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ORIGINAL ARTICLE

Vigilance and wake EEG architecture in simulated hyperammonaemia: a pilot study on the effects of L-Ornithine-L-Aspartate (LOLA) and caffeine

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Abstract Hyperammonaemia/mild hepatic encephalopathy (HE) can be simulated by the oral administration of a so-called amino acid challenge (AAC). This study sought to assess the effects of the AAC alone and in combination with either ammonia-lowering [L-ornithine-L-aspartate (LOLA)] or vigilance-enhancing medication (caffeine). Six patients with cirrhosis (5 males; 61.3 ± 9.2 years; 5 Child A, 1 Child B) and six healthy volunteers (5 males; 49.8 ± 10.6 years) were studied between 08:00 and 19:00 on Monday of three consecutive weeks. The following indices were obtained: hourly capillary ammonia, hourly subjective sleepiness, paper & pencil/computerized psychometry and wake electroencephalography (EEG) at 12:00, i.e. at the time of the maximum expected effect of the AAC. Results: On average, patients had worse neuropsychological performance and slower EEG than healthy volunteers in all conditions but differences did not reach significance. In healthy volunteers, the post-AAC increase in capillary ammonia levels was contained by both the administration of LOLA and of caffeine (significant differences between 10:00 and 14:00 h). The administration of caffeine also resulted in a reduction in subjective sleepiness and in the amplitude of the EEG on several frontal/temporal-occipital sites ($p < 0.05$; paired t-test). Changes in ammonia levels, subjective sleepiness and the EEG in the three conditions were less obvious in patients. In conclusion, both LOLA and caffeine contained the AAC-induced increase in capillary ammonia, especially in healthy volunteers. Caffeine also counteracted the AAC effects on sleepiness/EEG amplitude. The association of ammonia-lowering and vigilance-enhancing medication in the management of HE is worthy of further study.

Keywords Cirrhosis · Hepatic encephalopathy · Nutrition · Sleep

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Abbreviations

AAC	Amino acid challenge
EEG	Electroencephalogram
HE	Hepatic encephalopathy
HÖ	Horne Östberg
HRQoL	Health-related quality of life
KSS	Karolinska sleepiness scale
LOLA	L-ornithine-L-aspartate
MCS	Mental component score
PCS	Physical component score
PHES	Psychometric hepatic encephalopathy score
PSQI	Pittsburgh sleep quality index
SF-36	Medical outcomes short form

Introduction

Hyperammonaemia is a complication of liver disease and plays a central role in the pathogenesis of hepatic encephalopathy (HE) (Blei and Córdoba 2001). HE is characterized by impaired neuropsychiatric performance, psychomotor slowing (Sherlock et al. 1954; Vilstrup et al. 2014), and excessive daytime sleepiness (De Rui et al. 2013). Hyperammonaemia causes electroencephalographic (EEG) changes that are similar to those observed over the normal transition from wakefulness to sleep, thus also indicating that hyperammonaemia is associated with neural inhibition and reduced vigilance (Montagnese et al. 2011). Excessive daytime somnolence is positively correlated with the amount of slow activity in the wake EEG of patients with cirrhosis (Montagnese et al. 2009; De Rui et al. 2013).

HE can be precipitated by variceal bleeding, a complication of chronic liver disease/portal hypertension, which can be simulated by the administration of a so called amino acid challenge (AAC), a mixture of amino acids which mimics the composition of hemoglobin. This procedure has been used both to model HE and to predict the risk of its occurrence over time (Douglass et al. 2001; Romero-Gomez et al. 2004).

L-ornithine-L-aspartate (LOLA) lowers blood ammonia by partially correcting the dysfunction in glutamine synthetase flux and in the urea cycle which characterize patients with cirrhosis (Cash et al. 2010). Both intravenous and oral LOLA administration result in reduction of blood ammonia levels and improvement in neuropsychiatric performance (Kircheis et al. 1997; Poo et al. 2006).

Caffeine is a commonly utilized stimulant (Lieberman et al. 2002), exerting its action by antagonizing the effects of the neurotransmitter adenosine (Burke et al. 2015), which is implicated in sleep homeostasis and mediates the gradual increase in sleep propensity during the waking hours of the day (Porkka-Heiskanen et al. 1997; Holst and Landolt 2015).

The aim of this study was to assess the effects of induced hyperammonaemia (AAC) and the counter-effects of ammonia-lowering (LOLA) and vigilance-enhancing (caffeine) medication. This was obtained by measuring capillary ammonia levels, subjective sleepiness, neuropsychiatric performance and wake EEG topography in healthy volunteers and patients with cirrhosis after the administration of the AAC alone or in combination with LOLA or caffeine.

