Provas de Agregação em Bioengenharia

Summary of the Research Seminar

# Bioengineering Approaches for Cell Based Products

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## 1. PRELIMINARY NOTE

The oral presentation will consist of a research seminar where I will give an overview of the research and main achievements of the Cell Bioprocess Lab focusing on stem cell bioengineering. This is the strongest line of research in the lab I lead, and I will highlight the relevance of our contribution to this field. The seminar will be divided into two parts: Bioengineering integrated bioprocesses for manufacturing cell-based products and 3D advanced cell models.

## 2. SUMMARY of the RESEARCH SEMINAR

The last decades witnessed major changes in medical care for human health. Several diseases considered chronic until recently can now be cured. The completion of the Human Genome Project twenty years ago was a remarkable milestone, providing researchers with a comprehensive map of the human genome [1]. Furthermore, a better understanding of the mechanisms of disease at the cellular and molecular level, driven by rapidly evolving technological advancements, propelled targeted, more effective treatments. A new era of personalized and precise medicine started and, with it, a demand for a new generation of medicinal products [2-5].

From chemically synthesized drugs in the early 20$^{th}$ century through products made by cells (biopharmaceuticals) in mid-20$^{th}$-century, a new journey began a few decades ago with the use of genetic material and living cells as medicinal products (Advanced Therapy Medicinal Products – ATMPs) [6, 7]. This research seminar will focus on cell-based products, in particular stem cell-derived products, and on the main challenges related to their development towards translation into clinics.

Due to their complexity, the development of ATMPs brings manufacturing to centre stage. Compared with other biologics, such as recombinant proteins, virus-like particles (VLPs) and recombinant viruses, cell-based products have a significantly higher mass and surface area [8-10]. The transfer of operation units and process technologies, used for many decades in Biopharma industry, is not straightforward (e.g., sterile filtration at the end of purification cannot be done). Moreover, as living cells, their viability and other key biological features underlying the mechanism of action responsible for efficacy are highly dependent on temperature, pH, oxygen concentration, osmolarity and many other environmental factors [10]. These characteristics create many challenges in what concerns unit operation times in all steps of the production process (upstream and downstream). Bioprocess scalability is also a relevant issue to consider [11-13]. Growing from autologous to allogeneic therapies poses critical challenges concerning scalability. Still, both profit from the development of flexible production platforms to deliver high-quality cells in relevant quantities to satisfy clinical demand. Optimized manufacturing and better analytical tools will, hopefully, reduce the current cost of these therapies [14-19].

This seminar will discuss some of these challenges and highlight how the research in the Cell Bioprocesses lab I lead has been addressing them. I will present several case studies illustrating our contribution to the field of stem cell bioengineering, focusing on the development of scalable upstream and downstream processes yielding relevant cell numbers with the required quality attributes. The development of analytics to assist bioprocess development and how the integration of stem cell expansion and differentiation protocols with -omics data can impact cells maturity and functionality will be emphasised. Finally, I will explore how some of these tools and technologies were used to develop *in vitro* cell models, mimicking the behaviour of cells in tissues, while maintaining the system as simple as possible, to allow in-depth cellular and molecular interrogation, thus providing powerful tools for drug discovery and pre-clinical research.

## PART I:
## INTEGRATED BIOPROCESSES FOR MANUFACTURING STEM CELLS-BASED PRODUCTS

Stemness enables undifferentiated cells with the unique ability to develop into various specialized cell types. Stem cells have two key characteristics: self-renewal and differentiation capacity. The most common classification of stem cells is based on their potency and lineage progression, including five distinct types: totipotent, pluripotent, multipotent, oligopotent and unipotent. Classifications based on their origin, i.e., primary location, are also common (e.g., embryonic-, adult-, cord blood- stem cells) [20].

Yamanaka's findings in 2006 revolutionized biomedical research by demonstrating that pluripotent stem cells can be directly generated from fibroblast cultures, by the expression of defined transcription factors [21]. The era of induced pluripotent stem cells (iPSCs), as baptized by Yamanaka, started, and with it, the possibility to generate a virtually unlimited supply of cells with pluripotent potential, similar to that of embryonic stem cells (ESC) but not raising the same ethical concerns [22]. Despite the advances in our understanding of stem cell biology achieved in the last 15 years, especially those involving induced pluripotent stem cells, several challenges remain limiting the widespread clinical use of stem cell therapies.

