Preclinical development of the AMC-HepaRG-Bioartificial Liver
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Scaling-up of a HepaRG progenitor cell based bioartificial liver; optimization for clinical application and transport

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ABSTRACT

**Background and aims:** A new generation of bioartificial livers, based on differentiated proliferative hepatocyte sources has been developed. Several practicable and regulatory demands have to be addressed before these can be clinically evaluated. We identified three main hurdles: 1.) expansion and preservation of the biocomponent, 2.) development of scaled-up culture conditions, and 3.) transport of the device to the bedside. In this study we address these three issues for the HepaRG- progenitor cell line-loaded AMC-Bioartificial Liver.

**Methods and results:** HepaRG cells were expanded in large quantities and then cryopreserved or loaded directly into bioreactors. After 3 weeks of culture, key hepatic functions (ammonia/lactate elimination, apolipoprotein A1 synthesis and cytochrome P450 3A4 activity) did not differ significantly between the two groups.

Bioartificial livers were scaled up from 9mL to 540mL priming volume, with preservation of normalized hepatic functionality. Quantification of amino acid consumption revealed rapid depletion of several amino acids.

Whole-device cryopreservation and cooled preservation induced significant loss of hepatic functionality, whereas simulated transport from culture-facility to the bedside in a clinical-grade transport unit with controlled temperature maintenance, medium perfusion and gas supply, did not affect functionality.

In addition, we assessed tumorigenicity of HepaRG cells in immune-incompetent mice and found no tumor formation of HepaRG cells (n=12), while HeLa cells induced formation of carcinomas in eight out of twelve mice in 140 days.

**Conclusion:** The HepaRG AMC-Bioartificial Liver was scaled-up to a clinical size, and large-scale HepaRG expansion and cryopreservation, as well as transport to the bedside proved feasible without loss of functionality. For the future, the culture medium regimen can be modified by supplementing the critically depleted amino acids in a fed-batch set-up.
INTRODUCTION

Bioartificial livers (BALs) were conceived to improve the condition of patients suffering from Acute Liver Failure (ALF) and serve as a bridging-therapy to liver transplantation or spontaneous regeneration. They consist of extracorporeal bioreactors, loaded with liver cells and usually interfaced with the patient by means of a plasmapheresis circuit.

The AMC-BAL is a cartridge-based system with the distinct feature of direct contact between patient plasma and liver cells, in order to maximize mass-transfer (Fig. 1). The system was originally based on primary porcine hepatocytes and successfully applied in a series of 12 patients suffering from ALF [1, 2]. However, further clinical evaluation was abandoned upon the enforcement of a moratorium on xenotransplantation in the European Union, which includes primary-porcine hepatocyte-based BAL therapy. Furthermore, concerns about xenotransplantation and the negative outcome of a phase 3 clinical trial with a BAL system based on cryopreserved porcine hepatocytes have led to a set-back in the implementation of this technology [3, 4].

Figure 1. AMC-BAL. Schematic representation of the HepaRG BAL (A), with details (B-C) and hematoxylin/eosin-staining (D) showing medium/plasma inflow port (I), medium/plasma outflow port (II), gas inlet port (III) (gas outlet port at opposite position not visible), the non-woven matrix to which the cells attach (IV), gas capillaries (V) and inter-capillary space (VI) (through which the culture medium/plasma flows)
Alternatives to xenogeneic hepatocytes are human primary hepatocytes or proliferative cells of human origin. The latter are the most attractive option, since primary human hepatocytes are scarcely available. In recent years, a new generation of BAL systems based on proliferative human cells has been developed, of which several are under or near clinical evaluation [5-7]. The new generation AMC-BAL, now based on the human hepatic progenitor cell line HepaRG, is one of these systems [8-10].

Before these BALs can be clinically applied, several important challenges have to be addressed. First, the biocomponent has to be expanded and preserved in large amounts: estimations of the required cell mass to support ALF-patients range from 15g-300g for a 70 kg patient, amounting to ~2.5-50 m² of monolayer culture surface [11-13]. Once expanded, it is of paramount importance that cells can be preserved, for example by cryopreservation, to facilitate large-scale batch-wise production, characterization and validation, as required by the regulatory authorities for approval of the BAL as an advanced therapy medicinal product. Secondly, hardware and culture conditions have to be scaled-up and modified to meet clinical standards. Thirdly, BALs need to be preserved and shipped to the bedside without loss of functionality.