Materials and methods

Six consecutive patients (5 males; 61.3 ± 9.2 years; 5 Child A, 1 Child B) with biopsy-proven cirrhosis were enrolled. Aetiology was chronic hepatitis C in three patients and chronic hepatitis B, alcohol and metabolic syndrome in one each. Patients were excluded if they could not or did not wish to complete the proposed procedures, had misused alcohol in the previous 6 months, had overt HE or were on ammonia-lowering treatment, had a history of cardiovascular/cerebrovascular disease, neurological/psychiatric illnesses, or were taking psychoactive drugs. Six healthy volunteers (5 males; 49.8 ± 10.6 years) served as controls.

Participants were asked to keep regular sleep-wake schedules in the weeks prior to and during the study. They were also instructed to refrain from daytime napping and from changing their sleep-wake habits and their intake of caffeinated/alcoholic beverages over the whole study period. Adherence was confirmed by sleep diaries and by caffeine/alcohol intake records. A baseline sleep-wake profile was obtained, to include:

i) The Pittsburgh sleep quality index (PSQI) questionnaire (Buysse et al. 1989; Curcio et al. 2013). This is a self-administered questionnaire to assess sleep quality over the previous month, and to differentiate 'good' from 'poor' sleepers. The component scores are summated to provide the PSQI global score: scores of 5 or greater identify 'poor' sleepers, and the higher the score, the worst the sleep quality (range: 0–21).
ii) The Horne-Östberg questionnaire (HÖ) (Hörne and Östberg 1976). This one is used to assess diurnal preference and consist of 19 questions in which the subject is asked to indicate his/her timing of preferred physical, intellectual and social activity. The total score ranges from 16 to 86: scores ≤41 define evening (extremely evening ≤30) types, scores ≥59 define morning (extremely morning ≥70) types, and scores between 42 and 58 intermediate types.

Baseline Health-Related Quality of Life (HRQoL) was also assessed by the Medical Outcomes Short Form (SF-36) questionnaire (Ware and Sherbourne 1992). This is a self-administered instrument consisting of 36 items summarized in eight domains ranging from 0 (worst) to 100 (best). The domains refer to a set of physical functioning aspects (physical function, role physical, body pain, and general health), which are summarized in the Physical Component Score (PCS), and a set of mental functioning aspects (role emotional, vitality, mental health, and social function), which are summarized in the Mental Component Score (MCS). The summary indices PCS and MCS were obtained based on Italian reference weights (Apolone et al. 2000).

Experimental design

Experimental sessions were planned over three consecutive Mondays and consisted of the administration of an amino acid challenge (AAC) alone or in combination with L-ornithine-L-aspartate (LOLA) or coffee. The study was single-blinded.

Thus, enrolled subjects received the AAC plus placebo (oral and intravenous doses; Condition I), the AAC plus intravenous LOLA and oral placebo (Condition II), and the AAC plus caffeine and intravenous placebo (Condition III), in a randomized order (Table 1). The AAC was administered at 08:00 and expected to cause a peak in ammonia levels at 3–4 h from the administration. LOLA infusion was expected to control/contain hyperammonaemia after approximately 4 h from the beginning of its intravenous infusion, and therefore it was also administered at 08:00, to have its expected effect overlapping with the expected peak in ammonia levels (12:00). Caffeine increases vigilance within 60–90 min after administration and thus it was administered at 10:30, to have its expected effect coinciding with the expected peak in ammonia levels (12:00) (Table 1).

The AAC consisted of a banana-flavored mixture of 54 g of amino acids, i.e. the equivalent of haemoglobin contained in 400 ml of blood (Douglass et al. 2001). This was mixed with water in a large glass and ingested over 5 min.

LOLA was supplied as ampoules [20 g, i.e. four ampules of 10 ml each (Hepa-Merz; Merz Pharmaceuticals GmbH, Frankfurt, Germany)], brought to 500 ml with saline solution 0.9 %. The intravenous placebo consisted of 500 ml of saline solution (0.9 %). Both LOLA and placebo were administered continuously over a period of 3.5–4 h.

The administration of caffeine consisted of the intake of one double-espresso coffee (≅200 mg of caffeine on average, depending on brands). Variability was contained by obtaining the double-espresso coffee from the same machine in the same hospital bar, having verified that brands and doses did not change over the study period. The oral placebo consisted of one decaffeinated coffee (minimum amount of caffeine).

Outcome measurements

Measurements of ammonia levels, subjective sleepiness and neuropsychiatric status were obtained, in order to assess the effects/counter-effects of the challenge and the ammonia-lowering and vigilance-enhancing medication.

Ammonia levels

Capillary ammonia levels were obtained on an hourly basis from 08:00 until 19:00 on each of the three experimental days (Blood Ammonia Checker; Menarini Diagnostics, Firenze, Italy) (Huizenga et al. 1995).

