Blood Ammonia Levels are Labile and Responsive to Protein Intake in Patients with Compensated Cirrhosis without Overt Hepatic Encephalopathy

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BACKGROUND

Ammonia is metabolized by the liver into non-toxic substances. In cirrhosis, this capacity for ammonia metabolism is exceeded, resulting in hyperammonemia, associated with hepatic encephalopathy (HE). Data on blood ammonia levels in patients with cirrhosis, especially related to the effects of sample handling and processing, are critical and need more investigation.

AIMS OF THE STUDY:

1) To determine the level and interindividual variability of fasting spot venous ammonia in patients with cirrhosis who have not had an episode of overt encephalopathy
2) To determine the impact of a meal in the form of a standardized protein shake on the level of spot venous ammonia in patients with cirrhosis who have not had an episode of overt encephalopathy
3) To compare the local reference range in healthy volunteers (HV) using standardized procedures for blood sampling and sample handling vs. routine sample processing

METHODS

Study Population: Part 1 (Ammonia level and effect of a protein meal in cirrhotic patients)

Adult patients aged ≥ 18 to < 75 years with stable chronic liver disease with cirrhosis of any etiology who had not had an episode of hepatic encephalopathy requiring hospitalization were enrolled at three centers. Patients were eligible for enrollment regardless of gender or race/ethnicity. Key exclusion criteria were Child-Turcotte-Pugh score of > 9, history of liver transplant, body mass index < 18.5 or ≥ 40 kg/m^2, prior transjugular intrahepatic portosystemic shunt placement, treatment with systemic antibiotics (including rifaximin) within 4 weeks prior to enrollment, and current or prior use of laxatives (including lactulose) within 2 weeks prior to enrollment.

Part 1 design

After a fast (>4h from last meal) a venous blood sample was drawn for analysis of baseline spot venous ammonia. Subjects were then given a standardized meal (Ensure Enlive® protein shake containing 20g of protein), and spot ammonia was measured from repeat samples at 1h and 2 hrs after the meal. Subjects were observed in the unit for a total of 3 hours after the standardized meal to ensure no symptoms related to elevated ammonia emerged.

Study population: Part 2 (local reference range)

Approximately 30 healthy volunteers per study site were enrolled at 5 clinical sites. Male and female patients aged ≥ 18 to < 75 years with no chronic illnesses, or acute illness within 1 month prior to screening, no regular medications, or excessive alcohol use were eligible. Liver function tests (alanine aminotransferase [ALT], aspartate aminotransferase [AST], alkaline phosphatase [ALP], and bilirubin) had to be within the normal range.

Part 2 design

Samples for plasma ammonia were obtained after fasting (>4 hrs from meal) by venipuncture from a free-flowing venous blood sample (no tourniquet) and collected into two pre-chilled 4.0 mL NA/Hep specimen tubes. Samples were kept on ice after collection until immediate processing and analysis within 60 minutes from blood draw. Each site used their routine ammonia assay for analysis. Fresh samples were run in duplicate and compared to flash-frozen samples analyzed within 48 hours. The detailed process is outlined in Table 1.

Table 1: Ammonia sample processing

Blood draw from free-flowing venous sample (no tourniquet)
Collect into pre-chilled 4.0 mL Na/Hep tube
Keep on ice until immediate processing
Centrifuge at 3000 RPM at 4°C for 10 min
Pipet plasma into a 3.6mL Nunc starfoot cryovial kept on ice
Store sample on ice or 2-8°C refrigerator for max 20 min until analysis
Analysis within 60 minutes of blood collection

Statistical methods

A linear, mixed-effects model was used to estimate the significance of the described covariates. The model had a random effect by subject and fixed effects for time point and the current covariate tested. Significance for covariate effects was determined by minimum Akaike Information Criterion (AIC). The 95th percentile of a sample of approximately 30 healthy volunteers was used as the ULN for each site. All analyses were performed using R version 3.5.1 with the lme4 library.

RESULTS

Part 1

A total of 64 subjects with cirrhosis were enrolled at three sites. The mean (SD) age was 59.8 (7.0); range 44 - 71 years. The majority were male (58%). Mean (SD) MELD Score was 8.43 (2.21); range 6.43 - 14.86. Spot venous fasted ammonia at baseline was 33.71 (13.78; 18-85) µmol/L. Venous ammonia 1 and 2 hrs after the standardized 20g protein meal was 37.71 (13.86; 17-80) and 39.82 (15.20; 17-96) µmol/L, respectively; a 12 and 18% increase relative to baseline, respectively (Figure 1). The increase post meal was statistically significant (p<0.01). Inter-individual variability was 71% of the total variance (SD=9.3 µmol/L) while 29% was intra-individual variability (SD=5.9 µmol/L). Subjects with MELD>12 had a higher baseline ammonia level than those with MELD ≤12 (Figure 2). An increase of 1 point in MELD score correlated with 8% increased ammonia concentration without difference between time points. Ammonia concentrations did not appear to differ by sex or age. None of the subjects developed confusion or signs of HE.

