Hepatic Encephalopathy—A Guide to Laboratory Testing

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KEYWORDS

• Hepatic encephalopathy • Ammonia • Hyperammonemia • IL-6

KEY POINTS

- Laboratory testing for hepatic encephalopathy (HE) should be tailored to the clinical scenario.
- Blood testing of ammonia is not diagnostic for HE, but it can be helpful in supporting or excluding a diagnosis of HE when in doubt.
- Testing of blood ammonia requires careful adherence to laboratory protocols to minimize preanalytical variance and spuriously elevated values.
- Historically, ammonia testing has correlated only weakly with the severity of disease in type C HE; however, increasing data point to the prognostic value of ammonia, both in type A and type C HE.

HEPATIC ENCEPHALOPATHY—BACKGROUND

Hepatic encephalopathy (HE) is defined as brain dysfunction caused by liver insufficiency and/or portosystemic shunting, manifesting with a wide array of neurologic and/or psychiatric abnormalities that range from subclinical (or "minimal") alterations to coma.[1] Conditions in which HE occurs include decompensated cirrhosis (type C), acute liver failure (ALF) (type A), and portosystemic shunts (type B), which may be either iatrogenic (eg, surgical shunt or transjugular intrahepatic portosystemic shunt [TIPS]) or an inherent anatomic malformation. HE can be staged using the West Haven Criteria, and more recent updated terminology categorizes HE as either overt (stage 2–4) or covert (minimal to stage 1).

The pathophysiology behind HE is complex and multifactorial. Elevated blood ammonia, a neurotoxin, has long been known to be a necessary component of the syndrome. Ammonia crossing the blood-brain barrier, causing neurologic dysfunction, was thought to be a large part of the pathophysiology dating back over 120 years.

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Ammonia is produced by the deamination (via glutaminase) of nitrogen-containing amino acids in the intestine (both the microbiome as well as the enterocytes), liver, kidney, brain (astrocytes), and muscle. Conversely, ammonia can be consumed by the reverse process of amination of glutamate (via glutamine synthetase), forming glutamine in these same tissues. The resultant interorgan ammonia trafficking helps to explain the multifactorial nature and flux of hyperammonemia. The elimination of ammonia is achieved via the elimination of nitrogenous wastes, largely via urea (via the hepatocytic urea cycle by way of the kidney) or through the stool, either as a waste product or via bacterial protein synthesis. While the normal functioning liver largely succeeds in maintaining ammonia homeostasis, elevated blood ammonia concentrations can arise due to portosystemic collaterals shunting blood through or around the liver, comorbid renal dysfunction, and sarcopenia leading to decreased ammonia utilization and/or excretion.[1] It is important to keep in mind, however, that an elevated ammonia level, if confirmed, confers a diagnosis of hyperammonemia, which may have other nonhepatic causes. The syndrome of HE appears to require hyperammonemia, but more recent literature points to additional insults that may predispose patients with cirrhosis and portal hypertension to an overt HE event, such as increased permeability of the blood-brain barrier in patients with liver disease, possibly allowing increased NH4 into the central nervous system (CNS); similarly, increased oxidative stress, excess bile acids, and systemic inflammation may play a role in compromising mitochondrial energy production within the brain. Increased ammonia may lead to astrocyte dysfunction due to excess glutamine accumulation (via glutamine synthetase), leading to osmotic swelling, as well as direct mitochondrial toxicity, leading to bioenergetic failure.[2]

The diagnosis of HE is largely a clinical one, without the requirement for any confirmatory laboratory testing in the proper clinical scenario. Laboratory tests, however, can be critical in excluding other diagnoses in conjunction with clinical assessment. Herein, the authors will review the fundamentals of laboratory testing in the evaluation and management of patients suffering from HE.

AMMONIA

Technical Review

Ammonia is an unstable analyte, a byproduct of amino acid metabolism.[3] It exists in both a gaseous form (NH3) and a protonated or ionized form (NH4+). The interchange depends upon the pH of a solution, with more alkaline solutions shifting the balance to more NH3; however, the vast majority of ammonia exists as ammonium (NH4+) at physiologic pH (~98%). Due to its instability, proper specimen collection and analysis are important for accurate measurement of blood ammonia levels (**Table 1**).

