Project Final Report

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Name, title and organisation of the scientific representative of the project's coordinator:

Prof. Dr. Arti Ahluwalia – University of Pisa (UNIPI)

Tel: +39 050 2217062

Fax: +39 050 2217051

E-mail: arti.ahluwalia@unipi.it

Project website address: www.re-liver.eu

Table of Content

Section 1 – Final publishable summary report

Re-Liver

Logo:

Project title: **Bottom-up reconstitution of a biomimetic bioartificial liver**

Website: **www.re-liver.eu**

Contractors involved (Re-Liver consortium):

1 UNIPI: The University of Pisa, Prof. Dr. Arti Ahluwalia, Interdepartmental Research Centre "E. Piaggio", Largo Lucio Lazzarino 1, 56122 Pisa, Italy

Other partners and team leaders:

2 TECL: The Electrospinnig Company, Dr. Rob McKean

3 MEDI: Medicyte GmbH, Dr. Joris Braspenning

4 UNIMAN: The University of Manchester, Prof. Dr. Nicola Tirelli

5 GABOmi: GABO:mi mbH & Co. KG, Dr. Sibylle Gündisch

1.1 Executive summary

The Re-Liver project is based on a strong scientific interaction between two industry partners and two research institutes. The unique strategy of developing an organ-like product which can be used for in-vitro ADMET (Absorption, Distribution, Metabolism, Excretion and Toxicity) related investigations as well as for therapeutic applications (implantable ATMP: Advanced Therapeutic Medicinal Product, HemA), led to the development of several new innovations, methods and products.In order to provide a solid base for both aspects of the Re-Liver project, i.e. ADMET-related products and cell therapeutic products, an early step was to analyse human hepatic ECM in **WP1**. For this purpose a new method was developed to obtain hepatic extracellular matrix (ECM) in a reproducible manner. To analyse the viscoelastic parameters of the liver ECM, two mechanical testing methods, a) step reconstructed DMA (srDMA) and b) nanoepsilon dot (nano-$\dot{\varepsilon}$), were developed during the Re-Liver project. A new image analysis algorithm for the extraction of biochemical data from histological images of liver slices was also developed and implemented in an open source software.

WP2 dealt with the preparation of the "building blocks" for the construction of in vivo (implantable ATMP) and in vitro (ADMET) devices used in other WPs. We focused on two areas: 1) developing of electrospun scaffolds (3D synthetic mimics of the extracellular matrix, ECM) of different architectures, surface-functionalised or not, to be used in combination with hepatocytes for ADMET applications, and 2) developing of biocompatible, remodelable matrices (hyaluronic acid and fibrin) that can be formed in situ and possibly in the presence of cells such as autologous endothelial cells (ECs) for the production of implantable materials (ATMP applications) for liver tissue engineering.

WP3 dealt with cellular tools, i.e. cells, media as well as electrospun scaffolds, and with the development of a perfusion system to generate products that can be used for either (I) ADMET applications or (II) cell therapeutic applications. The aim of this WP was to develop and combine the tools with those developed in WP2 in the form of tangible products and outputs. A brief summary:

- Non-functionalized electrospun networks, which are mimetic of architecture and properties of hepatic ECM, were optimized for hepatocyte cell line proliferation and behaviour;
- Surface functionalized electrospun networks were evaluated for upcyte® hepatocyte adhesion and colonization;
- TECL Mimetix plates significantly enhanced HepG2 metabolic competency for ADMET;
- Two bioreactors were developed and patented (currently at the PCT stage);
- Several cell types were "upcyted" to generate liver organoids for ADMET and therapeutic applications;
- Liver organoids survived and proliferated in the bioreactor, LiveBox1, but not in static conditions;
- Therapeutic endothelial cells were generated which produce Factor VIII to treat haemophilia A;
- In situ gelling formulations used as injectable matrices for the matrix-guided implantation of endothelial cells (ECs);
- A chemically defined growth medium for the expansion of therapeutic endothelial cells was developed.

Marketing and exploitation plans have been generated and updated regularly as part of WP5 for the further development and exploitation of the products generated by the Re-Liver consortium.

WP4 focused on the in vitro and in vivo pre-clinical development of the two ATMPs. Liver organoids (LOs) were cultured in the LiveBox1 developed by UNIPI for evaluation of long term survival and metabolic performance of the LOs for ADMET applications. An inter-laboratory validation demonstrated good metabolic activity, stability and reproducibility of the LOs in the LiveBox1. The chemically defined medium developed in WP3 allowed a good expansion of the therapeutic ECs producing Factor VIII to treat haemophilia A. It could be nicely shown that expression of the therapeutic Factor VIII was retained during the expansion phase and upon implantation. Two ways of implantation and engraftment in animal models were investigated, endothelial cells in hydrogels, developed in WP2 by UNIMAN or therapeutic ECs implanted in a Cell Pouch™, in collaboration with Sernova (Cell Pouch™ manufacturer). The LOs were investigated for their engraftment as well in animal models. Liver organoids were implanted directly onto the mesentery using a fibrin glue or implanted using the biological vascularized scaffold (BioVaSc™) developed at the Fraunhofer IGB. In animal models, both therapeutic products, therapeutic endothelial cells producing Factor VIII and the LOs, were shown to be safe. Neither tumour formation could be observed nor did distribution of the cells within the organism occur indicating that cells stay at the site of implantation. These are very encouraging results for further preclinical and clinical development of the two ATMPs developed by the Re-Liver consortium.

1.2 Summary description of project context and objectives

Background and Aims

The aim of Re-Liver was to develop a bioartificial liver organoid constructing a synthetic equivalent of the human extracellular matrix (ECM) seeded with autologous or allogenic cells for the treatment of severe life threatening diseases. Beside the therapeutic application, cells containing bioartificial liver constructs were developed for in-vitro ADMET (Absorption, Distribution, Metabolism, and Excretion) applications.

The knowhow gathered within this project led to the development of several products for more predictive in vitro ADMET testing as well as the development of two different advanced therapeutic medicinal products (ATMP), therapeutic cells for treatment of haemophilia A (HemA) and liver organoids for acute liver failure (ALF) disease. Both products were developed for implantation by minimal invasive injection and will provide essential cell functions to treat the indicated diseases. The three year research project enabled this consortium to demonstrate pre-clinical proof of principle in animal studies and to investigate safety aspects allowing further (pre) clinical development. Although the long term goal is to utilize the engineered bioartificial constructs for therapeutic applications, within the time frame of the project the objectives of the Re-Liver proposal were:

- To reconstitute a 3D human hepatic ECM by re-designing and re-assembling its components using a bottom-up approach combining electrospun fibres which are modified to improve cell function and viability (WP1&2).
- To seed the reconstituted 3D ECM with human hepatic cells (upcyte® hepatocytes or other cell lines) to produce in a stable and reproducible manner products (plates and bioreactors) for in vitro applications (WP2&3).
- To develop angiogenic hydrogels to induce inosculation of microvessels with the host vasculature after implantation of Factor VIII-producing endothelial cells in animals (WP2, 3&4).
- To employ the liver organoids (LO) in a novel bioreactor device (LiveBox1) with integrated optical and environmental sensors for extensive testing of biological function and LO efficiency (WP1, 3&4).
- To investigate the optimal application route for such therapeutic products and the cellular capability to engraft. Two available options were investigated during the Re-Liver project, a) the hydrogels which allow an encapsulation of the endothelial cells as described in WP2 & 4 and b) implantation of the cells into a medical device (Cell Pouch™) or biovascular scaffolds (BioVaSc™) as described in WP4.
- To assess the immunogenicity and safety of bioartificial liver organoids as well as the autologous Factor VIII-producing endothelial cells (WP4).
- To develop a range of innovative commercial tools, technologies, devices and supporting products for use in in vitro (ADMET) and in vivo (therapeutic) applications based on the LOs and therapeutic cells (WP5).

The aim of WP1 was to provide data on the mechanical, structural and biochemical properties of the liver and of the materials developed in the project. The objective of this WP was to characterise and model human liver ECM mechano-architectural properties at different length scales. This defined the design criteria for constructing biomimetic synthetic replicas, which aim to match the structural and biological properties of the human matrix. The results achieved in WP1 were used in WP2 to fabricate the biomimetic replicas.

In particular in this WP the micro and milli-scale analysis of liver ECM establishes the architectural features and mechanical properties (e.g. pore size, fibre size and elastic modulus); while the nanoscale analysis returns the criteria for replicating chemical properties.