Previously, a laboratory-scale model of the HepaRG loaded- AMC-BAL was proven efficacious in prolonging survival time of rats after total liver ischemia [6]. In this study we address the abovementioned challenges for the HepaRG-AMC-BAL and provide additional safety data in preparation of clinical evaluation.

**MATERIAL AND METHODS**

**HepaRG cell culture**

HepaRG cells (Biopredic International, Rennes, France) were expanded as previously described [6]. Briefly, cells were cultured in supplemented Williams’ Medium E (HepaRG medium) in stacked monolayer culture vessels for two weeks and split in a 1:5 ratio. Where applicable, cells were cryopreserved in HepaRG medium, supplemented with 10% DMSO to prevent ice-crystal formation [14], in liquid nitrogen. Every cell-batch loaded in the 540mL- BALs passed a set of quality control tests: viability after thawing (trypan blue exclusion test >85%), microbial sterility, transcript levels of three hepatic genes (urea cycle enzyme arginase1 ARG1; detoxification enzyme cytochrome P450 3A4 CYP3A4 and hepatic transcription factor HNF4A), tested against a HepaRG reference batch) and morphology (>50% of culture surface covered by well delineated, granular hepatocyte clusters with bright canalicular structures).
**BAL hardware**

The 9mL- and 540mL-BAL cartridges were custom-made to specification (RanD, Medolla, Italy). The configuration of 9mL-BALs was described previously [6] (Table 1), and 540mL-BALs had a similar configuration, but with a priming volume of 540mL and 6254cm^2 of DuPont™ Spunlaced Nonwoven Fabric- matrix (DuPont, Wilmington, DE, USA), interlaced with 3800 gas capillaries for aeration (Fig. 1, Table 1).

The 540mL-BALs were produced and tested for biological and chemical safety in accordance with ISO10993-1 and ISO10993-4 quality standards and in compliance with Good Manufacturing Practice (GMP) (RanD, Medolla, Italy).

<table>
<thead>
<tr>
<th>Table 1A. Characteristics of the 9mL- and 540mL-BAL</th>
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<tbody>
<tr>
<td><strong>BAL type</strong></td>
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<tr>
<td>Internal volume (mL)</td>
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<tr>
<td>Matrix surface (cm^2)</td>
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<tr>
<td>Gas capillaries (cm per cm^2 matrix)</td>
</tr>
<tr>
<td>Certified for clinical testing</td>
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<tr>
<td>Cell mass loaded (g)</td>
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<tr>
<td>Total protein loaded (mg)*</td>
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<tr>
<td>Total protein day 22 (mg)*</td>
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</tbody>
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* data from cryopreserved 9mL-BALs from figure 2F

<table>
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<tr>
<th>Table 1B. Function and damage parameters of 9mL-BALs loaded with non-preserved cells (n=3) and 540mL-BALS loaded with cryopreserved cells, (n=9).</th>
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<tbody>
<tr>
<td><strong>9mL-BAL</strong></td>
</tr>
<tr>
<td>MEAN</td>
</tr>
<tr>
<td>Ammonia elimination (µmoles ∙ h^-1 ∙ g protein^-1)</td>
</tr>
<tr>
<td>Urea production (µmoles ∙ h^-1 ∙ g protein^-1)</td>
</tr>
<tr>
<td>ApoA1 synthesis (mg ∙ h^-1 ∙ g protein^-1)</td>
</tr>
<tr>
<td>Albumin synthesis (mg ∙ h^-1 ∙ g protein^-1)</td>
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<tr>
<td>AST leakage (U ∙ h^-1 ∙ g protein^-1)</td>
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<tr>
<td>LDH leakage (U ∙ h^-1 ∙ g protein^-1)</td>
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<tr>
<td>Lactate elimination (µmoles ∙ h^-1 ∙ g protein^-1)</td>
</tr>
<tr>
<td>Glucose consumption (µmoles ∙ h^-1 ∙ g protein^-1)</td>
</tr>
<tr>
<td>CYP3A4 activity * (µmoles ∙ h^-1 ∙ g protein^-1)</td>
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* (Testosterone 6β-hydroxylation)
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BAL culture

BAL culturing was performed in HepaRG medium as described previously [6]. Briefly, cells were brought into suspension in culture medium and loaded into the bioreactors, allowing 4 hours for attachment to the matrix before commencing perfusion. Medium flow inside the bioreactors was kept at 3 ml cm⁻² min⁻¹ and a mixture of 40% O₂, 5% CO₂ and 55% N₂ was perfused through the gas capillaries. The temperature was maintained at 36°C. Culture medium volume was 500ml for 9mL-BALs, and 90% of medium volume was replenished twice weekly. The recirculating culture medium volume for 540mL-BALs was 7L, of which 5L was refreshed daily.

