assessment of institutional readiness" being submitted to consortium management and made available for review on the HEAL secure online data storage space as of 31/07/2025.

This report presents a qualitative investigation into the institutional readiness of European hospitals to adopt the HEAL cell therapy—a novel HLA-homozygous human induced pluripotent stem cell-derived cardiomyocyte aggregate (hiPSC-CMA) product designed for the treatment of ischaemic cardiomyopathy and myocardial infarction-triggered heart failure.

The research is grounded in the Institutional Readiness (IR) framework, which assesses how well-equipped clinical institutions are to integrate disruptive health technologies such as cell and gene therapies. This study applied the IR framework to two large university-affiliated hospitals in Germany and the Netherlands. Through semi-structured interviews with cardiologists, cardiothoracic surgeons, and hospital pharmacy representatives, the study captured professional insights on infrastructural, clinical, organisational, and procedural requirements for future adoption of the HEAL therapy.

Key Findings

· Clinical Attitudes and Familiarity: Awareness of cell therapy was high across both sites, but the legacy of failed adult stem cell trials in cardiology continues to influence clinician scepticism. Optimism persists around iPSC-derived therapies, though confidence hinges on robust clinical data and regulatory approval.

· Evaluation Criteria: Respondents prioritised a combination of hard endpoints (e.g. mortality, readmission rates, LVEF improvements) and soft outcomes (e.g. patient quality of life, exercise tolerance) for assessing HEAL's clinical impact. Imaging techniques and long-term monitoring were noted as essential.

· Procedural and Technical Readiness: Both hospitals reported experience with complex interventions and ATMP handling (e.g. CAR-T), and infrastructure such as catheter labs, imaging, and cell facilities was already in place. However, delivery route, immune suppression protocols, and arrhythmia monitoring emerged as key concerns requiring careful clinical validation.

· Training and Collaboration: There was strong institutional capacity for cross-departmental collaboration, supported by previous experience with trials and regenerative medicine initiatives. Nonetheless, clinicians highlighted the need for targeted training and proctoring once the therapy becomes trial-ready.

· Patient Involvement: Both sites had processes in place for involving patient groups in trial design and consent review. While PROMs were familiar to most clinicians, knowledge of cardiac-specific measures was limited, underscoring the importance of expert guidance on QoL tool selection.

· Financial and Strategic Planning: One site (NL) demonstrated advanced costing and sustainability planning capacities, while the other (DE) appeared more reliant on centralised HTA processes. Both had experience running clinical trials, but institutional mechanisms for routine cost evaluation varied.

Institutional readiness to trial and adopt HEAL appears promising at both sites, particularly with respect to infrastructure, experience with ATMPs, and clinician openness to innovation. Nonetheless, the complexity of cell therapy delivery, the need for immune suppression, and questions around cost-effectiveness demand careful consideration in future trial design and health system planning. This report offers a foundation for HEAL developers and policymakers to refine clinical strategies, training plans, and implementation pathways as the therapy progresses toward human trials. The study's findings are limited by its focus on two clinical sites and the absence of real-world experience with the HEAL product, which is still in preclinical development. Insights are anticipatory rather than experiential, and while expert-based, cannot predict future adoption with certainty. Moreover, the limited range of staff roles interviewed (notably, the absence of hospital managers at both sites) constrains the organisational breadth of the assessment.

CATD

Task 5.4 Optimisation of methodology [M1 – M18]

The previously established GMP-compliant processes at CATD supported the manufacturing of iPSC banks with a maximum size of 71 cryovials. With the increasing demand for larger iPSC banks, particularly for the scope of the current project, this scale was no longer sufficient. The objective of the new process was to demonstrate the successful establishment of iPSC R26 banks of up to 360 cryovials, each containing 2 × 10^6 iPSCs. The revised process builds upon a cultivation strategy already established and routinely applied in the R&D department, where iPSCs are maintained as single cells prior to directed cardiac differentiation. This harmonization ensures consistency between R&D and GMP manufacturing processes and facilitates a smoother transfer of iPSCs into differentiation workflows and gene editing. The most critical change compared to the earlier process is the introduction of single-cell passaging and cryopreservation instead of working with cell clusters. To enable robust single-cell handling, three new GMP-grade reagents were implemented (**Figure 43**):

- iMatrix-511 MG as a defined recombinant culture matrix,
- Accutase GMP for enzymatic dissociation and single-cell preparation,
- Rho-kinase inhibitor Y-27632 GMP, which prevents apoptosis during single-cell culture and thereby increases cell viability.

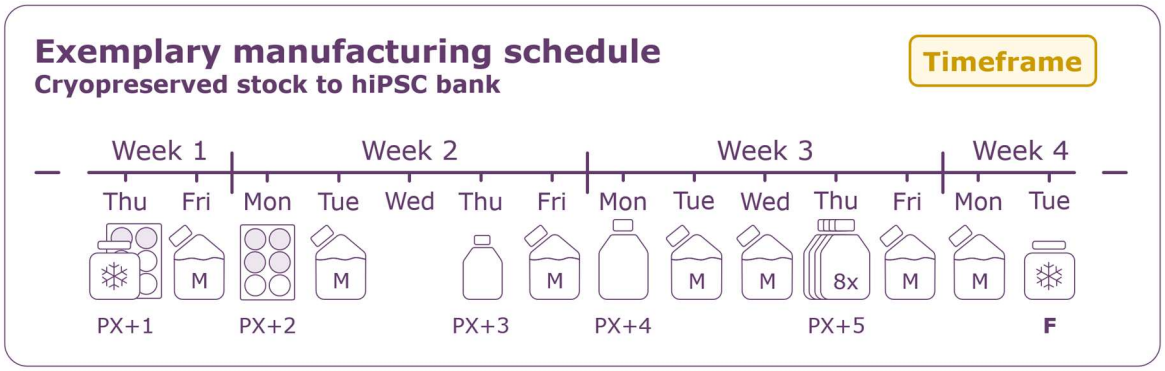


Figure 43: Timeline overview of the new process for GMP-compliant production of up to 360 cryotubes per cell bank.

