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# Liposome-supported peritoneal dialysis in the treatment of severe hyperammonemia: An investigation on potential interactions

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ABSTRACT

Peritoneal dialysis (PD) performed with transmembrane pH-gradient liposomes was reported to efficiently remove ammonia from the body, representing a promising alternative to current standard-of-care for patients with severe hepatic encephalopathy. In this study, we further characterized the properties of liposome-supported peritoneal dialysis (LSPD) by 1) assessing its in-use stability in the presence of ascitic fluids from liver-disease patients; 2) investigating its interactions with drugs that are commonly administered to acute-on-chronic liver failure patients; and 3) analyzing the *in vivo* extraction profile of LSPD. We found that LSPD fluid maintained its *in vitro* ammonia uptake capability when combined with ascitic fluids. The co-incubation of selected drugs (*e.g.*, beta-blockers, antibiotics, diuretics) with LSPD fluids and ammonia resulted in limited interaction effects for most compounds except for two fluoroquinolones and propranolol. However, considering the experimental set-up, these results should be interpreted with caution and confirmatory drug-drug interaction studies in a clinical setting will be required. Finally, metabolite-mapping analysis on dialysates of LSPD-treated rats revealed that the liposomes did not remove important metabolites more than a conventional PD fluid. Overall, these findings confirm that LSPD is a potentially safe and effective approach for treating hyperammonemic crises in the context of acute-on-chronic liver failure.

## 1. Introduction

Liposome-supported peritoneal dialysis (LSPD) has recently arisen as a promising strategy for emergency treatment of severe hyperammonemic crises and associated hepatic encephalopathy, one of the main complications of advanced chronic liver disease (ACLD) [1]. Taking advantage of a transmembrane pH-gradient, these scavenging vesicles efficiently removed ammonia in a rat model of secondary biliary cirrhosis, outperforming conventional peritoneal dialysis (PD) in lowering plasmatic ammonia levels and attenuating brain edema [2]. Additionally, LSPD did not trigger any complement activation-related pseudoallergy in pigs, a gold standard model for hypersensitivity reactions induced upon parenteral injection of colloids [2]. Furthermore, peritoneally administered liposomes with a size >500 nm showed low systemic availability in previous studies [1,3–5]. In light of these promising results, the present study was aimed at further characterizing the efficacy and safety pharmacology profile of LSPD, including its ammonia uptake capacity in the presence of ascites which commonly accumulate in cirrhotic patients, any potential drug-liposome interactions, and the overall metabolites extraction profile.

ACLD may decompensate in form of ascites, variceal bleeding, kidney dysfunction or hepatic encephalopathy with or without single or multiple organ failures, and includes the so-called acute-on-chronic liver failure which is associated with high short-term mortality [6]. In particular, the development of hepatic encephalopathy (HE) potentially leading to brain failure is strongly related to an increased risk of death. The clinical management of HE requires the rapid lowering of excessive ammonia levels, which play an important role in the impairment of the brain function [7]. The current standard of care for the prevention of HE relies on generic treatments, such as lactulose (non-absorbable disaccharide) and rifaximin (non-absorbable antibiotic) that reduce the absorption and the production of ammonia in the gut [8]. However,

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their effectiveness for the most severe cases of HE remains limited, and they can only be used as a supportive therapy [8]. Based on preclinical results, LSPD stands out as a promising therapy for acute HE; its mechanism of action following intra-peritoneal administration, relies on passive diffusion of ammonia from the blood into the abdominal cavity and into the liposome' core where it remains trapped. After a *ca.* 3 h dwell time, ammonia-loaded liposomes are removed by suction or gravity. Since ACLD patients will likely be under concomitant medication at the time of treatment, drug-drug interactions data will be critical prior to the first in human clinical trial. For instance, diuretics are commonly administered to prevent the formation of ascites, a frequent complication of cirrhosis and portal hypertension resulting in excessive fluid accumulation in the peritoneal cavity [9]. Typically prescribed diuretics include furosemide, spironolactone, amiloride and bumetanide [10]. Liver disease patients are also prompt to spontaneous bacterial peritonitis and may receive empiric antibiotic therapy (*e.g.*, fluoroquinolones). Additionally, β-blockers such as propranolol, carvedilol or nadolol may be administered for prophylaxis of variceal bleeding. Finally, since liposomes are known to cause anaphylactoid reactions in hypersensitive humans, antihistamines [11], corticosteroids, epinephrine and bronchodilator drugs may have to be given to prevent or manage allergic reactions in patients receiving LSPD [12]. In order to evaluate possible interactions with the aforementioned compounds, the ammonia capture capacity of LSPD was tested *in vitro* in the presence of a representative selection of different drugs (furosemide, norfloxacin, ofloxacin, sulfamethoxazole, propranolol, nadolol, dopamine, epinephrine, salbutamol). Moreover, to complement the safety assessment of LSPD, its metabolomic profile was established in comparison to a conventional PD fluid in healthy rats, where the metabolic wastes recovered in the peritoneal dialysate were identified using an untargeted metabolomic approach. This approach aimed at revealing on the one hand whether any vital endogenous molecules was significantly extracted during the dialysis session (safety endpoint), and on the other hand whether LSPD was able to remove other liver disease-related toxins besides ammonia (efficacy endpoint).

## 2. Materials and methods

### 2.1. Materials

Dipalmitoyl phosphatidylcholine (DPPC) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethyleneglycol)-2000] (DSPE-PEG) were supplied by Lipoid GmbH (Ludwigshafen, Germany). Cholesterol, dichloromethane ($CH_2Cl_2$), methanol (MeOH), acetonitrile (ACN), sodium citrate tribasic dehydrate, calcium citrate tribasic tetrahydrate, hydrochloric acid (puriss., ≥37%), propranolol hydrochloride, furosemide, norfloxacin, ofloxacin, sulfamethoxazole, epinephrine bitartrate, nadolol, dopamine hydrochloride and spironolactone were purchased from Sigma Aldrich (Buchs, Switzerland). Ammonium chloride, sodium chloride and calcium chloride dihydrate were obtained from Merck Millipore (Darmstadt, Germany), and magnesium citrate from Panreac Applichem (Darmstadt, Germany). Citric acid monohydrate and sodium hydroxide were supplied by Fischer Chemicals (Geel, Belgium), while xylitol and salbutamol sulfate were obtained from ABCR (Karlsruhe, Germany). Ammonium sulfate was purchased from Acros organics (Thermo Fisher Scientific, Waltham, MA). Conventional PD solution Physioneal-35 1.36% was provided by Baxter (Deerfield, IL). Ultrapure water was obtained from ultrapure water system “PureLab” (ELGA, Villmergen, Switzerland). Human ascitic fluids from liver disease patients (Supplementary Table S1) were obtained with the permission of the Bern cantonal ethics committee (protocol ID 2017-00970, Bern, Switzerland) from patients who had given informed consent.

