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Effect of albumin infusion on oxidative albumin modification and albumin binding capacity in chronic liver failure

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Abstract

Oxidative albumin modification and impaired albumin binding function have been described both in chronic liver failure and for therapeutic albumin solutions. The aim of the present study was to evaluate the effect of albumin infusion on redox state and binding function of circulating albumin. We studied 20 patients with cirrhosis who routinely received albumin infusions for prevention of post-paracentesis circulatory dysfunction or treatment of hepatorenal syndrome. We measured albumin fractions by redox state of cysteine-34 and albumin binding properties using dansylsarcosine as site II ligand. Therapeutic albumin solutions showed high contents of human nonmercaptalbumin-1 and human nonmercaptalbumin-2, exceeding the respective values in our patients with decompensated cirrhosis. An initial protocol for the first nine patients sampled at baseline, 24 h and 48 h after albumin infusion revealed no significant changes of oxidized albumin species or albumin binding properties. However, a modified protocol for the remaining 11 patients sampled at baseline, <1 h after and 24 h after albumin infusion revealed short-lived changes of oxidized albumin species while no changes in albumin binding properties were observed. In conclusion, therapeutic albumin infusion transiently changed albumin redox state but did not improve binding function of circulating albumin in chronic liver failure.

KEYWORDS
albumin binding capacity, human mercaptalbumin, human nonmercaptalbumin-1, human nonmercaptalbumin-2, neutrophil function

1 | INTRODUCTION

Albumin infusion has been shown to improve outcome in spontaneous bacterial peritonitis, to reverse hepatorenal syndrome combined with vasoconstrictors, and to prevent post-paracentesis circulatory dysfunction. These beneficial effects are associated with haemodynamic improvement reflected by neurohumoral changes such as a decrease in plasma renin activity.[1,2] As albumin is a multifunctional protein, its therapeutic effects in chronic liver failure are not only due to plasma volume expansion but also to improved nononcotic functions such as suppression of systemic inflammation[3] or prevention of complications during long-term administration.[4]

Albumin harbours two specific binding sites described by Sudlow: site I, which binds large heterocyclic compounds and dicarboxylic acids (such as bilirubin), and site II, which binds aromatic carboxylic compounds (such as benzodiazepines) as well as seven binding sites for non-esterified fatty acids.[5] Decreased binding of dansylsarcosine (DS)—a model ligand for the benzodiazepin binding site II—was found in patients with end-stage liver disease.[6] Interestingly, extracorporeal albumin dialysis using the molecular adsorbents recirculating system (MARS) has been found to improve DS binding,[7] while no such data exist for albumin infusion under the above-mentioned conditions. Further examples for impaired albumin function in cirrhosis include alterations in fatty acid binding (as estimated by electron paramagnetic resonance) and impaired metal binding (measured as ischemia-modified albumin).[8] Given the function of albumin to bind bacterial products, ROS and nitric oxide, the changes in its abundance and structure might contribute to neutrophil dysfunction observed in chronic liver failure.[3]

Impaired albumin function may be related to post-translational modifications like oxidative damage, which has been found in several disease conditions including chronic liver failure. Three fractions of albumin can be discerned according to the redox state of cysteine-34 (Cys-34): nonoxidized human mercaptalbumin (HMA) with Cys-34 as free sulfhydryl, reversibly oxidized human nonmercaptalbumin-1 (HNA1) with Cys-34 as mixed disulfide, and irreversibly oxidized human nonmercaptalbumin-2 (HNA2) with Cys-34 oxidized to sulfinic or sulfonic acid. We have reported marked oxidative albumin damage in decompensated cirrhosis and even more so in acute-on-chronic liver failure,[9,10] which was paralleled by an altered conformation[11] and related to an impaired albumin binding affinity for DS.[12] This finding among others has led to the concept of effective albumin concentration, which may further aggravate hypoalbuminemia observed in chronic liver failure.[13,14] Oxidative damage, as well as impaired DS binding, has been also documented in therapeutic albumin solutions, similar to the alterations observed in patients with decompensated cirrhosis.[15–17]

In the present study, we aimed to investigate the effect of albumin infusion on oxidative modification and binding properties of circulating albumin, as well as its circulatory effects estimated by neurohumoral parameters and innate immune effects, in patients with chronic liver failure who routinely received albumin infusion. In addition, we measured the content of oxidized albumin and albumin binding properties in therapeutic albumin solutions used for treating our patients.