Subjective sleepiness

Subjective sleepiness was also assessed on an hourly basis from 08:00 until 19:00 on each of the three experimental days, by completion of the Karolinska Sleepiness Scale (KSS). The KSS ranges from 1 to 9 (1 = very alert; 3 = alert; 5 = neither sleepy nor alert; 7 = sleepy but no effort to remain awake and 9 = very sleepy, fighting sleep, difficulty staying awake) (Åkerstedt and Gillberg 1990).

Neuropsychiatric assessment

Patients underwent complete neurological examination and overt HE was excluded according to the West Haven criteria (Conn et al. 1977; Vilstrup et al. 2014).

Neuropsychological evaluation The Psychometric Hepatic Encephalopathy Score (PHES) is paper-and-pencil test battery of five tests (Number Connection Tests A and B, Digit Symbol, Line Tracing, and Serial Dotting) (Weissenborn et al. 2001) covering domains, such as psychomotor speed and attention, which are known to be affected by HE. Each test is scored in relation to local norms adjusted for age and level of education (Amodio et al. 2008). Single scores were summated to obtain the PHES, or the rounded sum of the adjusted standard deviations from the norms. Based on

Table 1 Experimental Design

	Experimental session (randomized order)		
Study day Time of day[a]	1 Condition I	2 Condition II	3 Condition III
8:00	AAC (by mouth) Placebo (iv)	AAC (by mouth) LOLA (iv)	AAC (by mouth) Placebo (iv)
10:30	Placebo (by mouth)	Placebo (by mouth)	Caffeine (by mouth)
12:00	Neuropsychology Wake EEG	Neuropsychology Wake EEG	Neuropsychology Wake EEG
19:00			

AAC amino acid challenge, EEG *electroencephalogram,* LOLA *L-ornithine-L-aspartate*

[a] Capillary ammonia levels and subjective sleepiness were measured hourly from 08:00 until 19:00

Italian reference data, PHES was considered abnormal if ≤−4 (Amodio et al. 2008).

A computerised version of the Sternberg paradigm test was also administered (Sternberg 1966). Thirty-six consecutive pairs of numbers, with or without common digits, were presented on a computer screen and subjects were asked to press 1 on the keyboard if there were common digits (i.e. 5632, 694) and press 3 if there were no common digits (i.e. 41, 75). Both accuracy (% correct responses) and reaction times (ms, adjusted for accuracy) were obtained.

Neurophysiological evaluation Wake EEG was recorded at 12:00 h (time of expected ammonia peak after AAC) for 10 min, eyes-closed, using a 21-electrode EEG cap (10–20 system; ground: Fpz; reference: Oz). Each derivation had its own analogue-to-digital converter and was band pass-filtered between 0.33 and 70 Hz; sampling frequency 256 Hz; signal resolution 0.19 μV (Brainquick 3200, Micromed, Italy). Each recording was inspected by an operator (DR, SM) and a continuous 40-s section selected for subsequent spectral analysis and classification based on spectral indices (Amodio et al. 1999).

The 19 derivations (see Bersagliere et al. 2013 for electrode positions and labels) were re-referenced to the average of all derivations, detrended, and power density spectra were calculated using a fast Fourier transform routine (average of 392-s epochs with 1 s overlap, Hanning window, 0.5 Hz frequency resolution; MATLAB function "pwelch", The Math Works Inc., Natick, MA, USA). The peak of the power density spectrum within the frequency range 6–13 Hz was indentified in each derivation (MATLAB function "findpeaks") and assumed to represent the dominant EEG activity. The results obtained were verified by visual inspection. The power of the dominant activity was computed over a 2.5 Hz interval around the individual global peak (five bins of 0.5 Hz; ±1 Hz around peak), in each derivation.

Figure composition. Maps represent colour-coded power (dB; 0 dB = 1 μV^2) and frequency (Hz) values plotted at the corresponding position on the planar projection of the scalp model (function "topoplot" from EEGLAB; Delorme and Makeig 2004). Top view, nose facing up. Values between electrodes were interpolated.

Ethics

The study was approved by the Padova University Hospital Ethics Committee (2396P, 2011). All participants provided written, informed consent. The study was conducted according to the Declaration of Helsinki (Hong Kong Amendment) and Good Clinical Practice (European) guidelines.

Statistical analysis

Data are presented as mean +/- standard deviation (SD). The variables distribution was tested for normality using the Shapiro-Wilks' test. Comparisons between patients and healthy volunteers were performed by the Student t or Mann-Whitney U test, as appropriate. Ammonia levels, subjective sleepiness and neuropsychological data in the three sessions were compared by repeated measures ANOVA and the variables *condition* and *time* used as factors. Repeated measures ANOVA was performed both over the complete period of study (08:00–19:00) and over the period of maximum, expected effect of the AAC/counter - measures (10:00 – 14:00). The dominant frequency and log-transformed power were evaluated at each derivation: comparisons between the different conditions within one group (healthy volunteers or patients) were performed with two-tailed paired t-tests, and between groups with two-tailed unpaired t-tests. Due to the low number of participants, repeated measures ANOVA (factors derivation, condition) was not feasible. Significant differences at only a single derivation or at derivations not forming a cluster were considered spurious and not taken into account as an effect.

