3 Main S&T results/foregrounds

WP1 Consortium management

To ensure compliance with the Grant Agreement and Consortium agreement
Specific objectives:
Task 1.1: To secure day-to-day management of BALANCE including: monitoring of scientific progress; communication between partners and with the EC; financial management; reporting; decision making; conflict management

Monitoring of scientific progress:

At the start of the project a visit was made by representatives of partners 1 and 2 to RanD (Medolla, Italy) to discuss and settle the specifications of the bioreactor hardware (550 ml content, non toxic components and preferably mould made). Specifications and validation criteria were established, also for tubing systems and plasma filters. Agreement was obtained about the delivery of three bio-incubators (Performer O.LIVER) to connect the BAL to the patients circulation.

Multiple lab visits and telephone conferences have been done by the coordinator and his postdoc with partner 3 about the production of the research cell bank (RCB) and master cell bank (MCB). The GMP upscaling process was discussed and it was decided that upscaling by microcarrier culture would be the first choice. Finally partner 3 was not able to deliver sufficient amounts of GMP produced HepaRG cells for the planned pre-clinical experiments and the clinical trial. The coordinator and his legal office have extensively negotiated with partner 3, finally resulting in its partnership termination September 2014. It was decided that partner 1 and partner 5 together would take over the task of partner 3 to produce sufficient cell-BALS under non-GMP conditions for the pre-clinical experiments at partner 4. To prepare the planned clinical trial multiple meetings have been held with the professional organisations (CRO's Q-Serve and BD consultancy) about establishing a clinical protocol acceptable for the MHRA in the UK and to perform a risk analysis to be presented to the regulatory authorities in UK and the Netherlands. A clinical protocol was established by cooperation of the CRO's and partners 1 and 4.

In multiple discussions a standardization of the experimental model of paracetamol induced ALF in subjects was established. After the start of the first pre-clinical experiment in Edinburgh the coordinator has attended such an experiment.

At the AMC the coordinator chaired the bi-weekly research meetings of the combined AMC-partner 2-Balance project group.

Financial Management

As part of the financial management we:

- coordinated the well –defined amounts of work including final budget and payment terms
- distributed the received funding of the EU in a timely manner
- prepared the interim and final financial reports to the EC
- coordinated the preparation of statements and financial audit certificates from the partners for transmission to the EU
- distributed the items requested for reviews and audits , including the results of the financial audit prepared by an independent auditor

Communication between partners and with the EC:

To promote communication between partners and with the EC the Directory Board of the AMC has appointed at the start of the project a Balance project manager (Mrs Estella Koppel; later replaced by Mr Eelco Soeteman, because of maternal leave of Mrs Koppel). An AMC Medical research (AMR) financial manager (Mrs Rebecca Massonet; later Mrs Eugenie Quartier) was appointed as well.

Extensive email correspondence and telephone calls between the different workpackage leaders have supported the scientific coordination between partners.

In addition newsletters by the coordinator at the website were used for exchange of information

Multiple email and telephone contacts were established with our Project Officer (PO) at Brussels (dr T. Ingemansson).

Together with the AMC project manager and AMR financial manager reporting of the deliverables and the quarterly, the interim and end reports were coordinated,

After extensive consultation of the remaining 4 partners and our PO an amendment to the Balance project was established and approved by the EC.

Task 1.2: To organise meetings

The following meetings have been held and their minutes have been made and distributed:

- Kick-off meeting, whole consortium, Amsterdam, May 31st, 2012
- Management team, Maastricht, October 5th 2012
- Steering Committee, Advisory Board, full consortium Meeting, Feb 14, 2013, Schiphol
- Steering Committee, Advisory board, full consortium Meeting, Nov 21 2013, Paris
- Final full consortium Meeting Edinburgh July 1st 2015
- Multiple teleconferences

Task 1.3: To provide a secured web-based tool for supporting daily management and reporting, open communication between all consortium members and for dissemination of project results to the general audience

The website http://fp7balance.eu was launched August 2012 (Fig. 1). In collaboration with subcontractor HilhorstDesigns this website specially dedicated to BALANCE has been developed. This website contains both a publicly accessible part and a secured domain in which partners communicate via a forum about the progress and other matters related to the BALANCE research project. This part of the website is also used to distribute documents of general use within the consortium.