The fetal bovine serum (FBS) percentage was reduced from 10% to 5% for 540mL-BAL cultures after we confirmed that the FBS reduction did not affect functionality or growth rate (Fig. S1).

BAL loading optimization and biomass assessment

Laboratory scaled 9mL-BALs (n=3-5 per group) were loaded with HepaRG cells that were freshly isolated (non-preserved, 2g cells) or cryopreserved (2g, 1g or 0.5g cells). Hepatic functions were tested after 7, 14 and 21 days of culture. Aliquots of the loading suspensions and BALs, harvested after the last function test, were lysed in 0.2 M sodium hydroxide for total protein content determination, as described previously [6]. 540-mL BALs were loaded similarly, with 40 grams of cryopreserved HepaRG cells.

BAL cryopreservation

Four 9mL-BALs loaded with 0.5g of cryopreserved cells, were cultured for 21 days and tested for hepatic functionality and hepatic transcript levels. Subsequently, the BALs were flushed with HepaRG medium (cooled until 4°C) supplemented with 10% DMSO to prevent ice-crystal formation [14], sealed off and cooled down to -80°C at a rate of ~1°C/minute. After one week of cryogenic storage, BALs were thawed at room temperature under aeration, reperfused with culture medium (cooled until 4°C), and then maintained at 37°C. The medium perfusion rate was gradually normalized from 1/5th to normal speed over the course of 5 hours.

Tumorigenicity evaluation in mice

The welfare of the animals was maintained in accordance with the general principles governing the use of animals in experiments of the European Communities (Directive 2010/63/EU) and Dutch legislation (The Experiments on Animals Act, 1997). This included approval of the study by the local animal experimental committee (DEC-number 3517). Animal studies were performed under Good Laboratory Practice license and in adherence to the applicable EMEA and FDA guidelines.
Athymic Nude-Foxn1nu mice were randomized into three groups (n=12/group): subcutaneous injection with a suspension of 10^7 HepaRG cells (test group), 10^7 HeLa cells (positive control) or HepaRG medium only (negative control group). Tumor sizes were measured weekly with calibrated digital calipers, tumor volume was estimated using the formula: Volume = (length x width^2)/2. Follow-up was 140 days or until a humane endpoint, after which animals were sacrificed and examined macroscopic and microscopic for pathological changes. Microscopic evaluation included hematoxylin and eosin staining (4µm sections) of brain, kidneys, liver, spleen, lungs, axillary and brachial lymph nodes, gross lesions (including any remote nodules) and the injection site.

**Hepatic function tests**
Function tests were performed as previously described [15]. Briefly, BALs were exposed to HepaRG medium supplemented with 1.5 mM ammonia, 125μM testosterone, 2.27 mM D-galactose and 2 mM L-lactate. Medium volume was 7.8-9.1 mL/g biomass/hour test time (120 mL for 9mL-BALs and 1250 ml for 540mL-BALs) and samples were taken at 0, 30, 60, 120, 240, 480 and 1440 min. (9mL-BALs) or at 0, 15, 30, 60, 120 and 240 min. (540mL-BALs). Concentrations of L-lactate, ammonia, apolipoprotein A1 (ApoA1), 6b-hydroxytestosterone (as the metabolite through CYP3A4 activity), urea and glucose, as well as activities of aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) were quantified, and the metabolic or cell leakage rates were calculated as described previously [15].

**qRT-PCR**
Cells were harvested from 9mL-BALs at different time points as previously described [16], and RNA was isolated using the RNeasy mini-kit according to the manufacturer’s instructions (Qiagen, Hilden, Germany). Transcript levels of hepatic genes ARG1, CYP3A4 and HNF4A were determined by qRT-PCR using gene-specific RT primers and a touchdown qPCR protocol as described previously [17].