**Table 1**
Composition of citric acid solution, xylitol solution and final LSPD fluid.

| | Concentration (mM) |
|---|---|
| Citric acid solution | |
| Citric acid monohydrate | 600 |
| Magnesium chloride | 12 |
| Sodium chloride | 158.5 |
| Sodium hydroxide | 97.5 |
| Xylitol solution | |
| Sodium hydroxide | 65.5 |
| Xylitol | 135.0 |
| Calcium chloride dihydrate | 0.5 |
| Sodium chloride | 93.5 |
| Final composition of LSPD fluid | |
| Citrate | 25.2 |
| Sodium | 149.7 |
| Chlorine | 90.3 |
| Calcium | 0.4 |
| Magnesium | 0.5 |
| Xylitol | 118.0 |
| Lipids (DPPC, cholesterol, DSPE-PEG) | 8.4 |

### 2.2. Methods

#### 2.2.1. LSPD preparation by osmotic shock

LSPD fluid was prepared as described before [2]. Briefly, liposomes were prepared by lipid film hydration, dissolving a lipid mixture of DPPC, cholesterol and DPSE-PEG at 85.5:14:0.5 mol% in $CH_2Cl_2$/MeOH (95:5, v/v). After evaporation of the solvents with a rotary evaporator (KNF Neuberger, Freiburg, Germany), the formed lipid film was placed under vacuum overnight. The film was then rehydrated with ultrapure water for 1–2 h at 56 °C, and the liposomal suspension (100 mM lipids) was degassed under vacuum before being sterilized by autoclaving (20 min, 120 °C) with a steam sterilizer (Varioklav, H + P Labortechnik AG, Oberschleissheim, Germany). For the uptake experiments in ascites, a liposomal suspension in ultrapure water (130 mM lipids) of the same composition as above was obtained from Polymun Scientific (Klosterneuburg, Austria).

To create the osmotic shock, two parts by mass of liposomes were mixed with one part of a hyperosmolar citric acid solution (pH 2, 1050 mOsm/kg; detailed composition is listed in Table 1). After 40 min under orbital shaking at room temperature, the liposomes suspension was diluted by the addition of an alkaline xylitol-based solution (Table 1) at a mass ratio 1:6.94, in order to obtain 8.4 mM LSPD fluids. Both the citric acid and alkaline xylitol-based solutions were filtered (Vacuum filtration 500 “rapid”-Filtermax, PES membrane 0.22 µm pore size) and steam-sterilized prior to use. The pH and the osmolarity of the LSPD fluids 8.4 mM were of 6.00 ± 0.04 (C860 pH-meter, Consort, Turnhout, Belgium) and 358 ± 2 mOsm/kg (Osmomat 3000 basic freezing point osmometer, Gonotec GmbH, Berlin, Germany), respectively.

The hydrodynamic diameter of the liposomes was measured using the Mastersizer2000 laser diffraction particle size analyzer (Malvern Instruments GmbH, Herrenberg, Germany). The measurements are presented as volume distribution. Samples were analyzed in triplicate (Supplementary Fig. S1).

#### 2.2.2. In vitro uptake kinetics

*In vitro* drug and ammonia uptake kinetics were performed at 37 °C in side-by-side diffusion cells (PermeGear Inc., Hellertown, PA, Fig. 1A). In this experimental setup, the liposomes (2.1 mM) were physically isolated in one side of the dual-chamber system by a 0.1 µm pore size polycarbonate membrane (Sterlitech Corporation, WA). Both chambers contained 0.75 mM of ammonia and 1 µM – 1 mM of selected drug previously dissolved in HBS (15 mM, pH 7.4, 350 mOsm/kg).

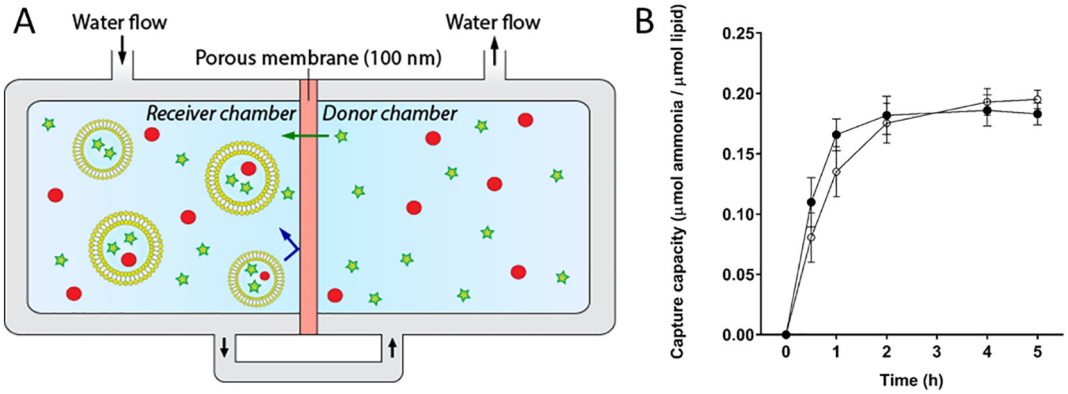


**Fig. 1.** (A) Evaluation of ammonia and drug uptake by transmembrane pH-gradient liposomes. The liposome-containing compartment (left) is separated from the liposome-free compartment (right) by a semipermeable membrane with 0.1 µm pores. The concentration of ammonia (0.75 mM, green stars) and drugs (1 mM, red dots) is equal at either side of the membrane at time 0. Scheme modified from [1]. (B) *In vitro* ammonia uptake by LSPD fluid (2.1 mM) in control buffer (open circles) and ascitic fluid (closed circles) during 5 h. Means ± SD ($n$ = 4–5). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

These drug levels, which are greater than those found in patients, were selected because the uptake assay was performed in a small closed compartment. With this experimental set-up, the use of very low drug concentrations such as those typically measured in plasma would result in such low amounts of captured drug that interferences with ammonia uptake would most probably not be detected. In patients undergoing an LSPD session, the transfer of the drug from the central compartment to the liposome core would lead to a displacement of the equilibrium, and attract drug from the peripheral tissues until exhaustion of the uptake mechanism. Therefore, the possibility of entrapping high amounts of drug *in vivo* due to the capture of the distributed drug was mimicked *in vitro* by using high concentrations. For the uptake experiment in the presence of ascites, ascitic fluids were added at a concentration of 37% v/v to both sides of the system and spiked with ammonia to reach a concentration of 0.75 mM. This concentration was chosen to assess the robustness of the formulation under extreme hyperammonemic conditions. Ascitic fluids were collected from four patients suffering from liver disease (Supplementary Table S1). Incubation under magnetic stirring was performed up to 5 h. At scheduled time points (30 min, 1, 2, 4 and 5 h), aliquots were withdrawn from the liposome-free compartment for ammonia and drug determination.