The adoption of single-cell passaging not only allows the generation of larger and more homogeneous iPSC banks, but also provides a significant advantage for the GMP-compliant production of iPSC-derived cardiomyocytes (iPSC-CM). Since iPSC-CM differentiation protocols typically rely on single-cell seeding to achieve reproducibility and consistency, aligning the banking strategy with this approach improves process robustness, reduces variability, and facilitates a more controlled manufacturing workflow for clinical and commercial applications.

Task 5.5 Distribution logistics [M24-M48]

Work is ongoing to establish optimal distribution logistics for cell handling. Data generated from current studies at CATD and UDUS are being used to determine whether cells should be cryopreserved or transported alive at 4 °C. Transport at 4 °C has already been shown to be effective for up to 24 h, as demonstrated in previous animal studies. Extended transport times are currently under investigation. Once the most suitable approach is defined, either 4 °C transport or cryopreserved shipment will be verified based on the upcoming lockdown runs.

1.2.3 Work Package 6

WP 6: Management and Dissemination; Lead Beneficiary MHH (and all partners)

The overall aim of WP6 is to ensure efficient delivery of the administrative, legal and financial aspects of the project.

MHH (and all partners)

Task 6.1 Coordination & Communication [M01-M42]

During this reporting period, one General Assembly meeting was held in February 2024. Originally planned to take place in Israel, the 3rd HEAL General Assembly Meeting had to be canceled again due to the ongoing political situation in the region and was subsequently relocated to partner UMCU in Utrecht, Netherlands. All partner Principal Investigators attended, with the meeting conducted in a hybrid format to allow staff from partner institutions to participate remotely. At the meeting, partners provided updates on their respective work packages and agreed on next steps. The discussions focused on the large animal experiments and the 12-month non-monetary project extension. An external speaker, Prof. Dr. Stefan Janssens from the Department of Cardiovascular Sciences at the University of Leuven, presented on “Innovation in Heart Failure Care: Renewed Focus on Translational Research.” His talk was highly relevant to the consortium, as he is also the lead PI subcontracted by UMCU for large animal experiments within the project.

The next General Assembly is planned for April 2026 in Vienna, Austria at the new partner SERO.

The meeting structure established during the first reporting period, combining in-person and online sessions, continued to be followed. Four quarterly online meetings and one in-person meeting were held this period. Meeting minutes are documented and securely stored on the project’s SharePoint platform (Academic Cloud), accessible to all partners. Smaller focus group meetings are held spontaneously between partners as needed.

In addition, a HEAL symposium was successfully organized in October 2024 during the German Stem Cell Network (GSCN) Annual Conference. This major scientific event gathers researchers, clinicians, industry experts, and students from Germany and internationally to discuss the latest advancements in stem cell biology and regenerative medicine, featuring presentations, workshops, poster sessions, and networking opportunities. The symposium highlighted key HEAL project achievements and fostered broader scientific exchange.

<u>Project Management and Documentation</u>

All project management documentation — including the data protection plan, dissemination plan, meeting minutes, templates, deliverable and milestone charts, and an overview Gantt chart — is securely maintained on the project’s SharePoint site, ensuring easy access for all partners.

The project coordinator closely monitors the timely delivery of milestones and deliverables through the Project Continuous Platform. Any deviations are thoroughly discussed within the consortium,

with adjustments made in line with the Risk Management Plan and approved by consortium members, ensuring proactive and transparent project management.

Laboratory Exchanges and Workshops

Research visits and small workshops are actively conducted among partners to share methodologies and introduce new protocols. Communication across the consortium remains exceptionally strong and effective throughout all project activities.

Recent visits comprised:

- Nicole Maeding (PMU/ SERO) and Soraia Martins (CATD) visited MHH to learn and perform immunophenotypic staining.
- Online workshops MHH and CATD to learn on protocol and methodologies
- Carlos Hernandez (MHH) vistied UMCU (subcontractor of UMCU) for large animal experiments

Project Extension

During this reporting period, a 12-month non-monetary extension was granted, enabling the consortium to pursue its research objectives more effectively within a manageable timeline, while closely monitoring progress in accordance with the established risk management plan.

Reason for request of extension:

Several delays have impacted on the progression across the consortium due to the complex interdependence of tasks between multiple partners. The following key challenges have contributed to the need for the requested project extension:

- Lengthy Material Transfer Agreement (MTA) processes significantly delayed the initiation and progression of key research activities.
- An extensive IP landscape and supply chain issues (particularly in Israel and other regions) caused substantial delays in establishing and functional testing of an IP-compliant hPSC culture medium and a cardiac differentiation medium, slowing down bioprocess development, optimization and upscaling.
- Extended time requirement for establishing the cultivation, analysis and differentiation of a common iPSC line across the consortium (including delayed adaptation to modified culture media) delayed progress bioreactor-based process development, upscaling and validation runs.
- Post-COVID supply chain disruptions caused delays in Deep Immune-Phenotyping Panel antibody availability; delay in cell sample availability for Immune-Phenotyping.
- Extensive experimental- and IP-related challenges in establishing a viable cryopreservation strategy for iPSC-cardiomyocyte aggregates slowed down establishing a clinically- and regulatory-viable cell product delivery strategy compliant with the clinical translation.
- Delays in hiPSC culture and differentiation protocol establishment delayed sample availability for genetic analysis requiring timeline adjustments.
- Extended discussions on rodent model selection and the delay in cell sample availability delayed safety/ teratogenicity studies.
- Substantially delayed regulatory approvals for large animal experiments (pig model) for myocardial infarction and transmural cell injection; ultimately resulting into assigning a subcontractor for the large animal experiments by Partner UMCU) and challenges in the production of cell samples for testing ,delayed the initiation and progress in functional and safety testing of the cell product.
- Prolonged discussions on TTP and IMPD due to delays listed above and evolving requirements delayed the completion of these documents and engagement with regulatory authorities (EMA/PEI).