**Ultra-Performance Liquid Chromatography-Tandem Mass Spectrometry**
Amino acids (AAs) were quantified in HepaRG medium of 540mL-BALs at 0 and 24 hours after medium refreshment at days 0, 2, 4, 7, 10, 14 and 21 of culturing. A mix of stable isotope-labelled internal standards was added to 50μl medium after which 500μl of acetonitrile was added while vortexing. The mixture was centrifuged for 10 min at 12,000 x g and the supernatant was taken to dryness under a nitrogen flow. The residue was resuspended in 200μl 0.01% heptafluorobutyric acid and 10μl was injected into the Ultra Performance Liquid Chromatography-Tandem Mass Spectrometer (Acquity-Quattro Premier XE, Waters, Milford, Massachusetts, USA) operated in the positive electrospray ionization mode using multiple reaction monitoring for the preselected analytes. The AAs were separated from interfering compounds by a linear gradient between solution B (acetonitrile/water, 4:1, v/v) and solution...
A (0.1% heptafluorobutyric acid). The gradient (0.4ml/min) was as follows: 0-2min 100% A, 2-5 min 50% A–50% B, 5-6min 100% B and 6-9min, equilibration with 100% A. All gradient steps were linear, and the total analysis time, including the equilibration, was 9min. Data processing was performed using Masslynx 4.1 software.

**Hypothermic culture**
9mL-BALs loaded with 0.5g of cryopreserved cells, were tested for hepatic functionality at day 21. Then temperature was reduced to 20°C or 30°C (n=4/group) for 24 hours. Next, the temperature was brought back to 37°C and hepatic functions were immediately tested.

**BAL transport unit**
The system for transportation of the 540mL-BAL cultures was custom designed and produced in an ISO 9100-certified facility (AMC medical innovations unit, Amsterdam, the Netherlands) (Fig. 4A). The design was evaluated through Failure Mode and Effects Analysis. The transport system utilizes disposable clinical-grade closed-circuit tubing-sets (RanD, Medolla, Italy). For the transport simulation, a 540mL-BAL cultured for 21 days was tested for hepatic functionality and then kept in the transport unit for 24 hours. In this time, the unit was loaded into a vehicle several times, driven over uneven terrain and left at 4°C for 16 hours. After this, hepatic functions were tested again and expressed as a percentage of the pre-transport tests.

**Statistics**
Data were collected, graphically represented and statistically analyzed using Prism 6.0 (GraphPad, La Jolla, CA, USA). Data are expressed as means ± standard deviations (SD). Multiple groups with repeated measures were compared with mixed-ANOVA analysis (alpha=0.05) and corrected for multiple testing using the Holm-Sidak method.

Hepatic functions and cell leakage rates of 9mL- and 540mL-BALs were compared by repeated T-tests and corrected for multiple testing using the Holm-Sidak method without assuming consistent SD (alpha=0.05).

**RESULTS**

**HepaRG cells can be loaded into BALs directly from cryogenic storage without loss of function and undergo two population doublings inside the AMC-BAL**
Laboratory-scale 9mL-BALs were loaded with HepaRG cells; one group with freshly isolated cells (non-preserved), and one group with cryopreserved cells (cryopreserved). Hepatic functionality was similar between cryopreserved cells and non-preserved cells after 21 days
of BAL culturing (Fig. 2A-D). Limited differences in functionality were measured at earlier time points: at days 7 and 14; non-preserved cells produced more of the hepatocyte-specific ApoA1 (p<0.01 and p<0.05, respectively) and had higher CYP3A4 detoxification enzyme activity at day 14 (p<0.05). Ammonia and lactate elimination did not differ significantly at any time point. The cell damage, as quantified by leakage of the intracellular enzyme AST, was also similar between both groups at all three time points (Fig. 2E).

Next, we compared BALs loaded with the original cryopreserved cell amount (2g) to BALs with half or one quarter of the loading mass (Fig. 2A-E). Strikingly, in both lower-cell mass groups, ammonia elimination capacities were at the level of control BALs from day 7, and lactate elimination was only significantly lower in the quarter-mass group at day 7. ApoA1 production was lower in both lower-cell mass groups at days 7 and 14, but not at day 21, and CYP3A4 activity was not different between the groups at day 21 (not measured at days 7 and 14) (Fig 2A-D). AST release remained lower at all three time points in the lower-cell-mass groups (Fig. 2E). Total protein analysis revealed that the cell mass loaded at half and one quarter of the original mass underwent approximately one and two population doublings respectively, while total protein content of control BALs remained stable (Fig 2F).