Specific aims:

- To define a protocol for the separation of ECM from cells that preserves the hepatic 3D architecture and the matrix constituents.
- To determine the mechanical properties, porosity, water content and permeability of the matrix.
- To quantify the protein content of the matrix.
- To define the architectural (e.g. pore connectivity and distribution) properties of the matrix.
- To model the fluid dynamics and mass transport in matrix and biomimetic replicas in flow conditions.

- To quantify, using the same methodologies, the properties of the biomimetic constructs

The aim of WP2 was to develop the chemistry and biomaterials techniques to produce cell-containing constructs, such as pseudo-lobules and bioartificial liver organoids, which have been used by the Re-Liver project partners (University of Pisa and Medicyte) in other WPs.

Extracellular matrices (ECMs) are composed of fibrillar (elastic) and viscous elements. The fibrillar components are of proteic nature (e.g. collagen, elastin), and are the major determinants of mechanical properties and cell adhesion. The viscous components are of a proteoglycan (glycosylated proteins) composition, with the specific presence of glycosoaminoglycans (e.g. hyaluronic acid), which mainly regulate hydration, osmotic pressure, transport properties in the matrix.

Electrospun materials can be viewed as artificial equivalents of the fibrillar components of natural ECMs, provided they mimic the organization of elastic fibres in a natural tissue (and thus providing adequate porosity, water swellability, etc.). Having in mind the ECM of the hepatic tissue, selected surface groups (sugars) were introduced onto the electrospun fibres in order to favour and modulate their colonisation by hepatocytes, which are known to recognize specific sugar units such as galactose.

In order to mimic also the viscous/viscoelastic components of ECMs, we have developed hydrogels based on hyaluronic acid/fibrin, which can in principle be infiltrated in the electrospun meshwork; if they are prepared in the presence of therapeutic cells, they can be seen as artificial hepatic lobules (pseudo-lobules), which can be seen both as ATMP or ADMET constructs. However, these gels can be seen as soft ECM-analogues in their own right. Appropriate liquid hyaluronic acid/fibrin mixtures can be injected, producing in situ conformal and possibly cell-laden implants. These matrices will be characterised by a high biodegradability/remodelling and by a rapid vascularisation, above all when guest cells, such as ECs, are included. These gels were developed only as ATMP.

The specific aims of WP2 were to develop

- Non-functionalized electrospun networks, which are mimetic of architecture and properties of natural hepatic ECM, as characterized in WP1.
- Surface functionalized electrospun networks to allow cell adhesion and colonization, in particular focusing on upcyte® hepatocyte culture.
- In situ gelling formulations based on hyaluronic acid and fibrin, predominantly used as injectable matrices for the matrix-guided implantation of endothelial cells (ECs).

The objective of WP3 was to characterize the growth conditions and development of a medium for therapeutic cells to achieve optimal production of therapeutic protein according to the criteria defined in WP5. Additionally, the fundamentals of bioengineered tissue, cells (WP3) and scaffolds (WP2) were combined (WP2 and WP3) and evaluated for functionality in the developed perfusion system (WP3). In parallel, a growth medium was developed which is chemically defined, which is essential for *in vivo* applications (WP4).

The specific aims of WP3 were to develop

- To develop optimal media conditions for stable production of Factor VIII by therapeutic endothelial cells.
- Production of upcyte® hepatocytes and liver cells in large scale to provide cells for studies in WP2 and WP3.
- Development of perfusion reactor for BLOs.
- Development of a chemically defined growth medium specific for endothelial cells for autologous cell treatment.
- Evaluation of perfusion reactor for survival and biological functionality of the liver in vitro organoids for ADMET applications.

The objective of WP4 was to employ technologies such as a perfusion system for evaluation of the BLOs produced in WP3. In a first approach, the liver organoids were evaluated for long-term survival and functionality for ADMET applications. In a second step, these bioartificial liver organoids were implanted in animal models to evaluate safety and efficacy of the liver organoids for therapeutic applications. In parallel, therapeutic endothelial cells producing Factor VIII were generated for the treatment of haemophilia A. Both ATMP products were evaluated in in vivo animal studies either implanted directly (with a hydrogel) or injected into a vascularized (biological) scaffold such as the Cell Pouch™ or BioVaSc™.

The specific aims of WP4 were:

- Validation of perfusion reactor for survival and biological functionality of the liver organoids for ADMET applications.
- Production of upcyte® hepatocytes, liver sinusoidal endothelial cells and mesenchymal stem cells in large scale to provide cells for generation of liver organoids.
- Development of an optimal isolation method for blood outgrowth endothelial cells and correction of the genetic defect.
- Evaluation of safety and efficacy of Re-Liver products, liver organoids for treatment of acute liver failure and therapeutic endothelial cells for treatment of HemA were evaluated in in vivo investigations.

The objective and aim of WP5 was to develop an exploitation and marketing strategy for the products developed during the Re-Liver project. Important aspects to consider are the intellectual property rights (IPR), which strategy to follow and how to handle foreground IPR. At first, the background IPR of each partner was identified, allowing to clearly separate it from any foreground IPR. A reporting system was established by GABO:mi, :milliarium - a web based project management-tool allowing an exchange of documents and confidential knowhow in a protected environment.

Very early within the project, a report dealing with the exploitation strategy for ADMET products was completed and continuously updated during the progress of the project. The same holds true for the exploitation of the ATMP, where an exploitation plan was developed and reported. As the Re-Liver consortium was successful in developing two advanced therapeutic medicinal products a plan was made for both of them. Beside the exploitation, a pre-clinical development plan for the two ATMPs was developed. Although for ATMPs biodistribution and safety are important aspects, we had to consider that these are very different products and the engraftment properties can vary significantly. These aspects were considered in each of the pre-clinical development plans.

Work strategy and general description

Background: Due the liver's central metabolic role, hepatic tissue and its engineered derivatives have a wide range of in/ex vivo and in vitro applications that include: the replacement of liver functions via transplantation of the whole organ or its lobes, or the use of bioartificial ex vivo devices (BALs); the treatment of metabolic disorders via implantable constructs; in vitro studies of toxicology, drug metabolism and tissue regeneration. The demand for liver-mimetic or replacement constructs is large and increasing. In the EU alone 6% of the population is estimated to suffer from chronic liver diseases (European Liver Patients Association. http://www.elpa-info.org/.), which are considered a major cause of death in the EU (European Commission Staff SO of the ECS. 2002. The Life of Women and Men in Europe: A Statistical Portrait. Publisher Office for Official Publications of the European Communities). At the same time, model systems are sought to study xenobiotic metabolism and possible toxicity of compounds (e.g. food additives or drugs) to hepatocytes, as well as systems that support viral infections.

Limitations: The possible applications are countered by A) the short supply of suitable donor livers, B) the poor reliability, consistency and predictivity of in vitro and ex vivo technologies capable of recapitulating liver functions in artificial constructs. As far as the latter point is concerned, significant developments are possible by using the most recent advances in biomaterial design, cell-based therapies, tissue engineering and bioreactor technology.

Obstacles for artificial constructs: A) The composition, microenvironment and microarchitecture of liver extracellular matrix (ECM) are essential features to preserve hepatocyte phenotype and enable a construct to perform the liver multiple functions. However, currently available products differ entirely in 3D organization and composition from the liver: for example, ex vivo BAL devices are based on polymeric flat membrane or hollow fibre reactors. B) The absence of a reliable and stable supply of hepatic cells is a crucial shortcoming which compromises attempts of standardization, clinical approval and ultimately commercial exploitation.

Key concepts underlying Re-Liver: A) human matrix as a design template for cellularized bioartificial liver, B) preparative techniques based on rapid prototyping and in situ (in vivo) gelation, C) standardized human cells as well as combinations of autologous and allogenic cells to reduce immunogenicity, D) minimally invasive implantation procedures, D) integration with angiogenic therapy to ensure rapid vascularization upon implantation.

Cells containing bioartificial liver constructs will be developed for in-vitro ADMET (Absorption, Distribution, Metabolism, and Excretion) applications and pre-clinically investigated, initially for treatment of metabolic disorders, such as haemophilia A.

Management structure and procedures

The project coordinator ensured the smooth operation of the project and guaranteed that all efforts were focused towards the objectives. He submitted all required progress reports, deliverables, financial statements to the European Commission, and, with the assistance of GABO:mi he was responsible for the proper use of funds and their transfers to participants. The Re-Liver office was established by and based at the coordinator in Heidelberg and at GABO:mi in Munich. The project office at the coordinator was concerned with the scientific management and the co-ordination of all research activities. The project office at GABO:mi was responsible for administrative, financial and contractual management and the organisational co-ordination of the project activities.