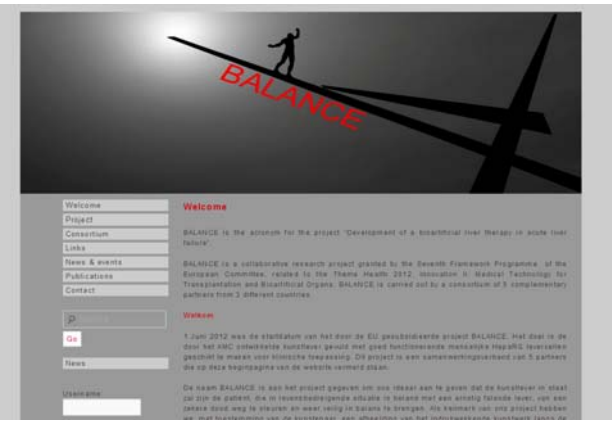

Figure 1: Balance website

The following deliverable reports have been finalized

D1.1 Annual financial and technical EC report

D1.2 Internal reports and minutes of consortium meetings

D1.3 Public website with secured log-in for BALANCE participants

WP2 Characterization and cell banking of HepaRG cells

To prepare the HepaRG cell line for cell-culture production and for certification

Specific objectives:

Task 2.1: To develop pathogen-free master and working cell banks

Two cell banks of HepaRG cells were generated by partner 3: a Research (or Working) Cell Bank (RCB), and a Master Cell Bank (MCB). The culture details of the cell banks were captured in a Standard Operating Procedure, and were approved by the other consortium partners. In total 57 vials of were frozen as RCB and for the MCB 102 vials, to be used for the planned pre-clinical experiments and future clinical trial. Both cell banks were tested for sterility, mycoplasma, endotoxin, viability after thawing and recovery and appeared to be safe and useful.

However both banks showed early transformation and could not be used for pre-clinical experiments nor clinical application.

Task 2.2. Assessment of stability and phenotype of HepaRG cells:

To be commercially successful, HepaRG should have stable phenotype at least up to passage 20. We evaluated properties of HepaRG cells incubated in monolayers at passages P16, P20 and P22 at week 1 and week 4 after complete differentiation. Functional stability of the cells in course of prolonged incubation is important since for commercial success of the project it is imperative to be able to maintain functional cells within bioreactor for few weeks.

a. Measure sugar and nitrogen metabolism (glycolytic Aldolase B enzyme, gluconeogenesis enzyme PEPCK, glycogen storage, ammonia elimination, urea cycle)

We evaluated sugar metabolism by assessing its two major components: glycolysis via aldolase B expression and glycogenesis via glycogen accumulation. Over P16, P20 and P22 at week1 and P16, P20 and P22 at week4. Nitrogen metabolism was assessed by evaluating mRNA levels of carbamoyl phosphate synthetase CPS1, a key enzyme in ammonia detoxification function of hepatocytes, measuring level of produced urea and glutamine.

b. Measure plasma proteins secretion with major interest onto proteins associated with inflammatory reaction (albumin, transferrin)

We assessed expression of albumin and transferrin in HepaRG cells P16-P22 at week 1 and week 4

c. Measure lipid metabolism

Lipid metabolism in HepaRG cells in tested conditions was evaluated by measuring transcription levels of an important enzyme in lipids transformation, *i.e.* Cytochrome P450 4F3 (CYP4F3).

d. Measure detoxification function (different CYP forms, GSTs, transporters and bile salt conjugation)

Detoxification is a very important function of liver and is performed by different phase I and phase II enzymes and transporters expressed in hepatocytes. We assessed transcription of CYP3A4, CYP2B6 and transporters BSEP and MRP2 as diverse markers of detoxification competence of HepaRG cells at different conditions. Phase I and phase II detoxification functions in HepaRG cell were also evaluated by direct measurement of oxidation of number of typical CYP substrates and by conjugation of paracetamol with glucuronide, sulfonate and glutathione.

Activity of MRP2 transporter, which is located at biliary side of polarised hepatocytes and is important for secretion of metabolites into biliary duct, was assessed by treating HepaRG cells with carboxydichlorofluorescein di-acetate (CDF-DA). CDF-DA, being a non fluorescent compound itself, after uptake by hepatocytes is hydrolyzed inside of the cells producing fluorescent metabolite. Fluorescent metabolite is secreted from hepatocytes via MRP2 receptors into biliary canaliculi where it accumulates and can be easily detected by fluorescent microscopy.

e. Characterize senescence properties: beta-galactosidase and telomerase enzyme activities, lipid droplets accumulation, the number of binucleated cells and the expression of the main known senescence/apoptosis

regulators, the tumour suppressors P16, P21 and P53 at both RNA and protein levels as well as the caspases 3 and 8 activity.

Senescence properties were evaluated by assessing b-galactosidase activity, lipid droplets accumulation (Oil Red O staining) and expression of senescence markers p53, p21 and p16 in HepaRG cells at passages P16, P20 and P22 at week 1 and P16, P20 and P22 at week 4.

f. Set-up FACS analysis with stem-cell and hepatocyte-specific antibodies for quick profiling of HepaRG cells.

Partner 5 tested and recommended a number of antibodies for distinguishing differentiated hepatocytes. These markers have been characterized using immunohistochemistry. However, as this is not a high-throughput technique, partner 3 has not further pursued this method.

Task 2.3: Assessing genotype and karyotype of monolayer cells at passage 16 and 20 (BP)

a. Define karyotype and ploidy status which are two critical parameters for establishing cell stability within passages. Classical karyotype and CGH array will be performed.