Combined, these results indicate that BALs can be loaded with a cryopreserved HepaRG cell mass corresponding to 25% of the final cell mass obtained after 21 days of BAL culturing, which is the optimal culture period. These conditions were applied to the HepaRG cultures in 540mL-bioreactors.

**HepaRG cells in 540mL-BALs exhibit all tested functions, but do not proliferate**

To accommodate the amount of cells required for clinical application, a scaled-up version of the AMC-BAL with a priming volume of 540mL was produced and tested after 21 days (Fig. 1, Table 1A). The tested functions were normalized to total protein content (Table 1A) and compared to those of the 9mL-non-preserved BALs at day 14 (Table 1B). Total protein-corrected hepatic functions, including ammonia and lactate elimination, urea, albumin, and ApoA1 synthesis and Cyp3A4 activity, did not differ between the two BALs. In addition, glucose metabolism remained comparable. The only difference found was that the leakage of AST and LDH into the culture medium was 3.5 and 7.1 fold decreased respectively, in the 540mL-BALs compared to the 9mL-BALs. Together, these results indicate that the performance of the 540mL-BAL compared to 9mL-BALs did not differ significantly (p=0.6312). Unexpectedly, total protein content after 23 days of culture was not increased relative to the starting amount, meaning that no net proliferation had occurred (Table 1A).
HepaRG-BAL substrate consumption changes over time

To assess culture medium depletion, we quantified the net change in glucose and AA concentrations in culture medium of 540mL-BALs under a regime of once-daily 5L medium change for 22 days. Glucose consumption increased over the culture period, but never exceeded 27% of the starting concentration (Table 2). After one week of culture, leucine and aspartate were consumed >50% of their starting concentration. Towards the end of the culture period, seven AAs had become depleted >50% in 24 hours: branched chain AAs leucine, isoleucine and valine, as well as aspartate, tryptophan and methionine (Table 2).

Figure 2. Culture time and biocomponent loading and preservation conditions in 9mL-BALs. Hepatic functions of BALs loaded with non-preserved HepaRG cells and cryopreserved HepaRG cells at different loading masses, measured at day 7, 14 and 21 (A-D). Testosterone 6β-hydroxylation as a measure of CYP3A4 activity, was not measured at day 14 for the half and quarter mass-loaded BALs (E). Total protein content of BALs loaded with different amounts of cryopreserved HepaRG cells, measured at 0 and 21 days (F).

$ p<0.05 $ vs non-preserved at same time-point; $ & p<0.05 $ vs previous time-point in same group; $ # p<0.05 $ vs cryopreserved
Table 2. Glucose, lactate and amino acid concentration in culture medium (given as % of start concentration after 24 hours of culture)

| DAY | gluc | phe | tyr | trp | ala | met | gly | val | ile | gin | asn | cit | orn | lys | arg | ser | pro | glu | asp |
|-----|------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
|     | start (µM) 10600 | 134  | 192 | 38  | 896 | 100 | 404 | 340 | 467 | 425 | 2754 | 52  | 3   | 11  | 432 | 283 | 272 | 242 | 333 | 158 |
| 1   | 100% | 100% | 100% | 84% | 102% | 88% | 105% | 104% | 57% | 77% | 117% | 121% | 121% | 565% | 113% | 99% | 129% | 100% | 131% | 62% |
| 3   | 95%  | 90%  | 99%  | 69% | 70%  | 75% | 110% | 89% | 64% | 73% | 91%  | 84%  | 77%  | 133% | 94%  | 92% | 101% | 84%  | 93%  | 54% |
| 5   | 92%  | 82%  | 99%  | 51% | 62%  | 75% | 87% | 84% | 42% | 56% | 103% | 88%  | 91%  | 154% | 95%  | 91% | 114% | 77%  | 104% | 30% |
| 7   | 90%  | 87%  | 90%  | 35% | 51%  | 63% | 90% | 74% | 17% | 36% | 111% | 70%  | 94%  | 226% | 106% | 94% | 120% | 74%  | 87%  | 12% |
| 10  | 89%  | 80%  | 86%  | 27% | 45%  | 47% | 88% | 64% | 9%  | 26% | 101% | 70%  | 76%  | 199% | 83%  | 79% | 98%  | 65%  | 84%  | 10% |
| 14  | 85%  | 72%  | 81%  | 13% | 47%  | 31% | 103% | 63% | 4%  | 22% | 106% | 62%  | 70%  | 279% | 82%  | 75% | 99%  | 77%  | 89%  | 17% |
| 21  | 73%  | 60%  | 70%  | 8%  | 41%  | 17% | 99% | 47% | 3%  | 17% | 74%  | 54%  | 86%  | 371% | 61%  | 52% | 63%  | 51%  | 67%  | 27% |

