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COVERT HEPATIC ENCEPHALOPATHY: DIAGNOSIS, REVERSIBILITY AFTER LIVER TRANSPLANT AND ROLE OF GUT MICROBIOTA

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1. ABSTRACT

Background. Hepatic encephalopathy (HE) produces a wide spectrum of nonspecific neurological and psychiatric manifestations. Mild HE that does not produce disorienting in time/space or asterixis is called covert HE (CHE). It occurs in 20%–80% of patients with cirrhosis. While significant progress has been made in understanding the importance of CHE, to date there is no consensus guidelines regarding the screening procedures for CHE and all the recognized techniques, although more or less sensitive and objective, require some kinds of equipment. Furthermore, the diagnosis of CHE can be confounded by other factors (as chronic alcohol misuse or HCV infection), which can cause cognitive alterations. It is also important to identify CHE in patients awaiting liver transplantation (LT) for the proper interpretation of cognitive disorders that may occure after transplantation. Infact, the influence of LT on mental performance is debated; as is the role of pretransplant HE. Notable, recent data on LT suggest that the cognitive dysfunction may not be totally reversible. Another important issue regards the treatment of CHE, in particular the effect of treatments on gut microorganism and ammonia production by microbial activity. HE treatment with prebiotics, antibiotics and probiotics, generally evidences a reduction of photogenic/ ammoniagenic bacteria and an increase in neurocognitive tests and mental status in patients. Nevertheless from a strictly microbiological point of view, little is known about the dynamics, interaction and metabolite production of the main bacterial groups in liver diseases.

Aims. The present study aims to: 1) test a simple verbal psychometric test, called Animal Naming Test (ANT_1), in the detection of CHE; 2) to evaluate the role of alcohol misuse, HCV infections, diabetes, aging and level of education as potential confounding factors in the diagnosis of CHE; 3) to evaluate the time course of the neuropsychological and electroencephalogram (EEG) features of patients with cirrhosis before and after LT with respect to prior HE and 4) to investigate how the HE treatments (by the use of lactulose, rifaximin and a probiotc mixture) affects gut microbial composition, determining changes in ammonia production.

Materials and methods. For *Aim 1*: 208 healthy subjects and 327 consecutive patients with cirrhosis underwent the ANT_1. Patients with cirrhosis were assessed by the Psychometric Hepatic Encephalopathy Score (PHES), a subgroup of 146 underwent also a quantified EEG and 95 the Critical Flicker Frequency. 202 patients were followed up for the occurrence of overt HE (OHE) and death. For *Aim 2*: a comprehensive neuropsychological profile and EEG spectral parameters were obtained in six age-matched groups of 30 subjects each: (i) HCV-related hepatitis without cirrhosis, (ii) chronic alcohol abusers, (iii) patients with HCV-related cirrhosis, (iv) alcohol-related cirrhosis, (v) cirrhosis not related to alcohol or HCV and (vi) healthy subjects. Cirrhotic patients were matched for MELD score. For *Aim 3*: the study population included 65 patients with cirrhosis on the transplant waiting list; 23 had a history of OHE. Each patient underwent an extensive psychometric assessment (10 tests, including paper and pencil tests and a computerized test) and an

EEG before and 9 to 12 months after LT. EEGs were analyzed spectrally, and the mean dominant frequencies were obtained. For *Aim 4*: independent batch culture fermentations with controlled pH (6.8) were inoculated with fecal samples from six patients with cirrhosis (age 66±3.3 years; Child-Pugh A n=5 and B n=1); average MELD score 9±2.8). Seven different treatments with lactulose, rifaximin and VSL#3 or their combination were performed. Microbial populations were enumerated using flow cytometry Fluorescent in Situ Hybridization, while an ammonia concentration was determined at 0, 4, 10 and 24 hours.

Results. *Aim 1:* in controls, the ANT_1 was found relevantly affected only by extremely low levels of education (<8 yrs) and old age (>80 yrs; p<0.001). Thus, an age and education adjusted criterion was obtained ($S\text{-}ANT_1$). Patients with CHE had significantly lower $S\text{-}ANT_1$ than the unimpaired ones (12±0.4 vs 16±0.7; p<0.001). By ROC analysis, two thresholds of 10 and 15 animals were obtained, producing a Scoring System (0=$S\text{-}ANT_1$>15, 1=$S\text{-}ANT_1$ 10-15, 2=$S\text{-}ANT_1$<10 sensitivity 83%, specificity 84%, respectively vs. PHES) that was correlated both to PHES (p<0.0001) and EEG (p=0.007). In the follow up, the $S\text{-}ANT_1$ resulted to have prognostic value on the risk of OHE and death, so that a prognostic index could be computed. *Aim 2*: the factor 'cirrhosis' was associated with low Phonemic Verbal Fluency (PVF) and Difference between Trail Making Test B and A (TMT B-A) (p<0.001). Chronic alcohol misuse was associated with low PVF, TMT (B-A), Memory with Interference Task at 10 (ITM 10) and 30s (ITM 30) (all p<0.05). An interaction was found between the factors 'cirrhosis', 'alcohol misuse' and tests (p<0.01). HCV hepatitis reduced ITM 10 (p<0.05), but no interaction was found between 'cirrhosis', 'HCV infection' and tests (p= 0.14). The EEG parameters were mainly influenced by 'cirrhosis' (p<0.05), and EEG alterations were more pronounced in patients with alcoholic cirrhosis (p=0.04). *Aim 3*: Patients with a history of OHE before LT had worse cognitive performances (p<0.001) and EEG performances in comparison with their counterparts with a negative history. They also showed greater cognitive improvement after LT (p<0.01); however, their global cognitive performance remained slightly impaired (p <0.01). After LT, EEGs normalized for 98% of the patients (p <0.01), regardless of any history of OHE. *Aim 4:* Lactulose treatment significantly increased total bacteria, *Bifidobacteria* and *Fecalibacterium prausnitzii* after 5 hours ($p\leq0.05$); in contrast Rifaximin and a probiotic mixture (VSL#3) have no significant effect. After 24 hours the combination of lactulose/Rifaximine/VSL#3 significantly increased total bacteria and *Bifidobacteria*. At time 5 h, lactulose significanly reduced ammonia, whereas rifaximin had no significant effect. VSL#3 alone had never significant affect in reducing ammonia, whereas at 24 h, in combination with lactulose and rifaximin, reduced significantly ammonia.

Conclusions. The ANT_1 is an easy first-line measure useful for detection of CHE. The diagnosis of CHE should considered the concomitant presence of alcohol misuse and low educational level, which had a synergistic effect with cirrhosis in damaging cognitive functions, and thus they should be considered as possible confounders when testing for CHE. After LT, patients with a history of HE showed

greater improvements than patients with a negative history, but their global cognitive function remained slightly worse; in contrast, EEGs normalized in both groups. Regarding HE treatments, microbial modulation by prebiotic, antibiotic and probiotic differently affect the population dynamics and metabolism. The strong increase in beneficial bacteria, reduction of ammonia and regulation of metabolite production seen using lactulose and its combination with VSL#3, emphasize the importance of gut microbiota handling in HE treatment.

2. RIASSUNTO

Stato dell'arte. L'encefalopatia epatica (EE) è caratterizzata da un ampio spettro di manifestazioni aspecifiche, sia neurologiche che psichiatriche. Un EE lieve tale da non indurre disorientamento spazio-temporale o asterissi è definita "EE non conclamata", e si manifesta nel 20%-80% dei pazienti con cirrosi epatica. Sebbene siano stati compiuti notevoli progressi nella comprensione dell'importanza dell'EE non conclamata, non esistono, ad oggi, linee guida condivise in materia di screening. Inoltre, tutte le tecniche di diagnosi universalmente riconosciute, anche se più o meno sensibili ed obiettive, richiedono l'utilizzo di una qualche forma di strumentazione. La diagnosi di CHE dovrebbe anche tener conto di altri fattori (come l'abuso alcolico o l'infezione da virus C), in grado di per sé di causare alterazioni cognitive, che potrebbero agire da fattori di confondimento. Un'altra problematica aperta in ambito di EE non conclamata è la reversibilità delle alterazioni cognitive, caratteristiche dei pazienti con storia di EE, dopo trapianto di fegato che è tuttora controversa. Studi recenti sembrano suggerire che le alterazioni cognitive caratteristiche dei pazienti con storia di EE non siano completamente reversibili. Anche il ruolo del microbiota intestinale nella patogenesi e quindi nella terapia dell'EE non è del tutto noto. Studi recenti hanno dimostrato che le terapie dell'EE, che si basano su prebiotici, antibiotici e probiotici, determinano una riduzione di batteri ammoniagenici e un concomitante miglioramento dei test neurocognitivi e dello stato mentale dei pazienti. Tuttavia, da un punto strettamente di vista microbiologico, poco si conosce circa le dinamiche e l'interazione tra i principali gruppi di batteri e come si modifica la produzione di ammonio a seguito della somministrazione di tali terapie.

Scopi dello studio. Il presente studio si propone di: 1) testare un test psicometrico verbale, chiamato Animal Naming Test (ANT_1), nella diagnosi di EE non conclamata; 2) valutare il ruolo dell'abuso alcolico cronico, dell'infezione da virus, del diabete, dell'invecchiamento e del livello d'istruzione come potenziali fattori di confondimento nella diagnosi di EE non conclamata; 3) di valutare l'andamento temporale neuropsicologico ed elettroencefalografico dei pazienti con cirrosi prima e dopo trapianto di fegato, in relazione alla presenza o meno di una storia di EE prima del trapianto e 4) di valutare come i trattamenti dell'EE (quali il lattulosio, la rifaximina e il probiotico VSL#3) modifichino la composizione microbica intestinale e determinino cambiamenti nei livelli di ammonio.

Risultati. *Obiettivo 1*: nei controlli l'ANT_1 è risultato influenzato in modo significativo solo da livelli di istruzione estremamente bassi (<8 anni di scolarità) e da un'età molto avanzata(> 80 anni; p<0,001). Sono, quindi, stati calcolati i punteggi aggiustati per età e livello d'istruzione (S-ANT_1). I pazienti cirrotici con EE non conclamata avevano uno S-ANT_1 significativamente inferiore dei pazienti senza EE (12±0,4 vs 16±0,7; p<0.001). L'analisi delle curve ROC ha permesso di ottenere due valori soglia dell'S-ANT_1, di 10 e 15 animali/minuto, ed è stato costruito un sistema di Scoring (0=S-ANT_1> 15, 1=S-ANT1 10-15, 2=S-ANT_1<10 con sensibilità 83% e specificità 84%) che è risultato ben correlato sia con la PHES (p<0,0001) che con

l'EEG (p=0.007). Nel follow-up, lo S-ANT_1 è risultato avere un valore prognostico sia sul rischio di EE conclamata che sulla mortalità ad un anno. *Obiettivo 2*: il fattore 'cirrosi' è risultato associato sia con il Phonemic Verbal Fluency (PVF) che con la Differenza tra Trail Making Test B e A (TMT) (B-A) (p<0.001). L'abuso alcolico cronico è risulato associato con un bassi punteggi al PVF, al TMT (B-A) e ai test di Memoria di interferenza a 10 (ITM 10) e 30 s (ITM 30) (tutti p<0.05). E' stata trovata un'interazione tra i fattori «cirrosi',' abuso alcolico 'e test (p<0,01). Il virus C determina una riduzione dell'ITM 10 (p<0,05), ma nessuna interazione è stata trovata tra i fattori 'cirrosi', 'infezione da HCV' e test (p=0.14). I parametri EEG sono risultati principalmente influenzati dal fattore 'cirrosi' (p<0,05). *Obiettivo 3:* i pazienti con una storia di EE, prima del trapianto, hanno avuto performance cognitive ed EEG peggiori rispetto alla controparte senza storia di EE (p<0.001). Ma, dopo il trapianto, hanno mostrato un miglioramento cognitivo maggiore (p<0.01); tuttavia, la loro performance cognitiva globale rimane lievemente compromessa (p<0,01). Dopo il trapianto, l'EEG si normalizza nel 98% dei pazienti (p<0.01), indipendentemente dall'avere una storia di EE. *Obiettivo 4*: il trattamento con lattulosio aumenta significativamente il numero totale dei batteri, dei *Bifidobatteri* e del *Fecalibacterium prausnitzii*, rispetto al controllo e dopo 5 ore (p≤0.05); al contrario sia Rifaximina che il probiotico VSL#3 non hanno alcun effetto significativo. Dopo 24 ore la combinazione di lattulosio/Rifaximina/VSL#3 aumenta significativamente il numero totale dei batteri e i *bifidobatteri*. Per quanto riduarda la produzione di ammonio, il lattulosio riduce in modo significativo l'ammonio, mentre la Rifaximina non ha effetti significativi. Il probiotico VSL#3 da solo non ha mai un effetto nel ridurre l'ammonio, mentre in combinazione con lattulosio e rifaximina a 24 ore lo riduce significativamente.

Conclusioni. L'ANT_1 è uno strumento utile e facile da somministrare per il rilievo dell'EE non conclamata. La diagnosi dell'EE non conclamata dovrebbe inoltre tener conto della concomitante presenza di un abuso cronico alcolico e di un basso livello d'istruzione, che possono avere un effetto sinergico con la cirrosi nell'alterare le funzioni cognitive, e dovrebbero quindi essere considerati come fattori di confondimento nella diagnosi di EE non conclamata. Dopo trapianto di fegato, i pazienti con una storia di EE prima del trapianto presentato nel post-trapianto un miglioramento maggiore rispetto pazienti senza storia di EE, ma la loro performance cognitiva globale rimane un po' alterata; al contrario, l'EEG si normalizza in quasi tutti i pazienti. Per quanto riguarda l'influenza delle terapie dell'EE sul microbiota intestinale dei pazienti con cirrosi, i prebiotici, gli antibiotici e i probiotici influenzano in modo diverso la dinamica e il metabolismo delle popolazioni batteriche. Il forte aumento dei batteri benefici e la riduzione dell'ammonio favoriti dal lattulosio e dalla sua combinazione con rifaximina e VSL#3, sottolineano l'importanza della manipolazione della flora intestinale nel trattamento dell'encefalopatia epatica.

3.INTRODUCTION

3.1 HEPATIC ENCEPHALOPATHY

Hepatic encephalopathy (HE) is a frequent complication and one of the most debilitating manifestations of liver disease, severely affecting the lives of patients and their caregivers. Unless the underlying liver disease is successfully treated, HE is associated with poor survival and a high risk of recurrence (1). Even in its mildest form, HE reduces health-related quality of life and is a risk factor for bouts of severe HE.

3.1.1 Definition and epidemiology

Hepatic encephalopathy is a brain dysfunction caused by liver insufficiency and/or portal-systemic shunting; it manifests as a wide spectrum of neurological or psychiatric abnormalities ranging from subclinical alterations to coma (2). This definition is based on the concept that encephalopathies are ‘‘diffuse disturbances of brain function’’ and that the adjective ‘‘hepatic’’ implies a causal connection to liver insufficiency and/or perihepatic vascular shunting (1).

In patients with cirrhosis, fully symptomatic overt HE (OHE) is an event that defines the decompensated phase of the disease, such as variceal bleeding or ascites (3). Overt hepatic encephalopathy is also reported in subjects without cirrhosis with extensive PSS. The prevalence of OHE at the time of diagnosis of cirrhosis is 10%–14% in general (4,5), 16%–21% in those with decompensated cirrhosis (3), and 10%–50% in patients with transjugular intrahepatic portosystemic shunt (TIPS) (6). The cumulated numbers indicate that OHE will occur in 30%–40% of those with cirrhosis at some time during their clinical course and in the survivors in most cases repeatedly (7). The risk for the first bout of OHE is 5%–25% within 5 years after cirrhosis diagnosis, depending on the presence of risk factors, such as other complications to cirrhosis (MHE or CHE, infections, VB, or ascites) and probably diabetes and hepatitis C (8,9). Subjects with a previous bout of OHE were found to have a 40% cumulative risk of recurring OHE at 1 year (10), and subjects with recurrent OHE have a 40% cumulative risk of another recurrence within 6 months, despite lactulose treatment.

3.1.2 Clinical presentation and classification

Hepatic encephalopathy produces a wide spectrum of nonspecific neurological and psychiatric manifestations (11). In its lowest expression, HE alters only psychometric tests oriented toward attention, working memory (WM), psychomotor speed, and visuospatial ability, as well as electrophysiological and other functional brain measures (12). As HE progresses, personality changes, such as apathy, irritability, and disinhibition, may be reported by the patient’s relatives, and obvious alterations in consciousness and motor function occur. Disturbances of the sleep-wake cycle with excessive daytime sleepiness are frequent, whereas complete

reversal of the sleep-wake cycle is less consistently observed (13). Patients may develop progressive disorientation to time and space, inappropriate behavior, and acute confusional state with agitation or somnolence, stupor, and, finally, coma. The recent ISHEN (International Society for Hepatic Encephalopathy and Nitrogen Metabolism) consensus uses the onset of disorientation or asterixis as the onset of OHE (13).

Hepatic encephalopathy should be classified according to all of the following four factors (11):

1) According to the underlying disease, HE is subdivided into I) Type A resulting from ALF; II) Type B resulting predominantly from portosystemic bypass or shunting; III) Type C resulting from cirrhosis.

2) According to the severity of manifestations. The continuum that is HE has been arbitrarily subdivided. For clinical and research purposes, a scheme of such grading is provided (**Table 1**). Operative classifications that refer to defined functional impairments aim at increasing intraand inter-rater reliability and should be used whenever possible.

3) According to its time course, HE is subdivided into I) Episodic HE II) Recurrent HE denotes bouts of HE that occur with a time interval of 6 months or less; III) Persistent HE denotes a pattern of behavioral alterations that are always present and interspersed with relapses of overt HE.

4) According to the existence of precipitating factors, HE is subdivided into I) Non precipitated or II) Precipitated, and the precipitating factors should be specified.

Table 1. HE classification according to the severity of clinical manifestations and clinical description

WHC including MHE	ISHEN	Description	Suggested operative criteria	Comment
Unimpaired		No encephalopathy at all, no history of HE	Tested and proved to be normal	
Minimal	**Covert**	Psychometric or neuropsychological alterations of tests exploring psychomotor speed/executive functions or neurophysiological alterations without clinical evidence of mental change.	Abnormal results of established psychometric or neuropsychological tests without clinical manifestations	No universal criteria for diagnosis. Local standards and expertise required
Grade I		• Trivial lack of awareness • Euphoria or anxiety • Shortened attention span • Impairment of addition or subtraction • Altered sleep rhythm	Despite oriented in time and space (see below), the patient appears to have some cognitive/behavioural decay with respect to his/her standard on clinical examination, or to the caregivers	Clinical findings usually not reproducible
Grade II	**Overt**	• Lethargy or apathy • Disorientation for time • Obvious personality change • Inappropriate behavior • Dyspraxia • Asterixis	Disoriented for time (at least three of the followings are wrong: day of the month, day of the week, month, season or year) ± the other mentioned symptoms	Clinical findings variable but reproducible to some extent
Grade III		• Somnolence to semi-stupor • Responsive to stimuli • Confused • Gross disorientation • Bizarre behavior	Disoriented also for space (at least three of the following wrongly reported: country, state [or region], city or place) ± the other mentioned symptoms	Clinical findings reproducible to some extent
Grade IV		Coma	Does not respond even to pain stimuli	Comatose state usually reproducible

3.1.3 Pathogenesis of hepatic encephalopathy: a complex network of interdependent organ systems

The complex HE pathogenesis is not yet fully elucidated. Ammonia was first implicated in the pathogenesis of HE. Ammonia is a by-product of nitrogen metabolism, and its formation in the body is predominantly a consequence of the action of the vast number of urease-producing bacteria located in the gut. Urease is a bacterial enzyme that catalyzes the hydrolysis of urea to carbamate and ammonia. Ammonia is absorbed into the hepatic portal circulation and transported to the liver where, under normal physiological conditions, it enters the urea cycle and is metabolized. Ammonia that bypasses this primary fate is subsequently 'picked up' and detoxified by glutamine synthetase (GS), an enzyme found in the hepatocytes surrounding the hepatic vein (as well as in muscle and astroglial cells), which catalyses the conversion of ammonia and glutamate to glutamine (14). Whilst the liver is critical in the homeostatic control of blood ammonia levels, other organs such as the brain, muscle and kidney are also known to play a role in regulating them. Insult to the liver, whether acute or chronic in nature, reduces its capacity to metabolize ammonia and this exerts an ammonia burden on extrahepatic tissues which can result in hyperammonaemia up to five times that of normal blood ammonia levels. In the context of liver failure, the brain, and more specifically, astrocytes, act as an alternative metabolic pathway for ammonia. In the brain, hyperammonemia promotes astrocyte swelling and impairment of neurotransmission. In chronic liver failure, astrocytes typically exhibit morphological features of Alzheimer type II astrocytosis, which include a large swollen nucleus, prominent nucleolus, margination of the chromatin pattern and significant enlargement of the cytoplasm. First evidence of the role of ammonia in the pathogenesis of HE date back to 1893, when Hahn and colleagues demonstrated the induction of an encephalopathic state in dogs following the formation of a surgical shunt, known as Eck's fistula, which served to divert nitrogen-rich blood from the portal vein directly to the inferior vena cava, therein bypassing the liver. Six weeks post-operatively, the dogs began to exhibit increased levels of aggression, irritability, ataxia, as well as experiencing seizures and eventually lapsing into coma especially following ingestion of an ammonia-rich meal (15).