Stability of abnormal HepaRG karyotype and ploidy of HepaRG cells in monolayer was assessed with classical karyotyping, chromosome painting and telomere staining, as well as with CGH arrays approach over a number of passages. It was established that HepaRG cells carry stable abnormal karyotype up to at least passage 22.

b. Measure sensitivity to TGF beta.

The TGF beta pathway has been selected as important for characterizing the capacity of cells to undergo apoptosis instead of to resist to cell death signals. TGF beta is one of the most powerful apoptotic molecules for normal hepatocytes. A loss of sensitivity to TGF beta generally accompanying transformation process is expected if cells undergo a transition from immortal to transformed status.

HepaRG response to TGF-b was evaluated and compared with that of primary hepatocytes.

c. Study transformation by growth on soft agar.

This well-known test has been previously applied to HepaRG cells; it is negative with immortal HepaRG cells.

Test of anchorage independent growth of HepaRG cells on soft agar has been accomplished.

d. Study of different oncogenes, liver tumour markers such as MALAT1 as well as characteristic miRNAs. Identification of immortalizing mutations in HepaRG.

We have accomplished evaluating expression of tumor marker genes MALAT1, c-myc, k-Ras, c-Met, h-Tert, since mutations in these genes are often associated with cancer. No abnormal regulation was detected.

Task 2.4: To determine tumorigenicity status in vivo

For regulatory purposes and to warrant optimal safety of the HepaRG cells to be applied in the clinical AMC-BAL, the in vivo tumorigenicity of high-passage HepaRG cells was tested in immunodeficient mice. As a positive control, a second group of mice was injected with the tumorigenic cell line Hela and a third group, as a negative control, with the vehicle (=culture medium). The injection and monitoring of the immunodeficient mice was performed by GLP accredited (required for regulatory purposes) TNO Triskelion, Zeist, the Netherlands. Subcutaneous inoculation of HepaRG cells did not result in tumor growth, whereas in the positive control group (Hela cells) the incidence of malignant tumors was statistically significantly increased. This indicates that the risk of HepaRG tumorigenicity in vivo is acceptable low.

The following deliverable reports have been finalized:

D2.4 Master and working cell banks
D2.5 Report on stability and phenotype of cells
D2.6 Report on genotyping and karyotyping of monolayer cells
D2.7 Report on tumorigenicity HepaRG cells in vivo

The milestone MS1 [Working cell bank : Master cell bank] was not reached, as the cell banks delivered by partner 3 showed early transformation and could not be used:

WP3 BAL hardware

To validate the safety of upscaled BALs for clinical testing, to develop transport, storage and incubation systems that guarantee optimal clinical performance and to prepare for certification of these hardware items.

Specific objectives:

Task 3.1: To produce the current bioreactor in laboratory and clinically relevant scale

The need of small bioreactors was lowered according to the project need (Fig. 2). At the end of the project still 5 small bioreactors are not purchased from RanD. The delivery is stopped and the final number purchased, used and paid for is 50.

Figure 2: The 9 mL and 550 mL AMC bioreactors

The clinical AMC bioreactor (not yet loaded with cells) and tubings (BAL AMC lines set) have been validated and released for clinical application by full documentation provided by our subcontractor RanD. Large bioreactor orders have been downsized from 75 to 44 due to amendments in planned studies and clinical project plan. As such orders have been placed for review studies and for pre-clinical studies under WP 4 amounting 29 large bioreactors. 15 are outstanding and as clinical trials will not start before end of BALANCE project further ordering has been halted.

Task 3.2: To validate the safety of upscaled BALs

According to the applicable regulatory requirements, biological and chemical safety of the clinical AMC bioreactor (not yet loaded with cells) and tubings (BAL AMC lines set) need to be demonstrated for clinical application. In accordance to ISO 10993 the BAL AMC lines set falls into the category of “External communicating device, circulating blood, limited contact duration”. Device classification: Class IIa, rule 2. The categorization of the AMC bioreactor alone is less clear, due to the multiple functions of the device (blood components collection and perfusion, unconventional oxygenation of the cells seeded inside) and the innovative therapy approach, i.e. bio-artificial liver support made by isolated cells in extracorporeal circulation.

The manufacturer of the bioreactors (RanD, Italy) has been subcontracted to assess the biocompatibility of AMC-bioreactor and the tubings. The main outcomes are that an oil extract and 0.9% NaCl extract of both items do not elicit acute systemic toxicity within 72 h in mice after intravenous and intraperitoneal injection (n=5/group). No leachables and chemical toxicity were found.

All items passed all the tests according to the required standards. The regulatory expert has agreed with the contents of all documents for regulatory purposes. Therefore the BAL AMC Lines Set and the AMC bioreactor (not with cells) meet the criteria for biological and chemical safety according to ISO10993-1 and ISO10993-4 quality standards and can be ordered upon our needs.

The physical and functional integrity and efficacy requirements of the bioreactor and tunings were also tested by RanD. All test results passed the acceptance criteria. Therefore, the BAL AMC Lines Set and the AMC bioreactor for cells are considered physically safe and functional for the intended use they are designed for.

