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Abstracts

Invited Speakers

INV02

Non-viral gene transfer for rare diseases

X M Anguela
1: Asklepios Biopharmaceutical

In vivo clinical investigation using non-viral vectors has mostly coalesced around treatment of cancer and as a vaccination tool. This is in part due to two main characteristics of non-viral vectors: short-lived transgene expression and their ability to elicit a strong immune response against the encoded protein combined with generation of an immunogenic milieu around the site of injection. However, for treatment of rare diseases, immunologically silent and long-lasting transgene expression is typically sought after. As such, viral vectors are currently the preferred choice as gene transfer tools for the treatment of rare, monogenic indications.
However, recent developments in the field of non-viral gene transfer are starting to broaden the therapeutic potential of such vectors. In this talk, we will summarize some of the latest developments in non-viral gene transfer tools for the treatment of rare disease. We will place special emphasis on the use of lipid nanoparticles containing RNA for the treatment of monogenic diseases, both in the context of standalone therapies and also in combination with viral vectors to mediate genome editing. We will also discuss the potential use of RNA-only vectors currently under preclinical development that might one day, if successful, circumvent the need for DNA-based viral vectors to achieve long-lasting expression in vivo.

INV04

CAR-based adoptive T-cell immunotherapies

P Menendez [1]
1: Josep Carreras Leukaemia Research Institute

Here, I will provide an introduction to the field of adoptive T-cell redirecting strategies in hematological and solid tumors. We will focus on how CAR-based immunotherapies have revolutionized cancer treatment and will discuss also the large window of opportunity existing to improve further such therapies in leukemias and solid tumors.

INV05

Crossing new frontiers in regenerative medicine: reprogramming technology in cell therapy and aging.

A Platero-Luengo [1]
1: Universidad de Sevilla

The revolutionary concept of ***nuclear cell reprogramming*** has redefined regenerative medicine in the 21st century. This new technology has emerged as a method of reversing the age and identity of virtually any cell to that of an embryonic-like stage. Under the action of the four reprogramming factors *Oct4*, *Sox2*, *Klf4* and *cMyc*, known as the Yamanaka Factors, cells undergo significant changes on the epigenome and chromatin architecture. This genomic reorganization leads to a gradual shut-down of the somatic program and the reactivation of pluripotency genes. During the reprogramming process, multiple hallmarks associated with aging appear to "reverse" such as telomere size, epigenetic marks, DNA damage, levels of oxidative stress, mitochondrial dysfunction, and gene expression profiles. This rejuvenation has been confirmed in a variety of cells and tissues, including those of human origin. Importantly, the transient expression of the Yamanaka Factors, known as ***partial reprogramming***, performs a first phase of epigenetic erasure without reaching pluripotency, which has been shown to be sufficient to promote rejuvenation and the appearance of intermediate progenitors. *In vivo* partial reprogramming has beneficial effects on organismal aging, including lifespan extension and tissue regeneration capacity. Furthermore, *in vivo* ***direct reprogramming*** from one cell type to another can be achieved through the action of lineage-specific transcription factors. We will discuss how cell reprogramming offers interesting possibilities to be explored for regenerative and healthy aging purposes.

INV07

Gene Editing 101: Cleaving DNA to correct mutations

F J Molina-Estevez [1 2]
1: Fundación para la Investigación Biosanitaria de Andalucía Oriental (FIBAO) 2: GENyO- Centro de Genomica e Investigacion Oncologica: Pfizer / Universidad de Granada / Junta de Andalucia

The therapeutic power of gene modification is a clinical reality in 2022. Not long ago, harnessing the perils of genotoxicity and miscarried ectopic expression of curative genes from viral vectors were major concerns in the field. However, pioneer works using nucleases in mammals three decades ago inspired emerging technologies to exploit DNA repair mechanisms in favour of a safer and more accurate approaches.

Genome editing allows to stimulate targeted DNA addition, to alter gene expression and/or to resource on the host cell promoters to drive the expression of corrective genes. Modular and programable nucleases are bringing gene modification down to nucleotide precision. Hence, its use is widespread from disease modelling to clinical trials. Therapeutic gene editing features tailored DNA modifications and is based on the use of modular or programable nucleases allowing to back-engineer single-nucleotide mutations or to stimulate safe-harbour targeted insertion of medicinal transgenes.

In this introduction to gene editing, we review nuclease-driven approaches, how many of these tools have reach clinical maturity, and how there is still room for growth in the field of medical gene editing.

INV08

Base and Prime Editing Strategies to Correct Mutations in Human Hematopoietic Stem Cells.

L Ugalde [1 2] B Olalla [1 2] S M Siegner [3] L Garcia-Garcia [1 2] A Clemens [3] L Alvarez [1 2] I Peña [1 2] M Perez [1 2] E Karasu [3] J Corn [3] J A Bueren [1 2] P Rio [1 2]
1: Division of Hematopoietic Innovative Therapies. Centro de Investigaciones Energéticas Medioambientales y Tecnológicas and Centro de Investigación Biomédica en Red de Enfermedades Raras (CIEMAT/CIBERER), Madrid 28040, Spain. 2: Advanced Therapies Unit, Instituto de Investigación Sanitaria Fundación Jiménez Díaz (IIS-FJD, UAM), Madrid 28040, Spain. 3: Department of Biology, ETH Zurich, Zurich, Switzerland

Base and prime editors hold several advantages in comparison to classical strategies since they do not rely on DSBs and allow the correction of specific mutations without utilizing HDR, which is inefficient in hematopoietic stem cells (HSCs). Base editing system contains a Cas9 nickase fused to a deaminase that, in combination with a sgRNA, favors the specific base conversion in a genomic target site. Cytidine base editors promote C to T base conversion and adenine base editors allow the conversion of A to G. Our results show that adenine base editing constitutes an efficient strategy to generate a specific A to G base substitution (85%) in *AAVS1* locus from healthy donor HSCs. Moreover, we have demonstrated the feasibility to efficiently correct a prevalent Fanconi anemia A stop codon mutation (c.295 C>T) in patient-derived HSPCs.

Additionally, prime editing is a versatile tool that allows targeted base conversions, insertions, and deletions, consisting of a Cas9 nickase fused to a reverse transcriptase, guided to a genomic site by a prime editing guide RNA. Several optimizations have been implemented to increase initial efficiencies, such as the use of a second sgRNA that promotes the generation of a nick in the non-edited strand (PE3) or the co-expression of a dominant-negative Mismatch Repair (MMR) protein (PE5). We have demonstrated that prime editing is an efficient approach to correct the c.295C>T mutation, reaching 42% editing. Overall, our results demonstrate that base and prime editing strategies constitute realistic approaches for the treatment of FA-associated mutations.

INV09

Genome-editing technologies in CAR-T cell therapy for cancer

S Guedan [1]
1: IDIBAPS

CAR-T cell therapy has shown tremendous promise for the treatment of B-cell malignancies, marking a new era in cancer therapy. However, several challenges remain for successfully applying CAR-T cells to solid tumors and other hematologic malignancies. In order to enhance the

therapeutic outcome of CAR-T cells it is important to design strategies to enhance T cell fitness, avoid tumor escape and control toxicities. Genome editing tools are a powerful platform to further optimize the activity of next generation CAR-T cells. Genome editing has been used to generate allogeneic "off-the-shelf" CAR-T cells, to eliminate detrimental genes for CAR-T cell development and function or to knockin the CAR or additional transgenes into desired loci of the T cell genome. Here, we will discuss some genome editing tools, protocols and strategies used to develop next generation CAR-T cells with improved therapeutic index.

INV10

Disease modelling by single-cell RNA sequencing (scRNAseq)

A Izeta [1]
1: Biodonostia Institute

The advent of single-cell RNA sequencing (scRNAseq) and additional single-cell omics technologies have provided scientists with unprecedented tools and datasets to explore biology at the cellular resolution. However, organs are complex and heterogeneous at the cellular level, single-cell experiments are costly, and extracting the enormous amounts of available information from published datasets is often difficult for researchers with limited computational expertise. In this educational session, I will introduce interested researchers to the disease modelling by scRNAseq field, using as a reference our work on how the different human skin cell types interact in homeostasis and what ceases to work in diverse dermatological diseases. The aims of this talk are to summarize the benefits and limitations of single-cell analysis, help the audience benefit from emerging insights from these novel techniques, and ultimately help accelerate translation of basic knowledge into novel diagnostic and therapeutic developments.

INV12

Imaging techniques for *in vivo* detection of advanced therapies in disease models

P Martin Duque [1]
1: Universidad de Zaragoza

Cancer is a disease that has been known for many centuries. The approaches used to eliminate it have been very numerous. But in addition to looking for new methods to eliminate tumors, another approach would be to find different ways to selectively deliver those therapies to the cancerous area.
Among the new areas of interest in oncology would be the use of molecular imaging. The visualization of therapies could shed light on their destinations and administration times, especially when they must be combined with others.
Our research in the search for new "Trojan horses" to bring various gene or cell therapies to tumors and imaging and visualization of these therapies (by PET, MRI, IVIS, etc.) can be extrapolated to other pathologies and development of diseases. We have shown it in diabetes and wounds but they might be used for other pathologies. All of those results will be presented in this talk

INV13

Lessons and challenges in the development of the vaccine MVA-COV2-S

M Esteban
1: Centro Nacional Biotecnología, CSIC, Madrid, Spain

The emergence of the COVID-19 pandemic caused a major health and economic burden to the world, with over 500 million infections and six million deaths. Within a short time the responsible viral agent, coronavirus SARS-CoV-2, spread rapidly to all continents due to an efficient transmission of the virus through the respiratory tract and the easy to infect a naïve immune population. Rapidly, the scientific community responded through the implementation of three strategies outlined by WHO to control a pandemic: quick diagnostic tests, antivirals and vaccines. A remarkable achievement was that within a year vaccination started, administering more than ten billion doses in what has become the largest vaccination program in history. Since then, more that 60% of the world´s population have received at least one dose of different COVID-19 approved vaccines. However, there is extreme inequity in the low- income countries where less than 10% of people have received the vaccines. From the beginning of the pandemic, our group of Poxvirus and Vaccines at the National Center of Biotechnology (CNB-CSIC) has actively participated in the development of a COVID-19 vaccine. Within a short time we generated a vaccine candidate, MVA-CoV2-S, and demonstrated in three preclinical animal models (mouse, hamster and macaques) that the vaccine was highly immunogenic and effective against SARS-CoV-2 infection. This rapid development was possible because we previously established a vaccine platform based on the highly attenuated poxvirus vector MVA (modified vaccinia virus Ankara) that we have used in the generation of vaccines against multiple pathogens (HIV, Ebola, chikungunya, zika, hepatitis C, malaria and leishmania), showing high immunogenicity and efficacy in animal models. In retrospect, in Spain we have learned important lessons from the pandemic, like preparedness for the making of human vaccines, the need for continuous surveillance of infectious agents, repositories and centers for rapid genomic analysis of variants of concern, active collaboration between research centers and hospitals, promote engagement of national industries to manufacture human vaccines, use of BSL-3 centers to address efficacy studies in animals, and make aware the society of the benefits of vaccines in the fight against SARS-CoV-2 and prevention of deaths. Questions like, when the pandemic will be over, consequences of long-COVID-19 in patients, duration of protective immune responses by current vaccines, newly developed vaccines against variants of concern, use of combined protocols of immunization and potential eradication of the virus are challenges being addressed.

INV15

Novel lentiviral pseudotypes for T and NK cell gene therapy and ‘nanoblades’ for efficient gene editing in hematopoietic gene therapy target cells and organoids

E Verhoeyen [1]
1: Inserm 2: CIRI; Inserm U1111

Chimeric Antigen Receptor (CAR)-T cell immunotherapy has modified the concept of treatment in hematological malignancies. In contrast to T cells, cord blood derived-NK cells (CB-NK) and CAR-

NK cells derived from CB-NK can be used “off-the-shelf” as immune cells with anti-tumor properties for the treatment of cancer patients. We have recently show that NK cells can be gene-modified by CAR-CD22 expressing LVs pseudotyped with baboon retroviral envelopes (BAEVs) at high efficiency and that these CAR+NKs show stronger anti-tumor activity than the original NKs. Moreover, in collaboration, we showed that cord blood derived NK enhanced the fitness of both CAR positive and CAR negative T cells, promoting lower levels of exhaustion and senescence.

We developed ‘nanoblades’ which are MLV- or HIV-derived virus like particle (VLP), in which the viral structural protein Gag has been fused to the Cas9, which are thus loaded with Cas9 protein together with gRNAs. To assure efficient nanoblade delivery of Cas9/sgRNA cargo into human T, B and HSCs we pseudotyped them with baboon retroviral, measles virus or/and VSV-G envelopes. We obtained efficient gene editing in T, B and HSCs. In addition, we inserted an expression cassette in a specific genomic locus of HSCs by combining nanoblades with AAV6 vectors carrying the donor cassette. Moreover, we have recently through bio-conjugation of AAV6 vectors with ligands increased AAV6 transduction of HSCs at high vector doses, while strongly reducing CD34+ cell toxicity. The introduction of a specific ligand on rAAV6 increased donor mediated gene knock-in and significantly increased HSC survival, an important feature for clinical translation of HSC gene editing strategies.
We will also report on the use of nanoblades for generation of CRISPR/Cas9 mediated knock-out of organoids.

INV19

Cancer Virotherapy with Oncolytic Adenoviruses

R Alemany [1 2]
1: Institut Catala d'Oncologia 2: IDIBELL-ICO

Lysis of tumor cells by oncolytic viruses is highly immunogenic and it has the potential to revert the immune suppression developed in the tumor microenvironment. Among different oncolytic viruses, human adenoviruses are non-enveloped DNA viruses with a cellular tropism and life cycle particularly suitable for oncolysis of tumors of epithelial origin. However, limited efficacy in clinical trials indicates that oncolytic adenoviruses need to be improved at different levels. Beyond targeting and intratumoral spread, humoral and cellular antiviral immune responses represent a major hurdle to overcome. Evading neutralizing antibodies to allow effective multiple systemic administrations and redirecting virocentric immune responses are pending issues to be solved.

INV21

Gene therapy for maturity-onset diabetes of the young type 3 (MODY3)

F Bosch [1]
1: Center for Animal Biotechnology and Gene Therapy

Maturity-onset diabetes of the young (MODY) are a group of monogenic diabetes characterized by onset of hyperglycemia at an early adult age. Among them, MODY3, the most common type of MODY, is caused by mutations in the gene encoding for the transcription factor hepatocyte nuclear factor 1A (HNF1A). Here, using a unique novel strategy based on the CRISPR/Cas9 technology, we generated the first MODY3 mouse model that presented beta-cell dysfunction and diabetes, faithfully mimicking the human clinical phenotype. MODY3 mice treated with AAV vectors encoding HNF1A showed markedly increased HNF1A protein content and upregulation of HNF1A target genes in islets, that resulted in counteraction of hyperglycemia. This study constitutes the first demonstration of genetic treatment for a monogenic diabetes and paves the way to clinical translation to MODY3 patients in the future.

INV22

Update on the clinical trial results of HSC-gene therapy for mucopolysaccharidosis type 1 Hurler

M E Bernardo [1]
1: SR TIget, Milan

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) +/- pre-/peri-transplant enzyme replacement therapy is standard of care for Hurler syndrome (MPS-IH). However, cognitive and

skeletal abnormalities progress over time after allo-HSCT, severely affecting patient's quality of life.
We report the interim results of a first-in-human phase I/II trial (NCT03488394) of 8 MPS-IH patients treated with autologous hematopoietic stem and progenitor cells (HSPC) genetically modified to overexpress human IDUA and followed-up for a median of 2 years after gene therapy (GT).
Patients (6 M, 2 F; median age at treatment 24 months) lacked a non-heterozygous-HLA-matched cord blood donor and displayed IQ/DQ>70. Primary endpoint of efficacy was blood IDUA activity up to supraphysiologic levels at 1y post-GT. Clearance of lysosomal storage material, skeletal and neurophysiological development were assessed as secondary and exploratory endpoints. Mean drug product $CD34^+$ cell dose was $20.9x10^6$/kg with a median vector copy number of 2.2 per genome. All patients had rapid hematologic recovery with median neutrophil engraftment on day +20 and short period of thrombocytopenia. The 5 patients positive for anti-IDUA antibodies before GT cleared them within 3 months after GT. The procedure was generally well tolerated. All patients showed sustained engraftment of gene-corrected cells with blood IDUA activity reaching supraphysiologic levels after GT in all patients, maintained at last follow-up. Urinary glycosaminoglycan (GAG) excretion levels reduced to normal or near-normal values by 1-year post-GT. IDUA activity in cerebrospinal fluid (CSF) became detectable by month 3 post-GT in all subjects accompanied by progressive decrease in GAG storage. With a median follow-up of 2 years, patients show stable cognitive and motor performances, reduced joint stiffness, improved or stable findings on brain and spine MRI and normal growth according to peers. HSPC-GT accomplishes extensive metabolic correction and initial clinical response with a favorable safety profile, highlighting its therapeutic potential for MPS-IH treatment.

INV24

AAV serotype associated impurities under a production platform

C Trigueros [1]
1: Viralgen SL

Adeno-associated viruses (AAVs) are among the most popular vectors for gene therapies due to its favorable tissue tropism and lack of pathogenicity. As recombinant AAVs are produced using living host cells some variability is involved in their manufacturing together with the presence of multiple contaminants such as empty capsids, immunogenic protein impurities and host cell DNA fragments.

Most of the AAV vector production to date has been performed at a small–medium scale using transfection-based production methods. In more advanced clinical trials or when moving to non-rare diseases, larger vector amounts are required. Viralgen is a CDMO that has developed a powerful and scalable platform for the production of AAV of diverse serotypes. Numerous batches have been already manufactured at 50, 250, 500 and 2000 liters in the past years, which have been used in several clinical trials around the world. This platform strategy has allowed us to generate a considerable amount of data, which results in an added value to try to understand the variability during the manufacturing processes, as well as the impurities associated with each serotype.

INV26

Non-viral engineering of therapeutic cells with the *Sleeping Beauty* transposon system

Z Ivics [1]
1: Paul Ehrlich Institute

Transposons are mobile genetic elements evolved to execute highly efficient integration of their genes into the genomes of their host cells. These natural DNA transfer vehicles have been harnessed as experimental tools for stably introducing a wide variety of foreign DNA sequences, including selectable marker genes, reporters, shRNA expression cassettes, mutagenic gene trap cassettes, and therapeutic gene constructs into the genomes of target cells in a regulated and highly efficient manner. Given that transposon components are typically supplied as naked nucleic acids (DNA and RNA) or recombinant protein, their use is simple, safe, and economically competitive. Thus, transposons enable several avenues for genome manipulations in vertebrates, including transgenesis for the generation of transgenic cells in tissue culture comprising the generation of pluripotent stem cells, the production of germline-transgenic animals for basic and applied research, forward genetic screens for functional gene annotation in model species and therapy of genetic disorders in humans. I will review the various parameters, considerations and the state-of-the-art in diverse genetic applications of *Sleeping Beauty*, one of the most widely used transposon systems currently available.

INV27

Towards the implementation of gene therapy in the clinical practice

Juan Bueren [2] Cristian Smerdou [1]
1: Cima Universidad de Navarra & Idisna, Pamplona, Spain 2: CIEMAT, CIBERER & IIS-FJD, Spain

After many years of efforts developed by the scientific community, including academic institutions and private companies, several gene therapy products have obtained marketing authorization in Europe. Furthermore, a large number of gene therapy trials are currently in advanced development stages. All this strongly suggests that in the coming years many gene therapy products will be available in different countries, including Spain. Consequently, in addition to the need to progress in developing more efficient therapies for debilitating diseases, further aspects will have to be considered to facilitate access of patients to these novel therapies. Indeed, the efficient implementation of gene therapy in Europe will rely on regulatory agencies and national health systems, but it will also depend on other factors, like strategic considerations from companies, hospitals, clinicians, scientists, and patient associations. To address challenges derived from the imminent approval of many gene therapy products, and to facilitate the access of patients to these therapies under conditions of quality, equity and with an optimal cost-effectiveness ratios, a multidisciplinary panel of experts from different areas, including gene therapy scientists, healthcare providers involved in rare diseases, healthcare managers, lawyers, economists, and patients, among others, have met to discuss these questions. Based on their deliberations, a document gathering multidisciplinary and comprehensive suggestions was elaborated. In addition to the Program of Advanced Therapies developed by the Spanish National

Health System, this document aims to facilitate a sustainable application of gene therapy in Spain. A summary of the main conclusions raised in this document will be presented.

INV28

Cell therapy based on the chimeric receptor NKG2D

A Perez Martinez [1]
1: Hospital La Paz

Natural killer group-2 member D (NKG2D) is an activating receptor expressed in cytotoxic lymphocytes that plays an important role in anti-tumor immunosurveillance. By recognition of the stress-inducible ligands MICA, MICB, and ULBP1-6, which are expressed on the surface of various tumor cells, including T, B, and myeloid leukemia blasts, the NKG2D receptor modulates lymphocyte activation and promotes target cell elimination. NKG2D chimeric antigen receptor (CAR) T cells have shown potent anticancer effects against various cancer types as pediatric sarcomas in preclinical studies and also have demonstrated safety and tolerability in relapsing/refractory (r/r) acute myeloid leukemia and myelodysplastic syndrome in adult patients. The use of allogeneic CAR T cells from healthy donors could be an attractive alternative because it presents many potential advantages, such as its immediate availability for all patients regardless of the hematological situation, standardization of the CAR T cell product, and reduced costs. However, the use of allogeneic T cells can cause undesirable graft versus host reactions and host immune rejection of infused non-HLA matched T-cells. We explore the use antigen-experienced memory T cells for CAR transduction as haploidentical $CD45RA^{+}$ depleted T cells. The immunoescape mechanisms to NKG2D approach may include release of NKG2DL, NKG2DL downregulation, NKG2D recognition on immune synapses, and inactivation by the immunosuppressive bone marrow niche, and leukemic cell clone selection.

INV32

Lentiviral Mediated Gene Therapy for Pyruvate Kinase Deficiency

J L Lopez-Lorenzo [1] A J Shah [2 3 4] S Navarro [5 6 7] O Quintana-Bustamante [5 6 7] J Sevilla [8] L Llanos [1] B Perez de Camino Gaisse [1] S Sanchez [1] B Glader [3 4] M Chien [3 4] B C Beard [9] K M Law [9] M Zeini [9] G Choi [9] E Nicoletti [9] G R Rao [9] M G Roncarolo [2 3 4] J A Bueren [5 6 7] J Schwartz [9] J C Segovia [5 6 7]

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Pyruvate kinase deficiency (PKD) is a rare inherited hemolytic anemia caused by mutations in the *PKLR* gene resulting in decreased red cell pyruvate kinase activity and impaired erythrocyte metabolism. Manifestations include anemia, reticulocytosis, splenomegaly and iron overload, and may be life-threatening. Current treatments are palliative with significant side effects. Preclinical studies in a clinically relevant PKD murine model demonstrated that infusion of gene-modified bone marrow (BM) cells may ameliorate PKD phenotype. Based on these preclinical data, a global Phase 1 clinical trial RP-L301-0119 (NCT04105166) is underway to evaluate the feasibility and safety of lentiviral mediated gene therapy. Six patients with severe PKD (defined as severe and/or transfusion-dependent anemia despite prior splenectomy) will be enrolled. Peripheral blood (PB) hematopoietic stem cells (HSCs) are transduced with PGK-coRPK-WPRE lentiviral vector (LV) and cryopreserved. Following release testing of the investigational product (RP-L301), patients receive myeloablative conditioning. RP-L301 is then thawed and infused. Patients are followed for safety assessments and efficacy parameters including PB and BM genetic correction and clinically significant improvement in anemia.

So far, two adult patients have been treated. Despite baseline hemoglobin (Hb) levels in the 7.0-7.5 g/dL range and substantial prior transfusion burden, both patients displayed normal-range hemoglobin (Hb), improved hemolysis markers, and have required no red blood cell transfusions post-engraftment. PB mononuclear cell VCNs for both patients were >2.0. No RP-L301 related serious adverse events have been observed after 18 months follow-up. In conclusion, , lentiviral gene therapy has shown to be safe and efficacious for the treatment of PKD.

INV33

Targeting the stem cell niche in the myeloid malignancies

S Méndez Ferrer [1]
1: University of Cambridge and NHS Blood and Transplant 2: On behalf of TAMARIN co-investigators

Haematopoietic stem cells (HSCs) reside in specialised niches that allow them to self-renew, proliferate, differentiate and migrate according to the organism's requirements. Our previous work showed that the brain regulates a peripheral stem cell niche in the bone marrow (Nature 452:442; Blood 133:224; Nat Comms 13:543), where mesenchymal stem cells (MSCs) play a key role in the normal HSC niche (Nature 466:829). Ageing is associated with an increased risk to develop myeloid malignancies, such as myeloproliferative neoplasms (MPNs) and acute myeloid leukaemia (AML). Our recent work showed that remodelling of bone marrow niches promotes myeloid cell expansion during premature or physiological ageing (Cell Stem Cell 25:407). Furthermore, AML cells co-opt energy sources and antioxidant defence mechanisms from HSC niche-forming MSCs to survive chemotherapy (Cell Metab 32:829), suggesting that adjuvant niche-targeting therapies could be therapeutic (Nat Rev Cancer 20:285). Indeed, damage to the HSC niche regulation is required for MPN progression (Nature 512:78). Along this line, the neuroendocrine regulation of bone marrow stem cells by noradrenergic signals (Haematologica 104:710) or by sex hormones (Cell Stem Cell 15:971) could potentially be harnessed to offer novel therapeutic approaches in MPN. The latter has been recently tested in a Phase II, multicentre, single arm clinical trial assessing tamoxifen's safety and activity in reducing molecular markers of disease burden in MPN (TAMARIN). The results suggest that tamoxifen can modulate unfolded protein response and inhibit mitochondrial respiration and pathogenic JAK-STAT signalling in a subset of potentially prospectively identifiable patients.

INV34

Correction of recessive dystrophic epidermolysis bullosa using genome editing strategies.

F Larcher [1]
1: CIEMAT

Genome editing technologies enable precise changes to DNA sequences and have the potential to treat genetic diseases. Epidermolysis bullosa (EB) is a group of rare genetic diseases characterized by dermal-epidermal adhesion defects. The recessive dystrophic subtype of EB (RDEB), which presents one of the most severe phenotypes, is caused by mutations in COL7A1, the gene encoding collagen VII, which is the main component of the anchoring fibrils that bind the dermis and epidermis. Gene editing approaches to correct pathogenic mutations in COL7a1 by NHEJ and, more precisely, HDR mechanisms have enabled the recovery of collagen 7 expression in patients' cells. Technical advances in delivery systems for CRISPR/Cas9 genomic nucleases in the form of ribonucleoproteins and template sequences using AAV vectors have achieved correction efficiencies that make it now possible to devise ex vivo gene editing-based therapeutic protocols that can be transferred to the clinic.