Later, some studies founded discrepancies in the direct correlation between ammonia concentration and the severity of HE in patients with cirrhosis (16). These findings have contributed to the general consensus that whilst ammonia has an irrefutable, and key role in the pathogenesis of HE, it may not be solely responsible for the neurocognitive sequelae and other factors might be contributing, such as inflammation and hyponatremia (17). Infection is an important precipitant of HE (16) and has been shown to worsen the progression of HE and cerebral edema in patients with ALF (18), and proinflammatory cytokines seem to act synergistically with ammonia in causing cerebral edema (19) in acute liver failure as well as cirrhosis (20). Overactive neutrophils with excessive degranulation activity and enhanced production of inflammatory cytokines may also play a role in this pathogenesis.

Additionally, alterations in toll-like receptor 4, a receptor responsible for recognition of gram-negative bacteria, may be at least partly responsible for the inflammatory state in the cirrhotic patient. Polymorphisms of this receptor that occur in cirrhotic patients may increase both the risk of infection and the risk of HE (21). Thus, ammonia is only a single component in a multifactorial disease process.

3.1.4 Gut microbiota: its role in hepatic encephalopathy

Gut-liver axis

The liver receives 70% of its blood supply from the intestine via the portal vein, thus it is continually exposed to gut-derived factors including bacterial components, endotoxins and peptidoglycans. The liver is of paramount importance in generating an effective innate immune response and is the first organ to encounter bacteria or toxins absorbed from the gut. Multiple hepatic cells, including Kupffer cells, sinusoidal cells, biliary epithelial cells and hepatocytes, express innate immune receptors known as pathogen-recognition-receptors (PRRs) that recognized and respond to bacteria express components (pathogen-associated molecular patterns [PAMPs]) from the gut (22). PRRs may be located on the cell surface (e.g.Toll-like receptor 4, TLR4), endolysosome (e.g. TLR9) or cytosol (e.g. nucleotide-binding oligomerization domain 1, NOD1). PAMP(s) recognition stimulates intracellular signaling pathways and transcription factors to induce a battery of genes encoding inflammatory molecules. PAMP-induced inflammatory response is indispensable for combating invading bacteria.

Cirrhosis is associated with both intestinal bacterial overgrowth (23,24) and dysbiosis, as autochthonous bacteria are decreased and potentially pathogenic bacteria increased (25,26). Several causes underlie such abnormalities in cirrhosis, including impaired gut motility, reduced bile flow, impaired gastric acid secretion, and altered IgA secretion and antimicrobial molecules (27;28). These abnormalities might primarily contribute to dysbiosis by facilitating the oral microbiome transition to small and large intestine, as bacterial species of buccal origin are enriched in the fecal microbiome of cirrhotic patients (26). Both animal and human studies shows that dysbiosis induces intestinal inflammation and gut leakiness. Therefore, dysbiosis-induced intestinal inflammation may contribute to pathological bacterial translocation (BT). The mechanism triggering intestinal inflammation in cirrhosis is unknown, but hitherto unidentified microbial products/metabolites might be involved. Conversely, a compromised intestinal defense might contribute not only to dysbiosis, but also to enhanced pathological BT. Thus, cirrhosis-associated dysbiosis contributes to intestinal homeostasis disruption by altering the intestinal immune system. Increased intestinal permeability allows microbial products/bacteria to translocate from the intestinal lumen to extraintestinal organs and spaces including mesenteric lymph nodes (MLNs), portal and systemic circulation. While microbial products likely escape from the intestinal lumen to the lamina propria via a paracellular route through disrupted tight junctions, living bacteria translocate via a transcellular route (transcytosis) (29). Translocation of viable bacteria is a hallmark

of cirrhosis, in particular during decompensation (29). In cirrhosis, impaired liver clearance contributes to an accumulation of PAMPs, and bacteria in the systemic circulation (24,30).
The liver forms a second vascular barrier for eliminating commensal bacteria that have escaped from the gut. In animal models of liver disease and gut dysfunction the liver is unable to capture escaped gut commensal bacteria, which then leak into the systemic circulation, resulting in a robust host nonmucosal immune response (30).

Danger-associated molecular patterns (DAMPs) originating from damaged liver tissue also increase during acute and chronic liver diseases (31). Microbial products and DAMPs activate receptors of the innate immune system contributing to liver disease progression and causing a systemic inflammatory response. Persistent stimulation of circulating immune cells increases plasma pro-inflammatory cytokines including IL-6, TNF-a and NO (32,33). Several of these molecules, namely TNF-a, can disrupt intestinal tight junctions augmenting gut leakiness. During decompensated cirrhosis, a further increase in intestinal permeability could contribute to enhanced translocation of PAMPs and viable bacteria (29) possibly leading to overt infections. As antibiotic intestinal decontamination normalizes the number of activated immune cells and their production of pro-inflammatory cytokines in experimental and human cirrhosis (34,35), the intestinal microbiota likely represents a driving force for systemic inflammation. Thus, recently it has been proposed that BT and systemic inflammation are the initial events leading to decompensation and organ failure in cirrhosis (36).Thus, the contribution of the gut microbiota to pathogenesis seems to become more pronounced with progressive disease and therefore represent an important therapeutic target in the management of cirrhosis and its complications, such as spontaneous bacterial peritonitis and hepatic encephalopathy (HE).

Changes in Gut Microbiota in hepatic encephalopathy

A firm scientific basis for a role for the gut microbiome in liver disease emerged in the middle of the last century with the recognition of the relationship between hepatic coma and the absorption of nitrogenous substances from the intestine (37). This was followed by the description of abundant coliforms in the small intestine of cirrhotic patients (38), and the role of bacteria was clinched by trials demonstrating that antibiotics led to clinical improvement in HE (39).

The relevance of certain enteric organisms to the pathogenesis of HE is supported by studies indicating the efficacy of the poorly absorbed antibiotic with activity against enteric organisms, rifaximin, in overt (40) and subclinical HE (41). However, it is important to note that the precise nature of the changes in the composition and/or metabolic activity of the microbiota that generate these benefits remains to be determined.
Molecular techniques, such as those based on sequence divergences of the small subunit ribosomal ribonucleic acid (16S rRNA) of bacteria (42) are now being directed at the more detailed investigation of the microbiota in liver disease. Recently

Zhang et al and Bajaj et al conducted studies in which they used a novel multitag pyrosequencing technique to characterize the microbiome from the fecal and colonic mucosal samples in patients with cirrhosis with and without HE and healthy controls (43,44,45). Zhang et al found over-representation of two bacterial families, Streptococcaceae and Veillonellaceae, in cirrhotic patients with and without MHE as compared with controls (45). They also showed that gut urease containing bacteria Streptococcus salivarius was absent in controls but was present in cirrhotics with or without MHE. The abundance of S. salivarius was significantly higher in MHE than non-MHE patients, and its count positively correlated with ammonia levels in patients with MHE (45). Bajaj and coworkers performed cognition testing, and inflammatory cytokines and endotoxin analysis in cirrhotics with and without HE (43). They found altered fecal flora (higher Veillonellaceae), poor cognition, endotoxemia, and inflammation in patients with HE when compared with those without HE. However, there were no significant differences in the other microbiome families between HE and no HE groups. This study, for the first time, also demonstrated a direct positive correlation between Porphyromonadaceae and Alcaligeneceae and poor cognitive function in cirrhotics. Alcaligeneceae degrade urea to produce ammonia, which likely explains the association of Alcaligeneceae with poor cognitive function. They also demonstrated that Enterobacteriaceae, Fusobacteriaceae, and Veillonellaceae were positively, and Ruminococcaceae negatively, related to inflammation. Network analysis comparison showed robust correlations, only in the HE group, between the microbiome, cognition and inflammatory cytokines (43).

3.2 COVERT HEPATIC ENCEPHALOPATHY

3.2.1 Definition

Mild HE that does not produce disorienting in time/space/identity or asterixis is called covert HE (2). This condition can be subdivided in an entirely asymptomatic condition, which is called minimal HE (MHE) and in a condition in which trivial signs of abnormality in attention or behaviour can be detected by trained medical staff or caregivers. This latter condition corresponds to stage I HE, according to West Haven classification (46).

Covert HE (CHE) occurs in 20%–80% of patients with cirrhosis (47,48,49).

3.2.2 Diagnosis

Testing for MHE and CHE is important because it can prognosticate OHE development, indicate poor quality of life and reduced socioeconomic potential, and help counsel patients and caregivers about the disease. The occurrence of MHE and CHE in patients with chronic liver disease seems to be as high as 50%, so, ideally, every patient at risk should be tested. However, this strategy may be costly, and the consequences of the screening procedure are not always clear and treatment is not always recommended. An operational approach may be to test patients who have problems with their quality of life or in whom there are complaints from the patients and their relatives (50). Tests positive for MHE or CHE before stopping HE drug therapy will identify patients at risk for recurrent HE. Furthermore, none of the available tests are specific for the condition (51), and it is important to test only patients who do not have confounding factors, such as neuropsychiatric disorders, psychoactive medication, or current alcohol use.

Testing strategies can be divided into two major types: psychometric and neurophysiological (52,53). Because the condition affects several components of cognitive functioning, which may not be impaired to the same degree, the International Society of Hepatic Encephalopathy (ISHEN) suggests the use of at least two tests, depending on the local population norms and availability, and preferably with one of the tests being more widely accepted so as to serve as a comparator. A listing of the most established testing strategies is given below. The test recommendation varies depending on the logistics, availability of tests, local norms, and cost (53).

1) Portosystemic encephalopathy (PSE) syndrome test. This test battery consists of five paper-pencil tests that evaluate cognitive and psychomotor processing speed and visuomotor coordination. The tests are relatively easy to administer and have good external validity (51). The test is often referred to as the Psychometric Hepatic Encephalopathy Score (PHES), with the latter being the sum score from all subtests of the battery. It can be obtained from Hannover Medical School (Hannover, Germany), which holds the copyright (Weissenborn.karin@mh-hannover.de). The

test was developed in Germany and has been translated for use in many other countries. For illiterate patients, the figure connection test has been used as a subtest instead of the number connection test.

2) The Critical Flicker Frequency (CFF) test is a psycho-physiological tool defined as the frequency at which a fused light (presented from 60 Hz downward) appears to be flickering to the observer. Studies have shown its reduction with worsening cognition and improvement after therapy. The CFF test requires several trials, intact binocular vision, absence of red-green blindness, and specialized equipment (48,54).

3) The Continuous Reaction Time (CRT) test. The CRT test relies on repeated registration of the motor reaction time (pressing a button) to auditory stimuli (through headphones). The most important test result is the CRT index, which measures the stability of the reaction times. The test result can differentiate between organic and metabolic, brain impairment and is not influenced by the patient's age or gender, and there is no learning or tiring effect. Simple software and hardware are required (55).

4) The Inhibitory Control Test (ICT) is a computerized test of response inhibition and working memory (56) and is freely downloadable at www.hecme.tv. The ICT test has been judged to have good validity, but requires highly functional patients. The norms for the test have to be elaborated beyond the few centers that have used it.

5) The Stroop test evaluates psychomotor speed and cognitive flexibility by the interference between recognition reaction time to a colored field and a written color name. Recently, mobile application software (''apps'' for a smartphone or tablet computer) based on the test has been shown to identify cognitive dysfunction in cirrhosis compared to paper-pencil tests (57). Further studies are under way to evaluate its potential for screening for MHE and CHE.

6) The SCAN Test is a computerized test that measures speed and accuracy to perform a digit recognition memory task of increasing complexity. The SCAN Test has been shown to be of prognostic value (58).

7) Electroencephalography examination can detect changes in cortical cerebral activity across the spectrum of HE without patient cooperation or risk of a learning effect (52). However, it is nonspecific and may be influenced by accompanying metabolic disturbances, such as hyponatremia as well as drugs. Possibly, the reliability of EEG analysis can increase with quantitative analysis. This specifically should include the background frequency with mean dominant frequency or spectral band analysis. Also, in most situations, EEG requires an institutional setup and neurological expertise in evaluation, and the cost varies among hospitals.

Although the above-described tests have been used to test for MHE and CHE, there is, most often, a poor correlation between them because HE is a multidimensional dysfunction (59). Learning effect is often observed with psychometric tests and it is unclear whether current HE therapy plays a role in the test performance. Therefore, interpretation of these tests and consideration of the results for further management need an understanding of the patient's history, current therapy, and effect on the patient's daily activities, if signs of HE are found.

3.2.3 Role of confounders in the diagnosis

Chronic alcohol misuse and HCV infection may act as confounders in the neuropsychological assessment for MHE, as they might directly impair brain functioning regardless of the liver disease they have caused (60,61). It is well known that chronic alcohol misuse is associated with specific changes in brain structure and function, which remain detectable long after patients have stopped drinking (62). The brain areas which are most susceptible to alcohol-related damage are the frontal lobes, periventricular structures and the cerebellum (63). The corresponding, damaged functions are executive function, working memory, strategy, switching, attention and psychomotor speed. Alcohol-related cirrhosis seems to be associated with more severe cognitive dysfunction compared to non-alcoholic cirrhosis based on some studies. The hypothesis that alcohol may have additional detrimental effect on the brain is supported by the more pronounced brain atrophy observed in patients with alcoholic cirrhosis compared to those with cirrhosis of different origin (64). Another potential confounder in the assessment of MHE is HCV infection. HCV infection has been found to be associated with fatigue, deficits in working memory and executive function (64). It has been hypothesized that these symptoms are caused by HCV colonization of microglia and/or interleukin activation (65). Forty-nine per cent prevalence of altered neuropsychological tests in patients with chronic HCV hepatitis has been reported (66); in contrast, Cordoba et al. (67) did not observe neuropsychological impairment in HCV-infected patients compared with patients with liver disease of other origin.

3.2.4 Role of liver transplantation

Generally, liver transplantation (LT) is thought to resolve cognitive deficits due to HE, but both new neurological diseases and an incomplete reversal of preexisting dysfunction can occur. It has been suggested that cognitive dysfunction after LT could be related to prior HE, anoxic/ischemic brain damage during surgery, osmotic myelinolysis, immunosuppressant toxicity, liver disease recurrence, comorbidities, and other metabolic alterations (68). Several studies have demonstrated substantial improvements in neuropsychological tests (69,70) and quality of life (71) after LT. Other studies have demonstrated improvements in cerebral function after LT with proton magnetic resonance imaging spectroscopy (72), positron emission tomography (73), and electroencephalograms (EEGs). Neuroimaging studies also support these findings (74). However, a number of studies have documented the persistence of various cognitive deficits within the months after LT (75,76). Only a few studies have analyzed the relationship between neuropsychological alterations and relevant presurgical variables to identify factors that place LT recipients at higher risk for cognitive dysfunction. Sotil et al. showed that transplant patients with a history of HE had worse neuropsychological performance as measured by the psychometric HE score and a lower critical flicker frequency in comparison with patients without a history of HE 18 months after LT (76).

3.3 TREATMENT of HEPATIC ENCEPHALOPATHY

3.3.1 General principles

At this time, only OHE is routinely treated (11). Despite its subtle nature, MHE and CHE can have a significant effect on a patient's daily living. Special circumstances can prevail where there may be an indication to treat such a patient (e.g., impairment in driving skills, work performance, quality of life, or cognitive complaints).

The first step in treatment of HE is identifying and treating precipitating causes which includes management of hypovolemia, gastrointestinal bleeding, infection, excessive diuretic use, diarrhea, vomiting, hyponatremia, hypokalemia or hyperkalemia, constipation, benzodiazepine use and noncompliance with lactulose or rifaximin therapy (77).

Current therapies for HE are based on ammonia-lowering with the hypothesis that the colon is the primary organ that generates ammonia. Most drugs have not been tested by rigorous randomized, controlled studies and are utilized based on circumstantial observations (2). These agents include nonabsorbable disaccharides, such as lactulose, and antibiotics, such as rifaximin. Other therapies, such as oral branched-chain amino acids (BCAAs), intravenous (IV) L-ornithine L-aspartate (LOLA), probiotics, and other antibiotics, have also been used.

3.3.2 Treatment of overt hepatic encephalopathy

Complex pathophysiology and limited understanding of HE at present has led to limited therapy for the management of HE (78). Although the evidence for ammonia is robust, the synergistic role of inflammation and infection in modulating the cerebral effects of ammonia has been shown to be important. The most commonly utilized pharmacological agents still include the non-absorbable disaccharides lactulose and lactitol. Recent literature has supported the role of lactulose in both primary, secondary and treatment of covert and overt HE. Although antimicrobial agents such rifaximin have had an established role in the treatment of encephalopathy recent metaanalysis has shown efficacy of lactulose similar to antimicrobials. However studies enrolled in most of these metanalysis are of poor quality that may have affect the overall results of these metanalysis. Recently combining the rifaximin and lactulose has shown promising results in the treatment of overt HE which needs further validation in multicenter trials (78). Till we have more definitive agents non-absorbable disaccharides still continues to be the first-line therapy for overt hepatic encephalopathy.

Non-absorbable Disaccharides

Disaccharides (lactulose and lactitol) get metabolized by the bacteria in the colon to acetic and lactic acid. This acidification of the colon not only creates a hostile environment for the survival of intestinal bacteria with urease activity involved in the production of ammonia in the gut, but also facilitates the conversion of NH_3 to non-absorbable NH_4^+. Both effects result in reduced levels of ammonia in

the colon and portal blood. Nonabsorbable disaccharides also cause a 4-fold increase in faecal nitrogen excretion due to their cathartic effect (79). The non-absorbable disaccharides have been a mainstay of therapy for HE for decades, and have been extensively studied in several small clinical trials. Oral lactulose was used in majority of these studies though some had used lactitol and lactulose enemas also (80,81).
Lactulose is the most commonly utilized nonabsorbable disaccharide for HE and it is generally used as initial treatment for OHE. Similarly lactitol is a disaccharide analog of lactulose which is neither absorbed nor broken down in the small intestine. A large meta-analysis of trial data did not completely support lactulose as a therapeutic agent for treatment of OHE, but for technical reasons, it did not include the largest trials, and these agents continue to be used widely (82). Though it is assumed that the prebiotic effects (the drug being a nondigestible substance that promotes the growth of beneficial microorganisms in the intestines) and acidifying nature of lactulose have an additional benefit beyond the laxative effect, culture-independent studies have not borne those out (83,84). In addition, most recent trials on lactulose have been open label in nature. Cost considerations alone add to the argument in support of lactulose (85). In some centers, lactitol is preferred to lactulose, based on small meta-analyses of even smaller trials (86,87). The dosing of lactulose should be initiated with 25 mL of lactulose syrup every 12 h until at least two soft or loose bowel movements per day are produced. Subsequently, the dosing is titrated to maintain two to three bowel movements per day. This dose reduction should be implemented. It is a misconception that lack of effect of smaller amounts of lactulose is remedied by much larger doses. There is a danger for overuse of lactulose leading to complications, such as aspiration, dehydration, hypernatremia, and severe perianal skin irritation, and overuse can even precipitate HE (88).