To study the effect of the addition of propranolol to ammonia-loaded liposomes, 0.23 mL were withdrawn from the cells after 2 h, and were replaced by 0.23 mL of propranolol solutions at different concentrations so as to obtain 1, 10 µM, 0.3 or 1 mM propranolol in the cells. Subsequently, the kinetics experiment was performed as described above.

### 2.2.3. Drug quantification

Drug concentrations in the aliquots withdrawn from the cells were determined by high-performance liquid chromatography (HPLC) equipped with a UV-detector (HPLC-UV, Dionex UltiMate 3000, Thermo Scientific, Sunnyvale, CA). Samples were spiked with spironolactone as internal standard solution for the measurements of all the drugs. Linearity was verified for concentrations between 7.8 µg/mL and 0.5 mg/mL. The limits of detection and quantification (LOD and LOQ, respectively) were set at signal-to-noise of 3:1 and 10:1, respectively. Quality controls (QC) samples were run during each drug quantification by using a drug solution at a known concentration within the calibration curve range. Analytical data are reported in Supplementary Table S2, and representative chromatograms are provided in Supplementary Fig. S2. HPLC was equipped with a Waters Xbridge C18 5 µm column. Mobile phases A (phosphate buffer 20 mM, pH = 3) and B (ACN) were injected at a flow rate of 1 mL/min with a constant ratio of 50:50 (v/v). Each run took no more than 10 min. Data were analyzed by Chromeleon® Chromatography Data System (Dionex, Thermo Scientific). For each drug, concentrations were determined according to the corresponding calibration curve and using the area under the curve (AUC) ratio between the studied molecule and the internal standard.

### 2.2.4. Ammonia quantification

Ammonia concentration in the samples from the diffusion cells was quantified by an enzymatic assay (AM1015 from Randox Laboratories, Crumlin, UK). Two hundred microliters of reagent solution were mixed with 20 µL of sample in a 96-well, flat bottomed, polystyrene plate. After 5 min of incubation, the samples absorbance was measured at 340 nm with a plate reader (InfinityM200Pro, Tecan, Männedorf, Switzerland). Two microliters of enzyme (glutamate dehydrogenase) were added to each well and after 5 min of incubation the absorbance decrease was assessed. Ammonia concentration was calculated according to the manufacturer's instructions. Each sample was measured in triplicate. To measure the ammonia levels in the ascites fluids from patients and in the ammonia uptake experiment in ascites fluids, the three hundred microliters of reagent solution, thirty microliters of sample, and three microliters of enzyme were used.

### 2.2.5. Statistical analysis of uptake data

Data from ammonia uptake experiments were analyzed by non-parametric statistical methods. Statistical two-group comparisons were assessed using a two-sided Mann-Whitney test (Prism 7.02; GraphPad Software). $p \leq 0.05$ was considered to be statistically significant.

### 2.2.6. Differential scanning calorimetry (DSC)

DSC measurements were performed with a Q200 calorimeter (TA Instruments, New Castle, DE) and the data were analyzed with Q Series software. Temperature and enthalpy calibration were performed using indium as reference. DPPC liposomes were prepared by adjusting the amounts of the usual protocol [2] in order to obtain pure DPPC liposomes. After 2 h incubation at 37 °C with 0, 10, 20 or 30 mM propranolol, 20 mM liposomes aliquots (with or without propranolol) of about 10 µL were weighed and hermetically sealed in TZero™ aluminum pans. The weight of each DSC pan was verified before and after the temperature scan to check for possible material leakage. After an initial isothermal period of 5 min, the samples were scanned at 5 °C/min (20 − 70 °C) and then cooled down.

### 2.2.7. In vivo metabolites uptake in healthy rats

*2.2.7.1. In vivo LSPD in healthy rats.* All animal experiments were performed in accordance with procedures and protocols approved by the cantonal veterinary authorities (Kantonales Veterinäramt Zürich,

license 2,012,189). Before the first experimentation, adult Sprague-Dawley rats (250–350 g, male; Charles River Laboratories) were allowed an acclimatization period of 1 week during which they had access to food and water *ad libitum* and followed a 12-h light/dark cycle. On the day of the experiment, the animals were anesthetized (isoflurane 2–2.5% in 0.8 mL/min oxygen flow) and slowly infused with an intraperitoneal injection at 60 mL/kg of LSPD fluid or control solution (Physioneal-35 1.36%, Baxter). The instillation was performed with a 20-gauge hypodermic needle. After 6 h of dialysis, the dialysate was withdrawn from the peritoneal cavity with a 22-gauge perforated silicone catheter (Venflon; Becton Dickinson, Fanklin Lakes, NJ) and an aliquot was immediately frozen in liquid nitrogen and kept at −80 °C until mass spectrometry analysis. A recovery period of 7 days was allowed, after which the experiment was repeated in a cross-over fashion. At the end of the second dialysis session, animals were euthanized by carbon dioxide asphyxia followed by a thoracotomy.

*2.2.8. Non-targeted metabolomic analysis*

*2.2.8.1. Sample preparation.* Dialysate samples collected upon *in vivo* LSPD sessions in healthy rats were thawed at 4 °C. For protein precipitation, a sample aliquot of 100 μL was mixed with 200 μL of LC-MS grade MeOH, vortexed for 15 s and centrifuged at 14,000 × *g* for 15 min to pellet the precipitate. The supernatant (50 μL) was transferred to a fresh tube, diluted with 950 μL MeOH and stored at −20 °C prior injection to the mass spectrometer.

*2.2.8.2. Mass spectrometry settings*. The platform consisted of an Agilent Series 1200 LC pump coupled to a Gerstel MPS2 autosampler and an Agilent 6550 Series iFunnel Quadrupole Time-of-Flight mass spectrometer (Agilent, Santa Clara, CA) equipped with an electrospray source operated in negative or positive mode. Each sample was injected into a continuous stream of organic phase flowing to the electrospray interface without prior chromatographic separation [13]. The flow rate was 150 μL/min of mobile phase consisting of isopropanol/water (60:40, v/v) buffered with 5 mM ammonium fluoride for negative mode and MeOH/water (60:40, v/v) with 0.1% formic acid at pH 3 for positive mode. Mass spectra were recorded in profile mode from *m/z* (mass-to-charge ratio) 50 to 1000 with a frequency of 1.4 spectra/s for 0.48 min using the highest resolving power (4 GHz HiRes). Source temperature was set to 325 °C, with 5 L/min drying gas and a nebulizer pressure of 30 psig. Fragmentor, skimmer, and octopole voltages were set to 175, 65, and 750 V, respectively. The mass spectrometer was run in both negative and positive modes to ensure the detection of structurally different metabolites. QC samples were used for quality assurance purposes and raw data were processed and exported for further analysis.