The biotech company Medicyte GmbH, filed for insolvency proceedings on May 26th 2015 with business ending on July 8th 2015. Insolvency receiver is the law firm Wellensiek in Heidelberg. This had no negative impact on the progress of Re-Liver because it happened towards the end of the project. Dr. Joris Braspenning remained the scientific coordinator of the project and finalised the periodic as well as the final report. But on a legal level Prof. Arti Ahluwalia from the University of Pisa has been appointed as the new project coordinator and new coordinating institution and will be responsible for the final transfer of funds to each participant.

The General Assembly (GA) was composed of all participants each of whom had one representative with the authority to vote. All other non-voting researchers working for this project joined the meetings and discussions. The GA met every six months during the funding period and the main tasks were to grant proper implementation of the participant's rights and obligations always in accordance with the contractual framework of the project and the Consortium Agreement. Furthermore, monthly web or phone conferences took place to discuss the process and open tasks on a regular basis. To facilitate the organisation and management, the scientific programme of the project was structured in work packages (WP) which together comprised the project. Each work package has been headed and coordinated by an experienced principal investigator as work package lead and a deputy leader. They were responsible for the management of their WPs. To ensure a high standard of research and monitor the progress of the project by taking part in the annual Governing Board Meetings a scientific advisory board was implemented.

1.3 Description of the main S&T results/foregrounds of Re-Liver

The original goal of Re-Liver was to engineer hepatic tissue constructs for ADMET, clinical applications and factor VIII cell therapy, using human livers as a design inspiration and a multi-faceted approach combining **new technologies** with **material and cell engineering**. The course of the project was changed when new studies were reported in the literature to show that endothelial cells and not hepatocytes produce factor VIII. This new finding forced the consortium to develop technologies for both hepatocytes and endothelial cells.

WP1

The aim of WP1 was to provide data on the mechanical, structural and biochemical properties of the liver and of the materials developed in the project. Part of the WP was addressed to modeling of flow and oxygen diffusion and consumption in 3D environments using computational methods.

The WP began with an in-depth microstructural analysis of human and pig liver extracellular matrix. For this purpose a new method for obtaining reproducible hepatic extracellular matrices (ECMs) was established. A liver ECM derived hydrogel was also developed as a Matrigel substitute for generating liver organoids. In parallel we also designed new techniques for characterizing the mechanical, biochemical and structural properties of liver ECM. The main advantage of the proposed techniques is their reproducibility, allowing precise quantification of parameters of interest. Of note are the two mechanical testing methods developed in Re-Liver and the new image analysis algorithm. The mechanical testing methods are: step reconstructed DMA (srDMA) and nanoepsilon dot method (nano-$\dot{\varepsilon}M$), both of which enable the precise evaluation of viscoelastic parameters of soft tissues. In parallel a new algorithm for the extraction of biochemical data from histological images of liver slices was developed and implemented in an open source software. From a scientific point of view it was also of interest to note that human livers cannot be used as a template for liver tissue engineering as it is not possible to obtain samples of healthy human liver and also because of the variability in donor livers. The Re-Liver consortium choose instead to use pig liver as a design template, given the similarity between porcine and human hepatic metabolism.

Having identified the structural features (pore size, fiber diameter, porosity, permeability, viscoelastic constants) necessary for designing human livers, new materials and fabrication technologies were developed to engineer liver constructs and vascularized hydrogels in WP2 and WP3.

In parallel fluid dynamic and mass transfer models were developed using Finite Element Methods (FEM) for designing bioreactors and informing cell culture experiments in WP3 and WP4. Specifically the models were focused on:

- investigation of flow in porous microspheres with the same structural characteristics as liver ECM in order to determine the ratio of diffusive to advective transport in bioartificial liver organoids;
- determination of optimal flow rates in bioreactors LiveBox1 and 2 to maximize nutrient turnover while maintaining shear stress at acceptable values for hepatocytes;
- experiments to determine oxygen consumption in liver organoids, for input to the models.

WP2

The WP2 focused on the development of chemical tools and on their use for the preparation of “building blocks”, i.e. materials that can be assembled in composite structures including in situ gelling biomimetic constructs for in vivo liver regeneration (ATMP) and for the development of in vitro (ADMET) tools. These “building blocks” are: 1) well-defined electrospun scaffolds (synthetic ECM mimics) capable of providing a suitable 3D environment (porosity and surface chemistry) for their adequate colonization with hepatic cells: this includes macroscopic and sub-mm sized samples (the latter prepared by laser machining of the former), 2) surface coating and bioconjugation tools for the chemical modification of the above materials to tune their physicochemical (e.g. swellability in water) and biological (selective cell colonization by incorporation of cell adhesive ligands) properties, 3) biocompatible, remodeling matrices (based on natural constituents hyaluronic acid and fibrin) suitable for the development of in situ gelling formulations.

In this regard we have successfully developed the following materials/techniques:

1) Two formats of synthetic polymer electrospun scaffolds without post-spinning funcationalisation:

A) The first format is composed of few-mm sized, non-surface-functionalised electrospun scaffolds made from Poly-L-lactide with fibre diameters of 2-4 microns and thickness of 50-100 microns (mimetic of hepatic lobules with regard to their internal morphology, surface chemistry and mode of cell attachment) in standard tissue culture plate formats (mainly 24- and 96-well plates) or as inserts for perfusion bioreactors. B) The second format is composed of sub-mm electrospun scaffolds of the same composition and architecture (mimetic of hepatic lobule dimension and internal morphology) that have been fabricated by using laser-machining techniques. For this process, different key parameters of the preparation process were evaluated: support substrate, scaffold thickness, shot frequency, gap distance, number of shots, and cut-out shape.

2) surface-functional electrospun constructs, where the fibres are coated with a layer of poly(glycerol monomethacrylate) (PolyGMMA) and further modified through the incorporation of galactose. This provides a suitable 3D environment (good diffusion of oxygen and cell nutrients inside the matrix, appropriate surface chemistry for adhesion) for the colonisation of these materials with hepatocytes. Upcyte® hepatocyte viability and bioactivity in these electrospun scaffolds have been studied. In particular, non-fuctionalised and galactose-functionalised scaffolds were seeded with upcyte® hepatocytes and cell metabolic activity (MTS) and CYP3A4 enzyme activity were measured to assess viability as well as biological functionality of the cells in these 3D synthetic environments.

3) A bioprinter, Sphyga (Spherical hydrogel generator), was designed for fabricating gel microspheres with encapsulated nano-scaffolds, sensors and cells. Sphyga is open source and was used to generate alginate and ECM gel spheres with upcyte hepatocytes and laser cut electrospun microscaffolds.

4) A library of hydrogels containing HA and fibrin, which differ in their ratio, their absolute concentration and the way they are modified to improve their affinity. Hydrogels showed varying mechanical properties and gelation kinetics depending on the concentration and composition of the gel components. The elastic modulus values obtained were similar to those reported for healthy (porcine) livers.

A few selected HA/fibrin hydrogel examples were used for the encapsulation of ECs (HDMECs provided by Medicyte) to produce homogenous dispersion of cells. These hydrogels were also loaded with angiogenic growth factor (FGF2). The viability of encapsulated cells was tested using the live/dead cell viability assays and their angiogenic effect was assessed by the formation of cellular junctions. The contraction of the cell-containing hydrogels and the migration of the encapsulated ECs were the two parameters monitored in order to select the best hydrogel for in vivo applications.

WP3

WP3 deals with the integration of cellular tools, i.e. **cells, media, scaffolds** and **bioreactors** that can be used for either (I) in vitro ADMET applications or (2) for in vivo cell therapeutic applications. The aim of this WP was to **develop** and **combine** these tools with those developed in WP2 in the form of tangible products and outputs.

Cells and Media

MEDI has developed a proprietary knowhow to induce cell proliferation in various mammalian cells such as hepatocytes and endothelial cells. The upcyte® process stands for upregulation of primary human cells, meaning induction of cell proliferation. As part of our work, several cell types have been upcyted and could be generated in large quantities in addition a profound quality control of the cells was performed. For the generation of liver organoids, three upcyte® cell types were generated namely, hepatocytes, mesenchymal stem cells and liver sinusoidal endothelial cells. The cells were generated, expanded in a standardized way and provided as working cell stocks to all partners of the Re-Liver consortium involved in cell culture work.