When obtaining a blood ammonia level, either venous or arterial sampling can be used. In theory, the ammonia level in a venous sample would be expected to be lower than in an arterial sample if the muscle, kidney, and brain are contributing to the utilization and disposal of ammonia before venous return. Accordingly, early laboratory investigations demonstrated a significant increase in arterial ammonia compared to simultaneous venous ammonia in a small study of 14 patients with cirrhosis ($P<.01$).[4] However, subsequent studies suggest that arterial levels are similar to venous samples and may not provide additional diagnostic nor prognostic capabilities, particularly in type C HE.[5,6] Other studies, particularly for type A HE in ALF, have suggested the use of arterial ammonia is strongly preferred; however, this was not confirmed in one of the largest retrospective analyses of ammonia in ALF.[5,6] Various society guidelines do not specify the source of the ammonia testing.[1,7,8]

Table 1
Clinical pearls on preanalytical factors associated with ammonia levels

Category	Publication	Outcome
Arterial vs venous source	Snady et al,[4] 1988	Arterial ammonia is significantly greater than venous ammonia (P<.01)
	Nicolao et al,[17] 2003	Arterial, venous, and partial pressure of ammonia were equally limited in their diagnostic and prognostic capabilities.
	Ong et al,[16] 2003	Venous sampling is adequate compared to arterial (r = 0.56 vs 0.61), and there is no advantage of using partial pressure of ammonia (in either venous or arterial samples).
Tourniquet use	Saleem et al,[48] 2009	Tourniquet time of more than 1 min increases hemolysis (OR 19.5, 95% CI 5.6–67.4)
Exercise (or clenched fist) and ammonia levels	Wilkinson et al,[49] 2010	Hyperammonemia occurs in exercise due to increased production from the contracting muscles. Ammonia levels in extreme exercise often surpass those in patients with cirrhosis.
Collection tubes	Goldstein et al,[10] 2017	Lithium heparin tubes were significantly less accurate than EDTA tubes. Lithium tubes may underestimate ammonia levels.
Temperature handling	Nikolac et al,[3] 2014	Samples centrifuged at 0°C had a 9.5% increase in ammonia level compared to 28% increase when centrifuged at room temperature (P=.033). Samples placed in ice immediately after centrifugation had significantly lower increase in ammonia level compared to samples stored at room temperature (13% vs 31%, P=.008)
Timing of specimen processing	Imbert-Bismut et al,[50] 2020	Ammonia samples take longer to arrive to the laboratory through pneumatic tube transport compared to walking the sample to the laboratory. Ammonia concentrations were not affected if the sample is processed within 1.75 h while at 4 °C
	Howanitz et al.[10]	Delay in processing of plasma samples by 24h at 4C increased ammonia levels by 37%
Other analytes may affect ammonia levels	Nikolac et al,[3] 2014	Various serum markers can lead to ammonia bias: ALT: r = 0.38, P = .001; RBC: r = 0.18, P=.025; GGT: r: 0.19, P=.015; WBC: r = 0.178, P=.024.
	da Fonseca-Wollheim,[51] 1990	In vitro formation of ammonia through deamination of glutamine by elevated plasma GGT can increase serum ammonia level by up to 30-fold.
Fasting vs. post-prandial ammonia levels	Bajaj et al,[12] 2020	Cirrhotic patients had a 12% (1h) and 18% (2h) post-prandial increase in ammonia relative to baseline.

Abbreviations: ALT, alanine transaminase; CI, confidence interval; EDTA, ethylenediaminetetraacetic acid; GGT,gamma-glutamyltransferase; OR, odds ratio; RBC, red blood cell; WBC, white blood cell.

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Additionally, studies have not confirmed the utility of testing the partial pressure of NH3 (representing the nonionized gaseous form) compared to total ammonia (representing gaseous NH3 and ionized NH4). Ultimately, the convenience and safety of venous sampling make this the preferred route for the majority of patients undergoing testing.