Current hurdles to the clinical translation of stem cell therapies include maintenance of the stem cell state, reproducible expansion of large numbers of stem cells, efficient control of the cell state, both pre- and post-transplantation. A deeper understanding of engineering fundamentals is essential to overcome such hurdles and guarantee robust, safe and cost-effective manufacturing of stem cells [23]. The identification of the best suited cell phenotype for each therapeutical application (progenitor cells *versus* immature cells *versus* mature cell phenotypes) and a better understanding of the mechanisms of action for cell replacement therapies *versus* paracrine based approaches will hopefully also contribute to overcome some of these hurdles [24-25].

In our lab, we have been developing robust and integrated platforms using scalable bioreactors for the expansion, differentiation and maturation of stem cell-based products; and evaluating cell purification strategies, while complying with good manufacturing practices (GMP) requirements. Several examples will be depicted focused on human Pluripotent Stem Cells (hESC, hiPSC), Cardiac Progenitor Cells, Neural Stem Cells, Mesenchymal Stem/Stromal cells (MSC) and Extracellular Vesicles. Due to their unique characteristics (easy scalability, hydrodynamically well-characterized, computer-control of culture parameters and non-invasive sampling), the "work horses" for bioprocess development in our lab are stirred-tank bioreactors.

Cell expansion protocols require a delicate compromise between the quantity (clinically relevant cell numbers are typically $10^9$-$10^{10}$ *per* patient, depending on the application) and the "maintenance of stemness", i.e. quality (viability, phenotype, karyotype, and differentiation potential). The goal in process development is to increase volumetric productivities (cell/mL) and reduce process time maintaining these cells' quality attributes. Depending on the cell type, our group and others have shown that using 3D aggregates [26-28] or microcarrier technology [29-30] and bioprocess intensification [31, 32], it is possible to scale up the production of stem cells in environmentally controlled bioreactors. Examples demonstrating the scalable production of MSCs will be presented and the major hurdles addressed, such as cell purification, highlighting how challenges were overcome [33-34]. For human pluripotent stem cells expansion, strategies based on the rational modulation of dissolved oxygen concentration and perfusion rate will be illustrating a total

cell expansion factor of 1100 in 11 days of culture, while cells maintained their self-renewal capacity, pluripotent phenotype and differentiation potential, as well as genomic stability [31].

As for differentiation and maturation steps in bioreactors, the focus of this seminar will be on the production of cardiac (Part I), hepatic and neural cells (Part II).

Despite many advances in the field, immature phenotypes of pluripotent stem cell-derived cardiomyocytes are a still major hurdle constraining their potential in cell therapy [35-37]. Many reports correlate immature cardiomyocyte-like phenotypes with low engraftment, poor survival rates and transient ventricular arrhythmias after transplantation [38]. We have been addressing questions related with scalable production of hiPSC-CM, namely studying the impact of the culture strategy (2D *versus* 3D) on differentiation paths and cellular molecular signatures, aiming to improve hiPSC-CM maturation *in vitro*. Several strategies have been explored by our group and others [39-43], such as co-cultures with other cell types (e.g., endothelial cells, vascular smooth muscle cells and mesenchymal stem cells), electrical and mechanical preconditioning, and genetic manipulation. Our major contribution to this field was focused on modulating cell metabolism. Using integrated "-omics" analyses, we identified major metabolic pathways involved in cardiomyocytes maturation. By manipulating the culture medium formulation, we demonstrated that it is possible to impose metabolic shifts from aerobic glycolysis (fetal-like CM) towards oxidative phosphorylation (adult-like). These shifts resulted in increased mitochondrial content and cellular ATP levels concomitant with upregulation of functional genes, improved sarcomere length and alignment and contractility of hiPSC-CM [44].

As the relevance of ECM (extracellular matrix) in hiPSC-CM maturation and functionality becomes evident, the protocols we developed to produce significant amounts of hiPSC-CM with improved mature phenotypes are currently being exploited in Margarida Serra Lab (iBET) and collaborators. They are exploring tissue engineering approaches in heart regeneration, namely for repopulation of decellularized heart [45], in biofabrication and 3D printing technologies, bioink materials and synthetic scaffolds [46].