2 | PATIENTS AND METHODS

2.1 | Patients

We enrolled consecutive patients who routinely received a commercial albumin solution following large-volume paracentesis ($n = 19$) or for treatment of hepatorenal syndrome ($n = 1$) at the Medical University of Graz from February 2017 to October 2019. According to our local SOP, patients received 10-g albumin per litre ascites removed in case the total volume exceeds 5 L for prevention of post-paracentesis circulatory dysfunction. Inclusion criteria were presence of cirrhosis, portal hypertensive ascites (SAAG >1.1), and paracentesis volume >5 L. Exclusion criteria comprised presence of hepatocellular carcinoma, advanced extrahepatic neoplasia, paracentesis for malignant ascites, and concomitant intrinsic renal disease. Informed consent was obtained in accordance with the declaration of Helsinki and with local laws. The study was approved by the local Institutional Review Board at the Medical University of Graz (EK # 29-040) and by the Austrian competent authority (EudraCT 2016-004012-35). The study was conducted in accordance with the Basic & Clinical Pharmacology & Toxicology policy for experimental and clinical studies.[18]

2.2 | Study design

Blood was sampled at baseline (before albumin infusion) and at two time-points after albumin infusion, that is, after 24 and 48 h for patients #1–9 (cohort 1) and immediately after albumin infusion and after 24 h for patients #10–20 (cohort 2). Plasma and serum samples were stored frozen for later determination of (i) oxidative albumin modification, (ii) albumin binding capacity (ABiC) for DS, and (iii) neurohumoral parameters. Total albumin was measured by bromcresol green assay on the

same day in the clinical laboratory and later in stored samples in the chemistry laboratory. In addition, in seven patients of cohort 1 fresh whole blood samples were processed on the same day to assess neutrophil function. All lab investigators were blinded to the clinical data of study participants.

Patients #1–16 received Albunorm 200 g/L (Octapharma, Vienna, Austria) whereas patients #17–20 received Humanalbumin Kedrion 200 g/L (Kedrion Biopharma, Vienna, Austria), as supplied by our hospital's pharmacy. Specifications of the respective commercial albumin solution including its shelf life was recorded. Small aliquots (1 ml, corresponding to 1% of the administered dose) of the infused albumin solution were removed for determination of its oxidation status and DS binding capacity.

2.3 | Oxidative albumin modification

Albumin was fractionated by high performance liquid chromatography (HPLC) to give three peaks according to Cys-34 in the free sulfhydryl form (HMA), as a mixed disulfide (HNA1), or in a higher oxidation state (HNA2), as previously described.[19] Briefly, 20 µl of diluted plasma were injected into the HPLC system using a Shodex Asahipak ES-502 N 7C anion exchange column (7.5 × 100 mm, Bartelt Labor- & Datentechnik, Graz, Austria) and 50-mM sodium acetate, 400-mM sodium sulfate, pH 4.85 as mobile phase. For elution, a gradient of 0%–6% ethanol and a flow rate of 1 ml/min were used. Detection was carried out by fluorescence at 280/340 nm. Quantification was based on peak area using Peak Fit software (SPSS Science, Chicago, IL).

2.4 | In vitro preparation of HSA and albumin fractions HMA, HNA1, and HNA2

EDTA plasma of four healthy donors was collected at the Division of Medicinal Chemistry as approved by the Ethics Committee of the Medical University of Graz (29–460 ex 16/17). The donors gave written informed consent prior to the collection of blood samples.

To prepare HSA, plasma of patients or healthy donors was diluted with 50 mmol/L potassium dihydrogenphosphate (pH 7.0) and applied to a HiTrapTM Blue HP column (GE Healthcare, Solingen, Germany). HSA was eluted with 50 mmol/L potassium dihydrogenphosphate solution containing 1.5 mol/L potassium chloride (pH 7.0) and the albumin containing fractions were collected. Pooled fractions were dialyzed against deionized water, sterile filtered, and the redox state was further assessed by HPLC. Samples were stored at −80°C until further analyses.

The albumin fractions HMA, HNA1, and HNA2 were prepared as described previously by our group.[11] Briefly, EDTA blood of the healthy donors was incubated with 1 mg/ml N-acetylcysteine for 1 h at room temperature (RT). This leads to a reduction of HNA1, resulting in mainly HMA. HMA was purified as described for HSA using a HiTrapTM Blue HP column. To obtain HNA1 or HNA2, HMA was incubated with 17 mmol/L cystine at 37°C for at least 24 h or 45 mmol/L hydrogen peroxide for 1 h at RT, respectively. Oxidation of Cys-34 was controlled by HPLC. Residual cystine or hydrogen peroxide was removed by dialysis against distilled water. Purified HMA, HNA1, and HNA2 were stored at −80°C.