Change of Repoting Periods:

In parallel of the consortium's granted request for a project extension an adjustment to the associated reporting periods was also granted. While the timeline for the second progress report remained unchanged, the deadline for the third progress report / final report was changed to 18 month instead of 6 month.

Respective Deliverabls and Milestones were granted an extension of in general 12 month.

- Original Deadline for the 3rd Progress Report/ Final Report: 28.02.2026
- Granted New Deadline for the 3rd Progress Report/ Final Report: 28.02.2027

Annex 1 and 2 was changed accordingly to the granted extension of the project.

Communication with the European Commission

Communication between the coordinating institution and the European Commission has been smooth and efficient. The coordinating institution, acting as the consortium's spokesperson, maintains regular contact with the assigned EU project officer to facilitate smooth project implementation.

Task 6.2 Dissemination & Communication [M01-M42]

During the second reporting period, the project website functioned as the main platform for disseminating information about HEAL. Designed to engage a wide audience, the site features multimedia content and comprehensive pages detailing the partnership, project concept, objectives, and activities.

The website is fully integrated with HEAL's social media channels to enhance outreach. A dedicated partner section offers direct access to key resources such as the project's SharePoint site, Academic Cloud, and Zenodo repository, enabling seamless and efficient information sharing among consortium members.

Website KPI[1]:

Site Visits:	950
Unique Visitors:	445 (an increase of 35%)
Engagement Rate:	29% (a decrease of 10% in engagement activity)

Traffic Sources:

Search Engines: 15%

Direct Traffic: 79%

Referral Traffic: 7%

[1] Website statistics from September 2024- September 2025

The website is regularly updated, and ongoing measures are being implemented to enhance usability and improve the relevance of content.

Social Media Presence:

The primary social media channel for the HEAL project is LinkedIn, where project updates, news, and information from consortium partners or other relevant sources are regularly shared. LinkedIn provides an effective platform to reach not only a professional audience and foster engagement within the scientific and healthcare communities, also to engage a broader audience outside the scientifical community.

Originally, the project also maintained an account on the platform formerly known as Twitter, now X. The HEAL consortium decided not to feed X account any longer. HEAL consortium doesn't want to support the current direction and see its core values apart from X's current content. Nevertheless, X account keeps existing and is not deleted at this stage of the project.

LinkedIn is in regards of KPI the strongest used social media platform.

The YouTube Channel is currently under construction to integrated it better within the project website, this intermediate unavailability should be solved latest during the first part of the third reporting period.

Currently, the consortium is exploring alternative social media platforms that could serve as effective channels for outreach and engagement. While a new platform has not yet been selected, the team is actively evaluating options to complement LinkedIn and ensure broad and impactful dissemination of project outcomes.

Social Media KPI:

LinkedIn Connections: 73

X Follower: 30

YouTube Follower: 20

Summary KPI:

Overall, the project's visibility is steadily increasing, as reflected in the growing number of connections and visitors to its communication platforms, including the website and social media channels. However, due to the inherently complex nature of the project's content, a certain level of scientific expertise is required for full comprehension. This explains the generally moderate growth in KPIs, as the project currently primarily appeals to a specialized and relatively small audience of scientific professionals in the field.

Nonetheless, the consortium remains committed to engaging a broader public audience whenever possible.

Newsletter/ Podcast:

Newsletter CATD: Cardiac Differentiation of iPSCs | Catalent Cell Therapy CDMO

EC: HLA-homozygous iPSC-cardiomyocytE Aggregate manufacturing technoLogies for allogenic cell therapy to the heart | HEAL | Project | Fact Sheet | HORIZON | CORDIS | European Commission

ISSCR: Member Spotlight: Robert Zweigerdt, PhD — International Society for Stem Cell Research

Newsletter INNO: InnoSer completes a major milestone in the HEAL project with validated tumorigenicity assay - InnoSer

Podcast by MHH: Stem Cell Podcast: Heart-Forming Organoids featuring Dr. Robert Zweigerdt Ep. 289: "Heart-Forming Organoids" Featuring Dr. Robert Zweigerdt - The Stem Cell Podcast

Podcast by UDUS: ImmunoChat with Nora Balzer “The future of Heart Repair: 3D Cell Models and Organoids” by Fabienne Becker ImmunoChat

Social Media posts (shared, reposted by HEAL LinkedIN):

- by CATD
- by UMCU
- by GSCN (promoting GSCN HEAL Symposium)
- by ISSCR (promoting Robert Zweigerdt/ MHH member speech and conference)
- by MHH
- by Joost Sluijter/ UMCU
- by INNO

Scientific Publications:

24, since the start of the project.

Dissemination and Exploitations:

The HEAL dissemination and exploitation plan was followed closely during this reporting period, with no major amendments. As in the first reporting period, HEAL partners remained highly active in promoting the project’s intermediate outcomes both within and beyond the scientific community.