*2.2.8.3. Data processing and statistical analysis*. Initial data processing and analysis was performed with Matlab R2010b (The MathWorks, Natick, MA), using functions embedded in the Bioinformatics, Statistics, Database, and Parallel Computing toolboxes. The mass spectrum was determined plotting the relative abundance as a function of the *m/z*. A cut-off to filter peaks of < 500 ion counts was applied, in order to avoid the detection of features that are too low to deliver statistically meaningful insights.

After each measurement, a list of ions with > 500 ion counts that match metabolites in the human metabolome database v3.6 [14] was obtained. Ions were putatively annotated based on accurate mass using a tolerance of 0.001 Da. Since this procedure does not allow to resolve metabolites with identical *m/z*, each ion can match one or multiple metabolites. The m/z ratio and the ion intensity value (indicative of relative abundance) were also included at every time point. Data quality was assessed by hierarchical clustering and visualized by principal component analysis (PCA).

Subsequently, treatment groups containing high-quality samples were compared after transformation of the values into their logarithm for clarity reasons (differential metabolites analysis). The following comparisons were tested based on the ratio (fold change, FC) of the peak intensity of each identified compound:

i). before and after each treatment: PD-6 h *vs.* PDi, LSPD 6 h *vs.* LSPDi
ii). between treatments: PD-6 h *vs.* LSPD-6 h

Differential metabolite abundance was modeled using mixed-effect linear regression with empirical Bayes variance estimation (moderated *t*-test), as implemented in the limma package [15] of the R environment for statistical computing [16]. In addition, the correlation structure of the data given by the 4 technical replicates per animal per treatment was taken into account using the "duplicateCorrelation" function. For each of the comparisons, a list of differentially abundant metabolites was generated after multiple testing correction of the *p*-values. Finally, multiple-comparison correction according to Benjamini-Hochberg was applied to adjust the p-values.

## 3. Results and discussion

### 3.1. In vitro uptake and interaction study

ACLD patients may develop ascites which is the accumulation of fluid in the peritoneal cavity, causing abdominal swelling. Therefore, the ammonia extraction efficacy of LSPD in ascitic fluids was studied *in vitro* to assess its in-use stability. The average ammonia concentration in the ascitic fluids of four patients suffering from liver cirrhosis (Supplementary Table S1) was 61 ± 18 μM. Exogenous ammonia was added such that the same concentration (0.75 mM) as in the ammonia-drug interaction experiments (see below) was reached. As illustrated in Fig. 1B, the liposomes remained stable upon exposure to the ascites and extracted ammonia to a level comparable to the control buffer. The ammonia capture capacity was consistent with previously investigated LSPD fluids [2]. In a hyperammonemic rat model of liver cirrhosis, we showed that on average 47 μmol of ammonia per kg bodyweight were removed from the body per dialysis session [2]. Taking into consideration the volume of distribution of ammonia, an LSPD session removed approx. 12%–35% of the total systemic ammonia in hyperammonemic rats [2].

Potential interactions between LSPD and selected drugs commonly prescribed to ACLD patients (Supplementary Table S3) were then investigated in the presence of ammonia. The goal was to determine whether such interactions could impact on the treatment efficacy (of both drug and/or LSPD), which would eventually translate to appropriate dosage adjustments. The selected drugs had a log P between −1.4 and 3.5 and importantly, a protonable nitrogen moiety that could theoretically favor a stable sequestration within the acidic core of the liposomes, and compete with LSPD's ammonia uptake [17–19]. The drug concentrations used in the *in vitro* set-up were substantially higher ($10^3$–$10^4$ fold) than those found in patients' plasma because, as discussed in the materials and methods section, the uptake assay was performed in a small closed compartment. Moreover, drug concentrations were slightly greater than that of ammonia to disfavor the uptake of the latter. Typically, for 2.1 mM lipids in the LSPD fluid, 1 mM drug and/or 0.75 mM ammonia were added to the system.

Fig. 2 shows drugs and ammonia uptake into the liposomes during 5 h of co-incubation. In the absence of drugs, around 70% of the initially added ammonia was scavenged by the liposomes by the end of the monitored period (red circle). Most ammonia was encapsulated within the first two hours, and then the uptake leveled off until the end of the observation period, which is consistent with the *in vitro* performance of LSPD fluids reported previously [2]. In general, the *in vitro* uptake of ammonia by LSPD was inversely related to the drug encapsulation level, likely due to a competition phenomenon between both molecules (*i.e.*, ammonia and drug). The tested compounds could be classified into two groups: those that slightly affected the ammonia