INV35

Computational biomechanics, 3D printing and hiPSCs: towards the generation of a biological ventricular assist device

M M Mazo Vega [1 13] O Iglesias-García [13] A Sánchez-Bueno [14] M Pérez Araluce [14] M E Fernández Santos [2] P Legazpi [2] A Sánchez de la Nava [2] S Janssens [10] F Fernández-Avilés [2] N Montserrat [12] M Serra [11] N Laita [3] M Rosales [3] E Peña [3] E Pueyo [3] M Doblaré [3] F Prosper [1] J Malda [6] J Gröll [9]
1: Clinica Universidad de Navarra 2: Fundación para la Investigación Biomédica del Hospital General Universitario Gregorio Marañón 3: Universidad de Zaragoza 4: Leartiker S. Coop 5: EBERS Medical Technology 6: Utrecht University 7: Boston Scientific 8: Technical University - TU Eindhoven 9: University Hospital Würzburg 10: Katholieke Universiteit Leuven 11: iBET 12: Institute for Bioengineering of Catalonia (IBEC) 13: CIMA 14: Universidad de Navarra

The explosive technological development in impacting areas such as human pluripotent stem cells (hiPSCs), biomaterials or additive manufacturing have put us closest to the aim of fabricating human tissues therapeutic tissues in the lab. However, there are crucial remaining factors, some technical, some economical, some regulatory, that hinder the final fulfilment of this ambitious aim. In the specific case of the myocardium, one such stepping stone is the relationship between cardiac muscle alignment and contractile output. In the project H2020 BRAV3, we are aiming at using computational modelling fed by cardiac-specific geometrical and mechanical data, to fabricate a biological ventricular assist device (BioVAD) to treat ischemic hearts. This BioVAD will supply new functional myocardium able to contract in the specific way a given myocardial ischemia requires (personalized). In order to do this, we are use a composite materials approach by which a melt electrospun scaffold in 3D printed in medical grade polycaprolactone, complying with the specific mechanical requirements of the heart. Human induced pluripotent stem cells are differentiated to the main cardiac phenotypes (cardiomyocyte, cardiac fibroblast, endothelium and smooth muscle), and employed to investigate the building of a functional BioVAD using either natural or synthetic biomaterials. At the same time, a large electromechanical bioreactor is designed and built, in order to promote the functional maturation of the fabricated BioVADs. All in all, the project results are advancing towards the aim of devising a new and potentially definitive therapeutic for myocardial infarction.

INV36

CAR-T cells for the treatment of multiple myeloma

C Fernandez de Larrea [1]
1: Hospital Clínic de Barcelona 2: IDIBAPS

Despite recent therapeutic advances, the prognosis of multiple myeloma (MM) patients when refractory to several drugs remains poor. Thus, new strategies using immunotherapy to improve outcomes are imperative. Chimeric antigen receptor (CAR) T-cell therapy is changing the treatment landscape of this disease, providing a very effective option for patients who are refractory to standard treatment. B-cell maturation antigen (BCMA)-targeted CAR T-cells have established outstanding results in heavily pre-treated patients. However, several other antigens are currently under investigation with promising results. Two products, Ide-cel and Cilta-cel have

been approved for clinical use. ARI0002h, our academic humanized CAR-T against BCMA, has shown very encouraging results both about efficacy and safety, with all 30 patients showing response, deeper clearance of bone marrow and extramedullary disease, as well as no neurotoxicity and low grade cytokine release syndrome. Unfortunately, relapses after CAR T-cell infusion are still reported. Hence, understanding the underlying mechanisms of resistance is essential to promote prevention strategies and to enhance CAR T-cell efficacy in this multiple myeloma.

INV43

Strategies to improve CAR-T cell therapy for solid tumors

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1: IDIBAPS

CAR-T cell therapy has achieved remarkable results by inducing high rates of sustained complete remissions in several hematologic malignancies, changing the paradigm of cancer treatment. Unfortunately, this success has not been recapitulated yet in the solid tumor setting, where objective clinical responses have been anecdotal and not durable. In spite of this, clinical experiences so far suggest that CAR-T cells are able to traffic to tumor sites, recognize and react against their target antigens, but they fail to persist, expand, and mediate productive and durable responses. Compared to hematologic malignancies, solid tumors pose extra barriers of complexity that CAR-T cells encounter upon arrival to tumors and need to overcome in order to be efficacious. Here, I will discuss such barriers and present new approaches to enhance the efficacy of CAR-T cells in the context of solid tumors, focusing on T cell extrinsic factors such as immunosuppression and tumor microenvironment.

INV47

Acute oxygen sensing in health and disease

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Oxygen (O_2) is needed for survival of most life forms on Earth and, therefore, the supply of sufficient O_2 to the tissues is a major physiological challenge. In mammals, a deficit of O_2 (hypoxia) triggers rapid cardiorespiratory reflexes (e.g. hyperventilation and increased heart output) that within a few seconds increase the uptake of O_2 by the lungs and its distribution throughout the body. These adaptive responses are essential for adaptation to high altitudes and for survival of patients with restrictions of gas exchange in the lungs. The prototypical organ within the "homeostatic acute O_2 sensing system" is the carotid body (CB), which contains sensory glomus cells expressing O_2-regulated ion channels. In response to hypoxia, glomus cells depolarize and release transmitters, which activate afferent fibers terminating at the brainstem respiratory and autonomic centers. The nature of the glomus cell O_2 sensor has been elusive but recent data have shown that these cells contain specialized mitochondria expressing high levels of specific enzymes and atypical electron transport chain (ETC) subunit isoforms. Glomus cells combine an accelerated ETC and high O_2 consumption with a cytochrome c oxidase having a low apparent

affinity for O_2. Hypoxia causes a slowdown of the ETC resulting in the accumulation of NADH and the production of H_2O_2, which are the signals that regulate membrane ion channels. Alterations of CB output can contribute to the pathophysiology of highly prevalent diseases such as respiratory depression, sleep apnea, or refractory hypertension. Disruption of the CB O_2 sensor may be also the cause of silent hypoxemia characteristic of some COVID-19 patients.

INV48

Engineered CRISPR prime editors with compact, untethered reverse transcriptases

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Prime editors (PEs) allow for the installation of a broad range of genetic edits. However, their large size makes them more challenging for research and therapeutic use. PE2, the most common PE system, relies on the use of a *Streptococcus pyogenes* Cas9 nickase (nSpCas9) fused to a pentamutant Moloney Murine Leukemia Virus reverse transcriptase (MMLV-RT). Here we report that that the separate expression of nSpCas9 and untethered MMLV-RT can still mediate efficient prime editing in human cells, which suggests that the RT functions *in trans*. We used this finding to rapidly engineer and test a variety of alternative RT domains, including a ~25% more compact variant of MMLV-RT that lacks the RNAse H domain as well as a bacterial group II intron maturase RT domain, that we engineered in a stepwise process in order to allow for prime editing in human cells. In sum, our findings improve the mechanistic understanding of prime editing, offer a platform for accelerated screening of new PE designs, and provide split and compact PEs with improved flexibility for expression and delivery in human cells.

INV49

Targeting and detection of cancer fusion oncogenes using CRISPR/Cas13

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Studies over the past decades have demonstrated the presence of specific fusion oncogenes (FOs) in almost 20% of human cancer. FOs provide unique diagnostic and therapeutic advantages because of their tumor-specific expression.

FO targeting diagnosis: Traditional diagnostic methods such as qRT-PCR, FISH, or NGS are routine in the clinic. However, those techniques are expensive, time-consuming, and contain multiple steps, requiring dedicated equipment and personnel.

FO targeted therapy: Many therapies currently used are non-selective, leading to severe side effects responsible for prolonged recovery, often followed by relapses.

In this sense, the identification of type II Cas13 nuclease, an RNA-targeting CRISPR enzyme, has opened up the possibility of selective cleavage of FO transcripts that could be used to develop new directed-therapies and highly-sensitive diagnostic options. Cell-based RNA targeting with Cas13 does not modify the DNA, like CRISPR/Cas9 system, and is associated with reversible and temporally controllable changes. Moreover, CRISPR/Cas13 system is correlated with high knockdown efficiencies and no off-target effects, contrary to shRNAs, offering unique advantages when used for therapeutic purposes. Diagnostic methods based on CRISPR/Cas13 provide rapid RNA detection with attomolar sensitivity and single-base mismatch specificity. We show our results in i) the set-up of a Cas13 system for selective degradation of FO RNA inducing efficient elimination of cancer cells, and ii) the development of a platform for highly-sensitive, specific, affordable, and instrument-free diagnostic test for FO detection by Cas13.

INV50

Genome editing approaches for beta hemoglobinopathies

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Beta-hemoglobinopathies are caused by mutations affecting adult hemoglobin β-chain production. The only curative treatment is allogenic hematopoietic stem/progenitor cells (HSPCs) transplantation, an approach limited by compatible donor availability and immunological complications. Therefore, transplantation of autologous, genetically modified HSPCs is an attractive therapeutic option. However, current gene therapy strategies based on the use of lentiviral vectors or CRISPR/Cas9 nuclease are not equally effective in all the patients and/or raise safety concerns. Base editing is a CRISPR/Cas9-based genome editing technology that allows the introduction of point mutations in the DNA without generating dangerous double strand breaks. Dr. Miccio will discuss base editing strategies aiming either to correct the genetic defect or target disease modifiers in patient cells and provide a safe and effective treatment for β-hemoglobinopathies.

INV53

Liver gene-editing based on nickase cas9 for the treatment of Primary Hiperoxaluria type I (PH1) is more efficient when using an all-in-one delivery system

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Targeted gene disruption mediated by programmable nuclease generates double-strand breaks (DSB) that are prevalently repaired by the error-prone non-homologous end-joining system (NHEJ). NHEJ introduces unpredictable deletions or insertions (indels) resulting in frameshift events or the introduction of early stop codon leading to the disruption of the gene function.

To minimize the variability of NHEJ *in vivo*, we co-injected two AAV-Cas9 nucleases carrying two different guides that target close regions in the *Hao1* gene in a mouse model of primary hyperoxaluria type I (PH1). NGS analysis showed an overall deletion of the sequence in between the two guide-targeted sequences, resulting in the complete elimination of the translated protein, as measured by western blot. However, this strategy might duplicate the chance of off-target events, a rising safety concern for clinical applications.

Thus, to generate a safer gene disruption, paired gRNAs were combined with the nickase variant of Cas9. Nickase Cas9 creates nicks instead of DSB, which are sensed by high-fidelity single-strand break repair pathways. Therefore, simultaneous nicks, appropriately spaced and oriented may lead to site-specific DSB and gene knockout.

Recently, we demonstrated that the simultaneous administration of two AAV-nickase Cas9, carrying the guides described above, mediated highly efficient disruption of *Hao1 in vivo*. By NGS analysis on-target, we determined that paired nickase Cas9 system do not lose the editing efficiency of paired nuclease Cas9. Additionally, in animals treated with only one guide we observed that individual nicks were faithfully repaired, as detected by NGS and normal target gene expression, thus minimizing potential off-target.

Moreover, we proved that the minimal AAV dose for targeted gene knockout can be reduced by combining nickase Cas9 and the two gRNAs in a single AAV vector (AAV-all-in-one). Additionally, we observed that the all-in-one system allowed the reduction of the vector dose to achieve a similar effect. More importantly, we established that *Hao1* disruption, mediated by AAV-all-in-one, at the minimal tested dose, is therapeutically relevant, in a mouse model of PH1.

Finally, CAST-seq quantitative analysis of eventual chromosomal rearrangements *in vivo* will further investigate the safety and clinical relevance of paired nickase system, for diseases that could benefit from permanent gene disruption like PH1.

INV54

In vivo gene editing for Mitochondrial NeuroGastroIntestinal Encephalomyopathy

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Mitochondrial NeuroGastroIntestinal Encephalomyopathy (MNGIE) is a rare mitochondrial disease caused by mutations in TYMP, a nuclear gene encoding thymidine phosphorylase, which results in a systemic accumulation of nucleosides (thymidine and deoxyuridine) and neurologic and gastrointestinal manifestations. In this study, we achieved an efficient integration of a human TYMP transgene into introns of the Tymp and Alb loci of hepatocytes in a murine model of the disease through the coordinated action of CRISPR/Cas9 and a TYMP cDNA template. CRISPR/Cas9 was delivered either as mRNA using lipid nanoparticles (LNP) or in an AAV2/8 viral vector; the latter was also used to package the TYMP cDNA. Insertion of the templates downstream of the Tymp and Alb promoters resulted in efficient transgene expression. Mice treated with a single i.v. dose of LNP carrying the CRISPR/Cas9 mRNAs showed a permanent (lifetime) reduction in plasma nucleosides, which was associated with the presence of human TYMP mRNA and functional enzyme in the liver. In mice with the edited Alb locus, the transgene expressed a secreted hybrid Alb-hTP protein, that was functional, with supra-physiological levels of TP activity in plasma. Gene editing was also detected, to a lesser extent, in mice receiving only the AAV vectors containing DNA templates, in the absence of Cas9, although with no detectable impact on plasma nucleoside levels. These results demonstrate the feasibility of liver-directed genome editing in the metabolic correction of MNGIE. Molecular analysis of on-target gene editing in the liver cells of mice treated long-term is currently ongoing.

INV55

Repurposing endogenous immune pathways to tailor and control chimeric antigen receptor T cell functionality

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Adoptive cell therapy based on chimeric antigen receptor-engineered T (CAR-T) cells has proven lifesaving for many cancer patients. However, its therapeutic efficacy is restricted to only a few malignancies, with solid tumors proving to be especially recalcitrant to safe therapy. Poor intra-tumor infiltration by T cells and T cell dysfunction due to an immunosuppressive microenvironment are key barriers against CAR-T cell success against solid tumors. Furthermore, boosting CAR-T cells uncontrollably would raise safety concerns. We present here T cell engineering strategies that illustrate how endogenous genes can be repurposed to improve persistence and potency of allogenic CAR-T cells in a tumor-specific manner using TALEN®-based gene editing.

INV56

Genome editing for improved allogeneic CAR-T cells

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CAR-T therapies have revolutionized cancer immunotherapy, achieving long-term responses in some B-cell malignancies. However, CAR-T cell approaches still present limitations for other hematological malignances as well as solid tumors, that compromise their therapeutic efficacy.

The immunosuppressive tumor microenvironment (TME) of solid tumors or the quality of T cells from heavily treated patients affect long-term persistence and functionality of CAR-T cells. Moreover, in some situations the fast and aggressive disease progression after relapse could hamper autologous CAR-T cell administration. Therefore, it is necessary to develop alternative strategies to make CAR-T therapies more efficient and accessible to all patients.

Allogeneic CAR-T cells are a valuable approach to reduce the risk of manufacture and/or therapeutic failure, as well as reduce the cost of the therapy. In this work we have explored the use of traditional CRISPR-based DNA-cleavage systems and novel DNA base editors, to deplete HLA-I and TCR complexes during CAR-T cell production, in order to avoid immune rejection and graft versus host disease. In addition, we have applied genome editing tools to introduce additional modifications in CAR-T cells to counteract immunosuppressive TME and to improve CAR-T cell efficacy. Moreover, we have combined genome editing technologies with virus-free gene-transfer strategies using *Sleeping Beauty* transposons, to generate highly innovative allogeneic CAR-T cells, that haven been fully characterized *in vitro* and *in vivo*. Finally, we have also implemented the generation of improved CAR-T cells at GMP level, in order to bring these innovative CAR-T cell product to the clinic.

INV60

Mechanistic insights into the response of Hematopoietic Stem Cells to Gene Editing

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Gene editing (GE) by artificial nucleases hold promise for gene therapy in Hematopoietic Stem and Progenitor Cells (HSPC). Despite rapid advances in GE-based therapies, a few challenges remain to be faced to improve GE efficiency and HSPC repopulating potential. We showed that the combination of nuclease-induced Double Strand Break with DNA repair template for Homology Directed Repair (HDR) delivered by AAV6 caused cumulative activation of the p53-mediated DNA Damage Response (DDR) pathway constraining HSPC proliferation and yield. Protracted DDR signaling leads to the establishment of cellular senescence, a condition of permanent cell cycle arrest. By integrating transcriptional analysis with innovative imaging-based

cellular assays we reported induction of cellular senescence markers and pro-inflammatory programs across edited HSPC subtypes and in vivo upon transplantation. Consistently, we found open chromatin at promoters of several senescence-gene categories and inflammatory genes of the IL1 axis and NF-kB pathway especially in HDR-edited cells. Mechanistically, we reported an ATM-p53 dependent activation of inflammatory cytokines in edited HSPC. Temporary inhibition of IL1 and NF-kB pathways at the time of GE increased edited HSPC clonogenicity in-vitro and long-term hematopoietic reconstitution in vivo with a concomitant decrease in senescence markers in edited HSPC. Our in vivo clonal tracking of HDR-edited HSPC revealed that IL1 inhibition improved polyclonal reconstitution preserving self-renewal and multi-potency of individual edited HSPC. Our findings define senescence and inflammatory programs as long-term consequences of CRISPR-Cas9 engineered human HSPC and pave the way for the development of novel strategies to overcome cellular barriers for efficient HSPC-based clinical applications.

Oral Presentations

OR01

Use and isolation of maternal milk exosomes: New vector for genetic hNIS therapy and diagnostic in oncology.

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Nowadays, exosomes are merging as potential therapeutic tools acting as nanocarriers of molecules and drugs due to its capacity to carry bioactive molecules that can be transferred into surrounding and distal cells. Maternal milk contains large amounts of exosomes with a major therapeutic or immunomodulatory potential

NIS gene allows the accumulation of iodine in the areas where it is expressed (using with the positron emitter isotope I^{124}, to be visualized by PET), or as a therapeutic gene for the treatment of tumors, allowing the accumulation of therapeutic doses of radioisotopes such as I^{131}. The high NIS expression on the breast while breastfeeding and the numerous exosomes obtained from maternal milk let us to think that the secretion of exosomes in maternal milk might lead to an endogenous NIS expression in those vesicles, and this could be translated into an antitumor and visualization potential or a therapeutic effect combined with I^{131}.

We studied the migratory properties of exosomes from maternal milk towards different types of solid tumors, with a great success, targeting specifically the metastatic nodules with sharp precision

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OR02

Lent-On-Plus: An all-in-one transactivator-free doxycycline inducible LVs ready for clinical applications.

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Tet-On systems induced by tetracycline and its derivatives are used for a variety of applications, including gene therapy. However, most of these Tet-On systems use rtTAtransactivator proteins (a TetR-VP16 chimera), which constitute a safety issue, since VP16 has been shown to cause different types of cellular toxicities by sequestering transcription factors and by activating cellular genes due to the binding of the TetR-VP16 protein to pseudo-TetO sites. In the present study we analysed the suitability of the Lent-On-Plus system (without transactivators) for clinical applications by studying doxycycline responsiveness, stability of the transgene over time and potential alterations of the cells RNA content on induced cells.For comparison, the same analysis was performed using the state of the art Tet-On 3G LVs from Takara bio. Our results show that the minimum dose necessary to achieve maximum GFP expression with the Lent-On-Plus system is 0.1ng/ml, while the Tet-On 3G system requires up to 3µg/ml, although the systems start to respond at 0.01ng/ml and 10ng/ml respectively. Additionally, transgene expression was stable for over 60 days in culture in cells transduced with the Lent-On-Plus system while around half of those generated with the Tet-On 3G LVs were silenced. More importantly, transcriptomic RNA-Seq analysis showed a large number of altered genes in cells generated with Tet-On 3G LVs, while these changes were minimal on cells generated with the Lent-On-Plus LVs.All this data together points to the Lent-On-Plus LVs as a safer alternative for gene therapy applications.

OR03

Using organoids to evaluate safety and potency of cell therapies

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Animal models currently used to test the efficacy and safety of cell therapies, mainly murine models, have important limitations as the underlying molecular and cellular mechanisms are often inherently different in humans, especially in the brain. Therefore, for translation to the clinic, the development of more complex and predictive models based on human cells may be crucial. Here, we have developed an *in vitro* model based on human forebrain organoids to study the differentiation potential of neural stem cells (NSC) and their safety and efficacy profiles as cell therapeutics. We generated brain organoids from iPSC lines and identified the formation of different cerebral tissues and cell types at different time points by the expression of typical markers from ventricular zone, ependyma, choroid plexus, cerebral cortex, microglia, oligodendrocyte precursors, astrocytes and neurons. After 4 months of culture, we transplanted different NSC lines transduced with EGFP into organoids. Live imaging of NSC-EGFP injected into organoids showed that cells integrated and migrated within the human tissue. We studied the differentiation potential, comparing these data with transplantation studies in the brain of NOD-SCID-γ mice. We found that NSC differentiate mostly into neuron and oligodendrocyte precursors both in mice and human organoid models although the number of neuroblasts were higher in mice models. Our results suggest that brain organoids can be used in the evaluation of

cell therapies, complementing the information obtained from animal models and increasing the predictability for future treatments, since it is a human model.

OR04

Development of biomimetic tympanic membrane substitutes for the treatment of chronic perforations

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Tympanic membrane perforations (TMP) represent a common cause of visit to the ear, nose, and throat specialist, usually due to recurrent infections or trauma. The rate of recovery depends on the TMP size and the presence of secondary infection. The current gold standard treatment (myringoplasty) presents relevant morbidity and costs. We aimed to develop and test a protein-based biomaterial to be used as a carrier in tympanic membrane (TM) regeneration. For this purpose, we designed scaffolds based on porcine gelatine and characterised them in terms of intrinsic properties (water uptake, water vapour transmission rate and degradation degree analyses), functional properties (puncture and mucoadhesion tests) and biocompatibility. The gelatine-based scaffolds showed adequate hydration and permeability properties, and did not degrade following 6-month exposure to water. The performance in the puncture and mucoadhesion tests was similar to native TM, and the scaffolds were fully biocompatible over one-week period. Encouraged by these results, we are currently conducting an *in vivo* study in a rat model of chronic TMP to study the integration, regeneration and functionality of the developed TM substitutes for a period of 8 weeks (N=4 per group). Outcome measures to monitor the evolution of the chronic perforations include the level of closure as measured by otoscopy and the detection of inflammatory markers in the blood at weeks 1, 4 and 8. Preliminary preclinical results will be presented at the meeting.

OR05

Using silent cells improves tumor homing and efficacy of of cell therapy for cancer

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Cell therapy for cancer is evolving rapidly. From the variety of strategies, cells can be used as carriers for delivering immunotherapeutic agents to the tumors. However, selection of the

appropriate cells to produce effective clinical outcomes is critical. We have studied a cohort of cancer patients treated with an immunotherapy consisting of mesenchymal stromal cells (MSCs) carrying oncolytic adenoviruses. The results indicated that patients treated with cells presenting deficit in stimulating the immune system showed better clinical outcomes.

We hypothesize that therapies based on cells presenting low pro-inflammatory profiles ('silent cells') upon systemic administration would result in better antitumor responses. We studied our hypothesis in immunocompetent mice treated with MSCs deficient for TLR4, MyD88 or MAVS as models of silent cells.

Interestingly, in vivo tumor homing after systemic administration of silent cells was significantly higher. This better homing to the tumor site was highly related to the mild immune response triggered by these silent cells in peripheral blood, evading the attack of the immune system against the carrier cell. As a result, the use of silent cells significantly improved the antitumor efficacy of the treatment in comparison to the use of wildtype MSCs. Moreover, it also increased the density of tumor-infiltrating T cells and induced the activation of the NF-κB pathway in the tumor.

In conclusion, while cell therapies in cancer generally aim to boost local immune responses in the tumor microenvironment, low systemic inflammation after systemic administration of the treatment may indeed enhance tumor-homing and the overall antitumor effect.

OR06

Expression of Galectin-3 inhibitors from a self-replicating RNA vector as treatment for pediatric osteosarcoma

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The outcomes of metastatic and nonresponder pediatric osteosarcoma (OS) patients are very poor and have not improved in the last 30 years. These tumors harbor a highly immunosuppressive environment, making existing immunotherapies ineffective. Here, we evaluated the use of Semliki Forest virus (SFV) vectors expressing galectin-3 (Gal3) inhibitors as therapeutic tools, since both the inhibition of Gal3, which is involved in immunosuppression and metastasis, and virotherapy based on SFV have been demonstrated to reduce tumor progression in different tumor models. In vitro, inhibitors based on the Gal3 amino-terminal domain alone (Gal3-N) or fused to a Gal3 peptide inhibitor (Gal3-N-C12) were able to block the binding of Gal3 to the surface of activated T cells. In vivo, SFV expressing Gal3-N-C12 induced strong antitumor responses in orthotopic K7M2 and MOS-J osteosarcoma tumors, leading to complete regressions in 47% and 30% of mice, respectively. Pulmonary metastases were also reduced in K7M2 tumor-bearing mice after treatment with SFV-Gal3-N-C12. Both the antitumor and antimetastatic responses were dependent on modulation of the immune system, primarily including an increase in tumor-infiltrating lymphocytes and a reduction in the immunosuppressive environment inside tumors. Our results demonstrated that SFV-Gal3-N-C12 could constitute a potential therapeutic agent for osteosarcoma patients expressing Gal3.

OR07

Hyperoxaluric phenotype improvement after *in vivo* lentiviral vector gene therapy in a preclinical model of Primary Hyperoxaluria Type 1

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Primary Hyperoxaluria Type 1 (PH1) is a rare genetic disease with hepatic origin caused by mutations in the *AGXT1* gene which codes for the liver enzyme alanine-glyoxylate aminotransferase. PH1 patients suffer from oxalate overproduction that can result in end-stage renal disease and life-threatening oxalosis. Thirty percent patients show infantile onset. The only curative treatment is liver and kidney double-transplant. Therefore, new therapeutic approaches are needed. *In vivo* lentiviral vector-based gene therapy has emerged as a promising therapy for liver monogenic diseases, especially in pediatric patients. A preclinical study of *in vivo* LV gene therapy in a PH1 mouse model has been conducted. Adult *Agxt1* KO mice were intravenously injected with different doses of a hepatocyte-specific LV expressing an EGFP reporter gene or an *AGXT* cDNA. Mice treated with the reporter LV revealed a transduction percentage up to 10%. To analyse the reversion of the PH1 phenotype, three weeks after LV injection of equivalent doses of LV expressing the *AGXT* cDNA, mice were subjected to an ethylene glycol challenge to induce overload in oxalate production. Treated mice showed a significant reduction in urine oxalate overproduction, prevention of weight loss, and no signs of nephrocalcinosis, achieving a partial pathological phenotype reversion of the PH1 pathology. Due to the cell-autonomous character of this disease, the observed phenotype reversion with such a limited number of corrected cells (estimated in 10%) opens the possibility of using LV for the treatment of PH1 and lays the foundations for future therapeutic approaches for PH1.

