Research Article
Cirrhosis and Liver Failure

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Clearance and production of ammonia quantified in humans by constant ammonia infusion – the effects of cirrhosis and ammonia-targeting treatments

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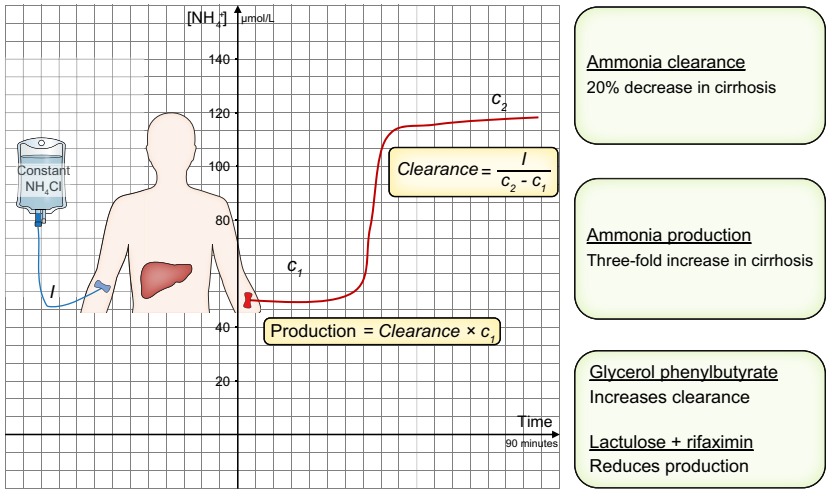
Graphical abstract



Highlights

- The constant ammonia infusion technique quantifies whole-body ammonia metabolism.
- The technique measures pathophysiological changes in ammonia metabolism and the effects of ammonia-targeting therapies.
- In patients with cirrhosis, ammonia clearance is decreased by 20% and ammonia production is increased three-fold.
- Treatment with glycerol phenylbutyrate increases clearance in healthy persons.
- Treatment with lactulose + rifaximin decrease production in patients with cirrhosis.

Impact and implications

High blood ammonia plays a key role in cirrhosis-related brain dysfunction. However, the relative roles of reduced ammonia clearance and increased ammonia production are poorly understood as is the action of ammonia-targeting treatments. This study presents a relatively simple test to measure ammonia metabolism. By using this test, it was possible to show that patients with cirrhosis exhibit decreased ammonia clearance and increased ammonia production compared to healthy persons, and to quantify the unique effects of different ammonia-targeting treatments. The test described herein may be used to examine a range of questions related to normal physiology, pathophysiology and the mechanisms of action of ammonia-targeting treatments.

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Clearance and production of ammonia quantified in humans by constant ammonia infusion – the effects of cirrhosis and ammonia-targeting treatments

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See Editorial, pages 266–268

Background & Aims: Hyperammonaemia is a key pathological feature of liver disease and the primary driver of hepatic encephalopathy (HE). However, the relative roles of increased ammonia production and reduced clearance are poorly understood as is the action of ammonia-targeting drugs for HE. We aimed to quantify whole-body ammonia metabolism in healthy persons and patients with cirrhosis and to validate our method by examining the effects of glycerol phenylbutyrate and lactulose + rifaximin treatment.
Methods: Ten healthy men and ten male patients with cirrhosis were investigated by 90-minute constant ammonia infusion to achieve steady-state plasma ammonia. Whole-body ammonia clearance was calculated as infusion rate divided by steady-state concentration increase and ammonia production was calculated as clearance multiplied by baseline ammonia concentration. Participants were re-investigated after the ammonia-targeting interventions.
Results: In healthy persons, ammonia clearance was 3.5 (3.1–3.9) L/min and ammonia production was 49 (35-63) μmol/min. Phenylbutyrate increased clearance by 11% (4–19%, $p = 0.009$). In patients with cirrhosis, ammonia clearance was 20% lower at 2.7 (2.1–3.3) L/min ($p = 0.02$) and production was nearly threefold higher at 131 (102–159) μmol/min ($p < 0.0001$). Lactulose + rifaximin reduced production by 20% (2–37%, $p = 0.03$). The infusion was generally well-tolerated apart from in one hyperammonaemic patient, with cirrhosis and possible bleeding unrelated to the infusion, who developed clinical HE that reverted when infusion was discontinued.
Conclusions: Whole-body ammonia clearance and production may be measured separately using the described technique. This technique identified a lower clearance and a higher production of ammonia in patients with cirrhosis, and showed that phenylbutyrate increases clearance, whereas lactulose + rifaximin reduces production.
Clinical trial number: ClinicalTrials.gov (1-16-02-297-20).

Introduction

Ammonia is continually produced and consumed throughout the human body during the metabolism of amino acids, purine and pyrimidine derivates, and polyamines.[1] Ammonia is neurotoxic but is effectively detoxified by transient enzymatic fixation to glutamate – yielding glutamine in a process catalysed by glutamine synthetase in the liver, muscles and brain – and ultimately by conversion to urea in the liver before final removal from the body.[1] Disturbances in ammonia metabolism are of paramount interest in hepatology because most liver diseases involve hyperammonaemia and because the frequent and devastating symptom of metabolic liver failure, hepatic encephalopathy (HE), is closely associated with ammonia.[2–5] Furthermore, the degree of hyperammonaemia seems to be of prognostic value in cirrhosis.[6–9] Rarer conditions, such as congenital disorders of urea cycle enzymes, are also associated with hyperammonaemia, with potentially fatal consequences.[1]

Even so, the clinical utility of blood ammonia sampling remains questionable;[7,10,11] possibly because ammonia metabolism is a continuous, dynamic and rapid balance between production and removal. Measurements of ammonia in the blood provide only a snapshot of this balance. This is a limitation to our understanding of the pathophysiology of cirrhosis and the mode of action of therapeutic interventions. Thus, an unmet need exists for a method allowing measurements of whole-body ammonia clearance and production.