Task 3.3: To assess optimal transport, storage and incubation conditions for large bioreactor

The processes for sterilization with ethylene oxide, the packaging & labeling as well as storage conditions of the bioreactors without cells were developed and tested positive according to EN ISO 11135-1 standard and EN ISO 10993-7 standard for ethylene oxide residuals limits.

Task 3.4: To prepare for certification of the large bioreactor

The regulatory expert has agreed with the contents of all documents describing the tests and data obtained under task 3.2 and 3.3, which is required for regulatory purposes. Therefore the BAL AMC Lines Set and the AMC bioreactor (not with cells) meet the criteria for biological and chemical safety, physical integrity and functionality.

A technical file including these outcomes has been drawn up, named R9800004 AMC Bioreactor ENG_3, of January 31st 2014. The technical file will be incorporated into the Investigational Medicinal Product Dossier (IMPD) to obtain approval for a clinical phase 1/2a study

Task 3.5: To adapt and purchase of a transport system of the large bioreactor

As described in D4.17, conditions were defined for the transport of the clinical AMC-BAL with cells from the production facility to eventual utilization site. These conditions were complex and comprised a temperature control, medium perfusion with gas supply for minimally 24 hours. The Medical Technical Department of the AMC (MIO) has produced a fully validated, according to all prior set specifications, transport system which has been used for the pre-clinical experiments in Edinburgh (WP5).

This stand-alone system supplied medium perfusion and gas to the HepaRG-AMC-BAL at a specified temperature. The gas flow and medium flow as well as the temperature were continuously monitored and these data were stored for further analysis (Fig 3).

Figure 3: Top view of the transport box with visibly the (a) inside box containing (b) bioreactor, (c) medium bag, (d) medium pump, and (e) the outside box containing (f) gas cylinder, (g) computer, and (h) batteries.

The transport system was tested and found to be stable for 24 hours at an environmental temperature of 4 °C and during a shipment with a ferry from partner 1 to partner 4 (Fig. 4).

Figure 4: Transport of the HepaRG-BAL in the transport box from partner 1 to partner 4 by car and ferry

Task 3.6: To adapt and purchase 3 large bioreactor incubators

The bioincubator system, needed for performing plasmapheresis and BAL perfusion in connection to the blood circulation was validated during the first half of the project period. Three devices (called Performer O.Liver), including the tubing and filter systems have been purchased from RanD (Fig 5). The O.Liver facilitates a blood circuit, plasma exchange part and the BAL circuit. The O.Liver and tubing system have been certified according to Medical Device Directive MDD 93/42/EEC and several CEI-IEL or EN-ISO safety norms.

The following deliverable reports have been finalized:

D3.8 52 large and 75 small bioreactors
D3.9 Biocompatibility data for IMPD
D3.10 Sterilization and packaging evaluation report
D3.11 Technical file on BAL for certification
D3.12 Description of BAL transport system and optimal preservation conditions
D3.13 Three incubators that facilitate the BAL during therapy

Figure 5: Performer O.Liver system

WP4 Production of HepaRG-bioreactor cultures

To select, develop and qualify the manufacturing process of HepaRG-bioreactor cultures
The production process of the HepaRG bioreactor cultures consists out of different stages:
-Expansion of cell masses
-Harvesting of the final cell mass
-Preservation of the cell mass and transport from BP to AMC
-Cell loading into the BAL
-Culture and testing of the bioreactor culture

Specific objectives:
Task 4.1: To transfer HepaRG technology and methods to partner 5
Transfer of laboratory procedures and specifications on monolayer and bioreactor culture of HepaRG cells to partner 5 has been done.
In addition, the appropriate cryopreservation scale of the cell masses (nr of vials, size of vials) was communicated.

Task 4.2: To develop a production process for HepaRG BAL cell cultures & Task 4.3: To verify cell function and stability before loading into the bioreactors
A production process of HepaRG cells in monolayer was developed by partner 5 who produced 11 batches of HepaRG (total 1.2581E+11 cells) in proliferating status with required specification parameters.
The work included following tasks: validation of high density cryopreservation process for HepaRG cells; production of 11 batches of HepaRG cells in proliferating status; cryopreservation of produced cells; validation of quality of cryopreserved cells for every batch according to cell number, cell viability, sterility and their ability to differentiate according to morphology, detoxification activity and expression of a number of hepatic genes, and delivering cryopreserved cells to partner 1 for BAL production.
A production process of HepaRG-bioreactor cultures was developed by partner 1 with most cost-efficient usage of culture medium.
Four verification runs of upscaled bioreactor cultures (550 mL) were performed (Fig 6 & 7).