OR08

Isogenic GAA-KO murine muscle cell lines mimicking severe Pompe mutations as preclinical models for screening of potential gene therapy strategies

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Pompe disease (PD) is a rare disorder caused by mutations in the acid alphaglucosidase (GAA) gene. Most gene therapies (GT) partially rely on cross-correction of unmodified cells through the uptake of GAA enzyme secreted by corrected cells. In the present study, different chimeric murine GAA proteins (IFG, IFLG and 2G) were designed with the aim to improve their therapeutic activity. Additionally, we generated isogenic murine GAA-KO cell lines resembling severe mutations from Pompe patients. All GAA-KO cells generated lacked of GAA activity, presented increased autophagy and glycogen content upon myotube differentiation as well as downregulation of mannose 6-phosphate receptors (CI-MPRs), validating them as models for PD. Phenotypic rescue analyses using lentiviral vectors point to IFG chimera as the best candidate in restoring GAA activity, normalizing the autophagic marker p62 and surface levels of CI-MPRs. Interestingly, in vivo administration of liver-directed AAVs expressing the chimeras further confirmed the good behaviour of IFG, achieving crosscorrection in heart tissue. In summary, we have generated different isogenic murine muscle cell lines mimicking severe PD phenotype and validated their applicability as preclinical models in order to reduce animal experimentation.

OR09

Effect of the disease stage over the phenotype and functionality of CAR-T cells in multiple myeloma patients

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Refractory or relapsed (R/R) multiple myeloma (MM) is nowadays an incurable disease. CAR-T immunotherapy against BCMA has proven its efficacy with complete response rates of 83-85%. However, despite the initial favorable response, all patients relapse with a progression free survival time of 8-12 months. We hypothesize that this lack of long-term response could be related to fitness and functionality of the CAR-T cells generated from these patients with R/R MM. Thus, we believe that CAR-T cells generated from patients in the early stages of the disease would be more functional.

The objective of this work is to perform a phenotypic and functional characterization of CAR-T produced from healthy donors and MM patients at different disease stage (MGUS, SMM, MM and R/R MM). Thus, we have generated BCMA-targeting CAR-T from each group of donor/patients

that were fully characterized using flow cytometry and functional assays including cytotoxicity, cytokine production and *in vivo* antitumoral efficacy. We observed that CAR-T cells produced from MM and R/R MM patients showed decreased memory phenotype, a reduced cytotoxicity against MM cell lines and lower production of IFNg, suggesting that long-term functionality of CAR-T cells from those patients would be compromised. Transcriptomic analyses by RNAseq corroborated functional results providing valuable mechanistic insights.

In conclusion, our results indicate that CAR-T generated from patients with later stages of the disease show phenotypes associated with worst prognosis and less functionality than those produced from healthy donors or patients in early stages of the disease.

OR10

Efficient treatment of cortical T cell acute lymphoblastic leukemia with T lymphocytes secreting anti-AntigenA T cell engagers

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The dismal clinical outcome of relapsed/refractory (R/R) T-cell acute lymphoblastic leukemia (T-ALL) highlights the need for effective targeted therapeutic strategies. Despite chimeric antigen receptor (CAR)-engineered T cells have revolutionized the treatment of B-cell malignancies, their clinical implementation in T-ALL is on its infancy. In this context, Antigen-A (AgA) represents a safe target for cortical T-ALL (coT-ALL) patients. T-ALL relapses are commonly very aggressive and hyperleukocytic, posing a challenge to recover sufficient non-leukemic mature T cells from leukapheresis in R/R T-ALL patients. Here, we report a comprehensive study comparing the efficacy of engineered T cells either expressing a second-generation AgA-CAR or Secreting AgAxCD3 T cell-engaging Antibodies (AgA-STAb). AgA-T cell engagers bind to cell surface expressed AgA and CD3 and specific T cell activation. We show that AgA-STAb recruitment of bystander T cells provides AgA-STAbs with enhanced *in vitro* cytotoxic efficiency than AgA-CAR T cells at much lower effector:target ratios. AgA-STAb cells are as effective as AgA-CAR T cells in cuttin-edge in vivo T-ALL patient-derived xenograft models. Our data suggests that AgA-STAb-T cells could be an alternative to AgA-CAR T-cells in coT-ALL patients, especially for those with aggressive and hyperleukocytic relapses with limited numbers of non-leukemic effector T cells.

OR11

Gene therapy for ALS by specifically overexpressing a pleiotropic chronokine, secreted α-Klotho (s-KL), in skeletal muscles

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Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disorder characterized by the loss of cortical and spinal motoneurons (MNs). Denervation of endplates and axonal retraction is thought to lead, in a "dying-back" pattern, to the death of MNs and subsequent muscle atrophy. ALS neuropathology is mainly associated with oxidative stress, inflammation, excitotoxicity, and mitochondrial dysfunction while chronokines like α-Klotho (α-KL) may counteract some of these pathways.

In the SOD1^{G93A} mouse model we have found decreased mRNA levels of α-KL in skeletal muscles, motor cortex and lumbar spinal cord. Furthermore, in rat spinal cord organotypic cultures, the overexpression of α-KL protects spinal MNs from glutamate-induced excitotoxicity.

Given the pleiotropic beneficial properties of α-KL, we hypothesized that boosting the expression in skeletal muscles through a gene therapy treatment would protect muscles from atrophy and prevent neuronal loss in SOD1^{G93A} mice. Our results show that the overexpression of secreted α-KL in muscles enhances motor function and delays disease onset as evidenced by rotarod and grip strength tests. Improvement of the functional outcome is corraborated by increased compound muscle action potential amplitudes of the tibialis anterior and the plantar interossei muscles compared to non-treated controls. Increased amplitude of motor evoked potentials also reflects the preservation of central connectivity between upper and lower MNs. All this correlates with a preservation of motoneurons, a higher number of innervated neuromuscular junctions and a heavier gastrocnemius muscle. These findings indicate that increasing the secretion of α-Klotho by muscles is a promising approach for promoting functional improvement in ALS.

OR12

Pathophysiological alterations of regulatory T cells in Parkinson's disease and generation of aSyn-CAR-Tregs as a novel therapeutic approach.

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Parkinson's disease (PD) is characterized by the progressive degeneration of nigrostriatal dopaminergic neurons, alpha-synuclein (aSyn) aggregation and chronic neuroinflammation. Recent studies show that aSyn aggregation leads to T-cell-mediated activation of the immune response, playing a major role in the regulation of the neuroinflammatory response and neurodegeneration. In particular, regulatory T cells (Tregs), which are the main physiological suppressors of the immune system, have been shown to be altered in PD patients, and their adoptive transfer reduces the neuroinflammation and protects against dopaminergic degeneration in preclinical PD models. In this work, we carried out a functional characterization of Tregs in murine models of PD. Interestingly, we found that the PD-associated mutation of LRRK2 (G2019S) decrease the population of C62L+ Tregs and the expression of CD73, both factors related with the maturity state and the suppressive capability of Tregs. In addition, with the aim to enhance the immunosuppressive effect of Tregs and to direct them to the damaged nigrostriatal pathway, we have generated CAR-T against aSyn aggregates (aSyn-CAR-T) that specifically recognize pathological aggregates of human aSyn and promote the activation of T cells. Moreover, experiments of adoptive transfer in a human-aSyn mouse PD model indicate that the transferred aSyn-CAR-T-cells remain in peripheral blood and are able to infiltrate the damaged nigrostriatal pathway. The identification of functional Tregs alterations associated with PD and the preclinical validation of the aSyn-CAR-Tregs outcome would be important steps to design new Tregs-based therapies in PD.

OR13

Correction of haematopoietic stem cells from Bernard-Soulier syndrome patients and disease models by gene therapy.

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Platelets recognize injured vascular vessels by the interaction between GPIB-V-IX platelet receptor and von Willebrand factor (VWF), contained in the exposed subendothelial matrix of the damaged area. The absence of this receptor impedes proper plug formation because platelets cannot recognize damaged vessels. This is the origin of an extremely rare disease denominated Bernard-Soulier Syndrome (BSS). Mutations of GPIB-V-IX receptor genes avoid its migration to the membrane and consequently clot formation.

Last decades, several gene therapy approaches for hematologic diseases have been successfully carried out. Our lab has developed a novel treatment for BSS subtype C based on the use of Lentiviral Vectors (LVs) to revert the disease originated by *GP9* mutations.

Firstly, we have reverted the disease phenotype on a *GP9*-Knock-out megakaryoblastic cell line (DAMI), which regularly expresses the GPIB-V-IX complex on its surface. Secondly, we have rescued a BSS model based on iPSCs from BSS patients. This model allows us to analyze our LVs behaviour along a differentiation process. Finally, we have isolated Hematopoietic Stem Cells

from BSS peripheral blood samples and treated them with our LVs. The reappearance of the complex after a differentiation towards megakaryocytic lineage indicates the reversal of BSS *in vitro*. All together our results demonstrated that we can revert GPIX expression on human BSS models.

Next, we will complete rescue experiments in a *GP9*-knock-out BSS animal model. We consider that all these results will allow us to propose an *ex vivo* curative LV-based gene therapy on Hematopoietic Stem Cells from BSS patients.

OR14

Towards Lentiviral Gene Therapy for *RPS19*-Diamond Blackfan Anemia Patients

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Diamond Blackfan anemia (DBA) is a rare bone marrow failure syndrome (BMFS) with an estimated prevalence of 7 cases per million, in which the hallmark of the disease is macrocytic anemia. Allogenic hematopoietic stem cell transplantation (HSCT) represents the only curative treatment for the patients with DBA. Mutations in ribosomal protein (RP) gene *RPS19* are present in around 25% of DBA patients. Therefore, here we aimed at the development of a clinically applicable lentiviral mediated *ex vivo* gene therapy to correct *RPS19*-haploinsuficient DBA HSCs. The therapeutic efficacy of *PGK.CoRPS19.Wpre** and *EF1α(s).CoRPS19.Wpre**-LVs was confirmed in primary $CD34^+$ cells from *RPS19*-deficient patients. Transduction of $CD34^+$ cells from these patients with the therapeutic LV was not toxic for the hematopoietic progenitor cells. Moreover, the therapeutic LV reverted the red blood cell differentiation defect characteristic of DBA $CD34^+$ cells, increasing the output of $CD71^+/CD235^+$ mature erythroid cells, both *in vitro* and *in vivo*, and preserved the hematopoietic repopulationin NSG mice. Additionally, preliminary safety studies, showed that the ectopic expression of *RPS19* did not produce evident changes in the repopulating function of healthy donors (HD) $CD34^+$ cells. These studies were also confirmed by examining the healthy status of transplanted immunodeficient recipients, and also by investigating the clonal repertoire of transduced HSCs in transplanted mice. Taken together, the preclinical studies conducted in this work support that the proposed gene therapy appraoch should constitute an efficient and safe approach for the treatment of RPS19 DBA patients.

OR15

Alterations in extracellular matrix deposition by wound bed fibroblasts during cutaneous wound healing of aged *Sox2*^{EGFP} mice

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Transcription factor Sox2 is a master regulator in adult stem cells and a key determinant of tissue homeostasis and regeneration. *Sox2* expression levels are reduced in aging. We have previously shown that the combination of aging together with *Sox2* haploinsufficiency (the existence of a single functional *Sox2* allele in *Sox2*^{EGFP} mice) generates an imbalance in the pool of adult stem cells in the skin, which in turn show an age-associated regenerative deficit. We hypothesized in this work that dermal stem cell deficit would also generate alterations in the wound healing capacity of these mice. We performed full-thickness splinted excisional wounds in the dorsal skin of aged (>19-month) *Sox2*^{EGFP} mice and *wt* littermates (N=4 per group). Differences in wound closure rates and morphometric analyses of wound closure at day 10 post-wounding were non-significant between old *Sox2*^{EGFP} and *wt* mice. However, we observed higher deposition levels of collagen in old *wt* mice. Moreover, the wound bed of *wt* mice presented long-shaped, fibrotic like cells, within a compact granulation tissue; whereas in the old *Sox2*^{EGFP} mice rounded fibroblasts were embedded in loose granulation tissue. Further, we identified a significant decrease in elastic fibers in healed wounds of old *Sox2*^{EGFP} mice. Altogether, our results demonstrate that old *Sox2*^{EGFP} mice present a deficit (delayed deposition) in extracellular matrix components such as elastic fibers and collagen by wound bed fibroblasts. These results may represent a novel example of dysfunction in the microenvironment preceding loss of regenerative capacity of the stem cell compartment of an adult tissue.

OR16

Genome editing for genodermatoses therapy: correction of a Netherton syndrome patient´s cells carrying a deep intronic pathogenic variation.

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The establishment of effective preclinical protocols for gene editing in skin is necessary for translation to the clinic.Precise CRISPR-mediated deletions can be generated very efficiently in the genome of patients' skin cells to correct the effect of different types of deleterious variations underlying genodermatoses. Gene correction by gene editing allows endogenous regulation of expression to be maintained while respecting the endogenous spatiotemporal expression patterns of the corrected genes. Deep intronic variants that disrupt transcription causing altered gene splicing can be addressed by making small deletions.

Netherton syndrome, an inherited form of ichtyosis that causes atopic skin manifestations, growth retardation and hair defects is associated with genetic variations in SPINK5, most of them resulting in absence of LEKTI, a protease inhibitor precursor protein encoded by this gene. We have characterized a novel pathogenic allele in a Netherton patient that consists of a rare deep intronic SPINK5 variation that, in the particular intronic genetic background of this allele, results in an out of frame pseudoexon retention. *Ex vivo* editing to remove the pseudoexon sequence was performed to restore proper SPINK5 splicing ,and thus LEKTI expression, in skin cells from this patient. Human skin tissue regenerated from the gene-edited cells showed LEKTI expression in suprabasal layers of epidermis and normal epidermal differentiation.

OR17

Correction of congenital dyserythropoietic anemia type II using lentiviral gene therapy

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Congenital dyserythropoietic anemia type II (CDAII) is a rare inherited disorder that affects red blood cell development. CDAII patients show hypercellular in the bone marrow, erythroid hyperplasia and the presence of binucleated erythroid cells. CDAII is caused by mutations in the *SEC23B* gene. SEC23B is involved in protein processing and Golgi-reticulum trafficking. CDAII management is generally limited to blood transfusion and iron chelation, being allogeneic hematopoietic stem cell transplant (HSCT) the only curative option. Consequently, autologous HSCT of genetically corrected cells can offer a definitive treatment for CDAII.

To develop a gene therapy for CDAII, we have designed two lentiviral vectors carrying the wild type or the codon optimized SEC23B cDNA (wtSEC23B LV and coSEC23B LV). We had generated *SEC23B* knock-out human hematopoietic progenitors (*SEC23B*^{KO} CD34^{+} cells) using the CRISPR/Cas9 system to assess the efficacy of the lentiviral vectors. *SEC23B*^{KO} CD34^{+} cells reproduced the erythroid defects observed in CDAII patients. Moreover, LV transduced *SEC23B*^{KO} CD34^{+} cells increased SEC23B protein expression and restored their normal capacity for *in vitro* erythroid differentiation. Similarly, *in vivo* erythroid differentiation in NBSGW mice showed a selection of the transduced cells, accompanied by a reduction in binucleated cells. Furthermore, SEC23B LV transduction of peripheral blood CD34^{+} cells from CDAII patients improved their erythroid differentiation potential with a reduction in the percentage of binucleated erythroid cells.

In summary, SEC23B LVs compensate for the SEC23B deficiency in *SEC23B*^{KO} CD34^{+} cells and in patients' hematopoietic progenitor cells, which opens the possibility of future treatment of CDAII by gene therapy.

OR18

Synapse topology and downmodulation events determine the functional outcome of anti-CD19 T cell-redirecting strategies

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Immunotherapeutic approaches focused on the redirection of T cell activity toward tumor cells are actively being investigated. Strategies based on the endogenous secretion of T cell redirecting bispecific antibodies by engineered T lymphocytes (STAb-T) are emerging as alternative or complementary approaches to those based on chimeric antigen receptors (CAR-T). The anti-tumor efficacy of bispecific anti-CD19 x anti-CD3 (CD19xCD3) T cell engager (BiTE)-secreting STAb-T cells has been demonstrated in several mouse models of B cell acute leukemia.

Here, we have investigated the spatial topology and downstream signaling of the artificial immunological synapses (IS) that are formed by CAR-T or STAb-T cells. Upon interaction with CD19-positive target cells, STAb-T cells form IS with structure and signal transduction which more closely resemble those of physiological cognate IS, compared to IS formed by CAR-T cells expressing a second-generation CAR bearing the same CD19-single-chain variable fragment. Importantly, while CD3 is maintained at detectable levels on the surface of STAb-T cells, indicating sustained activation mediated by the secreted BiTE, the anti-CD19 CAR was rapidly downmodulated, which correlated with a more transient downstream signaling. Furthermore, CAR-T cells, but not STAb-T cells, provoked an acute loss of CD19 in target cells. Such differences might represent advantages of the STAb-T strategy over the CAR-T approach and should be carefully considered in order to develop more effective and safer treatments for hematological malignancies.

OR19

Preclinical development of low-affinity HER2-specific CAR-T cells for the treatment of HER2+ breast cancer

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On-target off-tumor toxicity is a major limitation in the development of CAR-T cells for solid tumours. Here, we hypothesized that modifying T-cells with a low-affinity CAR may be a safe therapy for the treatment of patients with Her2-positive breast cancers. CARs that contain a scFv targeting HER2 with low affinity and differ in the transmembrane (TMD) and intracellular domains

(ICD) were designed and effectively expressed on the T-cells. The combination of the CD8α TMD with the 41BB ICD conferred the best antitumor effect with long-term persistence. Modifying the TMD of the Her2BBz-CAR strikingly impaired anti-tumour activity, suggesting a key role for the TMD on CAR-T cell function. Because high-affinity Her2-CART cells have induced severe toxicity in patients with cancer, we next compared the low-affinity Her2-BBz with a high affinity CAR. We demonstrated that high-affinity CAR-T cells can react to physiological levels of HER2 when cocultured with a panel of primary cell lines. By contrast, low-affinity Her2-CAR-T cells exhibited no reactivity, as evidenced by low of levels of CD107a expression and the inability to release cytokines and induce cell killing. Finally, using mice implanted with a HER2-low tumour in one flank and a HER2-high tumour in the other flank, we show that, while high-affinity CAR-T cells were able to completely eliminate all tumours, low-affinity Her2-BBz CAR-T cells could eliminate HER2-high tumours while sparing HER2-low tumours.

We conclude that the use of low-affinity Her2BBz-CAR-T cells could be a safe therapeutic option for patients with HER2+ breast cancer tumours.

OR20

Editing of Telomerase RNA Component gene in hematopoietic stem cells: An alternative tool to treat bone marrow failure in patients with autosomal dominant dyskeratosis congenita

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Dyskeratosis congenita (DC) is a very low prevalence disease included within inherited bone marrow failure (BMF) syndromes. It is caused by mutations in genes involved in telomere maintenance, leading to premature telomere shortening. One of the most prevalent mutated gene in DC is *TERC*, which encodes the telomerase long non-coding RNA component (TERC) and exhibits autosomal dominant inheritance (AD-DC). The allogeneic transplant of hematopoietic stem cells (HSCs) is the only curative treatment of the BMF. However, there are several limitations that hamper the success of allogeneic transplantation in a high percentage of DC patients. In order to develop an alternative curative treatment for BMF related to DC, we propose a gene therapy approach consisting of an autologous transplant of *ex vivo* corrected HSCs using CRISPR/Cas9 technology and adeno-associated vectors (AAVs). CRISPR/Cas9 system produced a double strand break (DSB) that would be repaired by homologous recombination (HR) using the template delivered by the AAV which contains the corrected *TERC* gene and the homology arms. HR efficiency has been analysed in healthy $CD34^+$ total cells and in colony forming units. RNA expression and maturation patterns have been studied to demonstrate that were not affected as consequence of designed editing approach. Next steps will be to demonstrate phenotypic correction on *TERC*-deficient models and HSC from *TERC*-deficient patients.

OR21

Studying Hematopoietic Stem Cell clonality in Gene Editing Strategies using DNA Barcoding

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Gene editing strategies through homology directed repair (HDR) have shown great promise for the treatment of hematologic diseases. However, due to the homogeneous modification of the cell genome in HDR gene editing, clonality studies of edited cells cannot be carried out. DNA barcoding offers a powerful approach for labeling individual cells to allow individual tracking of cell progeny. Here, we describe a barcoding donor template strategy to track the clonal outputs of gene edited human $CD34^+$ cells.

First, we developed a barcode adenoasociated vector serotype 6 library including a semi-random 65bp sequence oligonucleotide carrying the required sequences to knock-in the *PKLR* locus. This system allows the tracking of up to 10^5 different clones. We used this barcode library to study gene editing conditions, addressing both gene editing efficacy and clonality of modified hematopoietic progenitors. Two different culture media in combination or not with StemRegenin 1 (SR-1) were tested. A high *in vitro* targeting efficiency, up to 60% targeted hematopoietic progenitors was obtained. When these edited hematopoietic progenitors was transplanted into immunodeficient mice, gene editing efficiencies were slightly reduced. Analysis of clonal output *in vivo* showed up to 300 individually marked HSPCs, with 9 to 12 unique HSPCs clones per mouse responsible for 90% of the human hematopoietic engraftment. No evidence of progressive clonal dominance in any of the conditions tested was observed

Overall, DNA barcode is a powerful approach to study hematopoietic clonality of gene edited HSPCs and to optimize gene editing procedures to make it clinically applicable.

OR22

CRISPR-based strategies revert the $LMNA^{R249W}$ induced phenotype both in cultured cells and *in vivo*

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The *LMNA* c.745C>T p.R249W variant is the most frequent mutation found in LMNA-related congenital muscular dystrophy (L-CMD). L-CMD is an infantile, striated muscle, laminopathy characterized by prominent axial weakness, motor development loss, neck weakness and respiratory, cardiac and gastrointestinal alterations with fatal consequences. Since L-CMD is a rare genetic disease without cure, our main objective is to obtain effective therapies to fight

against it. We have explored the therapeutic value of two different CRISPR-based strategies. One consists on the use of sgRNAs specific for the *LMNA* c.745C>T mutation, while the other depends on the homology independent targeted insertion (HITI) of a wild type, exon3-12 cassette upstream of the *LMNA* c.745C>T mutation. We have studied both approaches in human and mouse cells as well as in an *Lmna*^{R249W} mouse model. In cultured cells, 745C>T-sgRNAs were highly efficient against the mutant allele and showed low activity for the wild type one. On the other hand, a low percentage of cells were edited by HITI. Importantly, for both approaches, AAV-mediated gene therapy studies showed significant survival improvement of the early lethality associated with the *Lmna*$^{R249/R249W}$ mice. In all the cases, mouse phenotype and cellular abnormalities induced by the LMNA-R249W mutation were reverted to a wild type status. These results confirm the therapeutic potential of these two CRISPR-based strategies for the treatment of L-CMD.

OR23

Feasibility of generating universal ARI-0001 T cells for the treatment of type B malignances

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Autologous T cells expressing the Chimeric Antigen Receptor (CAR) have been approved as medicaments against several type B malignancies and multiple myeloma. In spite of the impressive benefit, there are still important limitations. A main drawback comes from its autologous nature that impedes that all the selected patients can be treated and that also precludes the standardization as a medicament. Allogeneic off-the-shelf-CAR-T cells are an alternative to simplify this complex and time-consuming process. Here we investigated the feasibility of generating TCRKO ARI-0001 CAR-T cells using CRISPR/Cas9 as ribonucleoprotein to eliminate the TCRα-chain and lentiviral vectors expressing the CAR through the EFI1-a promoter. We first analysed the efficacy and potential safety issues that arise during disruption of the TCR gene. We found that efficient TCRKO leads to on-target large deletions mainly mediated by microhomology repair mechanism (alt-EJ), indicating a potential safety risk of this procedure that need monitoring. Importantly, TCRKO-ARI-0001 CAR-T cells maintain similar phenotype and antitumor efficacy compared to ARI-0001 CAR-T cells, with an unexpected increase in mitochondrial fitness. In summary, we describe a feasible platform for generating allogenic CAR T cells, a closer step in the roadway to generate a CAR-T therapy for patients with no possibility to obtain clinical approved ARI-0001 CAR T cells.

Poster Presentations

P01

Generation and proof-of-concept for allogeneic CD123 CAR-Delta One T (DOT) cells in Acute Myeloid Leukemia

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Chimeric Antigen Receptor (CAR)-T cells have emerged as a breakthrough treatment for relapse/refractory (r/r) hematological tumors, showing impressive complete remission rates in B-cell malignancies. However, around 50% of the patients relapse before 1-year post-treatment. T-cell “fitness” is critical to prolong the persistence and activity of the adoptively transferred product. Allogeneic T cells from healthy donors are less dysfunctional or exhausted than autologous patient-derived T cells, enabling a very attractive and cost-effective “off-the-shelf” therapy option. In this context, Delta One T cells (DOTs), a recently described cellular product based on MHC/HLA-independent Vd1^{+} gd T cells generated from the peripheral blood of healthy donors, represent a robust platform of allogeneic effector T cells. Here we generated and pre-clinically validated 4-1BB-based CAR-DOTs directed against the IL-3a chain receptor (CD123), a target antigen widely expressed on acute myeloid leukemia (AML) blasts. CD123CAR-DOTs showed vigorous, superior to control DOTs, cytotoxicity against AML cell lines and primary samples both *in vitro* and *in vivo*. Continuous administration of IL-15 supported the long-term persistence of a single-dose CD123CAR-DOTs in patient-derived xenograft models, sustaining their anti-leukemic efficacy as demonstrated in a re-challenge assay *in vivo*. Our results provide proof-of-concept for an allogeneic next-generation therapy based on CD123CAR-DOTs for r/r AML patients.

P02

CD34^{+}CD19^{-}CD22^{+} B-cell progenitors might underlie phenotypic escape in patients treated with CD19-directed therapies

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CD19-directed immunotherapies have revolutionized the treatment of advanced B-ALL. Despite initial impressive rates of complete remission (CR) many patients ultimately relapse. The fact that B-ALL patients successfully treated with CD19-directed T-cells eventually relapse, coupled with the early onset of CD22 expression during B-cell development suggest that pre-existing $CD34^{+}CD22^{+}CD19^{-}$ (pre)-leukemic cells could represent an “early progenitor origin-related” mechanism underlying phenotypic escape to CD19-directed immunotherapies. We demonstrate that CD22 expression precedes CD19 expression during B-cell development. $CD34^{+}CD19^{-}CD22^{+}$ cells are found in diagnostic and relapsed BM samples of ~70% B-ALL patients, and their frequency increases 2-fold in B-ALL patients in CR after CD19-CAR T-cell therapy. Important, the median of $CD34^{+}CD19^{-}CD22^{+}$ cells before treatment was 3-fold higher in those B-ALL patients who relapse after CD19-directed immunotherapy (median follow-up of 24 months). FISH analysis in flow-sorted populations and xenograft modeling revealed that $CD34^{+}CD19^{-}CD22^{+}$ cells harbor the genetic abnormalities present at diagnosis and initiate leukemogenesis in NSG mice. We suggest that pre-leukemic $CD34^{+}CD19^{-}CD22^{+}$ progenitors underlie phenotypic escape after CD19-directed immunotherapies. Our data reinforces the ongoing clinical studies aimed at simultaneously targeting CD19 and CD22 as a strategy to reduce CD19- relapses and encourages the implementation of such CD34/CD19/CD22 panel in flow cytometry clinical laboratories for initial diagnosis and subsequent monitoring of B-ALL patients during CD19-targeted therapy.