Because many factors may influence the ammonia level, it is important to have protocols in place to optimize accuracy in the laboratory measurement of ammonia. Erythrocytes have 3-fold higher concentration of ammonia, so a hemolyzed sample will significantly skew the result. Therefore, free-flowing blood is desired, and a tourniquet should not be used. Additionally, due to the muscle release of ammonia, patients should not clench their hand during blood draw.[3,9] Ethylenediaminetetraacetic acid (lavender top) is the preferred anticoagulant due to concerns of falsely low ammonia levels in the presence of heparin. The specimen tube is ideally pre-chilled and should be filled completely.[10] Since red blood cells are a potential source of ammonia diffusing into the serum, anticoagulated samples should be centrifuged to separate plasma as soon as feasible and ideally within 15 minutes of collection. The importance of promptly separating plasma from red blood cells to avoid spuriously elevated ammonia levels was demonstrated in a study of healthy volunteers noting a near 50% increase between results for heparinized plasma versus whole-blood specimens (30 vs 44 micromol/L, $P < .001$).[11] Following centrifugation, plasma is generally processed as a fresh sample but may also be frozen (−20C or −70C) for up to 24 to 48 hours. Fresh samples are preferred, as frozen samples often report a lower value.[12]

Once properly collected and processed, plasma ammonia can then be measured via either indirect or direct methods. In one indirect method using a point-of-care and dry-slide analyzer, free ammonia is liberated from the sample via alkalization. Ammonia then passes through a semipermeable membrane, changing the color of an ammonium indicator, which is then measured by reflectance spectroscopy. A second and more commonly utilized method directly measures ammonia through its reaction with α-oxoglutarate and reduced nicotinamide adenine dinucleotide phosphate (NADPH) forming glutamate and NADP+ and water. An absorbance spectrometer can assess NADPH or NADP levels, therefore determining the level of ammonia needed to complete this reaction. A final method also directly measures ammonia concentration using an ammonia avid membrane, which is typically created with a mixture of nonactin and monoactin antibiotics. Ammonia may be reported in conventional units of microgram/dL or in the International System of Units of micromole/L, and it is important to note the difference, with microgram/dL approximately 1.7-fold higher. Conversion calculators are available online.

A pertinent example of how the variability in measurement of ammonia across clinical sites may compromise care can be found in the STOP-HE trial. This randomized clinical trial investigating the use of ornithine phenylacetate for the treatment of overt HE narrowly missed its primary endpoint. A post hoc analysis performed after excluding 30 subjects, initially enrolled with hyperammonemia by local testing but subsequently found to have normal ammonia levels by centralized testing, (an exclusion criterion) appeared to demonstrate a statistically significant benefit for the intervention arm.[13] However, this analysis required retesting of frozen samples which are inherently less reliable than the results from fresh samples.

Diagnostic Capabilities

Given the complex interplay of ammonia and systemic inflammation in the pathophysiology of HE, hyperammonemia by itself cannot confirm a diagnosis of HE; however, it

is unusual to diagnose HE in the setting of a normal ammonia level. HE remains a clinical diagnosis; however, an elevated ammonia level may help confirm a diagnosis when there is high pre-test probability of HE diagnosis; and the higher the ammonia the level, the more likely the diagnosis.[14] Patients with decompensated cirrhosis can have a chronically elevated baseline ammonia level with minimal to no signs of encephalopathy; therefore, routine measurement for this population is discouraged. Similar to other analytes commonly measured in clinical practice (ie, ALT), there is no agreed upon value for the upper limit of normal (ULN) of ammonia in the healthy population, nor in the cirrhotic population. Comparison studies across centers have demonstrated significant differences between sites; however, the majority of healthy volunteers demonstrate ammonia levels well below the published ULN.[12] Furthermore, there is no diagnostic threshold for blood ammonia level in diagnosing and managing HE.[1,15] The degree of elevation of ammonia only weakly correlates with severity of symptoms in cirrhosis, presumably due to the synergistic effects of inflammation on the neuropsychiatric effects of ammonia.[2,16] Clinical staging of overt HE, therefore, does not rely upon nor require ammonia measurements but remains the responsibility of the trained clinician.