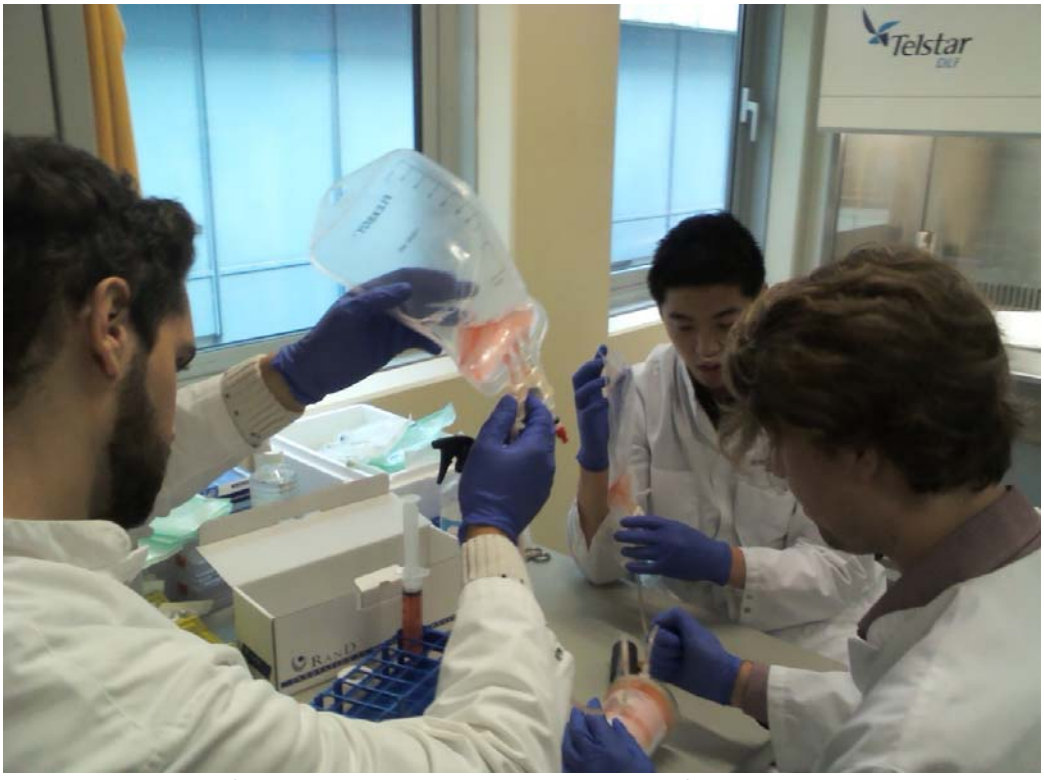

Figure 6: Loading of a 550 mL bioreactor with 30 grams of HepaRG cells.