Gluc, glucose; phe, phenylalanine; tyr, tyrosine; trp, tryptophan; ala, alanine; met, methionine; gly, glycine; val, valine; leu, leucine; ile, isoleucine; gin, glutamine; asn, asparagine; cit, citruline; orn, ornithine; lys, lysine; arg, arginine; ser, serine; pro, proline; glu, glutamate; asp, aspartate
Cryopreserved and hypothermic transport conditions compromise HepaRG-BAL functionality

Ideally, once differentiated, BALs would be cryopreserved and stored until required and shipped via existing cold-chain solutions to be thawed at the bedside. To explore this possibility, we adapted the protocol for cryopreservation of suspended HepaRG cells and stored fully differentiated 9mL-BALs at -80°C for one week. Unfortunately, after thawing, ammonia elimination and ApoA1 elimination capacity were lost almost completely and lactate elimination reversed into production (Fig 3A-C). Over the course of one week of BAL culturing after cryopreservation, ammonia- and lactate elimination capacity were partly restored. Transcript levels of the hepatic genes ARG1, Albumin (ALB) and CYP3A4 decreased to 0.7±0.26%, 5.2±4.1% and 1.3±0.6% of their pre-preservation levels, respectively. Transcript levels remained significantly decreased for up to seven days, indicating profound loss of phenotype (Fig. 3D).

Next to cryopreservation we explored preservation conditions at 4°C with and without active aeration, but these also led to unacceptable loss of function (data not shown). Therefore, we

**Figure 3.** Whole-BAL cryopreservation. A comparison of hepatic functions (A-C) and hepatic transcript levels (D) of 9mL-BALs tested at baseline (21 days of culture) and after cryopreservation. $ p<0.05$ vs baseline.
concluded that the HepaRG-BAL should be transported at (sub-) normothermic temperature, which also requires gas supply- and culture medium perfusion.

Next, we assessed the effect of 24 hours medium-perfused and oxygenated preservation at 20°C and 30°C on hepatic functions of differentiated 9mL HepaRG-BALs. The 20°C group
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showed a significantly decreased lactate elimination (72%), ammonia elimination (40%) and ApoA1 synthesis (41%), as well as an increased AST leakage (265%), indicating cell death (Fig 4A-D), compared to baseline BAL cultures maintained at 36°C. BALs incubated at 30°C maintained hepatic functionality, including lactate elimination, ammonia elimination, ApoA1 synthesis and CYP3A4 activity, as measured by testosterone 6β-hydroxylation (Fig. 4E). However, AST leakage was significantly higher (38%) than the baseline value for this group as well, indicating that although functionality is not impaired, there is more cell death compared to 36°C and thus transport at temperatures lower than 30°C is not opportune.

These data indicate that existing temperature-controlled infrastructure at 20°C does not suffice, and that HepaRG-BALs should be transported at 30-36°C.

The AMC-BAL can be transported safely in a novel transport unit, fit for clinical use
A dedicated transport unit was developed for the 540mL-BAL to maintain gas- and culture medium perfusion, as well as a temperature at 30-36°C during transport. To function autonomously, it requires an independent power source, the capacity of which is largely determined by the energy-consumption of the temperature-control system.

To meet the required transport conditions in the clinical setting, the transport system can operate autonomously for at least 48 hours, while logging culture conditions (Fig. 5A-C). As a proof of principle, one BAL was tested on hepatic functions before and after a 24-hour transport simulation, including a period of 12 hours at 4°C and exposure to realistic handling strain. Tested hepatic functions did not differ before or after the transport simulation (Fig 5B).