P03

Overcoming CAR-mediated CD19 downmodulation and leukemia relapse with T lymphocytes secreting anti-CD19 T-cell engagers

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Chimeric antigen receptor (CAR)-modified T cells have revolutionized the treatment of CD19-positive hematologic malignancies. Although anti-CD19 CAR-engineered autologous T cells can induce remission in patients with B cell acute lymphoblastic leukemia, a large subset relapse, most of them with CD19-positive disease. Therefore, new therapeutic strategies are clearly needed. We have compared the antitumor efficacy of engineered T cells either expressing a second-generation anti-CD19 CAR (CAR-T19) or secreting a CD19/CD3-targeting bispecific T-cell engager antibody (STAb-T19). We found that STAb-T19 cells are more effective than CAR-T19 cells at inducing cytotoxicity, avoiding leukemia escape *in vitro*, and preventing relapse *in vivo*. We observed that leukemia escape *in vitro* is associated with rapid and drastic CAR-induced internalization of CD19, that is coupled with lysosome-mediated degradation, leading to the emergence of transiently CD19-negative leukemic cells that evade the immune response of engineered CAR-T19 cells. In contrast, engineered STAb-T19 cells prevent the CD19 downmodulation observed in anti-CD19 CAR-mediated interactions. Although both strategies show similar efficacy in short-term mouse models, there is a significant difference in a long-term patient-derived xenograft mouse model, where STAb-T19 cells efficiently eradicated leukemia cells, but leukemia relapsed after CAR-T19 therapy. Our findings suggest that the absence of CD19 down-modulation in the STAb-T19 strategy, coupled with the continued antibody secretion, allows an efficient recruitment of the endogenous T cell pool, resulting in fast and effective elimination of cancer cells that may prevent CD19-positive relapses frequently associated with CAR-T19 therapies.

P04

Physiological (TCR-like) regulated lentiviral vectors: a platform for the generation of improved CAR-T cells

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Anti-CD19-CAR-T cells for B malignancies have provide impressive clinical responses. However, important limitations still remain due to severe adverse events and relapse of ~50% of the treated

patients. Several studies indicate that expressing the CAR following the natural behaviour of T cell receptor (TCR-like) can improve CAR-T cell's fitness and their antitumor efficacy. However, different levels of CAR basal expression are required depending on the antigen's density of target cells or CAR's affinity. Here, we propose to use different TCR-like lentiviral vectors (LVs) to drive diverse expression levels as a platform to express different CARs. First, using a synthetic *WAS*-gene promoter, we generated LVs for driven low/moderate transgene expression, following TCR-like pattern and which were applied to the ARI-0001 academic CAR to create AWARI-CAR-T cells. AWARI-CAR-T cells exhibited lower tonic signalling, higher proportion of stem/memory T_{SCM}, less exhausted phenotype and milder secretion of TNF-α and IFN-ɣ after efficient destruction of CD19+ lymphoma cells, both *in vitro* and *in vivo*. Moreover, we also showed their improved efficacy using an *in vitro* CD19+ pancreatic tumour model. We next designed a panel of novel LVs for transgene expression through different chimeric promoters (CD4, B2M and LCK loci) with different strengths. All the generated-LVs mimicked the TCR/CD3 pattern after stimulation at different levels, although the B2M-LV was selected for its strong basal expression and quicker downregulation upon T cell activation. In summary, we present here two different platforms for CAR expression on T cells at different levels and with a TCR-like behaviour.

P05

Use of anti-GD2 (Dinutuximab) as a target for CAR-T cells immunotherapy in Neuroblastoma

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Treating solid tumours with chimeric antigen receptors-modified T cells (CAR-T cells) has shown limited efficacy due to the lack of cancer-type specific antigens. Dinutuximab is a monoclonal antibody that recognizes a sphingolipid, disialoganglioside GD2, which has limited expression in normal tissues but is overexpressed in paediatric tumours, mainly neuroblastoma (NB). Dinutuximab, an anti-GD2 antibody, is currently standard of care in the treatment of NB. We are exploring the possibility of using Dinutuximab as target element for anti-NB-CAR-T cells. Previously, our group have explored the feasibility of producing CAR-T cells from PBMNCs, CD45RA+ post-apheresis fraction and cord blood using similar protocols of T cell transduction and expansion. In this work we performed a characterization of phenotype and functionality of anti-FITC-CAR-T cells derived from different sources, and the cytotoxic effect against anti-GD2-FITC labelled cell line. First, we analysed different anti-FITC-CAR-T cells products attending to CD4/CD8 composition, effector/memory phenotype, activation markers and VCN. Next, to test CAR-T cell efficiency, we performed cocultures of anti-GD2-FITC labelled cell line with PB/45RA/CB-derived anti-FITC-CAR-T cells at different ratios, in which we analysed the inflammatory cytokines release by LegendPlex and the expression of early activation markers as CD25 and CD134 in CAR-T cells by flow cytometry. Also, we analysed the percentage of dead NB by flow cytometry. *In vivo* experiments are currently on-going to test the efficacy of CAR-T cells in combination with GD2 treatment. Our strategy may complement the current use of Dinutuximab in the treatment of NB through its combination with a targeted CAR-T cell approach.

P06

Transposon-based CAR-T cells for lymphoma treatment

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TranspoCART is a project conceived to develop an innovative CART medicinal product based on a viral vector-free gene-transfer strategy, which is based on the use of transposons. A *Sleeping Beauty* transposon minicircle (SBmC) carrying the 4-1BB CAR endodomain, an anti-CD19 ScFv and a truncated EGFR -as a safety switch- was designed for the treatment of lymphoma patients. Our optimized manufacturing protocol reached transposition efficacies up to 44% of CAR^+ cells using PB cells from healthy donors and 50% from patients' samples. Extensive *in vitro* characterization of TranspoCART19 cells showed an enriched stem-cell memory/central memory phenotype, no signs of cell exhaustion and high level of specific cytotoxicity activity against $CD19^+$ cells. Dilution of SBmC and hyperactive form of transposase (SB100x) during cell manufacturing was confirmed, with no detectable levels of both components at the end of the expansion period. The *in vivo* antitumoral efficacy of TranspoCART19 cells was evaluated in NALM6 xenograft models using immunodeficient NSG mice. Treated animals showed improved survival compared to untreated mice, showing a similar efficacy compared to lentivirus-produced CART cells. Biodistribution and toxicity studies showed a safe integration profile of the transposon. Additionally, the efficacy of the safety switch construct was confirmed *in vivo* using the same xenograft model. Finally, scale-up and GMP validations of TranspoCART19 cell manufacturing were carried out in two independent GMP facilities, which demonstrated that the protocol is reproducible and highly efficient. These encouraging results will allow us to initiate a clinical trial with the TransproCART19 cell product.

P07

Creation of next generation CAR-T by Repurposing endogenous immune pathways

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Adoptive Cell therapy (**ACT**) is an emerging field that shows promise for refractory leukemia or lymphoma patients. Among these, genetically engineered T cells expressing **Chimeric Antigen Receptors (CARs)**, have highlighted as a successful therapy for type B malignancies. Although CAR-T cell therapies hold great promise, still face multiple challenges, including toxicity, inactivation by the tumour microenvironment and low persistence in patients. In the present study, we explore the application of genome editing (GE) approach based on **CRISPR/Cas9** to repurpose **TCR** and **PD-1**, two major players of the T cell activation pathway. Our final aim is to engineer the TRAC locus to avoid Graft versus host disease (GVHD) (generating universal CAR-T cells) and the PD-1 locus expressing **IL-15** (generating T cells that respond to PD-L1 increasing their activity). For proof-of-principle, we first designed a donor DNA to insert IL-15-2A-eGFP into PD-1 *locus*. We used PCR products for donor delivery and RNP nucleofection with CRISPR/Cas9. Our first results show the appearance of CD3- T cells due to the TCR Knock-out, and GFP+PD-1- T cells indicating a specific integration of the donor in the target *locus*. We observed a high proportion of death cells (from 50% to 12% alive cells) as consequence of GE, probably due to the use of PCR product as donors. The same donors will be delivered as AAV6 in next experiments as an alternative to reduce cytotoxicity of the approach.

This strategy allows generating **CAR-T cells with exhaustion/activation dependent IL-15 secretion**, which should lead to i**mproved therapeutic efficacy.**

P08

Generation of CAR-T cells for the treatment of squamous cell carcinomas in Fanconi anemia patients

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Fanconi anemia (FA) is an inherited disease associated with bone marrow failure and cancer predisposition. In particular, FA patients have a 600-fold increased incidence of head and neck squamous cell carcinoma (HNSCC). Current therapies for FA-HNSCCs are inefficient and toxic due to the hypersensitivity of FA patients to radio-chemotherapy. Thus, immunotherapy with CAR-T cells constitutes a promising therapeutic approach for these tumors. Since the generation of CAR-T cells requires a significant ex vivo manipulation of T cells, and given that FA cells are characterized by a significant fragility compared to healthy cells, we investigated the possibility of generating CAR-T cells from FA patients as an autologous cell therapy against HNSCCs. Peripheral blood T-lymphocytes from healthy donors (HD) and FA patients were transduced with a gamma-retroviral vector kindly provided by J. Maher, that expresses a second generation CAR against the EGF receptor (EGFR), highly expressed in HNSCCs. In addition, we generated a novel

lentiviral vector harboring the EGFR-CAR and an inducible suicide gene, Cas9. Although the rate of expansion of retroviral-mediated FA CAR-T cells was lower as compared to HD CAR-T cells, in both instances strong cytotoxic effects against FA-HNSCC cells were observed. Preliminary results obtained in lentiviral-mediated CAR-T cells confirmed the efficient generation and cytotoxic activity of FA CAR-T cells. Moreover, in this case the induction of Cas9 resulted in the eradication of FA CAR-T from in vitro cultures. Our studies demonstrate the feasibility of generating CAR-T cells from FA patients for the autologous treatment of HNSCCs.

P09

Development of 4th generation regulated CAR-T cells (iTRUCKs) targeting pancreatic solid tumors

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The efficacy of chimeric antigen receptor (CAR)-redirected T cells for treating solid tumors remains challenging. Main hurdles include heterogeneous antigen expression, inability of CAR-T cells to infiltrate into the tumor, inhibitory effect of tumor microenvironment and poor persistence of CAR-T cells . Arming CAR-T cells with immunostimulatory molecules (4th generation CARs/TRUCKs) could be determinant to achieve therapeutic efficacy against solid tumors. However, continuous expression of these potent molecules may cause unacceptable side effects on patients. In this work, we used a doxycycline (dox)-inducible, transactivator-free lentiviral vector (LV) (Lent-On-Plus) to generate clinically-relevant inducible TRUCKs. Previous publications showed that IL-18 releasing CAR-T cells present enhanced antitumor effect in complex tumor models, since IL-18 is a master regulator of the immune environment. Here, we describe *first-in-class* anti-CD19-CAR-T cells engineered to release IL-18 only in the presence of dox (αCD19-iTRUCK-IL-18). These iTRUCKs tightly controlled IL-18 expression upon dox addition and exhibited enhanced antitumor potency against a metastatic pancreatic ductal adenocarcinoma model expressing different levels of CD19. Interestingly, the presence of the inductor increases clinically-desirable TSCM/TCM phenotype and reduces exhaustion markers after multiple tumor re-challenges. Additionally, αCD19-iTRUCK-IL-18 were able to polarize M2 (pro-tumor) to M1 (anti-tumor) macrophages in a dox-dependent manner. Finally, the oral administration of dox to orthotopically implanted pancreatic tumor model mice and inoculated with αCD19-iTRUCK-IL-18 induced stronger antitumor responses compared with those without dox. These results showed that Lent-On-Plus LVs can generate iTRUCKs controlling functional IL-18 release, constituting a promising and safer strategy to externally control the activity and potency of TRUCKs.

P10

TCR/HLA-I double knockout CAR-T cells maintain lytic ability with reduced allogenicity

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Therapy based on T cells expressing chimeric antigen receptors (CAR) is a novel treatment for lymphoid neoplasms, with 5 medicaments already approved for liquid cancers. However, currently approved CAR therapies are based on autologous T cells, which increases the cost and reduces the efficacy of the treatment. On the other hand, several clinical trials involving CAR T cells have been impacted by T cell–intrinsic dependent factors, associated with their phenotypes, which could be overcome by the selection and combination of specific subsets of less-differentiated state for the final CAR production. In this project, we expect to generate off-the-shelf allogeneic CAR-CD19 T cells with a defined phenotype.

We generated universal antiCD19-CAR T cells, disrupting the B2M and TRAC genes in CAR T cells to avoid both graft versus host and host versus graft reactions, and then edited cells were isolated according to their phenotype, to obtain cells with a memory phenotype that increase the persistence, proliferation, and antitumor effect *in vivo*. Functionally, these cells are able to eliminate tumoral cells *in vitro* with the same efficacy as non-edited CAR T cells, and they do not seem to respond against allogeneic cells as cells with both HLA-I and TCR complexes do.

The combination of gene editing of CAR T lymphocytes by TCR and B2M loci disruption, with the isolation of specific less-differentiated T subsets is an *in vitro* procedure developed with the translational objective of improving the clinical results of CAR T cells infusions in patients with refractory or relapsing B neoplasms.

P11

Challenges of CAR-T cells in solid tumours: modelling immunotherapy with 3D osteosarcoma cultures

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Standard two-dimensional cell culture techniques do not reproduce the dynamic and complex progression of osteosarcoma (OS) and fail to mimic cell-to-cell or cell-to-microenvironment interactions. Current three-dimensional (3D) models, also called organoids or spheroids, provide powerful models for studying cancer treatment, including immunotherapies. Despite the efficacy of chimeric antigen receptor (CAR) T cells in haematological tumours, sarcoma patients remain resistant to this immunotherapy. To overcome these limitations, various 3D cell culture techniques have been developed. Here, we describe an approach to study NKG2D-CAR T cells against OS cell lines, such as 143B or MnnG-hOS. We facilitated cell-cell interactions in scaffold-

free wells with U-shaped bottoms. NKG2D ligands, tumour cytotoxicity, cytokines release and phenotype of CAR T cells were analysed in the organoid model. We showed that 3D cultures decreased NKG2D ligands, which are targets of our NKG2D-CAR T cells. Despite this ligand depletion, we demonstrate that NKG2D-CAR T cells were able to infiltrate OS organoids and induce anti-tumour activity. Infiltrating NKG2D-CAR T cells have different activation phenotype compared to non-infiltrating cells. In this regard, differences in cytokine expression were also observed in 3D co-cultures. In conclusion, OS organoids represent a valuable tool to study CAR T-tumour interactions because they represent a physiologically relevant model.

P12

Chronic antigen exposure induces CAR-T cell loss of function and a dysregulated gene expression profile leading to treatment failure in solid tumors

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CAR-T cell immunotherapy has achieved remarkable success in hematologic malignancies but remains ineffective in solid tumors. CAR-T cell dysfunction in the tumor microenvironment is a major hurdle for CAR-T therapy. Here we have established and validated an in vivo mouse model to study the T-cell intrinsic mechanistic pathways leading to CAR-T cell unresponsiveness. We have isolated tumor infiltrating T cells at two different phases: 1) after T cell activation that leads to tumor regression (named as “Effective CAR-T cells”) and 2) at a second phase in which persisting antigen leads to loss of T-cell functions and tumor progression (named as “Dysfunctional CAR-T cells”).

Dysfunctional TILs recapitulated features of T cell exhaustion including high level of PD-1 inhibitory marker expression and loss of Ki67 proliferation marker. Effective but not dysfunctional TILs displayed cytotoxic activity and released IFNγ when co-cultured with tumor cells ex vivo.

RNAseq analysis revealed extensive differences between effective and dysfunctional TILs with more than 2.000 differentially expressed genes. Dysfunctional CAR-TILs displayed a transcriptional profile overlapping with gene sets described in models of chronic viral infections and in dysfunctional human TILs. Surprisingly, while effective TILs retained their effector functions and were able to induce tumor regression in mice, T cell exhaustion gene signatures were also early enriched in effective TILs. Gene clusters and pathways possibly implicated in the dysregulated gene expression signature will be discussed. Altogether, our results suggest that CAR-T cell dysfunction needs to be addressed to unleash the full potential of CAR-T cell therapy in solid tumors.

P13

Elucidating the role of PD-1 genome editing in CAR-T cell function

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CAR-T cell therapy of solid tumours faces several hurdles, including dysfunction of the therapeutic T-cells. While inhibition of the PD-1/PD-L1 axis is being actively investigated, there is still controversy about the effects of long-lasting PD-1 genetic deletion on CAR-T cell function. Here, we hypothesized that consequences of the PD1-PDL1 axis on CAR-T cell therapy may depend on the CAR design and PD-L1 densities in the tumour. To test this, we generated a model of cancer cell lines expressing varying PD-L1 densities. PDCD1 was knocked out on CAR-T cells targeting HER2 or mesothelin. We show that even low expression of PD-L1 in tumour cells could significantly impair CAR-T cell effector functions *in vitro*, and that cytokine secretion was improved by genetic or pharmacological inhibition of the PD1-PD-L1 axis. *In vivo*, we observed that while CAR-T cells could induce complete responses (CR) in 87% of mice bearing PD-L1-negative ovarian tumours (PD-L1 KO), the percentages of CR in animals with PD-L1 expressing tumours (PD-L1 low, high or wild-type) were below 25%. *PDCD1* ablation in HER2-CAR-T cells showed a significantly enhanced antitumour effect compared to CAR-T cells alone or in combination with checkpoint inhibitors, with > 87% of animals with PD-L1-expressing tumours exhibiting CR. We confirmed our observations using CAR-T cells against other cancer types expressing HER2 or mesothelin. Our results show that, in a context where PD-L1 is expressed by tumour cells, the deletion of PD-1 in CAR-T cells can restore CAR-T cell functions, suggesting an overall beneficial effect on CAR-T cell therapy

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P15

CAR density influences CAR-T antitumoral efficacy and correlates with clinical outcome

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Recent studies have shown that control of CAR expression influences CAR-T efficacy. Therefore, we hypothesized that CAR density directly affects CAR-T cell function. In this study we characterized the *in vitro* and *in vivo* antitumoral efficacy of FACS-isolated subpopulations of CAR-T cells with different CAR densities targeting BCMA. CAR^{High}-T cells presented increased basal activation together with more differentiated phenotypes, increased cytotoxicity and cytokine production against multiple myeloma cell lines. This effect was also observed in the infusion products of a clinical trial, where products enriched in CAR^{High}-T cells presented increased cytotoxicity. After antigen-driven activation, higher presence of terminally differentiated effector cells was observed in CAR^{High}-T cells, along with increased exhaustion. *In vivo*, CAR^{Low}-T cells presented increased persistence, suggesting that higher CAR levels could reduce long-term efficacy. Transcriptomic analysis revealed completely different expression profiles, with increased tonic signal in CAR^{High}-T cells. We generated a gene signature associated to CAR density to annotate CAR^{High}-T cells in scRNA-seq analysis to further infer regulatory dissimilarities driven by different CAR densities, using novel computational methods. Finally, to evaluate the impact of CAR density in the clinical outcome of CAR-T therapies, we applied the inferred gene signature to score infusion products from a clinical trial, observing absence of complete responders in those products enriched on CAR^{High} signature.

Our data demonstrate that CAR density plays important roles in CAR-T activity with impact on clinical outcome. Comprehension of regulatory mechanisms driven by CAR densities at the single cell level offer an important tool for the development of improved therapies.

P16

Development and validation of a scalable method for the production of “off-the-shelf” exosomes-based products derived from CAR-T cells

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Adoptive cell therapies (ACTs) based on modified T cells with chimeric antigen receptors (CAR) have shown remissions in hematopoietic neoplasms but not in solid tumors. This is mainly due to the complexity of the tumor microenvironment in this kind of tumors. Unlike disseminated liquid tumors, solid tumors present an impenetrable physical barrier, which prevents the arrival of therapeutic cells to their targets, reducing the effectiveness of CAR-T cells in such type of tumors.

Recently, the peculiar properties of nanomaterials have made possible to improve current cell therapies against cancer, particularly exosomes have emerged as a potential therapeutic agent in the field of immunotherapy. One innovative application is the production of exosomes from CAR-T cells (EXO-CAR-T), which present CAR on their surface, allowing a specific lysis of the tumor. Two major challenges in developing exosome-based therapies should be considered; 1. The need to produce sufficient amount of exosomes with appropriate and intact biophysical properties. 2. The existence of HLA surface molecules on the EXO-CAR-Ts that may induce some type of rejection. With this in mind, we set up a large-scale EXO-CAR-T production and we evaluated the feasibility, safety and efficacy of the exosome's purification. Our preliminary data showed that CAR-T cell-derived exosomes purified from large-scale EXO-CAR-T production are functional, express exosomes markers and carry CARs on their surface.

P17

Exogenous miRNAs as theraputic agents against malignant melanoma.

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Micro RNAs (miRNAs) are non-coding RNA sequences with an aproximate length of 22 nucleotides that play an important role in regulating gene expression. Several miRNAs have been exposed as key mediators in cancer development, either by silencing proto-oncogenes or by inhibiting expression of genes involved in antiproliferative pathways. Some of the latter have been proposed as potential therapeutic agents against cancer. In this regard, we have studied for the first time the antitumour effect of several miRNAs previously sequenced from an invertebrate-derived sample. An *in* silico target analysis of these miRNAs revealed an interaction with proliferative and inflammatory key pathways. These sequences were later quimically mimicked and transiently transfected with lipofectamine in A-375 malignant melanoma cells. We have seen an inhibition of proliferation of up to 50% at day 3, which is caused by apoptosis induction as suggested by the annexin V assay. Once the mRNA targets of these miRNAs are confirmed, these results will open the door to the therapeutic use of exogenous miRNA in cancer treatment.

P18

Gene therapy with a viral vector expressing an anti-PD-1 antibody can be improved by reprogramming tumor associated myeloid populations

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Therapies based on PD1/PD-L1 blockade have shown remarkable clinical outcomes and durable responses, but are not efficient in a significant number of patients. One approach to increase the potency of immunotherapy is to modulate the tumor microenvironment (TME) by reprogramming myeloid cells to stimulate anti-cancer activity, since these cells constitute the major TME component. For this purpose, it is important to identify molecular signatures associated to cancer-promoting myeloid cells. In this work we defined the phenotype and proteome of tumor associated myeloid cells generated *ex vivo* using a lung cancer model (3LL). We identified differences in proteomic signatures between monocytic myeloid-derived suppressor cells (M-MDSC), granulocytic-MDSC, and tumor-associated macrophages (TAMs) related to lineage and cancer-driven polarization. The proteomic atlas of tumor-associated cells revealed important routes that could be altered to reprogram cancer-associated myeloid cells. Based on these data we evaluated the effects of several compounds at the differentiation, maturation, and immunosuppressive levels in MDSCs and TAMs. Furthermore, we confirmed the effects of selected compounds in myeloid cells from non-small cell lung cancer (NSCLC) patients *in vitro*. Finally, to assess this therapy *in vivo*, we evaluated the combination of two selected compounds, administered intraperitoneally, with immunotherapy based on a Semliki Forest virus (SFV) vector expressing an anti-PD-1 antibody locally in 3LL tumors in mice. This combination strategy showed potent antitumor effects, higher than the ones obtained with individual agents. Moreover, we observed similar effects when combining the most effective compound with systemic delivery of anti-PD-1 mAb.

P19

Lineage and Cancer-Polarized regulatory Interactomes in Myeloid Cells Modeling Immunosuppressive Tumor-Infiltrating Subsets

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Monocytic and granulocytic myeloid-derived suppressor cells together with tumor-infiltrating macrophages constitute the main tumor-infiltrating immunosuppressive myeloid populations. Due to the phenotypic resemblance to conventional myeloid cells, their identification and purification from within the tumors is technically difficult and makes their study a challenge. We differentiated myeloid cells modeling the three main tumor-infiltrating types together with uncommitted macrophages, using ex vivo differentiation methods resembling the tumor microenvironment. The phenotype and proteome of these cells was compared to identify linage-dependent relationships and cancer-specific interactome expression modules. The relationships between monocytic MDSCs and TAMs, monocytic MDSCs and granulocytic MDSCs, and hierarchical relationships of expression networks and transcription factors due to lineage and

cancer polarization were mapped. Highly purified immunosuppressive myeloid cell populations that model tumor-infiltrating counterparts were systematically analyzed by quantitative proteomics. Full functional interactome maps have been generated to characterize at high resolution the relationships between the three main myeloid tumor-infiltrating cell types. Our data highlights the biological processes related to each cell type, and uncover novel shared and differential molecular targets. Moreover, the high numbers and fidelity of ex vivo-generated subsets to their natural tumor-shaped counterparts enable their use for validation of new treatments in high-throughput experiments.

P20

Interference with senescence-regulating molecular pathways in human primary T cells to enhance effector activities

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Effector T cells are key mediators of immune responses in cancer and infectious diseases. As we age, we previously shown that a p38-regulated senescence pathway is activated which leads human T cells towards replicative senescence (Lanna et al. 2014. Nat Immunol. PMID: 25151490; Lanna et al. 2017. Nat Immunol. PMID: 28114291). Pharmacological Inhibition of the p38 MAP kinase with specific inhibitors can counteract T cell senescence, leading to increased effector activities in T cells. To identify in detail the molecular interactome pathways regulated by p38-driven senescence, differential quantitative proteomics were performed over purified human CD4 and CD8 T cells in the presence or absence of p38 inhibitors. Our results uncovered a regulated network of signalling and metabolic pathways which regulate T cell proliferation, senescence and T cell effector activities towards cancer and infectious diseases. Proliferation assays and phenotypic profiling was performed in human T cell lines and primary lymphocytes which corroborated results from high-throughput proteomics. Some novel key regulators were identified which could be pharmacologically targeted to revert human T cell senescence. Our results uncovered potential combination therapies that could improve conventional vaccines and cancer immunotherapies.