Rifaximin

Rifaximin is a nonsystemic antibiotic with a broad spectrum of antibacterial action covering Gram-positive and Gram-negative organisms, both aerobes and anaerobes, and it is structurally similar to Rifampin. By binding to bacterial DNA-dependent RNA polymerase, rifaximin inhibits bacterial RNA/protein synthesis. Structurally, the benzimidazole ring limits systemic absorption to 0.4%, with the primary mode of excretion via feces and low levels of drug excreted in urine or bile. Being virtually non-absorbed, its bioavailability within the GI tract is rather high with intraluminal and fecal drug concentrations that largely exceed the minimal inhibitory concentration values observed in vitro against a wide range of pathogenic organisms. Due to its low rate of systemic absorption, rifaximin appears to be relatively safe.

Rifaximin has been used for the therapy of HE in a number of trials (89) comparing it with placebo, other antibiotics, nonabsorbable disaccharides, and in dose-ranging studies. These trials showed effect of rifaximin that was equivalent or superior to the compared agents with good tolerability. Long-term cyclical therapy over 3–6 months with rifaximin for patients with OHE has also been studied in three trials (two compared to nonabsorbable disaccharides and one against neomycin) showing

equivalence in cognitive improvement and ammonia lowering. A multinational study (40) with patients having two earlier OHE bouts to maintain remission showed the superiority of rifaximin vs. placebo (in the background of 91% lactulose use). No solid data support the use of rifaximin alone.

3.3.3 Treatment of covert hepatic encephalopathy

Once patients with cirrhosis develop MHE, they are at a substantial risk of progression to OHE, resulting in a significant burden to the healthcare system and an increased risk of mortality (8;90). Studies have consistently shown that patients with MHE have a diminished quality of life, cognitive function, daily function, and driving impairment (47;91). Moreover, it has been demonstrated that even after therapy and improvement to normal mental status, a single episode of OHE may continue to have residual negative effectson cognitive function (92). Therefore, it would only seem logical to consider prophylactic treatment for HE in patients with cirrhosis.

Currently, there is a paucity of data regarding the role of prophylaxis in CHE. Although it is not standard to offer therapy for MHE and CHE, studies have been performed using several modes of therapy. The majorities of studies have been for less than 6 months and do not reflect the overall course of the condition. Trials span the gamut from small open-label trials to larger, randomized, controlled studies using treatments varying from probiotics, lactulose, and rifaximin. Most studies have shown an improvement in the underlying cognitive status, but the mode of diagnosis has varied considerably among studies. A minority of studies used clinically relevant endpoints. It was shown, in an open-label study (93), that lactulose can prevent development of the first episode of OHE, but the study needs to be replicated in a larger study in a blinded fashion before firm recommendations can be made. Studies using lactulose and rifaximin have shown improvement in quality of life (94,95) and also in driving simulator performance (96). Probiotics have also been used, but the open-label nature, varying amounts and types of organisms, and different outcomes make them difficult to recommend as therapeutic options at this time (97,98,99). Because of the multiple methods used to define MHE and CHE, varying endpoints, short-term treatment trials, and differing agents used in trials to date, routine treatment for MHE is not recommended at this stage. Exceptions could be made on a case-by-case basis using treatments that are approved for OHE, particularly for patients with CHE and West Haven Grade I HE.

3.3.4 Modulation of gut microbiota improves HE

Gut microbiota is clearly implicated in the development of HE, thus its modulation by various agents provides an opportunity to treat covert and overt HE. Successful modulation of gut microbiota leading to improvement in HE strengthens the belief that derangement in microbiota is certainly an important factor in development of HE.

Recent studies, which utilized multitag pyrosequencing technique to characterize the microbiome from the fecal and colonic mucosal samples in patients

with cirrhosis, suggested that probiotics, lactulose and gut specific antibiotic like rifaximin cause improvement in dysbiosis and HE. Such improvement relates to both changes in microbiota-associated metabolic function and improvement in dysbiosis.

Lactulose

Lactulose is used as standard therapy in HE. Traditionally, lactulose/lactitol has been the standard to which newer therapies have had to be compared. Its use was prompted by studies suggesting that colonic bacteria are the main ammonia producers in the body (100).

The mechanism of action through which disaccharides works is multifaceted. While intestinal "hurry" is their best-known mechanism for eliminating fecal waste products, including ammonia, DS are much more than simple cathartics. Upon entering the colon, DS are cleaved into monosaccharides by the bacterial flora, some of which (eg, *Lactobacilli* and *Bifidobacteria*) can then incorporate these monosaccharides into subsequent generations of bacteria, thereby gaining a growth advantage. The unincorporated monosaccharides are also utilized as fuel for the bacteria. This fermentation process generates lactic acid and hydrogen ions, thereby acidifying the fecal stream within the colon and causing subsequent protonation of ammonia molecules (NH^3) into ammonium ions (NH_4^+). Because the charged NH_4^+ is poorly absorbed across the colonocyte, the ion remains trapped within the colonic lumen. In addition, this protonation reaction can allow for movement of NH_3 from the bloodstream back into the colonic lumen in a classic example of stoichiometry ($NH_3 + H^+ \rightarrow NH_4^+$). Another mechanism of action that has been postulated for DS involves transformation of the fecal flora: reduction of urease-producing bacteria (which are not given a growth advantage with DS) in favor of the proteolytic species (eg, *Lactobacilli* and *Bifidobacteria*). In this regard, disaccharides can be considered a prebiotic—ie, a "meal" for the bacterial biomass.

In healthy individuals, lactulose therapy does not significantly change microbial composition using culture-independent techniques (101). In patients with cirrhosis, Bajaj et al demonstrated that, despite lactulose treatment in those patients who developed HE, there was an increase in dysbiosis, with a lower CDR and relative abundance of gram negative non-autochthonous bacteria (Enterobacteriaceae, Bacteroidaceae) (44). This is in contrast to earlier culture-based studies which showed increased autochthonous bacteria (Lactobacillaceae) after administration of lactulose in patients with cirrhosis (84). Lactulose withdrawal did not exert a very significant effect on the composition of the fecal microbiome except for the reduction of Faecalibacterium species, suggesting that changes in gut bacterial function rather than a change in the microbiome composition may be responsible for the effects of lactulose. In another study HE patients underwent characterization of their phenotype (cognition, inflammatory cytokines, in vivo brain MR spectroscopy), gut microbiome and urine and serum metabolome analysis while on lactulose and on days 2, 14 and 30 post-withdrawal. When patients with and without recurrent HE post-withdrawal were compared, brain MRS findings consistent with low grade brain edema

(increased glutamine/glutamate and decreased myo-inositol) were reported, with a relatively minor change in fecal microbiome (reduction in abundance of stool Faecalibacterium sp). HE recurrence was associated with altered choline metabolism by gut microbiome, resulting in low urine tri-methylamine oxide, high urine glycine and high serum choline, dimethylglycine, creatinine, which play an important role in development of HE (83).

Rifaximin

Rifaximin, a nonsystemic antibiotic with a broad spectrum of antibacterial action covering Gram-positive and Gram-negative organisms, is presumed to modulate intestinal bacteria, thereby reducing intestinal ammonia and toxin formation (102).

The effect of rifaximin therapy on the metabiome, i.e. the interaction between the phenome (cognition, liver disease severity and endotoxin level), microbiome and metabolome, was evaluated in patients with cirrhosis and MHE. There was no significant microbial change after treatment with rifaximin, apart from a modest decrease in Veillonellaceae and increase in Eubacteriaceae. A significant improvement in cognition, reduction in endotoxemia and a significant increase in serum long-chain fatty acids were seen after rifaximin therapy. A significant linkage of pathogenic bacterial taxa was demonstrated with the metabolites, especially those linked to ammonia, aromatic amino acids and oxidative stress, which shifted from a positive correlation to a negative correlation after rifaximin therapy, reflecting changes in bacterial metabolic function. These results suggest that the mechanism of action of rifaximin that lead to cognitive improvement could be associated with changing microbiota-associated metabolic function rather than just changing the numbers of beneficial or harmful bacteria (103).

Probiotics

Probiotics are live microorganism that, when admistered in adequate amounts, confer a health benefit on the host (104). Examples include strains of the genera *Bifidobacterium* and *Lactobacillus*.

Liu *et al.* for the first time demonstrated that cirrhotic patients with MHE had substantial derangements in the gut microbiota, with significant fecal overgrowth of potentially pathogenic *Escherichia coli* and *Staphylococcal spp*. Synbiotic treatment significantly increased the fecal content of non-urease—producing *Lactobacillus* species at the expense of these other bacterial species. Such modulation of gut microbiota was associated with a significant reduction in blood ammonia levels, endotoxemia and reversal of MHE in half of patients. The Child–Turcotte–Pugh functional class improved in nearly half of patients. This study demonstrated that treatment with synbiotics could be an alternative to lactulose for management of MHE in patients with cirrhosis. In this study, culture-based techniques were used for the characterization of gut microbiota (105).

Another recent study have demonstrated that over a 6-month period, treatment with probiotic VSL#3 significantly reduced the risk of hospitalization due to HE and significantly reduced Child-Pugh and MELD scores. Treatment with probiotic was also associated with improvements in rates of SIRS and plasma indole (106).

Despite a number of trials showing the efficacy of probiotics and synbiotics, their role in the management of HE is inconclusive and, currently, they cannot be recommended.

4. STUDY AIMS

1) Screening for Covert Hepatic Encephalopathy in routine clinical practice is a relevant issue. While significant progress has been made in understanding the importance of CHE, to date there is no standardized algorithm for diagnosis. There are, also, no consensus guidelines regarding screening for CHE and there is debate as to the utility of testing all cirrhotic patients as opposed to a limited testing strategy for those patients with evidence of cognitive impairment. The recent published HE guidelines suggest that, ideally, every patient at risk for CHE should be tested (2). Tools useful at this aim are the Psychometric Hepatic Encephalopathy Score (51), quantified EEG (107), *(*Critical Flicker Frequency (CCF) (54) or computerized tests (58,56). All these well recognized techniques, although more or less sensitive and objective, require some kind of equipment, even if this may be a simple pencil and a form of paper to be filled in. However, it is well known and accepted that some kind of evaluation of mental function can be obtained also by simple verbal questions, such as those concerning orientation in time/space/identity that are used to detect overt HE, which have obvious intercultural applicability and can be easily used in clinical practice. Therefore, this study focused on the applicability of a simple verbal psychometric test to assess routinely patients with cirrhosis who do not have overt HE. The Animal Naming Test (ANT), which is a semantic fluency test, seemed to have potential for this aim. The rationale of the choice of the ANT is that the knowledge of animal names is reasonably widely spread in humans of every culture, and might be only slightly influenced by age or education. Thus, the first aim of this study was to test the ANT in the detection of covert HE. Secondary aims were to: I) standardize the ANT in a sample of healthy subjects, II) produce a scoring system to assess the risk of CHE, and III) assess the prognostic value of ANT.

2) It can be difficult to differentiate between the effects of CHE and those resulting from other causes. For example, psychometric tests are able to detect functional deficits, but are unable to differentiate between different causes for these deficits. Therefore, the diagnosis of CHE should consider other factors which can cause cognitive impairment by itself and act as confounders. Recent studies show that chronic alcohol misuse, HCV infection and cirrhosis *per se* may cause cognitive alterations. Thus, the second aim of the present study was to assess the influence of alcohol misuse, HCV infection and cirrhosis *per se* on the neuropsychological and EEG profile and to evaluate the role of alcohol misuse, HCV infections as potential confounding factors in the detection of CHE-MHE. Parallel aims were evaluate also the role of diabetes, aging and a low level of educations as potential confounding factors.

3) The influence of liver transplantation on mental performance is debated, as is the role of pretransplant overt hepatic encephalopathy. It seems important to identify CHE in patients awaiting liver transplantation for the proper interpretation of

disorders occurring after transplantation. Recent data from studies evaluating liver transplantation suggest that the associated cognitive changes may not be totally reversible. Assuming that residual cognitive deficits after liver transplantation may reflect the extent of pretransplant morbidity, the third aim of this study was to evaluate the time course of the neuropsychological and EEG features of patients with cirrhosis before and after liver transplantation with respect to prior episodes of OHE.

4) Gut microorganisms may play a fundamental role in the pathogenesis and worsening of liver disease and pathology, where alteration of their composition and the production of toxic compounds may be considered as trigger actors. In particular, gut ammonia production by microbial activity is one of the main factors implicated in HE. Ammonia reducing strategies target mainly gut microbiota, by administration of prebiotics (lactulose), antibiotics (rifaximin) and probiotics (VSL#3). Although the role of ammonia in the pathophysiology of HE is well established, the factors governing its productions by the gut microbiota are poorly understood.

The forth aim of this study was to investigate how gut microbiota modulation by prebiotics, antibiotics and probiotic treatments affected microbial ammonia production and the relative abundance of important members of the gut bacteria, within the cirrhotic environment.

In summary, the aims of the present study were the follows:
1) test a verbal psychometric test, the Animal Naming Test, for the detection of CHE
2) estimate the role of alcohol misuse, HCV infection, aging, diabetes and a low level of education as potential confounding factors in the detection of CHE-MHE
3) evaluate the time course of the neuropsychological and EEG features of patients with cirrhosis before and after liver transplantation with respect to prior episodes of OHE, assuming that residual cognitive deficits after LT may reflect the extent of pretransplant morbidity
4) investigate how the use of lactulose, rifaximin or VSL#3 affects gut microbial composition, determining changes in ammonia and metabolites levels during HE treatment.

5. PATIENTS AND METHODS

5.1 *Aim 1: Animal Naming Test for detection of covert hepatic encephalopathy*

Three samples of subjects underwent the ANT (*see below*). The first one was composed by healthy Italian individuals and served to standardize the ANT, the second one by a consecutive sample of patients with cirrhosis who were enrolled either in the University Hospital of Padua or Rome, the third one by a consecutive sample of patients with inflammatory bowel disease (IBD) enrolled in the University Hospital of Padua.

ANT standardization sample. A convenience sample of 208 healthy volunteers (42% males) enrolled in general population after a structured interview to exclude alcohol misuse, consumption of psychotropic drugs, insulin-dependent diabetes or diseases that can damage cognitive functions. The sample was stratified according to age in order to have participants for each of the following age classes: 18-40, 41-60, 61–70, 71-80, >80 years. Four educational levels were considered: at least 5; at least 8; at least 13 years; and university degree (≥ 17 years). Following informed consent, each individual underwent the ANT both at 1 (ANT_1) and 2 minutes (ANT_2).

Control sample of hospitalized subjects with IBD. A group of 11 in-patients with IBD without liver disease -having the same exclusion criteria considered for the patients with cirrhosis- was enrolled, as a control group of hospitalized individuals with a chronic disease. Their age was 47±15 years (males 55%; educational level: at least 5 years 18%, at least 8 years 18%, at least 13 years 55%, university degree 9%).

Patients sample

Patients with cirrhosis. The patient population was recruited in two University hospital centers (Padua and Rome, Italy). It comprised 327 consecutive patients (69% males) with cirrhosis (172 from Padua, 155 from Rome; age 60 ± 13 years, mean ± SD; Child A: 40%, B: 37%, C: 23%). Their clinical and biochemical findings are shown in Table 2. Subjects were considered eligible if they had cirrhosis with or without clinical signs of HE. Exclusion criteria were age<18 yrs, neurological comorbidities (e.g. prior cerebrovascular disease and dementia), psychiatric disorders, alcohol misuse in the previous 6 months or use of sedatives (e.g. benzodiazepines, neuroleptic, antiepileptic and opiate drugs), heart, respiratory or renal failure that could confound the assessment of the mental state.

A subgroup of 202 patients with cirrhosis (153 from Rome and 49 from Padua) was available to be followed up. The patients were followed up for up one year. Both the first breakthrough of OHE and survival were considered. The patients who underwent liver transplantation in the follow up were censored on the day of transplant. The follow up for the first breakthrough of OHE was 216 (70-365) days (median and interquartile range); the follow up for survival was 328 (128-365) days. In one year, 65 patients died and 30 were transplanted; 77 had a breakthrough of OHE.

Table 2. Demographic, clinical and biochemical data of patients with cirrhosis and demographic data of healthy subjects

Healthy controls n=208			
Age [a] (years)	54 ± 19		
Males/females	87 (42%)/121 (58%)		
Age classes:			
18-40 years	60 (29%)		
41-60 years	60 (29%)		
61-70 years	38 (18%)		
71-80 years	28 (13%)		
>80 years	22 (11%)		
Education levels			
At least 5 years	59 (28%), *mean age±SD* 66±2		
At least 8 years	79 (38%), *mean age±SD* 49±2		
At least 13 years	39 (19%), *mean age±SD* 49±3		
University degree (≥17 years)	31 (15%), *mean age±SD* 49±3		
Patients with cirrhosis n=327			
Age [a] (years)	60 ± 13 years	MELD score	13 ± 6
Males	226 (69%)	Previous episodes of OHE	116 (35%)
Education levels		Ascites rating	
At least 5 years	34%	None	59%
At least 8 years	37%	Mild/Moderate	29%
At least 13 years	21%	Severe	12%
University degree (≥17 years)	8%		
Etiology of cirrhosis		Albumin (g/L)	35 ± 6.5
HCV-related	61%	Bilirubin tot.(mg/dl)	3.4 ± 6.1
Alcoholic	26%	INR	1.4 ± 0.34
Mixed	7%	Prothrombin time (%)	57 ± 21
Others	6%	Creatinine (mg/dl)	0.99 ± 0.8
Child-Pugh class		Sodium (mEq/l)	137 ± 5
A	40%		
B	37%		
C	23%		

Abbreviations: MELD (Model for End Stage Liver Disease); OHE, overt hepatic encephalopathy

[a] (mean±SD); [b] 1st: at least 5 years; 2nd: at least 8 years; 3rd: at least 13 years; 4th: university degree (≥17 years): [c] The grade of HE was determined according to the West Haven Criteria

Measures

The ANT: in a quiet room without distractions, the subjects were asked to list as many animals they could. All repetitions and errors were excluded from the calculations. The animals listed in 1 minute (ANT_1) were considered for patients and

healthy controls. In the latter for completeness of the standardization process, also the animals reported in 2 minutes (ANT_2) were calculated.

Clinical assessment of HE. The patients were examined by clinicians with high expertise in HE assessment. The assessment included full neurological examination, in particular to detect asterixis and grading of the neuropsychiatric abnormalities according to the West Haven criteria (46), using operative criteria (2). All patients without OHE underwent the PHES battery that was scored by the age and education-adjusted Italian norms (108). Patients were qualified as unimpaired (clinically normal, PHES normal), or as having CHE (MHE or grade I HE according to West Haven classification) or OHE (disoriented or presence of asterixis).

Neurophysiological assessment of HE. 146 patients from the Padua centre underwent also the digital EEG and 95 patients the CFF. Spontaneous closed-eyes resting EEG activity was recorded by digital EEG equipment (Brainquick 3200, Micromed, Italy) in the morning. A standard 21-channel cap (Micromed, Italy) was used, and the electrodes placed according to the 10–20 International System (109). The EEG tracing was assessed by spectral analysis after visual inspection to exclude artefacts. Spectral analysis was carried out on the derivation P3-P4 in the frequency range of 1–25.5 Hz. The EEG alterations were classified into three grades according to mean dominant frequency MDF (i.e. the mean frequency weighted by the power of each frequency band) and to the relative power of the theta and delta bands as follows: grade 1 – MDF > 6.8 Hz and theta relative power ≥ 35% –, grade 2 – MDF ≤ 6.8 Hz and delta relative power < 49% –, grade 3 – MDF ≤ 6.8 Hz and delta relative power ≥ 49%, as previously described (110). The CFF was measured by a portable, battery-powered analyzer (Hepatonorm Analyzer; R&R Medi Business Freiburg GmbH, Freiburg, Germany). The analyzer evokes an intrafoveal light stimulus with defined pulses of light at a wavelength of 650 nm, luminance of 270 cd/m2, and luminous intensity of 5.3 mcd. The frequency of the red light, which is initially generated as a high-frequency pulse (60 Hz) and which gives the patient the impression of a steady light, was reduced gradually until the patient had the impression that the steady light had changed to a flicker. The patient registers this change by pressing a hand-held switch. After a practice run, the test was repeated five times and an average value obtained; this was qualified as abnormal if <39 Hz (54).