Results

Demographic and baseline assessment variables for both patients and healthy volunteers are presented in Table 2. There were no significant differences in demographic variables, although healthy volunteers were slightly younger than patients. Similarly, there were no significant differences in sleep-wake indices, although patients had higher (worse sleep quality) average PSQI scores compared to healthy volunteers (7 ± 3.3 vs. 4 ± 3.5). The majority of patients were intermediate chronotypes, whereas healthy volunteers were mainly morning types. Albeit the differences were not significant, quality of life summary indices were lower in patients compared to healthy volunteers both in the physical (40 ± 8.4 vs. 52 ± 4.6) and mental component (49 ± 11.7 vs. 52 ± 5.5).

The experiments were performed without serious complications. However, 1 patient and 2 healthy volunteers complained of nausea in relation to LOLA administration.

Capillary ammonia levels

Figure 1 shows the time-course of hourly capillary ammonia levels in healthy volunteers (Fig. 1a) and patients with cirrhosis (Fig. 1b) in the three conditions: AAC (condition I), AAC + LOLA (condition II) and AAC + caffeine (condition III).

The AAC caused the expected, significant increase in ammonia levels, with maximum values around 4 h after intake, in healthy volunteers (Fig. 1a). Subsequently, ammonia

Table 2 Demographic, sleep-wake and quality of life indices of healthy volunteers and patients with cirrhosis

	Healthy volunteers ($n=6$)	Patients with cirrhosis ($n=6$)
Males (%)	5 (83)	5 (83)
Age (mean ± SD years)	49.8 ± 10.6	61.3 ± 9.2
Education (years)	15.5 ± 3.9	10.3 ± 3.3
HÖ (M/I/E)	4 / 1 / 1	1 / 4 / 1
PSQI	4 ± 3.5	7 ± 3.3
SF-36 PCS	52 ± 4.6	40 ± 8.4
SF-36 MCS	52 ± 5.5	49 ± 11.7

HÖ Horne-Östberg questionnaire of diurnal preference, *M* morning, *I* intermediate, *E* evening; *PSQI* Pittsburgh Sleep Quality Index; *SF-36* 36-item short form health profile questionnaire of health-related quality of life, *PCS* physical component summary, *MCS* mental component summary

concentrations abruptly dropped (from 12:00 to 13:00) to increase again slightly between 15:00 and 16:00. When either LOLA or caffeine were administered, the ammonia levels were noticeably blunted (from 10:00 to 12:00). Nonetheless, there were no significant differences between conditions, whilst the effect of time and the interaction condition*time were significant [condition: $F=3.15$, $p=0.07$; time: $F=8.61$, $p=0.00$; condition*time: $F=1.77$, $p=0.02$; Fig. 1a]. When statistical analysis was restricted to the time window where effects were expected (10:00–14:00 h), both condition and time were significant, with no interaction [condition: $F=10.35$; $p=0.00$; time: $F=11.70$, $p=0.00$; condition*time: $F=1.60$, $p=0.14$; Fig. 1a].

In patients with cirrhosis, the AAC caused a moderate elevation in ammonia levels, reaching the highest value at 11:00 (Fig. 1b). Ammonia concentrations subsequently remained almost constant until 13:00, and then progressively decreased. When LOLA was administered, a slight reduction (not significant) in AAC-induced hyperammonaemia was observed. The administration of caffeine did not result in apparent changes in AAC-induced hyperammonaemia. However, differences between conditions were not significant nor was the interaction, while the effect of time was significant [condition: $F=0.00$, $p=0.99$; time: $F=5.00$, $p=0.00$; condition*time: $F=0.80$, $p=0.70$; Fig. 1b]. The results did not change to any significant extent when analysis was restricted to the time window where effects were expected [condition: $F=0.11$; $p=0.89$; time: $F=0.84$, $p=0.50$; condition*time: $F=0.98$, $p=0.45$; Fig. 1b].