P21

Synergistic antitumour response with recombinant modified virus Ankara armed with CD40L and CD137L against peritoneal carcinomatosis

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Recombinant modified vaccinia virus Ankara (rMVA) is known to elicit potent antitumour immune responses in preclinical models due to their inherent ability to activate the innate immune system and the activation of adaptive responses mediated by the expression of tumour antigens and costimulus-providing molecules such as CD40L and CD137L. Here, we evaluated different rMVA vectors in preclinical peritoneal carcinomatosis models (ID8.OVA-*Vegf*/GFP and MC38). We compared rMVA vectors expressing a tumour antigen (OVA or gp70) either alone or co-expressed with CD40L or/and CD137L. In tumour-free mice, the vector coding for the triple combination was only slightly superior, whereas, in tumour-bearing animals, we observed a synergistic induction of T lymphocytes specific against vector-encoded and non-encoded tumour-associated antigens. The enhanced activation of the immune response was associated with improved survival in mice with peritoneal carcinomatosis treated with an rMVA vector encoding both CD40L and CD137L. Thus, the triple transgene combination in vaccinia viral vectors represents a promising strategy for the treatment of peritoneal carcinomatosis.

P22

Generation of CD19 CAR variants with NK cell specific motifs to determine the best choice for treating relapsed or refractory leukemias and lymphomas

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B-cell hematologic cancers such as leukemia and lymphoma are common forms of pediatric and adult cancers worldwide. Acute Lymphoblastic Leukemia (ALL) is the most common cancer among children with a prevalence of 20-25% of all cases. The survival rate for these patients at 5 years is 79.2%, but it is still an incurable disease in many patients. Chimeric Antigen Receptor (CAR) T cells therapy has arisen as a new alternative to conventional therapies in order to treat advanced refractory cancers. However, this therapy has some undesirable side effects such as cytokine release syndrome (CRS) and neurotoxicity. Also, despite the good results in clinic, some patients are refractory or relapsed after CART cell treatment. Moreover, another potential problem with this treatment could be the need of using allogenic T cells when is not possible to perform an apheresis to the patient, as allogenic T cells carry a risk of graft versus-host disease (GVHD). NK cells could be a good alternative for CAR based therapy, as they do not cause this kind of side effects, and exhibit a potent graft versus leukemia (GVL) effect without causing GVHD. In this

study, we adapted CD19 CAR to NK cells signaling motifs, obtaining different variants of the CAR ARI-0001. These variants were tested in adult peripheral blood and cord blood NK cells in order to determine the best choice to perform a clinical trial phase I to evaluate the safety and viability of allogenic CD19 CARNK cells for treating patients with previous autologous CART treatment.

P23

Non-clinical toxicology assays to demonstrate the safety of a cell medicinal product before first clinical use

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We demonstrated that clinical-grade human mesenchymal stromal cells derived from umbilical cord (MSC) and irradiated with 2 Gy (IR-MSC), display good viability and upregulate the expression of tumor suppressor genes 48 hours after irradiation (*TRAIL*, *DKK3* and *ANXA1*), enhancing the effect of radiotherapy in tumor-bearing mice. To prove the safety of IR-MSC administrated systemically, we carried-out two subchronic toxicity studies (90 days, single and repeat doses) following Good Laboratory Practices (GLP). 18 NSG mice (n=6) were injected intravenously with a single dose of 5000 IR-MSC, MSC or transformed MSC (tMSC, positive control) / g, a similar dose to that intended to be administered in a clinical trial. tMSC-injected mice showed a significant weight reduction compared to the MSC mice. Two tMSC-mice showed nodules in spleen and lung. One of them, presented an increase in size and inflammation in the spleen. No significant alterations were detected in the organs of IR-MSC and MSC mice. Next, we carried-out PCR for Alu sequences to determine the presence of human DNA in selected organs. Human DNA was detected in the organs of 3 mice injected with tMSC, showing that tMSC may be present at 90 days post-injection. In addition, we performed a repeat dose assay (days 1 and 3) using IR-MSC and MSC. The histological analysis showed that the intravenous administration of IR-MSC and MSC does not produce morphological alterations. Besides, human DNA was not detected in any organ. In conclusion, the results indicated that systemic administration of IR-MSC and MSC is safe.

P24

Intratumoral electroporation of a self-amplifying RNA expressing interleukin-12 induces antitumor effects in mouse models of cancer

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Vectors based on alphavirus self-amplifying RNA (saRNA) generate high and transient levels of transgene expression and induce strong innate immune responses, constituting an interesting tool for antitumor therapy. Administration of naked saRNA *in vivo* could have advantages compared to viral particles, but the high RNA instability makes this approach poorly efficient. A quick and simple strategy to increase RNA uptake by cells is electroporation. Here, we used Semliki Forest Virus (SFV) saRNA for local treatment of murine colorectal MC38 subcutaneous tumors. First, we optimized conditions for SFV saRNA electroporation in tumors using a saRNA coding for luciferase and evaluating *in vivo* expression by bioluminiscence. As proof-of-concept, the therapeutic potential of this approach was evaluated using saRNA coding for interleukin 12 (IL-12), a proinflammatory cytokine with potent antitumor effects. The delivery of SFV-IL-12 saRNA by electroporation led to an improvement in tumor control and higher survival compared to mice treated with electroporation or SFV-IL-12 saRNA as single therapies. Our results suggest that this combinatorial therapy enhances the overall antitumor effect by promoting antitumor specific immune responses and tumor cell death. Combination of local SFV-IL-12 saRNA electroporation with systemic PD-1 blockade led to an increase in antitumor effects, indicating that saRNA electroporation could benefit from already approved therapies. This therapy, which was also validated in a hepatic tumor model, suggest that local delivery of saRNA by electroporation could represent an attractive strategy for cancer immunotherapy. This approach could have an easy translation to the clinical practice, especially for percutaneously accessible tumors.

P25

Study of ferroptosis transmission by small extracellular vesicles in epithelial ovarian cancer cells

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Epithelial ovarian cancer (EOC) is the deadliest gynecologic cancer in women and current treatment involves surgical reduction of tumors followed by combination chemotherapy. Although treatment is effective, many EOCs recur and develop chemoresistance. In the last year, ferroptosis, a novel form of regulated cell death featured by the accumulation of iron and toxic species of lipid metabolism in cells, has emerged as a promising anti-tumor strategy for EOC treatment. This process has a high potential to become a complementary treatment to the current anti-tumour strategies to eliminate resistant cells and to avoid the relapse. Cancer cells, like other cells in the body, release small extracellular vesicles (sEVs) that allow the transport of substances from the cells themselves to communicate with their environment. Here, we shown the transmission of ferroptosis in epithelial ovarian cancer cells (OVCA) through sEV.

We evaluate the colony formation capacity, viability and ferroptotic parameters as MDA levels, GSH/GSSG ratio and intracellular Fe2+ levels on OVCA cells treated with supernantant (SN) and sEVs from F-OVCA cells. We found the sEV from F-OVCA treatment decrease the colony formation capacity, the cell viability and GSH/GSSG ratio and increase the MDA and Fe2+ levels. However, SN fraction doesn't induce ferroptosis process. Our results demonstrate the capacity of sEV to regulate ferroptosis by paracrine way in OVCA cells. Thus, we discovered the importance of the sEV in the communication between cells in OVCA which would mediate the ferroptosis process.

P26

Fueling Cancer Immunotherapy with Genome editing

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Genome editing-based technologies not only provide unprecedented opportunities to study the basic functionality of the cellular system, but also improve the outcomes of various clinical applications. In our team, we are developing genome editing tools to improve the immune system from both clinical and basic research perspective. During my talk we will discuss recent advances in the development of programmable nucleases, and their use for the improvement of immune cells. In addition, we will describe how different gene editing tools have enabled the use of healthy donor cells in CAR T therapy instead of autologous cells, minimizing the risk of graft-versus-host disease or rejection. Finally, we will talk about a complementary approach, based on extra vesicles (EVs), and how these EVs can overcome some of the challenges that currently limit cancer immunotherapy

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P27

Effective generation of tumor-infiltrating lymphocyte products from naïve and treated solid pediatric tumors using three-signal-based method

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Tumor infiltrating lymphocytes (TILs) have shown efficacy in some adult cancers but it has not been yet explored in **pediatric cancer**. Our group is assessing the feasibility of using TILs for the treatment of pediatric cancer. A total of 21 pediatric samples, treatment naïve or after chemotherapy, were received in the Advance Therapies Unit. After enzymatic digestion, tumor immune infiltrating profile were analyzed by spectral cytometry and also cultured to study their ability to effectively expand. TILs from resected tumors were cultured firstly using the standard method with IL2, but in order to optimize the TILs production time and to improve the successful expansion rate, we developed a new propagation method based on 3-signals-T-cell-activation. This new strategy differs from the traditional method in that cytokine exposure-signal was changed to IL7, IL15 and IL21, and the co-stimulation signals used were the agonistics of CD3 and CD28. We observed that adding costimulatory signal in phase 1 of culture lead to a faster increasing of absolute numbers and viability of TILs and a higher rate of TILs-successful expansion. This important issue will allow us to reduce the time of TILs manufacturing. Regarding TILs products, we observed an important decrease in CD4-phenotype-Treg cells with the new method but no differences were found in the expression of differentiation and activation markers or intracellular cytokines production. Our results shown that T cells from pediatric solid tumors could effectively be isolated and expanded and that the new expansion-method showed a more robust way to expand pediatric TILs.

P28

Mesenchymal stromal cells stably expressing CXCR4 and IL10 reduce Th1/Th17 differentiation and induce regulatory cells production preventing GvHD development

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Mesenchymal stromal cells (MSCs) currently constitute one of the cell type more frequently used in advanced therapies. While previous pre-clinical data have shown the efficacy of MSCs to revert graft versus host disease (GvHD) after allogeneic transplantation, only moderate therapeutic effects have been observed in clinical trials. With the purpose of increasing the anti-GvHD effect of these cells, adipose tissue derived human MSCs (Ad-MSCs) were transduced with a lentiviral vector carrying CXCR4 and IL10, two molecules involved in cell migration to inflamed sites and with potent anti-inflammatory properties, respectively. *In vitro* experiments showed that the stable expression of these molecules in Ad-MSCs (CXCR4-IL10-MSCs) efficiently enhanced the migration of MSCs towards SDF1 when compared to unmodified Ad-MSCs. CXCR4-IL10-MSCs also displayed enhanced capacity to inhibit the proliferation of activated T cells. Using a humanized GvHD mouse model generated by the transplantation of human peripheral blood mononuclear cells into immunodeficient NSG mice, a single dose of CXCR4-IL10-MSCs induced a decreased in the GvHD clinical score compared to unmodified Ad-MSCs and confirmed by histopatologic analysis in the target organs. Moreover, CXCR4-IL10-MSCs also induced a polarization from a pro-inflammatory (Th1 and Th17) to an anti-inflammatory profile ($CD3^{+}$-$IL10^{+}$) and a significant increase in the number of regulatory B and T cells, compared to unmodified Ad-MSCs. Taken together, our preclinical studies strongly suggest that the lentiviral-medicated expression of CXCR4 and IL10 should improve the clinical efficacy of MSC-based cell therapies to treat GvHD.

P29

Development of a gene editing approach for the treatment of *RPL5*-deficient Diamond-Blackfan anemia patients

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Diamond-Blackfan Anemia (DBA) is an inherited bone marrow failure (IBMF) syndrome mainly characterized by red cell hypoplasia, congenital abnormalities and increased risk of cancer. Among

the genes associated with DBA, *RPS19* is the most frequently mutated (25%) one, followed by *RPL5* (11%). Our laboratory has developed a lentiviral gene therapy approach for the treatment of *RPS19*-deficient patients. Here, we propose a homologous recombination (HR) gene editing strategy for the treatment of *RPL5*-deficient patients, since this protein might require a tight endogenous regulation due to its direct interaction with the MDM2, P53 master regulator. In particular, we have developed a single-stranded adeno-associated viral vector 6 (ssAAV6) harboring a codon optimized sequence of the *RPL5* cDNA. Designed sgRNAs were delivered as ribonucleoprotein, which facilitated the achievement of around 90% indels in $CD34^+$ cells, either from healthy donor cord blood (CB) or *RPL5*-deficient patient bone marrow (BM). To optimize the transduction, and thus the efficacy of HDR-mediated gene editing, healthy donor $CD34^+$ cells from BM and CB were transduced with *CoRPL5*-AAV6 at different multiplicities of infection (MOIs). RT-qPCR assay targeting *CoRPL5* sequence allowed us to detect the AAV sequence in up to 36% and 14% of CB-$CD34^+$ cells transduced at MOIs of 10^4 and $3x10^3$ genome copies per cell, respectively. Consistently, the efficacy of homologous recombination was 31% and 14% in these samples, respectively. Different strategies are under development to reduce toxicity and increase the gene editing efficacy in BM $CD34^+$ cells.

P30

Identifying SARS-CoV-2 'memory' NK cells from COVID-19 convalescent donors for adoptive cell therapy (RELEASE)

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COVID-19 disease is the manifestation of syndrome coronavirus 2 (SARS-CoV-2) infection, which is causing a worldwide pandemic. This disease can lead to multiple and different symptoms, being lymphopenia associated with severity one of the most persistent. Natural killer cells (NK cells) are part of the innate immune system, being fighting against virus-infected cells one of their key roles. Unlike T lymphocytes, NK cells are not known for their specific memory population. Nevertheless, studies regarding NK cell population in other viral infections, such as cytomegalovirus, have shown specific memory population of these cells. These NK cells are particularly re-activated when facing viral specific peptides. In this study, we determined the phenotype of NK cells after COVID-19 and the main characteristic of SARS-CoV-2-specific-like NK population in the blood of convalescent donors. CD57+ NKG2C+ phenotype in SARS-CoV-2 convalescent donors indicates the presence of 'memory'/activated NK cells as it has been shown for cytomegalovirus infections. Moreover, this “memory” population respond to the presence of SARS-CoV-2 peptides. Although the existence of this population is donor dependent, its expression may be crucial for the specific

response against SARS-CoV-2, so that, it gives us a tool for selecting the best donors to produce off-the-shelf living drug for cell therapy to treat COVID-19 patients under the RELEASE clinical trial (NCT04578210). NK cells from selected donors' have been isolated, frozen and storage. NK cell arm of RELEASE clinical trial has been initiated in several patients.

P31

Treatment of chronic limb threatening ischemia (CLTI) with autologous bone marrow-mononuclear cells: preliminary results of a clinical trial of an advanced therapy medicinal product

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Chronic limb threatening ischemia (CLTI) is a pathology in which the obstruction of the arterial blood flow entails, in addition to the high risk of loss of the extremity, a high mortality, decreased quality of life (due to pain, both at rest and during movement, ischemic ulcers, etc) and high costs for the national health system. When surgical treatment, both open and endovascular, is not possible, limb amputation is a very common alternative in these patients, although this procedure also carries a high perioperative mortality and a high rate of reintervention. Bone marrow mononuclear cells (BM-MNCs) constitute a heterogeneous cell population, with proangiogenic and immunomodulatory properties, which have been used for several years in regenerative medicine. BM-MNCs have been applied to the treatment of critical lower limb ischemia previously, although there are few studies dealing with CLTI, and the published results are very heterogeneous (mainly due to differences in the treatment protocol used). Taking this into account, we propose to develop a clinical trial to validate the safety and efficacy of the use of autologous BM-MNCs in the treatment of CLTI according to the route of administration (IA, IM or IA+IM). This CT was authorized by the Spanish Agency of Medicines and Medical Devices (AEMPS) in 2021 and three patients have been treated to date (each received cells by a different route of administration). None of the patients treated have suffered limb amputation. Moreover, they have shown improvement of symptoms and QoL.

P32

Correcting APDS2 syndrome-caused mutation for future gene and cell therapies

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APDS2 syndrome is a genetic disorder classified as a primary immunodeficiency and caused by mutations in *PIK3R1* gene. Such alteration results in a dysfunctional immune system, affecting NK cells among others. Giving the lack of specialized treatments against APDS2 syndrome, we aim to develop novel strategies to uncover effective specific-therapies directed to APDS2 patients by correcting the mutation in their own cells. For this purpose, we will use the CRISPR/Cas9 molecular tool to edit the genome of an APDS2-derived iPS cell line recently generated in our laboratory. According to the characteristics of the iPS cells, we will utilize nucleofection as the transfection method for CRISPR/Cas9 complex internalization, which supplies a less aggressive and more reproducible gene editing technology. Finally, we will employ corrected APDS2-derived iPS to differentiate them into hematopoietic stem cells expressing the CD34+ marker. These hematopoietic progenitors could be also differentiated into the different types of blood cells, including NK cells. To this extent, we will be able to obtain not only normal NK cells, but also a personal source of healthy transfusable components from the APDS2 patient. As a whole, our work represents an innovative therapy that will improve current treatments, providing an exceptional and powerful tool to study APDS2 syndrome as well as personalized gene and cell therapy. Moreover, our findings could be extrapolated to the research of similar pathologies, facilitating the development of advanced targeted gene and cell therapies.

P33

New regulatory region in WAS intron 1 involved in megakaryocytic differentiation

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Transcriptional regulatory sequences inside introns are often involved in cell-specific expression. Since the Wiskott-Aldrich syndrome (WAS) gene expression is restricted to hematopoietic cells, we performed an in silico analysis searching for regulatory regions in the first intron, followed by an in vitro analysis of these regions. We found and studied several transcription factor binding sites (TFBS) and proved the binding of Ets1, Stat1 and Hmg1, all involved in hematopoietic regulation. Interestingly, two of the Ets1 binding sites are placed in a palindromic fashion, a configuration previously shown to be associated with transcriptional silencing in tissue-specific genes. In order to investigate the role of this region in the transcriptional regulation of the WAS gene we: 1) analyzed the effect of disrupting the TFBSs on WAS gene expression levels, 2) investigated the effect of this intronic region on regulating the transcriptional activity of a strong, unrelated promoter and 3) analyzed the effect of this regulator on megakaryocytic differentiation.

Our results showed that the intron1 of the WAS gene acts as a transcriptional repressor, that this repression is important to control megakaryocytic differentiation and that Stat1, Hmg1 and especially Ets1 play a major role in this regulation. This findings should help to refine gene therapy strategies for WAS based on achieving physiological expression of the transgene.

P34

Bio-distribution evaluation of endothelial colony forming cells in a murine model of chronic limb-threating ischemia.

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Chronic limb-threatening ischemia (CLTI) is the most severe manifestation of peripheral arterial disease, and it is characterized by a chronic obstruction of the major systemic arteries. This debilitating disease affects over millions of people around the world. To date, the treatment available is surgical revascularization and, although many advances have been made in this approach, high percentage of CLTI patients cannot undergo surgery, mainly due to associated comorbidities. As an alternative, angiogenic cell therapy has arisen as a promising, safe and feasible strategy applicable to ischemic diseases. However, before any clinical translation, determining the route-dependent bio-distribution of the injected cells within the organism is crucial. In this sense, lipophilic DiR dye labelling allows a simple and quick detection of cells once administered, being compatible to *in vivo* imaging systems. Herein, a comparative study using pre-labelled endothelial colony forming cells (ECFCs) injected through different routes in a murine model of CLTI is shown, with the aim of evaluating the most suitable administration strategy and the bio-distribution of cells within time and the injection route. Results obtained showed that, after intramuscular administration of pre-labelled ECFCs, they remained around the injection zone. However, when the transplantation was intravenously, cells were translocated into other tissues. Therefore, ECFCs intramuscular administration promotes a more localized action in the injured area, as they remain distributed around the injection zone.

P35

Genetic rescue of a human model of Bernard-Soulier syndrome based on CRISPR-Cas9 gene therapy

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Bernard Soulier syndrome (BSS) is an autosomal recessive platelet disorder characterized by thrombocytopenia, large platelets and frequent bleeding. It is caused by mutations in one of the

three genes coding for the membrane glycoprotein complex in platelets GPIb-IX-V. The GPIb-IX-V complex is a receptor for Von Willebrand Factor and it is involved in platelet adhesion and aggregation. When severe bleeding occurs, platelet transfusion is the only available treatment for these patients and recurrent transfusions can induce platelet refractoriness. Restoration of the mutated gene in hematopoietic stem cells by gene therapy might represent a powerful treatment for BBS patients.

Induced pluripotent stem cells (iPSCs) are an excellent tool for human disease modeling and a platform where new therapies can be tested. We used an induce pluripotent stem cell line derived from a BSS patient containing a mutation c.259T>C in the *GP9* gene (hiPSC-BSS) to assess the potential of the CRISPR-Cas9 system to target and repair the endogenous gene by homologous recombination.

We started from a genetically heterogeneous pool of hiPSC-BSS cells transduced with CRISPR/Cas9. We used a single cell cloning system to obtain colonies from a single cell (*clones*), whose *GP9* locus was analyzed by sequencing. We achieved a homologous recombination (HR) efficiency of 2.3% in iPSCs (2 out of 75 clones were rescued). The two rescued clones were heterozygous for *GP9*, meaning that only one of the two alleles had been correctly edited. This condition should be sufficient to achieve the rescue of BSS disease.

P36

Novel *in vitro* approaches to study X-linked adrenoleukodystrophy.

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X-linked adrenoleukodystrophy (X-ALD) is a rare genetic disorder caused by mutations in ABCD1 gene, causing the absence or dysfunction of ALDP, a peroxisomal transmembrane protein responsible of transportation of very long-chain fatty acids (VLCFA) from cytosol into peroxisome. Consequently, there is an accumulation of VLCFA in plasma and tissues. The clinical spectrum of X-ALD patients is wide variable, ranging from several phenotypes without correlation with genotype. The most devastating phenotype is characterized by an aggressive inflammatory demyelination. Unfortunately, there is not an effective therapy for all affected patients, highlighting the need for research.

A therapeutical option could be the use of bone marrow derived-mesenchymal stem cells (BMSCs). BMSCs have been proposed as a therapeutical approach in many neurological diseases as they have beneficial effect both in direct (cell-to cell contact) and indirect (paracrine signaling) mechanisms.

Here, we are developing a novel in vitro model to study X-ALD from dental pulp stem cells (DPSCs). We could analyse that DPSCs from a X-ALD patient showed specific phenotypical differences in comparison to healthy DPSCs. Also, X-ALD DPSCs could be differentiated into neural-like cells. Immunocytochemistry experiments revealed that X-ALD neural-like cells showed specific mature neural markers expression. Electrophysiological assays were carried out to further characterize X-ALD neural-like cells. These cells exhibited significant differences in amplitude and kinetics of sodium and potassium currents. Interestingly, when X-ALD neural-like cells were directly co-cultured with BMSCs, they rescued a healthy phenotype. Further investigation of the effect of BMSCs on X-ALD cells may provide relevant insights into X-ALD research.

P37

Substrate Reduction Therapy for Primary Hyperoxaluria type I based on CasRx mediated GO mRNA cleavage

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CRISPR based technologies have offered novel opportunities for the development of new therapies that could overcome the traditional way to treat inherited monogenic diseases. Primary hyperoxaluria type 1 (PH1) is a rare inborn disorder where a mutated form of alanine glyoxylate aminotransferase (AGT) presents a reduction in its enzymatic activity that affects glyoxylate metabolism. AGT deficiency results in excessive hepatic oxalate production that accumulates in the kidney forming CaOx stones that lead to end-stage renal disease. A substrate reduction therapy (SRT) based on CRISPR-Cas9 editing of *Hao1* gene, results in the reduction of glycolate oxidase (GO) expression, has been previously shown to be an attractive alternative therapy for PH1. However, potential side effects associated to off target effects remains a concern for this type of therapeutic approaches.

Here we have explored the use of CRISPR/CasRx, a programmable RNA-guided, RNA-targeting CRISPR system with nuclease activity that allows target gene knockdown without altering the genome. First, *in vitro* studies were performed to select the most efficient guide-RNA (gRNA) targeting GO mRNA. We showed that CRISPR/CasRx system resulted in the reduction of the expression of the targeted protein, although simultaneous reduction of non-targeted exogenous protein was observed in mammalian cells, while endogenous controls remained unaltered. Lastly, the most efficient gRNAs that demonstrated lower collateral cleavage will be tested in a PH1 mouse model using an adenoassociated vector serotype 8 (AAV8) carrying CasRx and single or multiple targeting guides.

In summary, CasRx represents a very attractive system to reduce protein expression without altering the cellular genome.

P38

CCR2 expression is not necessary for the beneficial effects of mesenchymal/stromal cell-based therapy in DSS-induced colitis

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Mesenchymal stem cells (MSCs) are currently used in preclinical and clinical studies for treatment of immune-mediated disorders thanks to their immunomodulatory and regenerative tissue properties. Numerous immune responses and mechanisms of action have been described for the

immunomodulatory effects of MSCs although nowadays the precise mechanism of action remains to be fully defined. Previous data from the laboratory have demonstrated that in collagen-induced arthritis a transient increase of Ly6C-expressing monocytes was induced in peripheral blood upon infusion of MSCs that ultimately led to an increase of regulatory IL10-expressing macrophages in the draining lymph nodes. Hence, in this study, we aim to investigate whether the CCR2-mediated trafficking of immune cells is involved in the beneficial effects of MSC-based therapy. To address this, a clinically relevant mouse model of dextran sulphate sodium (DSS)-induced colitis using CCR2-deficient mice was used to study the trafficking of CCR2-expressing cell populations in vivo. Strikingly a single dose of allogeneic adipose-derived MSCs infused in DSS-induced colitic CCR2-deficient mice was as effective as in WT mice. These results suggest that in contrast to previously reported studies and to our initial hypothesis, the CCR2-mediated trafficking of immune cells is not involved in the mechanism of action mediated by infused MSCs in DSS-induced colitis.

P39

Mesenchymal stem/stromal cell-based therapy fails to provide clinical benefit in K/BxN serum transfer-induced arthritis

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Rheumatoid arthritis (RA) is an immune-mediated disorder caused by loss of immunological self-tolerance that generates systemic and chronic inflammation in synovial tissues that finally leads to cartilage and bone destruction. Despite advances in understanding the aetiology of RA and novel biologic drugs, a substantial number of individuals with RA remains intolerant or resistant to these therapies. Hence, development of new therapeutic approaches are highly needed and, in this sense, mesenchymal stem/stromal cells (MSCs)-based therapy has emerged as a new alternative treatment for RA patients thanks to their well described immunomodulatory properties. The majority of preclinical studies in MSC-based therapy have been conducted using the well-known collagen-induced arthritis (CIA) mouse model. However, the low incidence of the disease in commonly used mouse strains in immunological studies, the prolonged induction phase, together with the restriction of mouse strains that are susceptible to developing arthritis have led to the development of alternative experimental models of RA. In this sense, the K/BxN serum transfer-induced arthritis (STIA) model has been developed as a very useful in vivo model that mimics many human RA disease characteristics. In this study, we aimed to evaluate the immunomodulatory potential of allogeneic adipose-derived MSC-based therapy in the K/BxN STIA model. Unexpectedly, our data suggest that allogeneic adipose-derived MSC therapy was unable of modulating the progression of the disease despite various experimental parameters tested.