In cirrhosis, hyperammonaemia is thought to be the combined product of compromised liver removal, increased intestinal and renal formation in conjunction with portosystemic

Keywords: ammonia metabolism; hepatic encephalopathy; liver cirrhosis; lactulose; rifaximin; glycerol phenylbutyrate.
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shunting, and reduced muscular removal of ammonia due to sarcopenia.[12,13] To consider the relative importance of these effects, a whole-body picture is needed. Therefore, studies of the pathophysiology of hyperammonaemia and the action of attempted ammonia-lowering treatments should preferably examine whole-body ammonia metabolism, focusing on the balance between overall systemic clearance and production of ammonia; a balance that determines the ammonia concentration in the systemic circulation.

Therefore, this study aimed to assess whole-body ammonia metabolism in healthy persons and in patients with cirrhosis using an ammonia infusion technique. We developed a relatively simple test, based on a 90-minute constant infusion of ammonia to achieve plasma steady-state, which allowed us to measure the whole-body clearance of ammonia from the systemic circulation and the basal ammonia production rate released from tissues into the systemic circulation. We examined if the test may be used to quantify the specific pathophysiological changes in clearance and/or production in cirrhosis and the effects of ammonia-targeting therapies such as phenylbutyrate and lactulose + rifaximin. To test the ability of the methodology to detect different treatment effects, we chose dissimilar interventions for the two groups with distinctively different assumed ammonia-targeting modes of action: glycerol phenylbutyrate in healthy persons aiming to increase clearance and the drug combination of lactulose and rifaximin aiming to reduce production in patients with cirrhosis.

Materials and methods

Study population

Ten healthy men (mean age 39 years, range 22–66 years) and ten male patients with cirrhosis (mean age 58, range 43–73 years) were included. The healthy men were non-smokers without present or former alcohol consumption >12 g/day. The patients were recruited from the Outpatient Clinic at the Department of Hepatology and Gastroenterology, Aarhus University Hospital, Denmark. All had a clinical diagnosis of alcohol-related cirrhosis with two patients also having hepatitis C. One patient was treated with lactulose before entering the study, six were on proton pump inhibitors and three patients were being treated with beta blockers. The exclusion criteria in both groups were body mass index >30, estimated glomerular filtration rate <60 ml/min/1.73 m^2, Child-Pugh score >12, overt HE or more than one admission with HE within the past year, malignancy, other inflammatory disease or active infection, diabetes mellitus and treatment with rifaximin or branched-chain amino acids. Written informed consent was obtained from all candidates before inclusion. The study conformed to the ethical guidelines of the Declaration of Helsinki and Danish and European Union legislation on data protection. The protocol was approved by the Central Denmark Region Committees on Health Research Ethics (1-10-72-165-19) and the study was registered with ClinicalTrials.gov (1-16-02-297-20).

Study design

We conducted two experiments. *Experiment 1*: A study of ammonia metabolism in healthy persons and in patients with cirrhosis. *Experiment 2*: A study of the effect of ammonia-targeting treatment by two different mechanisms: 2.1) glycerol phenylbutyrate in healthy persons and 2.2) lactulose in combination with rifaximin in patients with cirrhosis. In these experiments, each participant served as his own control as concerns treatment effect.

All participants were investigated at 9 AM in the supine position after an overnight fast allowing only tap water. They were examined by standardised constant ammonia infusion (see below) at the Department of Hepatology and Gastroenterology, Aarhus University Hospital, Denmark. The participants avoided vigorous physical activity during the 48 h before the investigation. Seven out of ten healthy persons and eight out of ten patients with cirrhosis also consented to participate in *Experiment 2*, which was conducted at least three weeks after *Experiment 1* (the median time interval between the two experiment days was 42 days (range, 29–56 days)). *Experiment 2* was preceded by an ammonia-targeting intervention. The healthy persons were treated with the ammonia-targeting drug glycerol phenylbutyrate (Ravicti®, Immedica Pharma AB, Stockholm, Sweden). Glycerol phenylbutyrate was administered orally twice at a 2-h interval in doses of 4 g/m^2 body surface (total median dose: 18 g, range 15–19 g). The median time from first dose to initiation of the ammonia infusion was 3.5 h (range, 3.3–3.8 h) with the intention to reach the maximum active drug (phenylacetic acid) concentration during ammonia infusion.[14] In patients, the ammonia-targeting intervention comprised 2 weeks of treatment with lactulose (Orifarm, Leverkusen, Germany) 10-20 ml three times per day, adjusted to produce three semisoft stools per day, in combination with rifaximin (Xifaxan®, Norgine, Harefield, UK) 550 mg twice per day. The medication was handed out to patients. Treatment compliance was self-reported. One patient already on lactulose had a three-week wash-out period before the first day of investigation. In the rest of the patient cohort, lactulose and rifaximin treatment was initiated *de novo*.

