HEPATIC ENCEPHALOPATHY:
CLINICAL AND EXPERIMENTAL STUDIES

HEPATISCHE ENCEPHALOPATHIE: KLINISCHE EN DIEREXPERIMENTELE STUDIES

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voor mijn ouders

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Chapter 1

INTRODUCTION

The pathogenesis of hepatic encephalopathy is still not known. Ammonia was one of the first compounds considered as a cause of encephalopathy. Therapy, therefore, was aimed at decreasing pathological plasma ammonia concentrations in patients with hepatic encephalopathy. This approach, however, was often insufficient and new theories on the pathogenesis with implications for treatment were developed. The imbalance between aromatic and branched chain amino acids was thought to promote the accumulation of false neurotransmitters which in turn could lead to hepatic encephalopathy. However, the effect of therapy with branched chain amino acids was disappointing and the GABA-benzodiazepine theory was proposed. Reports on the beneficial effect of the benzodiazepine antagonist flumazenil on hepatic encephalopathy seemed to indicate a new therapeutic strategy.

CLINICAL ASPECTS

Before the pathogenesis and treatment of hepatic encephalopathy can be discussed, the clinical syndrome needs definition. Hepatic encephalopathy can develop during acute hepatitis (which is then called fulminant hepatic failure) or during chronic liver disease. In the case of chronic liver disease, hepatic encephalopathy occurs in both the acute form (episodes) and the chronic form (chronic portosystemic encephalopathy) with or without exacerbations (1). Several factors have been identified that precipipate or enhance hepatic encephalopathy; the most important are gastro-intestinal hemorrhage, azotemia, which may be caused by diuretics, sedatives, constipation, hypokalemic alkalosis, excess intake of dietary protein, alcohol abuse, hepatic parenchymal injury and infection (GASCAPAPI) (2-3). The clinical picture is independent of the precipitating cause and cannot be differentiated from that of other metabolic encephalopathies. Ultimately the problem becomes even more complex, since end-stage liver disease is often complicated by failure of other organ systems.

The severity of hepatic encephalopathy is graded on a scale of 0-4 (normal to coma) on the basis of clinical criteria of consciousness, intellectual function, behavior and neuromuscular abnormalities (table 1) (1,3-4). The flapping tremor was initially thought to be specific for hepatic encephalopathy but can also occur in other conditions (3). Because the clinical grading system is rather subjective, objective methods of

Table 1: Clinical grading of hepatic encephalopathy

grade HE	consciousness	intellectual function	personality/ behavior	neuromuscular abnormalities
0	normal	normal	normal	normal
1	inverse sleep pattern hypersomnia insomnia	impaired calculation short attention span mild confusion	depression or euphoria anxiety slowness	tremor impaired handwriting incoordination
2	lethargy	disorientation in time amnesia for past events	decreased inhibition inappropriate behavior	flapping tremor ataxia slurred speech grimacing
3	stupor, pre-coma arousable	disorientation in place/person	bizarre behavior incontinence	hyperactive reflexes Babinskis muscle rigidity
4	coma			coordinated response, flexion or extension to painful stimuli

According to Zieve, Conn and Opolon (1,3-4).

Table 2: EEG grading in hepatic encephalopathy (4-5)

grade HE	EEG characteristics
Parsons-Smith	
0	normal, regular alpha rhythm
A	suppression of alpha rhythm, frequent replacement by faster potentials generally flat and featureless
B	alpha rhythm unstable, disturbed by random waves at 5-7 c/s (most often temporal lobes) often underlying fast activity
C	alpha rhythm disturbed by 5-6 c/s waves in runs (particularly temporal and frontal)
D	5-6 c/s rhythms constant
E	2 c/s rhytm, preponderant frontally, spreading backwards
Opolon	
0	normal, regular alpha rhythm
1	irregular background activity (theta and alpha rhythm)
2	continuous theta activity, bursts of delta activity
3	prevalent theta activity polyphasic transient sharp and slow wave complexes
4a	continous delta activity abundant sharp and slow wave complexes EEG reactivity present
4b	slower activity no reactivity
4c	discontinuous activity with silent periods
4d	rare and flat activity
5	flat

quantitation were applied, the most important being the electroencephalogram and psychometric tests. Parsons-Smith et al. introduced the first system for grading hepatic encephalopathy according to the EEG (5); later others followed (4). The fewer waves per second, the more severe the encephalopathy (table 2). Triphasic waves, a feature of the EEG often encountered in severe hepatic encephalopathy, were once described as diagnostic for liver disease (6), but recently similar triphasic patterns have been associated with other metabolic encephalopathies (7). Although the EEG is less subjective than clinical grading, intraobserver and interobserver errors are still considerable. The usefulness of quantitative EEG analysis has been demonstrated in longitudinal studies (8-11). Because the method is objective, it seems especially of value for scientific investigations. Psychometric testing has been proposed for the detection of subclinical hepatic encephalopathy (12-13). This method requires patient cooperation and therefore cannot be used for quantitation of the more severe stages of

hepatic encephalopathy (grades 2-4).

GENERAL CONSIDERATIONS OF THE PATHOGENESIS

Two aspects seem important to consider in a study of the pathogenesis:

A. identification of the substrate that induces disease
B. the mechanism of action of that substrate.

A: Identification of the substrate

1. Specific factors. Zieve reported 3 requirements that have to be fulfilled before a compound or abnormality can be considered as a causitive factor: I. it should be present in abnormal amounts in the presence of hepatic encephalopathy; II. induction of the abnormality in an animal should induce coma; and III. correction of the abnormality should reverse coma (14). The role of ammonia, especially, as a specific toxin in hepatic encephalopathy has been intensively investigated with reference to these requirements. Increased plasma ammonia levels can be found during most clinical episodes of hepatic encephalopathy (15). Ammonia loading in laboratory animals and patients with liver cirrhosis has been shown to induce encephalopathy (16-18). However, therapy aimed at reducing ammonia levels is not always successful as treatment of hepatic encephalopathy. Furthermore, investigators have argued against a major role for ammonia (19), because low ammonia levels have also been found in severe encephalopathy (20).

2. Aspecific factors. In the past less attention has been paid to the role of complications, often found in severe liver disease, in the induction of encephalopathy. Renal failure is quite common in end-stage cirrhosis and fulminant hepatic failure (21-22). Electrolyte abnormalities (especially hyponatremia), hypoxia and acid-base disturbances may all be present (1,23-24). Nutritional deficiencies have been reported in, especially, chronic liver disease (25). The observation that zinc supplementation improved mild hepatic encephalopathy in some studies (26-29) supports a role for zinc deficiency in its pathogenesis. Furthermore, infections are often encountered (23). All of these factors are known to induce coma when liver disease is absent (30). Because

both the degree and combination of these complications vary markedly in liver disease, their contribution to the syndrome of hepatic encephalopathy is difficult to ascertain.

B. The mechanism of action of the substrate

In theory, toxins or deficiencies may influence central nervous system functioning at 3 levels (figure 1):

1. cell metabolism
2. electrophysiologic membrane function. Ion pumps maintain the concentration gradients of ions across the cell membrane, thereby affecting the generation and spreading of action potentials.
3. biochemical communication between neurons. Neurotransmisssion can be excitatory or inhibitory. Excitatory neurotransmission induces depolarization which could lead to an action potential in a neighboring neuron. Inhibitory neurotransmission causes hyperpolarization of the neuron, thereby making it more resistant to the induction of an action potential.

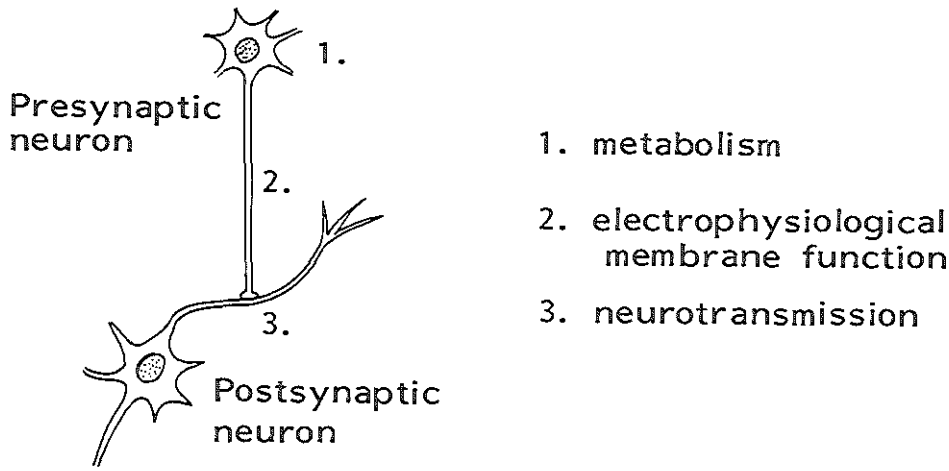

Figure 1: Central nervous system function: possible influences at 3 levels. Toxins or deficiencies may interfere with cell metabolism, electrophysiologic membrane function and neurotransmission.

The most important excitatory and inhibitory neurotransmitters in the central nervous system are glutamate and GABA (31), respectively.

Direct intervention at the neuronal level opens new therapeutic possibilities. Furthermore, direct correction of the defect in the central nervous system will also be the ultimate test of the relevance of the proposed theory on pathogenesis.

PATHOGENESIS AND IMPLICATIONS FOR THERAPY

Ammonia

Ammonia was the first toxin seriously considered as a factor in the pathogenesis of hepatic encephalopathy. It has been extensively studied with reference to the requirements of Zieve, as mentioned above. Ammonia was rejected as an important factor because poor correlations between plasma ammonia and the severity of hepatic encephalopathy were sometimes found (19). Furthermore, the convulsions observed in animals after a bolus injection of ammonia were thought to be incompatible with the sedation encountered in hepatic encephalopathy (19). Lastly, correction of hyperammonemia is not always successful as treatment of hepatic encephalopathy.

Nevertheless, correction of hyperammonemia is currently the most effective therapy we have. Ammonia is formed predominantly in the bowel by degradation of glutamine, which is used as an energy substrate in the intestinal wall, degradation of dietary proteins and degradation of urea by urease-producing enteric bacteria (32). Treatment consists of dietary protein restriction and administration of lactulose or related disaccharides and/or antibiotics. All treatment modalities diminish the formation of ammonia in the bowel. In most studies on lactulose, lactitol or the antibiotic neomycin, the success rate for the reversion of hepatic encephalopathy exceeded 60-70% (33). However, patients with nitrogen overload or patients with low-grade hepatic encephalopathy were usually selected for these studies. In clinical practice, therapy for encephalopathy in severe liver disease is often less successful.

False neurotransmitter theory

The false neurotransmitter theory, as postulated by Fischer and Baldessarini, was the first hypothesis for the pathogenesis of hepatic encephalopathy that explained the syndrome directly at the level of the central nervous system (34-35). They noticed the increase in the ratio of the plasma levels of the aromatic and branched chain amino acids, due to decreased catabolism by the liver of the aromatic and increased use in muscle of the branched chain amino acids. According to the theory, competition between the two classes of amino acids for transport across the blood brain barrier promotes uptake of aromatic amino acids. In the brain, the aromatic amino acids are decarboxylated, forming aromatic amines, and compete with Dopa for further metabolism. Instead of the catecholamines, false neurotransmitters (octopamine, tyramine and ß-phenylethanolamine) are formed. The false neurotransmitters bind to catecholamine receptors but then exert a minimal intrinsic effect. According to this theory a defect in catecholaminergic neurotransmission causes the clinical picture of hepatic encephalopathy.

The theory implies two possible therapeutic strategies: 1. branched chain amino acid infusion to restore the plasma amino acid balance and 2. direct intervention at the level of neurotransmission. Many clinical studies on the effect of branched chain amino acids in hepatic encephalopathy have been performed. Recent reviews of these studies could not demonstrate an important effect, i.e. reversal of hepatic encephalopathy, although restoration of the amino acid balance was achieved (36-37). L-Dopa and bromocriptine, a synthetic dopamine agonist, were administered to enhance catecholamine neurotransmission directly at the neuronal level. In acute hepatic encephalopathy no beneficial effect was found in a double-blind clinical trial (38). Controlled clinical trials on chronic portosystemic encephalopathy only indicated a positive effect of bromocriptine as adjuvant therapy for patients (mainly with neurological symptoms) who did not react to conventional therapy alone (39-40). Although rather attractive, the false neurotransmitter theory has therefore not been convincingly supported by direct intervention at the proposed neuronal level.

The GABA-benzodiazepine receptor theory

The second theory explaining hepatic encephalopathy at the level of altered neurotransmission with direct therapeutic implications was postulated by Schafer and Jones (41). This theory will be discussed in more detail, in view of the studies in this thesis.

Basic physiology. Gamma-aminobutyric acid (GABA) is the most important inhibitory neurotransmitter, accounting for 30-40% of the total neurotransmitter content in the brain (31). The GABA receptor is part of a supramolecular complex with separate receptor proteins for GABA, benzodiazepines and barbiturates (figure 1) (42-43). After the binding of GABA to its receptor, the chloride ionophore which is coupled to the GABA receptor opens and chloride ions diffuse into the cell, thereby causing hyperpolarization of the neuronal membrane (42). The binding of GABA to its receptor and thus its inhibitory action is modulated by the binding of benzodiazepine ligands to their own receptor on the same supramolecular complex. Benzodiazepine agonists

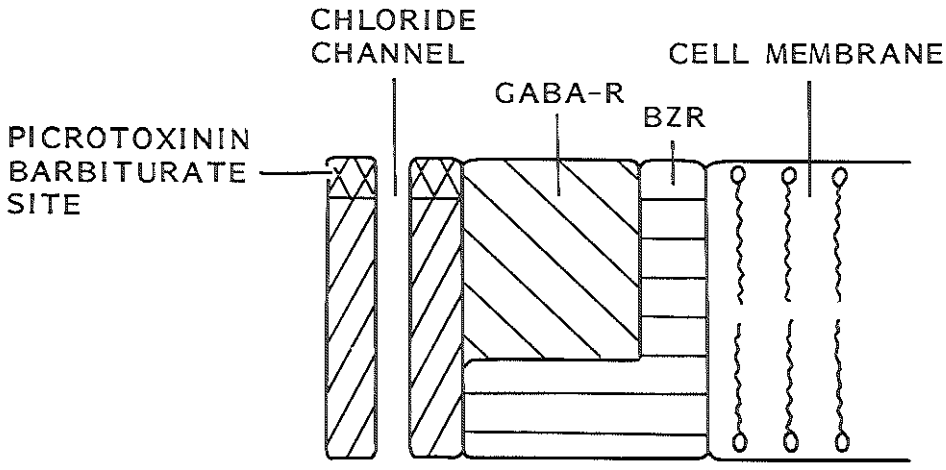

Figure 2: GABA-benzodiazepine receptor complex with binding sites for GABA (GABA-R) and benzodiazepines (BZR); the GABA receptor is coupled to a chloride ionophore (from: Bruun-Meyer SE. The GABA/benzodiazepine receptor-chloride ionophore complex: nature and modulation. Progr Neuropsychopharmacol Biol Psychiatr 1987;4:365-387).

enhance the binding of GABA to its receptor, whereas inverse agonists diminish it. Newly developed benzodiazepine antagonists, such as flumazenil, inhibit the binding of benzodiazepine agonists and inverse agonists but are not considered to have an intrinsic effect on their own (figure 3, table 3) (43).

The "GABA-theory" for the pathogenesis of hepatic encephalopathy implies elevated serum concentrations of gut-derived GABA, increased permeability of the blood brain barrier to facilitate passage of GABA into the central nervous system and an increased number of brain GABA and benzodiazepine receptors, which make the brain hypersensitive to the effect of GABA and benzodiazepines (41). The theory was supported by the finding of increased concentrations of GABA-like activity in serum (44), enhanced transfer of α-aminobutyric acid and GABA across the blood brain barrier

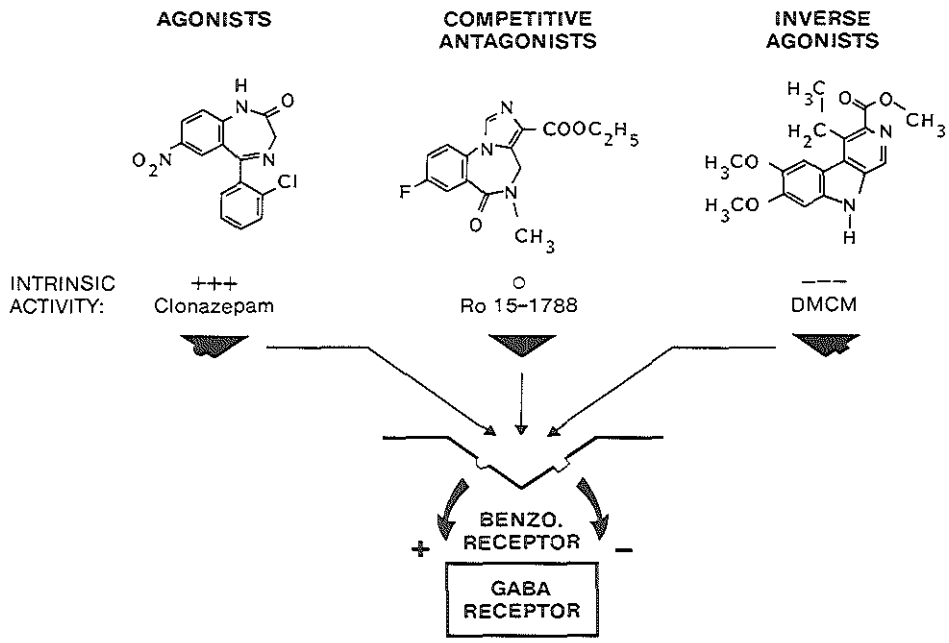

Figure 3: Interaction of the different benzodiazepine ligands with the benzodiazepine receptor and their subsequent influence on the binding of GABA to its receptor (from: Richards JG, Schoch P, Möhler H, Haefely W. Benzodiazepine receptors resolved. Experientia 1986;42:121-126).