In total, the consortium participated in more than 5 international conferences. Notably, the ISSCR held in Hamburg, Germany, in 2024 featured an informal HEAL side meeting, followed later in the year by the HEAL symposium at the German Stem Cell Network (GSCN) conference.

During the HEAL symposium at the GSCN on 26th September 2024 in Jena, Germany, four young investigators of the HEAL project presented their research to a broad international scientific audience. Complementing these presentations, an invited guest speaker, Prof. Eldad Tzahor from the Weizmann Institute of Science, Israel, delivered a keynote talk titled “Advancing cardiac therapeutics from animals to patients.”

The presentations included:

“Lineage-specific cancer-related mutations in human pluripotent and adult stem cells”- Jonathan Jung, HUJI

“HLA-homozygous GMP iPSCs show efficient cardiac induction on a new differentiation platform featuring scalability and enhanced robustness” -Soraia Martins, CATD

“hPSC-cardiomyocyte production: standardization and protein-free upscaling to 2000 mL”- Nils Kriedemann, MHH

“Optimization of cardiomyocyte cryopreservation and quality control aspects for potency and residual iPSC contaminations”- Fabienne Becker, UDUS

More details on the event can be found here: https://gscn-conferences.org/previous-gscn-conferences/gscn-conference-2024/program-speakers-2024

Figure 44: Presetations during GSCN conference 2024 – Robert Zweigerdt (MHH), Jonathan Jung (HUJI), Soraia Martins (CATD), Nils Kriedemann (MHH), Fabienne Becker (UDUS), Eldat Tzahor (keynote speaker, Weizmann Institute of Science, Israel) Foto Credit: Gaby Froriep

Project communication activities were primarily promoted through social media channels, the project website, newsletters, and press releases, enhancing visibility and engagement among diverse audiences.

Task 6.3: Progress monitoring [M01-M42]

Project progress is closely monitored by the coordinator, who proactively contacts partners whenever deviations are identified. These issues are subsequently discussed in consortium meetings to ensure transparency and keep all partners fully informed of the project's status.

During this reporting period, a non-monetary extension of the project was granted. Partners reported early deviations to the coordinating institution, and the European Commission (EC) project officer was promptly notified. Following the consortium's decision to submit an amendment request for this extension, the project officer provided strong support throughout the process.

Progress monitoring takes place through a range of consortium meetings, including General Assembly sessions, quarterly online meetings, and focused working group discussions addressing specific project tasks. For urgent matters, additional direct meetings are arranged as necessary.

All project milestones and deliverables are reported quarterly to all partners. Any deviations from the planned schedule are discussed collectively by the consortium. Minor deviations are generally managed directly between the involved partners, with continuous oversight by the coordinating institution.

To facilitate effective project management, the Risk Management Plan is actively employed, ensuring all partners share a common understanding of potential risks and mitigation strategies.

Moreover, the coordinating institution maintains regular and open communication with the EC project officer, adhering closely to their guidance to ensure smooth and successful project implementation.

Task 6.4: Risk management [M01-M42]

During the second reporting period, the HEAL Risk Management Plan played a crucial role in addressing and resolving deviations within the project. The most significant issue encountered was

the delay in achieving key milestones and deliverables, which represented ongoing deviations from the project's objectives.

Recognizing these challenges, the consortium, in close collaboration with the EC project officer, quickly identified that a project extension would be essential to ensure the successful completion of all goals. Consequently, a 12-month non-monetary extension was granted, providing the consortium with the necessary time to realign efforts and advance the project effectively.

Risk Identification and Monitoring

Throughout the implementation of the HEAL project, a comprehensive management process is in place to identify, monitor, and mitigate technical, management, and other risks that could potentially impact progress toward project objectives. The Risk Management Plan, established early in the project and disseminated to all partners, serves as a key tool guiding this process.

Potential risks include:

- Unexpected scientific findings or technical challenges
- Regulatory uncertainties and approval processes
- Communication or cooperation breakdowns among partners
- Failure to meet deliverables due to budget limitations or feasibility constraints
- Consortium partner withdrawals or operational disruptions
- Roles and Responsibilities

All partners bear the responsibility to promptly report any issues, including risks, delays, or changes in deliverable timelines and budget allocations, to their respective Work Package (WP) leaders and to the project coordinator. Effective communication ensures that potential problems are addressed early.

When delays or obstacles arise, the coordinator will organize task forces to develop and implement necessary corrective actions. If immediate resolution is not possible, the issue will be escalated for broader consortium discussion and action.

Continuous Adaptation of the Risk Management Plan

The Risk Management Plan remains a living document, continuously reviewed and updated as needed to reflect the evolving circumstances of the project. This adaptive approach ensures that risk mitigation strategies remain aligned with project needs and support the achievement of its objectives.

Task 6.5: Reporting and reviews [M01-M42]

The coordinating institution continuously reviews the progress of deliverables and milestones, ensuring that the project stays on track. During all consortium meetings, a comprehensive overview of the current status is presented, keeping all partners well-informed and aligned. In cases of deviations or delays, the coordinator maintains proactive and open communication with the affected partner and keeps the EC project officer closely updated. A real-time, up-to-date overview of milestones and deliverables is consistently maintained and accessible on the project's SharePoint platform, fostering transparency and collaborative oversight.

During the preparation of the first progress report, the consortium demonstrated excellent coordination and collaboration in consolidating content and finalizing contributions. This strong teamwork also played a crucial role in managing the coordination around the project's extension request, ensuring a smooth process.