Anthropometric measurements

Body composition was determined by bioelectrical impedance measurement (medical Body Composition Analyzer 515, Seca GmbH & Co. KG, Hamburg, Germany) except in one patient who had a pacemaker. BMI (kg/m^2) and body surface area (m^2) were calculated using standard formulas.[15] All patients were investigated for ascites by ultrasonography.

Experimental setup – the constant ammonia infusion technique

Arterial access was obtained using a sterile technique, after local infiltration anaesthesia with 0.5 ml of lidocaine 10 mg/ml (Aspen Pharmacare, Durban, South Africa), via ultrasonography-guided placement of a catheter (20 Gauge BD Arterial Cannula, BD, Franklin Lakes, NJ) in the right radial artery at wrist level. An isotonic ammonia-chloride solution of 152 mmol/L NH_4Cl (Addex®, Fresenius Kabi, Copenhagen, Denmark) in sterile water (Hospital Pharmacy Fyn, Odense, Denmark) was infused through a peripheral vein catheter at a constant rate of 0.25 mmol/kg/h (4.17 μmol/kg/min) for 90 min using a volumetric pump (Alaris GP Plus Volumetric Pump, BD). The infusion rate and duration were guided by two previous reports of ammonia infusion at a rate of ∼0.5 mmol/kg/min for

4 h in healthy persons[16] and 2 h in patients with cirrhosis[17] without measurable cerebral effects.

Ammonia sampling and analysis

Arterial blood for ammonia determination was drawn from the catheter via gas-tight adapters (Holdex Single-Use Holder PP, Greiner Bio-One GmbH, Kremsmünster, Austria). Samples were drawn three times over the hour before infusion start (as baseline) and at t = 0.5, 1, 2, 3 min and every 5 min from t = 0 min to t = 20 min and then every 10 min to t = 90 min. After infusion stop, samples were drawn at t = 90.5, 91, 92, 93 min and every 5 min to t = 110 min and then every 10 min to t = 130 min.

Blood was drawn to empty the catheter before each blood sampling and, after blood sampling, the arterial catheter was flushed with 1 ml of isotonic saline. Blood was sampled stasis free directly on cooled di-potassium EDTA 2 ml tubes (BD Vacutainer, BD, UK) and placed immediately on ice. Within 5 min from drawing, samples were centrifuged at 3,000 g, 4 °C for 5 min. Immediately after centrifuging, plasma was recovered by pipette and transferred to Inpeco aliquoting tubes (Sarstedt AG & Co. KG, Nümbrecht, Germany), which were stored at -40 °C until batch analysis the following day. Ammonia was analysed by enzymatic photometry with the reagents α-ketoglutarate, reduced NADPH analogue and glutamate dehydrogenase on Advia Chemistry XPT System and Atellica CH 930 Analyzer (Siemens Healthcare GmbH, Erlangen, Germany) at the Department of Clinical Biochemistry, Aarhus University Hospital, Denmark.

To limit the preanalytical factor of possible ammonia concentration drift due to plasma deamination processes following batch freezing and thawing,[18] the effect of batch ammonia analysis as compared to that of immediate analysis was assessed. Ammonia samples analysed after being frozen for 24 h were compared with ammonia samples analysed within 15 min of sampling. In healthy persons (n = 6), the mean±SD ammonia concentrations in batch-analysed samples was 4.5±1.5 μmol/L (min–max, 2–6) higher than in immediately analysed samples. In patients, the corresponding difference was 5.3±12.2 μmol/L (-6–35.5). All obtained ammonia concentrations were adjusted to account for this batch effect. Due to the higher batch effect variation in patients, individual batch effects were determined on the day of investigation. The higher variation in patients may be related to elevated levels of gamma-glutamyltransferase (mean 377, min–max 16–1,294; normal range 15–115).[18]

Additional biochemical analyses

The plasma concentrations of alanine aminotransferase, bilirubin, albumin, creatinine, haemoglobin, thrombocytes, leukocytes, c-reactive protein, chloride, potassium and arterial pH, base excess, bicarbonate levels and partial pressure of carbon dioxide were determined using routine analyses with accredited laboratory assays at the Department of Clinical Biochemistry, Aarhus University Hospital, Denmark.

Observation for adverse effects

Participants were observed for signs of physical or mental adverse effects to the ammonia infusion by a physician before, during and 2 h after infusion. Before and during infusion, patients were screened for cognitive impairment using the animal naming test.[19] Arterial blood pH and electrolyte measurements were monitored at t = 30, 60 and 90 min during ammonia infusion.

Calculations

Ammonia metabolism was quantified based on the formulas provided below, under the assumption of first-order kinetics.

At baseline spontaneous steady-state conditions, before the ammonia infusion, the net whole-body ammonia production (*P, μmol/min*) is the amount of ammonia released by the organs into the systemic circulation per minute, which equals the whole-body ammonia blood clearance (*Cl, L/min*) times the baseline steady-state ammonia concentration:

$$P(\mu mol/min) = Cl(L/min) \times c_1(\mu mol/L) \quad \text{(I)}$$

where c_1 is the mean plasma ammonia concentration of three baseline arterial blood samples drawn before infusion start.

During infusion, a new steady state is attained, and the arterial ammonia concentration increases from c_1 to c_2. Now, the amount of ammonia added to the circulation equals the ammonia infusion rate (*I*) plus the baseline production (*P*), and thus:

$$P(\mu mol/min) + I(\mu mol/min) = Cl(L/min) \times c_2(\mu mol/L) \quad \text{(II)}$$

c_2 being the mean of three arterial blood samples drawn at 70, 80 and 90 min after infusion start.