The potential of extracellular vesicles (EVs), including exosomes, for cardiac repair, is gaining significant attention in regenerative medicine. This research area propelled a joint

research line between Serra and my lab, to investigate the relevance of the parental cell line for scalable production of EVs, with improved bioactivity, in bioreactors [47, 48]. With a focus on -omics and profiting from all the investment in analytical tools implemented to support bioprocess development and characterization of stem cells and their secretome [49], we hope to continue our path contributing to the clarification of EV-mediated cell-cell communication mechanisms in the heart, enabling paracrine based therapies [50].

## Part II:
## BIOENGINEERING 3D CELL ADVANCED CELL MODELS

*In vitro* cell models can be traced back to the early 20$^{th}$ century. Over decades, they played a crucial role in advancing various fields including cell biology, pharmacology, and medicine. Late in the 20$^{th}$ century, the change from 2D to 3D cell models and the integration of bioreactors enabling reproducible and controlled changes of specific environmental factors, for which my work in long-term 3D neuronal and hepatocyte cultures in perfused bioreactors was one of the pioneers, gave the field expanded tools which are still growing.

As the techniques for generation of human iPSC lines are evolving, together with the development of small-scale bioreactors (e.g. AMBR) and organ-on-a-chip devices, *in vitro* disease modelling, drug discovery and screening using human cells are becoming a reality [51]. Applying engineering principles to 3D cultures will increase reproducibility and provide experimental control, improving functional readouts that will ultimately result in the adoption of these tools also by the regulatory agencies. Hopefully, the integration of these advanced models at the earliest stages of R&D and drug discovery, and also in pre-clinical toxicology, will improve the likelihood of successfully translating discoveries to first-in-man, also as a support to “quick-kill strategies” and decreasing high attrition rates that marked past decades [52, 53].

Many publications describe advanced 3D *in vitro* models using different nomenclatures such as spheroids, aggregates, organoids, microtissues, mini-organs, embryoid bodies and others [54]. For clarity and consistency with our publications, in this seminar we will keep the terminology 3D spheroids.

Although Pluripotent Stem Cells (PSC) are nowadays a strong component of the 3D advanced cell models, since 1996, we have consistently used primary cultures (murine and human) to generate those models [55-57]. The ability to maintain such 3D models in long-term cultures, with relevant physiological cell phenotypes, was leveraged by our competences in bioengineering.

In this research seminar I will focus on the development of (i) hepatic cell models and their applications pre-clinical research, toxicology and drug development and on (ii) neural cell models and their potential in pre-clinical research.

**Hepatic Cell Models**

Despite many advances on liver developmental biology and several publications reporting on stem cell-derived cultures of human hepatocytes with improved functionality [58], primary cultures of human hepatocytes (PHH) are still considered as the gold standard for testing liver toxicity and are essential for development of BAL (bioartificial liver) devices [59]. Despite the difficulties in obtaining freshly isolated hepatocytes and batch to batch variations (donor dependency) that impact metabolic profiles/phenotypes, we were able to address many drawbacks of PHH cultures, such as *in vitro* spontaneous de-differentiation. We demonstrated that perfusion bioreactor cultures of PHH spheroids could maintain liver-specific activities and architecture for more than 4 weeks [60]. Moreover, we developed a co-culture strategy for PHH spheroids and bone marrow-derived MSC that improved the longevity of the hepatocyte phenotype [61]. Key read-outs of hepatocytes functionality were shown, such as albumin and urea synthesis, cytochrome P450 (CYP) and drug transporter activities, and the formation of *bile canaliculi*-like structures. The latter organized in an open network, permitting secretion and therefore permitting repeated dose drug metabolism and toxicity studies. Many other studies using different hepatocytes cell sources, such as HepRG [62] and HepG2 cell lines [63], cryopreserved human hepatocytes and co-culture strategies with other liver cell types contributed to the advance of alternative liver cell models in the lab. Some examples will illustrate how, together with Catarina Brito lab (ITQB/iBET), Miguel Prudêncio lab (IMM) and Merck Global Health (Switzerland), we depicted mechanisms underlying the biology of

*plasmodium* hepatic infection. By partially overcoming the scarcity of models for liver-stage plasmodium infection, we contributed to the the discovery and profiling of novel drugs targeting *plasmodium* hepatic infection [64]. Recently, some of the knowledge, tools and technologies described above set the stage to improve the generation of hiPSC-derived mature hepatocytes at Margarida Serra Lab that, in collaboration with my lab, Prosper Lab (UNAV, Spain) and Batista Lab (IIS Aragon Inst, Spain), demonstrated the feasibility to produce hiPSC- Hepatocyte like cells in bioreactors [65] showing that the modulation of key environmental factors (e.g. dissolved oxygen) was critical to improve cells quality attributes of hPSC-derived hepatocyte-like [66].