P40

Enhanced therapeutic effect of mesenchymal stem/stromal cells overexpressing CXCR4 and IL10 in experimental colitis

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Inflammatory bowel diseases (IBD) consist on chronic inflammation of intestinal mucosa frequently associated to a significant increase in the risk of colitis-associated colon cancer. Multiple factors including environmental, genetic background and microbiota are related to its origin and aggravation. Current available treatments aim at reducing inflammation to avoid disease recurrence or to prolong clinical remission periods. Despite recent advances, no treatment is fully effective for many refractory patients, so that surgery remains as the only alternative. New therapeutic approaches are highly needed and, in this sense, mesenchymal stem/stromal cell (MSCs)-based therapy represents a promising option for unmet medical needs due to their immunomodulatory and regenerative properties. Preclinical and clinical data from animal models and clinical trials have demonstrated that MSC therapy is safe, although a significant heterogeneity exists in terms of therapeutic efficacy in the clinic. To enhance the therapeutic potential of MSC-based therapy, we have genetically modified adipose-derived MSCs with a lentiviral vector carrying the CXCR4 and interleukin 10 genes aiming to enhance their migration to inflamed tissues together with the improved anti-inflammatory potential. The therapeutic efficacy of CXCR4/IL10-expressing MSCs was tested using the dextran suphate sodium (DSS)-induced colitis model. A single dose of CXCR4/IL10-MSCs showed significant reduction in the disease activity index and incidence of colitis when compared with non-genetically modified MSCs suggesting that CXCR4/IL10-expressing MSCs may represent a more potent MSC-based cell therapy product for treatment inflammatory bowel diseases.

P41

Empty and Full Separation of Adeno-Associated Virus Vectors by Anion Exchange Membrane Chromatography

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Empty and Full Separation of Adeno-Associated Virus Vectors by Anion Exchange Membrane Chromatography

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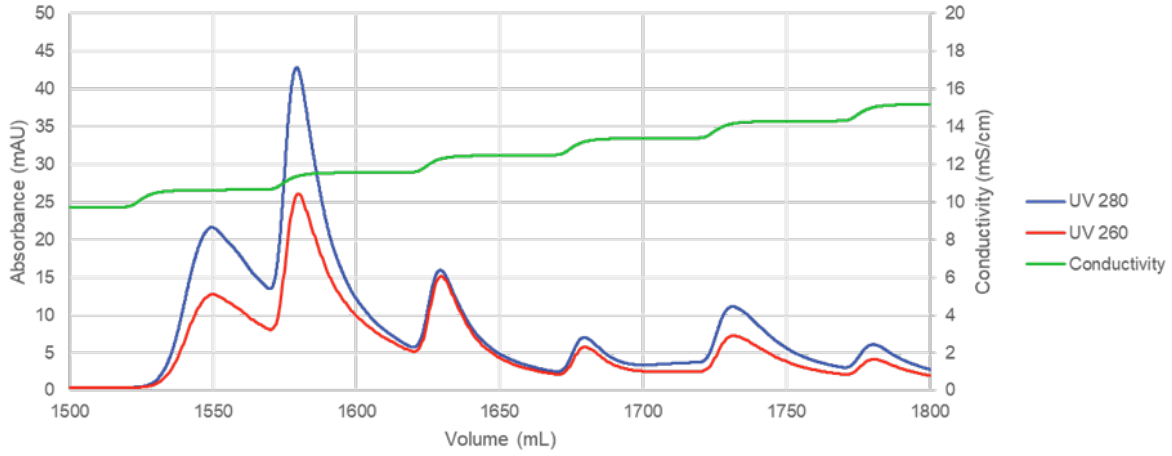
The approval of adeno-associated virus (AAV) gene therapies has led to increased research into AAV production and the burgeoning of promising clinical trials. However, significant challenges persist in AAV purification as AAV harvests typically contain a majority population of empty capsids that generate an immune response without delivering the therapeutic payload. In addition, subtle differences between therapies restrict platformability.

Here we assess the performance of anion exchange (AEX) membrane chromatography as the polishing stage of an AAV platform process following affinity purification. Utilizing a novel 1 mS/cm step gradient approach, we are able to demonstrate separation of empty and full AAV capsids of serotypes 5, 8, and 9 with the Mustang® Q XT membrane. This process maximizes the high flow rate benefits of membrane chromatography relative to traditional column chromatography, while providing improved separation. Distinct populations in the UV 260/280 chromatogram, analytical trends with PCR and ELISA tests, and capsid standards prepared by ultracentrifugation reaffirm separation. This technique is scalable between the 0.86 mL Mustang Q XT Acrodisc® unit and 5 mL Mustang Q XT capsule, and effectively clears residual host cell protein and DNA contaminants.

AAV Separation on 5 mL Mustang Q capsule



P42

Clarification with Pall Seitz® Depth Filters for Adherent and Suspension Adeno-Associated Virus Culture

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Clarification with Pall Seitz® Depth Filters for Adherent and Suspension Adeno-Associated Virus Culture

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In recent years, we have seen a rapid increase in the gene therapy market and the number of new therapies using adeno-associated viruses (AAV) as a viral vector. Our objective was to determine

the most efficient filter trains for the scalable clarification of both adherent-based and suspension-based AAV cultures.

Based on the analysis of data from >300 global studies, we examined throughput, recovery, flux and turbidity differences across multiple serotypes. The screening of Seitz filters for the AAV harvest resulted in a choice depending on two main parameters: the type of culture (adherent versus suspension) and the specific cell culture properties (including initial turbidity of the harvest, lysis and benzonase treatment).

Based on this analysis, for suspension AAV culture, we recommend evaluating the PDP8 filter in combination with the V100 or BIO20 filter. For adherent AAV culture, we recommend testing the PDK11 filter, a combination of PDP8 & V100 filter or the single layer V100 only for a very low turbidity harvest.

P43

Restoration of glyoxylate detoxification capacity of induced-hepatocytes after gene editing of the *AGXT* gene: A potential treatment for Primary Hyperoxaluria type I

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Nowadays the only curative treatment for many inherited metabolic liver disorders is liver transplantation. Due to the shortage of donor livers, new therapeutic approaches are needed. Liver cell replacement therapy appears as a promising alternative to organ transplant. Primary Hyperoxaluria Type 1 (PH1) is a rare genetic disorder characterized by the oxalate overproduction in the liver, resulting in renal damage. It is caused by mutations in the *AGXT* gene, codifying for alanine:glyoxylate aminotransferase (AGT), a hepatic enzyme involved in glyoxylate metabolism. We combined site-specific gene correction and hepatic direct cell reprogramming to generate autologous phenotypically healthy induced hepatocytes (iHeps) from PH1 patient-derived fibroblasts, avoiding the potentially tumorigenic step of iPSC.

First, we obtained specific *AGXT* gene corrected cells using two different CRISPR/Cas9 based strategies: 1) Accurate point mutation correction (c.853T-C) by homology-directed repair with a ssODN harboring the wild-type sequence; 2) Knock-in of an enhanced version of *AGXT* cDNA near the start codon of the endogenous gene. Then, we generated iHeps by lentiviral overexpression of hepatic transcription factors, directly from *AGXT*-corrected fibroblasts. iHeps showed a hepatic gene expression profile and hepatocyte functionality. Most important, *AGXT*-corrected iHeps exhibited *in vitro* reversion of oxalate accumulation compared to non-edited PH1-derived iHeps.

This innovative therapy set up a potential alternative cellular source to replace endogenous deficient hepatocytes with autologous functional corrected cells for genetic liver disorders. Moreover, patient-derived iHeps constitute a useful personalized *in vitro* disease modeling.

P44

Galectin-1 is required for the immune modulatory function of FOXP3^{+}CD4^{+} Treg cells in the intestine

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Galectin-1 is part of a family of carbohydrate-binding proteins, the so-called lectins, with high affinity for β-galactosides. Galectin-1 is widely expressed in many tissues and has immunomodulatory activity over different immune cell subsets that has been associated to induction of apoptosis in the target cells. Although, the immunosuppressive function of recombinant galectin-1 has been reported in many studies, the role of endogenous galectin-1 remains to be fully defined. In this study, galectin-1 deficient mice (*Lgals1*$^{-/-}$ mice) were used to investigate the *in vivo* function of galectin-1 in experimental colitis. *Lgals1*$^{-/-}$ mice showed enhanced susceptibility to experimental colitis with respect to WT mice as shown by the increased body weight losses and higher DAI compared to colitic WT mice. An increased plasticity of Th17 cells towards Th1 profile in DSS-induced colitic Lgals1$^{-/-}$ mice were determined. Strikingly, the exacerbated intestinal inflammatory responses in colitic Lgals1$^{-/-}$ mice was accompanied with increased frequencies of regulatory T cells in cLP. In addition, our results show that in the absence of endogenous galectin-1 an enhanced conversion of Foxp3^{+}CD4^{+} Treg cells into pathogenic T-Bet-expressing Foxp3^{+}CD4^{+} T cells was determined. Adoptive transfer of wild-type Foxp3^{+}CD4^{+} regulatory T cells into Lgals1$^{-/-}$ mice during DSS-induced colitis reduces the severity of the disease, achieving a similar intestinal inflammation as to colitic WT mice. Altogether, our results highlight the relevance of the expression of endogenous galectin-1 to prevent the development of aberrant pathogenic Treg cells during the course of an intestinal inflammatory response.

P45

Role of galectin-1 in regulatory T cell-based therapy in chronic colitis

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Cell therapy with regulatory T cells (Tregs) is under intense development thanks to their potential to maintain peripheral tolerance and their capacity to modulate unwanted immune responses. Galectin-1 (Gal-1) is a β-galactoside-binding protein that has broad anti-inflammatory and pro-resolving activities by targeting glycoproteins expressed by immune cells. We have previously demonstrated that Gal-1 is expressed by regulatory T cells and contributes to their immunosuppressive function *in vitro*. In this study, we analyzed the ability of galectin-1 deficient $CD4^+CD25^+$Foxp3 Tregs (*Lgals1*$^{-/-}$ Tregs) to suppress gastrointestinal immune responses using a T-cell transfer model of experimental colitis to study their functionality *in vivo*. *Lgals1*$^{-/-}$Tregs showed reduced immunomodulatory capacity compared to wild type Tregs, as suggested by the increase number of *Lgals1*$^{-/-}$ Tregs required to modulate intestinal inflammation induced by naïve $CD4^+$ T cells into *Rag-1*$^{-/-}$ mice. In addition, long-term survival of colitic mice treated with *Lgals1*$^{-/-}$ Tregs was compromised with respect to colitic mice treated with wild type Tregs mainly due to a systemic decline in *Lgals1*$^{-/-}$ Tregs, which paralleled to an increase in Th17 T cells frequencies in colon lamina propria and in spleen. These results indicate that galectin-1 is a key effector molecule in Tregs that is also required for long-term protection in chronic colitis. In summary, an adequate expression of galectin-1 should be analyzed in future cell therapy protocols with Tregs in inflammatory bowel disease.

P46

High value of [64Cu]$CuCl_2$ PET as a non-invasive tool to evaluate the restoration of physiological copper excretion after VTX-801 gene therapy in a mouse model of Wilson's disease.

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Wilson's disease (WD) is an inherited disorder of copper metabolism associated with mutations in ATP7B gene. We have shown that the administration of an adeno-associated vector (AAV) encoding a mini version of human ATP7B (VTX-801) provides long-term correction of copper metabolism in a murine WD model. In preparation of a future clinical trial, we have evaluated by Positron Emission Tomography (PET) the value of 64Cu biodistribution, excretion pattern and blood kinetics as pharmacodynamic biomarkers of VTX-801 effects. Six-week-old WD mice were injected intravenously with increasing doses of VTX-801 and three weeks or 3 months later with [64Cu]$CuCl_2$. Untreated WD and Wild type (Wt) mice were included as controls. Control WD mice showed increased hepatic 64Cu retention, reduced faecal excretion of the radiotracer and altered 64Cu blood kinetics (BK) compared to Wt mice. VTX-801 treatment in WD mice resulted in a significant reduction of hepatic 64Cu accumulation, the restoration of faecal 64Cu excretion

and the correction of 64Cu blood kinetics (BK). This study showed that VTX-801 restores physiological copper metabolism in WD mice, confirming the mechanism of action of VTX-801, and demonstrated the translational potential of [64Cu]$CuCl_2$-PET to explore VTX-801 pharmacodynamics in a minimally invasive and sensitive manner in WD patients.

P47

rAAV-mediated gene therapy in combination with short-term nitrogen-scavenger treatment corrects biochemical and behavioral abnormalities and increases lifespan in infant Citrullinemia Type 1 (CTLN-1) mice

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Citrullinemia type I (CTLN-1) is a rare autosomal recessive genetic disorder caused by mutations in the Argininosuccinate Synthase 1 (ASS1) gene that catalyzes the third reaction of the urea cycle. CTLN-1 patients suffer from toxic accumulation of circulating ammonia and urea cycle byproducts that causes metabolic encephalopathy and death at a very young age. Standard of care (SOC) management of CTLN-1 consists of daily nitrogen-scavenger administration and lifelong low-protein, high-calorie diet. Early restoration of hepatic ASS1 expression based on recombinant adeno-associated virus (rAAV) gene therapy represents an attractive prospect for treatment.
Vivet Therapeutics is developing VTX-804, a rAAV vector expressing the human ASS1 enzyme under the control of a liver-specific promoter. The long-term therapeutic efficacy of VTX-804 (6 months post-vector administration) was evaluated in 3-week-old CTLN-1 mice (*Ass1*$^{fold/fold}$) treated or untreated from birth to weaning with nitrogen-scavenger agents as per SOC in patients. Combination of VTX-804 with SOC restores CTLN-1 mice survival, normalizes weight gain and ammonia levels, while mice receiving VTX-804 alone present 90% survival, did not normalize weight gain and ammonia levels are normalized just transiently.
Similar to CTLN-1 patients; CTLN-1 mice showed several behavioral abnormalities such as anxiety and reduced welfare and innate behaviors that were notably improved when they were treated with VTX-804, and even more with the SOC in combination that improved rAAV-transduction and *hASS1* transgene expression at 3 and 6 months post-VTX-804 injection. Therefore, combination of the SOC prior to gene therapy administration represents a very promising therapeutic strategy for very young CTLN-1 patients.

P48

Optimizing the IdeS treatment regimen for enhanced adeno-associated virus transduction in the presence of neutralizing antibodies

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Pre-existing neutralizing antibodies (NAbs) to adeno-associated viruses (AAVs) remain an impediment for systemically administered AAV-mediated gene therapy treatment in many patients and various strategies are under investigation to overcome this limitation. Here, IgG degrading enzymes (Ides) derived from bacteria of the genus *Streptococcus* were tested for their ability to cleave human IgG and allow AAV-mediated transduction in individuals with pre-existing NAbs. Cleavage activity of three different Ides was evaluated *in vitro* in serum from different species. Passively immunized mice or non-human primate (NHP) with naturally occurring anti-AAV NAbs were used to define the optimal IdeS dose and administration window for AAVAnc80 and AAV8 vectors in mice and AAV3B in NHPs. The selected candidate, IdeS, was found to be highly efficient at cleaving human IgG, less efficient against NHP IgG and inefficient against mouse IgG. *In vivo*, we observed differences on how IdeS affected liver transduction in the presence of NAbs depending on the AAV serotype. For AAVAnc80 and AAV3B the best transduction levels were achieved when the vector is administered after IgG NAb digestion products are cleared from circulation. However, for AAV8 we only observed a modest and transient inhibition of transduction by IdeS cleavage products. Preconditioning with IdeS represents a unique treatment opportunity for patients primarily excluded from participation in gene therapy clinical trials due to elevated circulating anti-AAV NAb levels. However, careful determination of the optimal IdeS dose and timing for the administration of each AAV serotype is essential for optimal transduction

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P49

Terminal Schwann cells and Kranocytes connect neuromuscular junctions with the vascular network

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Neuromuscular junctions (NMJs) are the specific synapse sites between terminal nerves and muscle fibres. Two types of NMJ-capping cells [terminal Schwann cells (tSCs) and Kranocytes] cover these structures. tSCs, the glial component of the synapsis, surround the endplate and maintain the structure of NMJs. Kranocytes, also known as “perisynaptic-fibroblasts”, surround the tSCs, covering a wider tissue area. In response to nerve injury, Kranocytes migrate and create

bridges between endplates, which are later colonized by tSCs and axons, to promote the reinnervation process. To date, no study has addressed the interaction of NMJ-capping cells with the immediate muscle fibre microenvironment. Here, we addressed the question if NMJ-capping cells could send and receive information through other sources apart from nerve signalling, and specifically we analysed if they establish connections with the neighbouring capillaries. To this end, iDisco-clarified whole mount skeletal muscles from hindlimbs of C57BL/6 adult mice were analysed by immunofluorescence and confocal microscopy, as well as transmission electron microscopy. Both NMJ-capping cells (tSCs and Kranocytes) were found to establish connections to capillaries, independently to each other, suggesting they are exchanging different types of information. The contacts between tSCs and Kranocytes and the vascular system may represent a novel mechanism of information exchange between NMJs and the muscle fibres that is currently being investigated.

Key words: Neuromuscular junctions (NMJs), tSCs, Kranocytes, vascular network

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P50

Analysis of the neuroregenerative potential of human placental mesenchymal stem cells in a mammalian Central Nervous System model of axotomy.

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In the novel field of advanced therapies, stem cell therapeutics acquires great relevance in the last decades. The Central Nervous System (CNS) is a complex tissue composed of a vast number of diverse cells. Given their number and diversity, CNS pathologies and their therapies comprise challenging and promising fields of research. The retina is a specialized CNS extension encapsulated in the eyeball and easily accessible to experimental manipulation. Our group has studied the neuroregenerative potential of human placental stem cells (hPMSCs) in rat retina after traumatic neuronal injury. We have developed a co-culture in which dissociated neurons extracted from adult rats were cultured on a monolayer of hPMSCs. The experiments carried out under both normoxic and hypoxic conditions reveal that hPMSCs stimulate axonal regeneration of axotomized neurons compared to cultures in the absence of hPMSCs. In both conditions, we have measured the percentage of neurons that have regenerated their axons, the total length of these axon, and the average axon length per neuron demonstrating the ability of hPMSCs to increase axonal regeneration. Moreover, we have confirmed the synthesis of neurotrophic factors precursors in our hPMSCs co-culture system. We are currently focused in determining the influence of these neurotrophic factors and establishing the correlation with the axonal regeneration observed, as well as confirming neuronal function after axon regeneration.

P51

Investigation of RNA-misprocessing in neurological diseases for the development of novel diagnostic and therapeutic approaches

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RNA-processing is the molecular mechanism by which precursor messenger RNAs (pre-mRNAs) are capped, spliced and polyadenylated. It is a fundamental process that ensures a correct gene expression pattern and, as such, its misregulation has been implicated in a variety of human diseases.

In the lab, we are currently investigating RNA-misprocessing in cellular models, tissues and biofluids of patients with neurodegenerative diseases in order to develop: (i) personalized antisense RNA-based therapeutic strategies using RNA-targeting CRISPR-dCas13 system, (ii) sensitive RNA detection methods that could serve as future biomarkers.

In Frontotemporal dementia, we have combined bioinformatic and experimental approaches to characterize the molecular mechanism of a non-coding mutation in *GRN* gene, which leads to an aberrant splicing pattern that causes *GRN* mRNA degradation and progranulin haploinsufficiency. We are currently exploiting CRISPR-dCas13 RNA targeting system in order to identify sequences in *GRN* pre-mRNA whose targeting using antisense RNAs would restore *GRN* reading-frame. Based on this concept, we would like to develop a future personalized therapeutic approach for patients with this mutation.

Another aim in the lab is to perform and exploit computational analysis of RNA-seq data in order to investigate RNA-misprocessing events relevant for disease etiology and to develop sensitive RNA quantification methods which can be used as future biomarkers of neurological diseases. Specifically, we are developing targeted RNA-sequencing approaches to quantify cryptic polyadenylation events in *STMN2* and *HTT* genes in disease models of TDP43-proteinopathies and Huntington's disease.

P52

Molecular characterization of a non-coding mutation in *GRN* gene and investigation of CRISPR-dCas13 system for splicing modulation

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Mutations in granulin (*GRN)* gene are the second major genetic cause of frontotemporal lobar degeneration (FTD) and appear all throughout the gene. A non-coding mutation in the intron 7 of *GRN*, known as c.709-1G>A, has been reported to be specific of Basque population and some

other rare cases in the world. However, the molecular mechanism by which this intronic variant causes FTD is unknown. In this work, we establish the effect of *GRN* c.709-1G>A mutation *in silico*, *in vitro* and in patient samples. We observe that the mutation leads to an aberrant splicing pattern which causes *GRN* mRNA degradation and progranulin haploinsufficiency. Next, we investigate whether the mutation is suitable for splicing modulation approaches using RNA-targeting CRISPR-dCas13 system. Similar to already approved splice-switching antisense oligonucleotides, this system binds to specific sequences in a target pre-mRNA, sterically blocks access of RNA-binding proteins and therefore modulates RNA-processing pattern. We investigate the use of CRISPR-dCas13 system to develop a personalized therapeutic approach which restores *GRN* reading-frame in order to generate a shorter but partially functional progranulin protein that can rescue FTD phenotype.

P53

Characterizing novel markers for Kranocytes, the "forgotten" NMJ-capping cells

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Neuromuscular junctions (NMJs) enable nerve transmission to muscular fibres, triggering muscle contraction. NMJs are covered by terminal Schwann cells (tSCs) and mesenchymal cells known as Kranocytes. tSC functions are well characterised: they are essential in the maintenance of NMJ structure and in the reinnervation process after nerve injury. In contrast, little is known about the function of Kranocytes in homeostasis. In response to denervation, Kranocytes migrate earlier than tSCs and generate mesenchymal bridges that tSCs and axons will later colonize for reinnervation. Known kranocyte markers (CD34 and Tenascin C; the latter only in response to nerve injury) are not specific. To be able to isolate Kranocytes and make a deeper transcriptomic and functional characterization of this ill-known cell type, we first aimed to describe new membrane markers as a tool to isolate them. Skeletal muscles from the hindlimbs of C57BL/6 adult mice were analysed by confocal microscopy and flow cytometry. Immunofluorescence staining was performed on iDisco-clarified whole mount muscles. A screen of 50 antibodies yielded a new specific transmembrane glycoprotein marker for Kranocytes, Podoplanin. In muscle whole mounts, Podoplanin+ cells were limited to Kranocytes covering NMJs and lymphatic vessels. We are currently isolating Podoplanin+CD34+ cells by FACS, and will further characterise Kranocytes by single cell RNA sequencing. The identification of Podoplanin as a new and almost specific marker for Kranocytes will pave the way to shed light on the function of these cells in homeostasis and injury of skeletal muscle.

Key words: Kranocytes, neuromuscular junction (NMJ), podoplanin.

P54

Promoting brain regeneration through cellular reprogramming

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Although our knowledge about brain physiology has expanded significantly, most neurological conditions remain uncured. The mammalian brain has very limited regenerative capacity, since most of its cells are not replaced during normal or pathological situations. Neurons and their connections are established early in life and are designed to age with the individual.

In the lab we take advantage of the reprogramming technology to address regeneration. Nuclear cell reprogramming has emerged as a method that allows the age and identity of virtually any cell to be reversed to an embryonic-like stage by the action of the 4 Yamanaka Factors (4F). It has been shown that partial reprogramming (PR), the transient expression of the 4F, is sufficient to generate intermediate progenitors in some tissues, in addition to lead the cells to a youthful state. We use PR specifically in astroglia to revert these cells to a progenitor state with the aim of generating neural stem cell-like cells with the capacity to differentiate into induced neurons. Also, we propose to rejuvenate astroglia by PR in order to reduce age-related neuroinflammation to promote brain homeostasis and the maintenance of a healthy neuronal population.

P55

Direct reprogramming of adult human olfactory ensheathing glia (OEG) into neurons

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Different cell therapy strategies have been tried to heal Central Nervous System (CNS) injuries. A recent approach is the transplantation or *in vivo* generation of induced neurons (iNs) whose origin is direct reprogramming from mature glial cells. Direct cellular reprogramming consists in producing a differentiated cellular type from a somatic cell, without going through a pluripotent/stem cell stage. This has been achieved by transduction of viral vectors that express neurogenic transcription factors, with additional chemical manipulation.

We propose a unique cellular type as a candidate for direct reprogramming to iNs: olfactory ensheathing glia (OEG). OEG surrounds growing axons of sensory olfactory neurons and promotes axonal regeneration both in its physiological location (bulb and olfactory mucosa) as in *in vitro* and murine models of CNS injury. We suggest that engraftment of OEG and OEG induced neurons (OEG-iNs) would enhance neuroregeneration at the injured site. This would be due to the intrinsic axonal regenerative capacities of OEG, added to the presence of OEG-iNs which would mature to synapse forming neurons. OEG can be easily obtained from the patient´s olfactory mucosa and used for autologous transplantation.

We present the results of reprogramming adult human OEG into neurons (OEG-iNs), through the expression of the transcription factor NeuroD1. OEG-iNs have neuronal morphology, express general and mature neuronal markers and differentiate to glutamatergic neuronal subtype. Functionality has been achieved as OEG-iNs are capable of firing action potentials.

P56

Investigation of RNA misregulation in TDP-43 proteinopathies identifies novel targets for disease diagnosis and monitorization

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Amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) are two types of progressive and fatal neurodegenerative diseases. Also known as TDP-43 proteinopathies, they are characterized by nuclear depletion and cytoplasmic deposition of TDP-43, a RNA binding protein with a central role in RNA metabolism and processing. Therefore, multiple altered splicing events are expected upon TDP-43 nuclear depletion. In the present project, we have analysed transcriptome alterations in the context of FTD and ALS in order to identify RNA-misprocessing events relevant for disease etiology and diagnosis. First, we performed computational analysis of RNA-seq data in peripheral blood and cellular models from FTD patients and control subjects, which exposed a sort of splicing events and possible predictive signatures of disease development. Moreover, we have confirmed that cryptic polyadenylation of *STMN2* is a sensitive biomarker for TDP-43 proteinopathies with tissue specificity. Based on these results, we are currently developing a sensitive RNA quantification method, based on a targeted RNA-sequencing approach, in order to quantify in a high-throughput manner RNA misprocessing events which can be applied for disease diagnosis and monitorization.