Contrary to using elevated ammonia levels to diagnose HE, a normal ammonia level can often be used to exclude the diagnosis since the negative predictive value (NPV) of a normal ammonia concentration is high (NPV 0.81).[7,17] The American Association for the Study of Liver Diseases (AASLD) guidance states, "increased blood ammonia alone does not add any diagnostic, staging, or prognostic value for HE in patients with chronic liver disease. A normal value calls for diagnostic re-evaluation (GRADE II-3, A, 1)."[1] Additionally, more recently updated guidance from the European Association for the Study of the Liver (EASL) also recommends plasma ammonia testing primarily for its NPV in patients with cirrhosis.[8]

Clinicians less experienced in the diagnosis and management of overt HE may find the measurement of ammonia useful for confirming clinical suspicion, but routine measurement of ammonia for patients with cirrhosis presenting without an altered sensorium should be discouraged.[18] Studies observing trends in ordering blood ammonia have found that nearly a third of clinicians recommend routine ammonia testing in patients with grade 2 to 4 overt HE; however, of note, significantly fewer trainee physicians are likely to do so.[19] Current national trends suggest that practitioners are ordering ammonia testing at increasing rates for patients with compensated cirrhosis, quadrupling from 1.3 to 5.7 tests per 1000 inpatient days in 2007 to 2015 (P=.017); and a less pronounced increase for those with decompensated cirrhosis, rising from 7.7 to 13.1 tests per 1000 inpatient days.[20] This increase was postulated to be due to implementation of the electronic medical record and ability to routinely order these tests. However, despite the increases, the overall rates of ammonia testing appear to be low, representing only 1.9% and 4.5% of total hospitalizations in compensated and decompensated cirrhotic patients, respectively.[20] Finally, when clinicians do order ammonia levels in patients with HE, it often does not change management as 1 single-center study retrospectively examining total lactulose dosing determined. The investigators analyzed over 1200 patient admissions for HE, including 46% with the measurement of ammonia, and found that overall lactulose dosing did not differ between the groups.[15]

Prognostic Value

While ammonia cannot be used alone to diagnose HE, there is a growing literature supporting measurement of ammonia as a prognostic factor. This has been well established in patients with ALF.[5,21–23] Similarly, reduction in ammonia is associated

with better outcomes in patients with ALF.[24,25] However, utilizing ammonia for prognosis in patients with chronic liver disease has been considered controversial. Evidence now exists to challenge the AASLD guidance statement dismissing the prognostic value of ammonia for the diagnosis of HE.

In a large multicenter study of 726 stable outpatients with cirrhosis, investigators found a compelling increased risk of both hospitalization and mortality for patients with elevated ammonia (hazard ratio [HR] 2.13, confidence interval [CI] 1.89–2.40, $P<.001$; HR 1.45, CI 1.2–1.76, $P<.001$, respectively).[26] A cutoff of 1.4x the local ULN best predicted risk. It is important to note that both arterial and venous samples were utilized but were collected under a careful protocol at each site utilizing chilled collection tubes and rapid processing. The investigators also minimized variability across sites by utilizing internal controls at each laboratory and transforming each patient value to a multiple of the local ULN (termed AMM-ULN in the article).[26] For patients with documented HE, there are conflicting reports whether ammonia levels correlate with the severity of HE.[16,17] Other studies have found that ammonia can be an independent predictor of 28-day mortality and a lack of improvement to baseline ammonia by hospital day 5 is associated with high mortality.[27]

Another compelling example of the predictive capability of outpatient ammonia monitoring came from a retrospective analysis of the randomized controlled trial evaluating the ammonia scavenger, glycerol phenylbutyrate, for the prevention of HE.[28,29] The investigators collected fasting venous blood samples at baseline and days 7 and 14 of the 16-week trial. Ammonia levels were categorized in relation to the standardized ULN as follows: less than 1.0 x ULN, greater than 1.0 x ULN, less than 1.5 x ULN, greater than 1.5 x ULN. Patients with ammonia levels in the highest category (>1.5 x ULN) developed overt HE episodes at twice the rate of those with lower ammonia levels ($P = .002$).