For the 2nd Progress Report, the coordinator is responsible for gathering and consolidating both financial and technical inputs from all partners. The reporting on the Continuous Reporting Platform (Part A) was successfully completed by the end of August. Subsequently, financial statements have been requested and are being collected from partners, while the Technical Part B is currently undergoing finalization through collaborative efforts by all partners.

Once all components are finalized, the coordinator will upload the complete 2nd Progress Report to the Project Portal and ensure that it is submitted to the EU within the designated deadline.

In parallel to the report preparation, the consortium is actively preparing for the Progress Report Review Meeting scheduled for 19th November 2025. To ensure a smooth and comprehensive presentation of all work packages, preparatory meetings will be convened earlier in October 2025, allowing partners to align on key messages, address any last-minute issues, and rehearse their presentations.

This structured and collaborative approach to coordination and communication underscores the consortium's commitment to maintaining high-quality standards, meeting project timelines, and ensuring the successful delivery and impact of the HEAL project.

#§WRK-PLA-WP§# #@IMP-ACT-IA@#

1.3 Impact

HUJI:

HUJI recent research within WP3 is impactful. HUJI has determined, for the first time, the extent of cancer-related mutations in human pluripotent stem cells and their differentiated derivatives. HUJI have expanded the analysis to human adult stem cells, which has shown the lineage-specificity of mutations in human pluripotent stem cells. HUJI has generated a platform to categorize mutations based-on their predicted impact.

PMU:

Variabilities in the immune profile of iPS-CMs from different runs have the potential to influence the immune recognition and ensuing immune response. Therefore, this needs to be monitored and investigated for functional consequences which could impact graft acceptance and/or survival. PMU established an immunophenotyping strategy capable of identifying such immune profile variations as well as differences in the response to environmental cues (e.g. a proinflammatory environment). Correlation of phenotypic differences with immune responses are underway and will aid in quality assessment of the produced iPS-CM.

UDUS:

One of the main objectives is to analyze the capapilty of freezing cardiomyocyte aggregates or single cells instead of having to use freshly differentiated cells when needed for clinical application. As it is from the utmost importance to test the product before release for different release criteria, it is important to have the cells safely stored to be released to the clinic once cleared for all release parameters.

As some of these tests (e.g. mycoplasm tests) take more time than others, it is more feasible to cryopreserve the final product before release to ensure enough time for the quality assessment. It needs to be tested if our product in the form of cardiomyocyte aggregates, or single cells with a re-aggregation step, is compatible with freezing and thawing, while ensuring the viability and quality of the cells

1.4 Update of the plan for exploitation and dissemination of results (if applicable)

#§IMP-ACT-IA§#

INNO:

The validated in vivo tumorigenicity model will be offered as a commercial service by InnoSer to allow evaluation of broad cell therapy products. InnoSer already received two commercial requests that could not be services, but will be commercially viable after the validation of the model.

UDUS:

UDUS has already started small disseminations in the form of abstract submissions and presentations

4. Fabienne Becker, Stefanie Liedtke, Boris Greber, Nils Kriedemann, Ulrich Martin, Robert Zweigerdt, Gesine Koegler, Abstract 23 HLA-Homozygous iPSC from Cord Blood for the Generation of Cardiomyocytes for Allogeneic Cell Therapy to the Heart, Stem Cells Translational Medicine, Volume 12, Issue Supplement_1, September 2023, Page S25, https://doi.org/10.1093/stcltm/szad047.024
5. Fabienne Becker, Rigveda Bhave, Soraia Martins, Melanie Hühne, Boris Greber, Gesine Kogler, Abstract 12: Cord Blood Banking, Technical and Clinical grade GMP- Development of Advanced Therapy Products as HLA-Homozygous iPSC-derived Cardiomyocytes, Stem Cells Translational Medicine, Volume 13, Issue Supplement_1, September 2024, Page S14, https://doi.org/10.1093/stcltm/szae062.012
6. StN Series Talk Spotlight: Research “EU Project HEAL (HLA-homozygous iPSC cardiomyocyte aggregate manufacturing technologies for allogenic cell therapy to the heart)” by Fabienne Becker
7. 12th GSCN Conference HEAL Symposium Presentation “Abstract No. T25/P106. Fabienne Becker, Soraia Martins, Melanie Hühne, Boris Greber, Gesine Kogler. Optimization of cardiomyocyte cryopreservation and quality control aspects for potency and residual iPSC contaminations” by Fabienne Becker
8. Podcast Talk ImmunoChat with Nora Balzer “The future of Heart Repair: 3D Cell Models and Organoids” by Fabienne Becker
9. CARDDIAB Workshop (CARDiovascular Research in DIABetes) Presentation “HLA-homozygous iPSC-cardiomyocytE Aggregate manufacturing technoLogies for allogenic cell therapy to the heart (HEAL)” by Gesine Kögler

UDUS is planning to publish new findings toward GMP compliant cardiomyocyte freezing once sufficient qualitative and quantitative data has been acquired, focusing on new findings for cardiomyocyte aggregate and single cell freezing and classification of the data in the existing literature

10. Fabienne Becker, Soraia Martins, Carlos A. Hernandez-Bautista, Boris Greber, Robert Zweigerdt, Gesine Kogler, Improved Cryopreservation of Cardiomyocyte Aggregates Differentiated from GMP iPSC in a 3D Culture Format, Submitted at Scientific Reports

UOX:

Both Deliverable reports are suitable for the basis of peer-reviewed journal articles – one on meeting data protection requirements in allogeneic hiPSC cell therapy development and one on institutional readiness for novel cell therapies in cardiology/ cardiac surgery.

The work of adapting the written material, identifying suitable target journals, and preparing submissions for at least two manuscripts is planned to take place between 01/09/2025 and 31/12/2025 with the goal of having two papers under review by the start of 2026.