Table 3: Benzodiazepine receptor ligands

Benzodiazepine receptor agonists
diazepam,
N-desmethyldiazepam
clonazepam
oxazepam
midazolam
Benzodiazepine receptor antagonists
flumazenil (Ro 15-1788)
CGS 8216
Benzodiazepine receptor inverse agonists
DMCM (methyl-6,7-dimethoxy-4-ethyl-ß-carboline-3-carboxylate)
diazepam bound inhibitor (DBI)
octa-decaneuropeptide (ODN)
ß-CCB (butyl-ß-carboline-3-carboxylate)
ß-CCE (ethyl-ß-carboline-3-carboxylate)
RO 15-3505 (partial inverse activities)
RO 15-4513 (partial inverse activities)

(45-46) and increased binding of GABA and flunitrazepam to cerebral synaptosomal membranes in galactosamine-treated rabbits (47). Furthermore, visual evoked potentials were similar for rabbits treated with muscimol, a GABA agonist, or diazepam, a benzodiazepine agonist, and rabbits with galactosamine-induced liver failure (48).
However, the use of visual evoked potentials for studies on pathogenesis, thereby suggesting specificity of the method, has seriously been questioned (49-50). Furthermore, a study on visual evoked potentials in a rat model revealed differences in the VEP pattern during hepatic encephalopathy and after treatment with a GABA-mimetic drug (51). Confirmation of all other supportive evidence for the theory also could not be achieved. The GABA-receptor assay, used in the first clinical (44) and experimental (52-53) studies to measure plasma GABA levels, was found to overestimate the true levels of GABA as measured by HPLC due to interference of taurine and glutamine (54-55). Nevertheless, HPLC studies still revealed elevated GABA levels in clinical (56) as well as experimental (54,57) acute and chronic hepatic encephalopathy, but these findings contradicted the results of mass-fragmentography (58-59).

The concept of the increased permeability of the blood brain barrier for GABA, originally demonstrated in the galactosamine rabbit model of acute hepatic failure (45-46), also could not be confirmed in other animal models of acute or chronic hepatic encephalopathy (60-62). The only study confirming increased permeability for GABA concluded that it was an unspecific alteration in end-stage multi-organ failure (63). Furthermore, the idea that elevated plasma GABA concentrations induce elevated brain levels is not supported by the findings of normal GABA concentrations in cerebrospinal fluid in chronic hepatic encephalopathy (59,64-65), normal cerebral GABA concentrations in acute and chronic experimental hepatic encephalopathy (58,60) and normal post-mortem GABA concentrations in brains from patients with acute and chronic liver disease (66-67). Direct analysis of the extracellular brain fluid by in-vivo brain dialysis, to evaluate neurotransmitter access to neuronal synapses, also revealed normal GABA levels in portocaval shunted rats (68).

Further studies on the number of cerebral GABA receptors raised even more doubts on the significance of the GABA hypothesis. An increase in GABA receptors could be demonstrated in only 4 (47,69-71) of the 18 reported experimental studies on acute and chronic hepatic encephalopathy (47,52-53,57,60,69-79). Similarly, recent experimental studies on the number of benzodiazepine receptors (57,77) contradicted previous studies describing increased numbers (47,80). GABA and benzodiazepine receptor binding in brain tissue taken at autopsy from cirrhotic patients with hepatic encephalopathy was found to be unchanged in two studies (81-82); one study demonstrated unchanged numbers but an altered affinity of the GABA receptors (83). Recently, the finding of increased numbers of GABA and benzodiazepine receptors in the galactosamine rabbit model was retracted (84).

Therapy with a benzodiazepine antagonist. With the GABA theory fading, the concept of an endogenous benzodiazepine agonist that could cause hepatic encephalopathy was postulated (85). In a few case reports flumazenil, the first benzodiazepine antagonist in clinical practice, was said to ameliorate clinical hepatic coma (86-87). Furthermore, several benzodiazepine antagonists were found to be beneficial in rabbits and rats with acute hepatic failure due to galactosamine and thioacetamide (80,88-90). Because it is assumed that benzodiazepine antagonists do not have an intrinsic effect, a beneficial effect indeed points to the presence of endogenous benzodiazepine-like compounds.

Further evidence for the existence of a natural benzodiazepine ligand was obtained from in vitro electrophysiological studies on cerebellar membranes and radioreceptor assays of brain homogenates from rabbits and rats with toxin-induced liver failure (91-92). However, experimental studies on benzodiazepine antagonists also yielded negative results, particularly in rats with acute ischemic liver failure (93-94). Controversial findings were obtained on the effect of a partial inverse benzodiazepine agonist and a GABA receptor antagonist in this animal model (94-95).

In uncontrolled clinical studies on flumazenil for hepatic encephalopathy remarkably high success rates have been reported: in the two largest studies response rates of 60-

Table 4: Clinical studies on flumazenil for hepatic encephalopathy

	episodes studied				
		effect			screening
author, ref nr	total	≤1.0 mg[1]	>1.0 mg[1]	no effect	Bz
cirrhosis					
Bansky, 87	4	2		2	N
Bansky, 96	14	10		4	Y
Grimm, 97	9		6	3	±
Meier, 98	4		3	1	Y
Klotz, 99	2			2	Y
Ferenci, 100	1	1			Y
Pidoux, 101	6	4	1	1	Y[2]
Viel, 102	3	2		1	Y
acute hepatic failure					
Grimm, 97	11	4	2	5	±
Pidoux, 101	1	1			N
Sutherland, 103	1			1	N
unknown liver disease					
Scollo-Lavizzari, 86	1	1			N
Burke, 104	1	1			N
total	58	26	12	20	
no reversal HE after bolus		1	6		

Bz = benzodiazepine; Y = screening of plasma and/or urine was performed; N = no screening of plasma and/or urine was mentioned; ± = some of the patients were tested.
1. dose of flumazenil studied; 2. at least 3 patients had used benzodiazepines.

71% were found (96-97); considering all episodes studied, an effect occurred in 66% (table 4) (86-87,96-104). Antagonism of previously used synthetic benzodiazepines has been suggested (99). Some authors reported the previous use of these drugs (101), while others applied insensitive methods to screen for benzodiazepines in plasma or urine (97). Controlled studies on the effect of flumazenil are needed and particular attention should be directed toward the relation between a response to flumazenil and the previous use of benzodiazepines as detected by history and laboratory screening methods.

AIMS OF THE STUDY

1. To develop an objective method for measurement of the depth of hepatic encephalopathy for studies on its pathogenesis and therapy
2. to study the effect of flumazenil on hepatic encephalopathy
3. to evaluate the contribution of aspecific factors to the induction of hepatic encephalopathy.

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Chapter 2

OBJECTIVE MEASUREMENT OF HEPATIC ENCEPHALOPATHY BY MEANS OF AUTOMATED EEG ANALYSIS.

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Progress in hepatic encephalopahty and metabolic nitrogen exchange, A. Bengtsson, Jeppsson B, Almdal TH, Vilstrup H (Ed.), CRC Press, Boca Raton, USA (1991;59-66) under the title Spectral Analysis in Hepatic Encephalopathy with the following authors: C.C.D. van der Rijt, S.W. Schalm (part two).

ABSTRACT

Automated analysis of the electroencephalogram as an objective measurement of hepatic encephalopathy for the individual patient was investigated. In part one of the study the parameters mean dominant frequency, relative delta and relative theta activity were investigated. The mean dominant frequency of patients with grade 0 and 1 hepatic encephalopathy was in the normal range ($\geq$6.4 Hz); grade 1 hepatic encephalpathy, however, was characterized by abnormal relative power of theta activity of more than 35%. Twelve out of 34 patients with clinical grade 0 hepatic encephalopathy also had an elevated power of theta activity and probably therefore latent hepatic encephalopathy. Patients with grades 2, 3 and 4 hepatic encephalopathy had a low mean dominant frequency ($<$6.4 Hz) and could be identified by a biphasic power spectrum (theta and delta peaks, grade 2) or by a high power of delta activity ($\geq$70%, grade 3-4). In part two of the study the contributary value of the total power was investigated, especially for the differentiation between grades 3 and 4 hepatic encephalopathy. Grade 3 encephalopathy was characterized by a high total power ($>250\ \mu V^2$) and could be further differentiated from the grades 2 and 4. We conclude that automated EEG analysis based on the parameters mean dominant frequency, relative powers of the delta and theta bands and the total power is very suitable for objective classification of hepatic encephalopathy in individual patients.

PART ONE

Methods for the objective measurement of hepatic encephalopathy (HE) are badly needed, in particular for studies of its pathogenesis and evaluation of its treatment. Clinical grading (1), psychometric tests (2) and conventional electroencephalography (1,3) have been used for the assessment, but these methods are characterized by considerable inter- and intraobserver error. Quantitative EEG analysis has been shown useful in longitudinal studies of hepatic encephalopathy (4-7). Precise classification of individuals for the degree of encephalopathy by quantitative EEG analysis has not been described. Therefore we investigated the discriminatory value of the mean dominant frequency and of the so-called "power spectrum" in controls and patients with the different grades of hepatic encephalopathy.

MATERIALS AND METHODS

The study population comprised a group of 51 normal controls (median age 41 years, range 21-78) and a group of 66 patients with histologically confirmed cirrhosis of the liver (median age 60 years, range 21-75). The cirrhotic patients were seen by two independent physicians who assessed the clinical grade of hepatic encephalopathy (1); only patients with identical scores were included in the study. Thirty-four patients (age range 28-75 years) presented with grade 0, seven (45-73 years) with grade 1, thirteen (52-75 years) with grade 2, five (57-71 years) with grade 3, and seven (21-47 years) with grade 4.

During the investigation the patient lay in a quit room with eyes closed, but fully awake. When sleepiness was observed, an auditory stimulus was applied by the EEG technician, who carefully followed the the recording. Later the EEG record was seen by an electroneurologist and only records without technical artifacts and without evidence of sleepiness were accepted for analysis. The electrodes were placed according to the 10-20 system. During 3 epochs of 100 sec the variations in electric potential between 0.53 and 70 c/sec in leads T4-O2 and T3-O1 (tempero-occipital) were registered by an 8-channel EEG apparatus and stored on magnetic tape.

Subsequently the data were fed into a computer (PDP 11/34) at a sampling rate of 51.2 Hz and a sensitivity of 11 bits/5 V. To avoid aliasing (interference of high-frequency energy), the signals were filtered at 25.6 Hz. Each epoch of 100 sec was divided into 10 periods. The power spectrum was calculated for each period of 10 sec using Fast Fourier Transformation and subsequently the mean power spectrum for each 100 sec epoch was constructed. The frequency resolution was 0.1.

The parameters calculated for the range 1-25.6 Hz (to minimize the effect of slow eye movements) were the mean dominant frequency (MDF), the relative delta, theta and alpha activities. Usually the parameters of the 3 power spectra were avaraged. However, one spectrum was sometimes discarded in case of pronounced muscle activity in the beta frequency band or asymmetry between the leads T4-O2 and T3-O1.

The MDF was defined as

$$\frac{\sum_{i=1}^{n} (f_i S_i)}{\sum_{i=1}^{n} (S_i)}$$

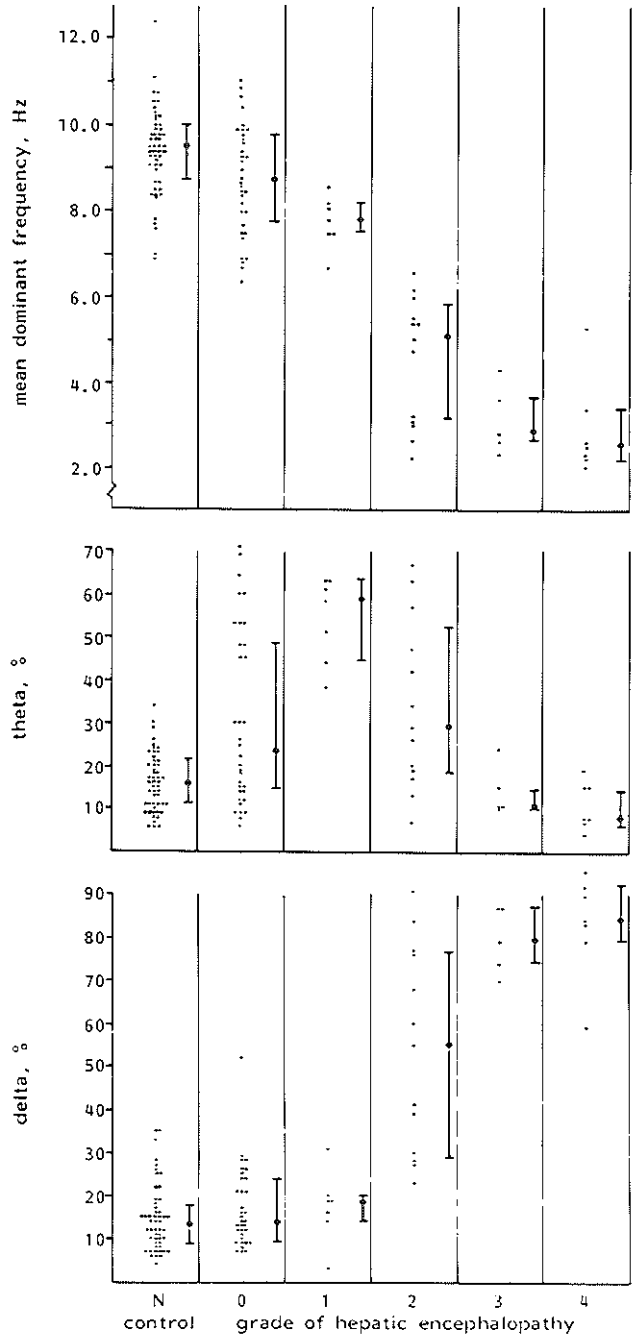
(f_i = frequency i, S_i = power of frequency i, n = the number of frequencies); delta activity comprised frequencies 1.0-3.5 c/sec, theta activity that of frequencies 3.5-8.0 c/sec, the alpha activity that of frequencies 8.0-13.0 c/sec, and the beta activity that of frequencies 13.0-25.6 c/sec.

RESULTS

In the control group age-related differences in MDF and the relative powers of delta and theta activity could not be shown.

Subsequently the values of the above mentioned parameters for the control group and for the 5 groups of patients with varying degrees of HE were compared. In figure 1 scatter diagrams illustrate the MDF and the relative powers of the delta and theta activities. The MDFs for the controls and the groups with grade 0 and grade 1 HE exceeded 6.4 Hz and clearly differed from those found for the groups with grade 2, 3 and 4 HE ($\leq$ 6.6 Hz). Only one MDF value (grade 2 HE) fell in the empirically defined normal range. Furthermore, a distinct slowing of the mean dominant frequency was observed with increasing grades of HE, but there was a large overlap of individual data in adjacent groups.

►Figure 1: Mean dominant frequency and quantitative measurement of theta and delta activities in normal controls and patients with varying degrees of hepatic encephalopathy grouped according to clinical assessment. The relative power of the theta activity probably identifies those with latent hepatic encephalopathy who were classified as patients with grade 0 HE. The power index of the delta activity can be used to identify patients with severe hepatic encephalopathy, who may clinically present as grade 2 HE. The quartiles in the figure are given to illustrate the trend of slowing activity in higher grades of hepatic encephalopathy.


mean dominant frequency, Hz
12.0
10.0
8.0
6.0
4.0
2.0
theta, %
70
60
50
40
30
20
10
delta, %
90
80
70
60
50
40
30
20
10
N
control
0
1
2
3
4
grade of hepatic encephalopathy

Although the MDFs of grade 0 and 1 HE patients did not differ from those of normal controls, the powers of the theta activity of grade 1 HE were clearly higher (>38%) than those of the control group (≤34%). For patients with clinical grade grade 0 HE, the powers of the theta activity ranged from control values to the values found for grade 1 patients. In controls and patients with grades 0 and 1 HE the delta activities were almost always ≤35%. The power of the delta activities found for groups 3 and 4 HE were ≥70%, with the exception of one value of 59% for a grade 4 person.

Individuals with grade 2 HE are characterized by an abnormally low MDF; further differentiation based on the powers of the theta/delta activity appears infeasible. Visual examination of the power spectra obtained for patients with grades 2, 3 and 4 HE revealed that 9 of the 13 spectra of grade 2 HE showed one peak in the theta and one peak in delta range. The other four had only a peak in the delta range and thus resembled the power spectra of patients with grades 3 and 4 HE (figure 2). The 9 patients of grade 2 HE with a biphasic power spectrum could be identified by the power of the delta activity of less than 70%.

DISCUSSION

This study describes an attempt to validate spectral analysis as an objective measurement of hepatic encephalopathy for the individual patient. Patients with grade 1 HE could be distinguished quite easily from the control group by means of their increased theta activity.

The finding of a wide scatter in the data for persons with clinical grade 0 encephalopathy was to be expected on the basis of the results of other studies. Conventional EEGs (8-9), psychometric tests (8) and biochemical monitoring (9) all demonstrated latent hepatic encephalopathy in clinically normal cirrhotic patients. If the range of the power of theta activity found for patients with grade 1 encephalopathy is used as criterion, then 12 of the 34 patients with clinical grade 0 HE could be classified as having latent encephalopathy.