**Neural Cell Models**

Neural cells were my first endeavour in this exciting field of 3D in vitro models. From the primary cultures of murine neurons and glial cells immobilized in Matrigel and perfused inside an NMR spectrometer to depict metabolic trafficking during ischemic episodes, in the mid-90s [55], to recent work with human iPSC-derived neurospheres, cultured for months in stirred-tank bioreactors that enable recapitulation of human neural microenvironment signatures, the research developed in my lab has provided important contributions to the field of neurosciences [67].

Exploring the potential of stem cell-derived human 3D neural models, we developed bioprocesses in perfusion stirred-tank bioreactors for the production of neurospheres that reproducibly differentiate into complex tissue-like structures containing functional neurons, astrocytes and oligodendrocytes and brain-like extracellular matrix secreted by glial cells ([67, 68]. We have adapted an extensive toolbox of analytical methodologies to 3D neural cell models, allowing molecular and phenotypic profiling and interrogation. Cell functionality was addressed by studying metabolic 10compartmentalization between neurons and astrocytes [69]. Neuronal activity was shown by spontaneous calcium transients, synaptic vesicle trafficking and release of neurotransmitters upon depolarizing stimuli, voltage-dependent potassium currents as well as glutamate-induced currents [67, 68]. In collaborative projects with Catarina Brito lab (ITQB/iBET), Manuel Carrondo Lab (iBET) and others, we demonstrated the valuableness of these CNS (central nervous

system) *in vitro* models. I will illustrate this with some examples demonstrating their use to assess viral vector tropism in human neural cells, assisting the development of gene therapy in the brain [70], as well as disease modeling tools for neurotoxicity [71] assays, and supporting the development of organ-on a-chip devices [72].

In conclusion, even if the core of my research is surely bioengineering, the exponential increase in biological knowledge we witnessed in the last three decades, to which I proudly contributed, made my career a very challenging and rewarding adventure. Although much progress has been achieved, several important challenges remain to be solved to ensure the successful translation of cell-based products to clinics. I will certainly continue to pursue some of them, focusing on better understanding the complex interplay of biochemical and biophysical clues in stem cells niches to support bioprocess development, scalability and standardization of protocols. All these goals will also depend on the development of robust bioanalytical methods for improved functional read-outs, that I intend to keep chasing.

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## 4. REFERENCES

[1] International Human Genome Sequencing Consortium. (2001) Initial sequencing and analysis of the human genome. Nature 409, 860–921

[2] Friedman A, Letai A, Fisher D et al. (2015) Precision medicine for cancer with next-generation functional diagnostics. Nature Reviews Cancer 15, 747–756

[3] Hamburg MA, Collins FS (2010) The path to personalized medicine. New England Journal of Medicine, Jul 22;363(4):301-4. Erratum in: N Engl J Med. Sep 9;363(11):1092

[4] Pardoll, D (2012) The blockade of immune checkpoints in cancer immunotherapy Nature Reviews Cancer 12, 252–264

[5] Hartmann J et al (2017) Clinical development of CAR T cells—challenges and opportunities in translating innovative treatment concepts. EMBO Molecular Medicine 9: 1183 - 1197

[6] Regulation (EC) No 1394/2007 of the European Parliament and of the Council of 13 November 2007 on advanced therapy medicinal products and amending Directive 2001/83/EC and Regulation (EC) No 726/2004; OJ L 324, 10.12.2007, p. 121–137

[7] Bashor, CJ, Hilton, IB, Bandukwala, H et al. (2022) Engineering the next generation of cell-based therapeutics. Nat Rev Drug Discov 21, 655–675

[8] Ozturk SS, Cell Culture Technology an overview in Cell Culture Technology for Pharmaceutical and Cell-Based Therapies, Sadettin Ozturk and Wei-Shou Hu (Eds), 2006, Taylor and Francis Group, ISBN 0-5247-5334-8

[9] Roldão A, Mellado MC, Castilho LR, Carrondo MJT, Alves PM (2010) Virus-Like Particles in Vaccine Development, Expert Review of Vaccines, 9(10): 1149 -76

[10] Serra M, Brito C, Correia C, Alves PM (2012) Process engineering human pluripotent stem cells for clinical applications, Trends in Biotechnology, 30(6): 350-9

[11] de Almeida Fuzeta M, de Matos Branco AD, Fernandes-Platzgummer A, da Silva CL, Cabral JMS. (2020) Addressing the Manufacturing Challenges of Cell-Based Therapies. Adv Biochem Eng Biotechnol; 171 :225-278.