UMCU:

UMCU is planning to publish new findings relating to the safety and retention of intracardiac iPSC-CMA transplantation when sufficient qualitative and quantitative data has been acquired.

PMU:

PMU already started small disseminations in the form of conference participations (Hyperflow Vienna 2023, Talk: 'Deep Immunophenotyping of human iPSC-derived Cardiomyocytes'; Austrotransplant Eisenstadt 2023, Poster: 'DEEP IMMUNE PHENOTYPING OF iPSC-DERIVED HLA-HOMOZYGOUS CARDIOMYOCYTES' Nicole Maeding, Anna Steinhuber, Rodolphe Poupardin, Nils Kriedemann, Robert Zweigerdt, Dirk Strunk; IGLD Leipzig 2024, Talk: 'Deep Immunophenotyping of iPSC-derived Cardiomyocytes by Spectral Flow Cytometry').

PMU is planning to publish data on iPS-CM immune phenotyping once comparison to differentiations from partners MHH and CATD could be generated.

SERO:

SERO started disseminations by

i) an accepted oral presentation at the ASSCR 2025, Vienna,

ii) a manuscript in preparation for Frontiers in Immunology with a planned submission on October, 7th 2025.

[OPTION for projects providing access to research infrastructure:

1.X Access to research infrastructure

UDUS:

As the largest EU allogenic cord blood bank with more than 28.000 stored cord blood transplants, we provide extensive experience in the distribution of these transplants world-wide, with extensive experience in automated processing, labelling cryopreservation, thawing and release of different products. In accordance, UDUS provides a GMP facility with multiple freezing devices and laboratories for future production and storage of the product, HLA laboratory for identity testing of products by HLA-sequencing, and FACS core facility for quality control by flow cytometry/sorting if appropriate.

#§PRO-GRE-PG§# #@FOL-UP-FU@#

2. FOLLOW-UP OF RECOMMENDATIONS AND COMMENTS FROM PREVIOUS REVIEW(S) (IF APPLICABLE)

After the review of the 1st progress report the EU recommended to have a deeper look in the following ascpects:

The consortium must intensify efforts to ensure timely project completion by overcoming bottlenecks and resuming delayed tasks, particularly by selecting a clinically meaningful cell product and delivery method.

To address these challenges, the consortium thoroughly reviewed and revised its deliverables, milestones, and overall project timeline to better reflect the current progress and realistic expectations. This revision involved re-prioritizing tasks, adjusting resource allocation, and incorporating contingency plans to mitigate risks and unforeseen delays.

During the 2nd reporting period, the consortium successfully requested and obtained a non monetary extension of 12 months. This extension provides the essential additional time required to complete critical research activities, further optimize protocols, and finalize regulatory preparations. It also allows for thorough validation of the clinically meaningful cell product and delivery methods, ensuring the robustness and safety needed for successful clinical translation.

By securing this extension, the consortium has reinforced its commitment to maintaining high scientific and quality standards while adapting to the complexities of advanced therapy development. This proactive approach enhances the likelihood of achieving all project objectives and delivering impactful therapeutic solutions within a feasible timeframe.

Addressing milestones and deliverables in a realistic manner, given the complexities of working with iPSCs and derived cells, is crucial. For example, cryopreservation challenges are critical for reaching various milestones and should be carefully addressed. Finalizing and establishing the protocol for differentiating and upscaling hPSC-CMAs early in the next reporting period is essential, as several deliverables depend on it. A more defined strategy for the role of genetically modified cell lines, especially the iCASP9 line, should be developed and communicated to meet project objectives.

The consortium fully appreciates these reviwers comments and their importance. This in mind, we have extensively progressed with our efforts in advancing the crypreservation chalanges by following different strategies in parallel. This the crypreservation of cell aggregates is a still unsolved chalange by the entire field of cell therapies, we have also tested the crypreservation of dissociated iPSC-CMs, such that they can be used for re-aggregation after thawing, ahead of the application to the heart. Moreover, we have successfully established a strategy for transportation of iPSC-CM aggregates in saline at 4°C for 24h; this is highly compatible with cells viability and closes an important (logistical) gap between the cell production side and the current animal experimentation side, equivalent to the future transportation to the bed sinde/ the patient in a hospital upon clinical translation.

Regarding the advancements in iPSC-CMA differentiation process development and upscaling, we have made substantial progress regarding a highly robust differentiaion protocol, avoiding previouf failure in achiving high CMs content in each differentiaion run. This protocol has now been sucessfully used to produce iPSC-CMs at two independent production sides i.e. at CATD and MHH followed by successful delivery of vital cells (via the advanced transportation strategy at 4°C) to UMCU for administration in the heart of the pig model.

Genetic modification of iPSC lines has also made substantial progress along projects Aims.

Together, we feel that – based on the outstanding collaboratinve spirit throughout the entire project and all partners - the project makes substantial progress on chalanging tasks within the restrictive timelines.