OTHER LABORATORY TESTING IN HEPATIC ENCEPHALOPATHY

When making a diagnosis of HE, it is important to consider a broad differential diagnosis and appropriately exclude other potential etiologies for altered mental status. Various laboratory tests to be considered can be found in **Table 2**. Additional

Table 2
Laboratory testing beyond ammonia for the evaluation of altered mentation in patients with cirrhosis

Higher yield	• Comprehensive metabolic panel including calcium, magnesium, and phosphate • Complete blood count • Thyroid-stimulating hormone (TSH) • Blood culture • Urinalysis with reflex to urine culture • Urine and/or blood toxicology screen • Blood alcohol level • Lactic acid
Lower yield	• Sedimentation rate (ESR) • C-reactive protein (CRP) • Thiamine • Vitamin B12 • Human immunodeficiency virus serology • Syphilis serology • Cerebrospinal fluid analysis (required if meningismus present)

considerations for laboratory testing in the diagnosis and management of HE are outlined in the following sections.

Thyroid Axis

Hypothyroidism is believed to cause hyperammonemia through the downregulation of ammonia metabolism to urea and possibly through concomitant myopathy.[30–33] In a multicenter prospective analysis (NACSELD-2), low thyroxine (as well as low maltose, high methyl-4-hydroxybenzoate sulfate, and high 3,4-dihydroxybutyrate) levels predicted advanced HE development.[34] In multivariable logistic regression analysis, thyroxine levels were an independent predictor of advanced HE (OR 0.67, CI 0.48–0.89, $P = .01$). Similarly, in a single-center prospective cohort study of 122 patients with hepatitis B virus (HBV)–related acute-on-chronic liver failure (ACLF), free triiodothyronine (FT3) level, and its change over time enhanced prediction of 90-day prognosis, with an area under the receiver operating characteristic curve of 0.892.[35]

Interleukin–6

Interleukin-6 (IL-6) is an important cytokine driving systemic inflammation, and its presence has been associated with both covert and overt HE. IL-6 serum levels may help diagnose minimal HE (MHE), as levels $\geq$ 8pg/mL discriminated against patients with and without MHE with a receiver operating characteristic of 0.751, whereas a level $\geq$ 7pg/mL had a sensitivity of 90% with an NPV of 93%.[36] Another study assessing risks of overt HE following TIPS, found a pre-procedure serum IL-6 level greater than 10.5 pg/mL predicted post-TIPS overt HE with an area under the curve (AUC) of 0.83.[37] IL-6 was an independent risk factor for overall overt HE (RR = 1.154, $P<.001$) and for stage 4 HE (coma) (RR 1.051, $P=.019$).[37] Similarly, in a study evaluating predictors of future overt HE, patients with an IL-6 above the median of 9 pg/mL developed overt HE much more often (35.6% vs 1.9, $P<.001$).[38] In a subset of patients without prior overt HE, the predictive performance of IL-6 was better than the model for end-stage liver disease (MELD) (AUC 0.966 vs 0.843) with the ideal cut off for IL-6 of 23.5 pg/mL, with sensitivity and specificity of 89.3% and 89.5%, respectively.[38]

Endotoxin and the Microbiome

The microbiome has been implicated in the pathogenesis of HE through the release of endotoxin as well as both increased intestinal ammonia release (via urease) and absorption (incorporated into amino acid and protein production). Endotoxemia is thought to occur in the setting of both intestinal dysbiosis and an impaired intestinal epithelial barrier ("leaky gut"). Studies have found that patients with liver disease have reduced variation in microbial species. This reduction in species diversity is even more pronounced in patients with HE.[39] One study found 8 fecal bacterial species were associated with overt HE, although these species could not predict future overt HE.[40] Endotoxin, or lipopolysaccharide, is released by gram-negative bacteria and induces an inflammatory cascade upon absorption into the liver via activation of Kupffer cells. Studies have found that patients with cirrhosis and HE often have elevated serum endotoxin levels compared to patients with cirrhosis without HE (0.27 ± 0.24, 0.059 ± 0.012, respectively, $P=.002$).[41] Short-chain fatty acids, products of bacterial metabolism, have also been implicated in HE, although studies are conflicting, and this association has not been well established. While 1 study found overt HE associated with lower levels of certain short-chain fatty acids, another study found no association.[39,40] Other investigators have utilized machine learning to create prediction models for the development of HE based upon oral or fecal microbiota, finding

fecal microbiota had superior discrimination ability when identifying patients with cirrhosis with or without overt HE by history.[42]