HepaRG cells are not tumorigenic in immunocompromised mice
Mice were injected subdermally with HepaRG cells (test group), HeLa cells (positive control) or vehicle (negative control group). After 6 days, nodules were observed in 0/12 vehicle control-injected animals, 8/12 HepaRG-injected animals and in 12/12 HeLa-injected animals (Table S1). After 14 days, all nodules had resolved in HepaRG-injected animals, indicating that the initial swelling reflects a temporary effect. In seven HeLa-injected mice the initial nodules regressed completely within 28 days. In the other five mice the initial nodules reduced in size, but still persisted at 28 days and increased later onwards.

After 140 days, nodules were observed in 0/12 negative controls and HepaRG-injected mice and in 8/12 positive controls. Four positive-control mice were sacrificed before the end of the study because of large nodule size or ulceration of the skin at the site of the nodule.
Upon pathological examination, the nodules in positive controls were confirmed to be carcinomas, as characterized by pleomorphic cells with a high mitotic activity, invading the surrounding tissue. In contrast, microscopic examination of negative control and HepaRG-injected mice did not reveal any treatment-related findings.

**DISCUSSION**

In this study we addressed three important hurdles that need to be overcome for clinical application of the HepaRG AMC-BAL system, and that largely apply to other (future) BAL systems and advanced cell-based therapies as well: expansion and preservation of the biocomponent, development of scaled-up culture conditions that are feasible in the clinical setting and transport of the BAL to the bedside.

Large-scale biocomponent culture and preservation are crucial for two reasons. On the one hand it is common practice and a requirement of regulatory authorities to facilitate sound characterization and safety testing on each batch of cells without time constrains. Secondly, long-term preservation significantly reduces complexity and costs of the production process, because cells can be expanded in large quantities at any site in the world, without the need to synchronize with the production of the final product. In the case of the AMC-BAL we successfully applied a scalable monolayer culture platform in combination with
cryopreservation after cell expansion, to generate cell mass that could be loaded into the bioreactor directly from cryogenic storage.

Once the expansion and preservation of the biocomponent is established, the culture process of the BALs has to be scaled-up in a way that is both practically and financially feasible. The scaled-up culture conditions were designed to be compatible with GMP standards, and the scaled-up BALs had protein-normalized functionality as predicted. We found that the amount of cryopreserved cells, a factor that contributes substantially to total costs, can be reduced at least four-fold without loss of functionality in 9mL-BALs, owing to significant proliferative activity (~2 population doublings). However, HepaRG cells did not proliferate inside the 540mL-BALs. Based on animal experiments and experience from liver resections, the estimations of required BAL-biomass vary between 2% and 40% of functional hepatocyte mass [11, 13], corresponding to approximately 15g-300g for a 70 kg patient [11]. On the basis of its matrix capacity, the 540mL-AMC-BAL can theoretically facilitate 900g of cell mass. Yet, the lack of proliferation of cell mass in the 540mL-bioreactor in the current study limited the cell mass to 40g, or 5% of functional liver cell mass. Although this is within the spectrum that is considered acceptable, 20% of functional hepatocyte mass would be more appropriate, since this was the amount successfully applied in the proof-of-principle study of the HepaRG AMC-BAL in rats [6]. We speculate that the difference in facilitating cell expansion in the 9mL- and 540mL-bioreactors may relate to the difference in dimension between the two bioreactor designs. Since cells have to spread over a larger distance after loading into the 540mL-BAL, the distribution is likely to be more heterogeneous, with a higher cell density at the periphery of the bioreactor. Because HepaRG cells exhibit contact inhibition [9], this may lead to clusters of cells in the periphery and empty areas in the center of the bioreactor. One possible way of resolving the inhomogeneous distribution of cell mass in the large bioreactor, is to carve-out additional loading channels from the matrix.