2.5 | Determination of binding properties by DS titration

Plasma samples were diluted with phosphate buffered saline (PBS) to a final concentration of 10-µM albumin; 100 µl of this dilution were applied to a flat-bottomed, half area, polystyrene, black 96-well plate (Greiner Bio-One, Kremsmünster, Austria). Successively, 100 µl of increasing concentrations of DS (Sigma Chemical) (0.625–640 µM) were added The plate was incubated for 20 min at 37°C. Fluorescence was measured using FLUOstar OPTIMA microplate reader (BMG LABTECH, Ortenberg, Germany) (excitation/emission: 355/460 nm). DS background fluorescence at various DS concentrations was subtracted from the respective fluorescence intensities and the resulting binding curves were fitted applying nonlinear regression using GraphPad Prism Software (Version 5; GraphPad Software, San Diego, CA, USA). K_d values were calculated at DS concentrations where half-maximal fluorescence was reached.

2.6 | Albumin binding function (ABiC)

This method is based on the measurement of the fraction of a site-specific fluorescence marker not bound by the albumin in the sample (unbound fraction). By the comparison of the unbound fraction of this marker to the same amount of a reference albumin, ABiC of the albumin in the sample can be expressed as a percentage of the binding capacity of the reference albumin.[6] For the ABiC assessment, all samples were diluted to the same albumin concentration of 150 µmol/L. The same volume and same concentration of the binding site II specific fluorescence marker DS were added to each sample. The unbound marker molecules were separated by ultrafiltration

(Centrisart I, Sartorius Göttingen; cutoff = 20 000 Da). Multiple aliquots of the albumin-free ultrafiltrate were added to a 96-well plate, and fluorescence was measured using FLUOstar OPTIMA microplate reader (excitation/emission: 355/460 nm) after the addition of an albumin solution as a fluorescence amplifier. In parallel, the same procedure was performed with a standard albumin as reference (standardized virus-inactivated human serum preparation from pooled human plasma, Biseko, Biotest® Pharma GmbH, Dreieich, Germany). The binding capacity for the marker DS was quantified according to the following equation:

$$\text{ABiC}(\%) = \text{Fluorescence in the ultrafiltrate of the reference} / \text{Fluorescence in the ultrafiltrate of the sample} \times 100.$$

2.7 | Neurohumoral parameters

Plasma renin and copeptin concentrations were measured by standardized immunoassays: Renin was measured using the chemiluminescent immunoassay Liaison Direct Renin (DiaSorin; Saluggia, Italy), and copeptin was measured using an immunoassay in a chemiluminescence-coated tube format (B.R.A.H.M.S., Kryptor, GmbH, Henningsdorf, Germany).

2.8 | Neutrophil function ex vivo

Peripheral blood was taken to the VACUETTE® tubes containing 3.8% sodium citrate (Greiner Bio-One, Kremsmünster, Austria). The Phagoburst® kit (Glycotope, Heidelberg, Germany) was used to determine the percentage of neutrophils that produce reactive oxygen species (ROS) in response to different stimuli and their intracellular ROS amount according to the manufacturer's instructions. In brief, 100 µl of the whole blood was mixed with 20 µl of either wash solution (no stimulus—shows neutrophil basal ROS production), N-formyl-met-leu-phe (fMLF; shows the ability of neutrophils to respond to the low physiological stimuli, reflecting their preactivated status), Phorbol-12-myristat-13-acetat (PMA; ROS production in the presence of a very potent stimulus, served as a positive control of the experiment, data not shown) or stabilized and opsonized (non-labelled) *Escherichia coli* suspension. The Phagotest® kit (Glycotope, Heidelberg, Germany) was used to determine the phagocytic capacity of neutrophils and percentage of nonphagocytic neutrophils according to the manufacturer's instructions. In brief, 100 µl of the whole blood was mixed with 20 µl of stabilized and opsonized FITC-labelled *E. coli* suspension in two tubes. One tube stayed on ice; another one was incubated for 10 min at 37°C. LSRII flow cytometer (BD Biosciences, San Jose, California, USA) with BD FACS Diva 6.2 software (BD Bioscience) were used to record 10 000 neutrophils. Further analysis was performed in FlowJoTM V10 software (BD Biosciences), and calculations of neutrophil functions were performed as previously described.[20]