[12] Want AJ, Nienow AW, Hewitt CJ, Coopman K. (2012) Large-scale expansion and exploitation of pluripotent stem cells for regenerative medicine purposes: beyond the T flask. Regen Med. Jan;7(1):71-84

[13] Rodrigues CAV, Fernandes TG, Diogo MM, da Silva CL, Cabral JMS (2011) Stem cell cultivation in bioreactors, Biotechnology Advances, 29, 6, 815-829

[14] Borys BS, So T, Colter J, et al. (2020) Optimized serial expansion of human induced pluripotent stem cells using low-density inoculation to generate clinically relevant quantities in vertical-wheel bioreactors. Stem Cells Transl Med. Sep;9(9):1036-1052

[15] Sebastião MJ, Marcos-Silva L, Gomes-Alves P, Alves PM (2021) Proteomic and Glyco(proteo)mic tools in the profiling of cardiac progenitors and pluripotent stem cell derived cardiomyocytes: Accelerating translation into therapy. Biotechnology Advances, 49, 107755

[16] Chilima TDP, Moncaubeig F, Farid SS, (2018) Impact of allogeneic stem cell manufacturing decisions on cost of goods, process robustness and reimbursement, Biochemical Engineering Journal, 137, 132-151

[17] Kimbrel EA, Lanza R. (2015) Current status of pluripotent stem cells: moving the first therapies to the clinic. Nature Reviews Drug Discovery, Oct;14(10):681-92.

[18] Badenes SM, Fernandes TG, Rodrigues CAV, Diogo MM, Cabral JMS. Microcarrier-based platforms for in vitro expansion and differentiation of human pluripotent stem cells in bioreactor culture systems. Journal of Biotechnology. 2016 Sep 20;234:71-82

[19] Stacey GN, Cao J, Hu B, Zhou Q. (2022) Manufacturing with pluripotent stem cells: Key issues for future research and development. Cell Proliferation Aug;55(8):e13301.

[20] Shah, A.A., Khan, F.A. (2021). Types and Classification of Stem Cells. In: Khan, F.A. (eds) Advances in Application of Stem Cells: From Bench to Clinics. Stem Cell Biology and Regenerative Medicine, vol 69. Humana, Cham.

[21] Takahashi K, Yamanaka S. (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. Aug 25;126(4):663-76.

[22] Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S. (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell. Nov 30;131(5):861-72.

[23] Madl CM, Heilshorn SC, Blau HM. Bioengineering strategies to accelerate stem cell therapeutics. Nature. 2018 May;557(7705):335-342.

[24] Tewary M, Shakiba N, Zandstra PW. (2018) Stem cell bioengineering: building from stem cell biology. Nature Reviews Genetics. 2018 Oct;19(10):595-614.

[25] Stem cells to Model and Treat Disease (2014) curated by Alnania R (Sc Ed) Chari S (Rev Ed), Cell Press Reviews, Publisher Elsevier, ISBN 978-0-12-420191-0

[26] Serra M, Brito C, Sousa MFQ, Jensen J, Strehl R, Hyllner J, Tostões R, Clemente J, Carrondo MJT, Alves PM (2010) Improving expansion of pluripotent human embryonic stem cells in perfused bioreactors through oxygen control, Journal of Biotechnology, 148: 208-215

[27] Christina Kropp C, Massai D, Zweigerdt R (2017) Progress and challenges in large-scale expansion of human pluripotent stem cells, Process Biochemistry, 59, Part B, 244-254

[28] Zweigerdt, R., Olmer, R., Singh, H. et al. (2011) Scalable expansion of human pluripotent stem cells in suspension culture. Nature Protocols, 6, 689–700

[29] Abranches, E., Bekman, E., Henrique, D. and Cabral, J.M.S. (2007), Expansion of mouse embryonic stem cells on microcarriers. Biotechnology and Bioengineering, 96: 1211-1221.