Cumulative ammonia levels (Area Under the Curve) were also determined. Total ammonia was higher in patients compared to healthy participants in all conditions, but the differences between patients and healthy participants were larger in Conditions II and III. Trends for statistical significance were observed (condition: $F=3.32$, $p=0.06$; subject category:

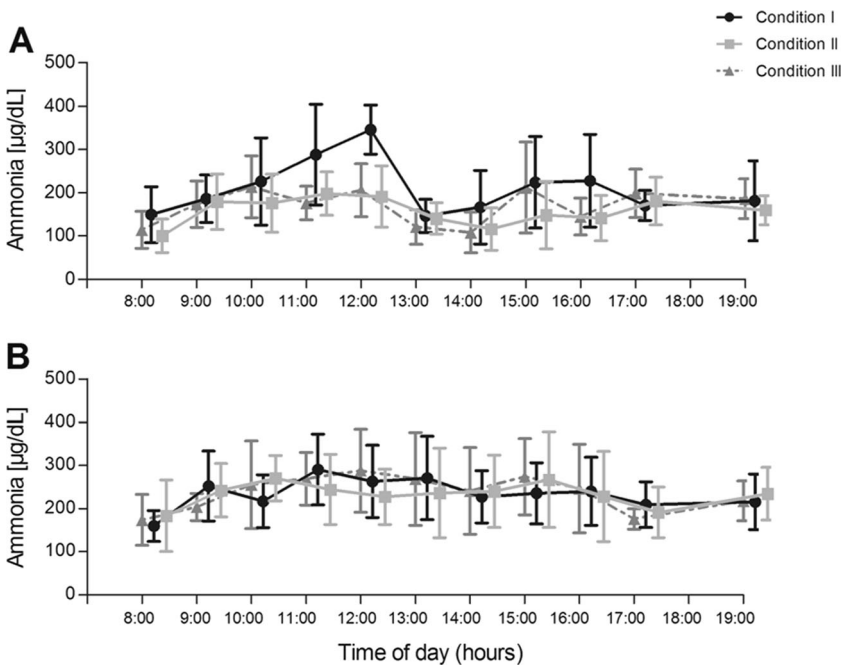

Fig. 1 Hourly capillary ammonia levels in healthy volunteers (**a**) and patients with cirrhosis (**b**), by condition. *Condition I*: AAC + placebo orally and intravenously; *black line*, *Condition II*: AAC + LOLA, intravenously + placebo orally; *gray line*, *Condition III*: AAC + placebo intravenously + caffeine orally; *gray broken line*

F = 4.87, $p = 0.05$; condition* subject category: F = 2.93, $p = 0.07$). Differences where more obvious and some became statistically significant when only the morning hours (8:00–12:00) where considered (condition: F = 5.74, $p = 0.01$; subject category: F = 4.14, $p = 0.07$; condition* subject category: F = 4.32, $p = 0.03$).

Subjective sleepiness

Figure 2 shows the time-course of sleepiness in healthy volunteers (Fig. 2a) and patients with cirrhosis (Fig. 2b) in the three conditions.

Limited changes were detected in subjective sleepiness in healthy volunteers on the day when the AAC was administered. Similarly, sleepiness was not altered when the AAC + LOLA were administered. The administration of caffeine was associated with slightly lower sleepiness levels compared to the other two conditions, especially between 12:00 and 14:00. However, there were no significant differences between conditions nor an effect of time [condition: F = 0.07, $p = 0.92$; time: F = 1.59, $p = 0.10$; Fig. 2a]. When statistical analysis was restricted to the time window where effects were expected (10:00–14:00 h), time was significant [condition: F = 0.66; $p = 0.52$; time: F = 3.91, $p = 0.00$; condition*time: F = 0.87, $p = 0.54$; Fig. 2b].

In patients with cirrhosis, virtually no modifications in subjective sleepiness were observed in any of the three conditions, and statistical analysis yielded no significant differences (Fig. 2b).

Neuropsychiatric assessment

Neuropsychological evaluation

On average, patients had worse neuropsychiatric performance than healthy volunteers, with worse PHES, and slower and less accurate Scan test performances (Table 3). However, none of the patients/healthy volunteers had abnormal psychometry. No significant differences in PHES or Scan scores were observed in relation to condition (Table 3).

Neurophysiological evaluation

On average, patients with cirrhosis had slower EEGs (Fig. 3a) compared to healthy volunteers. However, differences were not statistically significant and none of the EEGs was abnormal. Similarly, EEG frequencies did not differ in relation to the conditions (Fig. 3a).

On average, patients with cirrhosis had lower EEG amplitude (power of dominant activity) compared to healthy volunteers (Fig. 3b) but the variability was considerable and thus, differences not significant. In the patients, EEG power of the dominant frequency was similar in the three conditions (Fig. 3b). In contrast, in healthy volunteers the administration of caffeine together with the AAC resulted in reduced EEG power at several frontal and posterior derivations compared to administration of AAC alone.