Recent evidence has shown that endothelial cells and not hepatocytes as previously thought are the main cell source responsible for Factor VIII (FVIII) synthesis. For this reason, the consortium decided not to use liver organoids to treat HemA but to develop a single cell therapy based on endothelial cells only. As the human body consists of several types of endothelial cells (e.g. small or large vessel endothelial cells and liver sinusoidal endothelial cells) it was tested which cell type produced the highest level of FVIII. Compared to LSECs, small vessel endothelial cells from skin (HDMECs) and blood outgrowth endothelial cells (BOECs) were analysed. As liver sinusoidal endothelial cells (LSECs) and HDMECs can only be obtained from patients in an invasive way through biopsies, blood samples can be collected much less invasively and easier, this would be the preferred source and most patient friendly method.

As HemA patients do not express a correct copy of the gene encoding FVIII, these cells need to be genetically corrected to express transgenically functional FVIII. In an initial study, human dermal microvascular endothelial cells (HDMECs) were transduced with a lentiviral Factor VIII construct and cells having successfully incorporated the functional genetic information for Factor VIII were selcted. After selection, the Factor VIII secretion could be enhanced more then 25-fold compared to non-selected cell populations and increased up to an amount of about 200 mU. This enhancement of Factor VIII production would reduce the amount of therapeutic cells required for transplantation in order to cure HemA patients.

The generated transgenic FVIII cells were successfully expanded *in vitro* to test functionality in animal studies. First animal studies are planned and initiated. These studies were aimed at answering two fundamental questions: (I) functionality; transgenic Factor VIII endothelial cells continue to secrete FVIII after transplantation and (II) safety; the cells stayed at the site of injection and no tumour formation has been formed. These findings will be crucial for the future development of an ATMP for treatment of HemA. A further advantage of using endothelial cells only for therapeutic applications is the reduced complexity of the ATMP and, secondly, a clear risk reduction; it is known that many tumours are derived from hepatocytes, but hardly any tumours originate from endothelial cells.

Bioreactors

Two bioreactors for live imaging and 3D scaffold culture were designed, the first, LiveBox1 was for culturing 3D constructs in conditions of low shear stress and high nutrient turnover. The second, LiveBox2 was envisaged for clinical applications and contains an insert for electrospun scaffolds manufactured by partner TECL. The bioreactor designs were first optimized using computational methods (in WP1) and then fabricated using machining, millimolding and rapid prototyping. LiveBox1 was used for the culture of liver organoids while LiveBox2 was used to investigate the behaviour of upcyte® hepatocytes seeded on electrospun scaffolds.

Scaffolds

A study was undertaken to evaluate the suitability of electrospun scaffolds developed by TECL for hepatocyte culture and ADMET applications. Initial experiments suggested that upcyte® hepatocyte function does not improve substantially on the scaffolds. Furthermore the scaffolds are too fragile to withstand flow in the LiveBox bioreactors. Thus primary human hepatocytes and HepG2 cells were used instead. The cells were seeded on Mimetix plates for up to 21 days investigating metabolic function and morphology.

Tangible Outputs of WP3

- Liver organoids survive and proliferate in LiveBox1 but not in static conditions;
- Two bioreactors patented and currently at the PCT stage;
- The TECL Mimetix plate significantly enhances HepG2 metabolic competency (CYP activity, phase II enzyme activity) after 14 days and up to 28 days in culture;
- Several cell types were upcyted to generate liver organoids such as hepatocytes, liver sinusoidal endothelial cells and mesenchymal stem cells.
- Therapeutic endothelial cells were generated which produce Factor VIII to treat haemophilia A.
- A chemically defined growth medium for the expansion of therapeutic endothelial cells was developed.

WP4

WP4 involved the standardization of liver organoid (LO) production, the in vitro testing of LO functionality and the in vivo safety and efficacy studies for LOs as well as for FVIII expressing endothelial cells (ECs) aiming at the therapy of metabolic diseases and haemophilia A, respectively.

Liver organoids

LO production was established at MEDI using upcyte® hepatocytes, upcyte® LSECs and upcyte® MSCs in Matrigel based on a publication by Takebe and co-workers. Different conditions such as cell types, cell ratios, cell numbers, medium combinations, ECM compositions, static and dynamic culture were tested and a protocol could be established that led to reproducible and robust generation of LOs.

For the generation of LOs the originally described ECM consists of Matrigel™ which is animal derived and hence not suitable for clinical applications. Here, we have shown that Matrigel™ could be replaced by chemically defined agarose gels combined with growth and adhesive factors.

LOs could be generated at different scale (from 96-well up to 24-well plate) and were characterized with respect to histology and function. LOs showed significant liver like structure and function demonstrating potential to be ultimately used for both therapeutic and ADMET applications. A validation study between MEDI and UNIPI confirmed that LOs with similar morphology and function could be produced in both labs. LOs form under static conditions but need perfusion in order to maintain viable. For this reason the LiveBox1 system was used to culture the LOs for over 10 days.

For in vivo applications LOs were used either directly or integrated into the biovascular system BioVaSc from the Fraunhofer in Würzburg, Germany. For the safety study, LOs were generated and implanted into a small animal model and biodistribution of the containing cells was monitored using GFP and luciferase reporter. For this task the LOs forming cells were transduced with a luciferase-GFP construct and selected for expression using FACS sorting. From these reporter cells LOs were generated and transferred into the 3D-ratBioVaSc system that has been revascularized with upcyte® endothelial cells. LOs were then cultured within this system and characterized with respect to liver specific functions. For the biodistribution and small animal safety study these LOs were implanted into immunodeficient nude rats and mice. The biodistribution was monitored using in vivo bioluminescence imaging within a period of up to 12 weeks as well as using immunohistology and PCR from the transplant location as well as from representative internal organs (lung, spleen, and liver).

Although the longterm studies have not yet been completed with the first part of the experiments it could be shown that upcyte® cells within the LOs appear to be non-tumorigenic (long-term results to be confirmed) and do not spread. From the size of the luminescence signal it can be concluded that LOs were attached to the animal vascular system and that they were viable more than 30 days.

In the future, after more in depth pre-clinical studies, these minimal invasive implantations are then thought to be used for the treatment of metabolic disorders or as replacement for liver transplantation.

Haemophilia A

With respect to the development of a cell therapy for the treatment of haemophilia A endothelial cells were transduced with a FVIII coding construct, selected for FVIII expression followed by either direct implantation into a Cell Pouch™ system or inclusion in a hyaluronic acid-fibrin gel before implantation. The functional cell type for FVIII production has been changed from initially hepatocytes within bioartificial liver organoids (BLOs) to endothelial cells as the latter cell type has been recently found to be the predominant endogenous source for FVIII production. Another reason was that blood outgrowth endothelial cells (BOECs) can be used to treat the patients in an autologous setting avoiding immune reactions against the transplant.

In this preliminary study in small animals, following subcutaneous implantation of the Cell Pouch™, cells were transplanted into the Cell Pouch™ as single cell suspension and over time the resultant tissue within the Cell Pouches™ was analysed using immunohistochemistry. Preliminary assessment suggested that healthy ECs survived and expressed EC-specific markers as well as Factor VIII. No tumour outgrowth was detected in the animals used and the treatment was shown to be safe in the animals indicating proof-of-principle for this approach.

WP5

This WP focused on the exploitation and marketing of the products developed during the Re-Liver project.

All partners have screened the project for IPR-related issues including protectable foreground inventions and freedom to operate concerns. No freedom-to-operate issues have been identified during the first and second reporting period.

UNIPI filed an Italian national patent (reference MI2013001494) in September 2013 on an innovative cell culture support for their LiveBox1 bioreactor. UNIPI consulted the other partners and project coordinator, according to the rules agreed in the consortium agreement, and the consortium agreed that ownership of this patent was exclusively granted to UNIPI. No other patent applications for foreground results were filed by consortium members during the project. No cross-licensing of IPR was deemed necessary. TECL plans to file a patent on the microscaffold

technology developed during the first reporting period by the end of 2015 (after the project end) based partially on work done in Re-Liver and partially on work done in another unrelated project.