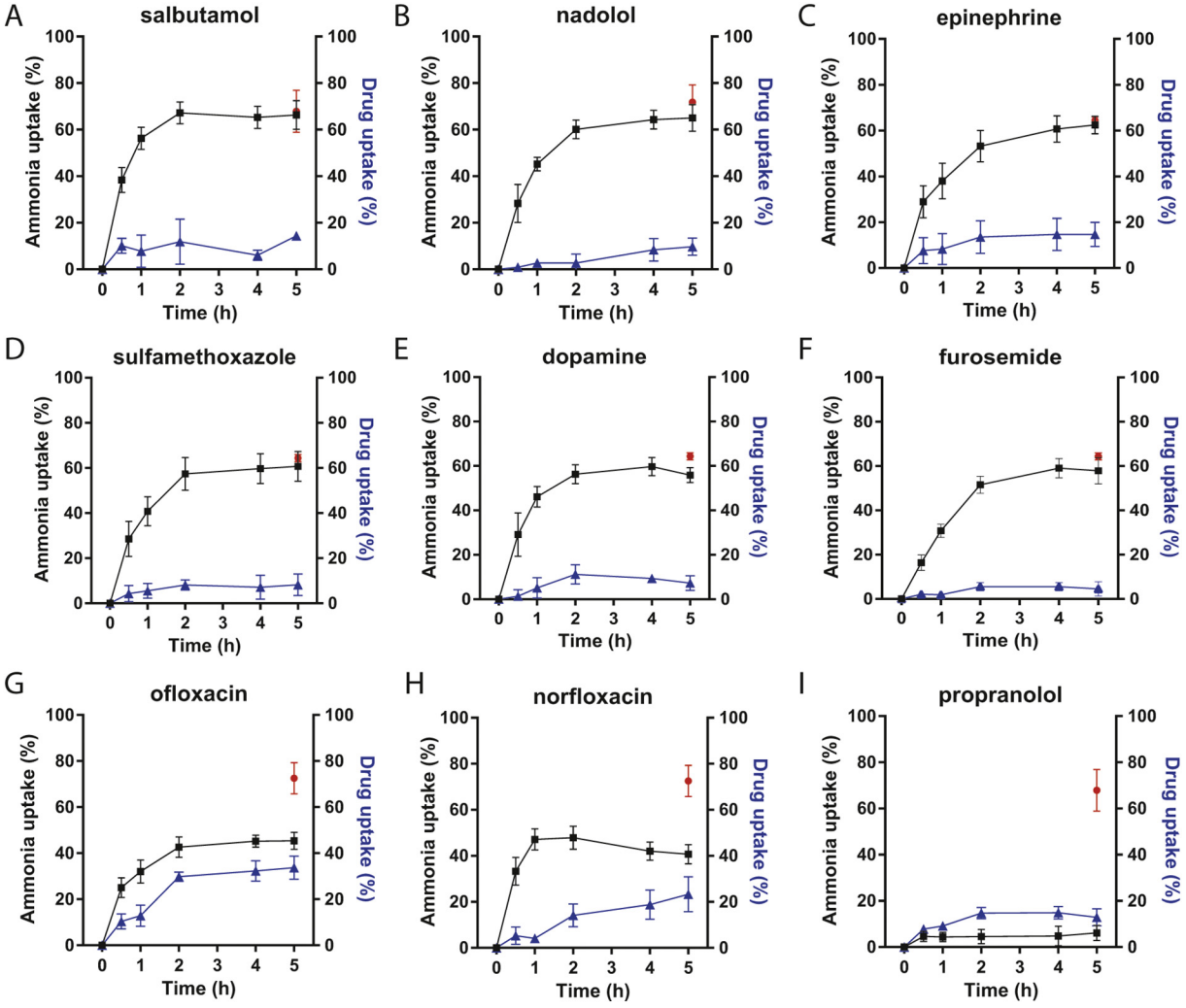


**Fig. 2.** *In vitro* ammonia and drug uptake by LSPD fluid (2.1 mM) during 5 h co-incubation. Red circle: ammonia uptake in the absence of drug after 5 h incubation; squares (left axis): ammonia uptake in the presence of the drug (1 mM), triangles (right axis): drug uptake in the presence of ammonia (0.75 mM). Uptake is expressed as percentage of theoretical maximum at 5 h (red circle). Means ± SD ($n$ = 6). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

uptake (weak competitors), and those that substantially reduced it (strong competitors). Propranolol was an exception as it was not taken up by the liposomes and yet it substantially affected the sequestration of ammonia.

The weak competitors were salbutamol, nadolol, epinephrine, dopamine, furosemide and sulfamethoxazole, which had a mild impact on ammonia uptake, slightly attenuating it by <10% after 5 h (Fig. 2A to F). These drugs were captured to a small extent by the liposomes (between 4 and 15% of initial drug amount), exerting only a minimal effect on ammonia extraction. Despite their high and comparable $pK_a$ values (between 8.9 and 9.8, Supplementary Table S3) which could have resulted in a stable entrapment within the liposome's core, salbutamol, nadolol, epinephrine and dopamine did not diffuse well across the lipid bilayer. Salbutamol was already shown [1] not to be preferentially taken up by liposomes despite its favorable logP and low molecular weight. Nadolol uptake (Fig. 2B) was the lowest among the weak competitors, which could be attributed to its classification as a water soluble yet poorly permeable BCS class III drug and the fact that its membrane passage primarily occurs through cation transporters [20,21]. Epinephrine and dopamine were weakly taken up by the liposomes as reported in the literature [22,23], which might be ascribed to their negative logP value. Their almost overlapping curves for both drug and ammonia uptake (Fig. 2C and E) are consistent with the structural similarity of these two drugs since they are both catecholamines. In the case of furosemide, the limited uptake (Fig. 2F) could be ascribed to the presence of a relatively high number of H-bond donor groups (4) in the molecule and to the fact that it has the highest polar surface area among the studied drugs [14], which hinder its permeation through the membrane.

Sulfamethoxazole is the only weak acid included in the study. At neutral pH, most molecules carry a negative charge on the sulfonamide group, which has a $pK_a$ of 5.6 (Supplementary Table S3). The remaining molecules with uncharged sulfonamide groups may cross the liposomal membrane but cannot remain trapped since both aniline and sulfonamide groups are mostly uncharged at pH 2. However, the negative charge on the sulfonamide group might interact with the polar heads of the phospholipid membrane. Hence, the small measured uptake (Fig. 2D) could in fact simply be an association with the membrane and not an entrapment within the acidic core of the liposomes.

The two quinolones ofloxacin and norfloxacin (Fig. 2G and H) were the most captured molecules among those tested (23–34%) and also reduced the ammonia uptake to a greater extent than the majority of the other drugs (ammonia uptake decreased by 27–32%, Fig. 3). The similarity of the effects observed with these two compounds is in agreement with their comparable structures, and their relatively high drug uptake could be explained based on their physico-chemical

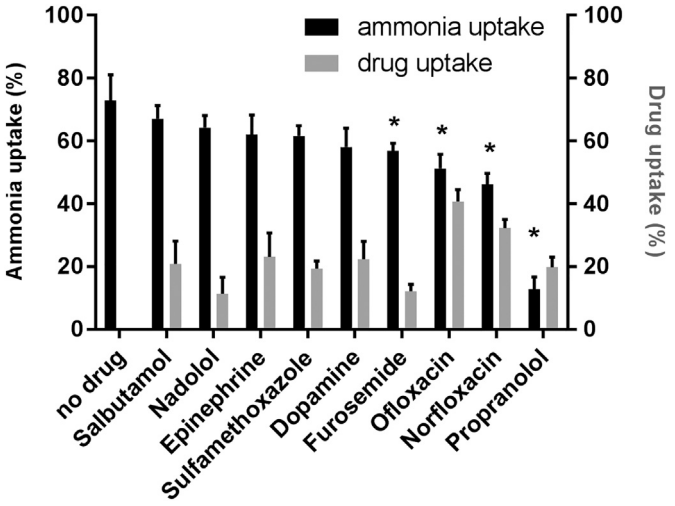


**Fig. 3.** *In vitro* ammonia and drug uptake by LSPD after 5 h co-incubation of ammonia and a single drug. The concentrations used were 0.75 mM (ammonia), 1 mM (drug) and 2.1 mM (lipids). The uptake is expressed as the percentage ratio between captured ammonia or drug and the theoretical total amount, and is presented as mean ± SD ($n = 6$). *p*-values were determined by two-sided Mann-Whitney test (*$p \leq 0.05$) between the uptake of ammonia in the presence and absence of drug. Data are extracted from Fig. 2.

properties. Indeed, a significant portion of these molecules is in a neutral or zwitterionic form at physiological pH [24,25], which are both more lipophilic than the charged species, therefore favoring the compound's passage through the membranes. Furthermore, they are the sole drugs (along with furosemide, as mentioned above) able to form an intra-molecular H-bond [26,27], which would enhance their permeability by "hiding" the hydrophilic regions of the molecules.