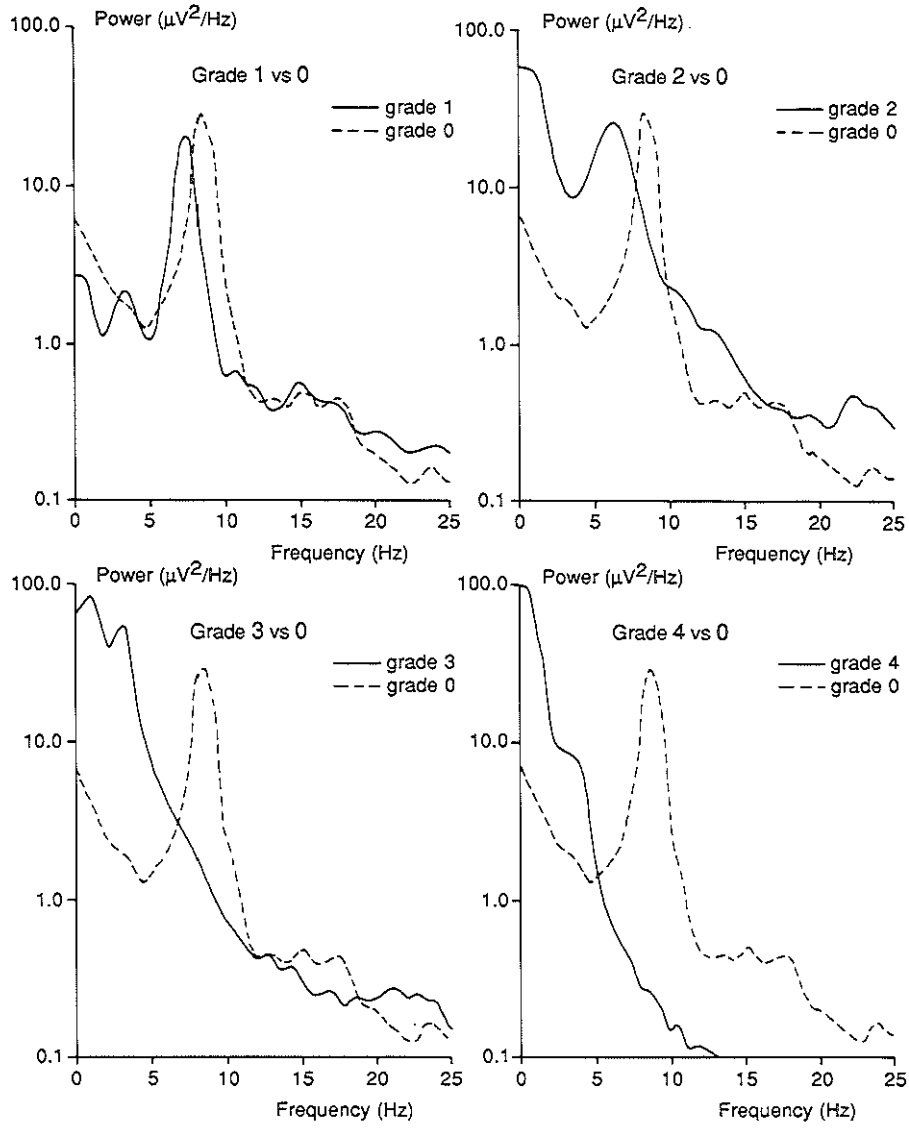


Figure 2: Examples of EEG power spectra of different grades of hepatic encephalopathy. The power spectra of patients with grade 0 encephalopathy resemble those of the controls.

The variation in the delta and theta components found for grade 2 HE was also large. Here again we face the problem; should we rely upon the clinical grading, and therefore conclude that the changes in the EEG really vary so much, or does the wide scatter of these measurements suggest clinical misclassification? The difference in MDF between the groups with grades 0-1 HE and those with grades 2, 3 and 4 HE suggests that clinical differentiation between subtle and severe grades of encephalopathy is reliable. Visual examination of the power spectra of clinical grade 2 patients indicated that 4 patients with only one peak in the delta band had a more severe encephalopathy that was comparable to that of patients with grade 3 and 4 encephalopathy.

The relative high MDF and low power of delta activity of one patient with grade 4 HE appeared to be due to superimposition of bilaterally synchronus activity with a frequency exceeding 14 c/sec, probably from a subcortical source, upon the slow waves of the delta activity. We consider this to be an exception and did not include these values when establishing the limits for each group. A reliable distinction between grades 3 and 4 HE could not be made with the theta and delta activities in our material, which included a relatively small number of patients with these grades of HE.

PART TWO

As described in part one, hepatic encephalopathy can be classified into four grades (0, 1, 2 and 3-4, respectively) using the parameters MDF and the relative theta and delta activities of the power spectrum. A distinction between the grades 3 and 4 hepatic encephalopathy could not be made. The EEG, however, is quite different for the grades 3 and 4, especially with regard to the amplitude of slow waves which are larger in grade 3 hepatic encephalopathy (1). We therefore investigated the contributary value of the total power of the EEG as measured by spectral analysis, in the classification of hepatic encephalopathy. To prevent clinical misclassification, the EEG grade of hepatic encephalopathy was used to group the measurements.

MATERIAL AND METHODS

Sixty eight electroencephalograms with power spectra from 25 patients (median age 50 years, range 27-70) with acute and chronic liver disease were selected for the study. EEG's were classified by an electroneurologist according to Opolon (1). In case he could not decide between two grades, the EEG was not included in the study. Ten EEG's were classified as grade 0 (age range 36-70 years), ten as grade 1 (32-63 years), fourteen as grade 2 (36-68 years), 16 as grade 3 (27-60 years) and 18 as grade 4 (27-60 years).

The technique of the EEG registration and quantitative analysis was similar to that described in part one of the paragraph. Since the amplitude of waves between the 2 hemispheres can differ, the power spectra from T4-O2 and T3-O1 were used seperately. Spectra were discarded when the corresponding EEG registration demonstrated technical artifacts or pronounced muscle activity. The total power of the spectrum was calculated for the range 1-25.6 Hz and defined as

$$\sum_{i=1}^{n} (f_i S_i)$$

(f_i = frequency i, S_i = power of frequency i).

RESULTS

Figure 3 illustrates the individual total powers of the spectrum for the different encephalopathy grades. An increase in power was demonstrated with increasing severity of encephalopathy, with a maximal power for grade 3. EEG's classified as grade 4 encephalopathy varied in power from values lower than those for grade 0 to values found with grade 2. Powers of grade 3 encephalopathy differed markedly from the other grades. Most values (22 from the 27) were greater than 250 μV^2, whereas just 2 of the grade 4 and 5 of the grade 2 values fell in that range.

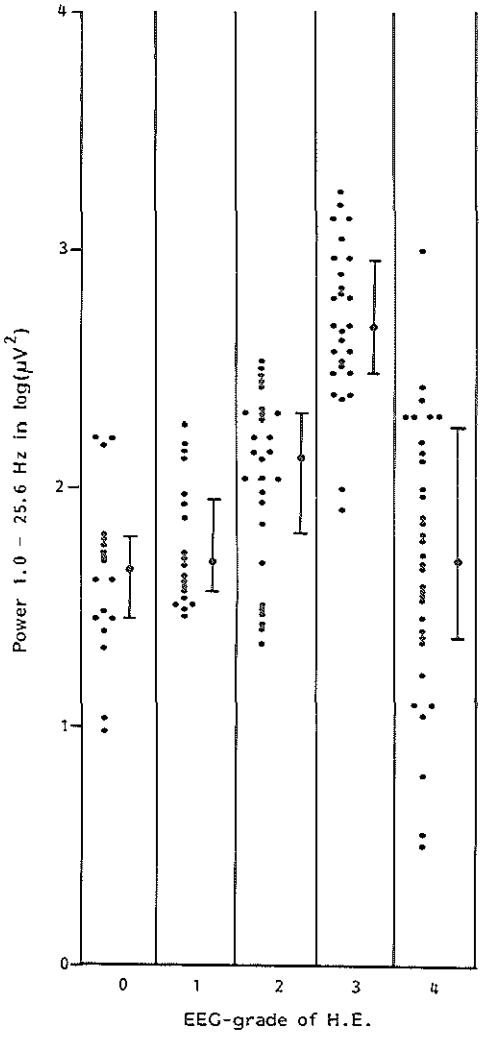


Figure 3: Total power of the spectra in different grades of hepatic encephalopathy grouped according to EEG evaluation. A total power $\geq 250\ \mu V^2$ seems to identify grade 3 hepatic encephalopathy. The quartiles are given to illustrate the trend of increasing power in higher grades of hepatic encephalopathy with a maximum at grade 3 hepatic encephalopathy.

Table 1: Proposed criteria for objective grading of hepatic encephalopathy by automated EEG analysis.

grade of encephalopathy	mean dominant frequency (c/sec)	power spectrum		
		power (uV^2)	% theta	% delta
0	≥6.4		<35	
1	≥6.4		≥35	
2	<6.4			<70
3	<6.4	≥250		≥70
4	<6.4	<250		≥70

DISCUSSION

Hepatic encephalopathy can be quantitated objectively by spectral analysis. As discussed in part one the mean dominant frequency could distinguish between the grades 0-1 and 2-4, respectively; a high (≥35%) relative theta activity appears specific for grade 1; and for the grades 3-4 encephalopathy a high (≥70%) percentage delta activity was demonstrated. With the introduction of the total power of the spectrum a further differentiation between the grades 3 and 4 seems possible.

Table 1 presents our proposed scheme for grading individual cirrhotic patients; the limits are derived from the results of this study. Since the criteria were chosen arbitrarily, the values must be tested in other groups of patients with hepatic encephalopathy. A more accurate distinction between the different grades may be found using statistical methods in larger groups of patients.

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Chapter 3

QUANTITATIVE EEG ANALYSIS AND SURVIVAL IN LIVER DISEASE

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ABSTRACT

A simple, objective method for measuring hepatic encephalopathy (automated spectral analysis of the EEG) was tested for its prognostic value in a group of 60 patients with cirrhosis of the liver and in 10 patients with acute hepatic insufficiency. A highly significant correlation was found between the grade of hepatic encephalopathy and survival. This study further validates the clinical value of objectively grading hepatic encephalopathy by spectral analysis.

INTRODUCTION

We reported on a simple automated EEG analysis test (spectral analysis) as an objective method to measure hepatic encephalopathy (1). With this test encephalopathy can be classified in 5 grades. The clinical significance of each grade of encephalopathy, however, is not fully determined. Accumulating experience suggests that this objective method of grading of encephalopathy is useful for monitoring medical therapy.

Theoretically, the grade of encephalopathy should have a prognostic significance, since hepatic coma is an important cause of death in liver cirrhosis (2-4) and acute fulminant hepatitis (5).

In this retrospective study we investigated the relationship between the grade of hepatic encephalopathy and life expectancy in order to validate further the clinical value of grading hepatic encephalopathy objectively by spectral analysis.

METHODS

Automated EEG analysis was performed in 60 patients with histologically confirmed cirrhosis of the liver, and in 10 patients with acute hepatic insufficiency. In the cirrhotic group 18 patients were under 46 years of age, 26 were between 46 and 65 years, and 16 patients were over 65 years. In 25 patients the cirrhosis was due to alcohol overconsumption, in 8 to viral infection, and in 27 patients the cirrhosis was

cryptogenic or of other etiology. The patients with acute hepatic insufficiency were younger than the cirrhotic patients: seven were less than 46 years of age, two were between 46 and 65 years, and one was over 65 years.

The technique of automated EEG analysis has been described previously by us (1). Variations in electric potential in the leads T4-O2 and T3-O1 (temporo-occipital) were registered during 3 epochs of 100 sec. For each 100 sec 10 periods of 10 sec were used to construct a mean power spectrum by the Fast Fourier Transformation method. To define the grade of hepatic encephalopathy the parameters calculated were: the mean dominant frequency, MDF, defined as

$$\frac{\sum_{i=1}^{n} (f_i S_i)}{\sum_{i=1}^{n} (S_i)}$$

(f_i = frequency i, S_i = power of frequency i, n = the number of frequencies), the relative power of the delta band (1.0-3.5 Hz), the relative power of the theta band (3.5-8.0 Hz) and the relative power of the alpha band (8.0-13.0 Hz). They were calculated for the range 1.0-25.6 Hz. The criteria to define the grade of hepatic encephalopathy are given in figure 1.

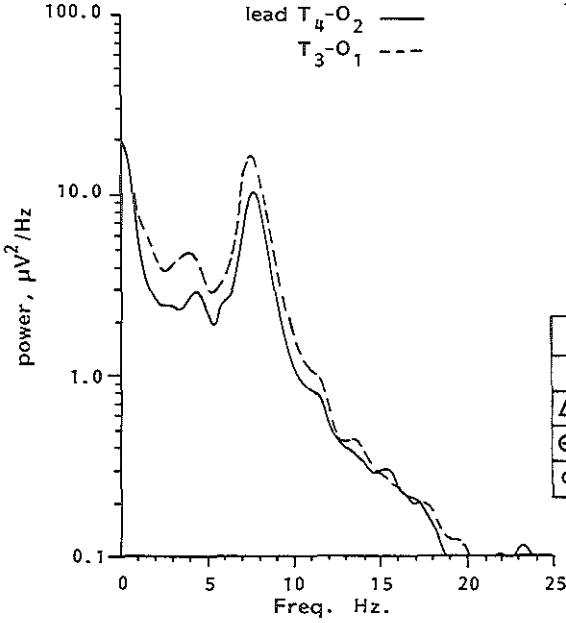
Using this scheme, the grade of hepatic coma was determined in the study population. In patients in whom spectral analysis was also performed during follow-up, the first test was used for this study.

Subsequently, survival curves for the grades 0, 1 and 2, and 3 and 4 hepatic encephalopathy were prepared using a modification of the Kaplan-Meier method (6). First the survival for each patient was determined in months. The percentages of patients that had survived 0, 1, 2,...,36 months were determined in relation to the number of patients at risk each month. Surviving less than 15 days corresponded to a survival of 0 months. This procedure explains survival less than 100% at month 0.

To determine the correlation between survival and grade of encephalopathy the log rank test (a standard test for comparing survival curves) was used (7).

RESULTS

Figure 2A gives the survival curves for the whole groups of patients with grades 0, 1 and 2, and 3 and 4 hepatic encephalopathy. The prognosis for patients with grade 3-4 hepatic encephalopathy was poor, the majority of the patients dying within 2 months. Exclusion of patients with acute hepatic encephalopathy, who all died within a month, did not change the survival curve for grades 3-4 encephalopathy markedly (figure 2B). There was a significant correlation ($p=0.001$) between survival and the hepatic encephalopathy grade in patients with cirrhosis of the liver, when all patients were included in the statistical test. In view of the small number of patients with prolonged



Grading hepatic encephalopathy

grade	MDF(Hz)	%Θ	%Δ
0	⩾6.4	<35	
1	⩾6.4	⩾35	
2 A	<6.4	Θ⩾Δ	<70
2 B	<6.4	Θ<Δ	<70
3-4	<6.4		⩾70

Leads	T_{4}-02	T_{3}-01	mean
MDF.[Hz.]	6.78	6.58	6.68
ΔAct. [%]	21.9	22.0	22.0
ΘAct. [%]	43.9	46.5	45.2
αAct. [%]	28.9	27.7	28.3
Enceph. grade	1	1	

Figure 1: Example of a report of spectral analysis to determine the grade of hepatic encephalopathy. The power spectra of the two symmetrical leads T3-O1, T4-O2 are shown on the left and the calculated measurements of the mean dominant frequency, the relative percentage of the powers of the delta, theta and alpha bands respectively on the right. In the upper right corner, our system for grading of encephalopathy is provided.

follow-up, differences between individual curves could not be tested. Visual inspection suggests that life expectancy in grade 1-2 is only somewhat inferior to grade 0, whereas the prognosis of patients with grade 3-4 is markedly different with a 1 year mortality of 82%.

DISCUSSION

Various methods are being advocated to measure hepatic encephalopathy objectively. Validation of these test methods, however, is difficult. Clinical experience (and logic) suggests that patients with hepatic encephalopathy have a limited life-expectancy, and that the higher the encephalopathy grade the poorer the prognosis. We reasoned that an objective test for assessment of hepatic encephalopathy, to be clinically useful,

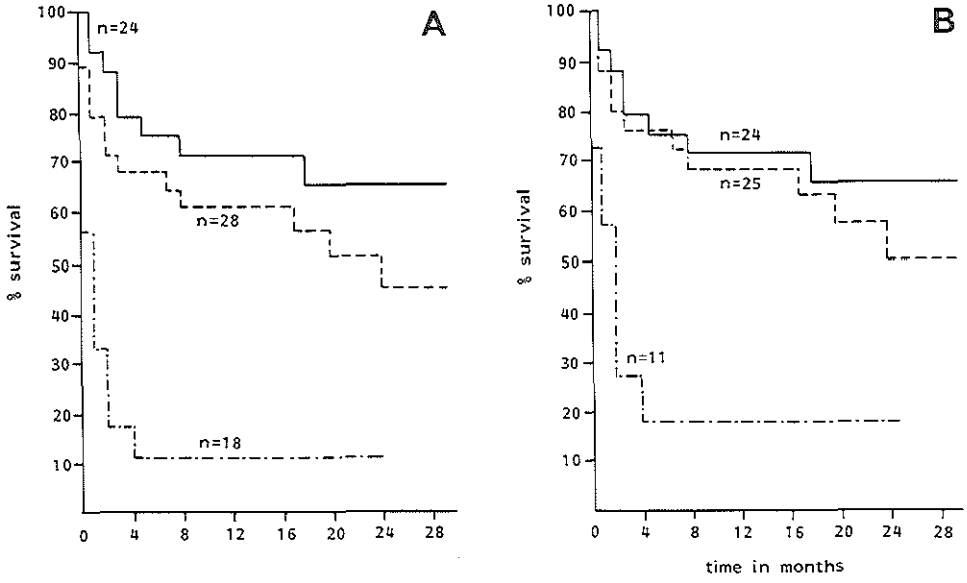


Figure 2: A. Survival curves constructed by the Kaplan-Meier method in acute and chronic liver disease according to grade of hepatic encephalopathy (———: grade 0 HE; ----: grades 1-2 HE; —— - —— grades 3-4 HE). B. Survival of patients with cirrhosis of the liver. A significant relation between survival and grade of encephalopathy was present (log rank test, $p=0.001$).

probably should be able to distinguish patients with a poor prognosis from those with a more prolonged life expectancy.