The exploitation strategy for ADMET products:

A report detailing the exploitation strategy for a high throughput ADMET testing tool using MEDI's upcyte® hepatocytes (background IPR), TECL's 96-well plate containing the Mimetix scaffold (background IPR) and the results of the tests optimising the combination of the two (foreground results) was completed in December 2013 (D5.2), and was revised regularly as further results were obtained. MEDI and TECL signed a commercial distribution agreement in December 2013 under which MEDI will distribute TECL's 96 well plate for 3D cell culture alongside its cell product lines. MEDI made some sales of the Mimetix plates but this marketing route was not successful, due in part to the lack of solid data showing an advantage in combining the two products. TECL is however marketing the Mimetix technology for other cell lines, such as HepG2, and primary hepatocytes through direct sales channels.

The route to market for UNIPI's LiveBox1 invention described above is through IVTech, a spin-off company from the University of Pisa founded in 23/01/14. Currently the company's CEO is Eng. Tommaso Sbrana, PhD in Biomedical Engineering. The mission of IVTech is to provide systems that enable the biologists to implement more relevant in-vitro models combining flow and 3D scaffolds. These new methods will allow a closer mimicking of patho-physiological environments to obtain more representative data of human response, with a consequent reduction of animal tests. Thanks to the strong know-how in designing in-vitro cell culture systems, IVTech follows the customers from the very first phases of an in-vitro model design to the subsequent technical support and follow up in using its systems. IVTech has already secured distributors in Italy, Germany, Austria and Switzerland and is coordinating an SME instruments proposal currently under phase 2 review.

The diagnostic tools for screening for inhibitory antibodies in HemA patient sera developed by MEDI were not found to be commercially attractive enough to merit exploitation following analysis of the market opportunity and need.

The exploitation strategy for ATMP products:

Two advanced therapeutic medicinal products (ATMP) could be developed from the results of the Re-Liver project.

First, liver organoids generated from upcyte® cells consisting of hepatocytes, mesenchymal cells and endothelial cells. This cell mixture spontaneously forms an organoid like structure which can be grown in a bioreactor like the LiveBox1 for multiple days. During cultivation in a bioreactor such as the LiveBox1 from UNIPI or the BioVaSc from Fraunhofer IGB these liver organoids demonstrated important liver functions such as albumin production as well as detoxifying activity as shown by CYP3A4 enzymatic activity. These upcyte® organoids can not only be used for ADMET related in vitro analysis, but could also be exploited as an ATMP considering the organoids could survive for longer periods and if they can be connected to a vascular system. One option for this could be to combine the LOs with the biological vascular scaffold (BioVaSc), a matrix with a vascular system, developed by the Fraunhofer IGB in Stuttgart (Walles and colleagues). The BioVaSc is generated from a decellularized porcine small bowel segment with preserved tubular structures of the capillary network within the collagen matrix. The BioVaSc technology enables the generation of various bioartificial tissues endued with a functional vascular network (Schanz et al. 2010). J Biotechnol. 2010 Jul 1;148(1):56-63: Vascularised human tissue models: a new approach for the refinement of biomedical research. A second option which does not use animal derived products is the LiveBox1 system in a high throughput parallel configuration. As the LiveBox technology is licensed by IVTech, a commercial agreement could be sought with the company.

Upcyte® liver organoids grown in the BioVaSc could be used to treat severe liver diseases like acute liver failure (ALF).

The second ATMP which could be developed are genetically corrected endothelial cells for the treatment of haemophilia A.

The commercial product that may arise from the genetically corrected endothelial cells could be combined with the Cell Pouch™ device technology from the company Sernova Corp. The corporations Sernova and Medicyte have established and have agreements in place including a collaboration agreement and term sheet which was completed

in 2012 to jointly commercialize a product for Haemophilia A involving genetically corrected BOEC cells placed within the pre-implanted Cell Pouch™. We anticipate through our relationship that Sernova and Medicyte (or its spin out, upcyte Technologies GmbH) will obtain the license rights to any new intellectual property developed as a result of this collaboration for the purpose of forming a joint venture, clinical development and marketing the product. As discussed, we anticipate that we may also license the HemA ATMP product to a large pharmaceutical marketing partner to further commercialize and distribute our product worldwide. We anticipate this product will be available for commercial application following successful completion of Phase I/II and Pivotal clinical studies and will be available for commercial sale early to mid 2020's.

Regulatory framework

TECL performed an analysis of the regulatory requirements for the scaffolds in the case that they would be included in the ATMP and determined that they would need to be producing in ISO class VII clean rooms and would require ISO13485 medical device quality certification. Although the consortium did not progress the electrospun scaffolds into an ATMP prototype, TECL did move into clean rooms in August 2014 and has achieved ISO 13485:2003 quality certification in March 2015. There are no specific requirements to market the Mimetix plates for ADMET applications.

MEDI has two products which have to fulfil specific regulatory requirements, the therapeutic cells themselves and the medium in which the cells are expanded (regulatory: manipulated) before implantation into the patient. During the project MEDI has been successful in developing a chemically defined medium to grow the therapeutic cells. The regulatory requirements for the medium are quite clear and production thereof has to be performed according to GMP-like standards such as additive substance for chemical entities.

Preclinical development

Since two ATMP products were developed, the ATMPs animal studies posed different requirements. For the liver organoids a biodistribution and safety study was performed at the Fraunhofer IGB institute in Wuerzburg. In this preclinical study, it was evaluated, if such bioartificial liver organoids can engraft in vivo and if such liver organoids are capable of forming any vascular structures and connect to the vascular system. To support the organoid vascularisation, a 3D-Matrix (BioVaSc, proprietary knowhow Fraunhofer IGB) which can be connected directly to the vascular system as described in Deliverable 5.3 and 5.4 was also evaluated. Although initial data indicate that engraftment of the liver organoids in the BioVaSc requires further optimization, implantation of the liver organoids for biodistribution and safety demonstrated that long term survival of liver organoids for more than 30 days is possible without formation of tumours or spreading of the cells within the organism. These results indicate the need for further development to improve engraftment, but are promising as functionality and safety of this ATMP could be shown.

For the therapeutic cells producing Factor VIII, an alternative strategy was chosen. For implantation of the cells, the Cell Pouch™ device technology from the company Sernova Corp which has been described in detail in D5.4. These studies have given insight into two fundamental questions: (I) functionality; the transgenic Factor VIII endothelial cells do secrete Factor VIII after transplantation and (II) safety; the cells stayed at the site of injection and now tumour formations could be observed. These findings are promising results for the future development of an ATMP to treat HemA.

1.4 The potential impact

Socio-economic impact and the wider societal implications of the project

Contribution to Community and social objectives

Social-economic impact Re-Liver

The impact of the Re-Liver project is quite high and based on the tight collaboration of the partners of this consortium. A strong interaction between industry and research institutes led to the development of several new innovations, methods and products.

Science, technology and innovation

The unique strategy of developing an organ-like product which can be used for in-vitro ADMET related investigations as well as for therapeutic applications, HemA resulted in a wide range of products. Even intermediate steps during development led to products to be used by the scientific community. A good example is the HisTOOLogy tool developed by the University of Pisa, for the characterization of the human and pig liver tissue. The tool uses a novel algorithm, based on k-means clustering, for the separation of different colours in histological images, and was used to better understand liver structure and architecture. The algorithm is open source, giving the scientific community the opportunity to share this new knowledge in the broadest range possible. UNIPI also developed and published new methods for viscoelastic testing of soft tissues and biomaterials using bulk and microscale (nano-intendation) compression. The nano-indentation method has been integrated into the Piuma nanoindentor commercialised by Optics11 (NL).

The next step of the development process was to modify the electrospun network to improve cell attachment and performance. Although during the course of the project, these steps have been adjusted, the University of Manchester was able to synthesize innovative compounds for coating of the electrospun fibre. These data have been partially published (Macromol. Rapid Commun. 2013). This led also to the development of a process to incorporate a fluorescent dye into the electrospun fibres to facilitate the investigation of cell penetration within fibre scaffolds of different architectures and depths using confocal microscopy. Company TECL incorporated the optimised network, or scaffold, into multi-well plates, using its proprietary technology (patent pending) to create Mimetix® plates (trademark registered in EU and USA) suitable for liver ADMET applications. Thorough investigations performed by MEDI, UNIPI and TECL, about using these plates for a variety of cell based assays, showed the difference in behaviour of different cell types such as cell penetration or preferred fibre diameter. The plates could be optimised in such a way, that TECL has been successful into significantly improving metabolic performance, for example CYP enzyme activity, of the cell line HepG2 for ADMET related applications. The HepG2 cell line demonstrates a poor metabolic performance in conventional (2D) cell culture, so this is attractive to pharmaceutical companies looking for cheap but effective high-throughput screening options. In parallel, another cell type has been extensively studied by this consortium, upcyte® hepatocytes. Upcyte® hepatocytes are genetically modified cells enabling normally non-proliferating primary hepatocytes to become proliferative. In this way, MEDI is capable to produce these cells on a large scale and to standardize ADMET related applications. The upcyte® hepatocytes were optimized to be metabolically competent in 2D allowing also high throughput screenings using these cells. Interestingly, the upcyte® technology has been successfully applied to different donors of hepatocytes offering for the first time new tools for the industrial customers to study the metabolism of newly developed compounds in a variety of donors. This is an important aspect, as donor specific metabolism is related to drug induced liver toxicity (Chen et al, doi:10.1016/j.jhep.2015.04.016) and any new tool to identify such side effects in an early stage will help to improve drug development and save enormous costs. Furthermore, such compounds would not enter the market reducing future drug induced liver injury, a clear benefit to the community.