P57

AAV-Mediated Expression of αKlotho Isoforms Rescues Relevant Aging Hallmarks in SAMP8 Mice

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Senescence represents a stage in life associated with elevated incidence of morbidity and increased risk of mortality due to the accumulation of molecular alterations and tissue dysfunction, promoting a decrease in the organism's protective systems. Thus, aging presents molecular and biological hallmarks, which include chronic inflammation, epigenetic alterations, neuronal dysfunction and worsening of physical status. αKlotho (KL) has been described as a powerful neuroprotector factor that delays aging consequences. It is mainly expressed in the kidneys and the choroid plexus in central nervous system (CNS) and presents two major splicing variants, a transmembrane isoform (m-KL) and a shorter secreted one (s-KL). Interestingly, Klotho expression decreases during non-pathological aging, which causes deregulation of key metabolic pathways and a decrease in the neuroprotection that this protein confers. In this context, we explored the AAV9-mediated expression of the two main isoforms of the aging-protective factor Klotho (KL) as a strategy to prevent these general age-related features using the senescence-accelerated mouse prone 8 (SAMP8) model. Our principal findings demonstrated that KL expression improved physical condition and cognitive performance without changes in age-related behaviors in treated SAMP8 mice. KL treatment rescues global epigenetic landscape and improves histological and biochemical markers related with CNS inflammation and cellular senescence. Moreover, at the bone level, KL treatment restores structural changes observed in SAMP8 tibia. In summary, our in vivo results showed, after a single treatment with AAVs-expressing KL in SAMP8 mice, an improvement of age-related deficits and relevant molecular aging-hallmarks such as epigenetic, inflammatory and bone alterations.

P58

3D bioprinting of a novel thermoplastic polyurethane material with potential for cartilage regenerative purposes

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In the last few years the 3D bioprinting has shown promising results in the biofabrication of artificial tissues for tissue engineering (TE). Cartilage, as an avascular and stratified tissue, presents a limited capacity of repair; therefore, a severe damage will often require surgical intervention. Many biomaterials, such as PLA, or PCL are being used to create scaffolds for cartilage TE, but, natural-based materials do not show enough integrity and synthetic-based materials do not have similar mechanical properties to cartilage such as friction and elasticity which limits their effectiveness and integration in the injury. Here, we evaluated the novel 1,4-butanediol thermoplastic polyurethane elastomer (b-TPUe) filament as a 3D bioprinting material for cartilage TE. The mechanical behaviour of b-TPUe in terms of friction and elasticity were examined and compared with human articular cartilage, PCL, and PLA. Moreover, infrapatellar fat pad-derived human mesenchymal stem cells (MSCs) were bioprinted together with scaffolds and in vitro and in vivo studied were performed. b-TPUe demonstrated a much closer compression and shear behaviour to native cartilage than PCL and PLA, as well as closer tribological properties to cartilage. Moreover, b-TPUe bioprinted scaffolds were able to maintain proper proliferative

potential, cell viability, and supported MSCs chondrogenesis. Finally, in vivo studies revealed no toxic effects 21 days after scaffolds implantation, ECM deposition and integration within the surrounding tissue. Our findings indicate that b-TPUe can be exploited for the automated biofabrication of artificial tissues with tailorable mechanical properties including the great potential for cartilage TE applications.

P59

Mineralized Eggshell Membranes as an Osteoinductive Biomaterial for Bone Regenerative Medicine

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Regeneration of damaged bone tissue focuses on the attempt to repair bone lesions that the body fails to heal using different combinations of synthetic and natural biomaterials. Hybrid biomimetic materials aim to replicate the organic-inorganic interactions of mineralized biological tissues. Eggshell membrane (ESM) can control the nucleation of calcite crystals during eggshell formation, constituting a potential organic template for the controlled precipitation of other mineral phases. Different biomimetic strategies have been studied to improve the properties of synthetic nanocrystalline apatites using a vapor diffusion seated drop (VDSD) crystallization methodology. This approach favors the microenvironment for the controlled precipitation of calcium phosphate (CaP), an essential mineral in bone tissue. In this study, a biological organic surface, such as eggshell membranes (ESMs), has been used to control the microstructural properties during the mineralization process. The results show that ESMs mineralized by VDSD give rise to a CaP/ESM biomimetic material that is similar in composition and properties to mineralized tissues. Moreover, in-vitro studies revealed that it is biocompatible and favors the osteogenic differentiation of hMSCs after 21 days in culture. We conclude that this new natural biomaterial could be developed for guided bone regeneration and restorative dentistry.

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P60

Pressure ulcers' treatment with a plasma scaffold containing mononuclear cells in an experimental model.

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Hard-to-heal wounds are an important health problem that affects a large number of persons influencing their life-quality. Pressure ulcers are one type of injury that include into this category of hard-to-heal wounds. Conventional methods for the treatment of these ulcers not always offer satisfactory results. In order to search for new therapeutic alternatives, an animal model of pressure ulcer was developed. To treat these experimental ulcers, a plasma support embedding mononuclear bone marrow cells were designed; these cells were obtained from luminescent strain FVB/N-Tg (β-Actin-luc)-Xen mice donor mice. To verify the effectiveness of the supports, three groups were formed: a *control group*, which did not receive any type of treatment, a *scaffold group* to which only the support without cells was applied, and a *scaffold + cells group* that was treated with the support containing luminescent mononuclear cells. The evolution of the wounds was followed for one week and samples were obtained from all groups on the third and seventh day and analyzed by histological techniques. In addition, the ulcers were evaluated macroscopically during this period. As preliminary results of this study, it can be concluded that plasma-embedded mononuclear cells accelerate the ulcer healing process in this experimental model. This improvement could be observed both by the histological study, where a more organized formation of dermal tissue is observed and where the presence of donor luminescent cells was verified, as well as macroscopically, where a reduction in the size of the ulcer is observed.

P61

Wound healing gene expression in a pressure ulcer animal model

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Hard-to-heal wounds are complex injuries with tendency to become chronic, between them, Pressure Ulcers (PUs) are ischemic injuries affecting skin and underlying tissue as result of intense and/or prolonged pressure, alone or in combination with shear and/or friction. PUs also has expensive time-consuming treatments and long-term hospitalizations. Advanced Therapies seems to be an interesting therapeutic approach to achieve a healing alternative treatment. In order to perform procedures those for methodological and/or ethical reasons are not viable in the clinical practice and to improve knowledge of this kind of lesions, pre-clinical pressure ulcer Swiss nu/nu mice model was developed using neodynium magnets. Afterwards, in order to get healing, the generated lesions were treated with a blood plasma-based scaffold combined or not with bone-marrow mononuclear cells isolated from luminescent strain FVB/N-Tg (β-Actin-luc)-Xen mice. Animals have been randomly assigned to three experimental groups (Control, Scaffold and Scaffold combined with cells). Lesion tissue biopsies were obtained after 3 and 7 days of treatment in order to investigate molecular response in the wound. The total RNA was isolated and each sample was reverse transcribed to complementary DNA. A wound-healing specific array (Wound Healing RT2 Profiler PCR Array, QIAGEN) was used for the molecular study of gene expression. Genes related with extracellular matrix, adhesion molecules, inflammatory cytokines, growth factors and transduction signals were included. After data processing using GeneGlobe software, it could be observed differences in terms of gene expression between experimental groups, being significant in some cases. Obtained results will be discussed in the poster.

P62

Trehalose cryopreservation of human mesenchymal stem cells from cord and adipose tissue

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Long-term cryopreservation of human mesenchymal stem cells (MSCs), for regenerative medicine applications, is highly important both in research and therapeutics. Current cryopreservation methods are based on dimethyl sulphoxide (DMSO) as cryoprotectant agents (CPAs), which has been shown to be toxic in clinical applications. Therefore, an alternative solution for cryopreservation of MSCs is required and we assessed the use of trehalose. The effects of trehalose as CPA at different concentrations, alone or in combination with ethylene glycol (EG) and glycerol (GLY)), was tested in MSCs from cord tissue through determination of cell viability, identity, proliferation and migration capacity. Although trehalose cryopreserved MSCs showed lower viability comparing with DMSO but cells maintained their functional properties, obtaining the best results when 0.5 M trehalose + 10% ethylene glycol (EG) was used to cryopreserve MSCs, also from adipose tissue. Cells viability, recovery capacity, stability and stress-related gene expression for both, MSCs from cord and adipose tissue, after cryopreservation with 0.5 M trehalose + 10% ethylene glycol (EG) were analysed and results will be presented. In conclusion, preliminary assays show that trehalose-based solution could be considered an effective and less toxic alternative to DMSO for cryopreservation in liquid nitrogen of MSCs.

P63

Preclinical safety and complementary efficacy assessment of an advanced medicinal product for the treatment of recessive dystrophic Epidermolysis Bullosa by gene editing

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Recessive dystrophic epidermolysis bullosa (RDEB) is a skin fragility disease caused by loss of functional type VII collagen (C7), a dermo-epidermal adhesive protein, leading to chronic blistering, progressive fibrosis and cancer. A premature termination codon-causing mutation in exon 80 of COL7A1 gene, c.6527insC, is highly prevalent (close to 50% of pathogenic alleles) in the Spanish RDEB patient population. Deletion of small, repetitive sequence-encoding exons in the triple helix-forming region of COL7A1 gene results in functional C7 proteins fit for anchoring fibrils formation at the dermal-epidermal junction.

We previously demonstrated in a proof of concept study that COL7A1 exon 80 deletion/skipping strategy based on the use of paired guideRNAs leading CRISPR/Cas9 action was an effective approach to rescue the RDEB phenotype (Bonafont et al, Mol. Ther. 2019; 27:986-998) Subsequently, an autologous bioengineered skin populated with RDEB cells (keratinocytes and fibroblasts) edited by exon 80 removal, an advanced medicinal therapy product (AMTP), received the orphan drug designation (EU/3/20/2253) by the EMA.

We show here the pre-clinical results of efficacy and toxicological studies, including tumorigenicity, histopathology, biodistribution and on-target/off-target genotoxicity which confirm that this cell-and-gene therapy AMTP is a safe and effective approach for patients living with this devastating genodermatosis, suitable to be explored in the context of a clinical trial.

P65

Placental MSCs and their exosomes as vehicles for the Na/I symporter (hNIS): A new agent for gene therapy and diagnostic.

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The Na/ I symporter gene (hNIS) is expressed in the thyroid and allows the accumulation of iodine from the diet. Moreover, it is widely used (i) as a reporter gene for molecular imaging (when the positron emitter isotope is I^{124} for PET or Tc^{99} for SPECT) or (ii) as a therapeutic gene for cancer therapy, mediated by the accumulation of I^{131}. An unresolved challenge is how to direct this gene specifically to the tumoral area.

As hNIS is expressed at the placental tissue (because it transfers iodine to the foetus from the maternal blood), in this work we decided to study whether placental MSCs and their derivatives (exosomes) (1) express hNIS endogenously and therefore transfers the imaging and therapeutic potentials when administered with radioactive iodine (2) are capable to reach the tumoral areas when they are intravenously injected due to the tumoral tissues extravasation, with great sucess.

Our findings highlight the possibility of the use of endogenous NIS expression as therapy and opening a wide range of new possibilities to treat and diagnose cancer

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P66

Coating an adenovirus with functionalized gold nanoparticles favors uptake, intracellular trafficking and anti-cancer therapeutic efficacy

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Adenoviral (Ad) vectors have proven to be important tools for gene and cell therapy, although some issues still need to be addressed. In the past years, several organic and inorganic materials have been developed to reduce immunogenicity and improve biodistribution of Ad vectors. Here, we investigated the influence of the functionalization of 14 nm PEGylated gold nanoparticles (AuNPs) with quaternary ammonium groups and an arginine-glycine-aspartic acid (RGD)-motif on the uptake and biodistribution of Ad vectors.

We report the formation of Ad@AuNPs complexes that promote cell attachment and uptake, independently of the presence of the coxsackievirus receptor (CAR) and αv β_3 and αv β_5 integrins, significantly improving transduction without limiting Ad bioactivity. Besides, the presence of the RGD peptide favors tumor targeting and decreases Ad sequestration in the liver. Additionally, tumor delivery of a coated Ad vector expressing the human sodium iodide symporter (hNIS) by mesenchymal stem cells induces increased accumulation of radioactive iodine (^{131}I) and tumor volume reduction compared to naked Ad-hNIS, highlighting the promising potential of our coating formulation in cancer gene therapy.

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P67

Development of two new cationic liposomes TROPY and SPIDER for the transfection of therapeutic oligonucleotides in mammalian cells

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There is a need for improving the delivery of oligonucleotides intended for gene therapy. Previously, we synthesized dioleyl pyridinium (DOPY) that was able to transfect cells with great efficiency. Now we describe the synthesis of two new cationic liposomes: trioleyl pyridinium (TROPY) and tetraoleyl pyridinium (SPIDER). Both syntheses were carried out in two synthetics steps. TROPY is a tris-pyridinium salt connected through a mesitylene spacer, SPIDER is a tetrakis pyridinium salt connected through a 1,2,4,5-tetramethylphenyl spacer. The pyridinium moieties in both liposomes bear an oleyl chain. We studied their biological activity in human prostate cancer PC3 cells. As a model for therapeutic oligonucleotides, we used a Polypurine Reverse Hoogsteen (PPRH) hairpin specifically directed against the promoter region of the antiapoptotic *survivin* gene,

either labelled with fluorescein or unmodified. The DNA binding capability was tested by gel agarose electrophoresis, observing a decrease in the amount of free oligonucleotide in the presence of liposomes. Tropy and Spider/PPRH particles were 142 and 198 nm in size, respectively, as determined by DLS. The internalization of the PPRH was followed by fluorescent microscopy, flow cytometry and confocal microscopy in PC3 cells, showing that PPRHs were delivered into cytosol and nuclei of the cells. Both liposomes had no intrinsic cytotoxicity up to 1.5 µg/ml. The decrease in viability of PC3 cells, due to the PPRH against *survivin*, transfected with either 1.5 µg/ml of TROPY or SPIDER was 84% and 91%, respectively, as determined by MTT assays.

P68

Targeting lung metastasis in triple negative breast cancer by CRISPR/Cas9 mediated *Tenascin-C* silencing with a chitosan-based vector

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Tenascin-C (TN-C) is a matricellular protein that is expressed in embryo with a very limited expression in the postnatal life. However, TN-C expression increases dramatically in some tumors. Clinically, this high expression is correlated with bad prognosis and metastasis in many solid tumors, including breast cancer. Even though improvements in survival have been made in early-stage breast cancer, the prognosis of the metastasized breast cancer has only improved negligibly. High TN-C expression is correlated with lower lung-metastasis free survival in breast cancer patients. It has been shown that the autocrine TN-C prepares the metastatic niche in the lungs, and also aids in the invasion into the pulmonary blood vessels in animal models. Therefore, in this study we investigated the in vitro effect of TN-C silencing by CRISPR/Cas9 system on the metastatic capacity of the most aggressive subtype of breast cancer, triple negative breast cancer (TNBC). Our study has shown that the TN-C CRISPR/Cas9 treated human cell line had a significantly lower metastatic capacity and had slower migration in the wound healing assay. To specifically target the lung metastatic niche, we used chitosan-based carrier system, which has previously been shown to aid in lung accumulation. The biodistribution study by near-infrared fluorescence imaging for the intravenous injection of oxytocin-grafted chitosan, a peptide-polymer conjugate system, has shown that the carrier system accumulated primarily in liver and then in the lungs. Considering the results herein, we developed a non-viral vector for TN-C editing by CRISPR/Cas9 in the lungs to decrease lung metastasis in TNBC.

P69

hokd as a potential tool for directed cancer gene therapy

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Owing to the difficult cancer staging, diagnostic, prognostic and treatment, researches in new therapies, more effective and with low side effects, are increasing. Currently, one of the most interesting therapies is suicide gene therapy. In the direct way, it consists in the introduction of a gene responsible of the production of a toxin, which will destroy the cell from his inside. In the present manuscript our purpose was to investigate the antitumor efficacy of *hokD* gene under the control of different promoters (induced or tumor-specific promoters) in cervix (HeLa) and breast (MCF-7) cancer cells. Experiments were conducted *in vitro* in 2D and 3D culture model and *in vivo* using NOD SCID mice. Our results showed that *hokD* gene expression under the control of tumor-specific promoter causes a drastic inhibition of HeLa and MCF-7 cells proliferation *in vitro* in both 2D and 3D models. Moreover, an important decrease on cell viability was observed by ATPlite analysis. Furthermore, *hokD* gene induced a severe loss of proliferation *in vivo* without any side effects in our animal model. Taking into account our results, this combination of gene and promoter could be a great option in future breast and cervical cancer therapies.

P70

Optimized *PKLR*-gene editing in human Hematopoietic Stem and Progenitor Cells to facilitate clinical gene editing therapy for Pyruvate Kinase Deficiency

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Pyruvate kinase deficiency (PKD) is an autosomal recessive hemolytic anemia caused by mutations in the *PKLR* gene. PKD is associated with reticulocytosis, splenomegaly and iron overload, and may be life-threatening in severely affected patients. Lentiviral gene therapy of Autologous Hematopoietic Stem Cell Transplantation (HSCT) has demonstrated curative properties (NCT#04105166). To develop a precise therapy, we developed a knock-in gene editing strategy by combining RNP electroporation and recombinant adeno-associated viral vector (rAAV6) donors. We obtained stable integration in up to 40% of colony forming units (CFUs) and efficient engraftment in primary and secondary NSG mice, in the absence of toxicity. Nevertheless, clinically relevant correction requires additional developments.

Different alternatives to optimize our original gene editing protocol have been explored in order to reach therapeutic levels. Firs, we assessed the addition of different compounds involved in AAV transduction (Bleomycin, Bortezomib, Teniposide, Daunorubicin and Polyvinyl alcohol), homology repair pathway (BRCA1 and MRE11A proteins) or in the maintenance of the stem compartment (SR1) during gene editing process. Some compounds gave moderate increases. However, best results were obtained when pre-stimulation of HSPC was increased to 48 hours and cell density during the editing process was reduced, reaching editing frequencies up to 70% in clonogenic progenitors. Edited HSPCs under this optimized protocol were infused into

immunodeficient mice and the specific integration was detected in up to 25% of the human cells three months after transplant.

These results confirm the feasibility *PKLR* gene editing in human HSPCs as a potential clinical application for the treatment of PKD.

P71

Editing of human Dyskerin Pseudouridine Synthase 1 gene in hematopoietic stem cells: A new tool to address the treatment of bone marrow failure in patients with X-linked dyskeratosis congenita

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Telomere biology disorders (TBDs) are caused by impaired telomere maintenance. Critically short telomeres limit the replicative cell capacity, leading to reduced tissue renewal. TBDs are variably characterized by bone marrow failure (BMF), cancer predisposition and multiorgan system complications, including organ failure and/or fibrosis. Progressive BMF is the most common life-threatening complication of TBD, affecting up to 80% of patients with classical dyskeratosis congenita (DC). X-linked dyskeratosis congenita (X-DC) is a TBD inherited BMF syndrome caused by mutations in the *DKC1* gene, which plays an essential role for telomerase function and rRNA pseudourydilation. The only curative treatment for the BMF of these patients is hematopoietic stem and progenitor cell transplantation (HSPCT). However, the poor outcomes of HSPCT, make necessary the development of alternative treatments. Here, we developed a gene editing approach based on the use of a rAAV donor vector and sgRNA/Cas9 ribonucleoprotein to generate specific *DKC1* cDNA transgene integration and functional expression in healthy cord blood derived $CD34^+$ progenitors, as proof of concept. Using specific sgRNAs, stable genome editing and *ex vivo* expansion rates have been analysed, either in liquid or in clonogenic cultures. These findings would propose a strategy that could be used as a therapy in patients. Next steps will aim the genetic editing of HSPCs from X-DC patients.

P72

CX3CR1 intron 4 targeting provides a novel safe harbour for gene editing therapies strategies on haematopoietic progenitor and stem cells

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Benefit of ongoing ex-vivo gene editing clinical trials using Haematopoietic Stem Progenitor Cells (HSPCs) is undeniable. However, there are still many concerns regarding editing efficiency, safety, post-transplantation transgene regulation and Central Nervous System (CNS) repopulation that need further refinement. We identified CX3CR1 as an interesting locus with a poised myeloid regulation. Herein, we identified a candidate gRNA targeting efficiently the 4th intron of this locus with CRISPR/CAS9 system on CD34+ HSPCs (>90% indels by ICE analyses), but maintaining unaltered CX3CR1 expression pattern. Next, we ranked different AAV6 donor templates exploiting different insertional DNA repair mechanisms to set an optimized targeting protocol on HSPCs. Robust gene editing was achieved, with >50% stable GFP expression on HSPCs. Notably, despite using a strong viral promoter in our reporter cassette, GFP and CX3CR1 retained a myeloid skewed pattern. Also, this inclusion of a strong promoter in our cassette leads to increased levels of CX3CR1 protein and mRNA in HSPCs and activated macrophages differentiated from the targeted HSPCs. However, neither expansion nor phenotype were barely affected. Since CX3CR1/CX3CL1 axis is involved in migration toward inflamed and damaged organs, we proposed that CX3CR1 increase could beneficially prime migration capacity in HSPCs and their derivates. Resulting on a improve recruitment towards inflamed tissues, including the CNS. In summary, our preliminary results encourage the use CX3CR1 4th intron as a safe harbour for CRISPR/CAS9 targeted insertion.

P73

Gene editing technologies for the improvement of the production of CD19-CAR-T cell-derived exosomes

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Chimeric antigen receptor (CAR) expressing T cells (CAR-T cells) have showed a great potential for the treatment of leukemias and lymphomas. However, CAR-T based therapies have also

reported some limited efficacy attributed to insufficient CAR-T cells penetrance and/or persistence and due to the immunosuppressive tumor microenvironment as well. As a complementary approach, preclinical research has demonstrated that nanotechnology based in secreted exosomes can overcome some of these challenges. CAR-T cell-derived exosomes have been proposed as an emerging and complementary approach, that retain most of the CAR-T cells features, including the capacity of release of high number of cytotoxic molecules, low immunogenicity and high biocompatibility. However, several limitations must be acknowledged and addressed before they can be effectively used in a clinical application. One of the main limitations in the development of exosome-based therapies is the difficulty in producing enough and efficient amounts of therapeutic exosomes. With the aim to solve this last limitation we developed an effective and safe platform, based on genomic editing techniques, to enhance exosomes production from CAR-T cells. Our study provided a proof of concept with preliminary results that demonstrate the utility of our approach. We demonstrated that the knockout of gene 1 induces an increase in the amount of exosome production, according to transmission electron microscopy (TEM), nanoparticle tracking analysis (NTA) and atomic force microscopy (AFM). We also showed that the obtained exosomes carry CAR molecules on their surface. All our data, open the door to combinatorial immunotherapy approaches pairing CAR-T with therapeutical EXO-CART.

P74

CRISPR/CAS9 approach targeting gene amplifications for efficient and selective elimination of cancer cells.

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Cancer has classically been considered as a group of diseases triggered by a series of (epi-)genetic and chromosomal alterations. Among them, oncogene amplifications are common in many cancer types and are powerful drivers of several human cancers. Indeed, they are also related to poor outcomes due to drug resistance, thus there is a need for developing novel targeted therapies, with improved efficacy and fewer side-effects. We have developed a novel therapeutic approach using CRISPR/Cas9 for the selective targeting of *NMYC* oncogene amplification in the neuroblastoma rare cancer develops in nerve tissue of children under age 5. The promising experimental results obtained in immunosuppress in vivo models have led us to expand our strategy to other type of lethal cancers with no treatment characterized by the presence of oncogene amplifications. Our preliminary CRISPR-based approaches have successfully targets different types of cancer xenografts in in vivo studies.

P75

In vivo gene editing based on CRISPR/Cas9 adenoviral vector delivery in humanized mouse models of Recessive Dystrophic Epidermolysis Bullosa Bullosa skin.

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Recessive Dystrophic Epidermolysis Bullosa Bullosa (RDEB), a devastating skin fragility disease characterized by recurrent skin blistering, scar formation and high risk of developing squamous cell carcinoma, is caused by mutations in COL7A1, the gene encoding anchoring fibril-forming *Collagen VII*. Deletion of small, in-phase exons encoding repetitive collagenous domain results in truncated Collagen VII fit for fibril formation. We have generated helper-dependent adenoviral vectors for CRISPR/Cas9 delivery to delete exon 80 of COL7A1, which contains a frameshift mutation that is highly prevalent in the Spanish population of RDEB patients. This NHEJ-based editing strategy proved to be highly effective for the restoration of Collagen VII expression when tested in keratinocyte and fibroblast cultures from patients. To model *in vivo* delivery of the viral vector into patient skin tissue, we used a humanized skin mouse model generated by grafting patient skin equivalents onto immunodeficient mice. Excisional wounds on regenerated patient skin grafts were filled with the adenoviral vectors embedded in a fibrin gel. We detected Collagen VII deposition in the basement membrane zone of the wounded areas treated with the vectors, suggesting that RDEB patient skin lesions can be directly treated by CRISPR/Cas9 delivery *in vivo*.

P76

Prime Editing as a potential strategy for the treatment of Autosomal Dominant Polycystic Kidney Disease in murine models

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Autosomal dominant polycystic kidney disease (ADPKD) is the most common inherited kidney disease, with a prevalence of 1 in 1000 live births. It is characterized by the appearance of cysts in the kidneys and by an increase in the size of the kidneys, leading irrevocably to end-stage renal disease.

Most of the mutations observed in the genes that cause this disease (*PKD1* and *PKD2*) are missense mutations, which makes possible the application of Prime Editing technology to correct these mutations. The correction of the ADPKD-causing mutation should allow a reversion of the ADPKD phenotype, since it has been demonstrated that the kidney is an organ that has plasticity, that is, the correct re-expression of the *Pkd1* and *Pkd2* genes in cystic kidneys results in a rapid reversal of ADPKD.

The overall objective is to use Prime Editing technology to correct the ADPKD-causing mutation in two different mouse models. To achieve this objective, the following specific objectives will be performed: 1) *In silico* design of pegRNAs; 2) *In vitro* testing of pegRNAs in renal cells of both mouse models; 3) Bioinformatics study and selection of the pegRNAs with the highest editing efficiency; 4) *In vivo* editing of the two mouse models with the selected pegRNAs.

Our work is aimed at laying the foundations of gene therapy in the field of renal genetics and specifically in ADPKD, which is the central axis of the project.

P77

Therapeutic extracellular vesicles (TEVs) to delivery Cas9-sgRNA complex for Ewing sarcoma treatment

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Ewing sarcoma is a rare and aggressive bone cancer affecting children and young adults. These tumours are characterised by chromosomal translocations that results in chimeric transcription factors (i.e. *EWSR1-FLI1)* that govern the tumorigenesis process. Previous studies carried out in our group have demonstrated that EWSR1-FLI1 gene inactivation using CRISPR-Cas9 tools produced cell growth arrest and senescence. However, delivery of Cas9 machinery to target cells in vivo is challenge and new approaches are necessary. Extracellular vesicles for Cas9 ribonucleoprotein delivery has emerged as an interesting tool due to its low immunogenicity and versatility. We have generated therapeutic extracellular vesicles (TEVs) containing Cas9 and specific sgRNAs designed to inactive EWSR1-FLI1 using a combination of molecular strategies that include the use of RNA binding proteins, ribozymes and protein dimerization domains. TEVs could be isolated from the culture medium of HEK293T cells transfected with the appropriate plasmids. Cas9 amount contain in TEVs was quantified by western blot using a Cas9 recombinant protein standard curve. *In vitro* assays showed that the addition of TEVs to the Ewing sarcoma cell line A673 produced around 50% gene editing at EWSR1-FLI1 gene after 72h demonstrating the effectiveness of this approach. We are improving this delivery system to increase their specificity to target Ewing sarcoma cells for its application *in vivo*. In conclusion, the use of TEVs to induce gene editing at least *in vitro* is efficient and would be readily applicable to other pathologies in which gene editing-based therapies could represent a therapeutic option.