This study provides evidence that measuring the grade of hepatic encephalopathy by means of automated EEG analysis has indeed a prognostic value for survival in cirrhosis of the liver. Ufer et al. (8) and Prytz and Sloth (9) published survival curves following the first episode of clinical hepatic coma irrespective of the grade. They reported an overall 1 month survival rate of 55-60%, and a 1 year survival of 20%. In patients who died following a coma episode, the grade of encephalopathy was said to be somewhat higher than that in patients who survived. In our study a 1 month survival of 55%, and a 1 year survival of 18% is found only for patients with grade 3-4 hepatic encephalopathy. It is therefore likely that patients with grade 3-4 hepatic encephalopathy according to spectral analysis are those with unequivocal clinical hepatic coma. The 1 month survival of 88% and a 1 year survival of 68% in the grades 1-2 hepatic encephalopathy are more favourable and do not differ markedly from cirrhosis without encephalopathy. However, identification of the group of patients with mild or subclinical hepatic encephalopathy by a simple and objective test appears very worthwhile, since medical treatment of latent hepatic encephalopathy may improve the quality of life in an important way.

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Chapter 4

QUANTITATIVE EEG ANALYSIS AND EVOKED POTENTIALS TO MEASURE HEPATIC ENCEPHALOPATHY

The contents of this chapter have been submitted for publication under the same title with the following authors: C.C.D. van der Rijt, S.W. Schalm.

Methods for the objective measurement of the depth of hepatic encephalopathy are important in scientific investigations and for the follow-up of the individual patient with liver insufficiency in general practice. Quantitative EEG analysis has been demonstrated to correlate with the severity of hepatic encephalopathy (1). Furthermore, the grade of hepatic encephalopathy as assessed by spectral analysis seems to have a prognostic value (2). In recent years new electroneurologic methods, in particular evoked potentials, have been investigated for the detection and quantitation of hepatic encephalopathy. Of the evoked potentials several modalities were advocated: flash visual evoked potentials (flash VEP's) (3-6), pattern reversal visual evoked potentials (7-10), somatosensory (10-12) and brainstem auditory evoked potentials (10) (SSEP's and BAEP's, respectively) and the measurement of the auditory P300 event-related potential, an electric counterpart of the reaction time (9,13).

With the introduction of these new methods evaluation of their value for clinical practice and scientific investigations seems indicated. The pattern reversal and the auditory P300 event-related potentials require patient participation and therefore can only be used for the detection of subclinical and mild hepatic encephalopathy. Flash VEP's (3-4,6), SSEP's (11) and BAEP's (14) have been studied for their correlation with the grades 0-4 encephalopathy, since they can be measured in all patients. No correlation with the depth of encephalopathy was found for the latencies of brainstem evoked potentials (14), whereas latencies of SSEP's (11) and flash VEP's (3-4,6) differed significantly between succeeding grades. The objectivity of flash VEP's, however, may be questioned, as three reported studies demonstrated different changes in wave pattern, especially with respect to peaks disappearing in higher grades of hepatic encephalopathy (3-4,6). According to our own experience on the use of flash VEP's in severe hepatic encephalopathy, the identification of distinct wave patterns is extremely difficult. A considerable inter- and intraindividual variation, indeed, has been reported (15).

The sensitivities of different methods for the detection of latent hepatic encephalopathy may be used to compare automated EEG analysis and different modality evoked potentials more directly. As far as we know, no study reported the correct sensitivity of the test, defined as the number of patients with abnormal test results in a group of patients with latent hepatic encephalopathy already diagnosed by a method considered as the

Table 1: Summary on the percentages of patients classified as having latent hepatic encephalopathy by various electrophysiologic methods in patients with chronic or acute liver disease without mental alterations.

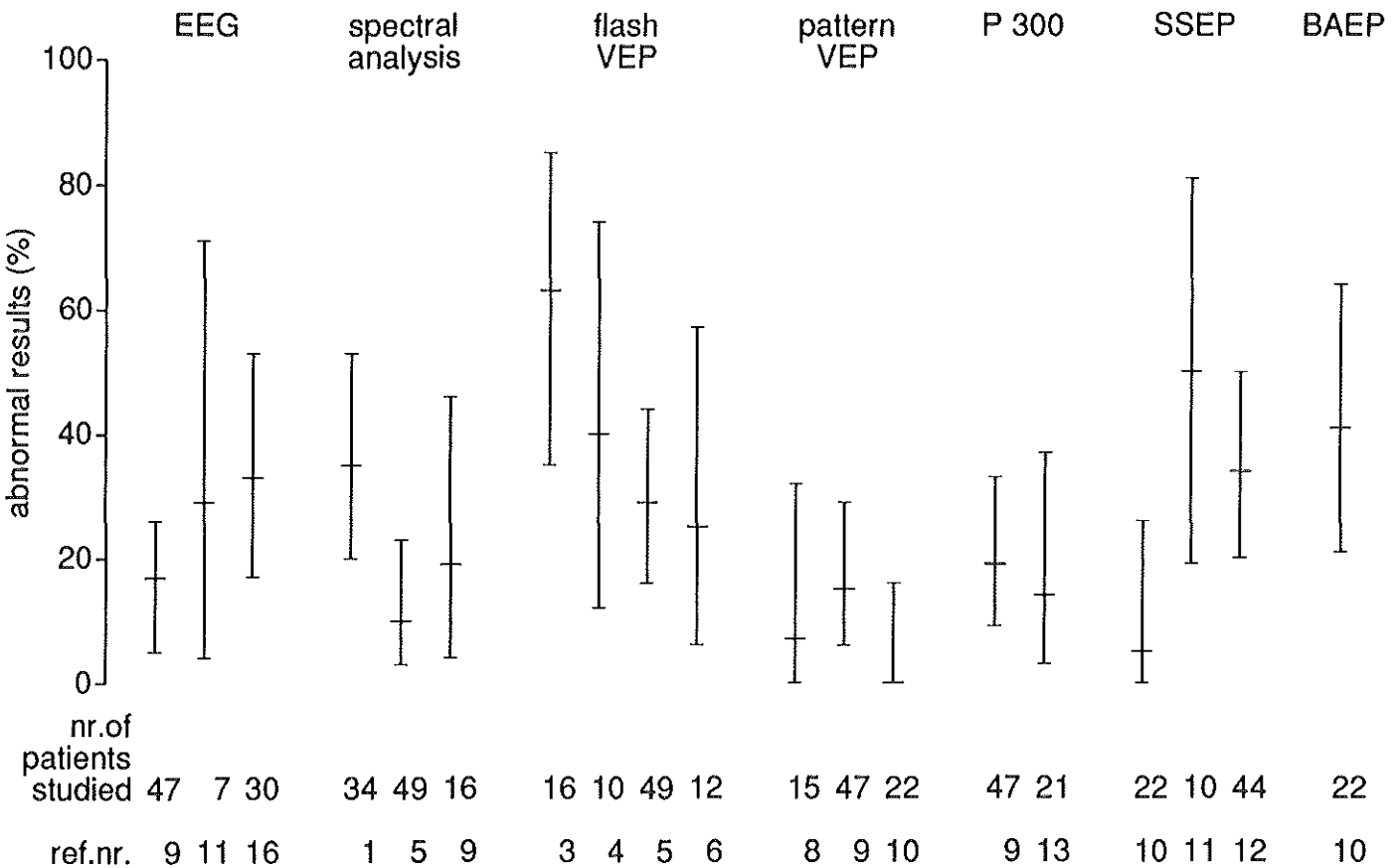
Method	% latent HE	95-percentiles[1]
EEG	21	12-31
spectral analysis	20	12-29
flash VEP	36	25-46[2]
pattern reversal VEP	10	4-18
P300	18	9-29
SSEP	28	17-38
BAEP	41	21-64

Patient groups from the studies used in figure 1 were combined with respect to the studied electrophysiologic method.

1: 95-percentiles were calculated using the formula $100[p \pm \{1.96\sqrt{(pq/n) + 1/2n}\}]$, if the product npq (n = total number of patients studied; p = proportion of patients with abnormal test results; q = 1-p) was greater than 15; otherwise standard curves were used.

2: $p < 0.05$ with respect to spectral analysis by χ^2-test.

golden standard, for instance psychometric tests. Only studies reporting on the percentage of cases with test results above the upper limit of normal, in groups of patients without clinical signs of hepatic encephalopathy are available. Studies on electroneurologic methods for patients without overt hepatic encephalopathy were selected and the percentage abnormal results was calculated for each test. Standard curves were used to determine the 95% confidence intervals. Figure 1 illustrates the results. The highest percentages were found for flash visual and somatosensory evoked potentials. After combining separate studies, only flash VEP's classified significantly more patients as having latent hepatic encephalopathy than spectral analysis of the EEG (table 1). However, different studies may include patient groups with different severity of the underlying liver disease and therefore different impairment of electroneurologic function. Just three studies reported on spectral analysis and evoked potentials together in the same patients (5,9,13); only two reported on the number of patients with abnormal test results indicating latent hepatic encephalopathy (5,9). The percentage of abnormal scores found by pattern reversal and auditory P300 event-related evoked potentials was similar to that of spectral analysis (9), whereas a higher percentage of patients with latent hepatic encephalopathy was detected by flash VEP's (5).


EEG
spectral analysis
flash VEP
pattern VEP
P 300
SSEP
BAEP
100
80
60
40
20
0
abnormal results (%)
nr.of patients studied
47 7 30 34 49 16 16 10 49 12 15 47 22 47 21 22 10 44 22
ref.nr.
9 11 16 1 5 9 3 4 5 6 8 9 10 9 13 10 11 12 10

It has to be stated that all known electrophysiologic methods are not specific with respect to hepatic encephalopathy. I would therefore conclude that there is not yet enough evidence to support the use of evoked potentials instead of spectral analysis for the monitoring of hepatic encephalopathy. Flash visual evoked potentials may be valuable for the detection of latent hepatic encephalopathy but results in more severe encephalopathy are contradictory. Studies on somatosensory evoked potentials are scarcely available; direct comparison with automated EEG analysis is awaited.

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◂Figure 1: Percentages of patients detected as having latent hepatic encephalopathy: percentages with 95-confidence intervals are given for different electrophysiologic methods in different studies.

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Chapter 5

FLUMAZENIL DOES NOT IMPROVE HEPATIC ENCEPHALOPATHY ASSOCIATED WITH ACUTE ISCHEMIC LIVER FAILURE IN THE RABBIT

The contents of this chapter have been published in Metabolic Brain Disease (1990;5:131-141) under the same title with the following authors C.C.D. van der Rijt, R.J. de Knegt, S.W. Schalm, O.T. Terpstra and K. Mechelse.

ABSTRACT

The effect of flumazenil, a benzodiazepine antagonist, on hepatic encephalopathy was studied in rabbits with acute hepatic failure induced by a two-stage liver devascularization procedure. The rabbits were randomized for treatment with 5 mg/kg of flumazenil or the placebo. The drug was administered at two easily recognizable time points in the course of the encephalopathy: firstly when the righting reflex was disturbed, secondly when the animal could no longer achieve to the sitting position. The response after flumazenil did not differ from that after the placebo, as measured by clinical evaluation and automated EEG analysis. Furthermore, the progression of the encephalopathy, as measured by the survival time after the first injection, was not affected by flumazenil.

INTRODUCTION

Benzodiazepine receptor antagonists have been reported to improve hepatic encephalopathy (HE) in experimental (1-4) as well as clinical (5-10) studies. The benzodiazepine receptor is part of the larger gamma-aminobutyric acid (GABA)-benzodiazepine receptor complex (11). It has been suggested that this receptor system may play a role in the pathogenesis of HE (12). Gamma-aminobutyric acid (GABA) is the most important inhibitory neurotransmitter, accounting for 30-40% of the total neurotransmitter content in the brain (13). The binding of GABA to its receptor, and thus its inhibitory action on neuronal membranes, is enhanced by the binding of benzodiazepines to their own receptors (11). Benzodiazepine antagonists inhibit the binding of benzodiazepines but, on their own, are not believed to have an important intrinsic effect on central nervous system functioning (11). Therefore, apart from offering a new therapeutic approach to HE, benzodiazepine antagonists may be important in studies on its pathogenesis: a beneficial effect may point to the presence of endogenous benzodiazepine-like compounds in HE, as has been recently suggested (14).

Various compounds that antagonize the benzodiazepine receptor have been studied in animal models of acute hepatic failure: flumazenil, CGS 8216 and Ro15-4513. All

ameliorated hepatic encephalopathy in rats and rabbits with galactosamine or thioacetamide-induced fulminant hepatic failure (1-4). However, a beneficial effect of CGS 8216 could not be confirmed in rats with acute ischemic liver failure (15). Furthermore, antagonism of the GABA-receptor itself also did not affect the course of hepatic encephalopathy in this animal model (16). Therefore, further experimental controlled studies on the effect of a benzodiazepine antagonist in hepatic encephalopathy are needed. We studied the effect of flumazenil in our recently described rabbit model of acute ischemic liver failure (17). We measured the response by clinical evaluation of HE, spectral analysis of the EEG and determination of the survival time.

MATERIAL AND METHODS

Animals: Sixteen New Zealand white rabbits with acute ischemic liver failure were used. Four healthy control rabbits were studied to assess the intrinsic effect of flumazenil in comparison to that of diazepam.

Rabbits with acute hepatic encephalopathy: Before surgery the rabbits received doxycycline, in dosages increasing to a maximum of 25 mg, in their drinking water for about 5 days. Food was withdrawn 24 hr before surgery, except for glucose drinking water ad libitum. Anesthesia was induced with Hypnorm (0.6-0.8 ml im; 10 mg of fluanison and 0.2 mg of fentanyl base/ml). After intubation muscle relaxation was achieved with 1.0 mg of Pavulon iv, and anesthesia was maintained with N_2O:O_2 (2:1) and 0.025 mg of fentanyl iv, whenever indicated. A Ringer-lactate solution (20 ml/hr) and Haemacel (10 ml/hr) were administered via a cannula in a marginal ear vein during the operation. To prevent acidosis, the infusion regimen was changed to 8.4% sodium bicarbonate at a rate of 15 and 30 ml/hr during and immediately after construction of the portacaval shunt (PCS), respectively. To monitor arterial blood pressure, blood gases and pH, a 5 Ch catheter was inserted in the right femoral artery. Fifty mg of amoxicilline were given as antibiotic prophylaxis.

The surgical procedure was performed as previously described (17). After severing the attachments of the liver, a small-diameter (5mm) side-to-side PCS was constructed with 7-0 prolene within 15 minutes. A loose ligature was placed around the hepatoduodenal ligament and threaded through a subcutaneous plastic tube. Three

silver electrodes were placed directly on the dura mater through small burr holes in the skull. Two were implanted 5 mm to the right of the sagittal suture, 3 mm anterior and 11 mm posterior to the coronal suture, respectively, and the third was implanted 5 mm to the left of the sagittal suture and 3 mm anterior to the coronal suture.
Postoperatively the rabbits were given glucose water ad libitum, followed by a normal diet. The second day after surgery 50 mg of amoxicilline was given iv and acute liver ischemia was induced by tightening the loose ligature around the hepatoduodenal ligament. To prevent hypoglycemia, an infusion of 10% glucose (5 ml/hr) was started, the dose being adjusted to maintain normoglycemia, which was controlled hourly. Body temperature was measured every 2 hr and remained normal throughout the experiment. Before the induction of liver ischemia and at regular intervals in the course of the experiment, spectral analysis was performed and arterial blood samples were taken. Every half-hour the rabbits were taken out of their restraining boxes for evaluation of the stage of HE. Two easily recognizable clinical stages of HE could be identified in most animals. Stage A was characterized by a disturbed righting reflex: the animal not getting up immediately when placed on its side. At stage B the rabbit lay in the cage and could not come to the sitting position, not even after stimulation, and usually did not even lift its head. In some cases stage B was preceded by a period of agitation or marked ataxia. When stage A or B was identified the evaluation was repeated 10 min later for confirmation.

The rabbits were randomized for treatment with flumazenil or placebo. Flumazenil or the vehicle, 5 mg/kg, was injected at a rate of 1 mg/kg/min. The effect of flumazenil on HE was evaluated clinically before and 10 minutes after the injection as well as by spectral analysis before and 5 min after the injection and by determination of the survival time after the first injection of flumazenil or placebo. Liver failure was confirmed by a rise in arterial ammonia levels and a decline in clotting factors. Autopsy studies of all rabbits were performed to verify that the hepatoduodenal ligament was adequately clamped.

Control rabbits: Hypnorm, 0.5 ml/kg, was administered intramuscularly and electrodes were placed on the dura mater, as described above. The effect of flumazenil was assessed in a randomized cross-over study performed at least 1 week after the surgical procedure. For this purpose 5 mg/kg of flumazenil or the vehicle was injected at a rate

of 1 mg/kg/min. After a wash-out period of one week the other compound was given. The effect was assessed by clinical evaluation of the animal before and 10 minutes after the injection and by spectral analysis before and 5 min after the injection. A similar cross-over study was carried out to evaluate the effect of 5 mg/kg of diazepam versus that of 0.9% saline.

Spectral analysis: The variations in potential between the electrodes over the right hemisphere were registered by an EEG apparatus (Ahrend-Van Gogh, Amsterdam, The Netherlands) and fed into a computer at a sampling rate of 51.2 Hz and a sensitivity of 11 bits/5V. A power spectrum was established, as described earlier (18). The frequency resolution was 0.1 Hz. The mean dominant frequncy (MDF), the power and the percentages of the delta, theta, alpha and betha frequency bands were calculated over the frequency range 1.0-25.6 Hz.

Chemicals: Flumazenil was kindly provided by Hoffmann-La Roche & Co, Mijdrecht, The Netherlands. Just before the experiment it was suspended in sterile distilled water at 37°C with Tween 80 (1 drop per 5 ml). Diazepam was solubilized in water with alcohol (10%) and propylene glycol (40%). Clotting factors were assessed by means of the Normotest (Nyegaard, Oslo, Norway). Arterial ammonia levels were measured by an enzymatic method using glutamate dehydrogenase.

Statistics: The Wilcoxon two-sample test was used for statistical comparisons. The effects on the MDF and survival times were analyzed with the paired- or non-paired Student's t-test, whenever indicated; for analyzing the survival times logarithmic transformation was
used.

Table 1: Clinical recovery after flumazenil or placebo.

	Flumazenil	Placebo
stage A	0/8	0/8
stage B	1/6	1/5

RESULTS

Rabbits with hepatic encephalopathy: The degree of liver failure, as measured by the rise in arterial ammonia levels and the decline in Normotest values, in the group of rabbits treated with flumazenil was similar to that found for the placebo group (figure 1).