A very different behavior was observed with propranolol, which lowered the ammonia uptake more markedly than the other molecules (6% of ammonia uptake after 5 h incubation; Fig. 2I) even though it was not extensively captured by the liposomes (13% of the initial drug amount). In this case, a destabilizing effect on the liposomes could explain the low ammonia uptake rather than a competitive diffusion across the phospholipid membrane. Indeed, propranolol was previously reported to destabilize cellular and artificial membranes [28]. Propranolol (at a molar drug–to-lipid ratio of 1.5) disrupted the outer phospholipid monolayer and led to the formation of "worm/thread-like micelles" and eventually spherical micelles [29]. Entrapped ammonia in transmembrane pH-gradient liposomes could therefore have been released from the liposomes during this process, explaining the limited ammonia uptake in presence of propranolol. In order to verify this hypothesis, liposomes were loaded with ammonia during 2 h incubation, at the end of which propranolol or salbutamol were added to the system. Salbutamol was chosen as a negative control since it was the compound that least affected ammonia uptake (Fig. 3). Four different concentrations of propranolol were tested (1 and 10 μM, 0.3 and 1 mM) and compared to the controls (no drug or 1 mM salbutamol). Fig. 4A shows that unlike the control experiments, the addition of propranolol at high concentrations triggered ammonia release from the liposomes. However, this was not observed at lower propranolol concentrations, indicating that the destabilizing effect of propranolol was concentration-dependent.

The hypothesis was confirmed by DSC analysis. For this purpose, DPPC liposomes were incubated for 2 h at 37 °C with different concentrations of propranolol and their thermogram was recorded (Fig. 4B). Pure DPPC liposomes were used instead of LSPD liposomes because the presence of cholesterol abolishes the pre-transition peak [30], and could mask a possible effect of propranolol. It should be noted that DPPC is anyhow the main component of the liposomes in LSPD solution (85 mol%). In these experiments, the propranolol-to-lipid molar ratio was in a close range (0.5–1.5) to that used in the uptake experiment (0.15–0.5, Fig. 2I), but the drug and lipid concentrations used were higher to increase the magnitude of the endothermic peaks. DPPC exhibited pre-transition and transition peaks at around 38 and 41 °C, respectively, in accordance with literature [31]. The addition of propranolol resulted in lowering of the pre-transition peak temperature by 5 °C at the highest tested drug concentration. A pre-transition peak is usually associated with the formation of ripples in the membrane upon an increase in temperature [32]. Specifically for DPPC, this has been explained in terms of structural changes in the lamellar lattice [33]. It was demonstrated that the presence of compounds in the membrane typically shifts the pre-transition peak to lower temperatures [34]. This phenomenon was observed at high propranolol concentrations (> 20 mM), which showed a clear destabilizing effect on the membrane, and produced a shift and shape change of the pre-transition peak.

In light of these interactions studies, it appears that most of the tested drugs (namely salbutamol, nadolol, epinephrine, dopamine, furosemide and sulfamethoxazole) would not significantly affect the performance of LSPD nor the bioavailability of the drugs themselves when administered concomitantly. These *in vitro* experiments revealed a potential interaction between LSPD and norfloxacin, ofloxacin and propranolol. However, it should be noted that the concentrations used were many folds higher than those expected in humans. As shown with propranolol the competitive effect for LSPD's ammonia uptake was dose-dependent and therefore an *in vitro* drug-drug interaction will not necessarily be clinically relevant. Further investigations should be conducted to assess the significance of the observed effects under physiological conditions. In all cases, propranolol could be replaced by nadolol for the prevention of variceal hemorrhage [35], given its extremely limited interactions with LSPD. Regarding the antibiotics that are administered to patients presenting ascites to prevent and treat spontaneous bacterial peritonitis, norfloxacin is only recommended for the specific case where ascitic fluid protein is below 1.5 g/dL [36]. Moreover, the use of quinolones is nowadays inadvisable due to the emergence of resistance [37]. They can be replaced by equivalent trimethoprim-sulfamethoxazole (low uptake, Fig. 2D) [38] or the outperforming third generation cephalosporin ceftriaxone, which is a large (555 g/mol) and polar molecule that should not be taken up by LSPD [39]. In all cases, the latter is currently considered the agent of choice in the treatment of spontaneous bacterial peritonitis [40]. Finally, higher LSPD doses or repeated PD sessions could be investigated in

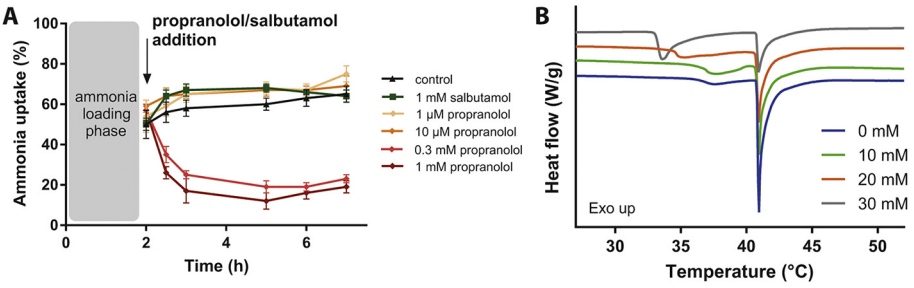


**Fig. 4.** (A) *In vitro* ammonia uptake/release kinetics of ammonia-loaded liposomes after addition of four different propranolol concentrations. Salbutamol (1 mM) and fresh buffer were used as negative controls. Means ± SD ($n = 6$). (B) Thermograms of 20 mM DPPC liposomes incubated for 2 h at 37 °C with increasing concentrations of propranolol (0–30 mM). Thermograms have been offset on the y-axis for clarity.