Revision Deliverables:

Del. Number	Del. Name	Status	Comments of Experts (1st PR)	Revision by Consortium
D3.1	Validated Nude Rat Assay for Tumorigenicity	Request for revision	The deliverable is incomplete. A new NSG mouse model has been proposed, with experiments starting in January 2024. Initial validation steps are adequate, and the switch is deemed suitable. The deliverable may be accepted upon submission of validation data.	Tumorigenicity has been evaluated in NSG mice with Hela cells and undifferentiated pure iPSCs as positive controls and spiking experiments were performed to validate assay sensitivity. Data will be presented at the 2nd Progress Report Review Meeting in November 2025.
D3.2	Characterisation of arrhythmic events in long-term studies upon intramyocardial delivery and absence of life-threatening ones; SOP for iPS-CMA delivery for optimized retention	Request for revision	This task has been substantially delayed, awaiting optimization of iPSC differentiation to cardiomyocytes and cryopreservation. Progress is being made.	This deliverable is scheduled for completion by the end of October 2025, with the analysis to be presented at the 2nd Progress Report Review Meeting in November 2025.
D3.3	Genetic integrity of iPSCs after mass production identifying genomic aberrations to enable cell line selection	Request for revision	This deliverable has been delayed due to final product development issues. Assays for assessing genetic integrity are well-described but need to be performed on GMP iPSC lines after scalable expansion. Progress is being made.	The analysis of iPSCs after mass production has been completed, showing no evidence for further genetic instabitlity during cell mass production. Further analysis of cells prior to their potencial clinical use, we be completed during the last phase of the project.
D5.2	Final R&D SOP for optimised methodology for iPSC expansion and differentiation to iPS-CMA's	Request for revision	Given that the final product has not been developed yet, the SOP is in a very preliminary form and requires substantial revisions. The delivery of the final SOP is delayed by 18 months and should be resubmitted when available.	We have made substantial progress towards establishing a final differentiation protocol for iPSC-CMA production; finalizing the resptive SOPs is in progress as well.

Revision Milestones:

MS Number	MS Name	Achieved	Comments of Experts (1st PR)	Revision by Consortium
MS1	First iPS-CMA batch for WP2/3 testing available	Partially	The milestone has been partially completed due to delays in final product development. However, cells have been shipped to consortium partners.	Despite the unfortunate delay (explained elsewhere), this milistone has been achieved in the meantime.
MS5	TPP for the IMP drafted (Version 0)	Partially	Assurance was provided during the review meeting, that the internal report has been generated, but evidence of work is lacking	Gaps in the IMPD have been identified which includes clarity on key regulatory questions such as product viability, formulation handling, sterility testing, identity criteria, viral/genetic safety, residual iPSCs, proliferation, and biodistribution. Thes questions will be addressed at a meeting with the PEI in late 2025.
MS6	FTO strategy agreed	Partially	The milestone can be considered partially achieved as the report lacks a clear description of the agreed FTO strategy discussed during the review meeting. However, the consortium indicates that this aspect has been completed internally hence evidence should be provided.	A comprehensive IP Landscaping/FTO report has been completed following continuous updating throughout the project lifespan as described in task 5.1. HEAL consortium members have now identified IP which can potentially be used in the project. They have also flagged competing or overlapping IP held by others which should be evaluated to determine how to optimally proceed with technology development within the project and beyond.
MS7	Scope and respondents for infrastructure	Partially	The purpose of this milestone remains unclear. Assurance was given during the review meeting that the MS is achieved, but evidence of work is lacking.	The Milestone, which mainly concerns defining the scope of Task 5.3, is achieved since the deliverable led by UOXF—comprising two comprehensive reports—has been completed. Contributions from partners and an external advisory panel further enhanced the quality and thoroughness of the data protection and institutional readiness studies, with documented evidence supporting the successful fulfillment of this milestone.
MS8	Feedback from PEI regarding cell type for tumorigenicity studies	No	Completion of this milestone is delayed.	Feedback on protocol discussions with PEI on the exploitation of Induced pluripotent stem cells for clinically applicable heart repair was reviewed by the consortium. This included previous scientific advice

				consultations with PEI describing the reprogramming of human peripheral CD34+ cells into induced pluripotent stem cells (hiPSCs), the isolation and purification of these cells, the differentiation of hiPSCs into cell (hiPSC)-derived cardiomyocytes (hiPSC-CMs) and the purification and characterization of these differentiated cells. The respective meeting minutes/ protocols on the HEAL consortium's respective previous scientific advice consultations with the PEI have now been reviewed and included in the TPP development where appropriate. Additional gaps identified will be the subject matter for follow up scientific meetings with the PEI in late 2025 including specific cell type for tumorigenicity studies.
MS11	HLAh iPSC line(s) carrying iCASP9 in the AAVS1 locus	Partially	Milestone partially completed; ongoing challenges with CDK1-iCASP9 strategy due to off-target integration.	No futher ongoing challenges. Targeting repeated and successfully completed. 3 HLAh iPSC lines carrying iCASP9 in the AAVS1 generated.
MS13	Development Plan is prepared	Partially	Assurance was provided during the review meeting, but evidence for the work performed is missing.	D4.1- Report on the Development Plan has been completed. In collaboration with the partners through regular interactions, a Development Plan describing the path to solve the identified weaknesses is now well established. Due to complexities of the IMP class definition of the final product desired characteristics defined in the TPP were delayed by 12 months. As a result, delays are foreseen in finalising IMPD and IB documents (D4.3) due to changes in the timeline for the required updated information from partners required to finalize their SOPs. Through the requested project extension, there is confidence that we can stay on track to follow and complete the actions outlined in this deliverable
MS14	PoC for iPS-CMA closed-loop bioprocessing in 5l scale	No	The project has made significant advancements, reaching the 2-liter scale successfully. However, achieving the 5-liter scale is delayed.	Milestone not achieved yet, delayed until 31 December 2025.

3. EXPLOITATION PRIMARILY IN NON-ASSOCIATED THIRD COUNTRIES (IF APPLICABLE)

Not applicable.

#§IMP-ACT-IA§# #@CON-MET-

4. OPEN SCIENCE

The interim research results from HEAL are published as open access articles in high-impact, peer-reviewed journals with international visibility. The selection of journals depends on the nature and scope of the findings.