[30] Serra M, Correia C, Malpique R, Brito C, Bjorquist P, Carrondo MJT, Alves PM (2011) Microencapsulation technology: a powerful tool to integrate expansion and cryopreservation of pluripotent human embryonic stem cells, PLoS ONE, 6(8): e23212

[31] Abecasis B, Aguiar T, Arnault E, Costa R, Gomes-Alves P, Aspegren A, Serra M, Alves PM (2017) Expansion of 3D human induced pluripotent stem cell aggregates in bioreactors: bioprocess intensification and scaling-up approaches, Journal of Biotechnology 6:81–93

[32] Cunha B, Aguiar T, Carvalho Sofia B., Silva Marta M, Gomes Ricardo A., Carrondo MJT., Gomes-Alves P, Peixoto C, Serra M, Alves PM (2017) Bioprocess Integration for Human Mesenchymal Stem Cells: From Up to Downstream Processing Scale-Up to Cell Proteome Characterization, Journal of Biotechnology, 248: 87-98.

[33] Cunha B, Aguiar T, Silva MM, Silva R, Sousa MF, Peixoto C, Serra M, Carrondo MJT, Alves PM (2015) Exploring continuous and integrated strategies for the up- and downstream processing of human mesenchymal stem cells, Journal of Biotechnology, 213:97–108.

[34] Cunha B, Peixoto C, Silva M, Carrondo MJT, Serra M, Alves PM (2015) Filtration methodologies for the clarification and concentration of human mesenchymal stem cells, Journal of Membrane Science, 478:117-129.

[35] Yoshida, Y., Yamanaka, S. (2017) Induced Pluripotent Stem Cells 10 Years Later: For Cardiac Applications. Circulation Research, 120, 1958–1968

[36] Shiba, Y., Gomibuchi, T., Seto, T., Wada, Y., Ichimura, H., Tanaka, Y., Ogasawara, T., Okada, K., Shiba, N., Sakamoto, K., et al. (2016) Allogeneic transplantation of iPS cell-derived cardiomyocytes regenerates primate hearts. Nature 2016, 538, 388–391.

[37] Zhang, Y., Mignone, J., MacLellan, W.R. (2015) Cardiac Regeneration and Stem Cells. Physiol. Rev. 95, 1189–1204.

[38] Cheng Y-C, Hsieh ML et al (2023) Combined Treatment of Human Induced Pluripotent Stem Cell–Derived Cardiomyocytes and Endothelial Cells Regenerate the Infarcted Heart in Mice and Non-Human Primates, Circulation, 148, 18, 1395–1409

[39] Abecasis B, Gomes-Alves P, Rosa S, Gouveia PJ, Ferreira L, Serra M, Alves PM (2019) Unveiling the molecular crosstalk in a human induced pluripotent stem cell derived cardiac model, Biotechnology and Bioengineering, 2019, 116:1245-1252

[40] Abecasis B, Canhão PGM, Almeida HV, Calmeiro T, Fortunato E, Gomes-Alves P, Serra M, Alves PM (2020) Towards a microencapsulated 3D hiPSC-derived in vitro cardiac microtissue for recapitulation of human heart microenvironment features, Frontiers in Bioengineering and Biotechnology, 8: 580744

[41] Tenreiro, M.F., Louro, A.F., Alves, P.M. et al. (2021) Next generation of heart regenerative therapies: progress and promise of cardiac tissue engineering. NPJ Regenerative Medicine Med 6, (1):30

[42] Mummery, C.L., Zhang, J., Ng, E.S., Elliott, D.A., Elefanty, A.G., Kamp, T.J. (2012) Differentiation of human embryonic stem cells and induced pluripotent stem cells to cardiomyocytes: A methods overview. Circulation Research, 111, 344–358.