This example shows the importance of highly predictive systems and applications to study liver toxicity and liver metabolism of newly developed compounds. As the cell based products are more thought to be used in early screens, higher sophisticated models are necessary to study slow metabolising compounds or compounds which are toxic to other liver cells which are non-hepatocytes (DeLeve DOI 10.1007/978-1-4419-8327-5_2). To setup such as systems different cell types were combined to generate an organ-like structure. As described by Takebe et al (doi:10.1038/nature12271), 3 different types of stem cells were capable of self-assembly into an organ-like structure, which they called liver bud. We were interested to see, whether differentiated cells were also capable to do so. For this purpose, we combined differentiated upcyte® cells of liver sinusoidal endothelial cells together with hepatocytes

and the mesenchymal stem cells. Indeed, also these cells formed an organ-like structure which we analysed thoroughly. The results of these investigations are going to be published in the open access journal PLOSone and be made available to the scientific community. During the course of the study, it turned out that the liver organoid (LO) could not be cultivated under static conditions and cells became necrotic. To solve this, we combined the LivBox1 developed and patented by UNIPI and the LOs to study if longer cultivations are possible. Initial studies were performed for up to 10 days although the aim was to develop a new system allowing the LOs to survive for up to 30 days. Such a system could be a new application to study subchronical liver toxicity in vitro, studies which are normally performed in animal models. It is known that animal models are not always predictive for human metabolism of new compounds. A human cells based model would be of significant benefit, particulary if a variety of donors, each metabolising differently, can be offered. Clearly these studies would not be high throughput but applied as low throughput during the medicinal chemistry phase at a later stage during development. The initial interlab validation, demonstrated the robustness of the system but further validation is needed to get such a system accepted by the regulatory bodies as a standard for ADMET application during drug development and safety assessment.

The development of the LiveBox1 and LIveBox2 bioreactors by UNIPI and premarket testing of these products by the consortium led a group of young engineers from UNIPI to found a start-up company, called IVTech. The company secured the intellectual property rights of the products and initiated the production and sales.

Besides the methods and products developed for the ADMET applications, several products and methods were established during the development of 2 advanced medicinal products. Initially it was thought to develop a liver organoid based product to cure two liver related diseases, HemA and metabolic liver disease e.g. liver failure. Scientific papers appearing at the start of the project published that not hepatocytes but endothelial cells are the predominant producers of the clotting factor VIII. The consortium recognized this as opportunity to develop 2 separate products, one based on genetically corrected autologous endothelial cells producing FVIII and a liver organoid for the treatment of liver diseases.

Important for the cell based therapy is the amount of cells needed to treat the disease. For the treatment of HemA to achieve a constant level of FVIII to be protective against (internal) bleedings is about 5% of normal levels. To achieve these levels about 1 billion cells are needed that produce this level of FVIII in a 70 kg person. To this amount of cells, cell culture medium is required. The regulatory body for Germany, the Paul Ehrlich Institute, dislikes any medium which contains undefined animal components such as foetal calf serum. The initial growth medium for the endothelial cells contains 5% FCS, so a new medium without any animal components needed to be developed. Such media is called a chemically defined medium and can be produced according to GMP guidelines, an important requirement for development of an ATMP. In a systematic screening and testing strategy, MEDI was able to identify protein based supplements which can replace FCS without any negative growth effects on the endothelial cells. First industrial companies have already shown interest to produce and commercialise this chemically defined medium for therapeutic applications.

Next to the medium, the cell source is an important aspect to consider. Initially, we started our endothelial cell work with endothelial cells isolated from the skin. Taking these biopsies is an invasive step, which we would like to avoid especially in bleeders as patients. A new source to isolate endothelial cells could be identified in blood samples. Blood samples can be much easier collected then skin biopsies and are definitively much less invasive, a procedure which is of clear benefit for the patient. The isolation of these endothelial cells from blood, so called blood outgrowth endothelial cells or BOECs could be established at MEDI in a standardised manner and the new technique could be further optimised to increase the number of cells as starting point. The higher number of cells after isolations has two advantages, smaller samples can collected from the patient and to generate the cell amount needed for treatment less population doublings and thus less time is required both in favour for the patient.

For the implantation and engraftment of the therapeutic cells, a new hydrogel has been developed by UNIMAN. This injectable material presents an innovative way to promote interactions between two well-known and clinically employed biomaterials, i.e. hyaluronic acid and fibrin. The result is a material that should be regarded as inherently safe, which is injectable, can be formed in the presence of cells, is rapidly biodegraded by these cells and possibly control their inflammatory activation; in short, an ideal provisional matrix for in vivo tissue engineering. This formulation has sufficient novelty and inventiveness to justify a patent filing, as agreed with the UNIMAN IP agency (UMIP). Additional physico-chemical and in vivo data are being produced as a spin-out of Re-Liver; they will be combined with those produced in the frame of the project and protection will be sought in 2016.

For the LOs, a chemically defined medium was already developed by MEDI so development of the liver organoids for therapeutic application could focus on the improvement of cells survival and cell performance during cultivation. A significant portion of knowhow has been collected during the development of the LOs for long term ADMET applications. This knowhow was transferred to the Fraunhofer Institute IGB in Wuerzburg. This Institute is focussing on the development of new cell based therapies and offers safety studies and Proof of Concept studies to investigate functionality and safety of the ATMP. A second reason why we decided to collaborate with this institute is their BioVaSc platform. The BioVaSc offers a vascularized matrix on which the LOs can be seeded and it has been tested if the LOs do attach or even are capable to grow and survive in the matrix. Initial studies demonstrated a poor survival of the LOs in the matrix which needs further optimisation.

New science and innovation networks

In addition to the benefits received by TECL from collaboration with Re-Liver partners, the company has established a new grant-funded consortium with Aurelia BioScience Ltd and Takeda (UK) to develop a new 3D cell-based assay format, building on the micro-scaffold technology developed in Re-Liver (InnovateUK project 43569-311233). The Re-Liver grant also helped TECL to build a consortium for the EU FP7 funded HESUB project (601700) to develop scaffolds for use in bioreactors to expand stem cells for therapeutic use.

The Re-Liver project has helped to increase the visibility of UNIPI in both innovation and excellent science. UNIPI benefitted from the project, forming long-lasting collaborations with partners and new networks and grants on: i) in-vitro systems (new grants for bioreactor development), ii) cell-imaging (grant for setting up a university imaging network) and iii) micromechanics (collaboration with Optics11). Furthermore, the university is proud to have generated a new spin-off company, IVTech, as a result of the R&D stemming from the project.

Re-Liver has primarily allowed UNIMAN to improve their understanding of the commercial and technological landscape in the field of artificial organs. This activity has allowed interactions with AstraZeneca in terms of a number of in vitro cellular models (PhD studentships and a large grant funding the NorthWest Centre for Advanced Drug Delivery), while is forming the basis for a long-term activity on a new platform of injectable hydrogel formulations that are currently evaluated also for plastic surgery applications and are the subject of Wellcome Trust fellowship proposal (Dr. Jason Wong, Manchester).

The Re-Liver project allowed MEDI to expand their product portfolio, deepen our knowledge about advanced therapeutic medicinal products and important aspects of their development. The strong interactions between all Re-Liver partners helped to speed up the development process which led to the high output of products. A clear benefit for MEDI was definitively the formation of new collaborations (e.g. Sernova Corp.) and scientific networks (Fraunhofer Institute, University of Heidelberg) based on the scientific data generated. Also MEDI has been able to receive several new national and international grants and funding for further development of the liver organoids for ADMET as well as therapeutic applications. The Re-Liver grant also helped MEDI to build a new consortium (e.g. with Sernova) for a Horizon 2020 call to further develop the Factor VIII producing endothelial cells for therapeutic use to treat Haemophilia A.