At stage A the effect of flumazenil or placebo could be studied in all 16 rabbits, although the time between the induction of liver ischemia and the diagnosis of this stage of HE varied considerably: 1.5-11.0 hrs for the flumazenil-treated and 1.5-10.5 hrs for the placebo group. The righting reflex did not normalize in any of the rabbits after administration of either flumazenil or the placebo (table 1). Two rabbits treated with flumazenil and three rabbits receiving the placebo died before stage B was

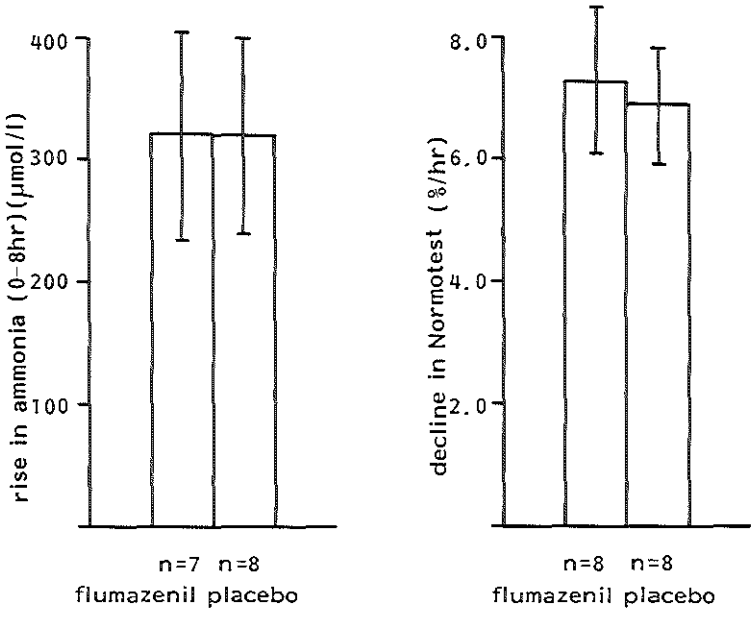

Figure 1: Parameters of acute hepatic failure: on the left, the rise in arterial ammonia levels during the first 8 hours after induction of liver ischemia; on the right, the hourly decrease in clotting factors, as assessed by Normotest. The results are expressed as mean ± SEM.

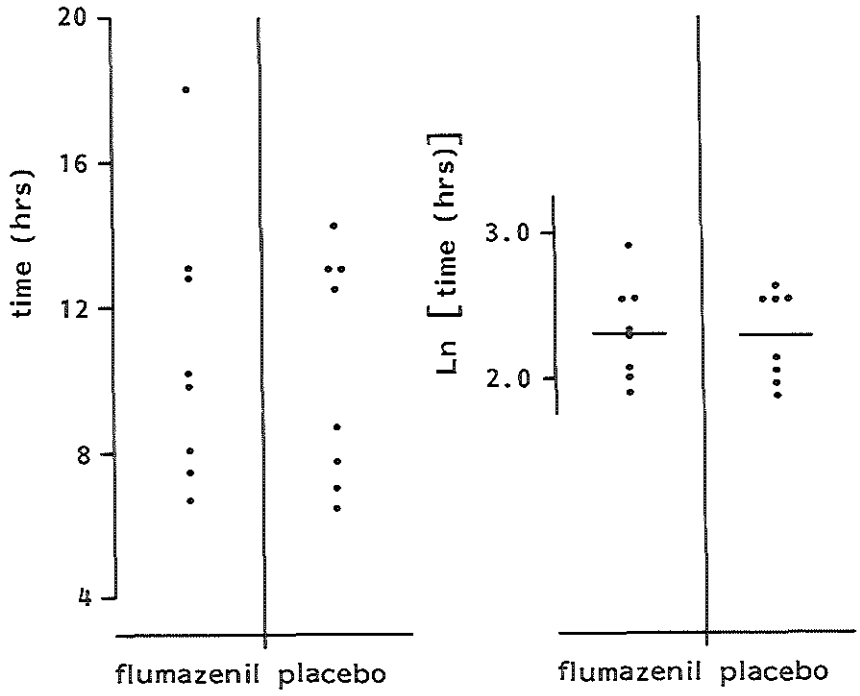

Figure 2: Survival times after the first injection of flumazenil or the placebo: on the left, absolute values; on the right the same survival times after logarithmic transformation to obtain a normal distribution (——=mean).

identified or confirmed (most of them were agitated just before death). Therefore, at stage B 6 rabbits could be given flumazenil and 5 rabbits the placebo. In each group 1 animal died within 10 minutes of the injection and 1 animal regained the ability to sit (table 1).

The survival times after the first injection of either flumazenil or the placebo are shown in figure 2. Logarithmic transformation of the data was used to obtain a normal distribution. As the mean ratio of the survival times for the two groups after administration of the first injection was 1.04 (with a 95%-confidence interval of 0.72 to 1.49), progression of HE was similar for flumazenil and the placebo.

Table 2: Changes in MDF (Hz) after treatment of hepatic encephalopathy with flumazenil or placebo.

		stage A		stage B	
	baseline	before	after	before	after
Flumazenil	5.33 ± 0.40 (8)	4.69 ± 0.22[a] (8)	4.55 ± 0.90[b] (8)	4.20 ± 1.20 (6)	4.28 ± 2.27 (5)
Placebo	6.00 ± 1.14 (8)	5.38 ± 0.77[c] (8)	4.93 ± 1.06[a] (8)	4.92 ± 1.41[b] (5)	3.60 ± 0.74[c] (4)

a: difference with respect to baseline: $p<0.01$
b: difference with respect to baseline: $p<0.02$
c: difference with respect to baseline: $p<0.05$
Results are expressed as mean ± S.D.; the number of rabbits is given in parentheses.

Table 3: Changes in the parameters of spectral analysis for control rabbits after treatment with flumazenil or diazepam.

rabbit	MDF (Hz)	power (%)	delta (%)	theta (%)	alpha (%)	beta (%)
flumazenil						
1	+0.1	+0.53	-0.3	-1.8	+0.7	+1.4
2	+0.5	-43.67	-3.9	+0.5	+0.8	+2.6
3	+0.4	-20.59	-5.6	+2.7	-1.2	+3.6
4	+0.5	-13.41	-6.0	+2.0	+2.2	+1.8
diazepam						
1	-1.7	+484.62	+25.3	-17.3	-5.8	-2.2
2	-0.4	+713.36	+15.6	-16.5	-2.7	+3.6
3	-0.5	+612.85	+8.4	-5.7	-4.4	+1.5

Results are expressed as the changes with respect to the baseline values after diazepam or flumazenil minus these changes after the placebo; the change in power is expressed as the percentage change assuming that the power at baseline was 100%.

The effects of flumazenil and placebo on the MDF's are shown in table 2 and figure 3. At stage A the MDF's were already significantly decreased with respect to baseline values (paired Student's t-test). At this stage neither flumazenil, nor the placebo induced normalization to the baseline values (table 2). At stage B the MDF's were decreased further, except in one animal with an MDF higher than the baseline value; in this animal a further increase occurred from 5.9 to 8.1 Hz after flumazenil, due to a peak in the power spectrum around 15 Hz. A similar effect did not occur in other animals.

At both stages of HE the changes in MDF with respect to the values before injection were calculated for each rabbit. The changes did not differ between flumazenil and placebo. At stage A the mean difference between flumazenil and placebo was 0.31 Hz with a 95%-confidence interval of -0.54 to 1.16 Hz. At stage B the mean difference was 1.07 Hz with a 95%-confidence interval of -0.68 to 2.82 Hz. There were also no differences in the effects on power and the delta, theta, alpha and beta percentages of the power spectrum after the first as well as the second injection.

Control rabbits: The effects of flumazenil and the placebo were studied in four rabbits and the effects of diazepam and physiologic saline in three rabbits. After flumazenil behavioral changes did not occur and spectral analysis showed only minimal changes (table 3). A similar dose of diazepam induced marked lethargy: the animals could be placed on their sides and they slept for the following hour. Electroencephalography revealed a high amplitude rhythm with an increase in background delta activity. These changes were confirmed by the marked increase in power and % delta activity as measured by spectral analysis. The MDF, the % theta and the % alpha activity decreased (table 3).

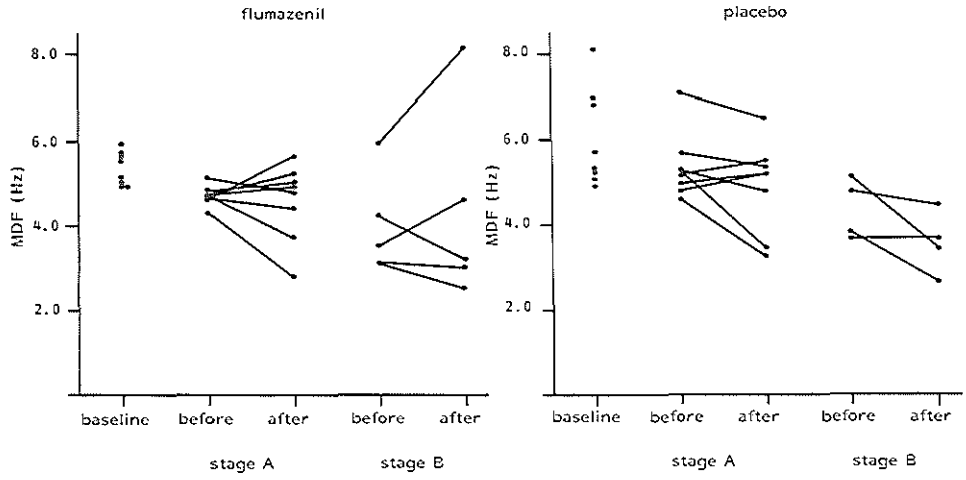

Figure 3: Changes in MDF after treatment of hepatic encephalopathy with flumazenil or placebo: on the left, rabbits treated with flumazenil; on the right, rabbits receiving the placebo.

DISCUSSION

We could not demonstrate a beneficial effect of flumazenil on hepatic encephalopathy in our animal model of acute hepatic failure. Our findings are in accordance with the results from a rat model of acute ischemic liver failure (15). An important role for an endogenous benzodiazepine-like compound in this animal model therefore seems unlikely. Our findings are contrary to the results of experimental controlled studies on benzodiazepine antagonists in galactosamine and thioacetamide-induced acute liver failure (1-4).

In the galactosamine animal models the effect of a benzodiazepine antagonist was investigated only during mild hepatic encephalopathy. All rats apparently regained normal clinical status (1) and neurological response scores improved in 75-100% of the rabbits (2). Disturbed visual evoked responses normalized in both studies (1,2). We could not demonstrate a clinical effect of flumazenil in any of the rabbits in stage A. The upper limit of the 95%-confidence interval for this response in 8 rabbits, 36.9%, is therefore still much lower than the responses observed in the galactosamine models (1,2). Furthermore, the MDF after flumazenil neither normalized to the baseline value nor showed an improvement significantly different from that in the animals receiving the placebo. Regarding the 95%-confidence intervals for the difference in effect on the MDF between flumazenil and placebo (-0.54 to 1.16 Hz at stage A and -0.68 to 2.82 Hz at stage B) a small effect of flumazenil cannot be excluded. Nevertheless, a discrepancy exists between the results in the galactosamine animal models (1,2) and the results in our model of acute ischemic liver failure.

We used a highly reproducible rabbit model in which acute hepatic failure was induced by a two-stage liver devascularization procedure. Liver failure was confirmed by a decrease in clotting factors and a rise in arterial ammonia levels. All animals developed encephalopathy, as confirmed by the occurrence of two easily recognisable features of the encephalopathy: a disturbed righting reflex in all and loss of the ability to maintain posture. The survival of the animals after induction of liver ischemia was similar to that reported after total hepatectomy (19), which confirms the suitability of this animal model. Recently, the usefulness of an ischemic animal model for the study of hepatic

encephalopathy has been questioned (20); however, scientific evidence is not available for this statement.

The concept of investigating the effect of a benzodiazepine antagonist on hepatic encephalopathy originated from studies on galactosamine-induced liver failure in rats and rabbits, in which increased numbers of brain GABA and benzodiazepine receptors were demonstrated using ligand-membrane binding essays (1,21-22). Both flumazenil and CGS 8216 were found to have a beneficial effect in these animal models (1,2) and antagonism of endogenous benzodiazepine-like compounds was suggested. However, later studies on the numbers of GABA and benzodiazepine receptors in different animal models of hepatic encephalopathy, including the rat model of acute ischemic liver failure, yielded no changes in GABA and benzodiazepine receptors (23-28). Furthermore, the finding of increased numbers of brain GABA and benzodiazepine receptors in the galactosamine rabbit model has recently been retracted (29). Nevertheless, increased GABA-ergic tone is still postulated in this animal model (29). A beneficial effect of a benzodiazepine antagonist on hepatic encephalopathy may be found only in an animal model in which the influence of the GABA-benzodiazepine receptor complex on central nervous system function is enhanced.
Another consideration seems worthwhile. Flumazenil may not only bind to central benzodiazepine receptors. A beneficial effect of flumazenil upon blood pressure homeostasis was demonstrated in an animal model of hemorrhagic shock (30). A drop in blood pressure has been reported in galactosamine-induced liver failure (31,32) and may have contributed to the encephalopathy in this animal model of acute hepatic failure.

Differences in the types and doses of benzodiazepine antagonists might also explain the discordance between different studies. In the only published study on rabbits (with galactosamine-induced liver failure) a dose of 1-2.5 mg/kg of flumazenil was used to ameliorate hepatic encephalopathy (2). We used a higher dose: 5 mg/kg. This dose has been proven to antagonize marked benzodiazepine-induced slowing of the EEG in healthy rabbits, without causing any changes when given alone (33). To confirm the absence of an intrinsic benzodiazepine-like effect of flumazenil when given at this rather high dose, we compared the effects of a dose of 5 mg/kg of flumazenil and diazepam in healthy rabbits. Contrary to diazepam, flumazenil induced neither behavioral changes

nor slowing of the electroencephalogram. Therefore, our negative findings cannot be explained easily by dose differences.
We studied the effect of flumazenil in both early and late hepatic encephalopathy. Since no effect of flumazenil could be detected in stage A, which occurred early in the course of the experiment, when brain edema was almost certainly absent, our negative findings cannot easily be explained by the presence of brain edema.

Flumazenil has been suggested to be of benefit in the treatment of hepatic encephalopathy in some uncontrolled clinical studies (5-10). However, negative results have now also been forthcoming (34-36). Furthermore, GABA and benzodiazepine receptors were found to be unchanged in autopsied brain tissue from cirrhotic patients with hepatic encephalopathy (37). At present, no unequivocal evidence for an increased GABA-ergic tone is present in human hepatic encephalopathy. Our negative findings on the effect of flumazenil in our animal model of hepatic encephalopathy warrant further caution for its postulated benefit in clinical hepatic encephalopathy.

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Chapter 6

FLUMAZENIL THERAPY FOR HEPATIC ENCEPHALOPATHY: A DOUBLE-BLIND CROSS-OVER STUDY

The contents of this chapter have been submitted for publication under the same title with the following authors: C.C.D. van der Rijt, S.W. Schalm, J. Meulstee, Th. Stijnen.

ABSTRACT

The effect of flumazenil, a benzodiazepine antagonist, on hepatic encephalopathy was investigated in a double-blind cross-over study. The eighteen patients who participated in the study had undergone conventional therapy for at least 24 hours; pre-study screening did not reveal neurological or non-hepatic metabolic disorders that could have induced encephalopathy. The first 9 patients were studied for two 3-day periods , separated by one wash-out day. In each period a single injection (1.0 mg) of flumazenil or the placebo was followed by a continuous infusion of 0.25 mg/hr for three days. The effect was studied by means of clinical evaluation, EEG grading and quantitative EEG analysis before and 15 minutes after the single injection and at 24, 48 and 72 hours. The last 9 patients were only studied for 2 days to determine the immediate effects of flumazenil and the placebo given in randomized order on 2 consecutive days. Cross-over analysis could be performed in 16 cases for the study of the immediate effect and in 8 cases for the study of the steady-state effect. There was a trend toward a decrease in the clinical grade of hepatic encephalopathy 15 minutes after bolus injection of flumazenil ($p=0.06$). However, neither EEG grading nor quantitative EEG analysis changed significantly. Furthermore, the effect of flumazenil on the clinical grade, the EEG grade and spectral analysis was not significantly different from that of the placebo during the 3 days of infusion. In a separate open study 13 patients, who were excluded from the double-blind study largly because of renal failure or evidence of recent use of benzodiazepines, underwent evaluation to determine the immediate effect of 1.0 mg of flumazenil. The mean dominant frequency increased significantly in this group of patients; an effect on the clinical and/or EEG grades of encephalopathy was found only for patients who had previously received benzodiazepines. Our study does not support a therapeutic effect of flumazenil on hepatic encephalopathy itself; a beneficial effect may however occur when benzodiazepines contribute to the encephalopathy.

INTRODUCTION

Flumazenil is the first benzodiazepine antagonist to be used in clinical practice. Its use has also been suggested for the treatment of hepatic encephalopathy since the

publication of several uncontrolled clinical studies on the effect of flumazenil in acute as well as chronic liver failure (1-7). In the two largest studies, response rates of 60 and 71% were reported (1-2).

Studies on flumazenil therapy for hepatic encephalopathy arose from the idea that altered GABA-ergic neurotransmission is of prime importance in the pathogenesis of hepatic encephalopathy (8). After controversial findings on the number of GABA and benzodiazepine receptors (9-19), GABA metabolism (17,20-24) and brain GABA concentrations (17,22), interest focussed on the role of a putative endogenous benzodiazepine agonist in liver disease (25). Not only did experimental studies support the presence of benzodiazepine-like agents in the brains of animals with acute hepatic failure (26-28), but such agents were also reported to be isolated from serum and CSF from patients with hepatic encephalopathy (25,29-30). Benzodiazepine agonists enhance the binding of GABA to its receptor and thus the inhibitory effect of GABA on central nervous system functioning. Benzodiazepine antagonists, such as flumazenil, inhibit this effect of benzodiazepine agonists (31). Therefore, if benzodiazepine agonists play an important role in the pathogenesis of hepatic encephalopathy flumazenil should ameliorate hepatic coma.