2.9 | Neutrophil function in vitro

ROS production was measured essentially as previously described.[21] Human neutrophils from healthy volunteers ($n = 3$) were isolated with Percoll and resuspended in PBS++ (Dulbecco's PBS supplemented with Ca^{2+} and Mg^{2+}, 1 g/L D-glucose and 4-mM sodium bicarbonate) to a concentration of 6.25×10^6 cells/ml. ROS production was measured indirectly using chemiluminescence in 5×10^5 freshly isolated neutrophils per well at 37°C in luminescence-grade 96 well plates (Nunc, Thermo Fisher Scientific) in a Lumistar Omega luminescence microplate reader (BMG Labtech, Offenburg, Germany). Neutrophils were treated with isolated HSA or in vitro prepared HMA, HNA1, or HNA2 fractions (3.5 mg/ml) either without stimulus or stimulated with heat-killed, serum-opsonised *E. coli* as indicated, and incubated with 150-µM luminol and 18.75 U/ml horseradish peroxidase for analysis of total ROS (sum of intracellular and extracellular ROS). Data output was recorded as relative light units. Relative light units per second (RLUs/s) or total RLUs integrated over the indicated periods of time were analysed. ROS production were normalized to PBS (0) and PMA (1) stimulation.

2.10 | Statistical analyses

Continuous variables are presented as median (Q1, Q3). Outcome variables were compared by Mann–Whitney test, Wilcoxon test, or Friedman test with Bonferroni correction for pairwise comparisons as appropriate. The relation between variables was assessed by Spearman correlation coefficient. A P value below 0.05 was considered statistically significant. Statistical analyses were performed using SPSS version 26.0 software.

3 | RESULTS

We included 19 patients with refractory ascites receiving albumin after large-volume paracentesis and one patient

TABLE 1 Patient characteristics.

	Patients #1–9[a]	Patients #10–20[b]	$p_{\text{Mann–Whitney}}$
Age (years)	58 (50, 69)	57 (54, 65)	0.766
Sex (male)	78%	64%	0.642[c]
Aetiology of cirrhosis Alcohol Other	89% 11%	73% 27%	0.591[c]
MELD	15 (13, 26)	14 (11, 24)	0.552
Bilirubin (mg/dl)	2.4 (1.3, 14.6)	2.8 (1.3, 24.5)	0.824
Creatinine (mg/dl)	1.0 (0.8, 1.1)	1.0 (0.7, 1.3)	0.882
INR	1.64 (1.35, 1.83)	1.38 (1.14, 1.92)	0.295
AST (U/L)	38 (31, 125)	45 (26, 74)	1.000
ALT (U/L)	24 (20, 70)	17 (13, 36)	0.230
GGT (U/L)	168 (52, 381)	132 (65, 182)	0.552
AP (U/L)	131 (74, 249)	126 (96, 174)	0.710
Albumin (g/dl)	3.9 (3.5, 4.4)	3.3 (3.1, 3.6)	0.095
HMA (%)	53 (48, 58)	55 (50, 61)	0.766
HNA1 (%)	37 (34, 41)	37 (30, 39)	0.656
HNA2 (%)	10 (8, 12)	9 (6, 13)	0.882
ABiC (%)	55 (48, 62)	54 (43, 65)	0.824
PRA (μIU/ml)	672 (256, 4876)	337 (273, 732)	0.656
Copeptin (pmol/L)	25 (23, 32)	29 (21, 56)	0.656
Paracentesis volume (L)	8.5 (5.9, 12.0)	8.3 (5.9, 11.1)	0.720
Albumin infused (g)	100 (40, 100)	60 (40, 100)	0.603

Note: Continuous variables are given as median (Q1, Q3).
Abbreviations: ABiC, albumin binding capacity; ALT, alanine aminotransferase; AP, alkaline phosphatase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transpeptidase; HMA, human mercaptalbumin; HNA1, human nonmercaptalbumin-1; HNA2, human nonmercaptalbumin-2; INR, international normalized ratio; MELD, model for end-stage liver disease; PRA, plasma renin activity.
[a]Patients #1–9 were sampled at baseline, after 24 h and after 48 h.
[b]Patients #10–20 were sampled at baseline, immediately (<1 h) after albumin infusion and after 24 h.
[c]Fisher's exact test.

TABLE 2 Characteristics of the infused therapeutic albumin solution.

	All patients	Patients #1–9	Patients #10–20	*p* value[a]
HMA (%)	44 (39, 50)	46 (41, 50)	41 (36, 51)	0.497
HNA1 (%)	41 (38, 44)	39 (37, 44)	42 (38, 44)	0.604
HNA2 (%)	15 (14, 16)	14 (14, 15)	16 (13, 21)	0.243
Interval infusion-expiry (months)	31 (28, 32)	31 (31, 32)	31 (27, 51)	0.842

Note: Data are given as median (Q1, Q3).
[a]#10–20 *versus* #1–9, Mann–Whitney test.

who received albumin for treatment of hepatorenal syndrome. Patient characteristics are summarized in Table 1.