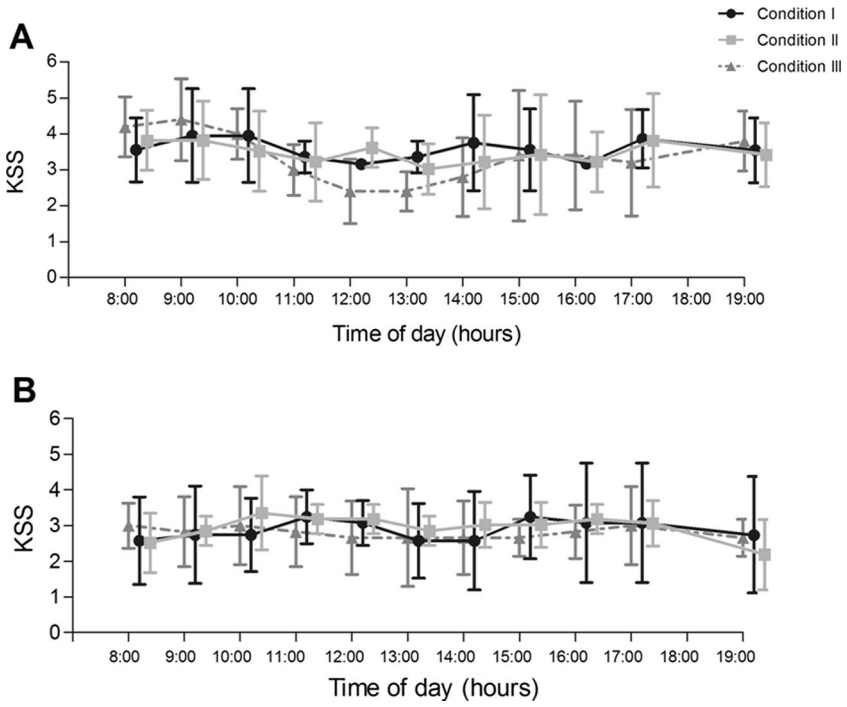

Fig. 2 Hourly subjective sleepiness ratings (Karolinska Sleepiness Scale) in healthy volunteers (**a**) and patients with cirrhosis (**b**), by condition. *Condition I*: AAC + placebo orally and intravenously; *black line*, *Condition II*: AAC + LOLA intravenously + placebo orally; *gray line*, *Condition III*: AAC + placebo intravenously + caffeine orally; *gray broken line*

Table 3 Neuropsychiatric variables in healthy volunteers and patients with cirrhosis, by Condition

	Healthy volunteers ($n=6$)			Patients with cirrhosis ($n=6$)		
	Condition I	Condition II	Condition III	Condition I	Condition II	Condition III
PHES	4.4 ± 0.8	4.6 ± 0.5	4.6 ± 0.5	1.5 ± 1.2	1.8 ± 1.1	1.8 ± 1.6
Sternberg reaction time (ms)	1083 ± 28	1076 ± 202	1134 ± 159	1474 ± 20	1584 ± 243	1570 ± 154
Sternberg accuracy (%)	92 ± 5	90 ± 3	92 ± 7	86 ± 6	84 ± 6	86 ± 8

Condition I: AAC + placebo by mouth and intravenously

Condition II: AAC + LOLA intravenously + placebo orally

Condition III: AAC + placebo intravenously + caffeine orally

PHES psychometric hepatic encephalopathy score

Discussion

Capillary ammonia

In healthy volunteers, the AAC produced an increase in capillary ammonia concentrations 3–4 h after administration, which is in substantial agreement with previous studies (Douglass et al. 2001; Bersagliere et al. 2012; Casula et al. 2015). This was followed by an abrupt decrease in ammonia levels, most likely in relation to the fact that ammonia is a high extraction molecule and its hepatic removal is flow-dependent. A second, smaller peak in capillary ammonia concentrations was observed in the afternoon hours (15:00–16:00), also in line with previous observations (Bersagliere et al. 2012) and most likely in relation with large intestine (as opposed to small intestine) absorption of the AAC.

The administration of AAC combined with LOLA, which has been reported to exert a favorable influence on HE by virtue of its blood ammonia-lowering effects, contained the increase in capillary ammonia levels, and the expected AAC-induced peak in ammonia concentration around 12:00 was not observed. LOLA also appeared to completely neutralize the smaller, afternoon ammonia peak, thus confirming its ammonia scavenging properties (Kircheis et al. 1997; Bai et al. 2013).