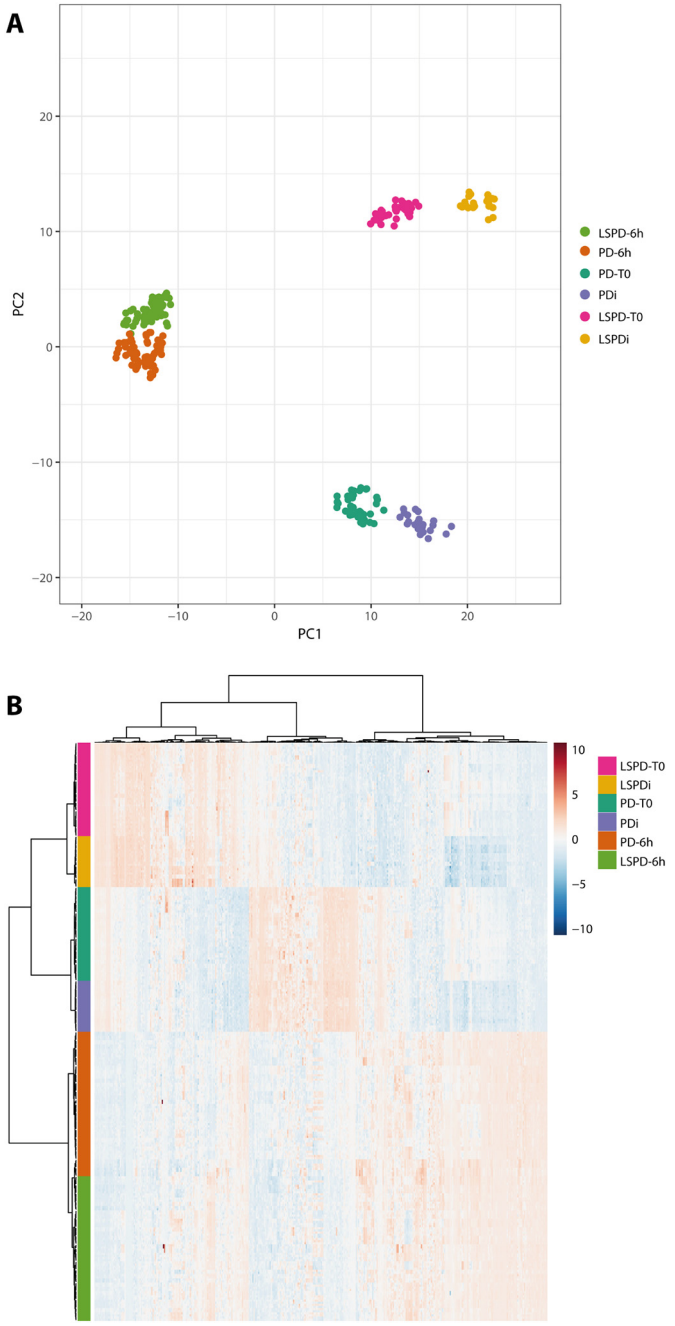


**Fig. 5.** (A) Sample similarity analysis. Distance multi-dimensional scaling plot based on quantile normalized data. PC = principal component. PC1 is associated with the time after administration, PC2 with the treatment effect. No outliers could be seen, samples perfectly clustered according to their treatment and time, meaning that the difference in metabolites level correlated with their group. (B) Heatmap of sample-metabolite matrix. Displayed are all 390 metabolites (columns) of all measurements (rows). The intensity of each metabolite is represented by the colour scale (top right of the graph). The dendrograms on the axes give information about the similarity between metabolites (x-axis) or measurements (y-axis). The height of the branch points is proportional to the difference between the two groups.

order to compensate for the lower ammonia uptake in quinolone-treated patients.

### 3.2. Metabolites' extraction profile in healthy rats

Confirming that LSPD does not remove important endogenous compounds from the patient's body is a crucial aspect to ensure the safety of the liposomal formulation. To this end, the extraction profile of LSPD fluids was assessed in comparison to a commercially available PD solution. The whole metabolites footprint of 8.4 mM LSPD fluid or a conventional PD solution (Physioneal 35 Glucose 1.36%) was analyzed by mass spectrometry (MS) immediately after and at 6 h post-intraperitoneal instillation.

Data were initially normalized (quantile normalization) to remove systematic errors or technical variations between the samples (Supplementary Fig. S3). Subsequently, their quality was assessed by hierarchical clustering and visualized by principal component analysis (PCA) to detect potential outliers, label-swaps and other irregularities. PCA allows for a convenient visualization of the sample-sample distances by projecting the high-dimensional coordinates into the two-dimensional plane of maximal separation. In the PCA plot (Fig. 5A) no outliers could be identified and samples perfectly clustered in the 6 groups they belonged to: freshly prepared LSPD or PD fluids (LSPDi and PDi group), dialysate samples recovered immediately after peritoneal instillation (LSPD-T0 or PD-T0) or at the end of the dialysis session (LSPD-6 h or PD-6 h). Principal component 1 (PC1, x-axis) is associated with the time after administration and PC2 (y-axis) with the treatment effect. The initial distinction between LSPD and control treatment at time 0 vanished after 6 h of dialysis, suggesting a similar dialysate composition as time progressed, and therefore a comparable extraction profile of the two peritoneal fluids.

Differentially abundant metabolites (DAM), *i.e.* compounds that are significantly enriched or depleted in one group *versus* another, were identified by statistical analysis and FC evaluation. The arbitrary limit used as discriminating threshold was fixed at a false discovery rate (FDR) of 0.01 and a FC of 1.2, which would correspond to a value of 0.83 for a strongly depleted compound. The relative abundance of aconitic acid, a dehydrated form of citric acid, significantly decreased after 6 h LSPD, likely due to its diffusion out of the peritoneal space during the dwell time. For the same reason, glucose and lactate levels were reduced in PD-treated rats, since these two compounds are among the main components of the control PD fluid.

Overall, in the comparison LSPD-6 h *vs.* PD-6 h, both LSPD and PD treatments were found to perform similarly and significantly extracted around 185 metabolites in the peritoneal space, including several amino acids, a few bile acids and lipid derivatives (Supplementary Tables S4 and S5). The analysis of the DAMs showed that 28 metabolites were preferentially extracted by LSPD (Table 2), and that they were mainly lipophilic ones. For purposes of comparison, it is important to mention that the average volume recovery after PD was comparable for LSPD and control-treated groups. Moreover, only compounds that exhibited a strong increase in concentration when comparing LSPD-6 h *vs.* LSPDi were selected, in order to remove from the analysis the substances that were initially present in the LSPD fluid. Among the most extracted components, lysophospholipids could be identified, which are structurally related to the liposomes' phospholipids and would therefore readily associate with the lipid bilayer. Interestingly, cholanoic acid and its derivatives are relevant compounds to be removed since elevated levels have been measured in hepatic failure patients [41].