By subtracting Equation I from II, the ammonia clearance (*Cl*) was calculated as:

$$Cl(L/min) = \frac{I(\mu mol/min)}{c_2 - c_1(\mu mol/L)} \quad \text{(III)}$$

P can then be calculated from Equation I.

The apparent volume of distribution (V_d) of ammonia was calculated from the individual arterial decay curves following termination of ammonia infusion:

$$Vd(L) = \frac{\Delta AUC(\mu mol/L \times min) \times Cl(L/min)}{c_2 - c_3(\mu mol/L)} \quad \text{(IV)}$$

ΔAUC being the area under the decay curve minus the basal area from the x-axis to the last blood ammonia concentration measured after infusion stop during the time period from t = 90 to t = 130 min. The area under the decay curve was calculated using the trapezoidal rule; c_3 being the arterial ammonia concentration at t = 130 min. Of note, Equations I-III assume steady-state ammonia concentrations in the blood, so the distribution volume does not affect the calculations.

Statistical analyses

Data were analysed using Stata v.14.2 (StataCorp, CollegeStation, TX) and presented as means with 95% CIs unless otherwise stated. The dependent and independent sample *t* test was used for paired and unpaired observations, respectively. For skewed data, non-parametric Wilcoxon–Mann–Whitney test was used for comparisons between groups.

Correlations were investigated by Spearman's rank correlation coefficient (ρ). The significance level was set at 0.05 in a two-tailed test. Graphs were prepared in Stata and Prism v. 9.2 (GraphPad Software, San Diego, CA). Sample size was guided by power calculations with SD and sample means extracted from tentative calculations based on the ammonia infusion curves previously reported.[16,17] With SD estimated to 0.6, a sample size of n = 10 per group was required to detect a mean difference in clearance of 0.6 L/min between healthy persons and patients with cirrhosis; and a sample size of n = 8 healthy persons was required to detect a 20% improvement in clearance with a correlation between paired observations of 0.5, power 0.80 and a significance level of 0.05.

Results

Study participants

Clinical, anthropometric and biochemical characteristics are displayed in Table 1. Liver laboratory tests were different between the groups. C-reactive protein was within the normal range in both groups but slightly higher among patients. Furthermore, among patients, total muscle mass was reduced to 26 (21–31) *vs*. 31 (29–34) kg, $p = 0.02$. Patients had a median Child-Pugh score of 7 (min–max, 6–9) and two had moderate ascites.

Safety of ammonia infusion

Apart from one incident mentioned below, adverse or HE relevant events, including change in mental state or physical or mental fatigue, were not observed during ammonia infusion in either group. One patient with Child-Pugh 9 cirrhosis developed grade 2 HE at the end of ammonia infusion with lethargy, asterixis, nausea and non-bloody vomiting. After discontinuation of infusion, subsequent analyses showed that ammonia had increased to 348 μmol/L (baseline 124 μmol/L) and revealed a baseline haemoglobin of 3.9 μmol/L. He recovered from HE within 2 h. The patient refused endoscopic examination and treatment but consented to a 24-hour admission for blood transfusion and remained clinically stable during follow-up in the outpatient clinic. The case was interpreted as unidentified gastrointestinal bleeding occurring prior to the study and thus unrelated to the infusion, accompanied by hyperammonaemia. This patient was not included in *Experiment 2.* The incident was reported as a serious adverse event to the Central Denmark Region Committees on Health Research Ethics, January 15th 2022. For the rest of the experiments, plasma ammonia concentration was monitored in real time before and during infusion.

Ammonia infusions were associated with negligible but consistent changes in arterial pH and plasma chloride, potassium, carbon dioxide and standard bicarbonate (Table S1).

Ammonia concentration profile

The arterial plasma ammonia concentrations for *Experiment 1* are presented in Fig. 1 with individual curves provided as Figs. S1 and S2. Before infusion, arterial ammonia concentrations were lower in healthy persons (14 [10–19] μmol/L) than in patients with cirrhosis (53 [32–74] μmol/L, p <0.0001). Upon initiation of ammonia infusion, the ammonia concentration rapidly increased, reaching an approximated steady-state after about 40 min. During infusion, the final arterial ammonia concentration was lower in healthy persons (117 [101–133] μmol/L) than in patients with cirrhosis (180 [127–233] μmol/L, $p = 0.007$).

As seen in Fig. 1 and Figs. S1 and S2, the ammonia concentration profiles in patients were more heterogenous than those of healthy persons, and in two patients (ID 17 and 20),

Table 1. Clinical, anthropometric and biochemical characteristics.