P78

Detection of Ewing sarcoma fusion oncogenes variants using CRISPR/Cas13

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Ewing sarcoma is an aggressive tumor type that arises mainly in children and adolescents. It is the second most prevalent bone tumor, with a 70–80% survival rate for individuals with standard-risk and a localized tumor; however, this rate decreases to 30% for those patients with metastatic disease. Ewing sarcoma cells are characterized by a balanced chromosomal translocation in which a member of the *FET* gene family is fused with an *ETS* transcription factor. *EWSR1–FLI1* is the most prevalent fusion oncogene (FO) (85%), although there are numerous variants of this FO with different partner genes. The detection of FOs associated with Ewing sarcoma by traditional diagnostic methods such as qRT-PCR, FISH, or NGS is a routine in the clinic. However, those techniques are expensive and time-consuming, requiring multiple steps and dedicated equipment and personnel. In this context, the identification of a novel RNA-targeting CRISPR enzyme, Cas13, allows the selective detection of FO transcripts oppening up the possibility of the development of new diagnostic options. The CRISPR/Cas13 system has already been used to develop viral detection systems, providing rapid RNA detection with attomolar sensitivity and single-base mismatch specificity. We have optimized a highly sensitive, specific, affordable, and instrument-free diagnostic test for FO detection by Cas13.

P79

Screening of epigenetic modulators in Ewing's Sarcoma using CRISPR/Cas9

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The epigenome is highly deregulated in cancer and, as it consists of reversible modifications at the cellular DNA level, it is considered an attractive therapeutic target. Childhood cancers are characterized by lower mutagenic profiles than adult cancers. Few mutations drive malignant transformation. The paediatric tumour Ewing's Sarcoma (ES) is characterized by the EWSR1/FLI1 fusion gene, as a consequence of the translocation t(11;22) (q24;q12). Its product acts as a transcription modulator that has been shown to be a key player in the dysregulation of the epigenome of these neoplastic cells. We hypothesized that high-throughput CRISPR targeting of epigenetic regulators will enable us to define a set of genes that are essential to ES tumorogenicity. We have identified genes whose absence inhibits cell proliferation of ES-derived cell lines. This approach allows the identification of possible candidates that could be targetable to inhibit tumor progression.

P80

Generation of a dual-fluorescent reporter cell line to test different delivery approaches for the CRISPR/Cas system

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One of the most widely used gene-editing tools is the CRISPR/Cas system, which emerged as an immune prokaryotic defense system against bacteriophages. It is a highly versatile system suitable for multiple applications, including different diagnostic strategies and targeted therapies. However, one of its major concerns is the delivery of the ribonucleoprotein elements when applied for *in vivo* experiments and gene therapy strategies. In fact, the core of the CRISPR/Cas research is focused on developing appropriate delivery methods and creating reliable models that allow to determine the efficiency of the system when applying these delivery strategies. For this reason, we have generated a dual-fluorescent reporter cell line that has been created through a CRISPR/Cas9-loxP system, with the aim of assessing the efficiency of multiple methods to deliver the CRISPR/Cas elements, such as transient transfection or stable lentivirus infection. Confocal microscopy and flow cytometry analysis were employed to confirm the editing activity through GFP activation when the protein Cas9 was differently delivered. Besides, our preliminary results reveal that the targeted gene-editing events were successfully detected in our *in vitro* model. In conclusion, this reporter cell line may help to further study and analyse the efficiency of the CRISPR/Cas system in delivery experiments for complex *in vivo* models, which would increase its translational potential when applied for gene therapy strategies.

P81

Response to oncolytic virus in canine patients with spontaneous gliomas

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Gliomas are the most frequent malignant intracranial tumors in dogs, presenting a poor therapeutic response to the treatments. The use of oncolytic adenovirus to treat gliomas has shown promising results in murine models and human clinical trials. Due to the poor prognosis of canine patients with gliomas, we propose using oncolytic adenovirus ICOCAV15 to improve their well-being and survival.

The ICOCAV15 will produce a lysis of tumor cells, and will activate immune response, thereby reducing tumor burgeon in patients with gliomas.

Eight companion dogs with spontaneous gliomas diagnosed by the Neurology Department at the UAX Veterinary Hospital were included.

Surgery by craniotomy, a biopsy of the tumor was taken for diagnosis. ICOCAV15 was intratumorally administered in 6 dogs. Routine blood analysis was performed during follow-up. Tumor samples (pretreatment and post-mortem) were obtained to detect CD3, IBA1 and Calprotectin by immunohistochemistry. Follow-up by MRI to determine RAVNO criteria. This study was approved by the Ethics Evaluation Committee of the Official College of Veterinarians of Madrid (COLVEMA).

ICOCAV15 manage 2/6 CR (6 and 13 months) and 2/6 SD (6 and 27 months). Median survival time was 247 days in treated patients (n=6, two remain alive), and 76 days in the control group (n=2, one remains alive). An increase of CD3, IBA1 and Calprotectin was observed in ICOCAV15-treated tumors.

This proof of concept shows a longer survival time, higher immune infiltration, and absence of secondary effects in intracranial ICOCAV15-treated patients; therefore, ICOCAV15 should be further explored as a treatment in veterinary neuro-oncology.

P82

Oncolytic virus as immunotherapy treatment in canine cancer patients

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The use of oncolytic viruses is an innovative approach that produces the lysis of tumor cells and induces antitumor immune responses. The intratumoral therapy with oncolytic virus received the approval from the FDA and EMA with Imlygic for human melanoma patients. Our goal is to determine safety and efficacy of the oncolytic canine adenovirus (Ad) ICOCAV15 in canine patients.

Eight dogs diagnosed with carcinoma and adenocarcinoma were intratumorally treated with ICOCAV15. To evaluate the safety of the treatment, blood count, biochemistry and coagulation test were determined during follow-up. RECIST criteria were used. Neutralizing antibodies against virus were assessed. Immune infiltration, vascularization and viral presence in the tumor were determined by CD3, CD4, CD20, CD31 and Ad using immunohistochemistry. The compassionate use of ICOCAV15 was approved by the Ethics Evaluation Committee of the Official College of Veterinarians of Madrid (COLVEMA).

All dogs maintained good quality of life during follow-up, and some of them increased the median survival time (MST) compared to dogs treated with chemotherapy. No side effects were detected. Two patients showed partial response (PR), and the rest showed stable disease (SD) at different times during the study. ICOCAV15 was detected during follow-up inside the tumor, and antiviral antibodies were detected in all patients. Tumor immune infiltration increased after viral administration.

Therefore, we suggest that ICOCAV15 intratumorally administrated, should be proposed as a new tool to the treatment of carcinoma because of its safety, it is well tolerated by dogs and shows promising results.

P83

Exploring the pore-forming toxin aerolysin as a candidate transgene for oncolytic adenoviruses

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Oncolytic viruses (OVs) have regained clinical interest in the oncology field for their potential to restore host anti-tumour immunity in addition to directly replicating in and killing cancer cells, making them ideal partners for combination with other immunotherapies. Among OV vector platforms, oncolytic adenoviruses are one of the most widely studied for their tumour selectivity, safety and transgene-arming capacity. However, one of the major challenges remaining is the fact that highly immunogenic viral epitopes often give rise to biased immune responses against them over tumour epitopes, a phenomenon known as viral immunodominance. Here we show that aerolysin from *Aeromonas hydrophila* is a strong immunogenic cell death (ICD) inducer that could enhance anti-tumour efficacy if armed in oncolytic adenoviruses. We found that vaccination with CT26 cells treated with aerolysin-containing supernatants controlled tumour growth not only in the vaccination site but also prevented a tumour rechallenge in the other flank of BALB/c mice. Thus, the absence of secondary tumour suggested that an immune response was triggered by tumour cells undergoing ICD. We expect to take benefit from aerolysin as a transgene for oncolytic therapy, taking into account that the oncolytic virus will restrict the toxicity within the tumour. Moreover, the virus-independent ICD caused by a secreted pore-forming toxin in the neighbouring non-infected cells may diminish the viral immunodominance.

P84

Suicide gene therapy: *ldrB* genes from *E.coli* as a potential tool against cancer

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Cancer is one of the diseases with the highest mortality at present. Due to this it is necessary to develop new more effective therapies with fewer side effects. The use of suicide gene therapy can be a novel tool for this. Here, we evaluate the cytotoxic impact of ldrB toxin of Escherichia coli. For that purpose, colorectal, breast and cervix cancer cells were transfected under the control of TRE3G promoter inducible by doxycycline. Our results showed a decrease in cell proliferation in the HeLa, MCF7 and HCT116 cancer cell line. On the other hand, the mechanism of action of cell death is revealed through the development of a western blot protocol, showing the presence of proteins involved in pyroptosis in the transfected cells with respect to the control. Scanning electron microscopy shows that ldrB toxin generates a decrease in cell density and creates large pores in the membrane that can lead to cell death. The appearance of large-sized pyroptotic bodies is also observed, as well as the rupture of the membrane. Taken together, our results provide proof of the antitumor effect of this toxin in colorectal, breast and cervical cancer, and a sample of the potential of suicide gene therapy as an anticancer tool.

P85

Secretome from porcine cardiosphered derived-cells decreased the pro-inflammatory profile of porcine M1 macrophages in vitro.

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Activated macrophages are usually divided into M1 (pro-inflammatory profile) and M2 (anti-inflammatory profile), based on surface marker expression and cytokine secretion. Both are related to inflammatory diseases such as myocardial infarction (AMI), being key in both the initial inflammatory response and subsequent wound healing. However, the prolonged presence of M1 can lead to expansion of the infarcted area, thus delaying the repair phase.

Administration of the secretome from stromal cells has been suggested as a promising therapeutic approach for inflammatory diseases, as they appear to ameliorate the inflammatory environment by modulating the activation state of macrophages.

Pigs are widely used as animal models in the field of cardiovascular diseases, due to their similarity to the human heart. Therefore, we used porcine macrophages to study the immunomodulatory capacity of secretome from porcine cardiosphere-derived cells (S-CDCs) *in vitro*.

Porcine macrophages were isolated from the peripheral blood of healthy pigs and polarized to M1 by adding hGM-CSF to the medium. On day 7 of culture, surface receptor expression and cytokine expression were measured by flow cytometry and qPCR in response to 24 hours of LPS + IFNg stimulation and S-CDCs treatment.

M1-polarized macrophages with hGM-CSF and stimulated with LPS + IFNg, and M2-polarized macrophages with hM-CSF and stimulated with IL-4 were used as controls.

After treatment with S-CDCs, M1 markers such as SLA-II and TNFa decreased whereas M2 markers such as CD206/CD163, Arg-1 and IL-10 increased.

In conclusion, the administration of S-CDCs may have a beneficial effect on the inflammatory phase of AMI.

P86

Positive angiogenic effect of secretome from menstrual blood-derived mesenchymal stromal cells (MenSCs) on HUVECs

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Mesenchymal stromal cells isolated from menstrual blood (MenSCs) currently represent a valuable therapeutic tool due to their biological activity, including angiogenic properties, and for being a source of mesenchymal stem cells (MSCs) with easy isolation, high proliferation rate, low immunogenicity, and no ethical conflicts. The secretome from these cells shares the therapeutic capacity of their progenitor cells with additional advantages such as being a more stable treatment with lower immunogenicity. Thus, the aim of this work was to evaluate the functional effect of the secretome on human umbilical vein endothelial cells (HUVEC). For this purpose, proliferation was analyzed by CCK8 and functional angiogenesis assays (migration, wound closure, and tube formation) were performed. All assays were performed with a secretome concentration of 100 µg/mL and 24 hours of co-culture. M199 +1% FBS medium was used as negative control (NC) and supplemented endothelial cell growth medium (EGM2) as positive control (PC). The results obtained were statistically compared with the NC. A significant increase in proliferation rate (p value = < ,01), migration rate (p value < ,001), and tube formation (p value < ,01) was observed. The wound assay showed a significantly higher closure capacity (p value < ,0001), similar to that observed in CP. All the results obtained demonstrate the angiogenic properties of the secretome from MenSCs and corroborate the role of the secretome as a potential tool in regenerative therapies.

P87

Proteomic profile on remote myocardium after administration of secretome from menstrual blood derived cells in swine infarct model

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The swine model of myocardial infarction is a great animal model to mimic this pathology and evaluate the efficacy of different therapies. After myocardial infarction induction, proteomic changes are developed in the remote and infarcted myocardium, contributing to the pathogenesis and the poor prognosis. In this scenario, the evaluation of stem cell therapy effects let shed light on new therapeutic strategies. Therefore, in this work, we improve the therapeutic potential of menstrual blood stromal cells (MenSCs) by priming with IFNγ and TNFα (MenSCs*) and evaluate

its effect on remote myocardium after their secretome administration (S-MenSCs*). For this purpose, myocardial infarctions were generated induced by 90-min balloon occlusion of the mid-LAD. Then, and by intrapericardial administration, animals received S-MenSCs* (AMI/S-MenSCs*, n = 4) and vehicle (AMI/Placebo, n = 4). Seven days post-therapy, hearts were harvested and infarcted tissues were collected for proteomic analyses. Comparative results showed 64 genes differentially expressed (FDR < .05) after S-MenSCs* treatment in remote myocardium. STRING web tool was used for functional enrichment analysis, revealing that differentially expressed genes are related to Gene Ontology Biological Process and Reactome Pathways such as *Muscle contraction* (GO:0006936), *Striated muscle contraction* (GO:0006941, SSC-390522) (FDR < .05). These results suggest that the secretome content of MenSCs* could regulate the gene expression of protein related to contractile fibers of cardiac tissue, contributing to alleviate cardiac disfunction. In conclusion, the therapeutic potential of S-MenSCs* in remote myocardium of myocardial infarction patients may reduce cardiac remodeling and so, improving their prognosis.

P88

Bioengineered 3D models of pancreatic cancer as personalized medicine platforms

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Pancreatic cancer (PC) represents the seventh leading cause of cancer death worldwide and its incidence is increasing every year. Survival in this type of tumor is less than 5% due to lack of early diagnosis, rapid disease progression, high rate of metastasis and treatment failure. In PC, the stroma constitutes more than 90% of the tumor mass and is composed of many different elements. This highlights the need to create new 3D in vitro models incorporating the tumoral microenvironment (TME), as tumor progression and response to treatments depend to a large extent on it. These models may help us to understand and overcome the resistance of PC to several therapeutic strategies. Thus, the incorporation of dECM-based bioinks provides 3D models of a more physiological TME giving them their characteristic complexity and allowing interactions between cells and with the ECM. This makes it possible to mimic characteristics of native tumors: development and metastasis, and treatment outcomes. In addition, organoids are good personalized medicine platforms, useful in pharmacotyping studies because they show parallel sensitivity and resistances in patients. Some treatments strategies, as CAR-T therapy, find many challenges to be effective in solid tumors. Because PC organoids can mimic the native tissue, they can facilitate the identification of novel antigens which could serve as targets for CAR-T therapy and also can help us to better understand resistance to it.

P89

Pre-stimulation of CACs with atherosclerotic factors potentiate their regenerative properties in critical limb ischemia.

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Cell therapy mediated by circulating angiogenic cells (CACs) has been postulated as a promising cardiovascular therapy due to their angiogenic properties, and therefore a good alternative treatment against critical limb ischemia (CLI). However, major efforts are still required to understand their mechanisms of action in order to enhance their regenerative effect. According to previous results of our research group, the incubation *ex vivo* of CACs with factors secreted by atheroma plaques (AP) promotes activation and mobilization of these cells. For that reason, we aimed to evaluate whether pre-stimulated CACs *ex vivo* with AP secretomes could potentiate the regenerative properties associated with CACs *in vivo*, in a murine model of CLI, with a 21 days follow up. The results obtained show an improvement in blood flow and a decrease in the progression of the disease when the CLI mice were treated with CACs, and even more when cells had been previously stimulated with atherosclerotic factors. In addition, our proteomic analysis identified more than 3000 proteins in the ischemic tissue, and the expression patterns in mice treated with CACs stimulated or not with AP factors were totally different. According to functional classifications, altered proteins were associated with different processes such as necrosis, immune response and development of vasculature. In conclusion, atherosclerotic factors cause changes in the regenerative role of CACs.

P90

3D-Bioprinted CSC-based malignant melanoma models as a new tool for antitumoral drug screening

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Mimicking the cancer microenvironment still remains challenging but it is acquiring a vital importance due to its influence on tumor progression and metastasis. 3D bioprinting is an emerging tool that enables the creation of tissue models by combining different cell types and supporting matrices, providing a platform to represent the great tumor heterogeneity. In this work, we have developed two 3D melanoma tumor model based on cancer stem cells (CSCs), employing the extrusion bioprinting method. For that, we embedded cancer stem cells isolated from a MM established cell line or a primary-patient derived cell line, fibroblasts, mesenchymal stem cells, and endothelial cells, within a hydrogel representing the three human skin layers, in

order to mimic melanoma and its tumor microenvironment. TME-cells showed high proliferation and metabolic activity, and actively remodeled their niche. MM models displayed similar rheological properties that skin and were able to support an early onset of vascularization. Besides, MM models displayed different response to vemurafenib compared with cell cultures, allowing the detection of chemoresistant tumors. Moreover, MM hydrogels supported tumorigenesis in murine xenotransplant achieving more mimetic *in vivo* models. To our knowledge, it is the first time that a novel tri-layered bioprinted 3D CSCs- based melanoma model has been developed, with potential alternative to small animal models and use for oncological research and analysis of tumor response to new personalized therapies against melanoma.

P91

Tumor-on-a-chip for metastasis study: closer to precision medicine

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For decades, numerous studies have been carried out to generate a mimetic model for the study of metastasis, the main reason for most cancer deaths, to resolve the unknowns surrounding this disease. To better understand this process of cell dissemination, more realistic models capable of faithfully recreating the tumor microenvironment (TME) completely are needed. Therefore, new tools known as tumor-on-a-chip and metastasis-on-a-chip have recently been proposed. These tools incorporate microfluidic systems and small culture chambers in which the TME can be faithfully recreated thanks to 3D bioprinting. In this work, a literature review has been carried out on the different phases of metastasis, the unknowns that remain for its complete understanding, and the use of new models for the study of this disease. The aim is to provide an overview of the current landscape and the great potential of these tumor-on-a-chip systems for *in vitro* translational research into the molecular basis of this pathology. In addition, these models will allow progress towards personalized medicine by generating chips from patient samples that mimic the original tumor and the metastatic process in order to perform an accurate pharmacological screening and establish the most appropriate treatment protocol.

P92

Establishing the optimal lymphatic endothelial progenitor cell (LEPC) source for cellular therapy and tissue engineering approaches

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The lymphatic system is crucial for maintaining homeostasis, nutrient transport, and immune surveillance within adult tissues, and is also relevant in the promotion of cancer metastasis. Lymphatic endothelial progenitor cells (LEPCs) promote lymphangiogenesis *in vitro* and *in vivo*, and represent a suitable cell source for the *in vitro* engineering of lymphatic vessels. Although LEPC

express transmembrane glycoproteins such as Podoplanin, CD31 and LYVE-1, the optimal strategy for the extraction of LEPCs from solid tissues and their subsequent use for cellular therapy remains to be established. In this work, we aimed to establish a reliable source of LEPCs to be used in tissue engineering and cell therapy, as well as characterize their lymphangiogenic behaviour *in vitro*. To this end, primary cells were extracted from the bone marrow, dermis and vascular stromal fractions (VSF) of C57BL/6 adult mice (N=22) and analysed for the expression of Podoplanin, CD31 and LYVE-1 by flow cytometry, at day 0 and after 4 days of culture in the presence of lymphangiogenic medium. The results showed that VSF and bone marrow outperformed the dermis as the optimal tissue sources of LEPCs. The establishment of a reliable LEPC tissue source will favour the development of patient-specific cellular therapies.

P93

Development of customized bioinks for dynamic 3D-printed cancer models

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Use of three-dimensional (3D) bioprinting for the *in vitro* engineering of tissues is booming. Numerous commercial biocompatible bioinks are available, with suitable mechanical and rheological characteristics. However, cell-laden bioinks based on a single polymer do not properly mimic the complex extracellular environment needed to tune cell behavior, as required for *in vitro* tumor modelling. In addition, processes such as cell migration should be dynamically monitored. Solid tumor micromodels based on printed decellularized extracellular matrices (dECMs) have the advantages that (i) the biomolecule-rich matrix of dECM allows cell growth in a natural 3D environment; and (ii) microtumors with a combination of dECM scaffolds and tumoral cells allow cancer monitoring in a more realistic 3D microenvironment. To develop melanoma and breast cancer models, we have exposed porcine skin and breast tissues to diverse decellularization strategies. Effective tissue decellularization was confirmed by histological analysis and DNA quantification. The resulting dECMs were grinded and digested into thermally-crosslinkable hydrogels that were rheometrically characterized. A number of tumoral cell lines and accompanying cells were cultured and labelled individually to allow dynamic cell monitoring within the bioprinting assays. The decellularized porcine dermis and breast were thus effectively turned into printable dECM inks that exhibit suitable rheological and biocompatible properties. Additionally, complex and manipulable 3D cellular models have been developed for melanoma and breast cancer modelling. Our results suggest that *in vitro* 3D printed dECM can be used as a powerful tool in tissue engineering and disease modelling, to make progress towards understanding cellular behavior and drug responses.

P94

Novel culture conditions for the improvement of the in vitro expansion of human Spermatogonial Stem Cells. Future stem cell therapies to restore fertility in prepuberal boys enrolled in our experimental fertility preservation program with patients with cancer or genetic syndromes.

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Some of the cancer treatments and genetic syndromes, such as Klinefelter Syndrome, can cause fertility problems in adulthood in prepuberal children. Prepuberal patient do not have the option to cryopreserve the sperm so, to the date, there is no alternative to restore the fertility in the future. The aim of this experimental fertility program is to preserve testicular tissue (with Spermatogonial Stem Cells (SSCs)) to expand in vitro these stem cells for future autologous transplantation of the tissue or expanded SSCs in the adulthood. We collected and processed testicular biopsies of both adults and children (oncologic and KS). Each biopsy was divided into 3 fragments: for histological study, for clinical use and for research. We observed that adult patients, all oncologic prepuberal patients and 20% of Klinefelter Syndrome prepuberal patients express both VASA and MAGEA4 germ cell markers. Firstly, we expanded human male fetal germ cells (hPGCs) in vitro under several culture conditions. Our findings provide a 2D culture system to expand hPGCs that could be useful to study propagation to SSCs. Secondly, we pursued in vitro expansion cultures of adult SSCs using a modification of our previous culture conditions for hPGCs. We observed an increase of GPR125 + cells (3,3%) in comparison with the control culture condition (1,5%) after 28 days in culture. After these preliminary data in adult SSCs, we will use this novel culture condition for improvement of in vitro expansion of prepuberal SSCs for future cell therapy.

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A comparative study of cell culture conditions during conversion from primed to naive human pluripotent stem cells

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The successful reprograming of human somatic cells into induced pluripotent stem cells (hiPSCs) represented a turning point in the stem cell research field owing to their ability to differentiate into any cell type and lack of ethical issues. In the mouse, PSCs are thought to exist in a naive state, the cell culture equivalent of the immature, pre-implantation embryo whereas in humans, PSCs are in a primed state, which is more committed pluripotent state than naive state. Recent studies have focused on capturing a similar cell state in human, since their earlier development stage and lack of cell-of-origin epigenetic memory make the better candidates for further re-differentiation and use in disease modelling, regenerative medicine and drug discovery. In this study, we compared and evaluated the successful establishment and maintenance of primed hiPSC and human embryonic stem cell (hESC) lines with three different naive conversion media both feeder and feeder-free conditions. In addition, we compared the directed differentiation capacity of primed and naive cells in the three germ layers. We characterized these different cell states with commonly used pluripotent and lineage specific markers, and showed that, in general, naive culture medium 1 (in both feeder and feeder-free system) confers greater hiPSCs and hESCs viability and highest naive pluripotency markers expression. This medium also allows for better differentiation of cells toward endoderm.

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Therapy free of cells *vs* human mesenchymal stem cells from umbilical cord stroma to treat the inflammation in OA

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In the last decade, extracellular vesicles in combination with the use of miRNAs to modify post-transcriptional expressions of multiple genes have shown their utility in new therapies to treat inflammatory diseases. This work delves into the anti-inflammatory effect of extracellular vesicles derived from mesenchymal stem cells previously modified so that the expression of miR-21 is inhibited. We compare the efficacy of two treatments, mesenchymal stem cell with their miR-21 inhibited through lentiviral transfection and their derived extracellular vesicles, against inflammation in a new OA animal model. The modified mesenchymal stem cell and their extracellular vesicles were intraperitoneally injected in an OA animal model by twice. One month after treatment, the animals were eutanized to check which therapy was the most affective to reduce inflammation compares with animals untreated. Treated OA model sera were analyzed for cytokines and chemokines. Subsequently, different organs were analyzed to validate the results

obtained. Extracellular vesicles were the most efective treatment to reduce SASP as well as chemocines and cytokines in serum of OA animals compared with mesenchymal stem cells alone in a statistically significant way. Besides, miR-21 is acting through ERK1/2 pathway which is envolved into inflammation. Syndecan 1 seems to be involved into this communication between SASP and inflammation as well as extracelular vesicles production, which make it a good candidate to generate a new anti-inflammatory therapy.

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Upregulation of the Yamanaka´s factors by single adenoviral gene cellular reprogramming.

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The induction of pluripotency by enforced expression of different sets of genes in somatic cells has been achieved with reprogramming technologies firstly described by the Yamanaka's group.

Methodologies for generating induced pluripotent stem cells are as varied as the combinations of genes used. It has previously been reported that the adenoviral E1a gene can induce expression of two of the Yamanaka factors (c-Myc and Oct-4) as well as epigenetic changes.

Here, we demonstrate that E1a-12S over-expression is sufficient to induce pluripotent-like characteristics closely to epiblast stem cells in mouse embryonic fibroblasts through the activation of the pluripotency gene regulatory network. These findings provide empirical evidence that expression of one single factor is sufficient for reprogramming to the epiblast stage.

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