Statistical analysis

In healthy sample, analysis of covariance (ANCOVA) was used to evaluate if age, level of education and gender were predictors of the ANT and to evaluate the effect of education levels and age classes on the ANT. The Newman-Keuls test was used for post hoc comparisons. The thresholds of normality for the ANT_1 and ANT_2 in healthy subjects were fixed at the 2.5^{th} percentile. In patients with cirrhosis, general linear model was used to evaluate if a history of OHE (dichotomous variable ‘yes’ or ‘no’) and alcohol etiology (dichotomous variable ‘yes’ or ‘no’) were predictors of ANT_1, adjusting for age (in years) and education (years of education). Receiver operating characteristic (ROC) curves analysis and the Youden test was

performed to determine the best threshold for the ANT_1. The sensitivity, specificity, positive (PPV) and negative predictive values (NPV) for the detection of subjects with CHE *vs*. unimpaired by the ANT_1 were calculated. The correlations between the ANT and the EEG and the CFF were assessed using Pearson *r* correlation. Survival analysis was performed by the Kaplan Mayer method and comparisons were performed by the Log-rank test or the χ^2 test, when appropriate. Multivariate survival study was performed by the Cox model. Results are expressed as means ± SD, unless otherwise specified.

5.2 *Aim 2: Confounders in the detection of covert hepatic encephalopathy*

Patients

One hundred and fifty patients were studied (males 68%; age 52 ± 9 years). They were retrospectively selected from a database of 1200 subjects studied in a tertiary referral centre for hepatic encephalopathy (HE) of the Department of Medicine of Padova University Hospital, Italy. The selection was performed to obtain six groups of individuals matched for age and education and, as far as patients with cirrhosis were concerned, the severity of liver disease was assessed by the MELD score. Patients were selected consecutively until reaching 30 per group. In line with an exploratory study design, the number of 30 subjects per group was chosen arbitrarily. The following six groups were defined: (i) 30 non-cirrhotic patients with chronic HCV hepatitis, (ii) 30 noncirrhotic chronic alcohol misuser, (iii) 30 patients with HCV-related cirrhosis, (iv) 30 patients with alcohol related cirrhosis, (v) 30 patients with cirrhosis without a history of alcohol consumption and without HCV infection, (vi) 30 healthy subjects without history of alcohol consumption and without HCV infection. This way, it was possible to compare the main groups alcohol/nonalcohol, cirrhosis/no cirrhosis, HCV/no HCV, of 1:1 or 1:2 and calculate the interactions between them. Of the patients with cirrhosis not related to alcohol or HCV infection: 18 had HBV-related cirrhosis, three had HBV–HDV-related cirrhosis, five had primary biliary cirrhosis, one had haemochromatosis, one had Budd–Chiari syndrome and two had cryptogenic cirrhosis. The diagnosis of cirrhosis was based on case history, clinical examination, biochemical and ultrasound findings and was confirmed by biopsy where needed. Specifically, the diagnosis of HCV-related cirrhosis was based on positive anti-HCV-ELISA and fibrosis score ≥4 on liver biopsy, based on the Metavir classification (21). The diagnosis of HCV-related hepatitis without cirrhosis was based on positive anti-HCV-ELISA/HCV-RNA PCR and fibrosis <4 on liver biopsy. The absence of HCV hepatitis was assessed by antibodies test. The presence of chronic alcohol misuse was confirmed by quantity and frequency questionnaires, and only patients under the care of an alcohol specialist centre were considered. All patients with chronic alcohol misuse had a daily alcohol intake of more than 30 g/day in females and 60 g/day in males. Patients with alcohol-related cirrhosis had been abstinent for more than 6 months, and the absence of alcohol consumption was declared by the patient and confirmed by a relative. The

groups were selected so that their demographical variables were matched (Table 3); in addition, patients with cirrhosis were also matched (MELD score) (Table 2). The exclusion criteria were the presence of significant neurological and psychiatric disease (mood or personality disorders), current OHE, use of psychoactive and antihypertensive medication.

Table 3. Demographic variables of the six groups of subjects

Cirrhotic groups

	HCV-cirrhosis	Alcohol-related cirrhosis	non alcohol/HCV cirrhosis
Age *(years)*	54 (±8)	52 (±8)	50 (±11)
Education *(years)*	8 (±3)	8 (±3)	8 (±3)
Males:females (n)	19:11	19:11	23:7

Non cirrhotic groups

	HCV hepatitis	Chronic alcohol abusers	Healthy subjects
Age *(years)*	49 (±9)	53 (±9)	53 (±9)
Education *(years)*	8 (±3)	8 (±4)	8 (±3)
Males:females (n)	20:10	18:12	15:15

Psychometric assessment

All patients underwent comprehensive neuropsychological examination by an experienced neuropsychologist. The following battery of psychometric tests was administered: (i) The Phonemic Verbal Fluency (PVF) is a verbal and executive function test, in which subjects are asked to generate as many words as possible, starting with a given letter (C, P, S) within a time span of one minute for each letter. (ii) The Trail Making Test A (TMT A) is a psychomotor speed test with visual search and attentional components, in which subjects are required to connect 25 numbered circles in sequential order, as quickly as possible. (iii) The Trail making test B (TMT B) is a psychomotor speed test with visual search and higher attentional components compared to TMT A, implicating divided attention and task switching, in which subjects are required to connect 25 circles containing numbers from 1 to 13 and letters from A to N in sequential and alternating order (1-A-2-B-3-C. . .), as quickly as possible; (iv) The Difference TMT B minus TMT A (or TMT (B-A) was obtained subtracting performance time of TMT A from performance time of TMT B. This provides an estimate of the exceeding time required to perform TMT B compared to TMT A, which is independent of the motor and search components of the task, which are comparable in TMT A and TMT B. Therefore, it is an estimate of the higher selection and attention abilities required to perform TMT B. (v) The Digit Span (DS) is a short-term memory test, in which the subject is required to repeat a list of numbers in increasing order. (vi) The Memory with Interference Task at 10 and 30 s (ITM 10 and ITM 30) is a working memory test, in which subjects are required to memorize three letters and to repeat them after they have been busy with an interfering task (counting two by two). (vi) The Immediate and Delayed Story Recall

Memory (ISRM and DSRM) is a memory tests, in which subjects are required to repeat a story immediately after it has been told, and then again after some 4 min. The diagnosis of MHE was defined by the presence of two abnormal psychometric tests (TMT A and TMT B) and/or abnormal slowing of the EEG (22). While the detection of MHE was based on two psychometric tests, a comprehensive neuropsychological battery was administered to fully characterize the neuropsychological profile of the patients, and thus to assess any possible form of cognitive impairment. The TMT A and the TMT B were performed using the forms and the reference values standardized in our Unit (23). For the other tests, Italian procedures and reference values were utilized (24). Ninety-five per cent of patients had not undergone previous psychometric testing and were administered one of the available versions of the tests, randomly chosen; the remaining 5% had psychometric testing over the previous 6 months and were given a different version compared to baseline. In this subgroup different versions of the tests were utilized. Test performance was expressed by age and education-adjusted Z values. In addition, the mean Z score of all psychometric tests (MZPT) was used as overall cognitive index.

EEG assessment

Both patients and healthy subjects underwent recording of spontaneous, wake, eyes-closed EEG activity, immediately after the psychometric evaluation, by using a 21-electrode EEG cap (*for the procedure's description see above*).

Statistical analysis

Results are expressed as mean ± SD. The distribution of data was analysed by Kolmogorov–Smirnov test: all psychometric tests and EEG parameters fitted a Gaussian distribution. All the neuropsychological tests were expressed as age and education-adjusted Z scores. In that way, psychometric tests were homogeneously expressed and comparable, so that the neuropsychological profiles could be analysed by repeated measure ANOVA. Two-way ANOVA was used to assess the influence of different factors on the neuropsychological profile. The Z score of neuropsychological tests was used as within condition and the aetiology of cirrhosis (three levels: alcohol, HCV, no alcohol/HCV) or the presence of cirrhosis (two levels: present vs. absent as between condition,) were used as between condition respectively. Two-way ANOVA using aetiology (two levels: alcohol vs. HCV) and cirrhosis (two levels: present vs. absent) was used to assess the effect on EEG spectral parameters. One-way ANCOVA was used to assess the role of aetiology within cirrhotic patients, adjusting for the severity of liver disease evaluated by the MELD score. Post hoc comparisons were carried out by the Tukey test.

5.3 *Aim 3: Reversibility of Cognitive Impairment after Liver Transplantation*

Patients

The present study consisted of prospective assessment of neuropsychological

and neurophysiological function before and after LT. Out of consecutive 278 patients with liver cirrhosis evaluated in the tertiary referral centre for HE of the Padova University Hospital, 246 were placed in the waiting list for LT. Of these, 28 patients died on the waiting list and 91 patients were successfully transplanted. Of the latter, 10 patients died during the first year after LT (6 within one month - for sepsis, 2 in the period ranging between six and nine months, due to cardiovascular events). Nine patients were lost to follow-up and 7 patients were excluded (2 because of alcohol intake, 3 for relapse of liver disease and 2 for cerebrovascular events immediately after LT). 65 patients (54 males; age [mean ± SD] 51±8) were included.

Prior to LT, patients with severe renal failure, use of psychoactive drugs, free of alcohol intake less than 6 months, inability to participate in psychometric tests, overt or anamnestic neurological diseases (except HE), such as cerebrovascular events, dementia or Parkinson's disease were excluded. After LT, patients were excluded if they showed recurrence of severe liver disease, alcohol misuse relapse, use of psychoactive drugs, cerebrovascular events, pontine myelinolysis and central nervous system infections.

To assess the role of previous HE on the post transplant cognitive profile the following variables were collected: number of OHE episodes, grade of HE based on the West Haven criteria. Model for End-Stage Liver Disease (MELD) scores were calculated at the time of neuropsychological and neurophysiological examination before LT. The indication for LT was end-stage liver disease in 52 patients and hepatocellular carcinoma in 13. Immunosuppressive therapy at the time of post-LT assessment was tacrolimus as monotherapy (n=34; dose 4±3 mg/die) or in association with mofetil mycophenolate (n=23; dose 1000 mg/die) or cyclosporine (n=8; dose 180±47 mg/die). Patients who completed the post-LT evaluation had good liver (AST 50.4 ± 78 UI/l; Bilirubin total 0.9 ± 0.5 mg/dl; Albumin 41.3 ± 7.3 mg/dl; Prothrombin time 82±16%) and renal function (creatinine 1.23 ± 0.35 mg/dl).

Methods

All patients underwent an extensive neuropsychological evaluation and an EEG spectral analysis 2-8 months prior to and 9-12 months after LT. Furthermore, in a subgroup of 11 patients the measures were also performed 3 and 6 months after LT to test the time course of the psychometric and EEG findings. The demographic and clinical characteristics of patients included are illustrated in Table 4.

Table 4. Demographic, clinical and biochemical characteristics of the total sample of patients studied before liver trasplantation (n=65), and according to the presence of previous episodes of OHE.

	Total sample n=65	**Prior OHE n=23**	**No prior OHE n=42**	*p* value
Demographics				
Age (years) [a]	51 ± 8	51 ± 8	50 ± 9	0.53
Males/Females (n)	54/11	20/3	34/8	0.53
Etiology of cirrhosis (n)				
Virus-related	38	11	27	0,04
Alcoholic	13	4	9	
Mixed	9	7	2	
Others	5	1	4	
Child-Pugh class				
A	4	2	2	0,44
B	55	20	35	
C	6	3	3	
MELD score	13±3	13±2	13±5	0.94
Ascites rating (n)				0.57
None	37	14	23	
Mild/Moderate	20	8	12	
Severe	8	3	5	
AST (UI/l)	80 ± 44	79 ± 47	81 ± 42	0.8
Bilirubin tot.(mg/dl)	4.4 ± 4.8	4.05 ± 4.5	5.2 ± 5.4	0.3
Albumin (mg/dl)	31.2 ± 4.7	31 ± 3.9	31 ± 5.8	0.83
Prothrombin time (%)	58 ± 17	56 ± 17	59 ± 15	0.6
Creatinine (mg/dl)	0.98 ± 0.28	0.98 ± 0.28	0.99 ± 0.29	0.97
Glucose (mmol/l)	6.6 ± 4.5	6.8 ± 5.6	6.5 ± 3.8	0.87
Sodium (mEq/l)	137 ± 6	137 ± 5	138 ± 4	0.5
Previous OHE (n)	23			
One episode of OHE (n)	11			
≥ 2 episodes of OHE (n)	12			

[a] (mean±SD)

Neuropsychological assessment

All patients underwent neuropsychological assessment comprising 1) an extensive paper and pencil psychometric battery, and 2) the computerized SCAN test.

The psychometric tests battery comprised the following paper and pencil tests: Trial Making Test A (TMT A), Trial Making Test B (TMT B), Digit Span (DS), Phonemic Verbal Fluency (PVF), Symbol Digit Test (SDT), Memory with Interference Task at 10 and 30 seconds (ITM 10 and ITM 30) and The Immediate and Delayed Story Recall Memory (ISRM and DSRM). The tests were chosen to cover

different cognitive domains: attention, memory and executive functions (Table 2). Two tests accounted for more than one domain: the Trail Making Test B and The Symbol Digit Test. Psychometric tests were expressed as age- and education-adjusted Z scores, i.e. in units of standard deviations stratified on the basis of age and education level in the reference population of normal individuals. For each test, a Z score ≤ -2 was considered abnormal. For each individual, we calculated both the number of abnormal tests and the mean Z psychometric index (ZPSI) were calculated. ZPSI was used as an overall synthetic index of cognitive performance.

The computerized Scan test is a digit recognition task based on the Sternberg paradigm (111). It has previously been shown to be useful in patients with cirrhosis (112). The subject is presented with 36 consecutive pairs of numbers, which may or may not have common digits. The subject is asked to press 1 on the keyboard if there are digits in common between the two numbers (i.e. 4983, 691) and 3 if there are not (i.e. 481, 7562). The number of correct responses, expressed as a percentage of the total number of stimuli (accuracy), and the accuracy-adjusted, average ScanRT (ms) are calculated.

MHE was diagnosed in patients with cirrhosis in the pre-LT examination. The diagnosis was defined by the presence of 2 or more impaired psychometric tests between TMT A, TMT B, SDT and/or abnormal slowing of the EEG, in the absence of a known cause of impairment (11). To compare cognitive performance before and after LT in patients with MHE we considered, after LT, the alterations on the same psychometric test used to detect MHE before LT (at least at two tests altered between TMT A, TMT B and SDT). While the detection of MHE was based on three psychometric tests (TMT A, TMT B and SDT), we decided to administer a complete neuropsychological battery, to assess any possible cognitive abnormality in the post-transplant course.

Statistical analysis

Continuous variables are reported as mean ± standard deviation. Categorical variables were reported as absolute number and/or percentages. All the neuropsychological tests were expressed as age and education-adjusted Z-scores. The chi-square test or Fisher's exact tests were used to study the existence of significant differences between nominal variables. Student *t* test was used to compare means. Univariate regression analysis was used to identify those pre-transplant factors that were associated with post-transplant cognition. ANOVA for repeat measures was used to compare the mean Z score of psychometric tests of patients with a history of prior OHE with patients without.

5.4 ***Aim 4: Modulation of gut microbiota in HE***

Independent batch culture fermentations with controlled pH (6.8) were inoculated with fecal samples from six cirrhotic patients (age 66±3.3 years; Child-Pugh A (n=5) and B (n=1); average MELD score 9±2.8). All patients with cirrhosis

did not take any antibiotics for two months prior to collection of the faecal samples, had not consumed pre or probiotic supplements.

Freshly voided stool samples were collected in sterile plastic pots on the day of inoculation of the batch vessels. Samples were diluted (1:10 w/w) with sterile reduced phosphate buffered saline (PBS) (0.1 M, pH 7.0) and homogenized in a stomacher (Seward, Norfolk, UK) at normal speed for 2 min. Each vessel was inoculated with 15 ml (10% v/v) of the homogenized fecal slurry.

Batch culture fermentations

Anaerobic, pH controlled batch culture fermentations were used to assess the effect of prebiotic, probiotic and antibiotic on composition of gut microbiota and fermentation characteristics. Water jacketed fermenter vessels (300 ml) were aseptically filled with 135 ml of presterilized basal nutrient medium. The temperature of each batch vessel was maintained at 37°C by means of a circulating water bath. The pH was maintained at 6.8 using an Electrolab pH controller. Batch cultures were run for a period of 24 h and 5 ml samples were obtained from each vessel at 0, 5, 10 and 24 h.

Seven different treatments with lactulose, rifaximin and VSL#3 or their combination were performed. Conditions tested: no treatment (ctrl), lactulose (1%), rifaximin (616μg/ml), lactulose and rifaximin, VSL#3 (initial[] of 108cell/ml), VSL#3 and lactulose,VSL#3 and rifaximin,VSL#3 and lactulose and rifaximin.

Microbial populations were enumerated using flow cytometry Fluorescent In Situ Hybridization (FISH) at 0, 4, 10 and 24 hours. Ammonia concentration was determined at 0, 4, 10 and 24 hours.

Fluorescent in-situ hybridization (FISH)

Changes in the fecal bacterial populations for each batch were assessed using fluorescent in-situ hybridization (FISH). Samples (375 μl) obtained from each vessel at each time point were fixed overnight in 1.125 ml of 4% (w/v) filtered paraformaldehyde (pH 7.2). The fixed cells were centrifuged at 13,000 g for 5 min, washed twice with filtered phosphate buffered saline (PBS) (0.1 M, pH 7.0), resuspended in 300 μl of a mixture of PBS/ethanol (1:1 v/v) and stored at −20 °C until further analysis. Oligonucleotide probes targeting specific regions of the 16SrRNA gene labelled with the fluorescent dye Cy 3 were used (Sigma–Aldrich Ltd., UK). The bacterial groups were selected based on their predominance and contribution to the colonic microbiota. The probes used were specific for total bacteria, *Bifidobacterium spp.*, *Lactobacillus/Enterococci* and *Fecalibacterium prausnitzii*.

6. RESULTS

6.1 *Animal Naming Test for detection of CHE*

Standardization sample

The characteristics of healthy subjects sample are summarized in Table 2. Males and females were homogeneously distributed throughout the age classes (χ^2= 2.9, p=0.4); in contrast, an inverse association between age and education levels (χ^2 =58,p<0.001) was detected, as expected, because high education was rare in old individuals and low education was rare in young individuals due to the calendar effect (since the opportunities for studying increased with time).

In healthy individuals, the ANT_1 and the ANT_2 were found to be strictly correlated (r=0.88, p<0.01) and both tests were found to be correlated with age and education, but not with gender.

On closer inspection, education produced a roof effect over 8 years, so that only subjects with education <8 years were found to list a significantly lower number of animals at 1or 2 minutes than subjects with education ≥8 years. Similarly, subjects with age>80 years were found to list a significantly lower number of animals than subjects≤ 80 years, whereas the effect of age was negligible, if any, in subjects with age≤70 years. Therefore, fixing the lower limit of reference at the 2.5th percentile, the resulting limits are reported in Table 5. The age-education adjusted equivalent score (A-ANT_1) is therefore easily obtained, giving a bonus of 5 animals for people with education≤7 years and age≤80 years, of 8 animals for people with education ≤7 years and age>80 years, and of 2 animals for people with age>80 years and education >7 years, and considering 12 as the cut off of normality for the A-ANT_1.

However, for simplicity, the following model for a simplified ANT_1 equivalent score (S-ANT_1) was implemented: S-ANT_1=ANT_1 for all subjects with education ≥ 8 years, S-ANT_1=ANT_1+3 for subjects with education<8 years and age≤ 80 years; S-ANT_1 =ANT_1+6 for subjects with education<8 years and age>80years.

Table 5. ANT_1's and ANT_2's limits of normality equivalent scores

Limits of normality ANT_1 and ANT_2				
	ANT_1		**ANT_2**	
	Age≤80 years	Age>80 years	Age≤80 years	Age>80 years
Primary school	7	4	10	7
School > 7 years	12	10	19	13
Equivalent score				
A-ANT_1 (limit of reference ≥12)	Education≤7 years and age Age≤ 80 years: +7 animals Education≤7 years and age Age>80 years: +8 animals Education>7 years and Age>80 years: +2 animals			
S-ANT_1 (limit of reference ≥10)	Education≤7 years and age Age≤ 80 years: +3 animals Education≤7 years and age Age>80 years: +6 animals			

Control group of patients with IBD.

On average, the S-ANT_1 performance was comparable of that of healthy subjects in these patients (Table 6) and, notably, none of them had abnormal S-ANT_1 values.