Economic

The economic impact of the Re-Liver project can be measured by the new jobs which were created by the different partners. In total 11 additional jobs were generated throughout the project duration and several postdoctoral scientists and PhD students could train and development their skills. Several workshops and trainings were organized and upon need, short term exchange programs were initiated to exchange knowhow, organize trainings of students and to transfer new methods and knowledge.

The broad range of applications as well as working on in vitro applications next to advanced medicinal therapeutic products, allowed scientists, general staff along to the PhD students collect an enormous knowhow resulting in highly-skilled workers for the market offering them ideal career opportunities.

But training and knowledge transfer were not only exchanged within the consortium through exchange programs or workshops organised by UNIPI and MEDI but also synergistic effects with external partners and other facilities were initiated. Several students of the Fraunhofer Institute were trained to handle the cells from MEDI needed to produce the LOs. In close collaboration with Fraunhofer, a PhD student from MEDI trained herself in seeding the BioVaSc with LOs. The collaboration will continue after the Re-Liver project has ended and seeding and cultivation of LOs will be developed further to improve LO attachment and growth of the organoids. Also with Sernova, the provider of the

Cell Pouch™ a collaboration could be set up, people of Sernova were trained as well to handle the endothelial cells as well as to perform quality controls assays before implanting the cells in their Cell Pouch. This project will also continue beyond Re-Liver.

Social

The social benefits of Re-Liver arise from both the ADMET as well as therapeutic products. For ADMET, the improvement of the drug development process by offering new and more predictive tools to identify side effects such as drug induced liver injury at an early stage or how compounds are metabolised in the liver offer a clear benefit for the European community. The benefits for the therapeutic products are clear benefits for patients. New treatments become available for HemA and the current hard to treat acute liver failure. A significant improvement in the quality of life of patients especially for HemA patients due to the continuous protection instead of thrice weekly injections with fluctuating levels of FVIII.

Within the consortium, the cultural diversity has led to improved understanding positively affecting attitudes and behaviour. In addition, having English as the common language has led to improvement of language skills for several PhD students and scientists.

The dissemination activities during the Re-Liver project may have led to a better understanding of the products developed by potential customers. Several of the activities have also given the community an awareness of the benefit of science for patients and society.

Main dissemination activities and exploitation of results

The main objectives of WP6 were to disseminate the results generated in Re-Liver and to establish a training program for Re-Liver members and the outside community.

Dissemination:

First of all, the website of Re-Liver (www.reliver.eu) has been launched (**D6.1**) in January 2013, giving insights into the concept, objectives and impact of Re-Liver and providing an overview of internal and external meetings. The project website has been divided into a password protected members area and a publicly accessible part.

The public part has been regularly updated concerning for example the latest news about the project and dissemination activities like posters or publications from all partners. A downloadable version of the project flyer can be found on the website, giving general information and contact of Re-Liver. In the separate section “News and Dissemination” documents like press releases and applications notes have been published.

The password-protected Re-Liver iWeb platform (:milliarium) has been regularly updated with internal documents like meeting minutes and presentations on results and was helpful to monitor the overall progress of the project.

To further disseminate the research efforts and results to the scientific community as well as the general public the Re-Liver team published several articles, two more general ones (one for example published in “The Parliament Magazine”) and 11 scientific publications so far (please see ECAS, two publications are still pending due to outstanding journal requests). At least four additional publications are in preparation at the moment (**D6.3**).

Dissemination material like project flyers and posters have been regularly provided to the public during for example scientific conferences, other meetings and/or exhibitions. For project presentations, posters have been designed and a project presentation template has been provided to all partners reflecting the Re-Liver design and colour code. The project logo as well as the website has been displayed on all dissemination material like presentations, flyer and website if applicable.

Main dissemination activities:

- The internal and external part of the website has been regularly updated throughout the project duration providing insights into project news.
- The password-protected members area was intensively used to exchange internal documents and to oversee the whole progress of the project.

- Dissemination material like project flyers and posters have been regularly provided to the public during for example scientific conferences, other meetings and/or exhibitions.
- Five oral presentations were held by UNIPI in front of a wider public (hospital staff, high schools, at open days).
- Summarizing, the consortia has already disseminated and is still going to disseminate the results of this project as widely as possible to the scientific community and to the wider public.

Table 1 Overview about all dissemination activities in ECAS.

	Posters	**Oral presentations**	**Exhibitions**	**Workshops**	**Publications**
MEDI	5	3	13	1	0
UNIPI	12	21	0	3	8
TECL	9	2	5	0	0
UNIMAN	0	2	0	1	1
Total	26	28	18	5	9

Training:

In total five workshops have been organized (three by UNIPI, and one by UNIMAN and MEDI each), whereas three of them took place already during the first period. In the second period, the first workshop at UNIPI was held in July 2014 and was focused on the use of the LiveBox bioreactors and Sphyga. The second workshop on bioreactors was held in July 2015.

Additionally individual trainings and for example post doc exchanges took place. In particular staff from MEDI held an one week training at UNIPI in order to transfer know how on liver organoid generation and set up the inter-laboratory validation experiments already described in **D4.1**.

As part of the dissemination and training programme, all involved researchers from UNIPI disseminated the project by giving talks about their work to students. For example, Prof. Arti Ahluwalia gave a talk about liver tissue engineering and the project, her colleague Prof. Giovanni Vozzi gave a course on material fabrication and has shown the Re-Liver concept in his lessons and Prof. Paolicchi has described the results of liver decelluarisation in his lessons on clinical pathology.

Exploitation of results

The Re-Liver consortium was able to develop a variety of new products and tools as described in the science, technology and innovation part (1.4).

To provide a clear overview, most of the exploitable products and their status of exploitation are listed in the Table 2 below and are also shown in the graphical overview below the table.

Products	**Description**	**Status of exploitation**
Mimetix 3D multiwell plates	The data generated during this project could nicely show the plates to be a good tool for different models using a range of liver cells, including HepG2, upcyte® hepatocytes and primary hepatocytes.	The Mimetix 3D multiwell plates have entered the market. http://www.electrospinning.co.uk/shop/

LiveBox1	The bioreactor, LiveBox1, is a transparent chamber designed for inter-connected dynamic cell cultures. LB1 is featured with a flow inlet and outlet for the perfusion of cell culture media. The clamp system provided with the LB1 assures the watertight closure of the system in both static and dynamic conditions (up to 1 mL/min).	The LiveBox1 is produced and commercialised by the spin-off IVTech stemming from the University of Pisa. The LiveBox1 has entered the market. http://www.ivtech.it/Products/LiveBox1-%28LB1%29/
upcyte® cells	Using the unique upcyte® technology, primary cells are driven into proliferation, thus allowing controlled and reversible bypass of cell cycle control mechanisms without inducing immortalization, uncontrolled cell growth or changing the typical phenotype.	The upcyte® cells are produced and commercialised by the spin-off upcyte® technologies stemming from the company Medicyte. http://www.upcyte.com/products/human-upcyte-hepatocytes.html
Cell culture media	Different cell culture media have been developed for growing the cells used in this project	Media for cultivation of upcyte® cells are produced and commercialised by the spin-off upcyte® technologies stemming from the company Medicyte. http://www.upcyte.com/products/media.html
Cells and LiveBox1	The bioreactor LiveBox1 is perfectly suited to cultivate complex 3D cell structures, like the organoids.	Since both products are on the market, a co-commercialisation cannot be excluded. The companies IVTech and upcyte® technologies may consider entering into such discussions.
Cell mixtures to generate liver organoids (LOs) for ADMET applications	Initial studies performed within the Re-Liver project have shown that the 3 cell types needed to generate LOs, can be premixed and after thawing are capable of forming a LO.	Premarket-phase. The company upcyte® technologies currently evaluates this mixed product and has provided first batches to beta-testing customers.
Therapeutic cells for treatment of HemA	Blood outgrowth endothelial cells producing the therapeutic clotting factor VIII to treat severe HemA could be successfully generated.	Preclinical development. Therapeutic cells are currently evaluated in Safety animal studies. Further Proof of Concept studies are needed in animal models before entering the clinical stage.
Hydrogel formulations for therapeutic cell engraftment	A library of hydrogels has been developed and characterised. Mechanical properties and contraction of the gels, cell encapsulation and migration have been studied.	The formulation is ready and Standard Operation Procedure has been sent to MEDI. Gels have been used for in vivo experiments.
Chemically defined media for expansion of therapeutic cells.	Chemically defined do not contain any undefined animal components and can therefore be produced according the GMP regulations.	Media formulation available for transfer to a third party GMP contract manufacturer.
Liver organoids for	Within the Re-Liver project could be shown	Preclinical development. Therapeutic

treatment of acute liver failure (ALF)	that the 3 cell types needed to generate LOs, can be produced in sufficient large quantities for such a therapeutic application by applying the upcyte® technology.	cells are currently evaluated in Safety animal studies. Further Proof of Concept studies are needed in animal models before entering the clinical stage.