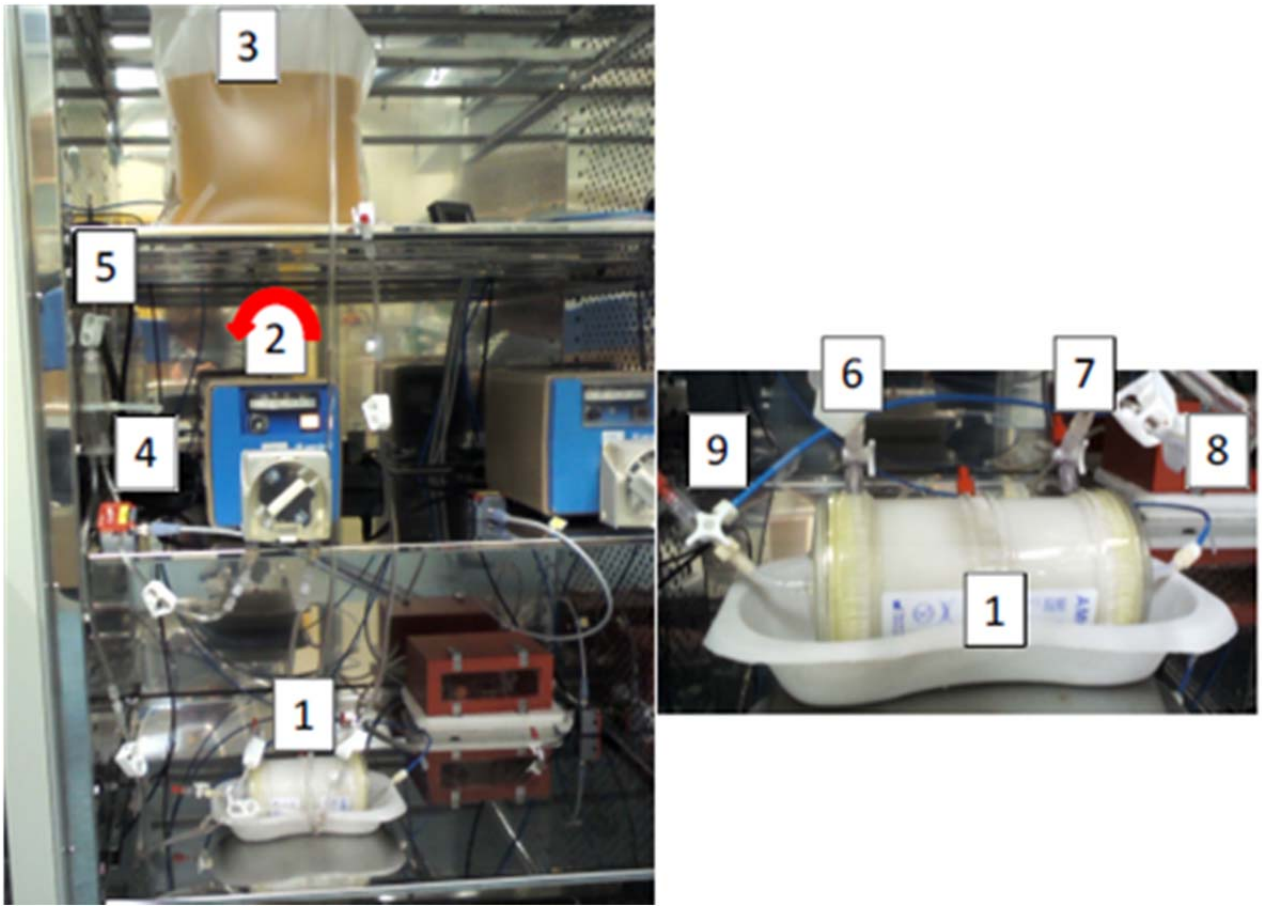

Figure 7: Left: Large incubator with one clinical BAL: 1, BAL; 2, medium pump; 3, medium reservoir; 4, medium flow sensor; 5, bubble catcher.
Right: Detail of the BAL: 6, medium in-port; 7, medium out-port; 8, gas in-port; 9, gas out-port.
Of the four pilot BALs the first two became infected with bacteria. Only one of these BALs was infected early (after 7 days); of the other infected BAL most experiments were completed. The BALs were loaded with 30 gram of cells with a viability of 92.3±4.0%. We assessed total protein content, functionality, cell damage and metabolic parameters, and the transcript levels of three hepatic genes. In total we successfully produced 3 pilot BALs and 6 BALs for pre-clinical experiments. The functionality and differentiation status of the BAL cultures was sufficient for the envisaged pre-clinical experiments.

Task 4.4: To specify preservation conditions

The HepaRG cells need to differentiated into the bioreactor during 2-3 weeks at the production site. Subsequently, the HepaRG-BAL needs to be transported from the production facility to the utilization site (normally the hospital) under well-defined conditions. We tested different optional preservation conditions (varying in temperature, presence/absence of medium/gas supply) on 9 mL HepaRG-BALs. It was found that low temperature was detrimental to the BALs. Therefore it was concluded that optimal preservation conditions comprised temperature control, with medium perfusion and gas supply.

Task 4.5: To assess stability of bioreactor cultures loaded in BALs before and after ALF treatment

Different types of stability were assessed:
1. Stability of the cells in monolayer in time
2. Stability of the bioreactor cultures (9 mL) in time
3. Stability of the bioreactor cultures in the presence of human plasma

Ad 1. The stability of the HepaRG cells in monolayer was assessed by comparison of the functionality data at passages 16, 18 & 19 and 22 & 23.
We assessed protein content, ammonia elimination and urea production. The data confirmed that the HepaRG cells showed stable phenotype at least until at passage 20.

Ad 2. The stability of the bioreactor cultures (9 mL) was tested at regular intervals after seeding with 25% of cell mass (cryopreserved HepaRG cells) up to 35 days. In general, the functionality, as measured for urea production, ammonia elimination and apolipoprotein synthesis, became maximal between 14-21 days after seeding and

remained at this high level. The cell leakage remained constant during the whole culture period. The lactate elimination started to decline however after 21 days, while the glucose consumption increased, indicating that the metabolism changes into a type more characteristic for cancer cells, known as the Warburg effect. So the bioreactor cultures are optimal between 14-21 days after seeding. The expression levels of hepatic genes showed a trend of increase between 14 and 35 days, but significance was not reached.

Ad 3. Four 9 mL HepaRG-BALs were exposed to human plasma (from healthy subjects) for 16 hours. Their functionality was subsequently tested during 24hours in test medium (post-plasma) In the post-plasma phase the cell leakage (aspartate aminotransferase) was significantly increased and the cells produced lactate instead of eliminating it. In addition some hepatic genes were significantly decreased in their gene expression levels. Parameters not affected included ammonia elimination, urea production, lactate dehydrogenase leakage, and glucose consumption. Yet, the effect of plasma exposure to the cells was far more dramatic after culturing in monolayer, so BAL culturing protects the HepaRG cells against the cytotoxic effects of plasma.

The following deliverable reports have been finalized
D4.14 Report on transfer of technology and qualified QC tests
D4.16 Specification of serum content utilization in the medium
D4.17 Report on preservation conditions
D4.18 Report on stability of bioreactor cultures
D4.151 Large scale production process of HepaRG-cells
D4.152 Production process of HepaRG-BALs for pre-clinical experiments.