Table 6. S-ANT_1 in healthy subjects, in patients with IBD and in patients with cirrhosis, subdivided on the basis of HE severity

	Healthy sample n=208	**IBD patients** n=11	**Patients with cirrhosis** n=327			
			Unimpaired on PHES n=169	**Covert HE** n=126		**Overt HE** n=32
				Pooled N=126	**MHE** n=76	**Grade I** n=50	
S-ANT_1	23±0.5 [a]	25±2.5	16±0.7 *	12±0.4*§	13±0.5*§ #	11±0.6*§ #	4±0.9*§°

Abbreviations: HE, hepatic encephalopathy; MHE, minimal hepatic encephalopathy, ANT_1, Animal Naming Test at 1 minute; [a] mean±standard error

* $p<0.001$ versus healthy subjects (Tukey's adjustment for multiple comparisons)

§ $p<0.05$ versus unimpaired HE (Tukey's adjustment for multiple comparisons)

# $p<0.01$ MHE *vs*. grade 1 HE

° $p<0.01$ *vs* unimpaired and *vs*. CHE (Tukey's adjustment for multiple comparisons)

Patients with cirrhosis

The clinical and demographical characteristics of the patients are reported in Table 2. One hundred and sixty-nine (52%) patients with cirrhosis were found to be unimpaired on PHES, 32 (10%) were found to have OHE on clinical examination (n=18 grade 2, n=14 grade 3), and 126 (38%) were found to have CHE (MHE or grade I HE). Of these, 50 (15%) had grade I HE and 76 (23%) had MHE on PHES. The S-ANT_1 was found to be correlated with MELD score (r=-0.16, $p<0.025$) and S-ANT_1 was lower in patients with a history of previous OHE than in those who did not have OHE (12±0.6 vs. 14±0.5,$p<0.03$). Adjusting for MELD score, the S-ANT_1 was comparable in subjects with cirrhosis of different etiology (alcoholic cirrhosis=13.6±0.6, viral=13.1±0.5, other etiologies=13.6±0.9; $F_{2,256}=0.6$,$p=0.5$). The S-ANT_1 performance was lower in patients with cirrhosis than in healthy subjects (and subjects with IBD), and, within the patients with cirrhosis, the patients with CHE resulted in between the unimpaired ones and those with OHE (Table 6). In addition, the subjects with grade 1 HE on clinical examination resulted to have lower S-ANT_1 than those with only MHE on PHES (Table 6).

The EEG was found to be altered in 42% (n=47) of the 146 patients who underwent EEG examination and the CFF was found to be altered in the 24% (n=23) of the 95 patients who underwent CFF examination. There were correlations between S-ANT_1 and EEG spectral parameters (MDF r=0.23 $p<0.001$; delta relative power r=-

0.17 p=0.04; theta relative power r=-0.23 p=0.005; beta relative power r=0.26 p<0.001); however, the S-ANT$_1$ did not result to be related with CFF.

A-ANT$_1$ ≤ 16 resulted to provide the optimal cut-off to discriminate the patients with CHE *vs.* the unimpaired ones by ROC curve analysis (sensitivity 79%-CI$_{95\%}$ 68-83;specificity 59%-CI$_{95\%}$ 51-67). Using the S-ANT$_1$, the optimal cut-off value to detect patients with CHE *vs*. unimpaired ones was S-ANT$_1$<15 on ROC curve analysis (sensitivity=83% CI$_{95\%}$=75-89;specificity=47% CI$_{95\%}$= 40-55) (Fig. 1). The ROC curves obtained respectively from the A-ANT$_1$ and the S-ANT$_1$ were comparable (Fig. 1). In addition, S-ANT$_1$ < 10 was found to have quite satisfying values of specificity (84% CI$_{95\%}$=78-90). Sensitivity/specificity, PPV and NPV of the S-ANT$_1$ are shown in Table 7. This produced a three step Scoring System of simple clinical use, which is easy to memorize (Table 8).

Applying the above Scoring System based on S-ANT$_1$, 30% (n=97) of patients had Score 2, 39% (n=128) had Score 1 and 31% (n=102) had Score 0. This Scoring System was found to be correlated with PHES (r=-0.40 p<0.0001) and the MDF of the EEG (r=-0.18 p<0.05), but not with CFF (r=0.11,p: n.s). A negative correlation was found with Child Pugh score (r=0.22 p<0.01), and a trend for a correlation with MELD score (r=0.12 p=0.059). The ANT$_1$ Scoring System was found a good predictor of CHE (χ^2=15 p=0.0005), also if it was considered in its wider meaning as altered PHES/EEG or HE grade I (Table 8).

Figure 1. ROC curves of ANT$_1$ in detecting Covert HE versus Unimpaired HE.

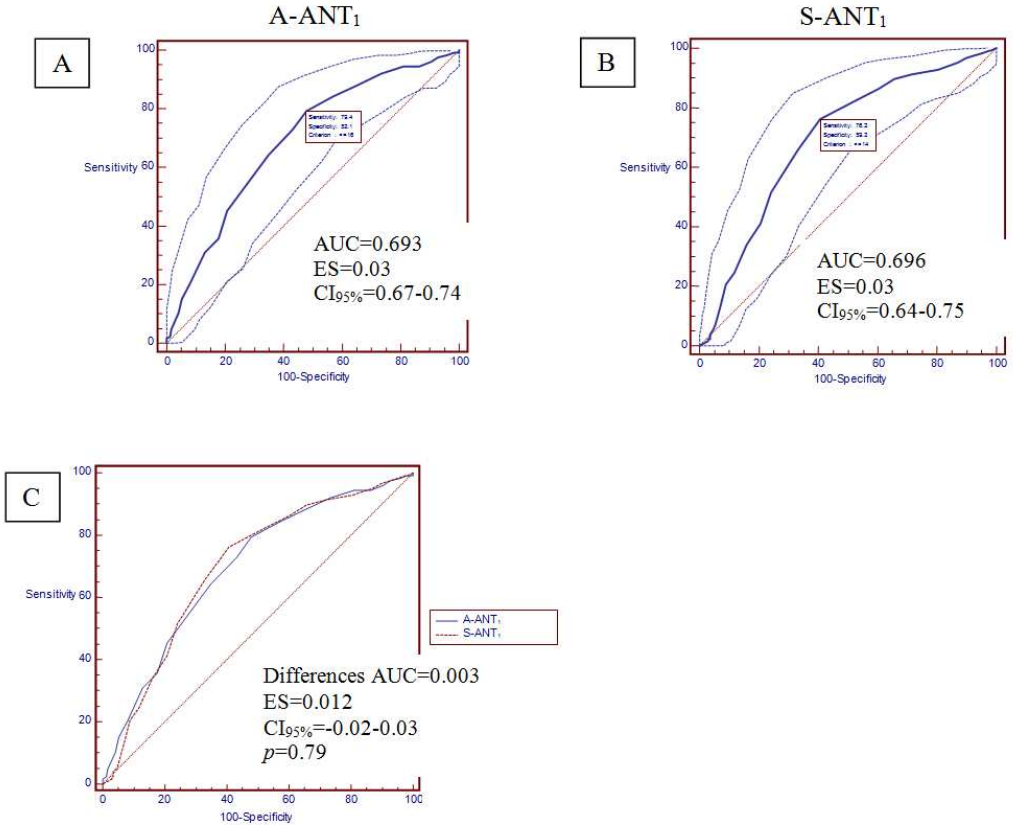


Table 7. Sensitivity/specificity, positive predictive value (PPV) and negative predictive value (NPV) of the S-ANT$_1$

	Sensitivity	Specificity	PPV	NPV	+LR	-LR
CHE *vs.* unimpaired	23%($CI_{95\%}$=16-30)	91%($CI_{95\%}$=87-95)	66%($CI_{95\%}$=58-74)	61%($CI_{95\%}$=52-66)	1.9	1.6
OHE *vs.* CHE	72%($CI_{95\%}$=16-30)	77%($CI_{95\%}$=12-44)	44%($CI_{95\%}$=27-61)	92%($CI_{95\%}$=83-100)	0.8	10.8
Grade I* *vs.* MHE	38%($CI_{95\%}$=5-21)	87%($CI_{95\%}$=46-75)	66%($CI_{95\%}$=53-79)	68%($CI_{95\%}$=55-81)	1.9	0.5
Grade I* *vs.* unimpaired + MHE	38%($CI_{95\%}$=26-51)	90%($CI_{95\%}$=86-94)	43%($CI_{95\%}$=21-65)	88%($CI_{95\%}$=73-100)	0.8	7

	Sensitivity	Specificity	PPV	NPV	+LR	-LR
EEG Altered vs. Normal	26%($CI_{95\%}$=13-36)	95%($CI_{95\%}$=91-99)	76%($CI_{95\%}$=64-88)	73%($CI_{95\%}$=60-86)	2.4	2.7
CFF Altered vs. Normal	9%($CI_{95\%}$=0-21)	92%($CI_{95\%}$=86-98)	25%($CI_{95\%}$=7-43)	76%($CI_{95\%}$=56-93)	0.3	3.1

Abbreviations: CHE, covert hepatic encephalopathy; OHE, overt hepatic encephalopathy; MHE, minimal hepatic encephalopathy; PPV, positive predictive value; NPV, negative predictive value; LR, likehood ratio; EEG, electroencephalogram; CFF, Critical Flicker Frequency. * Based on West Haven Criteria

Table 8. ANT$_1$ Scoring System

Score	S-ANT$_1$	Interpretation
0	> 15 animals	**Probably normal** (PHES or EEG abnormal=31%)
1	> 10 and ≤ 15 animals	**Possibly abnormal** (PHES or EEG abnormal =55%)
2	≤ 10 animals	**Probably abnormal** (PHES or EEG abnormal =78%)
Correction grid*		
If education < 8 years	ANT$_1$ row values + 3 animals	
If both education < 8 years and age > 80 years	ANT$_1$ row values + 6 animals	

*If patients had less than 8 years of education a bonus of 3 animals should be added to the raw score. If patients had both less than 8 years of education and more than 80 years old a bonus of + 6 animals should be added to the raw score.

Prognostic study

The S-ANT$_1$ score resulted to have prognostic value on the risk of a breakthrough of OHE in the follow up, and S-ANT$_1$≤10 had also prognostic value on the one-year risk of mortality. On multivariate analysis, S-ANT$_1$ score '2' resulted to have independent prognostic value on the occurrence of a bout of OHE in addition to

previous episodes of OHE (Table 9). Further, the S-ANT_1 improved the prognostic value of the MELD score on mortality at one year, because the higher numbers of animals reported in one minute the lower the risk of death (Table 9). Finally, using S-ANT_1 and the history of previous OHE, a Prognostic Index of the risk of OHE (HEPI) could be derived [HEPI =10*EXP (-0.0711 x S-ANT1+0.6826 x previous OHE (inserting '0'=for no previous OHE and '1' for previous OHE)]. The HEPI was found to be a strong predictor of OHE on Cox's method (p<0.00001). The HEPI is about 2 for individuals without previous history of OHE and normal S-ANT_1 (giving a probability of OHE at 6 months of about 20%), is about 5 for individuals with either low S-ANT_1 or previous OHE (giving a probability of OHE at 6 months of about 30-35%), and≥10 for individuals with both low S-ANT_1 and previous OHE (giving a probability of OHE at six months≥50%).

Table 9. Predictors of breakthrough of OHE and death at one year

	Follow up study	
	Risk of breakthrough of OHE	**Risk of death**
univariate model	MELD: HR=1.06 ($CI_{95\%}$:1.02-1.10),p<0.001 S-ANT_1: HR=0.92 ($CI_{95\%}$:0.88-0.96),p<0.001 S-ANT_1 score*'1'*: HR=1.24 ($CI_{95\%}$:0.70-2.18), p=0.46 S-ANT_1 score*'2'*: HR=2.38 ($CI_{95\%}$:1.39-4.10), p<0.001 Previous bout OHE:HR=2.20($CI_{95\%}$:1.39-3.48), p<0.001	MELD: HR=1.07 ($CI_{95\%}$:1.02-1.11),p<0.001 S-ANT_1: HR=0.93 ($CI_{95\%}$:0.89-0.98), p<0.001 S-ANT_1 score *'1'*: HR=0.97 ($CI_{95\%}$:0.52-1.80),p=0.93 S-ANT_1 score *'2'*: HR=1.81($CI_{95\%}$:1.02-3.21),p<0.05 Previous bout OHE: HR=1.40 ($CI_{95\%}$:0.85-2.30), p<0.18
multivariate model*	S-ANT_1 score *'2'*: HR=2.10 ($CI_{95\%}$:1.20-3.63), p<0.02 Previous bout OHE: HR=1.79 ($CI_{95\%}$:1.10-2.91), p<0.01	S-ANT: HR=0.95 ($CI_{95\%}$:0.90-0.99),p<0.02 MELD: HR=1.06 ($CI_{95\%}$:1.02-1.10),p<0.01

* Variables selected by backward procedure using Newton Raphson algorithm

6.2 Confounders in the detection of CHE

The three groups of patients with liver cirrhosis (alcohol-related, HCV-related and non alcohol-HCV related) did not significantly differ regarding liver dysfunction, ascites rating and prevalence of diabetes mellitus (Table 10). They differed with regard to AST and creatinine level (higher in the HCV-cirrhosis group and in the alcohol-cirrhosis group, respectively). The diagnosis of MHE, based on the EEG spectral analysis was higher in the alcohol-related cirrhosis group (53% *vs* 27%; p=0,04). Also in the case of MHE detected by the alteration of two psychometric tests (27) the prevalence was higher in patients with alcohol-related cirrhosis (53% *vs* 7% in HCV-cirrhosis and 27% in non HCV-alcohol cirrhosis groups; p=0,0005). The prevalence of previous episodes of OHE was similar in the three groups, slightly higher in the no HCValcohol cirrhosis group but without reaching statistical significance (p=0,42).

Table 10. Clinical and biochemical variables of patients with liver cirrhosis

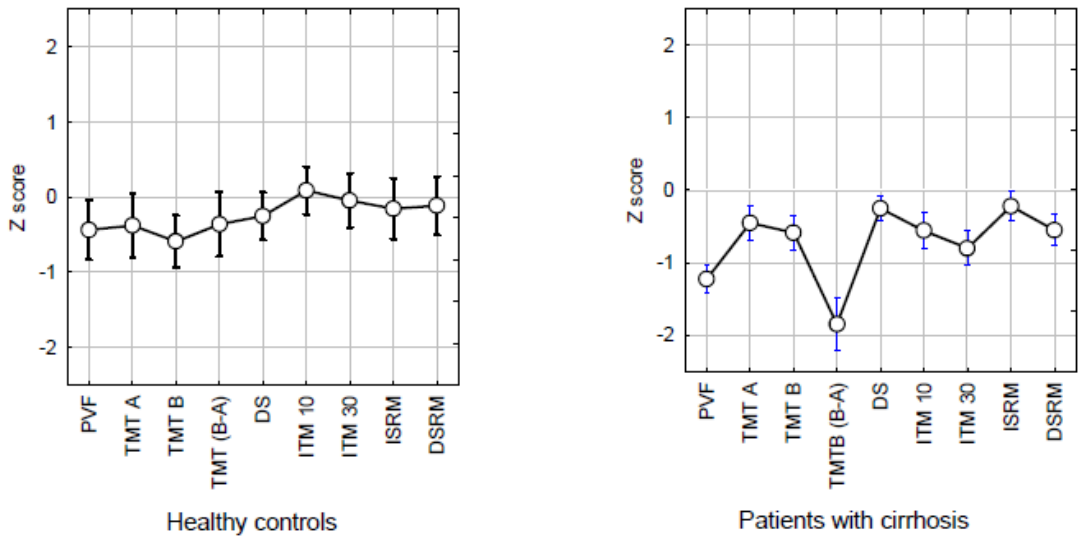
Patients with cirrhosis (n=90)				
	HCV-related (n=30)	**Alcohol-related (n=30)**	**Non alcohol/HCV (n=30)**	**p value**
Ascites rating (n)				0,2
None/mild	20	16	17	
Moderate	7	7	11	
Severe	3	7	2	
HE grading (n)				0,39
Grade 0	26	22	23	
Grade 1	4	8	7	
Child-Pugh class (n)				0,34
A	1	0	0	
B	18	18	23	
C	8	10	5	
Previous episodes of HE (%)	15	27	29	0,42
MHE-EEG [a] (%)	8 (27%)	16 (53%)	5 (27%)	0,04
MHE-F [b] (%)	2 (7%)	16 (53%)	5 (27%)	0,005
AST (U/l)	101±58	49±23	72±48	0,0005
Bilirubin (µmol/l)	57±53	88±87	64±8	0,28
Albumin (mg/dl)	32±5	31±4	31±6	0,71
Prothrombin time (%)	61±13	56±16	54±18	0,24
Creatinine (mg/dl)	0.91±0.23	1.20±0.84	0.90 ±0.19	0,07
Diabetes (n)	7(29%)	4 (17%)	3 (12%)	0,29

HE, hepatic encephalopathy; MHE, minimal hepatic encephalopathy
[a] The diagnosis of MHE was basis on EEG spectral analysis: MDF < 6.8 Hz or THETA ≥ 35 Hz [25]
[b] The diagnosis of MHE was basis on altered TMT-A and TMT-B [27]

Neuropsychological profile

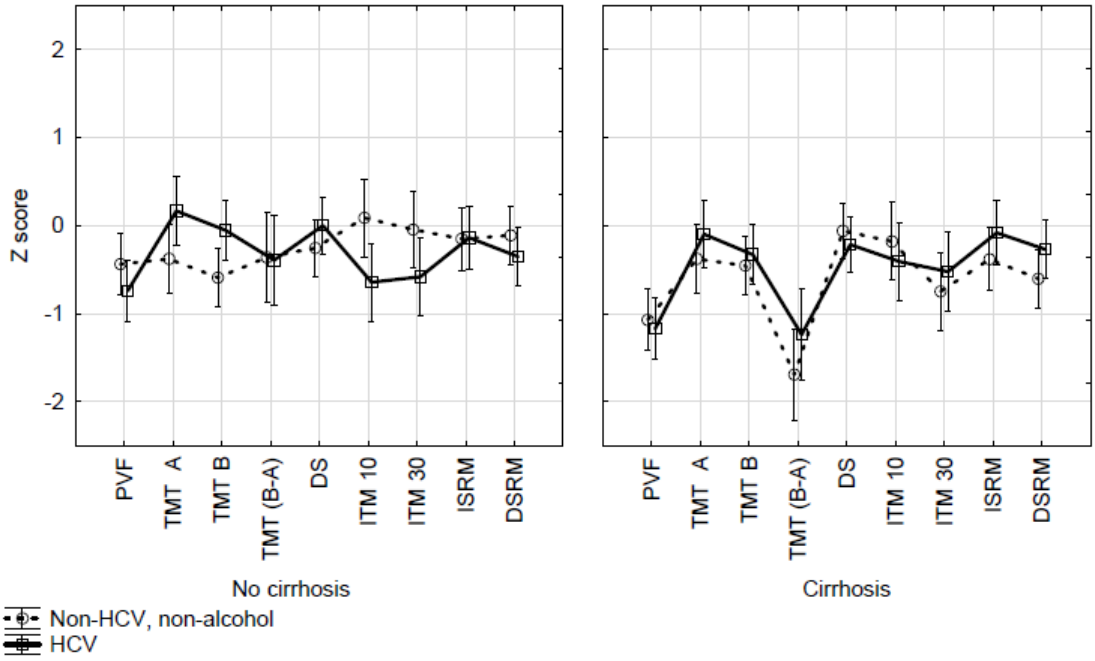
In control subjects, the profile of test performance was homogeneous ($F_{8,232}$=1.6, p=0.11), as expected in subjects where cognition is preserved in all domains. In contrast, in patients with cirrhosis it was heterogeneous ($F_{8,712}$=23.8 p<0.001), with an interaction condition (healthy normal vs. cirrhosis) *per* tests ($F_{8,1424}$=6.4 p<0.001) (Fig.2), showing impaired executive functions, evidenced by worse performance in PVF and TMT (B-A). In contrast, only a trend for ITM 10 and ITM 30 reduction was detected in patients with cirrhosis.

Figure 2. Z scores of psychometric tests in control healthy subjects and in patients with cirrhosis. The profile of psychometric test performance is homogeneous in control healthy subjects (A) and heterogeneous in patients with cirrhosis (B), with massive reduction of the TMT (B-A) and PVF, which reflecting executive functions.