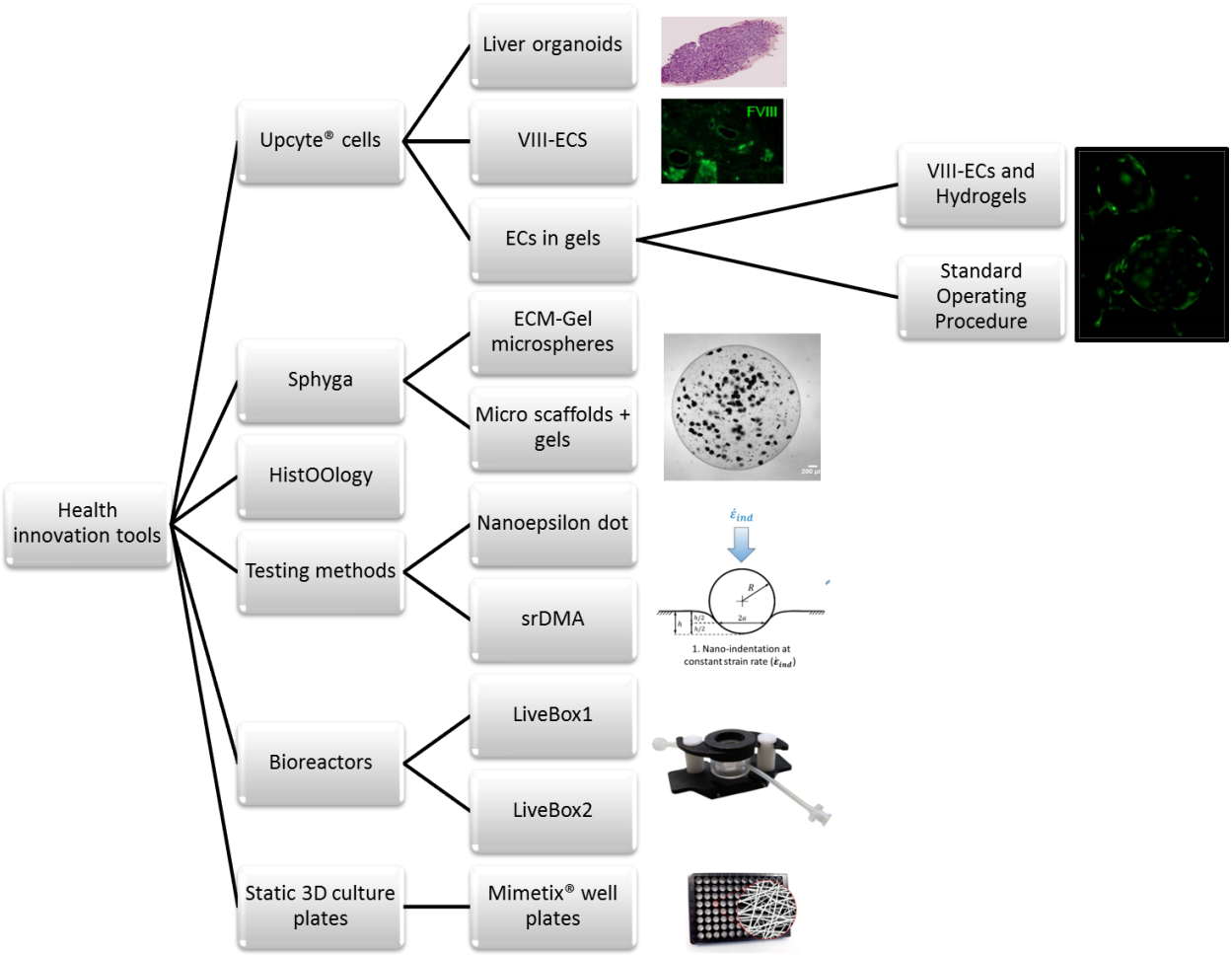

Figure 1 Graphical overview about exploitable foreground

The Re-Liver consortium will **continue to advertise/publish its outcomes beyond the project duration**, promoting further exploitation of the results obtained. Detailed exploitation plans for the ADMET and ATMP products have been described as deliverable.

The activities organised by the project and the outcomes obtained have also led to **new ideas for research proposals** and plans to better integrate existing projects. As a result of this, funding could be obtained or collaborations with third parties could be initiated by several of the partners.

Partner	**Funding projects or joint R&D projects with (industrial) partners**
TECL	HESUB EU FP7 grant
TECL	Collaboration with industrial partner Micronanics Ltd.
TECL	Collaboration with industrial partner Pharmacelsus GmbH

UNIPI	H2020 Mari Curie ITN grant
UNIPI	New national and internal grant approved
MEDI	Collaboration with industrial partner Sernova Corp.
MEDI	Collaboration with partner Fraunhofer IGB branch Wuerzburg.
MEDI	New national grant approved with TU Ilmenau and University of Heidelberg
MEDI	New grant approved (Eurostars) with industrial partner Mimetas.
MEDI	New grant approved Horizon 2020, HemAcure
UNIMAN	FP7 UNIVAX grant
UNIMAN	TSB national grant with AstraZeneca
UNIMAN	Industrial grant from AstraZeneca

Outlook and future research

The strong interaction between industry and research institutes within this Re-Liver project led to the development of several new innovations, methods and products important for future research and project continuation. New tools such as HisTOOLogy developed by the university of Pisa has been provided to the scientific community for further improvement or personal adaptation to enable the tool to be used to characterize other tissue beside liver as well. The experimental know how generated with hepatocytes grown in Mimetix plates will be further extended with additional cell types offering a platform technology for culturing a broad range of cell types in an 3D environment. Also the bioreactors developed will support future tissue engineering methods enabling the scientific community to further improve the physiological relevance of in vitro cell based assay systems and making them better comparable to the in vivo situation in human.

Beside the scientific improvements made for the in vitro and ADMET related products also for the therapeutic application significant results were obtained. For the implantation and engraftment of the therapeutic cells, a new hydrogel has been developed by UNIMA. This injectable material presents an innovative way to promote interactions between two well-known and clinically employed biomaterials, i.e. hyaluronic acid and fibrin. The result is a material that should be regarded as inherently safe, which is injectable, can be formed in the presence of cells, is rapidly biodegraded by these cells and possibly control their inflammatory activation; in short, an ideal provisional matrix for in vivo tissue engineering. The Re-Liver project allowed deepening our knowledge about advanced therapeutic medicinal products and important aspects of their development. The strong interactions between all Re-Liver partners helped to speed up the development process which led to the high output of products. A clear benefit was definitively the formation of new collaborations (e.g. Sernova Corp.) and scientific networks (Fraunhofer Instute, University of Heidelberg) based on the scientific data generated. Together with the BioVaSc system of the Fraunhofer, a vascularized matrix was available for implantation of the liver organoids. A project which will be continued as the scientific coordinator, Dr. Braspenning, has moved to the translational centre for tissue regeneration of the Fraunhofer institute. In addition, a new consortium (e.g. with Sernova) could be set-up to apply for a Horizon 2020 call to further develop the Factor VIII producing endothelial cells for therapeutic use to treat Haemophilia A. The grant application has been selected to receive funding and negotiations for the grant agreement between partners have been initiated.

Section 2 – Use and dissemination of foreground

2.1 Plan for use and dissemination of foreground (including socio-economic impact and target groups for the results of the research)

Section A

List of Scientific Publication

For more information please see the ECAS system.

List of Dissemination Activities

For more information please see the ECAS system.

Section B

For more information please see the ECAS system.

Section 3 – Report on societal implications

Please see the ECAS system.