However, although quite remarkable success rates were found in uncontrolled clinical studies, the lack of an effect of flumazenil on hepatic encephalopathy has also been reported (32-33). Controversial results were also obtained in animal experiments: beneficial effects of a benzodiazepine antagonist in galactosamine and thioacetamide-induced hepatitis (34-36) and negative findings for ischemic liver failure (37-38). Therefore, controlled clinical trials were considered necessary.

We studied the effect of flumazenil on hepatic encephalopathy in patients with acute hepatitis and chronic liver disease, using a double-blind cross-over design. We measured the response by means of quantitative EEG analysis, an objective assessment of brain dysfunction in liver disease (39).

PATIENTS AND METHODS

Before the start of the study the protocol was reviewed and accepted by the Medical Ethics Committee of our hospital. Informed consent was obtained from patients with low grades of hepatic encephalopathy before the start of the study and from patients with severe encephalopathy during the study when the encephalopathy became less severe.

Patients:

Between February 1987 and February 1990, 54 patients with histologically confirmed acute or chronic liver disease were screened for eligibility for our study on the effect of flumazenil on hepatic encephalopathy. Only patients with an elevated arterial blood ammonia level (>30 umol/l) and encephalopathy diagnosed objectively by spectral analysis (39) after at least 24 hours of standard therapy were included. Standard therapy consisted of protein restriction and bowel cleansing (lactulose with or without neomycin). Exclusion criteria were: structural neurological disorders of possible importance as far as the encephalopathy was concerned found by careful neurological examination and, when indicated, CT-scanning; intoxication; marked abnormalities in blood biochemistry other than parameters of liver function (glucose, electrolytes, urea and arterial blood gas analysis); previously diagnosed endocrinological disease that could have induced encephalopathy; hypersensitivity to benzodiazepines; and (recent) use of benzodiazepines. HPCL plasma analysis for benzodiazepines was performed

►Legend to table 1:

1. underlying liver disease: C cirrhosis; C* acute exacerbation of cirrhosis; F fibrosis; H hepatitis; HCC hepatocellular carcinoma
2. etiology of the underlying liver disease; AFLP acute fatty liver of pregnancy
3. clinical grade and EEG grade of encephalopathy before treatment
4. Glasgow Coma Scale, calculated from the sum of the eye response (1=no response; 2=open in response to pain; 3=open in reaction to verbal command; 4=open without stimulus), the motor response (1=no response; 2=extension in response to pain; 3=flexion in response to pain; 4=appropriate motor response to pain; 5=execution of commands) and the verbal response (1=no response, 2=grunting in response to pain; 3=incomprehensible speech; 4=comprehensible speech); t=score of motor and eye response only because of intubation of the patient.
5. evidence for recent use of benzodiazepines as obtained from history and by HPLC plasma analysis. Cl chlordiazepoxide; D diazepam; M midazolam; O oxazepam; L lorazepam; # impossible because of technical artefacts; midazolam could not be detected by HPLC; ? small peak in the HPLC spectrum which could not be identified with certainty.

Table 1: Initial features of patients included in the study.

			liver disease		encephalopathy				benzodiazepines[5]			
nr	sex	age	type[1]	etiology[2]	duration	clin.gr	EEG gr[3]	GCS[4]	history	plasma		comments
double-blind study												
1	F	55	H	unknown	6 day	0	2	13	-	-		
2	F	22	H	AFLP[1]	1 day	1	2	13	-	-	?	
3	F	49	C'	nonA-nonB	3 days	1	3	13	-	-	?	deteriorating renal function
4	F	61	C	PBC	3 months	1	2	13	-	-		
5	F	65	C	unknown	13 years	1	2	13	-	-		porto-systemic shunt (PSS)
6	F	18	H	auto-immune	3 days	1	2	13	-	O		
7	M	38	C	hepatitis B	5 days	2	2	13	-	-		partial hepatectomy after HCC
8	F	61	C	unknown	1 year	3	3	9	-	-		urinary infection; hypothyroidism
9	M	46	C'	alcohol	12 days	3	4	12	-	-		died on the last day of the study
10	M	53	C	alcohol	1 month	2	4	13	-	-		urinary infection; spinal tuberculous abces
11	M	59	C	alcohol	5 months	1	2	13	-	-		
12	F	23	H	hepatitis B	1 day	4	3	6	-	-		
13	M	62	C	unknown	6 weeks	1	2	13	-	-	?	
14	M	55	C	alcohol	7 days	1	1	13	-	-	?	
15	F	63	C	PBC	5 weeks	1	2	13	-	-		
16	F	42	H	toxic	1 day	4	3	9	-	-		
17	F	55	C	PBC	1 month	1	2	13	-	-		
18	M	67	C	alcohol	14 months	1	2	13	-	-		
open study												
1	F	17	H	toxic	5 days	4	4	2'	-	-		respiratory infection
2	M	65	C	alcohol	14 days	2	2	13	-	D		retroperitoneal abces
3	M	18	H	unknown	2 days	4	4	6	D	#		
4	M	63	C	unknown	2 days	4	3	9	-	-	?	renal failure
5	F	54	F	congenital	1 day	1	2	13	-	-		PSS; no protein restriction
6	M	33	C	alcohol	8 days	4	2	8	CI	CI		renal failure; urinary infection
7	M	65	C	alcohol hepatitis B	26 days	4	4	5	-	D		renal failure; urinary infection
8	F	69	C'	unknown	4 days	1	2	13	-	-		renal failure; obstipation
9	F	46	H	hepatitis B	4 days	3	3	6'	D,M	-		
10	M	40	C/HCC	hepatitis B	10 days	3	-	13	-	-		renal failure
11	M	61	C	hepatitis B	11 days	2	3	13	-	-		renal failure
12	F	34	C	alcohol	13 days	1	2	13	CI	CI		
13	F	33	C	alcohol	2 days	4	3	4	L	-		carbamezapine for epilepsy
14	M	48	H	unknown	6 days	2	3	11	-	N		renal failure

before acceptance into the study.

Eighteen patients were considered eligible for the double-blind cross-over study. The characteristics of these patients are given in the tables 1 and 2. Two patients (cases 6 and 12) were withdrawn after the first study day because of liver transplantation, leaving 16 patients for the final cross-over analysis. HPLC plasma analysis failed to show unequivocal evidence for the use of benzodiazepines by these patients.

Table 3 summarizes the reasons for exclusion of the other patients. Fourteen of the excluded patients participated in the open study (tables 1,2).

Table 2: Laboratory data for groups before initiation of the study.

	study I (n=18)		study II (n=14)	
	mean	SD	mean	SD
Hb (mmol/l)	7.2	1.2	7.2	1.6
WBC (10^9/l)	8.8	6.2	16.3	12.6
platelets (10^9/l)	133	101	160	95
antithrombin3 (%)	0.27	0.16	0.24	0.15
urea (mmol/l)	6.4	6.1	17.8	16.7
creatinine (μmol/l)	111	57	274	250
Na (mmol/l)	136	7	137	8
K (mmol/l)	3.9	0.5	4.2	0.6
Ca (mmol/l)	2.37	0.15	2.33	0.17
PO4 (mmol/l)	0.80	0.37	1.32	0.72
protein (g/l)	64	12	62	11
albumin (g/l)	27	5	25	5
bilirubin (μmol/l)	244	228	333	228
alk. phosph. (U/l)	183	141	156	53
ASAT (U/l)	188	271	138	103
ammonia (μmol/l)	113	40	110	49

Treatment and evaluation:

Double-blind cross-over study: From the start of the study in February 1987 until November 1988 the first 9 patients were studied for two 3-day periods separated by one day to minimize carry-over effects. The order in which flumazenil and the placebo were administered was randomized. In each period a first dose of 1.0 mg of flumazenil in 10 ml of vehicle or 10 ml of the vehicle alone was given intravenously (0.1 mg/min.) to study the immediate effect. Four hours later a loading dose of 0.5 mg was followed immediately by a continuous infusion of 0.25 mg/hr for three days to study the

Table 3. Reasons for exclusion from the double-blind trial.

total number excluded: 36 reason	number
history of hypersensitivity to benzodiazepines	1
previous or current use of benzodiazepine	15
use of non-benzodiazepine sedatives	2
renal failure	14
severe hyponatremia	1
hypoxia	4
neurological abnormalities	5
possible Korsakoff's syndrome	2
incomplete standard therapy	3
no patient consent	2
transplantation	3

potential long-term effect. Standard therapy was continued during the study period. The depth of encephalopathy was assessed by clinical grading, conventional EEG grading and spectral EEG analysis before and 15 minutes after the first injection. The same 3 features were assessed 24, 48 and 72 hours after the start of each of the two study periods, and the average values of these measurements at 24, 48 and 72 hours were calculated.

From November 1988 until February 1990, the cross-over study was simplified by focusing on the immediate effect of flumazenil. One dose of flumazenil (1mg/10ml) and one dose of the placebo (10ml) were given on two consecutive days. The depth of encephalopathy was assessed before and 15 minutes after injection.

Open study: Fourteen patients excluded from the controlled study participated in an open study. One mg of flumazenil was injected at a rate of 0.1 mg/min. Clinical evaluation, EEG grading and spectral analysis were performed before and 15 minutes after injection.

Assessment of hepatic encephalopathy

Clinical grading. Grade 0 hepatic encephalopathy was defined as the absence of at least 3 of the following abnormalities: inverted sleep pattern, disturbed memory, impaired calculation (serial 7's) and slowness of speech; grade 1 as the presence of at least two of these abnormalities (one abnormality in association with a flapping tremor was also classified as grade 1); grade 2 as the presence of at least 2 of the following: lethargy,

disorientation in time and flapping tremor; grade 3 as the presence of at least two of the following: a state in which the subject must be stimulated repetitively to open his eyes or execute commands, disorientation in terms of place and disorientation with respect to person; and grade 4 as coma.

EEG grading. Grade 0 hepatic encephalopathy was defined as the presence of a background activity consisting of alpha rhythm; grade 1 as an alpha rhythm with some scattered theta waves; grade 2 as a background activity of theta rhythm intermixed with some delta and alpha frequencies; grade 3 as a background of polymorphic delta activity of high amplitude with spontaneous variability; and grade 4 as delta activity of relatively small amplitude (40).

Spectral analysis. Automated EEG analysis was performed, as described earlier (39). Variations in electric potential in the leads T3-O1 and T4-O2 (temporo-occipital) were registered for 100 seconds. Using Fast Fourier analysis a power spectrum was constructed from which the mean dominant frequency (MDF) was calculated for the range 1.0-25.6 Hz. to measure the response to treatment with flumazenil or placebo.

HPLC plasma analysis

An HPLC system was used to detect benzodiazepines in plasma by means of a Chromspher C8 column and a detector with a 254 nm filter. Two analyses were performed with eluents consisting of H_2O, methanol and triethylamine (450:550:5), acidified to pH 6.6 with 30% acetic acid and H_2O, methanol, acetonitrile and triethylamine (600:200:200:5), pH 5, respectively. Only peaks characteristic of known benzodiazepines were considered evidence for recent use of benzodiazepines.

Statistical analysis

Statistical analysis for assessment of the effect of flumazenil in the double-blind cross-over study was performed according to Armitage et al. (41). The effect of flumazenil on the clinical and EEG grades of encephalopathy was tested by the two-sample Wilcoxon rank test, the effect on the MDF by the Student's t-test. Differences between the 2 study periods with respect to laboratory data were evaluated by the paired Student's t-test. In the open study the paired Student's t-test was used to test the effect of flumazenil on the MDF.

RESULTS

Double-blind cross-over study

The results of assessment of hepatic encephalopathy before and 15 minutes after injection of 1.0 mg of flumazenil or the placebo are shown in figure 1. Sixteen patients received flumazenil as well as the placebo; two patients could be studied for just one day (one received flumazenil and one the placebo). In most cases the hepatic encephalopathy was generally mild clinically but electroencephalography revealed marked slowing, i.e. grades 2-4 hepatic encephalopathy, in all subjects. Clinical assessment as well as EEG grades of hepatic encephalopathy were somewhat higher for the group of patients who received the placebo in the first and flumazenil in the second period than for those who were treated in the reverse order.

A period effect over the 2 study periods could not be demonstrated for either the clinical grade of HE or the mean dominant frequency, but the EEG grade of HE exhibited a significant reduction in severity over the 2 periods ($p=0.02$). Biochemically, there was a trend toward lower ammonia levels in the second period but the decrease was not significant (table 4). A significant interaction between period and effect could not be demonstrated for either the EEG grade or the clinical grade and MDF. Cross-over analysis of the data was therefore justified.

Fifteen minutes after injection of flumazenil the clinical grade of HE had decreased in 6 patients; after the placebo a decrease was found in 2 cases and a rise in clinical grade in 2 others ($p=0.06$). However, EEG readings did not reveal any changes in the grade

Table 4: Laboratory data of the double-blind cross-over study.

	period 1 (n=16)		period 2 (n=16)		
	mean	s.d.	mean	s.d.	p
Na	134	7.3	135	7.2	0.66
Ca	2.05	0.14	2.14	0.23	0.09
PO4	0.82	0.34	1.03	0.28	0.01
urea	7.1	6.2	8.1	8.1	0.20
ammonia	103	40	89	34	0.06
PH	7.48	0.05	7.45	0.03	0.07
PO2	11.7	2.8	12.0	3.2	0.81
PCO2	3.8	0.6	3.9	0.6	0.62
glucose	8.3	5.3	8.5	4.9	0.81

of encephalopathy 15 minutes after the injection of either flumazenil or the placebo. Furthermore, there were no differences in the changes in MDF with respect to baseline values between flumazenil and placebo ($p=0.21$, 95% confidence interval of -0.42 up to 0.68 Hz). Three of the 6 patients with a possibly lower clinical grade of HE 15 minutes after a single injection of flumazenil belonged to the first group of 9 patients who were studied for a longer period. None of these three patients showed clinical deterioration four hours after the first injection of flumazenil. Furthermore, a beneficial effect of the benzodiazepine antagonist also could not be demonstrated during the 3-day infusion periods in 8 patients. There were no differences in effect on either the clinical and EEG grades of encephalopathy or the MDF's between flumazenil and placebo ($p>0.05$) (fig. 2). The mean difference in the changes in MDF with respect to

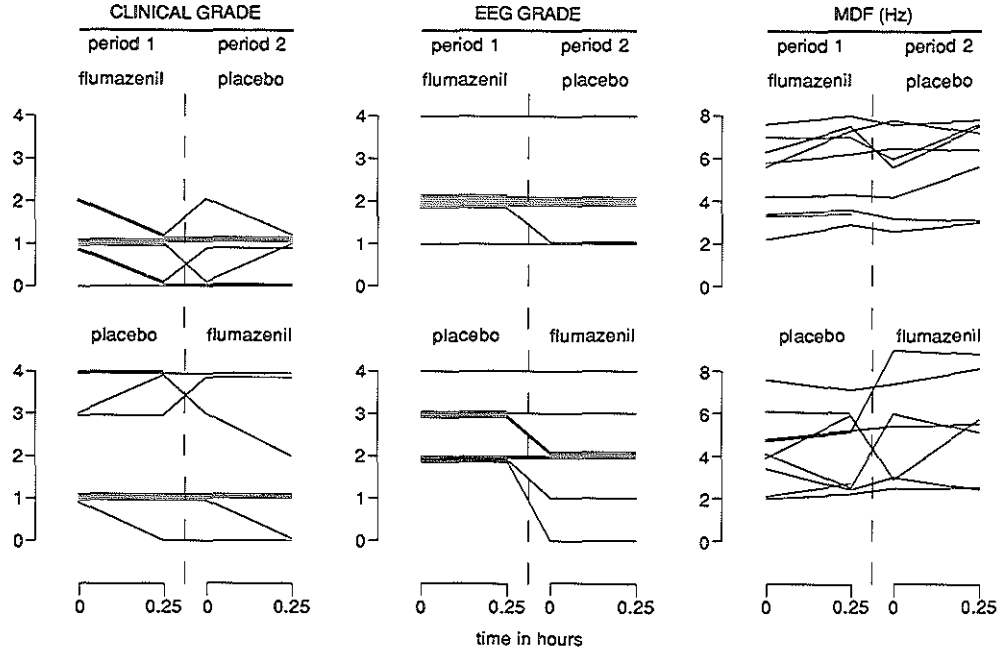

Figure 1: Assessment of each case of hepatic encephalopathy before and 15 min after the single injection of flumazenil or the placebo during the double-blind study: on the left, the clinical grade; in the middle, the EEG grade; on the right, the mean dominant frequency (MDF); top panel, subjects who received flumazenil in the first period; bottom panel, subjects who received the placebo in the first period.

baseline values induced by the infusions of flumazenil and placebo was -0.44 Hz with a 95%-confidence interval of -2.22 up to 1.34 Hz.

Open study

Hepatic encephalopathy was more severe in these patients than in the subjects of the double-blind study (figure 3). Three patients improved clinically after injection of flumazenil: 1 subject went from grade 4 to 2 and two from grade 3 to 2 HE. In contrast to the results of the controlled study, the EEG grade also improved in 2 cases. Furthermore, the MDF increased significantly ($p=0.02$) with a 95% confidence interval of 0.29 up to 1.31 Hz. Of the 4 patients who were also given the placebo, 1 subject improved clinically from grade 3 to 2. All patients who exhibited a change in the clinical and/or EEG grade had previously been treated with benzodiazepines.