	Healthy persons (n = 10)	Patients with cirrhosis (n = 10)	*p* values
Cirrhosis parameters			
Child-Pugh score[#]		7 (6–9)	–
MELD-Na score		12 (10–14)	–
Ascites (0/1/2/3)		8/0/2/0	–
Body composition			
Body weight (kg)	84.6 (78.6–90.6)	77.0 (66.9–87.3)	0.17
BMI (kg/m^2)	24.9 (23.3–26.5)	25.0 (21.8–28.2)	0.98
Body surface area (m^2)	2.1 (2.0–2.2)	1.9 (1.8–2.1)	0.08
Body fat percentage (%)*	24.5 (21.1–27.9)	21.8 (14.5–29.2)	0.44
Total muscle mass (kg)*	31.4 (29.0–33.8)	25.8 (21.0–30.6)	**0.02**
Total body water (L)*	47.1 (44.1–50.1)	42.9 (37.0–48.8)	0.15
Extra cellular volume (L)*	19.5 (18.3–20.8)	19.9 (17.2–22.7)	0.74
Biochemistry			
Alanine aminotransferase (U/L)	20 (14–26)	33 (23–43)	**0.02**
Bilirubin (μmol/L)	12 (8–17)	23 (15–31)	**0.02**
Albumin (g/L)	40 (38–42)	28 (23–34)	**0.0002**
Creatinine (μmol/L)	75 (67–84)	66 (55–77)	0.16
Haemoglobin (mmol/L)	8.5 (8.3–8.7)	7.1 (6.0–8.2)	**0.02**
Thrombocytes (x10^9/L)	236 (195–277)	87 (58–116)	**<0.0001**
Leukocytes (x10^9/L)	5.0 (4.3–5.7)	5.2 (4.0–6.3)	0.81
C-reactive protein (mg/L)[#]	4 (4–4)	4.2 (4–8.2)	**0.01**

MELD-Na, model for end-stage liver disease-sodium.
Values are given as mean (95% CI).
Independent sample t-test was used for comparisons between the groups. For skewed data, Wilcoxon–Mann–Whitney test was used.
[#]Given as median (range).
*Only given for nine out of ten patients with cirrhosis as measurement of bioelectrical impedance was contraindicated in one patient (with a pacemaker).

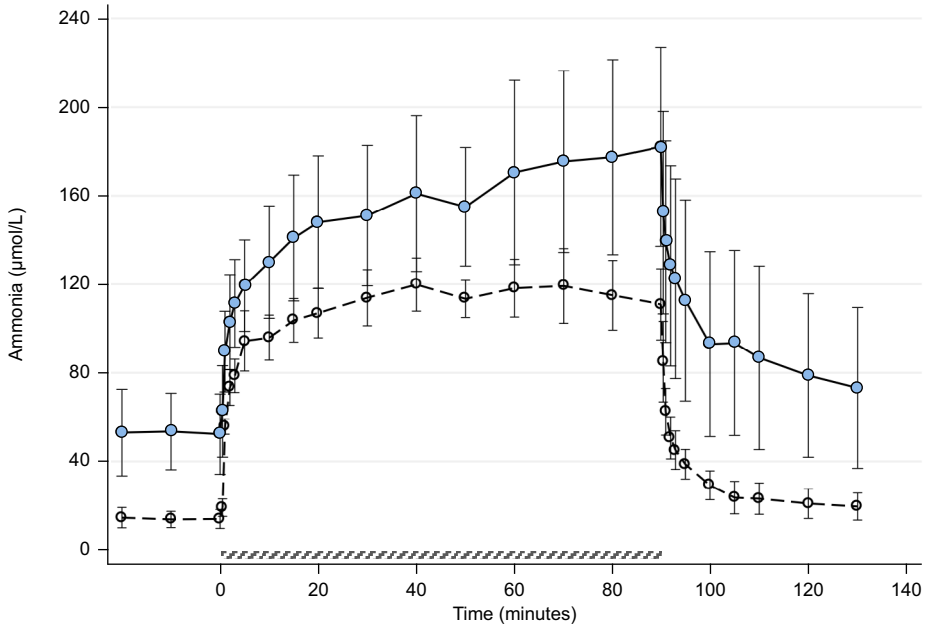

Fig. 1. Ammonia concentration profiles. Arterial plasma ammonia concentration excursions before, during and after ammonia infusion for healthy persons (white circles) and patients with cirrhosis (blue circles). Mean values with their 95% CIs are shown for the sampled time points. The shaded bar indicates timing of ammonia infusion.

ammonia concentration tended to slowly increase during the attempted steady-state period.

Following termination of the ammonia infusion, arterial plasma ammonia levels declined by more than 50% within a few minutes, declining more slowly thereafter and reaching close-to-baseline values within 40 min (Fig. 1).

Experiment 1. Ammonia metabolism in healthy persons and patients with cirrhosis

The results of *Experiment 1* are presented in Table 2. In healthy persons, ammonia clearance was 3.5 (3.1–3.9) L/min and ammonia production was 49 (35–63) µmol/min. Patients with cirrhosis exhibited lower ammonia clearance, at 2.7 (2.1–3.3) L/min (p = 0.02), and higher production, at 131 (102–159) µmol/min (p <0.0001), than healthy participants. Excluding the patient who developed HE changed neither of these results.

V_d of ammonia was non-significantly lower in healthy persons than in the cirrhosis group (13.6 [11.1–16.0] *vs.* 17.1 [13.6–20.7] L, p = 0.08). Ammonia clearance, production and V_d did not correlate with any of the body composition measures, including total muscle mass, nor with biochemical or clinical makers of liver disease severity. Bearing in mind that the study was not powered for subgroup analysis, it is noted that no difference was observed in ammonia clearance or production between proton pump inhibitor-treated and -untreated patients (p = 0.13 and p = 0.41, respectively) or between beta blocker-treated and -untreated patients (p = 0.44 and p = 0.47, respectively).

Experiment 2.1. Effect of ammonia-targeting intervention by glycerol phenylbutyrate in healthy persons

Administration of glycerol phenylbutyrate increased ammonia clearance in 6/7 healthy persons with a mean increase of 12% (4–20%, p = 0.009) (Fig. 2 and Table 3), as also reflected by a smaller increase in ammonia concentration during infusion in *Experiment 2* (increase 91 [79–102] µmol/L *vs.* 102 [87–118] µmol/L in *Experiment 1*; p = 0.007). The basal production rate was increased from 40 (26–53) to 56 (42–71) µmol/min (p = 0.002). The increase was proportional to the marginal increase in baseline ammonia from 12 (7–16) to 15 (12–17) µmol/L (p = 0.04). It is uncertain if this was coincidental or related to the lack of fasting because of the oral administration of glycerol phenylbutyrate. V_d was unchanged by the intervention (p = 0.51).