Publications have appeared in the following journals:

- Advanced Healthcare Materials
- Bioactive Materials
- Biology Open
- bioRxiv
- Cancer Biology and Therapy
- Disease Models & Mechanisms
- Humanities and Social Sciences Communications
- Journal of Cardiovascular Translational Research
- Journal of General Physiology
- Nature Biotechnology
- Nature Cell Biology
- Nature Protocols
- Scientific Reports
- Stem Cell Reports
- Stem Cell Research & Therapy
- Stem Cells Translational Medicine
- The American Journal of Human Genetics

Target Audience: The academic and industrial research community, clinicians, supply chain actors.

Central knowledge repository: The Consortium uses Zenodo as data repository built on open source software that accepts all forms of research output of the project from data files to poster files. The platform is open for all researcher and HEAL partner have a special access to the HEAL group on Zenodo.

Data Security: A Data Security Plan was created and shared with the consortium. The plan is based on “Guidelines on FAIR Data Management in H2020”[2]. The Plan is continually revised in the consortium.

#§CON-MET-CM§# #@WRK-PLA-WP@#

5. DEVIATIONS FROM ANNEX 1 AND ANNEX 2 (IF APPLICABLE)

5.1 Tasks/objectives

MHH:

WP1 Upscaling of iPSC-CM production

Deliverable D1.4/ D4 Closed system iPS-CMA manufacturing 5l scale (Due Date: month 36, 31.08.2025, future submission: month 45, 31.05.2026)

The deliverable is delayed due to extensive technical challenges related to process upscaling to the 5L scale, some of which were unforeseen despite prior experience in this area.

[2] V3.0 26.07.2016 Annex 1.

Milestone 14 PoC fir iPS-CMA closed-loop bioprocessing in 5l scale (Due Date: month 18, 28.02.2024, future submission: month 40, 31.12.2025)

Please see reason of deviation in explanation of deliverable D1.4.

WP 5 Cost Effectivenss, HTA and Explotation

Deliverable D5.2/ D23 Final R&D SOP for optimised methodology for iPSC expansion and differentiation to iPS-CMA's (Due Date: month 36, 31.08.2025, future submission: month 45, 31.05.2026)

The deliverable is postponed due to the extended time required for complex process modifications and optimizations, which involve input from multiple project partners including CATD. Additionally, this task is dependent on the delayed work in D1.4 / D4 as described above.

PMU:

WP 2 Immunogenicity

Deliverable D2.2/ D9 Immune response data predicting immune-suppression requirements of HLA-matched iPS-CMAs (Due Date: month 32, future submission: month 40)

- Structural changes leading to change from beneficiary PMU to Serologis and following setup phase of the new laboratory caused substantial delay in associated lab work.
- Acquisition and typing of HLA-matched/-mismatched PBMC donors was delayed.

Deliverable D2.3/ D10 Machine learning correlation algorithm to correlate immune phenotype and function in innate and adaptive immune assays (Due Date: month 36, future submission: month 44)

- Deliverable D2.3 relies on the input of data included in D2.2 and is therefore similarly affected

SERO:

- Deliverables D2.2 and D2.3 received new due dates (31.04.2026 and 31.08.2026) due to the granted cost-neutral extension. They were therefore transferred to SERO and are not part of this reporting period.

INNO:

WP 3 Objective 5. Complete regulatory compliant toxicology, biodistribution and tumorgenicity studies:

Task 3.5 Regulatory compliant tumorgenicity studies

Validation of the toxicology and tumorigenicity testing was delayed. Study designs needed to be approved by the project consortium as they were evaluated for their compliance with regulatory advise. This will not affect the general timelines of the project and effective final product toxicology and tumorigenicity evaluation will only be performed on the optimized cells project in the end phase of the project. The final tumorigenicity experiment needed to be performed on the final cell product and hence these experiments were not feasible until the product was fully validated. The tumorigenicity model and assays are now ready to test the final CMA cells product. Suicide gene experiments and tumorigenicity assays are scheduled to start in Q4 2025.

EATRIS:

Milestone 8 Feedback from PEI regarding cell type for tumorgenicity studies (Due Date: month 12, 31.07.2023, future submission: month 38, 31.10.2025)

Feedback on protocol discussions with PEI on the exploitation of Induced pluripotent stem cells for clinically applicable heart repair was reviewed by the consortium. This included previous scientific advice consultations with PEI describing the reprogramming of human peripheral CD34+ cells into induced pluripotent stem cells (hiPSCs), the isolation and purification of these cells, the differentiation of hiPSCs into cell (hiPSC)-derived cardiomyocytes (hiPSC-CMs) and the purification and characterization of these differentiated cells. The respective meeting minutes/ protocols on the HEAL consortium's respective previous scientific advice consultations with the PEI have now been reviewed and included in the TPP development where appropriate. Additional gaps identified will be the subject matter for follow up scientific meetings with the PEI in late 2025 including specific cell type for tumorigenicity studies.

5.2 Use of resources *(n/a for MSCA and Lump Sums)*

PMU:

The change of partner PMU to Serologis became retroactively active 01/01/2025. All costs declared are for the period until the end of works (31/12/2024) as agreed with the Amendment. No further costs are claimed from HaDEA (project HEAL) for work done by PMU after 31/12/2024.

BI:

The partner BI reported their expense during the first reporting period.

#§WRK-PLA-WP§#

HISTORY OF CHANGES		
VERSION	PUBLICATION DATE	CHANGE
1.0	15.12.2021	Initial version (new MFF).
1.1	01.05.2023	Minor updates in Part A. Added section 1.4 on updates to the plan for exploitation and dissemination of results.