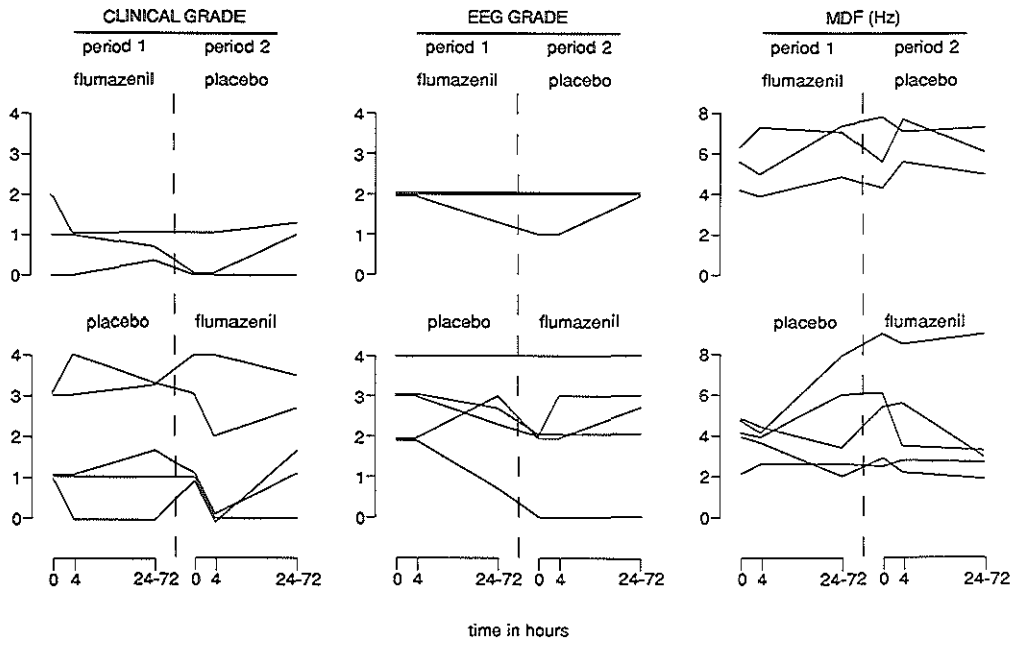

Figure 2: Assessment of each case of hepatic encephalopathy before and during the continuous infusion of flumazenil and the placebo during the double-blind cross-over study: on the left, the clinical grade; in the middle, the EEG grade; on the right, the mean dominant frequency (MDF); top panel, subjects who received flumazenil in the first period; bottom panel, subjects who received the placebo in the first period.

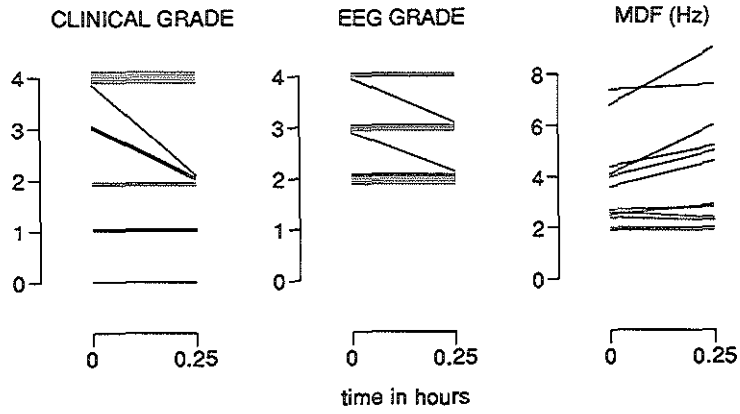

Figure 3: Assessment of each case of encephalopathy during the open study before and 15 minutes after administration of 1 mg of flumazenil: on the left, the clinical grade of encephalopathy; in the middle, the EEG grade of encephalopathy; on the right, the mean dominant frequency (MDF).

DISCUSSION

The findings of our controlled double-blind study do not support a major effect of the benzodiazepine receptor antagonist flumazenil on hepatic encephalopathy. Although the clinical grade of HE decreased in 6 patients 15 minutes after the injection of flumazenil, the effect did not reach statistical significance. Furthermore, the effect was still present 4 hours after the injection in the 3 patients who were studied for a longer period. Since flumazenil is rapidly eliminated from the body and the duration of action is much shorter than four hours, even in liver disease (42), the absence of subsequent deterioration does not support a dose-effect relationship. Evaluation of the clinical grades of HE during the 3-day infusion periods also did not indicate an effect of flumazenil. The same applies for quantitative EEG analysis and conventional EEG readings. These results are in accordance with those of animal studies on acute ischemic liver failure (37,38). However, they contradict the findings of uncontrolled clinical studies that demonstrated a remarkable effect of flumazenil on hepatic encephalopathy (1-7). Interestingly, in our open study we also found a significant effect

of flumazenil, as measured by quantitative EEG analysis. These patients had been excluded from the controlled trial, partly because of recent use of benzodiazepines.

Controlled studies of hepatic encephalopathy are hampered by the variety of metabolic disorders encountered in acute hepatic failure as well as cirrhosis of the liver, renal failure being the most common (43). Some metabolic factors, especially uremia, may lead to hepatic encephalopathy but can also induce encephalopathy in the absence of liver disease. In addition to metabolic complications, the recent use of benzodiazepines often also leads to (hepatic) encephalopathy (44). The causes of encephalopathy in liver disease are therefore widely varied, and strict selection criteria are needed for controlled clinical trials. Of the 54 patients evaluated, only 18 were found to be eligible for the study. Similar figures have been reported by others (45).

Because of the difficulty in randomizing sufficient patients for a clinical trial on hepatic encephalopathy, we decided to use a cross-over design for the double-blind study. The main problems of such a design are differences in the assessments between the two periods due to time and/or carry-over effects (41). In our study we could not find a period effect for the clinical grade and the MDF, but the EEG grade of HE was significantly lower in the second period. In theory, an important carry-over effect must have been absent since the elimination half-time for flumazenil in liver disease has recently been reported to be 2.0-6.6 hr (42); a wash-out period of 24 hrs should therefore be enough to prevent carry-over. We could not demonstrate an interaction between the drug effect and the period. Statistical analysis using the results from both periods of the cross-over study was therefore justified (41).

The controlled double-blind study consisted of patients with cirrhosis or hepatitis and generally mild hepatic encephalopathy. The findings for this group contradict the results of several uncontrolled studies (1-7). A response rate of 60-71% was found for the two largest series (4-5). In the uncontrolled studies the effect of flumazenil was assessed by clinical observation and electroneurology (EEG or evoked response examinations). The dose associated with immediate amelioration of hepatic encephalopathy was 0.2-1.0 mg in most studies (1-4, 6-7). This dose is the same as that used to reverse benzodiazepine-induced sedation after anesthesia or intoxication (46). We administered a dose of 1.0 mg to study the immediate effect of flumazenil.

The continuous infusion of flumazenil gave plasma levels between 10 and 20 ng/l (Dr. H. Drost, Utrecht), the concentration range recommended for antagonism of benzodiazepines (47). Grimm el al. (5) used a dose of 2-15 mg/15 min-3 hrs; the onset of clinical improvement occurred in 5 out of the 12 cases after administration of 0.3-1.1 mg. Our negative results cannot therefore be explained by simple dose differences.

Screening for recent use of benzodiazepines is important to distinguish between an effect of flumazenil on hepatic encephalopathy itself and antagonism of benzodiazepines taken previously. Plasma analysis for the presence of benzodiazepines together with a carefully taken history and study of medical reports is essential. Some of the improvement in hepatic encephalopathy reported after injection of flumazenil (2,33) may therefore be attributable to the use of insensitive methods for detection of benzodiazepines. The results of our open study indeed suggest that an effect of flumazenil may occur only when benzodiazepine-induced sedation contributes to the encephalopathy. However, "endogenous" benzodiazepines, possibly diazepam and desmethyldiazepam, have recently been reported to be found in plasma and brain extracts from patients and animals with and without hepatic encephalopathy (29,48-49). The question arises whether screening for benzodiazepines by plasma analysis before the start of the study actually excluded all possible responders. We excluded 15 patients from the primary study because of recent or current use of benzodiazepines. Twelve of them were immediately identified by history and the study of medical reports; plasma analysis for benzodiazepines was the sole reason for exclusion in only one case (nr. 2 in the open study). Small peaks in the plasma HPLC spectrum that could not be identified with certainty were found for 5 patients included in the double-blind cross-over study. Therefore, our negative findings cannot be explained simply by a selection bias.

We studied a selected group of patients with predominantly mild hepatic encephalopathy for which standard therapy had been unsuccessful. Extrapolation of our results to patients with hepatic encephalopathy in general is therefore not justifiable. In our study the number of patients with alcoholic liver disease was limited. According to reported effects of alcohol on the GABA-benzodiazepine receptor system (50), alcoholism may affect the response to flumazenil. In several of the open studies the percentage of patients with alcoholic liver disease was higher than in our study (2,4).

In conclusion we could not demonstrate a clinically important effect of flumazenil on hepatic encephalopathy in a small double-blind cross-over study of a highly selected patient population. Flumazenil may be effective against hepatic encephalopathy induced by benzodiazepines. More, and preferably larger, controlled studies of different groups of patients are needed to reach a consensus about the effectiveness of flumazenil in hepatic encepalopathy.

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Chapter 7

PHARMACOKINETICS OF FLUMAZENIL IN LIVER DISEASE

The contents of this chapter have been accepted for publication in the European Journal of Clinical Pharmacology under the title "Pharmacokinetics of flumazenil in fulminant hepatic failure" with the following authors: C.C.D. van der Rijt, R.H. Drost, S.W. Schalm, M. Schramel.

ABSTRACT

We studied the pharmacokinetics of flumazenil, a benzodiazepine antagonist, in 4 healthy volunteers, 3 patients with acute hepatitis and 3 with liver cirrhosis. One and 2.0 mg of flumazenil were administered intravenously to patients with liver disease and control subjects, respectively. Mean residence times and elimination half-lives in liver disease (3.0-9.4 hr and 2.0-6.6 hr, respectively) were 2-6 times the values found for volunteers (1.0-1.5 hr and 0.85-1.2 hr, respectively); the plasma clearance rates were decreased 3-5 times from 700-850 ml/min for controls to 120-320 ml/min in liver disease.

INTRODUCTION

Flumazenil, the first benzodiazepine antagonist in clinical practice, is recommended for selective reversal of benzodiazepine-induced sedation in anesthesia, intensive care patients and cases of intoxication (1). Increased sensitivity to benzodiazepines has been demonstrated in liver disease (2-3); moreover use of benzodiazepines can precipitate hepatic encephalopathy (4). Flumazenil may therefore be particularly important as an antagonist of benzodiazepines for patients with liver disease. Furthermore, flumazenil is under investigation for the treatment of hepatic encephalopathy itself (5-7).

In healthy volunteers flumazenil is rapidly eliminated from the body by the liver (8-11). Therefore, it is reasonable to assume impaired elimination in liver disease. We studied the pharmacokinetics of flumazenil in healthy volunteers and patients with severe liver disease.

SUBJECTS AND METHODS

Subjects: Six patients with decompensated severe liver disease participated in the study: 3 with acute hepatitis and 3 with liver cirrhosis, as confirmed by biopsy. Patient characteristics and markers of the severity of the underlying liver disease are given in table 1. The etiology of the liver disease was toxic in case A, auto-immune in C,

primary biliary cirrhosis in D, hepatitis B in F and unknown in cases B and E. The patients were treated with the following medications: ranitidine (A,B,D), prednisone (D), diuretics (furosemide and amiloride for D; spironolacton for E) and folic acid (C). Four healthy volunteers without a history of liver disease were also studied (subjects G-J). They did not use any medication except oral contraceptives (subject I) and showed no signs of alcohol abuse. Patients with liver disease received a 1.0 mg dose of flumazenil, which was injected over a 10 min period. Blood samples were taken from the contralateral arm before injection and 0, 2, 5, 10, 20, 30, 60, 120, 180 and 240 min after administration. The healthy volunteers received a higher dose of 2.0 mg of flumazenil within 5 min. This higher dose was used to ensure that the plasma concentrations of flumazenil in these healthy subjects would exceed the detection limit of the assay for the first 3 hours after injection. Blood samples were collected before injection and 0, 2, 5, 10, 20, 30, 45, 60, 120 and 180 min after administration.

Assay method:

Sample preparation: Bond Elut C_{18} disposable reversed phase columns (Betron, Rotterdam, The Netherlands) were pre-washed with 2.0 ml of methanol, 2.0 ml distilled water and 2.0 ml of Sörensen buffer (pH 7.4). After transfer of 500 μl of plasma and 100 μl of internal standard (50 ng of Ro 15-3505) they were washed twice with 1.0 ml of distilled water. Flumazenil was eluted with 0.5 ml of methanol. The eluate was evaporated under a stream of nitrogen at 40°C and the residue was dissolved in a mixtute of toluene-methanol (96:4). Aliquots were then analyzed.

Instrumentation: Gas chromatography was performed on a 30 m x 0.25 mm ID fused silica column with a chemically bonded stationary phase Sil 5 CB (J.W. Interscience, Breda, The Netherlands). Splitless injection and nitrogen-phosphorus sensitive detection were applied. Initial oven temperature was 120°C for 0.6 min, followed by a rise of 30°C/min to 280°C; the final stage of 280°C lasted 7 min. Injector and detection temperatures were 280°C and 300°C, respectively. Helium was used as carrier gas at a gas flow of 3 ml/min.

The limit of quantification was 1 ng/ml.

Data analysis: Pharmacokinetics were analyzed by the Nonlin-computer program. The individual plasma concentration-time curves were fitted to 2 exponential terms for most subjects; just for subject H mono-exponentional fitting was the best option. Using the

fitted plasma concentration-time curve the following parameters were calculated: the elimination half-life ($t_{½}$) from the time constant in the second exponential term; the clearance (Cl) from the ratio dose/area under the curve (AUC); the mean residence time (MRT) from the ratio of the area under the "concentration x time versus time" curve (TAUC) to the AUC; and the volume of distribution from the product of the MRT and the clearance (12,13).

RESULTS

The individual values for the pharmacokinetics of flumazenil are given in table 1. Rapid pharmacokinetics were demonstrated for the healthy volunteers with elimination half-life times of about 1 hour and slightly longer mean residence times. For the patients with liver disease $t_{½}$ varied between 2 and 6.6 hours, i.e. up to 6 times the values determined for controls, with the highest values found for the patients with acute liver insufficiency. Mean residence times were similarly prolonged in liver failure. The plasma clearance rates for patients were decreased 3-5 fold: from 700-850 ml/min for controls to 120-320 ml/min in liver failure. No differences in the volumes of distribution could be demonstrated between controls and patients with liver disease. For patients with liver disease, the clearance rate of flumazenil was related to the plasma antithrombin III level, a parameter for the assessment of liver function ($r_s = 0.943$, $p < 0.05$).

DISCUSSION

We have demonstrated markedly impaired pharmacokinetics of flumazenil in severe liver disease, in cirrhosis as well as hepatitis. Our findings for severe liver disease are similar to published values for Child C liver cirrhosis (14). As in our study plasma clearance rates showed about a 4-fold decrease and elimination half-lives about a 4-fold increase in patients with Child C liver disease (14). In a study on pharmacokinetics in less severe liver disease, plasma clearance rates were about 2 times decreased and elimination half-lives about 2 times increased (15). Our assessment of the elimination kinetics of flumazenil for healthy volunteers is again similar to that reported by others (8-11).

Elimination kinetics of flumazenil have been demonstrated to be linear over a wide dose

Table 1: Individual parameters of liver disease and disposition kinetics of flumazenil.

	acute hepatitis			liver cirrhosis			healthy volunteers			
subject	A	B	C	D	E	F	G	H	I	J
sex	F	M	F	F	F	M	F	F	M	F
age (y)	18	18	18	61	65	38	28	28	36	36
albumin (g/l, N 36-48)	31	31	30	21	25	31	47	44	49	44
antithrombin III (U/l, N>85)	0.10	0.01	#	0.52	0.23	0.20	1.09	1.12	1.23	1.12
bilirubin (μM, N<12)	196	556	457	393	42	602	11	7	11	7
ASAT (U/l, N<30)	94	330	304	109	50	109	18	16	16	18
pharmacokinetics										
$t_{½}$ (hr)	2.25	6.56	5.59	2.32	2.08	3.16	1.21	0.74	0.99	0.85
MRT (hr)	2.98	9.39	8.08	3.28	2.98	4.53	1.45	1.07	1.10	1.05
Cl (ml/min)	261	144	122	322	272	218	858	724	720	694
V_{ss} (l)	46.5	81.0	59.1	63.3	48.5	59.1	74.5	46.6	47.5	43.5
V_{ss} (l/kg)	*	*	*	0.88	0.97	0.86	1.26	0.78	0.63	0.79

not detectable in plasma; * body weight not available for cases with severe disease

range (2.5-40 mg iv) (8-11). Therefore, we felt it was justified to give the control subjects a double dose, thereby enhancing the sensitivity of the analysis.

Flumazenil is eliminated from the body almost exclusively by the liver (8). The high clearance rate in healthy subjects suggests that extraction of the drug by the liver is at least partly blood flow-dependent (8-9). The pharmacokinetics of drugs for which the hepatic clearance is determined by liver blood flow in the healthy state may become dependent on liver function in the event of liver disease (16). The correlation between the plasma clearance of flumazenil and the plasma levels of antithrombin III in patients with liver disease supports the relation between clearance and liver cell function. A relation between the plasma clearance rate of flumazenil and the Child Pugh score has

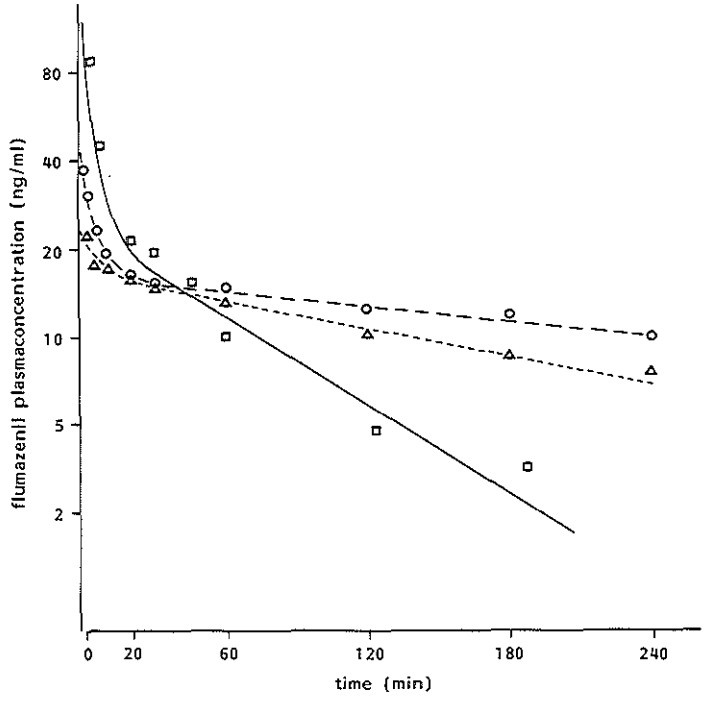

Figure 1: Plasma concentration-time curves: a patient with acute hepatitis (subject C, -----), a patient with liver cirrhosis (subject F, ...), a volunteer (subject J, ———).

also recently been demonstrated (14).