Part 2

The demographics of the healthy volunteers at each study site are shown in Table 2. Correlation between paired fresh samples was excellent (coefficient 0.95) (Figure 4), whereas correlation between the average of the two fresh samples and the frozen sample was only moderate (coefficient 0.62) (Figure 5).

Table 2. The demographics of the healthy volunteers at each study site.

	A (N=29)	B (N=30)	C (N=30)	D (N=32)	E (N=29)
Female	12 (41.4%)	16 (53.3%)	24 (80.0%)	23 (71.9%)	19 (65.5%)
Male	17 (58.6%)	14 (46.7%)	6 (20.0%)	9 (28.1%)	10 (34.5%)
Age Mean (SD)	39.0 (14.5)	27.2 (2.9)	26.7 (5.9)	35.2 (14.3)	44.8 (14.1)
Age Range	19 - 72	22 - 34	18 - 45	18 - 66	23 - 70

Table 3. The mean (SD) varied across the local labs despite strict sample processing and analysis processes. The upper limit of normal (ULN) value, defined as the 95th percentile of a sample of approximately 30 healthy volunteers was in most cases clearly lower than the laboratory's routinely used ULN.

Site	Mean (SD)	Min	Max	Median	95th percentile	Routine ULN
A	21.33 (12.17)	5	62	20	38	32
B	23.85 (5.74)	12	35	24	34	47
C	12.32 (6.78)	5	28	12	25	52 (F), 60 (M)
D	16.94 (9.13)	5	41	15	33	30
E	22.87 (6.89)	10	45	21	37	72

Figure 1. Ammonia concentration after consumption of a 20g protein meal in patients with cirrhosis.

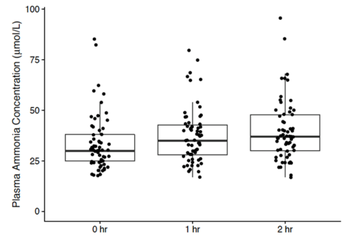


Figure 2. Baseline ammonia in subjects according to MELD score.

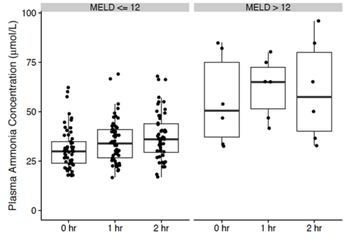


Figure 3 Plasma ammonia reference range based on 30 healthy volunteers using standardized sample processing and analysis procedures. Red bar denotes the ULN using 95th percentile.

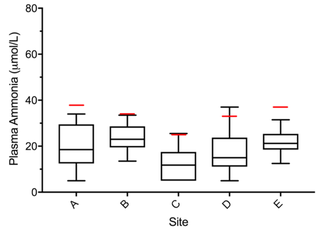


Figure 4. Correlation of paired ammonia samples analyzed fresh within 60 minutes from blood (correlation coefficient 0.95).

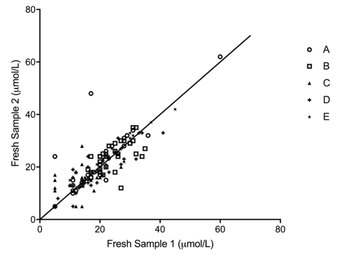
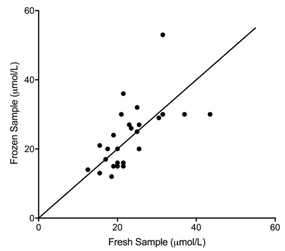


Figure 5 Correlation of ammonia samples analyzed fresh within 60 minutes from blood draw (shown mean of two paired fresh samples) with samples analyzed after a freeze-thaw cycle (correlation coefficient 0.62).



CONCLUSIONS

- Venous ammonia is elevated in a subset of patients with compensated cirrhosis without a history of overt HE and increases significantly after a meal containing 20g of protein for at least two hours.
- Sample handling and processing have a major impact on ammonia levels and are critical for data quality.
- Normal range determined using healthy volunteers and strict sample processing and analysis procedures can differ significantly from normal range provided by the kit manufacturer.
- Correlation between paired fresh samples is excellent but freezing affects ammonia levels.
- Age and gender do not appear to influence ammonia level, but higher MELD score is associated with higher baseline ammonia.