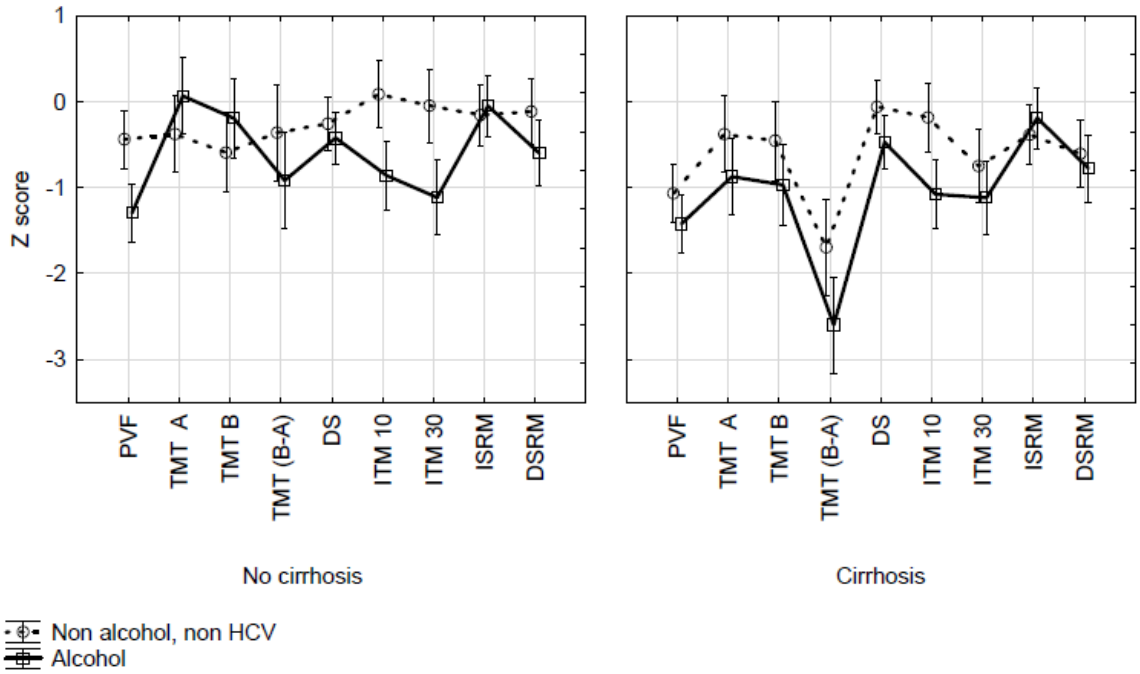
The performance of psychometric test was heterogeneous in subjects with HCV hepatitis ($F_{8,232}$=2.7 p=0.008), since it differed across the domains. An interaction condition (healthy normal vs. HCV hepatitis) *per* tests ($F_{8,994}$=2.4 p<0.02 Fig 2) was found, showing reduced ITM 10 in subject with HCV hepatitis (p<0.05). However, there was no interaction between the factors 'cirrhosis' *per* 'HCV infection' *per* tests ($F_{8,928}$=1.5 p=0.14); indeed, the cognitive profile of patients with HCV-related cirrhosis was comparable that of non-alcohol and non-HCV-related cirrhosis (Fig. 3).

Figure 3. Psychometric tests performance in patients with HCV hepatitis and HCV related cirrhosis. With respect to healthy controls (A), the subjects with HCV hepatitis had reduced ITM 10. No interaction was found between the factor 'cirrhosis' and the tests (B).



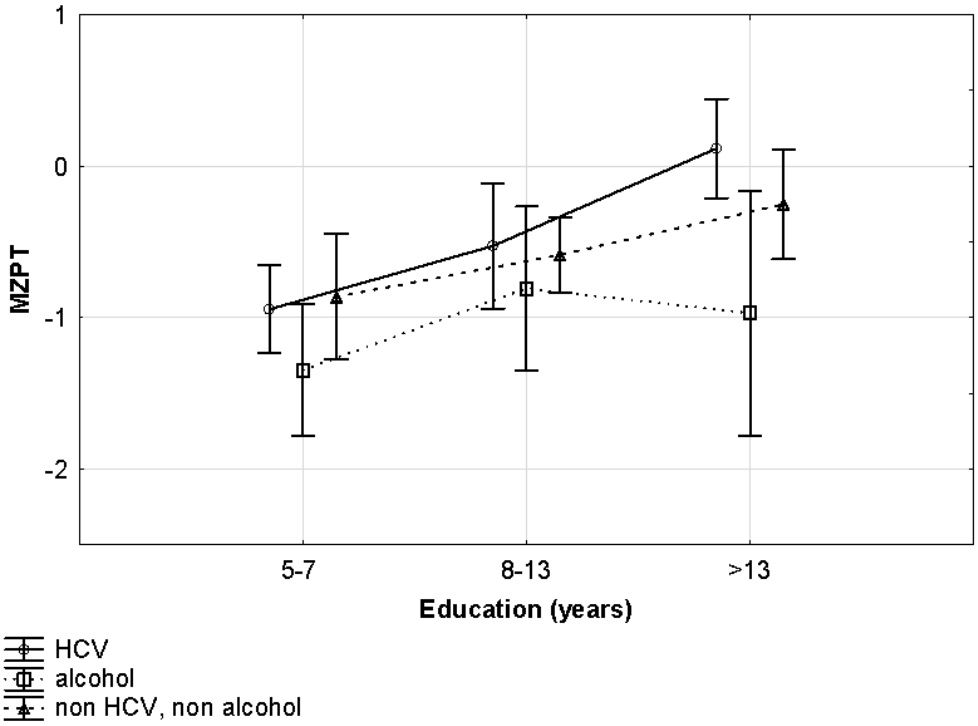
The performance of psychometric tests was heterogeneous also in non-cirrhotic alcohol misusers ($F_{8,232}$=7.7 p<0.001), with an interaction condition (healthy controls vs. non-cirrhotic alcohol misusers) *per* tests ($F_{8,994}$=3,9 p<0.01) and reduction of PVF, TMT (B-A), ITM 10 and ITM 30 (all p<0.05) (Fig. 4), therefore showing a decay in the domains of selection and switching, as well as in working memory. An interaction was observed between the factors 'cirrhosis' *per* 'alcohol misuse' *per* tests ($F_{8,928}$=2.5 p<0.01), so that patients with alcoholic cirrhosis showed lower performance in PVF, TMT (B-A), ITM 10 and ITM 30 (all p<0.05) compared to patients with non-alcohol non-HCV cirrhosis (Fig. 4).

Figure 4. Psychometric tests performance in patients with chronic alcohol misuse (A) and cirrhosis alcohol-related (B). With respect to healthy controls (A), non-cirrhotic alcohol misusers had reduced PVF, TMT (A-B), ITM 10 and ITM 30. (B) Patients with alcoholic cirrhosis showed lower performance of PVF, TMT (B-A), ITM 10 and ITM 30 (all $p<0.05$) than patients with non-alcohol non HCV cirrhosis.



At multiple regression analysis, the overall cognitive performance of patients with cirrhosis, expressed like the mean Z score of all psychometric tests (MZPT), was shown to be influenced by the etiology of cirrhosis ($F_{16,505}=1.7$ $p<0.05$) and this effect remained unchanged also after the correction for the MELD score (Fig. 5).

Figure 5. Psychometric performance in patients with cirrhosis, corrected for the MELD score. (A) Z scores of psychometric tests are lower in patients with alcoholic cirrhosis ($F_{16,504}$=1.7 $p<0.05$). (B) Patients with alcohol related cirrhosis showed lower MZPT than patients with HCV and non HCV-non alcohol cirrhosis, also in the case of correction with the MELD score ($F_{2,63}$ $p=0.01$).



EEG profiles. The MDF, the theta and the beta relative power of quantified EEG were influenced by the factor 'cirrhosis' ($p<0.05$) (Table 11). Alcohol misuse *per se* did not influence EEG spectral indices. However, alcohol misuse was associated with a trend for reduction of MDF and increase of theta and delta relative powers. HCV infection did not appear to be associated with EEG changes (Table 11).

Table 11. EEG variables in the various groups studied

	Patients without cirrhosis			Patients with cirrhosis		
	Healthy controls	**Alcohol abusers**	**HCV hepatitis**	**Non alcohol-non-HCV related**	**Alcohol related**	**HCV related**
Spectral EEG measures						
MDF (Hz)	11.36±1.38	11.78±1.63	10.98±0.98	**9.59±1.94***	**8.96±1.98*[a]**	**10.09±1.98*[a]**
Absolute power (μV^2)	89.64±127.96	80.39±84.96	45.13±30.08	153.23±159.74	143.68±100.06	107.50±81.40
Delta (%)	9.23±9.41	5.64±3.21	7.31±2.57	9.23±9.41	**12.78±13.55 [a]**	7.36±3.91
Theta (%)	14.17±5.81	15.52±12.23	16.10±6.12	**28.92±19.62***	**34.36±16.96 *[a]**	**25.90±17.92 *[a]**
Alfa (%)	47.24±15.84	47.90±14.13	51.82±12.32	46.85±20.16	38.45±17.91	47.01±16.82
Beta (%)	29.37±12.43	30.92±14.09	24.48±10.43	**14.97±9.56***	**14.60±7.91*[a]**	**19.71±14.79***

Note: mean values ± SEM. *****p < 0.05 *vs.* Controls; **[a]** p<0.05 *vs.* subjects without cirrhosis and the same exposure (alcohol, HCV, or non alcohol/HCV)**; [b]** p<0.05 *vs.* subjects with cirrhosis without exposure to alcohol or HCV

Other possible confounders

Since age and education impinge on cognitive decline, we considered the overall cognitive performance adjusted for age and education (MZPT), comparing that of the older patients with cirrhosis (4^{th} percentile) to that of the younger patients with cirrhosis (1^{st} percentile), adjusting for liver dysfunction and etiology. Three educational levels were considered: 5-7 years; 8-12 years and > 13 years. MZPT was found to be predicted by education ($F_{2,66}$=8.1 p<0.001) and etiology ($F_{2,66}$=4.2 p<0.02), with a lower performance in alcoholic cirrhosis. While we found an addictive risk of low education in explaining low cognitive performance (or, vice versa, the protective effect of high education), older age did not seem to be a risk factor *per se.* In contrast to psychometric tests, the EEG indexes did not show an association with education or etiology or age.

The presence of diabetes in patients with cirrhosis was not associated with impaired psychometric performance (*p*=0.29), or low EEG MDF (*p*=0.33).

6.3 Reversibility of Cognitive Impairment after LT

Cognitive function and EEG features before LT

The prevalence of altered psychometric paper and pencil tests before LT is shown in Table 12 and the spectrum of cognitive and EEG alterations before LT are shown in Table 13. Detailed neuropsychological evaluation showed that 42% of patients had one or more abnormal cognitive tests and 12% failed six or more tests, indicating broad neuropsychological impairment (Table 13). Neuropsychological tests before LT documented abnormalities in several domains; the most highly affected were attention and executive function, represented by the TMT B (impaired in 38%

of patients), the TMT B-A (in the 37%) and the SDT (in the 31%) (Table 12). Memory tests were less impaired (DS and ISRM were altered in 5% of patients, DSRM in 6%, ITM 10 and ITM 30 respectively in 14% and 11%). The Scan test was abnormal in 19 patients (29%) and the EEG was abnormal in 22 patients (34%).

MHE was diagnosed in 21 patients (32%) (Table 13). The clinical and biochemical characteristics of patients with and without MHE are presented in Table 4. The two groups were comparable in terms of age, years of education, MELD score. 23 patients (35%) had a history of OHE; the clinical and biochemical characteristics of patients with and without a history of OHE are presented in Table 4 and revealed no statistically significant differences in age, gender, presence of diabetes mellitus and degree of liver dysfunction. However, there was a statistically significant difference in aetiology of cirrhosis, where viral plus alcohol-related aetiology was more frequent in the prior OHE group (chi-square, p=0.084) (Table 14). As expected, in the group of patients with a history of OHE, MHE was more common (61% *vs.* 39%, p=0.03). None of the patients showed OHE at the time of the evaluation before LT. Four patients (all of them with prior OHE) had a bout of grade 2 OHE in between the assessment and LT, and recovered completely (2 episodes were precipitated by constipation, 1 by urinary tract infection and 1 by excessive diuretics). None of the analyses (*vide infra*) changed when these four subjects were excluded.

Table 12. Prevalence of abnormalities on psychometric tests and corresponding neuropsychological domains in the total sample of patients and according to the presence of previous episodes of OHE, before and 9-12 months after LT.

Cognitive Domain	**Test**	**Pre-LT Total sample (n=65)**	**Post-LT Total sample (n=65)**	*p* values	**Pre LT No OHE (n=42)**	**Post LT No OHE (n=42)**	*p values*	**PreLT Prior OHE (n=23)**	**Post LT Prior OHE (n=23)**	*p* values
Attention	**TMT A**	14 (22%)*	12 (18%)	0,48	2 (6%)	7 (22%)	**0,035**	10 (43%)	5 (26%)	0,22
	TMT B	25 (38%)	22 (34%)	0,56	7 (21%)	10 (32%)	0,25	14 (61%)	10 (59%)	0,89
	TMT B-A	24 (37%)	4 (6%)	**0,0001**	7 (21%)	0 (0%)	**0,0017**	13 (57%)	3 (18%)	**0,006**
	SDT	20 (31%)	10 (15%)	**0,01**	7 (21%)	4 (13%)	0,33	10 (43%)	5 (28%)	0,29
Memory	**DS**	3 (5%)	0 (0%)	0,6	0 (0%)	0 (0%)	1,00	3 (17%)	0 (0%)	**0,038**
	ISRM	3 (5%)	1 (2%)	0,25	0 (0%)	0 (0%)	1,00	2 (12%)	0 (0%)	**0,086**
	DSRM	4 (6%)	3 (5%)	0,76	0 (0%)	1 (6%)	0,11	3 (18%)	0 (0%)	**0,033**
	ITM 10	9 (14%)	5 (8%)	0,18	1 (4%)	2 (6%)	0,67	8 (44%)	3 (17%)	**0,047**
	ITM 30	7 (11%)	5 (8%)	0,47	2 (7%)	2 (6%)	0,85	5 (28%)	2 (11%)	0,15
Executive function	**TMT B**	25 (38%)	22 (34%)	0,56	7 (21%)	10 (32%)	0,25	14 (61%)	10 (59%)	0,89
	SDT	20 (31%)	10 (15%)	**0,01**	7 (21%)	4 (13%)	0,34	10 (43%)	5 (28%)	0,29
	PVF	9 (14%)	7 (11%)	0,52	4 (14%)	3 (9%)	0,47	5 (28%)	4 (22%)	0,63

NOTE: * Number (percentage) of patients with altered test. Abbreviations: TMT A, Trial Making Test A; TMT B, Trial Making Test B; DS, Digit Span; PVF, Phonemic Verbal Fluency; SDT, Symbol Digit Test, ITM 10 and ITM 30, Memory with Interference Task at 10 and 30 seconds; ISRM and DSRM, Immediate and Delayed Story Recall Memory; OHE, overt hepatic encephalopathy

Table 13. Prevalence of cognitive and EEG abnormalities before and 9-12 months after LT

	Pre LT (n=65)	**Post LT (n=65)**	*p* values
Number of altered tests:			<0.05
≥ 1 test	27 (42%)*	15 (23%)	
≥ 2 tests	22 (34%)	7 (11%)	
≥ 3tests	17 (26%)	5 (8%)	
≥ 4 tests	12 (18%)	4 (6%)	
≥ 5 tests	9 (14%)	2 (3%)	
≥ 6 tests	8 (12%)	0 (0%)	
Scan test	19 (29%)	10 (15%)	0,017
Cognitive impairment°	19 (30%)	6 (9%)	< 0.05
EEG	22 (34%)	1 (1.5%)	< 0.05
MHE	21 (32%)	7 (11%)	< 0.05

NOTE: * Number (percentage) of patients with altered value; ° Cognitive impairment is represented by at least at two tests altered between TMT A, TMT B and SDT. Abbreviations: MMSE Mini Mental State Examination; EEG electroencephalogram; MCI Minimal Cognitive Impairment; MHE Minimal Hepatic Encephalopathy

Table 14. Comparison of clinical and biochemical characteristics of patients according to the presence of MHE and of a history of OHE before LT

	Before LT						**After LT**
	MHE (n=21)	**No MHE (n=44)**	*p* values	**Prior OHE (n=23)**	**No prior OHE (n=42)**	*p* values	**Delta ZPSI**
Age (years)	50,6 ± 9,9	50,6 ± 8,2	1	51,5± 7,5	50,3± 8,8	0,59	*p*=0,073
Sex (M/F)	81%/9%	84%/16%	0,86	87%/13%	82%/18%	0,64	
Age of education (years)	7.9 ± 3,3	9,9 ± 3,5	0,2	8,3 ± 3,3	9,3 ± 3,3	0,28	*p*=0,52
Etiology of cirrhosis Viral Alcohol HCV+alcohol Other	 13(62%) 6 (29%) 2 (9%) 0 (0%)	 25 (57%) 7 (16%) 7 (16%) 5 (11%)	0,26	 12/52% 3/13% 7/30% 1/4%	 22/65% 6/18% 2/6% 4/12%	**0,084**	
Alcohol etiology	6 (29%)	7 (16%)	0,49	8/43%	10/24%	0,11	*p*=0,04
MELD score	9,7 ± 4,4	10,8 ± 5,3	0,62	10,6± 4,3	11,3± 5,5	0,59	*p*=0,18
Creatinine (mg/dl)	0,9 ± 0,2	1,0 ± 0,3	0,39	0,9± 0,3	0,9± 0,3	0,63	*p*=0,48
Bilirubin (mg/dl)	3,8 ± 2,6	4,1 ± 4,5	0,86	4,4± 3,2	4,2± 4,7	0,82	*p*=0,26
Diabetes	5 (25%)	6 (15%)	0,33	4 (19%)	6 (18%)	0,93	
Prior OHE	11 (61%)	12 (31%)	**0,03**				*p*=0,0014
> 1 episode of prior OHE	7 (39%)	5 (!3%)	**0,05**				*p*=0,0073
MHE				11 (48%)	7 (26%)	**0,03**	

NOTE: Mean standard deviation for continuous variables; number of patients (n/%) for categorical variables. Abbreviations: MELD Model for End stage Liver Disease; OHE Overt Hepatic Encephalopathy, MHE Minimal Hepatic Encephalopathy

Cognitive and EEG assessment after LT

At the end of follow up, there was a significant cognitive improvement in transplanted patients (Table 13). The ZPSI improved significantly (-0.9 ± 0.94 to -0.2 ± 0.47 $p<0.05$). The degree of improvement in global cognitive function (delta ZPSI) was significantly higher in patients with a history of OHE ($p=0.0073$), and especially in those who had had more than one episode of OHE ($p=0.0014$) (Table 14). A relationship was observed between delta ZPSI and the aetiology of cirrhosis, with patients with alcohol-related and HCV plus alcohol cirrhosis showing higher improvement (F=2.93; $p=0.04$) (Table 14). There was no association between delta ZPSI and age, educational level, MELD score, bilirubin and creatinine levels (Table 14). After LT, cognitive impairment was significantly lower than prior to LT (30% *vs* 9%; $p<0.05$), but it not disappear completely. To evaluate the possibly predictors of the presence of cognitive impairment after LT we performed a univariate regression

analysis considering the following variables: age, MELD, prior episodes of OHE, the presence of MHE at the time of first evaluation, alcoholic aetiology and diabetes. Only age (57±5 *vs* 50±9 years) was significant (p=0.08). Thus, we did hot perform multivariate analysis.

The number of psychometric tests altered after LT significantly decreased (p<0.05), however, after LT, 23% of patients had more than one test altered and 8% had ≥ 5 (Table 13). Furthermore, alterations in those psychometric tests used to detect MHE were still present in some patients after LT. Respectively, the TMT A was altered in the 18% of patients after LT *vs* the 22% before LT (p=0.48) , TMT B in the 34% *vs* 38% (p=0.55) and the SDT the 6% *vs* 37% (p=0.01) (Table 12).

Quantified EEG showed a significant marked improvement: both MDF and relative power of theta significantly improved (10.8±0.66 *vs*. 9.14±1.8 Hz and 17.5±5.3 *vs.* 30±16.7% respectively; all p<0.001). The EEG normalized in the 99% of patients and only one patient remained unchanged and abnormal.