Experiment 2.2. Effect of ammonia-targeting intervention by the combination of lactulose and rifaximin in patients with cirrhosis

Two weeks of treatment with lactulose and rifaximin reduced ammonia production in 7/8 patients with cirrhosis by 20% (2–37%) p = 0.03 (Fig. 3 and Table 3). Ammonia clearance and V_d did not change significantly (p = 0.08 and p = 0.07, respectively). Baseline ammonia levels were unchanged by the intervention (p = 0.3, Table 3).

Discussion

We describe and suitability test a novel method to quantify whole-body ammonia clearance and production. The constant ammonia infusion technique was able to quantitatively describe the pathophysiology of hyperammonaemia in patients with cirrhosis, who exhibited moderately lower ammonia clearance and considerably higher production than healthy persons. The method was also able to describe and quantify how two different ammonia-targeting treatment principles affected ammonia metabolism through distinct mechanisms. Thus, glycerol phenylbutyrate acutely augmented clearance in

Table 2. Ammonia metabolism in healthy persons and patients with cirrhosis – before intervention.

	Healthy persons (n = 10)	Patients with cirrhosis (n = 10)	*p* values
Ammonia levels (μmol/L)			
Arterial, baseline	14 (10–19)	53 (32–74)	**<0.0001**
Arterial, infusion	117 (101–133)	180 (127–233)	**0.007**
Whole-body ammonia metabolic parameters			
Ammonia clearance (L/min)	3.5 (3.1–3.9)	2.7 (2.1–3.3)	**0.02**
P, Ammonia production rate (μmol/min)	49 (35–63)	131 (102–159)	**<0.0001**
Ammonia V_d (L)	13.6 (11.1–16.0)	17.1 (13.6–20.7)	0.08

Values are given as mean (95 % CI). V_d: Volume of distribution.
Independent sample *t* test was used for comparisons between the groups.

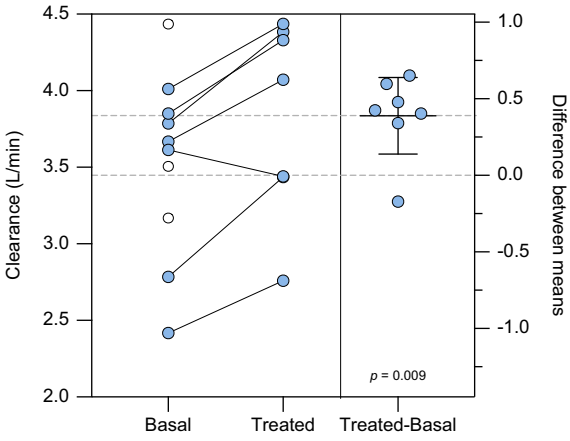

Fig. 2. Effect of glycerol phenylbutyrate on ammonia clearance in healthy persons. Estimation plot of clearance before intervention during basal conditions (basal) and after ammonia-targeting intervention with glycerol phenylbutyrate (treated) in healthy persons (n = 7). Dotted lines indicate the basal and treated group mean clearance and differences between means. Individual and group mean differences and 95% CIs are shown. Ammonia clearance of the participants who were examined only on Day 1 (basal conditions, n = 3) are shown (white dots). Dependent sample *t* test was used for statistical comparison.

healthy persons, whereas lactulose + rifaximin diminished ammonia production in patients with cirrhosis.

This is the first human study of the constant ammonia infusion technique to quantitate whole-body ammonia metabolism in healthy persons, to describe pathologic changes in cirrhosis and unravel the mechanisms of ammonia-targeting treatments. Only few comparable studies exist. A similar technique was used in a historic study of 19 mongrel dogs and eight patients with various liver diseases conducted in 1969 but never repeated.[20] It reported a whole-body ammonia clearance of 4.4 L/min, which is higher than 2.7 L/min in our patients who all had cirrhosis. The differences may partly be explained by case mix, a shorter 60-minute infusion time with larger deviations from certain steady state and less strict handling of blood samples. Furthermore, a study from 1979 followed disappearance of plasma ammonia after a bolus and calculated a combined mean whole-body clearance of 4.7 L/min for patients with cirrhosis and healthy persons.[21] Whereas our infusion-stop data clearly demonstrated both fast and more slowly exchanging distribution volumes, the bolus method will be biased by the fast-exchanging compartments and will therefore overestimate clearance, which probably explains the difference between our observations. Two studies used a constant infusion of ammonia to examine neuropsychological effects of induced hyperammonaemia in healthy persons and patients with cirrhosis[16,17] but did not calculate metabolic values. From these published data, we tentatively calculated a 3.6 L/min clearance in healthy persons and 2.8 L/min in patients with cirrhosis; which is close to our findings.[16,17] Taken together, despite the paucity of previous publications on the issue, the available data support the plausibility of our measurements.