The following milestones have been reached:
MS2 First HepaRG-BAL for pre-clinical study
MS4 selection monolayer culture system
MS5 Manufacturing process bioreactor cultures: no 'qualified' procedures as this was related to GMP-procedures

WP5 Safety and efficacy in subjects with ALF

To obtain proof of principle for safety and efficacy in a pre-clinical model of ALF

Specific objectives:

Task 5.1: To develop a reproducible invasively-monitored model of paracetamol-induced ALF in subjects;

We successfully established a pre-clinical model of ALF. The pilot studies were a great success and gave us a good understanding of the upper and lower limits of paracetamol dosing for ALF in study subjects. In the pilot phase of the project 17 female weight-matched subjects were exposed to varying toxic doses of paracetamol ranging from 300-1500 mg/kg. Not only were protocols for management and sampling tested and refined, but also these studies helped us identify and improve on potential technical and surgical limitations and bottlenecks.

We have established protocols for the connection of the subjects to plasmapheresis systems and have further improved anesthetic protocols and interventional management. Following careful evaluation of the pilot phase results, and the presented variability of the subject subjects coupled with the added complexity and physiological stress of plasmapheresis-based therapy; we decided to expose the subjects to a subacute dose (1g/kg bw) for the main study phase. This was in order not to further complicate management of the subjects during therapy, which could confound and mask therapeutic efficacy through deteriorating health.

Task 5.2: To assess clinical and biochemical parameters in paracetamol-induced pre-clinical models under ICU monitoring and therapy conditions;

We have established a multifactorial monitoring system including manual and machine-assisted recording of physiological readings and the 1-4-hourly sampling of biofluids (Blood and Urine). The former include urine output and intracranial pressure, while the latter among others includes heart rate, mean arterial pressure, body temperature, CO2 end tidal volume, bispectral index and respiratory rate. Blood gases, in critical phases of the studies, taken every hour and supplied us with acid/base, oximetry, oxygen, electrolyte and metabolite parameter readings. We had a plethora of plasma biochemistry tests done by ourselves and contracted professional analytical services. These parameters included plasma paracetamol concentration, levels of ammonia, total and conjugated bilirubin, albumin, alkaline phosphatase (ALP), alanine transaminase (ALT) and aspartate aminotransferase (AST), creatinine, urea, lactate dehydrogenase (LDH), phosphate, triglycerides, creatine kinase and coagulation panels including prothrombin time, activated partial thromboplastin time and fibronectin. We also had routine haematology done, which gave us blood cell counts and characteristics.

The completed main study of 23 subjects (9 Controls, 8 Empty BALs and 6 Filled BALs) profile differential effects from the stress of plasmapheresis and anti-coagulant treatment on the poisoned subjects; and the successful reduction of renal and liver impairment, through additional BAL treatment. Compared at end-point to controls, characteristically plasmapheresed subjects show higher physiological stress (heart rate, body temperature and venous saturation), whereas lower urine output and intracranial pressure suggest pronounced morbidity. Biochemical data however supports beneficial impact of the filled versus empty BAL on renal function (creatinine, phosphate and urea levels), and global tissue damage (LDH), although, predominantly on liver (AST). All plasmapheresed subjects have successfully completed their full duration of treatment and thus non-inferiority of the filled over the empty BAL is documented.

Task 5.3: To assess efficacy/ safety of HepaRG-BAL in three groups of 7 subjects

The study-specific results shown document the physiological stress of plasmapheresis and group-specific impact on bodily and tissue-specific functions. Indeed, in comparison between treatment groups, it becomes clear how strong the relative beneficial impact of the bioreactor on the emergence of global and specific injury and dysfunction was. Importantly, non-inferiority of filled BAL treatment is evident. Furthermore, it appears that pronounced kidney, muscle and liver damage were slowed down or even reversed in the filled BAL subjects. We look forward to further studies on the biobank samples and outstanding results of histological screening, cytokine and biomarker screens will give us clarity on the trajectory and severity of injury in all subjects and the full beneficial impact of the treatment.

Statistical assessment of post-treatment parameters documented the following points:

- Plasmapheresis exposes the subjects to significant physiological stress, characterized by heart rate, body temperature and intracranial pressure fluctuations.
- Model-specific profound third spacing incorporates albumin fluctuations that influence relative plasma marker levels and makes normalization necessary.
- In doing so, more profound kidney and muscle impairment/ damage is indicated in empty BAL subjects.

- Further data inclusion and refinement with subsequent informed statistical assessment will improve an appreciation of the detailed effects.

When all the outstanding support data is present, it will allow us to do a first pass assessment and grouping of the baseline liver injury in the various groups. Based on this, we will perform second pass analysis to assess impact of treatment on progression and severity of injury in comparison between groups. A third pass will then be performed to assess causality between early markers and outcome/therapy efficacy to inform further investigation, funding applications and publications.