OTHER EXPERIMENTAL LABORATORY WORKS

Interest in alternative means of measuring ammonia has led to ammonia breath testing. In 1 study, an elevated vaporized ammonia level of $\geq$165 ppb had an AUC of 0.86 in distinguishing between patients with cirrhosis versus healthy controls (95% CI: 0.79–0.93), and a breath ammonia level $\geq$ 175 ppb could distinguish between cirrhotic patients with and without HE with an AUC of 0.83 (95% CI: 0.73–0.94).[43] This may be an avenue for future research, especially as a potential point-of-care test in the outpatient setting. A recent proof-of-concept publication of a product called Wize Sniffer, an electronic semiconductor gas sensor, found that TGS2602, which detects ammonia, hydrogen sulfide, ethanol, and hydrogen, had an AUC of 0.864 (95% CI 0.662–1, $P = .00$) in differentiating between cirrhosis with and without HE at a value of 0.065.[44]

Microbially derived metabolites have been found to be associated with overt HE.[34] In 1 multicenter inpatient cohort, high methyl-4-hydroxybenzoate sulfate and 3,4-dihydroxybutyrate levels, both bacterial metabolites, along with low thyroxine, lysophospholipids, and isoleucine, were associated with overt HE with an AUC 0.87 to 0.9. Other studies investigating metabolites have found that in comparing patients with hepatitis B cirrhosis with MHE versus no HE, 27 small-molecule metabolites were found to be potential biomarkers for MHE[45]

Research focused on the CNS has observed that biomarkers of various neurons may be implicated in HE. Glial fibrillary acidic protein (GFAP) elevation in the serum is a marker of astrocyte injury. Gairing and colleagues[46] found that serum GFAP was elevated in cirrhotic patients with covert HE compared to those without (median GFAP 163pg/ml [interquartile range (IQR) 136;268] vs 106 pg/mL [IQR 75;153, $P<.001$). However, not all neurologic metabolites are diagnostically helpful. Serum S100 B is expressed in astrocytes and other glial cells. One study found S100 B to be less effective than ammonia in diagnosing covert HE.[47]

SUMMARY

Laboratory testing for the diagnosis and management of HE is largely focused on identifying possible precipitating factors and excluding other conditions. HE remains both a clinical diagnosis and one of exclusion. The nature and extent of laboratory testing will vary according to clinical circumstances. The testing of ammonia levels in the blood can be helpful when the diagnosis is in question but is neither required for confirmation in the proper clinical context nor is ammonia testing necessary for staging or prognosis with a few key exceptions: ALF, and possibly ACLF, where it can correlate with intracranial hypertension and mortality risk. Monitoring of the patient receiving treatment for HE does not require repeated ammonia testing unless the diagnosis is called into question or if treating patients with known or suspected intracranial hypertension. An argument can also be made to follow blood ammonia levels when treating patients with therapies specifically targeted at reducing hyperammonemia. More recent literature is lending support to the prognostic capabilities inherent in ammonia levels in the blood of cirrhotic patients, both in predicting future HE events and in determining outcomes in hospitalized patients. If ammonia levels are to be tested and relied upon, one must have strict protocols in place to control preanalytical factors and collection methods to avoid common pitfalls in the measurement of this labile analyte. Newer and novel biomarkers are being explored for the diagnosis and management of HE, including IL-6 and other inflammatory markers, and results are encouraging. Further studies investigating the

utility of other laboratory-based testing to diagnose, stage, or predict HE, including evaluating severity of systemic inflammation, fecal microbiota, bacterial metabolites, and neurologic biomarkers are encouraged.

CLINICS CARE POINTS

- Ammonia remains key to the pathophysiology of hepatic encephalopathy
- False elevations of ammonia occur with delays in centrifugation and processing, storing blood at room temperature, utilizing a tourniquet and/or clenching fist for blood draw, and in patients with significantly elevated ALT and/or GGT given their intrinsic enzymatic acitivites
- Ammonia tends to be higher in arterial and post-prandial blood samples

DISCLOSURE

R.T. Frederick serves as an investigator for Salix Pharmaceuticals/Bausch Health, Mallinckrodt, Astra Zeneca, River 2 Renal; a consultant for Mallinckrodt, Seal Rock Therapeutics, Tennor Therapeutics.

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