The administration of AAC combined with caffeine also contained the increase in capillary ammonia levels in the morning, while the effect was less obvious in the afternoon. This was a somewhat surprising finding, as caffeine is not expected to have direct ammonia-lowering properties. However, caffeine – and methylxanthines in general - has diuretic properties, most likely mediated by inhibition of phosphodiesterases in the proximal tubule (Osswald and Schnermann 2011). Renal ammonia excretion has been shown to be enhanced by volume expansion (Jalan and Kapoor 2003). Therefore, it is possible that the simultaneous administration of fluids (saline as the intravenous placebo) and caffeine may have enhanced renal ammonia excretion, thus

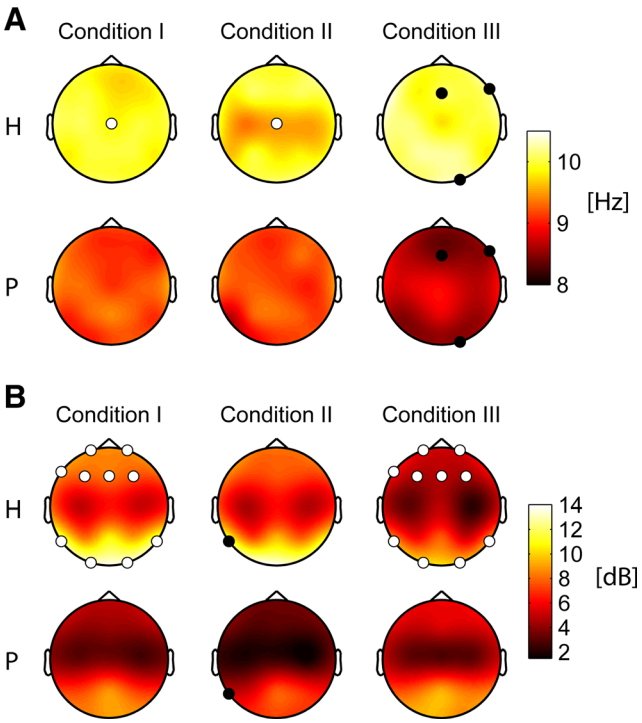

Fig. 3 **a** Topographical maps of the frequency [Hz] of the dominant EEG activity in healthy controls (H; $n=6$) and patients (P; $n=6$). Average values were interpolated and color-coded in the range of 8–10.5 Hz. The white dot represents a significant difference (two-tailed paired t-test, $p<0.05$, $n=6$) between Conditions I (AAC) and II (AAC plus LOLA). However, as this was a single derivation, significance was considered spurious (please also refer to the section Statistical Analysis). Black dots indicate significant differences (two-tailed unpaired t-test, $p<0.05$, $n=6$) between healthy volunteers and patients. However, as these were non-contiguous, single derivations not forming a cluster, significance was considered spurious. **b.** Topographical maps of spectral power [dB] of the dominant wake EEG activity in healthy controls (H; $n=6$) and patients (P; $n=6$). Geometric mean values were interpolated and color-coded in the range of 1.5–14 dB (0 dB = 1 μV^2). White dots represent significant differences (two-tailed paired t-test, $p<0.05$, $n=6$) between Conditions I (AAC) and III (AAC plus caffeine); these were several, contiguous derivations forming a cluster. The black dot represents a significant difference (two-tailed unpaired t-test, $p<0.05$, $n=6$) between healthy volunteers and patients; as this was a single derivation, significance was considered spurious. *AAC* amino acid challenge, *LOLA* L-ornithine L-aspartate

containing the effect of the AAC on ammonia levels. This may be also interesting in terms of the pathophysiology of dehydration/diuretic overdose as a precipitating factor for HE (Strauss and da Costa 1998).

In patients with cirrhosis, the administration of AAC also produced an increase in ammonia levels, reaching its highest value around 11:00. In line with previous observations, ammonia levels did not decrease to any significant extent during the subsequent hours (Bersagliere et al. 2012). This is in line with the following assumptions: (i) hyperammonaemia after the AAC derives from amino acids absorption and oxidation, thus the increase in ammonia levels after the AAC can be comparable in healthy volunteers and compensated patients and (ii) generated ammonia is removed largely by the liver urea cycle in a flow-dependent fashion, thus explaining prolonged hyperammonaemia after the AAC in patients compared to healthy volunteers.

In patients, the administration of LOLA contained the AAC-induced morning ammonia peak, to some extent (11:00–13:00), but the effects were not major. These results are only partly at odds with previous reports, especially if we keep into account of the total amount/length of administration of the drug. Literature data indicate that in patients with cirrhosis LOLA exerts it full ammonia-lowering effect after several consecutive days of daily iv administration (Stauch et al. 1998; Kircheis et al. 2002; Schmid et al. 2010), with significant results reported from day 2 on (Kircheis et al. 1997). Therefore, it is possible that our measurements did not extend long enough to detect the ammonia-lowering effects in patients, whose urea cycle/skeletal muscle ammonia-removal mechanisms may be impaired and/or already activated, and thus less sensitive.

In patients, caffeine had no effect on the increase in capillary ammonia levels due to the AAC. Functional renal dysfunction is extremely common in patients with cirrhosis and is characterized by impaired free water excretion, decreased renal perfusion and decreased glomerular filtration rate (Arroyo et al. 2002). Therefore, while patients enrolled in the study had normal urea and creatinine levels, subclinical renal dysfunction/reduced renal reserve may account for a reduced sensitivity to caffeine as a diuretic, and thus for a reduced excretion of ammonia via the kidney compared to healthy volunteers.