Analysis of the infused commercial albumin preparations revealed marked oxidative albumin modification, with low content of nonoxidized HMA and high contents of the oxidized HNA1 and HNA2 species (Table 2). Notably, the fraction of oxidized albumin species of commercial albumin was higher than that of the circulating albumin of our patients. No difference was detected between the albumin preparations from two different suppliers used in our study (data not shown).

Following interim analysis of patients #1–9 (cohort 1), no differences were detected between T0 (baseline) and T1 (24 h after albumin infusion), neither for oxidized albumin species nor for albumin binding properties as assessed by ABiC or K_d values (Table 3). Therefore,

TABLE 3 Albumin fractions, albumin binding capacity and neurohumoral parameters before and after albumin infusion (cohort 1, patients #1–9).

	T0 (baseline)	T1 (after 24 h)	T2 (after 48 h)	$p_{Friedman}$
Albumin (g/dl)	3.9 (3.5, 4.4)	4.1 (3.7, 4.3)	4.1 (4.0, 4.4)	1.000
HMA (%)	53 (48, 58)	52 (47, 62)	52 (40, 59)	0.895
HNA1 (%)	37 (34, 41)	38 (31, 41)	38 (33, 48)	0.459
HNA2 (%)	10 (8, 12)	11 (8, 11)	8 (8, 12)	0.651
ABiC (%)	55 (48, 62)	58 (52, 66)	50 (44, 54)	0.135
K_d plasma	26 (21, 39)	27 (19, 36)	28 (22, 36)	0.772
PRA (μIU/ml)	672 (256, 4876)	716 (149, 4049)	1187 (338, 5662)	0.651
Copeptin (pmol/L)	25 (23, 32)	26 (16, 43)	30 (25, 36)	0.651

Note: Data are given as median (Q1, Q3). None of the variables is statistically different between T0-T1-T2 (Friedman test).
Abbreviations: ABiC, albumin binding capacity; HMA, human mercaptalbumin; HNA1, human nonmercaptalbumin-1; HNA2, human nonmercaptalbumin-2; PRA, plasma renin activity.

TABLE 4 Albumin fractions, albumin binding capacity and neurohumoral parameters before and after albumin infusion (cohort 2, patients #10–20).

	T0 (baseline)	T1 (after <1 h)	T2 (after 24 h)	$p_{Friedman}$
Albumin (g/dl)	3.3 (3.1, 3.6)	4.2 (3.8, 5.9)***	4.0 (3.7, 5.0)	0.001
HMA (%)	55 (50, 61)	55 (51, 56)	55 (48, 58)	0.307
HNA1 (%)	37 (30, 39)	34 (33, 38)	32 (30, 37)*	0.020
HNA2 (%)	9 (6, 13)	11 (10, 14)*	11 (7, 14)	0.038
ABiC (%)	54 (43, 65)	55 (50, 62)	57 (44, 64)	0.670
K_d plasma	20 (15, 33)	20 (16, 29)	16 (14, 31)	0.459
PRA (μIU/ml)	337 (273, 732)	133 (49, 392)**	319 (125, 1005)#	0.002
Copeptin (pmol/L)	29 (21, 56)	28 (20, 52)	33 (17, 52)	0.150

Abbreviations: ABiC, albumin binding capacity; HMA, human mercaptalbumin; HNA1, human nonmercaptalbumin-1; HNA2, human nonmercaptalbumin-2; PRA, plasma renin activity.
*$p < 0.05$ *versus* T0.
**$p < 0.01$ *versus* T0.
***$p < 0.001$ *versus* T0.
#$p < 0.05$ *versus* T1 (Friedman test, pairwise comparison with Bonferroni correction).

assuming that the postulated effects of albumin infusion were only short-lived, the protocol was modified for the subsequent patients (patients #10–20, cohort 2), collecting T1 samples within 1 h after the end of albumin infusion. In this subcohort, a significant increase at T1 *versus* T0 was observed for total albumin and HNA2 levels; again no changes were observed for ABiC or K_d values (Table 4). As expected, plasma renin concentration decreased significantly at T1 in cohort 2 but did not change in cohort 1, while no significant changes were observed for plasma copeptin concentration (Tables 3 and 4).

In addition, we analysed the effect of albumin infusion in the whole cohort comparing baseline to timepoint 24 h after infusion (which was available in both cohorts), allowing comparison with higher statistical power ($n = 20$). Again, no significant effect was found with exception of a small reduction in median HNA1 from 37% to 36% (Table 5). Since we observed no effect (not even a trend) of albumin infusion on ABiC or on K_d, we decided to stop enrolment after patient #20.