[43] Henning Kempf, Ruth Olmer, et al (2014) Controlling Expansion and Cardiomyogenic Differentiation of Human Pluripotent Stem Cells in Scalable Suspension Culture, Stem Cell Reports, Volume 3, Issue 6, 1132-1146

[44] Correia, C., Koshkin, A., Duarte, P. et al. (2017) Distinct carbon sources affect structural and functional maturation of cardiomyocytes derived from human pluripotent stem cells. Scientific Reports 7, 8590

[45] Tenreiro MF, Almeida HV, Calmeiro T, Fortunato E, Ferreira L, Alves PM, Serra M (2021) Interindividual heterogeneity affects the outcome of human cardiac tissue decellularization, Scientific Reports, 11, 20834

[46] Almeida HV, Tenreiro MF, Louro AF, Abecasis B, Santinha D, Calmeiro T, Fortunato E, Ferreira L, Alves PM, Serra M (2021) Human Extracellular-Matrix Functionalization of 3D hiPSC-Based Cardiac Tissues Improves Cardiomyocyte Maturation", ACS Applied Bio Materials, 4(2), 1888–1899

[47] Costa MHG, Costa MS, Painho B, Sousa CD, Carrondo I, Oltra E, Pelacho B, Prosper F, Isidro IA, Alves PM, Serra M. (2023) Enhanced bioprocess control to advance the manufacture of mesenchymal stromal cell-derived extracellular vesicles in stirred-tank bioreactors. Biotechnology and Bioengineering, 120(9):2725-2741.

[48] Louro, AF, Paiva, MA, Oliveira, MR, Kasper, KA, Alves, PM, Gomes-Alves, P, Serra, M (2022) Bioactivity and miRNome Profiling of Native Extracellular Vesicles in Human Induced Pluripotent Stem Cell-Cardiomyocyte Differentiation, Advanced Science, 9 (15)

[49] Sebastião MJ, Serra M, Gomes-Alves P, Alves PM (2021) Stem Cells characterization: 'Omics reinforcing analytics. Current Opinion in Biotechnology, 71, 175-181

[50] Sebastião MJ, Gomes-Alves P, Reis I, Sanchez B, Palacios I, Serra M, Alves PM (2020) Bioreactor-based 3D human myocardial Ischemia/Reperfusion invitro model: a novel tool to unveil key paracrine factors upon AMI, Translational Research, 215:5774

[51] Ingber, D.E. (2022) Human organs-on-chips for disease modelling, drug development and personalized medicine. Nature Reviews Genetics 23, 467–491

[52] Jensen C, Teng Y. (2020) Is It Time to Start Transitioning From 2D to 3D Cell Culture? Frontiers Molecular Biosciences. Mar 6,7:33.

[53] Brassard JA, Lutolf MP (2019) Engineering Stem Cell Self-organization to Build Better Organoids. Cell Stem Cell. Jun 6,24(6):860-876.

[54] Pașca SP, Arlotta P, et al (2022) A nomenclature consensus for nervous system organoids and assembloids. Nature. 2022 Sep,609(7929):907-910.

[55] Alves PM, Flögel U, Brand A, Leibfritz D, Carrondo MJT, Santos H, Sonnewald, U (1996) Immobilization of Primary Astrocytes and Neurons for NMR Monitoring of Biological Processes in Vivo, Developmental Neuroscience, 18:478-483.

[56] Miranda JP, Leite SB, Muller-Vieira U, Rodrigues A, Carrondo MJT, Alves PM (2009) Towards extended functional hepatocyte in vitro cultures, Tissue Engineering, Part C, 15:1-11.

[57] Tostões RM, Leite SB, Miranda JP, Sousa MFQ, Wang DIC, Carrondo MJT, Alves PM (2010), Perfusion of 3D encapsulated hepatocytes – a synergistic effect enhancing long term functionality in bioreactors, Biotechnology and Bioengineering, 108:41–49.

[58] Baxter M, Withey S, Harrison S, Segeritz CP, Zhang F, Atkinson-Dell R, et al. (2015) Phenotypic and functional analyses show stem cell-derived hepatocyte-like cells better mimic fetal rather than adult hepatocytes. Journal of Hepatology, 62: 581–589

[59] Zuang, V., Dura, A., et al., Non-animal Methods in Science and Regulation - EURL ECVAM Status Report 2020, EUR 30553 EN, Publications Office of the European Union, Luxembourg, 2021, ISBN 978-92-76-28395-9.

[60] Tostões RM, Leite SB, Serra M, Jensen J, Bjorquist P, Carrondo MJT, Brito C, Alves PM (2012) Human liver cell spheroids in extended perfusion bioreactor culture for repeated dose drug testing, Hepatology, 55(4):1227-1236.