Although some relation exists between ammonia blood levels and the grade of HE and prognosis in patients with cirrhosis,[6–10] it has been difficult to establish a clinically important role of single blood ammonia measurements in patients.[11,22] Since blood ammonia concentration is a product of the balance between clearance and production, separate measurement of clearance and production will provide a better pathophysiological understanding. In our patients, ammonia clearance was ~20% lower and ammonia production nearly threefold higher than in healthy persons (Table 2). Mathematically speaking, basal ammonia concentration will increase by 300% after a threefold increase in production and by a further 25% after an additional 20% clearance reduction, together explaining the fourfold increase in ammonia observed in patients with cirrhosis (Table 2). From this perspective, increased ammonia production was quantitatively the more important cause of hyperammonaemia, which supports this as a rational therapeutic target. The effect of reduced ammonia clearance was possibly lower than expected based on the known deficiencies in urea synthesis and hepatic glutamine synthesis in patients with cirrhosis; and future studies may explore whether reduced clearance plays a more dominant role in more advanced disease.[1,23,24] It should also be kept in mind that production (μmol/min) and clearance (ml/min) cannot be compared directly because ammonia removal from plasma in μmol/min (clearance multiplied by concentration) will increase with increasing ammonia concentrations. Thus, during metabolic stress and high ammonia production situations, *e.g.* bleeding and intestinal dysbiosis, a moderate reduction in clearance may have a clinically important impact on the systemic ammonia concentration.

Healthy persons were studied after a short-term intervention with phenylbutyrate, which is used to lower ammonia in hyperammonaemic urea cycle disorders[25] and thought to increase ammonia elimination by renal glutamine excretion after phenylacetylglutamine formation. In accordance with this expected mode of drug action, treatment increased ammonia clearance (Table 3 and Fig. 2). This supports the utility of the method for ammonia metabolism studies.

In cirrhosis, it remains a paradox that anti-HE treatments like lactulose, rifaximin, L-ornithine-L-aspartate and certain newer trial drugs believed to target ammonia may have beneficial

Table 3. Ammonia metabolism – before and after ammonia-targeting intervention.

	Healthy persons (n = 7)			Patients with cirrhosis (n = 8)		
	Before intervention	After intervention (glycerol phenylbutyrate)	*p* value	Before intervention	After intervention (lactulose + rifaximin)	*p* value
Ammonia levels (μmol/L)						
Arterial, baseline	12 (7–16)	15 (12–17)	**0.04**	46 (31–60)	42 (25–58)	0.29
Arterial, infusion	114 (96–132)	105 (93–118)	0.05	164 (122–205)	177 (122–231)	0.28
Whole-body ammonia metabolic parameters						
Ammonia clearance (L/min)	3.4 (2.9–4.0)	3.8 (3.2–4.4)	**0.009**	2.9 (2.3–3,6)	2.6 (2.1–3.2)	0.18
P, Ammonia production rate (μmol/min)	40 (26–53)	56 (42–71)	**0.002**	128 (98–158)	103 (75–132)	**0.03**
Ammonia V_d (L)	12.4 (10.3–15)	14.6 (7.7–21.5)	0.51	18.5 (15.0–22.0)	15.4 (11.8–18.9)	0.07

V_d: Volume of distribution.
Values are given as mean (95% CI).
Dependent and independent sample *t* test was used for paired and unpaired observations, respectively.

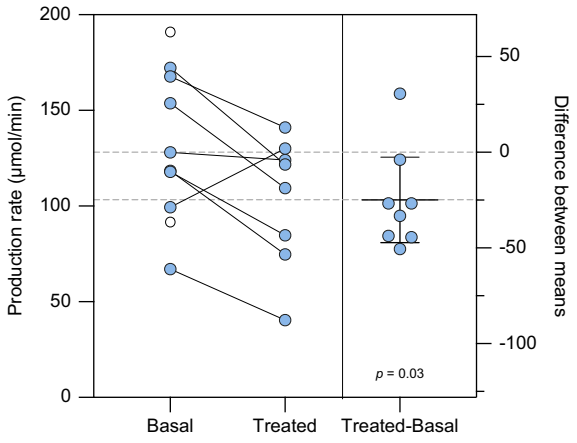

Fig. 3. Effect of lactulose in combination with rifaximin on ammonia production rate in patients with cirrhosis. Estimation plot of baseline endogenous ammonia production rate before the intervention during basal conditions (basal) and after ammonia-targeting intervention with lactulose in combination with rifaximin (treated) in patients with cirrhosis (n = 8). Dotted lines indicate basal and treated group mean production and differences between means. Individual and group mean differences and 95% CIs are shown. Individual ammonia production rates of patients with cirrhosis who were examined only on Day 1 (basal conditions, n = 2) are shown (white dots). Dependent sample *t* test was used for statistical comparison.

clinical effects without systematically changing ammonia levels.[26–29] This emphasises the need for a better physiological understanding of their mode of action. We examined the effect of lactulose + rifaximin and showed that the primary effect was a ~25% reduction in ammonia production, occurring without discernible changes in ammonia concentration (Table 2), which is in accordance with the assumed, but hitherto undocumented, mode of action of these drugs. We speculate that diminished ammonia production may reduce the ammonia load to the brain, especially in conditions in which transient ammonia fixation into glutamine in the reduced muscle mass may be exhausted. Notably, the treatment only reduced ammonia production by 20%, leaving room for additional treatments that target ammonia production. The data from *Experiments 2.1 and 2.2* showed that the constant ammonia infusion technique can quantify the pathophysiologic defects in whole-body ammonia metabolism in cirrhosis as well as the effects of drugs.