Interestingly, the difference in HNA2 plasma concentration before (T0) and 24 h after albumin infusion correlated significantly with the HNA2 content of the infused albumin solution in the whole cohort ($n = 19$, $R_{SPEARMAN} = 0.80$, $p < 0.001$) (Figure 1). However, no significant correlations were found between the difference in HMA plasma concentration before/after albumin infusion and the HMA content of the infused albumin solution.

Analysis of the infused albumin solutions revealed no significant correlations between albumin binding

TABLE 5 Albumin fractions, albumin binding capacity and neurohumoral parameters before and after albumin infusion (whole cohort, patients #1–20).

	T0 (baseline)	T1 (after 24 h)	$p_{Wilcoxon}$
Albumin (g/dl)	3.6 (3.3, 4.3)	4.0 (3.8, 4.3)	0.067
HMA (%)	54 (50, 59)	53 (48, 60)	0.588
HNA1 (%)	37 (33, 39)	35 (31, 39)	0.024
HNA2 (%)	9 (7, 12)	11 (8, 13)	0.161
ABiC (%)	55 (45, 65)	58 (48, 64)	0.268
K_d plasma	25 (17, 31)	19 (15, 34)	0.381
PRA (μIU/ml)	354 (278, 1691)	450 (147, 1814)	0.147
Copeptin (pmol/L)	26 (22, 41)	29 (16, 49)	0.159

Abbreviations: ABiC, albumin binding capacity; HMA, human mercaptalbumin; HNA1, human nonmercaptalbumin-1; HNA2, human nonmercaptalbumin-2; PRA, plasma renin activity.

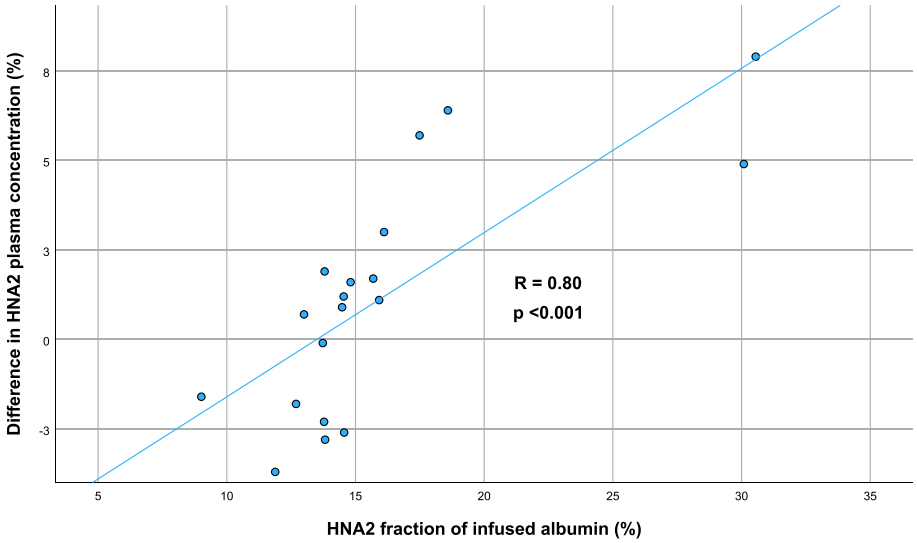

FIGURE 1 Relation of the difference in HNA2 plasma concentration before and 24 h after albumin infusion to HNA2 content of the infused albumin solution (whole cohort, $n = 19$*). *HNA2 content of infused albumin missing for one patient.

characteristics (expressed as K_d of the DS binding assay) and the fraction of HMA ($R_{SPEARMAN} = 0.132$), HNA1 ($R_{SPEARMAN} = -0.135$), or HNA2 ($R_{SPEARMAN} = 0.053$), indicating that the redox state of albumin is not an important determinant of its binding properties.

We also assessed neutrophil function ex vivo in seven patients of cohort 1. Significant differences were detected only in the number of neutrophils, which produced ROS in response to *E. coli*. This parameter increased 24 h after albumin infusion and returned to the baseline level after 48 h (Table 6). The percentage of neutrophils with ROS production without a stimulus and in response to fMLF, intracellular ROS level of neutrophils, neutrophil phagocytic capacity, and the percentage of nonphagocytic neutrophils were unaltered (Table 6). Furthermore, we assessed the influence of albumin fractions on ROS production of isolated neutrophils from healthy patients in vitro (Figure 2). HNA2 increased total neutrophil ROS production without an additional stimulus ($p < 0.01$). In combination with heat killed *E. coli* as a stimulus, HSA and HNA1 significantly increased total ROS production ($p = 0.02$ and $p < 0.01$ respectively).