[61] Rebelo SP, Costa R, Silva MM, Marcelino P, Brito C, Alves PM (2017) Three-dimensional co-culture of human hepatocytes and mesenchymal stem cells: improved functionality in long-term bioreactor cultures, Journal of Tissue Engineering and Regenerative Medicine, 11(7): 2034-2045.

[62] Rebelo SP, Costa R, Estrada M, Shevchenko V, Catarina C, Alves PM (2014) HepaRG microencapsulated spheroids in DMSO-free culture: novel culturing approaches for enhanced xenobiotic and biosynthetic metabolism, Archives of Toxicology, 89(8): 1347-1358.

[63] Arez F, Rebelo SP, Fontinha D, Simão D, Martins TR, Machado M, Fischli C, Oeuvray C, Badolo L, Carrondo MJT, Rottmann M, Spangenberg T, Brito C, Greco B, Prudêncio M, Alves PM (2019) Flexible 3D Cell-Based Platforms for the Discovery and Profiling of Novel Drugs Targeting Plasmodium Hepatic Infection, ACS Infectious Diseases,5(11): 1831-1842

[64] Khandelwal, A, Arez, F, Alves, PM, Badolo, L, Brito, C, Fischli, C, Fontinha, D, Oeuvray, C, Prudencio, M, Rottmann, M, Wilkins, J, Yalkinoglu, O, Bagchus, WM, Spangenberg, T (2022) Translation of liver stage activity of M5717, a Plasmodium elongation factor 2 inhibitor: from bench to bedside, Malaria Journal, 21 (1)

[65] Isidro IA, Vicente P, Pais DAM, Almeida JI, Domingues, M, Abecasis B, Zapata-Linares N, Rodriguez-Madoz JR, Prosper F, Aspegren A, Alves PM, Serra M (2021) Online monitoring of hiPSC expansion and hepatic differentiation in 3D culture by dielectric spectroscopy. Biotechnology Bioengineering, 118, 3610–3617.

[66] Almeida JI, Tenreiro MF, Martinez-Santamaria L, Guerrero-Aspizua S, Gisbert JP, Alves PM, Serra M, Baptista PM. (2022) Hallmarks of the human intestinal microbiome on liver maturation and function. Journal of Hepatology, 76(3):694-725

[67] Simão D, Pinto C, Piersanti S, Weston A, Peddie CJ, Bastos AEP, Licursi V, Schwarz SC, Collison LM, Salinas S, Serra M, Teixeira AP, Saggio I, Lima PA, Kremer EJ, Schiavo G, Brito C, Alves PM (2015) Modelling human neural functionality in vitro: 3D culture for dopaminergic differentiation, Tissue Engineering – Part A, 21(3-4): 654-668.

[68] Simão D, Silva MM, Terrasso AP, Arez F, Sousa MFQ, Mehrjardi NZ, Saric T, Gomes-Alves P, Raimundo N, Alves PM, Brito C (2018) Recapitulation of Human Neural Microenvironment Signatures in iPSC Derived NPC 3D Differentiation, Stem Cell Reports, Vol II, 1–13

[69] Simão D, Terrasso Ana P, Teixeira Ana P, Brito C, Sonnenwald U, Alves PM (2016) Functional metabolic interactions of human neuron-astrocyte 3D in vitro networks, Scientific Reports, 6:33285

[70] Simão D, Pinto C, Fernandes P, Peddie CJ, Piersanti S, Collinson LM, Salinas S, Saggio I, Schiavo G, Kremer EJ, Brito C, Alves PM (2016) Evaluation of helper-dependent canine adenovirus vectors in a 3D human CNS model, Gene Therapy, 23:86-94.

[71] Terrasso AP, Pinto C, Serra M, Filipe AE, Almeida S, Ferreira AL, Pedroso P, Brito C, Alves PM (2015) Novel scalable cell based-model for in vitro neurotoxicity testing: combining human differentiated neurospheres with gene expression and functional endpoints, Journal of Biotechnology – 3D cell culture special issue, 205:82-92

[72] Materne EM, Ramme P, Terrasso AP, Serra M, Alves PM, Brito C, Sakharov DA, Tonevitsky AG, Lauster R, Marx U (2015) A multi-organ chip co-culture of neurospheres and liver equivalents for long-term substance testing, Journal of Biotechnology, 205:36–46.