It should be kept in mind that the technique measures the resulting net sum of numerous metabolic processes in several organs that produce and bind ammonia. This was also reflected in the ammonia concentration decay curves that did not fit even complex compartmental models (not shown). In accordance, in both groups, clearance by far exceeded hepatic perfusion, reflecting extrahepatic ammonia removal from the blood.[30]

Other ammonia metabolism tests have been described.[31–33] Best known is the glutamine challenge test in which blood ammonia is followed after an oral glutamine load.[33,34] At best, the test is semi-quantitative, relying on intestinal uptake and glutaminase activity. The constant ammonia infusion technique, in contrast, makes direct use of the substrate under investigation.

Some considerations and limitations apply to the study. (A) The ammonia clearance is so high that it will also depend on cardiac output.[30] Cardiac output may be increased in some patients as part of the hyperdynamic circulation.[35] As the clearance measure reported here may to some extent be maintained by this mechanism, the derangement of the enzymatic processes behind ammonia removal may be slightly underestimated. (B) The method assumes steady-state conditions,[30] which was successfully achieved in most but not all patients (Fig. S2). This may have caused overestimation of ammonia clearance and hence of production (Equation III). However, even in the most extreme example (ID 17), overestimation will only be approx. 10%, which may be considered acceptable. Furthermore, a lower infusion rate may have yielded closer to steady-state conditions in patients with the lowest clearance. (C) The result of the constant ammonia infusion technique likely depends on changing metabolic conditions.[36,37] Thus, feeding state, protein catabolism and muscle activity were standardised in our study. The method may be used to describe and quantitate the effects of these factors. (D) Changes in splanchnic blood flow may theoretically affect systemic ammonia clearance. In normal physiology, splanchnic blood flow is increased by feeding and reduced during exercise. To control for these effects, participants were fasted for 12 h and abstained from exercise for 48 h before their examination. The ammonia infusion itself is unlikely to alter hepatosplanchnic haemodynamics as it remains unchanged even during high-rate amino acid infusion.[38] (E) It may be considered whether the methodology could employ enteral ammonia administration. We emphasise that our aim was to quantitate

ammonia clearance from systemic blood and ammonia release ('production') from the tissues into systemic blood. Doing so requires knowledge of the rate of ammonia infusion into the blood stream (Equations I-III). After enteral administration, ammonia would be metabolised in the intestines and partly removed by the liver before entering systemic circulation. Therefore, the calculations described by Equations I-III would not be possible. Consequently, our method requires intravenous administration of ammonia to system (F) The concentration of ammonia in systemic circulation is determined by influx to and removal from the systemic blood by a number of tissues, and the methodology presented herein measures the combined contributions of all tissues including the hepatosplanchnic system, muscles and kidneys. However, the method does not separate the effect of single tissues on ammonia metabolism, which needs to be studied separately if their effect on whole-body systemic clearance and production is to be determined. (G) We assumed first-order kinetics of ammonia removal from the bloodstream. Therefore, a shift towards zero-order kinetics (saturation) would challenge our calculations. However, the achieved steady states without appreciable accumulation of ammonia speaks against this possibility. Still, prolonged infusions might dynamically alter ammonia metabolism by depletion of carbon skeletons in muscles and stimulation of ureagenesis.[1,23,24]

The handling of samples and analysis of ammonia should be considered a particular source of bias. The measurement of ammonia demands great diligence in terms of standardised conditions, blood collection on ice and rapid processing.[22,39] In the present study, efforts were made to limit preanalytical factors. Also, the calculation of clearance (Equation III) is robust towards minor post sampling ammonia concentration drift as long as sampling factors remain static within the subject.[18] The calculation of production is more sensitive to such factors and, in our study, we adjusted accordingly to account for the effect of batch analysis on ammonia concentration drift.

The ammonia-chloride infusion was safe and generally well-tolerated. No alteration in mental state was observed. This is in line with two other studies that used twice the infusion rate and specifically studied cognitive function.[16,17] The one patient who developed HE probably had an occult gastrointestinal bleed unrelated to the infusion and the ammonia infusion most likely played an additional role in HE development. Due to this incident, as a safety measure, future studies in patients with more advanced disease may employ a lower ammonia infusion rate. A lower infusion rate will not change the estimation of parameters provided the ammonia concentration increase during infusion can be measured accurately and the assumption of first-order kinetics is valid. Anyhow, ammonia concentration should be monitored in real time during infusion in these patients to avoid ammonia levels exceeding 250 μmol/L even for shorter time periods.

The constant ammonia infusion technique creates opportunities for future studies of normal physiology and pathophysiology – not least studies on the effects of ammonia-targeting therapies – and may be employed in human and animal experiments alike.

In conclusion, the constant ammonia infusion technique is a feasible method for description and quantification of whole-body ammonia metabolism in humans. The technique may provide new insights into human physiology and pathophysiology and elucidate the effects of therapeutic HE interventions.

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Abbreviations

HE, hepatic encephalopathy; V_d, volume of distribution.

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Conflict of interest

The authors declare no conflicts of interest that pertain to this work.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Peter Lykke Eriksen, Hendrik Vilstrup and Peter Ott conceived and designed the study. Experiments and data collection were performed by Peter Lykke Eriksen and Lars Djernes. Data were analysed by Peter Lykke Eriksen. The first draft of the manuscript and figures were prepared by Peter Lykke Eriksen. All authors contributed to the editing and revision of the manuscript and approved the final version.

Data availability statement

Individual ammonia concentration curves of the study participants have been provided as supplementary material.

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Supplementary data

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