4 | DISCUSSION

This is the first study of the effect of albumin infusion on oxidative albumin modification and ABiC for DS in chronic liver failure. Infusion of therapeutic albumin solutions produced a transient increase of HNA2, which correlated with the HNA2 content of the infused albumin, a small decrease of HNA1 at 24 h, while HMA levels remained unchanged. In contrast to the improvement of ABiC by 6-h MARS sessions (from 69.7% to 78.5%),[5] no significant change in ABiC was observed in our study immediately after albumin infusion (54% vs. 55%, Table 4) or 24 h after albumin infusion (55% vs. 58%,

TABLE 6 Neutrophil function before and after albumin infusion (cohort 1, patients #1–7).

	T0 (baseline)	T1 (after 24 h)	T2 (after 48 h)
Nonphagocytic cells (% of *E. coli* batch control)	109 (57, 157)	87 (33, 157)	123 (76, 662)
Phagocytic capacity (% of *E. coli* batch control)	113 (95, 221)	128 (73, 211)	124 (68, 159)
ROS production (no stimulus) (%)	1.5 (1.2, 1.9)	1.3 (1.2, 1.5)	1.4 (1.4, 1.5)
ROS production (no stimulus) (GMFI)	146 (90, 170)	118 (97, 270)	150 (95, 230)
ROS production (fMLF) (%)	1.9 (1.3, 2.7)	2.1 (0.6, 2.7)	2.8 (1.3, 24.7)
ROS production (fMLF) (GMFI)	141 (96, 171)	154 (111, 216)	123 (97, 189)
ROS production (*E. coli*) (%)	97.5 (96.6, 98.1)	99.2 (98.3, 99.8)*	98.9 (93.7, 99.5)
ROS production (*E. coli*) (GMFI)	533 (347, 857)	750 (488, 967)	553 (449, 1180)

Note: Data are given as median (Q1, Q3).
Abbreviations: fMLF, N-formyl-met-leu-phe; GMFI, geometric mean fluorescence intensity; ROS, reactive oxygen species.
*$p < 0.05$ *versus* T0 (Friedman test, pairwise comparison with Bonferroni correction).

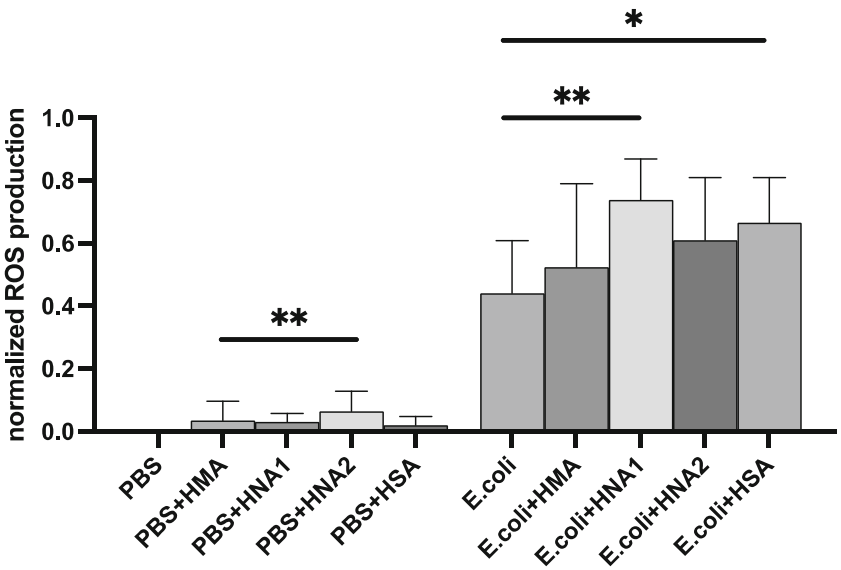

FIGURE 2 Normalized total ROS production of isolated neutrophils from healthy volunteers. Neutrophils were treated with isolated total human serum albumin or in vitro prepared HMA, HNA1 HNA2 fractions (3.5 mg/ml) either without stimulus (PBS) or stimulated with heat-killed, serum-opsonised *Escherichia coli*. HNA2 increased total neutrophil ROS production without an additional stimulus, whereas with heat-killed *E. coli* as a stimulus, HSA and HNA1 significantly increased total ROS production (statistical test: linear mixed effect model).

Table 5). Likewise, no changes in binding affinity (K_d) for DS were found.