The following deliverable reports have been finalized:
D5.19 Establishment of a reproducible invasively-monitored pre-clinical model of paracetamol induced ALF
D5.20 Report on variability of survival time of paracetamol induced ALF in subjects
D5.21 Data from statistical analysis, report completed

The following milestones have been reached:
MS6 safety and efficacy in subjects with ALF

WP6 Preparation clinical trial

To obtain regulatory approval for the planned phase I/II a study and to make practical preparations for clinical testing

Specific objectives:

Task 6.1: To prepare IMPD and QA/QC documents for approval of future clinical trial

The AMC-BAL, based on the improved HepaRG cell line, has been developed as a novel treatment to bridge ALF patients in the future. At the start of Balance we had the intention to treat 10 ALF patients with the HepaRG BAL to show safety and feasibility. However, due to the impossibility to obtain in time sufficient cell numbers under GMP conditions the clinical trial had to be abandoned. Nevertheless a huge effort has been given to prepare the documents necessary for approval by the regulatory bodies: the CCMO in the Netherlands and the MHRA in the UK. In a series of meetings partners 1, 2 and 4 under the supervision of a professional CRO (Q-Serve) have agreed on the following organization arrangements or documents.

-Case report forms (CRF) for all data collection during the trial to be stored and consulted in an electronic database accessible via the website

-An extensive risk assessment of the BAL preparation, BAL transport and patient treatment

-A list of specifications of the clinical HepaRG-BAL
-An Investigational Medicinal Product Dossier (IMPD)
-Standard operating procedures (SOP's).of BAL loading&culturing and quality control procedures
.

Task 6.2: To complete the study protocol

In a series of meetings partners 1,2 and 4 under the supervision of a professional CRO (Q-Serve) have agreed on a clinical protocol including patient informed consent paper and the members of a data monitoring board.
A future clinical Phase I/IIa trial will have directly profit from all these preparations

The following deliverable reports have been finalized:

D6.22 Report on organizational arrangements for future clinical trial
D6.23 Documents for regulatory approval for future clinical Phase I/IIa study

WP8 Dissemination and exploitation

The overall aim of WP8 was to adequately inform the external stakeholders of BALANCE of the results obtained and to effectively translate the main deliverables of BALANCE into solutions for healthcare and related markets. As we were forced to abandon plans for a clinical trial, dissemination also focused on creating awareness and the need for future funding.

Specific objectives:

Task 8.1: To disseminate results obtained to external stakeholders

The dissemination strategy of BALANCE targeted various groups: the scientific community healthcare providers, patients and the general public. The specific dissemination activities are summarized in table A2 of this report. Some examples are given here as illustration.

Figure 8: articles in AMC magazine for public and patients (2012), magazine for liver patients (2014) and magazine for hepatologists (2012), stand at AMC public day (6 October 2013).

Task 8.2: To plan exploitation of main deliverables and to attract financing for next (clinical)phases

Partner 2 has set up a plan to exploit project deliverables in order to try to secure follow up financing for subsequent development of the BAL system or to find a partner willing to take over the further development and subsequent commercialisation. In order to do so partner 2 has defined the attractiveness of the market, the needed follow on finance and described this in a Proposition for Investment. In addition, a Business Plan was finalized. On the basis of these documents potential investors, companies and Health Insurers have been approached and will be approached as financing future activities of BAL investigation is an ongoing activity. More details are provided in deliverable D8.29 (exploitation plan).

Task 8.3: To secure intellectual property rights

Deliverable D8.31 is related to any possible inventions made during the BALANCE project defined as patentable by an independent patent lawyer. Furthermore, already existing granted patents at the start of BALANCE on the BAL hardware had to be maintained. The already existing patent application on the improved BAL in combination with the improved HepaRG cell line had to be guided through the granting procedure (PCT, national country filing and granting per jurisdiction). During the BALANCE project these procedures have been taken care of.
No new patentable inventions were defined during the BALANCE project.

Existing granted patents were maintained (on the BAL system, internally numbered P039969, have been maintained in DE, ES, FR, UK, IT, AU, CA, JP and US. This patent will expire in October 2016).

The filed application on the BAL and cell line combination, internally numbered P6025187, has been filed in EP (as PCT) and in national phase in DE, ES, FR, UK, IT. Furthermore, this PCT patent was granted for its claims related to the improvement of the cell culture system in the US in December 2014. Subsequently, divisional claims have been filed in the US for the use of the BAL system in relation to the 3-dimensional cell culture format, which Divisional Application is still pending. Granted patent of this file will protect the BAL with cell line up to 2030.

Preparation phase I/IIa clinical trial in humans

For regulatory approval of an anticipated Phase I/IIa clinical trial in humans, a significant risk may encompass that the approval trajectory takes more time than estimated. Therefore, we started early in the project with preparations. Setting up study protocols and documents were partly outsourced to subcontractors. As such also an outline for a Quality Manual System (QMS) for approval of the required Manufacturing License for partner 2 was set up in close collaboration with subcontractor Xendo, hired by partner 2. A series of template Standard Operating Procedures (SOP) and documents was made for describing: Organizational chart, Job descriptions, Training procedures, Documentation control, CRO qualification, Audit plan.

The following deliverable reports have been finalized

D8.28 Report on dissemination to stakeholders and the general public
D8.29 Exploitation plan main project deliverables
D8.31 New patent applications