In addition to the required cell mass, the feeding regimen is also a major determinant for practical and financial feasibility of the upscaling process of BAL cultures. We explored glucose and AA depletion in the 540mL-BAL under the currently applied regimen. All AAs were increasingly consumed, except for glycine and ornithine, indicating change of the energy metabolism of the BAL in time, which are unrelated to cell mass changes, as the protein content remained stable. As a result, substrate requirements changed in time. Since hepatic functions were expressed at their expected levels, we conclude that the abovementioned culture regime suffices, and that the depletion of several AAs was not yet critical. AAs can be used for protein synthesis or energy metabolism. Seven AAs were depleted for more than 50% within 24 hours towards the end of the culture period: tryptophan, methionine, valine, leucine, isoleucine aspartate and alanine. All of these are considered essential, except for aspartate and alanine. Aspartate can be deaminated into oxaloacetate, which can in
turn be used as an energy source in the citric acid cycle and alanine can be deaminated, yielding pyruvate as a source of energy. Tryptophan, leucine and isoleucine can in turn function as substrates to produce alanine. As these AAs get depleted, glucose consumption increases. This analysis of medium requirements enables the development of a fed-batch culture protocol [18], in which limiting supplements can be replenished into the system (semi-)continuously, thus reducing the total amount of culture medium and handling required and possibly at the same time, improving functionality.

HepaRG cells, like other highly functional proliferative hepatocytes, such as stem cell derived hepatocytes, require time to differentiate and once fully differentiated, can be kept in culture for several weeks until needed [7, 15]. The implication of this culture scheme is that differentiated BALs need to be transported to the clinic from the production site under conditions warranting safe and reproducible preservation. To facilitate transport of the BAL system to the bedside, we successfully developed and tested a dedicated transport unit that can autonomously support a pre-validated and functional BAL for 48 hours. In addition, we explored hypothermic and subnormothermic conditions and cryopreservation of entire BALs. In this study, cryopreservation of entire differentiated 9mL-BALs led to major loss of functionality, and even after 7 days post-thawing, functions were not restored. The fact that transcript levels of hepatic genes had not restored indicates that cryopreservation and thawing did not only induce cell death, but also dedifferentiation. Others have described that control over the rate of cooling is of major importance for the successful cryopreservation of alginate-encapsulated cells of the proliferative HepG2 liver cell line [19]. To gain precise control over the rate of cooling in the entire bioreactor is challenging, due to the dimensions and relative solidity of the device. Conceivably, a higher level of control over the rate of cooling can be achieved by perfusion of the aeration-capillaries with a cooling-agent. This would require the development of new technologies and still, the cells furthest away from the capillaries may be affected by freezing or thawing injury. We believe it is uncertain that this issue can be overcome in the foreseeable future. An alternative strategy would be to focus on pre-differentiation of HepaRG cells before cryopreservation. This would eliminate differentiation time, so the BAL could be loaded with cells whenever required, just as the classical primary hepatocyte based systems.

Finally, the safety of BAL application should be extensively scrutinized prior to clinical evaluation. Despite the cancer-related origin of HepaRG cells [9], we found no signs of in vivo tumorigenicity of HepaRG cells, which is in accordance with previous findings [20]. We chose for a 140 days follow-up to balance between the increased sensitivity that might be obtained using a longer test, against the likelihood of false-positive results due to spontaneous tumor formation, in accordance with the appropriate FDA guidance [21]. When HepaRG cells are applied in a BAL in combination with a double filter setup of 0.45 uM and 0.2 uM pore
size the risk of primary tumor initiation is negligible. Furthermore, materials and production procedures for the 540mL-BALs comply with GMP standards, a requirement for clinical application. HepaRG culture medium contains the xenogeneic compound FBS, however, this does not prohibit clinical application when it is certified free of prion diseases. Although not required by law before clinical application, we believe it is preferable to gain experience with the device at its final scale in a large-animal model and to generate additional safety data to maximize safety and prospects of clinical testing.

In summary, we have successfully overcome three hurdles towards clinical application of the new generation HepaRG-AMC-BAL: the preservation of HepaRG cells, upscaling of BAL cultures, and transport of BALs to the bedside. The optimization of bioreactor culture still calls for further research, particularly focusing on expansion and preservation of the biomass inside the bioreactor.
REFERENCES

Chapter 7

SUPPLEMENTAL INFORMATION

Figure S1. Reduction of FBS in culture medium of 9mL-BALs to 5%. Hepatic functions (A-D) and enzyme leakage (E) of 9mL-BALs loaded with cryopreserved HepaRG cells, cultured in medium with 5% or 10% FBS. N.m. not measured.