Subjective sleepiness

In healthy individuals, subjective sleepiness tends to decrease over the morning hours, from get-up time to the early afternoon hours, when a peak of sleepiness is observed. This phenomenon is more or less obvious depending on sleep-wake habits and on diurnal preference (Turco et al. 2015). In the healthy volunteers enrolled in this study, limited changes were observed in subjective sleepiness in the morning hours of the days when the AAC and the AAC plus LOLA were administered, thus suggesting an AAC-related increase in subjective sleepiness, which was not counteracted by LOLA. This is expected, as the cerebral effects of ammonia are due to alterations in cerebral metabolism which do not immediately benefit from a reduction in blood ammonia levels (Butterworth 1998). In contrast, the administration of AAC plus caffeine seemed to produce a transient decrease in sleepiness (10:00–14:00), somewhat restoring its physiological time-course, and counteracting the effect of the AAC alone. However, no condition effect was observed.

In patients with cirrhosis, subjective sleepiness did not fluctuate to any significant extent in any of the three conditions, possibly indicating that the AAC-sleepiness inducing effect was counteracted by neither LOLA nor caffeine. Caffeine is an antagonist of the adenosine receptor (Ribeiro and Sebastiao 2010). Adenosine is implicated in sleep homeostasis and mediates the gradual increase in sleep propensity during the waking hours of the day (Porkka-Heiskanen et al. 1997; Holst and Landolt 2015). Its importance is supported by animal studies showing that extracellular cerebral adenosine increases during the waking hours and decreases during subsequent sleep (Porkka-Heiskanen et al. 2000). Interestingly, an abnormal decrease in brain levels of the adenosine receptor A1AR has been documented in patients with hyperammonaemia (Boy et al. 2008). This may explain the lack of effect observed in patients with cirrhosis after caffeine administration since caffeine alertness-increasing properties are mediated by its adenosine receptor antagonist abilities.

Neuropsychiatric assessment

No significant differences in neuropsychological performance of healthy volunteers or patients with cirrhosis were observed in the three conditions. None of the healthy volunteers or the patients had abnormal performance on any of the tests or an abnormally slow EEG in any of the conditions. This is in line with previous findings (Romero-Gomez et al. 2004; Bersagliere et al. 2012) and with the fact that the patients enrolled in this study were well-compensated, with no history of overt HE nor signs of milder forms of HE on testing. However, patients performed consistently less well than healthy volunteers on all tests in all conditions and their EEGs were slower, suggesting either a higher sensitivity to the AAC or some degree of cirrhosis-related neuropsychiatric impairment. Of interest, EEG amplitude of healthy volunteers was higher than that of patients, which is in line with our previous observation that the AAC has more pronounced effects on amplitude in healthy volunteers and on frequency in patients (Bersagliere et al. 2013). However, limited conclusions in this respect can be drawn from the current study due to the absence of a baseline EEG. In healthy volunteers, the administration of caffeine together with the AAC seemed to

contain the AAC-related increase in EEG amplitude in several frontal and posterior derivations. These findings fit with previous studies showing that the administration of caffeine decreases the amplitude of alpha activity over frontal and posterior areas of the scalp in healthy volunteers (Künkel 1976; Etevenon et al. 1988). Thus, caffeine seems to exert its psycho-stimulant effects in both physiological and pathological conditions. Both the behavioural and neurophysiological effects of caffeine were considerably blunted in patients, possibly suggesting that timing and dose may need adjustment. Clearly, given the limited number of subjects enrolled, the present study has an exploratory nature. However, further work along these lines seems worthy, especially as caffeine has been shown to have positive effects on fibrosis (Modi et al. 2010) and even on liver-related mortality and the likelihood of developing hepatocellular carcinoma (Setiawan et al. 2015). For example, the acquisition of information on caffeine intake and/or its modulation may be considered in the following scenarios: 1) standard history-taking in patients with cirrhosis and HE, especially when tested for HE; 2) provision of advice on caffeine intake and timing, similar to that provided to drivers or insomniacs; 3) coffee intake could be considered amongst the measures caregivers are advised to proceed with at home when they have the impression that an episode of overt HE may be developing (for example administer coffee if patient is still fully conscious but confused, and administer an enema if bowel emptying has not been regular).

In conclusion, both LOLA and caffeine contained the AAC-induced increase in capillary ammonia, especially in healthy volunteers. Caffeine also counteracted the AAC effects on sleepiness/EEG amplitude. Therefore, the association of ammonia-lowering and vigilance-enhancing medication in the management of HE is worthy of further study.

Compliance with ethical standards

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