The collected data were also visualized as a heatmap, displaying the abundance of the 390 metabolites detected by MS in each sample across all treatment comparisons and allowing visual comparison among the six studied groups (Fig. 5B). On the y-axis the measurements were sorted out by treatments. The upper half of the graph shows that both LSPD-T0 and LSPDi exhibited a comparable profile, analogously to PD-T0 *vs.* PDi. This similarity was expected since after injection and immediate withdrawal, most of the peritoneal content corresponded to the freshly injected fluid. In contrast, LSPDi and LSPD-T0 showed very different profiles compared to their counterpart PDi and PD-T0. Metabolites that appeared relatively abundant for one fluid were present in a small extent in the other, which is consistent with the difference in compositions between the two fluids. On the contrary, no clear

**Table 2**
Differentially abundant metabolites, with corresponding FC and FDR, in the comparison LSPD-6 h *vs.* PD-6 h. Each *m/z* ratio might correspond to more than a metabolite and give therefore rise to uncertainty about compound identification.

| Metabolites (putative annotation) | FC | FDR |
|---|---|---|
| LysoPE(0:0/20:0) | 2.26 | 3E-30 |
| LysoPE(0:0/16:0) | 2.19 | 2E-34 |
| Italidipyrone | 1.69 | 2E-37 |
| LysoPE(0:0/20:2(11Z,14Z)) | 1.64 | 9E-11 |
| Deferoxamine | 1.54 | 8E-16 |
| LysoPE(0:0/22:4(7Z,10Z,13Z,16Z)) | 1.50 | 2E-09 |
| LysoPE(0:0/18:2(9Z,12Z)) | 1.47 | 9E-11 |
| LysoPE(0:0/20:1(11Z)) | 1.46 | 2E-10 |
| Vomifoliol 9-[glucosyl-(1- > 4)-xylosyl-(1- > 6)-glucoside] | 1.45 | 4E-37 |
| Alpha-Linolenic acid | 1.43 | 4E-10 |
| Hordatine B | 1.38 | 1E-11 |
| 3a,7a,12b-Trihydroxy-5b-cholanoic acid | 1.36 | 5E-03 |
| Linoleic acid | 1.35 | 7E-06 |
| Docosahexaenoic acid | 1.34 | 8E-08 |
| Docosapentaenoic acid | 1.33 | 2E-07 |
| 3b,12a-Dihydroxy-5a-cholanoic acid | 1.32 | 7E-03 |
| Sulfolithocholylglycine | 1.27 | 1E-04 |
| Eicosadienoic acid | 1.25 | 4E-06 |
| 8,11,14-Eicosatrienoic acid | 1.24 | 3E-05 |
| Hypogeic acid | 1.24 | 1E-05 |
| Indoxyl sulfate | 1.24 | 8E-07 |
| Cortexolone | 1.23 | 3E-05 |
| Deoxycholic acid glycine conjugate | 1.23 | 4E-04 |
| 12-Ketodeoxycholic acid | 1.23 | 8E-04 |
| 3b-Hydroxy-5-cholenoic acid | 1.21 | 9E-07 |
| Aspidospermatine | 1.21 | 8E-04 |
| Taurocholic acid | 1.21 | 2E-03 |
| 7-ethyl-10-[4-(1-piperidino)-1-amino] carbonyloxycamptothecin | 1.20 | 1E-06 |

**Table 3**
Relevant metabolites for ACLD and CKD, with their corresponding FC and FDR, identified in the comparison LSPD-6 h *vs.* PD-6 h.

| | FC | FDR |
|---|---|---|
| ACLD | | |
| Tyrosine | 0.99 | 8E-01 |
| Glutamine | 0.74 | 2E-17 |
| Glutamic acid | 0.96 | 6E-01 |
| Phenylalanine | 0.93 | 1E-02 |
| Creatinine | 0.88 | 1E-05 |
| CKD | | |
| Methylhistidine | 0.91 | 1E-03 |
| Betaine | 0.90 | 6E-05 |
| Indoxyl sulfate | 1.24 | 8E-07 |
| Cresol sulfate | 1.13 | 2E-02 |
| Hippuric acid | 0.83 | 3E-10 |
| Creatinine | 0.88 | 1E-05 |

separation in the metabolite composition was found between LSPD or conventional PD the after a dwell time of 6 h. Although some metabolites varied in intensity, no visible trend was observed, confirming that in general the two peritoneal fluids extracted compounds in a comparable way at 6 h postdose.

Among the DAMs in the comparison LSPD-6 h *vs.* PD-6 h, relevant metabolites in the context of liver-related diseases such as ACLD [42] and chronic kidney disease (CKD) [43] were identified. Table 3 reports some critical disease markers detected in the dialysate samples with their respective level. All values were close to the threshold (FC = 1.2 or 0.83), suggesting that the two fluids acted comparably in scavenging these metabolites. LSPD was able to extract ten important metabolites in ACLD and CKD in a similar way to the control. Among them, the removal of indoxyl sulfate and cresol sulfate could be beneficial since excessive blood levels of these two metabolites might result in further progression of kidney disease [44]. Furthermore, glutamine extraction could be advantageous since its metabolization in the brain into glutamate and ammonia leads to free radicals production and mitochondrial abnormalities [45]. Although glutamine and creatinine seem to be better extracted by the control, the ammonia uptake by LSPD could have shifted the production equilibrium of these two metabolites and therefore reduced their total levels in the peritoneal space.

## 4. Conclusions

The transmembrane pH-gradient liposomes maintained their ability to entrap ammonia in the presence of human ascitic fluids but also with the concomitantly added drugs salbutamol, nadolol, epinephrine, dopamine, furosemide and sulfamethoxazole. Both tested fluoroquinolones and propranolol seemed to interfere with the LSPD's ammonia uptake capacity. However, considering that the *in vitro* drug concentrations used in this experimental set-up were 3–4 orders of magnitude greater than actual levels found in patients, these results should be interpreted with caution. If the current work was able to highlight potential interactions, confirmatory drug-drug interaction studies in a clinical setting will still be required for a restricted selection of drugs. In all cases, multiple LSPD sessions or the replacement of these medications represent viable alternatives for the treatment of ACLD patients. Additionally, metabolomic analysis of dialysates in rats revealed that, generally, LSPD exhibited an extraction profile similar to that of a commercial PD solution, which represents an additional confirmation of its safety. Overall, these findings suggest that LSPD is a valid strategy for the safe and effective treatment of hyperammonemic crises in the context of ACLD.

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## Competing interests

Vincent Forster, Meriam Kabbaj and Jean-Christophe Leroux are cofounders of Versantis AG, a company based on LSPD technology.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jconrel.2018.03.030.

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