The concept of effective albumin concentration has been postulated by Jalan and Bernardi[13] and further elaborated in a recent study by Baldassarre et al.[14] Analysing human albumin by HPLC/MS, they quantified the native albumin fraction (%) and the effective albumin concentration (g/dl), which was closely associated with albumin binding function and short-term survival in chronic liver failure. Consistent with our findings, their ancillary study of eight patients receiving albumin infusion showed only transient increases in total albumin concentration and no change of native albumin abundance.[14] Since irreversibly oxidized HNA2 is believed to be degraded more rapidly than native albumin,[22] a high content of HNA2 in therapeutic albumin solutions may explain the transient increase in total plasma albumin observed in both studies. On the other hand, early decline of plasma levels of both native and oxidized albumin may be related to rapid loss into the extravascular pool. Interestingly, in contrast to the modest changes observed after albumin infusion, a recent study in patients with neurological disorders reported long-lasting increases in HNA2 levels by plasma exchange but not by immunoadsorption, presumably

related to the high volume of albumin substitution in plasma exchange.[23]

High content of oxidized albumin in therapeutic albumin solutions has been previously reported by Bar-Or et al.[15] In their study, analysis by mass spectrometry revealed oxidative albumin modification at Cys-34 in 57% of commercial albumin *versus* 23% of plasma albumin of healthy volunteers. This finding was confirmed by our group using HPLC.[16] Consistent with these findings, Baldassarre et al.[14] reported that the fraction of native albumin was lower in commercial albumin (39%) *versus* albumin of healthy controls (49%). Importantly, in our study, the fraction of oxidized albumin expressed as the sum of HNA1 plus HNA2 was higher in therapeutic grade albumin (56%) even when compared with plasma from patients with decompensated cirrhosis (46%). It should be noted that HNA1 was related to inflammatory responses in vitro in leucocytes from both healthy volunteers and patients with cirrhosis.[24]

Our finding of increased ROS production without a stimulus in response to HNA2 supports the notion that therapeutic albumin, due to its oxidative modifications, could have proinflammatory effects. On the other hand, albumin may have beneficial immunomodulatory effects in cirrhosis by scavenging ROS. Impaired ROS production has been found in cirrhosis[25] and albumin was able to prevent unstimulated (resting) ROS production of neutrophils from patients with cirrhosis ex vivo.[26] In our study, intracellular ROS production upon *E. coli* stimulation increased 24 h after albumin infusion, possibly reflecting improved innate immune function as a first line of defence in cirrhosis. The increase of in vitro ROS production of healthy neutrophils stimulated with *E. coli* in response to HSA and HNA1 confirmed the results observed ex vivo in our patients. Taken together, our findings point towards an induction of ROS production through albumin upon physiological stimulation, whereas oxidized albumin may act proinflammatory by increasing unstimulated ROS production. The mechanisms and consequences of modulating neutrophil ROS production in response to albumin need to be studied in more detail to explore a putative "janus face" of albumin supplementation in cirrhosis.

In order to confirm the beneficial oncotic effect of albumin infusion in our study, we also assessed the neurohumoral parameters renin and copeptin before and after albumin infusion. We found a significant reduction of plasma renin concentration in cohort 2 where plasma was sampled immediately after albumin infusion, indicating a transient improvement in circulatory function. These findings are consistent with the well-known beneficial effects of albumin infusion on the neurohumoral axis in decompensated cirrhosis.[1,2]

Our study has some limitations: First, the overall sample size was small and the protocol was not uniform as we decided to change the timing of T1 for cohort 2, since we did not observe any significant changes on interim analysis of cohort 1. Second, the commercial albumin solution changed during the period of enrolment due to preference of our hospital's pharmacy, though no difference in the quality of both preparations was evident. Third, using the site II ligand DS, only one facet of albumin binding function was assessed. Fourth, as 80% of our cohort had alcohol-associated liver disease, we cannot extend our conclusions on other aetiologies such as viral hepatitis or metabolic dysfunction-associated steatotic liver disease.

In conclusion, infusion of therapeutic albumin solutions changed albumin redox state with a transient increase in the fraction of irreversibly oxidized HNA2 and failed to improve binding function of circulating albumin in patients with chronic liver failure. Based upon the findings of this small proof-of-concept study, the development of improved therapeutic grade albumin preparations containing lower oxidized fractions and better binding properties may be contemplated.

AUTHOR CONTRIBUTIONS

Rudolf E. Stauber, Vanessa Stadlbauer, Karl Oettl: Study design; manuscript writing. **Florian Rainer, Vanessa Stadlbauer, Rudolf E. Stauber**: Patient recruitment. **Margret Paar, Karl Oettl**: Assay of albumin fractions and binding function. **Irina Balazs, Angela Horvath, Nicole Feldbacher, Vanessa Stadlbauer**: Assay of neutrophil function. **Andreas Posch, Florian Rainer, Rudolf E. Stauber, Vanessa Stadlbauer**: Data management; statistical analysis. All authors: final manuscript review.

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CONFLICT OF INTEREST STATEMENT

All authors declare that they have no conflicts of interest related to the study.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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