Relation with previous episodes of OHE

Patients with a history of OHE showed worse cognitive performance before LT and greater cognitive improvement after LT (p< 0.01) compared to their counterparts with a negative history (overall mean Z score -1.45 ± 1.14 *vs* -0.49 ± 0.55 p< 0.001) (Fig. 6). Their performance remained slightly worse than that of patients without a history of OHE, but still within the normal range (overall mean Z score -0.41±0.47 *vs* -0.11±0.50; p< 0.01) (Fig. 6). Before LT, patients with a history of OHE showed a worse performance in all psychometric tests, and thus in several cognitive domain: attention, memory and executive functions (p<0.001) (Fig. 7). After LT, both groups showed considerable improvement in almost all psychometric tests, except TMT A (mean Z score after LT -0.17± 1.15 in the no-Prior OHE group *vs* -0.55±0.89 in the Prior-OHE group), the TMT B (-1.38±1.47 *vs.* -2.28±1.24 p<0.05), the TMT B-A (-0.13± 0.9 *vs.* -0.58± 1.35). Patients with a history of OHE showed a significant improvement after LT in those psychometric tests exploring memory function (p<0.001), indeed almost all patients normalized (Table 13); in contrast, their performance in those tests exploring attention and executive functions was lower than patients without a history of OHE (p<0.001) and they did not improve significantly (TMT B -2.3±1.24 *vs* -1.4±1.5; SDT -0.6±1.2 *vs* -0.01± 0.9; both p<0.05*)* (Fig. 7).

Patients with a history of OHE also showed more significant EEG slowing before LT than their counterparts with a negative history (MDF 8.8 ± 1.9 *vs.* 9.6 ± 1.6 Hz; theta relative power 31.8±15.9 Hz *vs* 26.7±15.3 Hz) without reaching statistical significance (p=0.36). After LT the EEG became comparable in the two groups (MDF 10.8 ±0.85 *vs.* 10.8 ± 0.6; theta relative power 18.4±7.1 *vs.* 16.6 ± 4.2 p< 0.01) (Fig. 7).

Figure 6. Comparison of cognitive and EEG performance between prior-OHE group and No prior-OHE group before and 9-12 months after LT. (A) Before LT the overall Z score of all psychometric tests (ZPSI) was significantly lower in the prior-OHE group (-1.45 ± 1.14 vs -0.49 ± 0.55 $p<0.001$) and had a greater improvement after LT than No prior-OHE group but their performance remain lower (0.41±0.47 vs -0.11±0.50; $p< 0.01$). (B) EEG spectral analysis: before LT the MDF was lower in the prior-OHE group (MDF 8.8 ± 1.9 vs 9.6 ± 1.6 Hz $p=0.36$) and after LT they became comparable (p<0.01).

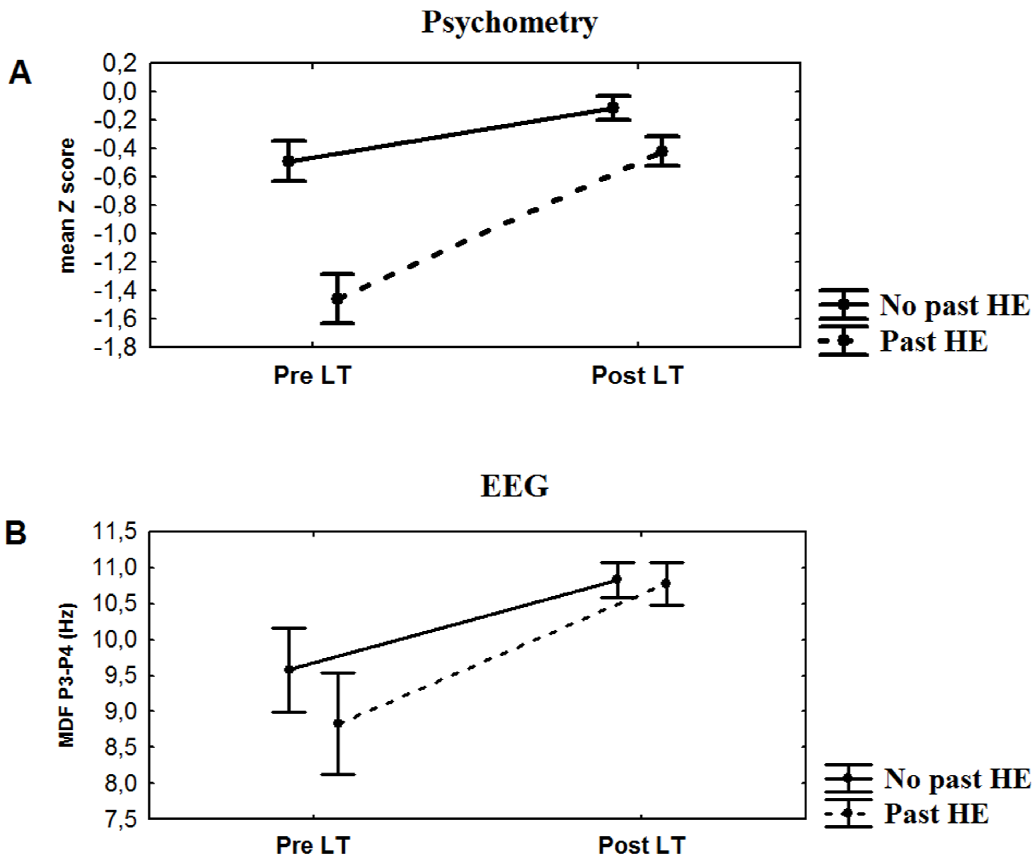
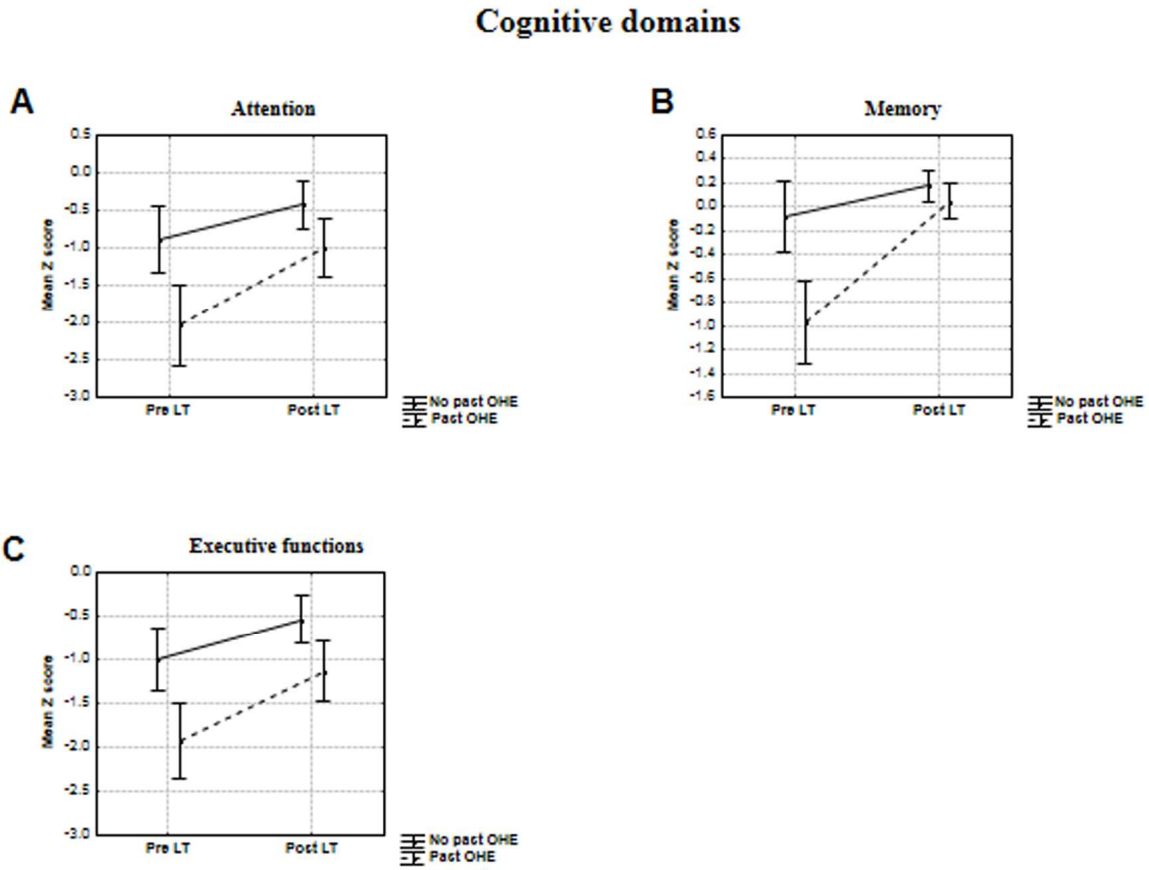


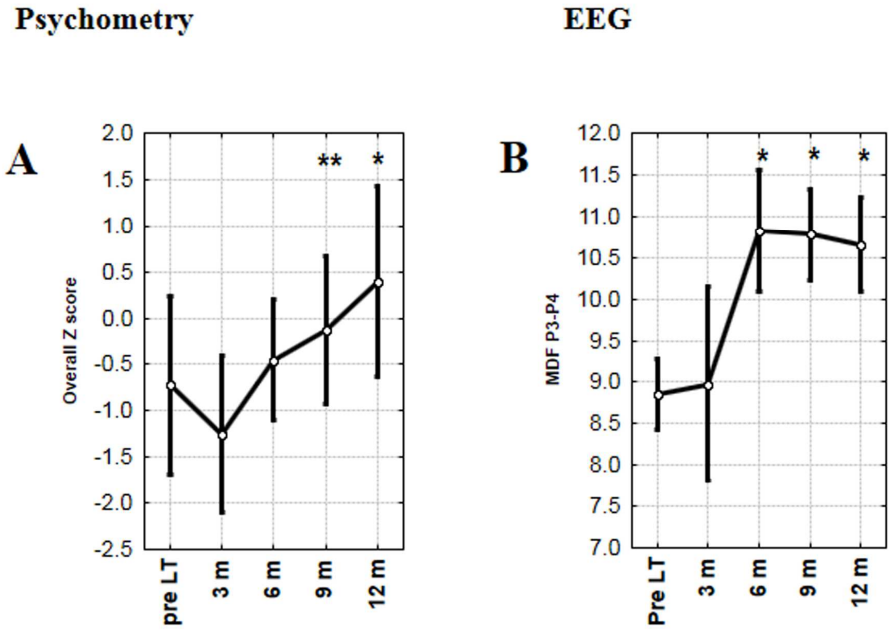
Figure 7. Cognitive function before and after LT, according to the presence of a history of OHE. (A) and (C) Cognitive domain of attention (explored by TMT A, TMT B, TMT B-A, SDT) and executive function (explored by TMT B, SDT and PVF) were more impaired before LT in patients with prior-OHE, after LT their performance remained lower than patients without prior-OHE. (B) Cognitive domain of memory (explored by DS, ISRM, DSRM, ITM 10 and ITM 30), before LT, were more impaired in prior-OHE patients but after LT their performance became comparable to No-prior-OHE patients.



Three-monthly cognitive and EEG assessment after LT

In a subgroup of 11 patients evaluated every three months after LT, the ZPSI slightly worsened immediately after LT and subsequently, steadily improved (Fig.8). ZPSI at 9 and 12 months was significantly better than at 3 months (p<0.05 and < 0.001 respectively). On EEG spectral analysis, the MDF remained unchanged at 3 months and significantly improved from 6 months onwards (p<0.001). Patients who had experienced previous episodes of OHE showed worse EEG profile than their counterparts with a negative history at all time points, although statistical significance was not reached (p=0.75).

Figure 8. Neuropsychological and EEG performance prior to and at 3, 6, 9 and 12 months after LT in a subgroup of 11 patients. (A) The overall Z score of psychometric tests worsened at 3 months after LT and improved from 6 months onward. Cognitive performance (ZPSI) at 9 and 12 months was significantly better than a 3 months (p<0.05 and < 0.001 respectively).(B) At the EEG spectral analysis the MDF remained unchanged at 3 months, steadily improved at 6 months and then remained stable (p<0.001) .

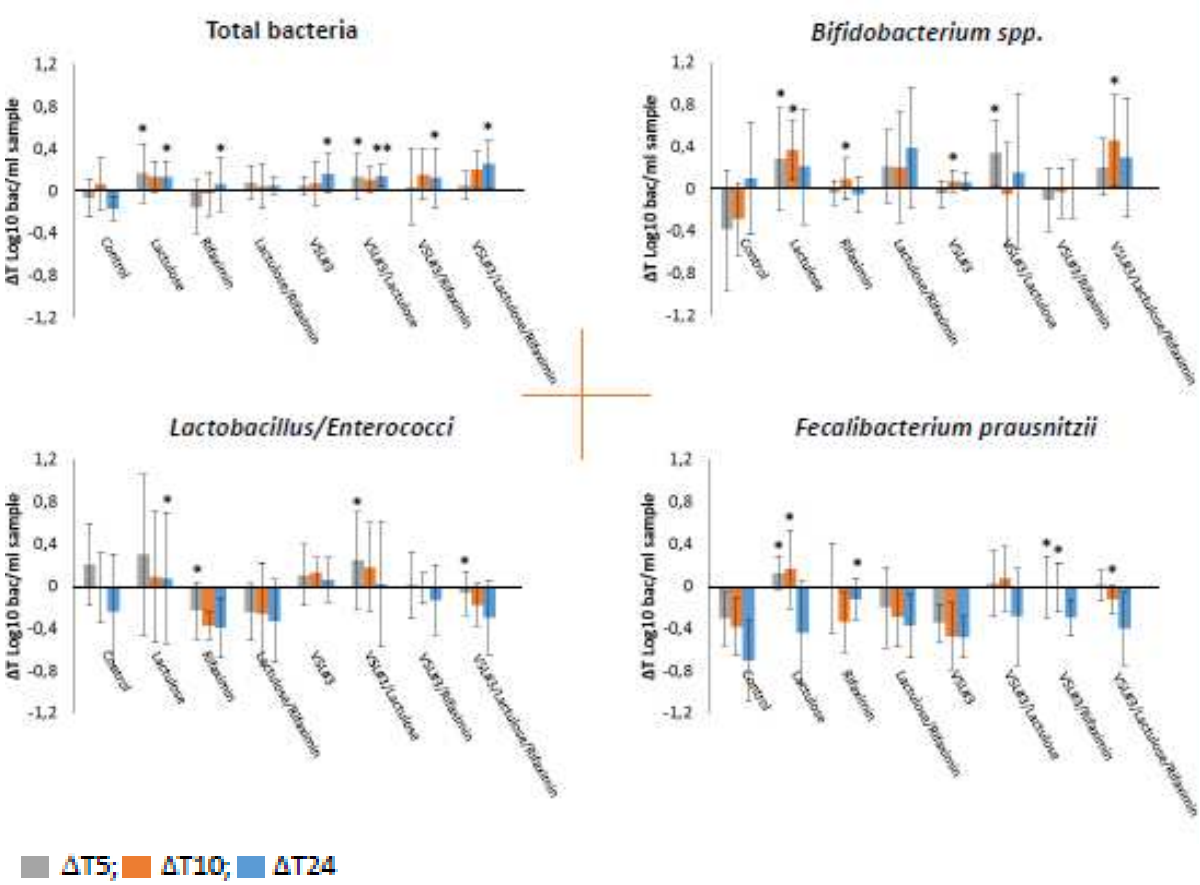


6.4 Modulation of gut microbiota in HE

Bacterial enumeration

Lactulose treatment significantly increased total bacteria, *Bifidobacteria* and *Fecalibacterium prausnitzii* after 5 hours ($p \leq 0.05$) (Fig.9); in contrast Rifaximin and VSL#3 have no significant effect. After 24 hours the combination of lactulose/Rifaximine/VSL#3 significantly increased total bacteria and Bifidobacteria. In contrast, Rifaximin and VSL#3 alone have not significant effect (Fig.9).

Figure 9. Average bacteria count differences, between times 5, 10, 24 and 0 for the considered FISH probes (mean ± sd, n=6)



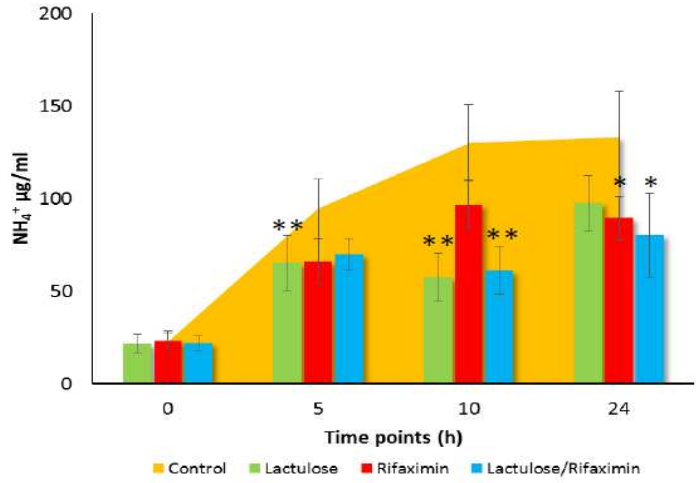
Average bacteria count differences, between times 5, 10, 24 and 0 for the considered FISH probes (mean ± sd, N=6). *p-value≤0.05,**p-value≤0.01,paired t test, treatment vs ctrl.

Ammonia level assay

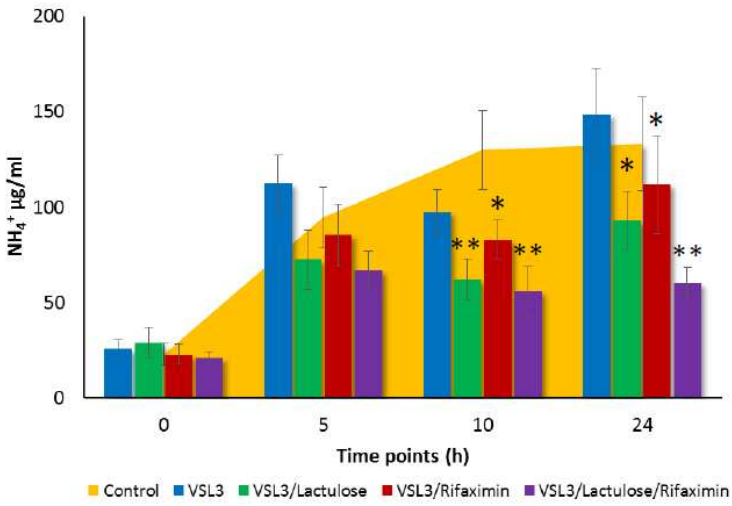
At time 5 h, lactulose significanly reduced NH_4^+, whereas rifaximin had no significant effect. Rifaximin reduced NH_4^+ at time 24 h, also in combination with lactulose (Fig. 10). VSL#3 alone had never significant affect in reducing NH4+, whereas at 24 h in combination with lactulose and rifaximin reduced significantly NH4+ (Fig. 10).

Figure 10. NH_4^+ concentration assessed by colorimetric method on the batch culture fermentation supernatant (mean±sd, n=6, in triplicates)

Lactulose and Rifaximine



VSL#3



*p-value≤0.05 **p-value ≤0.01paired t-test, treatment vs ctrl

7. DISCUSSION

This study analysed various aspects of hepatic encephalopathy, focusing on its mildest form, which is called Covert HE. In particular, a new tool to screen for CHE in patients with cirrhosis, the role of possible confounders in the detection of CHE, the reversibility of cognitive impairment owned by CHE after liver transplantation and the modulation of gut microbiota by HE treatments was analized. More in detail this study: *1)* investigated the applicability of the Animal Naming Test in patients with cirrhosis with various expression of HE, founding that the ANT_1 was an easy first-line tool to detect covert HE, simple to score and closely associated with the degree of HE and risk of bouts of OHE in the follow up; *2)* demonstrated that a history of alcohol misuse and HCV infection hamper cognition, where alcohol was also found to have a synergistic role with cirrhosis in compromising cognitive function and thus should be considered in the diagnosis of CHE; *3)* showed that both cognitive dysfunction and EEG measures significantly improved one year after transplantation but cognitive impairment did not disappear completely; patients with previous OHE had greater improvement one year after transplantation, but their overall cognitive performance remained slightly lower than that of the patients without previous OHE; *4)* showed the beneficial effects in gut microbiota during HE treatment, by a strong increase in beneficial bacteria, reduction of ammonia and regulation of metabolite production using lactulose treatment and its combination with Rifaximin and VSL#3.

In order to investigate the usefulness of the ANT to detect Covert HE, firsly we standardized the ANT in healthy subjects, obtaining the resulting thresholds of normality. The values of reference for ANT in healthy subjects from our sample closely corresponded to those derived from standardization samples of thousands of individuals from Italian population and to be dependent of age and education (113,114,115). In addition, the reference values for the ANT that were found in our study are perfectly in line with wide epidemiological studies in many Western Countries: Netherland (*116),* Spain (117), Portugal (118), France (119), Sweden (120), Canada (121,122) and USA. Notably, animal fluency was found to be independent of gender in agreement with the majority of studies with the exception of the ones by Rosselli M et al. (*123)* and Raoux N. et al. (119). This is an advantage in comparison with the semantic verbal fluency for other categories of objects were found to be influenced also by gender, as it is reasonable for categories of objects with which there is different familiarity between males and females. The strict relationship between ANT_1 and ANT_2, that was proved, justify the use of the only ANT_1 in clinical practice, for simplicity. Further, the study of the features of ANT_1 in relation to education and age showed relevant ceiling effects concerning education (which significantly influenced the results only in very low educated subjects) and age (which significantly influence the results only in relevantly aged people). Therefore, a simplified adjustment of ANT_1 for age and education was possible ($S\text{-}ANT_1$), avoiding the complex standardization system that is usually required for psychometric testing. The agreement between our standardization and previous

studies on ANT strengthens the rationale of the proposal of two thresholds of 10 and 15 animals in one minute for the S-ANT_1. The sample of patients with cirrhosis that we studied was rather typical for subjects referred to a tertiary care center, with a prevalence of CHE of 38% (124) and with prevalence on patients with viral-related cirrhosis slightly higher than the one of patients with alcoholic cirrhosis as is typical of South Europe. The S-ANT_1 resulted very easy to administer, without any complain by the patients, and rapidly provided a piece of information correlated with the ones obtainable by much more cumbersome techniques, such as paper and pencil psychometrics or quantified EEG, even though the cognitive domain explored by the ANT_1 concerns semantic fluency which, in principle, should not be the more proper to detect the first cognitive defects caused by HE (12). However, if the goal of the examiner is to screen for a degree of HE that is just before frank disorientation, the use of a widely applicable test is reasonable and relatively low sensitivity can be preferred. Notably, despite the ANT_1 was not chosen as a tool of extreme sensitivity, also patients with cirrhosis who had PHES>-4 showed a lower ANT_1 performance than healthy subjects or subjects with IBD. This finding was also confirmed after excluding individuals with a history of alcohol misuse, which may act as a confounder in the assessment of cognitive performance in patients with cirrhosis. At any rate, a slowing of cognitive processes in patients with cirrhosis even before reaching the threshold for MHE was also observed in other previous studies (125) and supports the idea of a continuous spectrum of cognitive decline in patients with cirrhosis (126). In addition, the lower performance of the S-ANT_1 in patients with MHE compared with those with grade 1 HE suggests the heterogeneity of patients labeled as having CHE and provides evidence that the S-ANT_1 can detect it. An adequate performance of the ANT_1, which is a semantic fluency test, requires efficient organization of verbal retrieval and recall, as well as self-monitoring aspects of cognition (the participant must keep track of responses already given), effortful self-initiation, and inhibition of responses when appropriate (127) and these cognitive skills requires not only memory abilities, but efficient executive functions. Therefore, it is not surprising that the presence of CHE, which it is well-know to impaired executive functions (128), would affect semantic fluency. As well as mostly recommended neuropsychological tools using to diagnose CHE, i.e. the PSE-syndrome test battery, the Inhibitory Control Test (ICT) and the Scan battery, the ANT_1 explores cognitive functions related to prefrontal cortex/anterior cortical areas, which are particularly vulnerable in CHE. Accordingly, the use of ANT_1 results to be legitimate, in principle, as a simple tool for at least first-level assessment CHE in patients with cirrhosis. This was further proved by: *i)* the correlation of ANT_1 with liver dysfunction (MELD score), *ii)* the lower values of ANT_1 in subjects with a history of previous bouts of OHE, *iii)* the correlation with PHES and the quantified EEG. The lack of a correlation between the ANT_1 and the CFF might indicate that CFF reflects a psycho-physiological domain poorly related to semantic fluency, thus supporting the opinion that high-quality assessment of CHE is preferably multidimensional. Notably, the S-ANT_1 at a threshold of 10 animals and 15 animals

(with specificity of 84 % and sensitivity of 83%, respectively) was able to provide a good classification criterion and produce a meaningful Scoring System for clinical practice. Further, the threshold of 10 animals was found to have prognostic value on the risk of bouts of OHE in the follow up, which is additive to that of a history of previous overt HE. A prognostic index on the risk of OHE (HEPI) composing the information obtained by S-ANT$_1$ and the history of previous OHE was also computed and resulted to be effective. Possibly, even some prognostication about survival seemed to be possible. However, it should be validated in independent prospective studies in other populations. Thus, the S-ANT$_1$ Scoring System resulted to be a useful and easy tool for first-line assessment of HE in clinical practice, at least where accurate formal neuropsychological testing and/or neurophysiological tools are not available.

Investigating the role of confounders in the detection of covert HE, this study confirmed that cirrhosis per se, and therefore HE, is the most relevant factor hampering cognition and, on closer inspection, executive function in patients with HCV-, alcohol-related and non-alcohol/non-HCV cirrhosis groups. At any rate, also a history of alcohol misuse and HCV infection were found to hamper cognition. Both alcohol and HCV impinged on working memory, and alcohol impinged on executive function. Alcohol was found to have a synergistic role with cirrhosis in compromising cognitive function. The EEG was considerably influenced by cirrhosis per se, thus supporting its role in detecting MHE. Alcohol misuse slightly worsened EEG findings in patients with cirrhosis; in contrast, HCV infection had no additive effect. Consequently, the diagnosis of MHE, based on both EEG spectral analysis and psychometric tests was more common in the alcohol-related cirrhosis group. The finding that in patients with cirrhosis, regardless of other confounders, the most impaired tests were TMT (B-A) and PVF is in agreement with previous studies suggesting that prefrontal cortex – and thus executive function – is particularly vulnerable in these patients (13). This is supported by neuroimaging data (10), and suggests the presence of a disorder of the prefrontal/anterior cingulate cortex and basal ganglia circuits. This finding also supports the present consensus on the neuropsychological tools to be used to diagnose MHE, i.e. Psychometric Hepatic encephalopathy score (PHES), which studies cognitive functions related to ‘anterior’ rather than ‘posterior’ cortical areas, as well as the Scan battery, the Inhibitory Control Test (ICT) and the Stroop test. In contrast, the good performance in verbal memory tests concerning the recall of a short story explains the apparent cognitive normality of several cirrhotic patients on routine clinical interview, where only verbal abilities and memory are required. The finding that non-cirrhotic alcoholic misusers showed an impairment in PVF, TMT (B-A), IMT 10 and IMT 30 is in accordance with the ‘Frontal lobe hypothesis’, stating that chronic alcohol misuse impairs executive functions. More precisely, cognitive flexibility, speed in the allocation of attentional resources, shifting ability, speed in information processing, inhibition of perseveration errors, perceptual motor speed, abstraction and planning abilities, and suppression of irrelevant information, which are all related to prefrontal cortex

functioning (64). Indeed, the TMT (B-A) reflects the extra cost of performing a test (TMT B) that has a higher requirements in terms of switching and sustained attention compared to TMT A. Switching and sustained attention, as well as PVF, pertain to prefrontal cortex function. The effect on IMT 10 and IMT 30 concerns working memory in a verbal task that also implicates prefrontal cortical function. The observation that patients with alcohol-related cirrhosis exhibited worse performance in PVF, TMT (B-A), ITM 10 and ITM 30 is in agreement with studies suggesting that chronic alcohol misuse has additive damaging effect on the brain of patients with cirrhosis (128). This finding suggests that the diagnosis of MHE with psychometric tests sensitive to prefrontal dysfunction, such as the ICT, the Stroop and the Scan tests might be biased by chronic alcohol consumption. Notably, the PHES – i.e. the most accredited tool for the detection of MHE (51)– also reflects prefrontal functions and thus might be biased by alcohol misuse, at least to some extent. Recently, however, Goldbecker et al. (129) produced data suggesting that the confounding effect of alcohol misuse on PHES and ICT is negligible. The effect of HCV infection was significant on working memory, in that patients with HCV hepatitis had reduced ITM 10 compared to healthy subjects. This finding is in agreement with those of Forton et al. (130), who reported impairment in working memory, and with subsequent, similar observations by Weissenborn et al. (131) and Fontana et al. (132). These might be caused by HCV brain colonization (65) or, indirectly, by pro-inflammatory cytokines activation. HCV infection did not have a confounding effect in patients with cirrhosis, in agreement with the study by Cordoba et al. (67). This may relate to the fact that in these patients any direct effect of HCV might be masked by the more relevant impact of cirrhosis and HE per se. Cognition as a whole was also influenced by education in patients with cirrhosis, even if education-adjusted norms were used. This suggests that highly educated patients may have higher resilience, because of a higher cognitive reserve and that they may maintain adequate cognition despite brain damage, as observed in Alzheimer Disease (133). The finding that quantified EEG indices were found considerably influenced by the factor ‘cirrhosis’ and only marginally by alcohol misuse per se, is in line with previous findings (7).

Looking on the effects of successful liver transplant on cognitive function and EEG features, this study showed, firstly, that both cognitive dysfunction and EEG measures significantly improved one year after transplantation but, despite the significant improvement, cognitive impairment did not disappear completely, while the EEG normalized in almost all patients. Secondly, patients with previous OHE had worse both psychometric and EEG performance before LT and greater improvement one year after transplantation, but their overall cognitive performance remained slightly lower than that of the patients without previous OHE. Thirdly, the group of patients who were evaluated every three months showed early worsening of psychometric performance and stepwise psychometric improvement from 6 months onward, while the EEG ameliorated from 3 months and remained substantially stable from 6 months onward. At the time of the assessment before LT, our liver transplant

candidates showed impairment in several domains and the most highly affected ones were attention and executive functions, while memory tests were less impaired. In accordance with previous studies (134), MHE had a high prevalence in our sample of patients (32%) and a relevant proportion of MHE patients (61%) had had previous episodes of OHE. To detect cognitive alterations, we used several paper and pencil tests and the Scan test, a well validated computerized test, previously proved to be a good index of cognitive impairment in cirrhotic patients for its ability to explore executive functions and response speed, which are crucial features of MHE. At the second assessment, that was performed 9-12 months after LT, cognitive performance improved significantly but, nonetheless, the improvement was not generalized to all cognitive domains, in line with previous studies (14), so that some cognitive impairment was still present in the 9% of patients. After having excluded from our sample the patients who had developed major neurological damage, our results showed that the cognitive improvement was more pronounced in those patients who had had prior OHE, in particular in those who had had more than one episode of OHE. This finding may be not surprising, considering that, as *per* definition, liver transplant should remove HE. However, their performance remained slightly impaired, notwithstanding the patients with a history of OHE showed greater cognitive improvement after LT compared to their counterparts with a negative history. A possible explanation could be the maintenance of slight brain damage resulting from bouts of OHE. At this regard, it has been reported that HE may cause brain damage, including neuronal loss, and animal models provide convincing evidence that several neuronal cell death mechanisms are activated in HE (75). Despite this, the loss of neurons resulting from episodic HE seems to be mild. Nevertheless, brain tissue loss, even when it is mild, can produce a decline in cognitive function, as was suggested by the correlation between brain atrophy and psychometric test results (135). In addition to a direct link to HE *per se*, studies in experimental animal models provide convincing evidence that neuronal cell death mechanisms similar to those established in stroke and traumatic brain injury are activated in brain by liver failure *per se*. An alternative hypothesis is that subjects with other factors impinging on brain function, such as the presence of brain atrophy of any reason (128) or slight brain damage resulting from chronic alcohol misuse, HCV infection, vitamin or other micro-nutrient deficiency, diabetes etc. present residual cognitive impairment after LT, since this was not caused by liver disease *per se*, and, therefore could not be reversed changing the liver. In addition, such slightly damaged brain might have been more susceptible to the action of the mechanism causing HE, so that OHE might have occurred more easily. Another interesting finding is that, one year after LT, both patients with and without a history of OHE showed considerable improvement in almost all psychometric tests, except in TMT A and TMT B, which resulted to be the most impaired tests after transplantation. This might mean that attention and executive functions are less susceptible to the removal of the cirrhotic liver. So other confounders could affect the brain, independently of HE. We know that alcohol impairs cognition and causes brain

atrophy, which affects mostly the frontal lobes and the cerebellum, but in our sample it did not seem to have significant impact. Another hypothesis is that other factors, like the immunosuppressive therapy, play a role in maintaining lower performance in attention and executive functions.Age was found to be the only likely predictor of cognitive dysfunction one year after transplantation. Aging is associated with the enlargement of brain ventricles and with the occurrence of white matter lesions that, in turn, are closely associated with the development of mild cognitive impairment and dementia (136). Furthermore, previous studies have shown that the size of brain ventricles in cirrhotic patients after LT is larger than that of controls (135), supporting the notion that cirrhosis acts synergically with aging to cause central brain atrophy, and consequent enlargement of brain ventricles (128). Similar to cognitive performance, quantitative EEG resulted altered in 22 patients (34%) before LT, but, differently from psychometric performance, the EEG showed a significant marked improvement and normalized in the 99% of patients, one year after LT. Only in one patient the EEG remained unchanged with respect to the baseline. This observation is in line with the one by Ciancio et al (137), who found that psychometric performance of transplant recipients does not improve as well as the EEG does. A key question is why quantified EEG improves massively after transplantation while the cognitive performance is not complete. One explanation is provided by the fact that the EEG reflects the oscillation of postsynaptic potentials of pyramidal cortical cells. This is sensitive to the influence of energy-providing metabolic pathways, electrolyte balance and the clearance of toxic substances. Therefore, it is not surprising that after LT, and thus after the removal of pathogenic metabolic factors related to the presence of cirrhosis, electrogenesis was normalized. In contrast, psychometric tests investigate cognition, which is an integrated complex of functions, influenced by factors such as age, education, type of job (blue collars/white collars), subjective ‘intelligence’ and connectivity across brain areas. All such factors are substantially independent of liver function and cannot be modified by the removal/replacement of the cirrhotic liver. In addition, cognitive impairment in patients with liver cirrhosis may have a different origin, as mentioned before. Deficiencies of water-soluble vitamins, in particularly thiamine, and minerals (zinc, magnesium, and iron) can produce changes in mental function (138). Also, inflammation is a factor causing of cognitive abnormalities. Montagnese et al. (139) showed that C reactive protein and TNFα concentrations were independent predictors of abnormal psychometric tests, but not of EEG alterations, in patients with cirrhosis this suggesting that neuropsychological and EEG abnormalities have partially different biochemical correlates. Furthermore, patients with a history of OHE showed more EEG slowing before LT than their counterparts with a negative history but, differently from psychometric performance, after LT the EEG became comparable in the two groups. This represents a further proof that the discrepancies between EEG and psychometric abnormalities, often observed in patients with cirrhosis depend, at least to some extent, on the different pathways leading to such abnormalities. In fact, as mentioned before, while the EEG reflects the cortical electric activity. Psychometric tests

measure cognitive function, which reflects much more complex and integrated phenomena. Another important finding of this study is the worsening of the overall cognitive performance immediately after transplant (at 3 months). This might be explained by the enormous impact that the transplant procedure, high dose of immunosuppressant therapy and surgery complications have on patients. From 3 months onwards, psychometric and EEG performances improved significantly. These findings seem to suggest that LT is able to significantly improve patients' cognitive function in the long term, despite the seriousness of surgery, possible perioperative complications, and the neurotoxicity of the immunosuppressant therapy.

In order to evaluate microbial modulation by prebiotic, antibiotic and probiotic treatment in stool samples of cirrhotic patients which were inoculeted in a 24-hour batch culture fermentations model at controlled pH (6.8), this studied showed that ammonia production is reduced by all the treatments in a time dependent and combination manner, except for VSL#3 alone. In effect, VSL#3 ammonia removal was augmented by the presence of prebiotic and antibiotic. Over 24 hours ammonia removal was retained although the efficacy of lactulose was greater at Time 5 and Time 10**.** Thus ammonia reduction appears to be directly linked to increase in relative abundance in Bifidobacteria, better induced by prebiotic and antibiotic. This was in line with previous studies that demostrated that probiotics and synbiotics may exhibit efficacy in the treatment of HE by modulating the gut microbiota. They improve derangement in microbiota by decreasing the counts of pathogenic bacteria and thus improving the endotoxemia, HE and the liver disease (105). Several mechanisms may be involved for such improvement and may include: (i) modulation of the gut microbiota may reduce total ammonia in the portal blood by decreasing bacterial urease activity, decreasing ammonia absorption by decreasing pH, decreasing intestinal permeability, and by improving nutritional status of gut epithelium, (ii) they may decrease endotoxemia and proinflammatory milieu resulting in improvement in inflammation and oxidative stress, and liver disease severity, and finally (iii) they may decreasing uptake of other toxins, such as, indoles, oxindoles, phenols, mercaptons, etc. (140). However, opinion is divided on the clinical significance of these apparent benefits, with one recent systematic review concluding that probiotics had a clinical benefit (141), whereas another concluded that although probiotics appeared to exert a significant effect on blood ammonia levels, probiotics were not effective against clinically meaningful end points (142) It should be noted that a separate systematic review that focused exclusively on MHE concluded that prebiotics, probiotics, and synbiotics were effective for this indication, but that lactulose was superior to both probiotics and synbiotics (99). Each of the major reviews on the topic be moaned the challenges that the interpretation of available data presented, in large part because of limitations in study design and performance.

The effect of treatments on *Fecalibacterium prausnitzii* was also analysed in this study, since recently, it has been demonstrated that levels of F. prausnitzii, a major member of the *Clostridium leptum group* (143) and one of the most prevalent bacteria within the human gut, decreases significantly in patients affected by liver

cirrhosis (26). Therefore, it has been suggested that *F. prausnitzii* could have a role in gut homeostasis, taking into account its immunomodulatory activities (144). Rifaximin alone had no significant effect on major bacterial groups and these results are in agreement with those obtained in a previous study by Bajaj et al in patients with cirrhosis (103). Rifaximin was associated with improved cognitive performance and reduction in endotoxemia in patients with cirrhosis and MHE but only modest change in the stool microbiota characterization with a modest reduction in *Veillonellaceae* and a trend towards increased *Eubacteriaceae*, without an overall significant change in microbiota composition at the phylum or order level (103). Another interesting study of the same group showed that there was no significant difference in the stool flora between patients with HE on lactulose compared with those additionally on rifaximin (43). Thus this study showed that gut microbiota modulation is a potential target for relieving the symptoms of HE by regulating colonic ammonia production. Combination strategy, with *Bifidobacteria* increase, represents the best ammonia reducing condition. In general lack of a direct correlation between microbial modification and ammonia reduction for the tested treatments, suggest a modulation in ammonia production rather than increased size of the "colonic ammonia sink" via microbial biomass alone, as a possible mode of action. The data pave the way to further investigations on gut metabolic activity and microbial cross-talkin the presence of lactulose, rifaximin and VSL#3.

8. CONCLUSIONS

In conclusion, the present study showed that:

1) The S-ANT$_1$ is an easy and useful tool to for first-line assessment of HE that is related to more complex techniques and has prognostic implications;
2) HCV infection, alcohol misuse and low educational level are risk factors for cognitive dysfunction. Alcohol misuse and low educational level had a synergistic effect with cirrhosis, and thus they should be considered when testing for MHE;
3) Cognitive and EEG performance significantly improved one year after LT, but while the EEG normalized in the 99% of patients, cognitive dysfunction did not fully recover. A history of OHE is associated with lower cognitive performance one year after LT, despite cognitive improvement is relevant. Therefore, this study has added to our awareness the risk of cognitive persistent deficit associated with a history of OHE before LT.
4) Microbial modulation by prebiotic, antibiotic and probiotic treatment differently affect the population dynamics and metabolism. The strong increase in beneficial bacteria, reduction of ammonia and regulation of metabolite production seen using lactulose and its combination with VSL#3, emphasize the importance of gut microbiota handling in HE treatment.

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