To our knowledge, dose-response studies on the use of flumazenil to antagonize benzodiazepines in liver disease have not yet been published. Plasma concentrations of flumazenil between 10 and 20 ng/ml have been recommended for antagonism of benzodiazepines in healthy subjects (17). For situations in which prolonged antagonism of benzodiazepines is indicated, an infusion rate of 1.44 mg of flumazenil/hr has been calculated (17). Assuming a similar flumazenil plasma concentration-effect relationship in individuals with and without liver disease, a lower rate of infusion of flumazenil would suffice for antagonism of benzodiazepines in patients with severe liver disease. According to the equation dose = clearance x plasma steady state concentration, an infusion rate of 0.1-0.3 mg/hr would then be sufficient to reach steady state concentrations of 15 ng/ml, assuming clearance rates of 120 and 320 ml/min, respectively. Higher infusion rates would indeed be recommended for patients with less severe liver disease. However, because the elimination of benzodiazepines in liver disease is markedly impaired (18), treatment with flumazenil may be necessary for longer periods than in health.

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Chapter 8

FACTORS CORRELATED WITH ENCEPHALOPATHY IN LIVER DISEASE: A LOGISTIC REGRESSION ANALYSIS

The contents of this chapter have been submitted for publication under the same title with the following authors: C.C.D. van der Rijt, P. Mulder, S.W. Schalm, E.P. Krenning

ABSTRACT

Studies on the pathogenesis of hepatic encephalopathy often reveal discrepancies between the levels of specific toxins and the presence of encephalopathy. In order to investigate the contribution of the metabolic disturbances frequently encountered in complicated liver disease to the pathogenesis of encephalopathy in liver disease, we performed a case-control study. A comparison between twenty six patients with liver disease and encephalopathy and 26 controls with liver disease without encephalopathy of their vital signs (temperature, heart rate and blood pressure), complications of portal hypertension, alcohol consumption and, predominantly routine, laboratory determinations was carried out. There was no significant difference in vital signs between the two study groups. Patients with encephalopathy had more severe ascites and consumed more alcohol. Of the 43 laboratory determinations studied, 19 were significantly different between the two study groups; they included indicators of liver cell damage, liver protein synthesis, liver detoxification, infection and renal function as well as zinc, glucose, vitamin, thyroid hormone, cortisol and prolactine levels. Starting with variables with a p-level <0.005 in the univariate analysis, logistic regression analysis was performed using a backwards selection procedure. The granulocyte count, zinc concentration, massive ascites and T3 level were selected as predictive variables. Starting with these variables, the contribution of the variables with p-levels between 0.05 and 0.005 was tested using a forward procedure. Heavy alcohol consumption contributed significantly to the model and replaced the variable granulocytes. The possible relationships between the selected variables and encephalopathy in liver disease, whether directly causal or an epiphenomenon, are discussed.

INTRODUCTION

Encephalopathy is a serious complication of acute and chronic liver disease. Many studies on the pathogenesis of hepatic encephalopathy have focused on specific toxins that are not adequately metabolized by the diseased liver (ammonia, GABA, mercaptans, free fatty acids). However, several discrepancies have been found between the plasma levels of specific toxins and the grade of hepatic encephalopathy.

In acute hepatic failure as well as chronic liver disease, complications can occur in several organ systems; renal failure is especially common (1-2). But acid-base disturbances, electrolyte abnormalities, infections and oxygen deficiency are also often encountered (3-5). Nutritional deficiences have been found, especially in chronic liver disease (6). Since each of these complications alone can induce encephalopathy (7), they may also contribute to the encephalopathy in liver failure, possibly together with specific toxins. The impact of a combination of factors has received little attention. Logistic regression analysis has been used in epidemiology to study the predictive value of a combination of variables for the occurrence of a disease state. To gain more insight into which factors are related to the encephalopathy of liver disease in clinical practice, we performed a case-control study of patients with liver disease with and without encephalopathy. A set of independent variables that were closely correlated with encephalopathy was selected by logistic regression analysis.

PATIENTS AND METHODS

Patients: Two groups of patients with acute or chronic liver disease were selected for the study. The first group consisted of 26 cases of liver disease and hepatic encephalopathy; the second group comprised 26 controls with liver disease but without hepatic encephalopathy. The two groups of patients were similar in age (47 ± 17 yr (group 1) vs. 49 ± 16 yr (group 2); mean $\pm$ S.D.) and sex (males:females; 17:9 (group 1) vs. 19:7 (group 2)). Four of the 26 subjects from group 1 presented with hepatitis, 1 with steatosis of the liver and 20 with cirrhosis. Histological confirmation was available for all but 3 patients with cirrhosis and 1 patient with unknown liver disease. For the patients in the 2nd group, the underlying liver disease was hepatitis in 3, chronic hepatitis or steatosis of the liver in 3 and cirrhosis in 20 subjects (3 without histology). The etiology of the liver disease was alcoholic (group 1 vs 2: 9 vs 7), viral (6 vs 6), auto-immune (1 vs 3), toxic (1 subject in group 2), primary biliary cirrhosis (1 vs 2), secondary biliary cirrhosis (2 in group 2), Wilson's disease (1 in group 2), poly-arteritis nodosa (1 in group 2) or unknown/cryptogenic (9 vs 3). Two patients in each group had received a surgical portocaval shunt. All subjects were hospitalized at the time of the study. There was no significant difference between the two groups in the duration of admission before entrance into the study (4.3 ± 6.0 days (group 1) vs. 3.3 ± 3.5 days

(group 2); mean ± S.D.).

Study-design: A case-control study was performed between November 1987 and April 1989. Every patient with known acute or chronic liver disease and clinically evident encephalopathy admitted by the Department of Internal Medicine II was considered a case. When such a patient was identified, the next patient with liver disease but without encephalopathy admitted to our hospital was included in the study as a control. The selection of patients was irrespective of sex, age, severity and etiology of the underlying liver disease. Only patients with a malignancy, if already known during the selection procedure, were excluded from the study. The study did not interfere with the therapeutic strategy for the patients.

The presence of clinically evident encephalopathy was assessed by the first author. Temperature, pulse rate and blood pressure were recorded on the day of the study. The medical records were studied to evaluate the complications of portal hypertension; ascites was graded as absent, present or massive on the basis of physical examination and echography; esophageal varices, graded as absent or present, were evaluated by means of esophagoscopy or radiology; portal collaterals, graded as absent or present, were investigated by echography; and the length of the spleen was measured by echography. Drugs used by the patients were recorded, with special attention to the actual use of lactulose and/or neomycin and diuretics. The number of alcoholic beverages consumed each week was also noted (table 1).
On the day the patient was evaluated for the presence or absence of encephalopathy, blood was drawn for a number of, predominantly routine, laboratory determinations (table 2). They represented assessment of a wide range of metabolic functions, including hepatic function itself (liver cell damage, protein synthesis and detoxification). A list of the causes of metabolic encephalopathy, as published by Plum and Posner (7), was used as a reference for the choice of variables.

Clinical grading of encephalopathy: Grade 0 encephalopathy was defined as the absence of at least 3 of the following abnormalities: inverted sleep pattern, disturbed memory, impaired calculation (serial 7's) and slowness of speech; grade 1 as the presence of at least two of these abnormalities (one abnormality in association with a flapping tremor was also classified as grade 1); grade 2 as the presence of at least 2 of

the following: lethargy, disorientation in time and flapping tremor; grade 3 as the presence of at least two of the following: a state in which the subject must be urged repeatedly to open his eyes or execute a command, disorientation in place and loss of self; and grade 4 as coma.

Table 1: Patient characteristics

	group 1	group 2		group 1			group 2		
	frequency	frequency	p	mean	SEM	nr	mean	SEM	nr
hepatic encephalopathy									
grade 0		26							
grade 1	5								
grade 2	7								
grade 3	4								
grade 4	10								
lactulose and/or neomycin	22	14	0.04						
diuretics	9	13							
glucocorticoids	4	3							
dopamine	0*	0							
alcohol (drinks/week)			0.01						
0	12	21							
1-14	3	3							
>14	8	1							
unknown	3	1							
ascites			0.003						
absent	4	14							
present	9	9							
massive	13	3							
esophageal varices									
absent	5	7							
present	16	15							
unknown	3	3							
venous collaterals									
absent	12	17							
present	10	9							
unknown	4	0							
spleen (in cm)				15	1	(22)	13	1	(26)
temperature (°C)				36.5	0.1	(26)	36.8	0.1	(26)
heart rate (bpm)				90	3	(26)	83	3	(26)
blood pressure (mm Hg)									
systolic				128	4	(26)	140	5	(26)
diastolic				75	3	(26)	82	3	(26)

*: 2 patients had been treaten with dopamine the day before inclusion.

Statistical analysis: The difference in each variable between the two groups of patients (group 1 with and group 2 without hepatic encephalopathy) was tested by the two-sample Wilcoxon test or the Chi-square test. Variables that were significantly different were selected for logistic regression analysis in an attempt to discriminate between the two groups of patients. Each group was assumed to be a random sample of an underlying population, I and II, respectively. In the analysis the probability that a patient belonged to either of the two populations (say population I) was expressed as a logistic discriminant function of the explanatory variables $x_1,...,x_n$:

$$P[pop.I \mid x_1,...,x_n] = \{1 + \exp(-ß_0 - ß_1x_1 - ... - ß_nx_n)\}^{-1}$$

The ß-coefficients were estimated by means of a maximum likelihood estimation method. Variables with $p<0.1$ in the likelihood ratio test were selected for the model. A ß-coefficient can be interpreted as being the logarithm of the odds ratio related to an increase of one unit in the corresponding x-variable. The odds is defined here as the probability of belonging to population I versus the probability of belonging to population II; the odds ratio is the factor by which the odds must be multiplied when a variable increases by one unit, the other variables remaining constant.

RESULTS

According to table 1, patients with hepatic encephalopathy presented with more severe ascites, consumed more alcohol and were treated with lactulose and/or neomycin more often than the controls. No significant differences were observed in the parameters of portal hypertension other than ascites.

There was a significant difference in the results of nineteen of the 43 laboratory determinations performed between cases and controls (table 2). They represented parameters of liver cell damage (ASAT), liver synthesis (Thrombotest, Normotest, antithrombin 3), liver detoxification (ammonia, bilirubin), infection (total leukocytes, granulocytes, monocytes), renal function (creatinine) and endocrinologic function (T3,

►Legend to table 2:
*: plasma calcium level corrected for albumin
levels of significance 1):$p<0.05$; 2):$p<0.01$; 3):$p<0.005$; 4):$p<0.001$

Table 2: Laboratory measurements in the case-control study

	group 1			group 2			
	mean	SEM	nr	mean	SEM	nr	p
Quantitative liver function							
liver cell damage:							
ASAT (U/l)	207	45	(26)	99	19	(26)	1)
ALAT (U/l)	385	156	(26)	87	17	(26)	
alk.phosphatase (U/l)	135	10	(26)	185	23	(26)	
synthesis:							
albumin (g/l)	26	1	(26)	30	1	(26)	1)
total protein (g/l	64	2	(26)	68	2	(26)	
Thrombotest (%)	21	2	(25)	42	3	(26)	4)
Normotest (%)	28	3	(25)	57	5	(26)	4)
antithrombin 3 (U/ml)	0.34	0.04	(25)	0.60	0.06	(26)	3)
detoxification:							
NH3 (μM)	118	11	(26)	74	6	(26)	3)
bilirubin (μM)	258	41	(26)	103	29	(26)	4)
bile acids (μM)	134	21	(25)	102	16	(26)	
Quantitative non-hepatic metabolic function							
hematology							
hemoglobin (mM Fe)	7.4	0.2	(26)	7.6	0.2	(26)	
thrombocytes (10^9/l)	133	20	(25)	152	21	(25)	
leukocytes (10^9/l)	13.2	1.6	(26)	7.5	0.9	(26)	4)
granulocytes (10^9/l)	11.1	1.4	(25)	5.5	0.8	(25)	4)
lymphocytes (10^9/l)	1.5	0.2	(25)	1.5	0.2	(25)	
monocytes (10^9/l)	0.9	0.1	(25)	0.6	0.1	(25)	1)
plasma viscosity (Cst)	1.25	0.04	(22)	1.32	0.01	(26)	
FDP's (% >10 mg/l)	29			40			
chemistry							
urea (mM)	13.7	2.4	(26)	7.3	1.0	(26)	
creatinin (μM)	187	37	(26)	89	6	(26)	1)
K (mM)	4.2	0.2	(26)	4.1	0.1	(26)	
Na(mM)	137	1	(26)	139	1	(26)	
Ca(mM)*	2.35	0.04	(26)	2.35	0.02	(26)	
PO4 (mM)	1.19	0.12	(26)	1.03	0.03	(26)	
zinc (μg/100ml)	48	4	(22)	66	4	(25)	3)
Mg (mM)	0.90	0.04	(25)	0.83	0.02	(26)	
osmolality (mosmol/kg)	292	4	(26)	286	2	(26)	
glucose (mM)	6.2	0.3	(26)	5.4	0.5	(26)	2)
pO2 (kPa)	11.2	0.6	(25)	12.4	0.5	(25)	
PCO2 (kPa)	3.9	0.1	(25)	4.2	0.1	(25)	
PH	7.46	0.01	(25)	7.45	0.01	(25)	
vitamin B1 (μM)	161	19	(22)	108	10	(26)	
vitamin B2 (μM)	0.33	0.02	(23)	0.31	0.01	(26)	
vitamin B6 (μM)	147	15	(21)	96	8	(24)	3)
vitamin B12 (nM)	912	39	(21)	655	55	(25)	3)
folic acid (nM)	15	3	(20)	19	3	(25)	
endocrinology							
T3 (nmol/l)	0.47	0.06	(25)	1.21	0.11	(26)	4)
T4 (nmol/l)	61	8	(26)	91	7	(26)	3)
T3/T4	0.008		(25)	0.014		(26)	4)
TSH (mU/l)	4.04	2.51	(25)	1.90	0.23	(26)	
cortisol 9.00 hr.A.M.(nM)	699	95	(23)	457	43	(26)	1)
prolactin (μg/l)	16.2	3.2	(26)	8.0	0.7	(26)	1)
plasma benzodiazepine (% positive)	32			31			

T4, cortisol and prolactin) as well as zinc, glucose and vitamin (B6 and B12) concentrations.

Simultaneous inclusion of all variables that yielded a significant difference of $p<0.05$ between the two groups of patients in the logistic regression analysis could not be justified in view of the total number of patients and the risk of overidentification. The first selection of variables to be excluded from further analysis was based on theoretical grounds; glucose and vitamins B6 and B12 were rejected as variables for further analysis because their levels did not indicate hypoglycemia, hyperglycemia or deficiences, respectively. Next, variables were selected on the basis of a p-level <0.005 in the univariate analysis. Among the variables thus under consideration were thrombotest, normotest and antithrombin 3, all of which represent liver protein synthesis, leukocytes and granulocytes, which represent infection, and the variables of thyroid function, T3, T4 and the ratio T3/T4. Further selection, therefore, was performed by applying logistic regression analysis to these three sets of variables. Thrombotest from the first set, granulocytes from the second set and T3 from the last performed the best. Ultimately, 7 variables were included in the logistic regression analysis: ascites, thrombotest, ammonia, bilirubin, granulocytes, zinc and T3. Starting with a full model including all of these variables it appeared from a backwards selection procedure that hepatic encephalopathy was best predicted by the presence or absence of severe ascites, granulocytes, zinc and T3 (table 3). The predictive value of these variables together was 93.2%

The predictive values of variables with p-values in the univariate analysis between 0.05 and 0.005 were investigated in a second logistic regression analysis. Starting with the variables selected by the first analysis, the contribution of the variables alcohol, albumin, ASAT, creatinine, cortisol and prolactine was tested using a forward procedure. Only the frequent use of alcohol contributed significantly to the model, even replacing the variable granulocytes. The presence of severe ascites, heavy drinking and low levels of zinc and T3 predicted the correct group for 95% of patients (table 3).

Table 3: Results of logistic regression analysis

variables studied	ß-coeff	SE	odds ratio	p
1. Backward selection procedure for variables with p<0.005				
included:				
massive ascitis	2.59	1.56	13.29	0.06
granulocytes	0.32	0.24	1.38	0.06
zinc	-0.08	0.04	0.93	0.01
T3	-7.01	3.10	0.0009	0.0003
excluded				
mild ascitis				
ammonia				
bilirubin				
thrombotest				
2. final model				
included				
massive ascitis	2.82	1.76	16.74	0.06
T3	-8.61	4.07	0.0002	0.0003
heavy drinking	4.91	3.43	135.49	0.03
zinc	-0.05	0.04	0.95	0.05
excluded				
thrombotest				
mild ascitis				
ammonia				
cortisol				

ß-coefficient = logarithm of the odds ratio; SE = standard error of the ß-coefficient; odds ratio = the factor by which the odds must be multiplied when the corresponding variable increases by one unit; p = significance of the likelihood ratio test.

DISCUSSION

We performed a case-control study to gain more insight into the factors related to hepatic encephalopathy. Because several discrepancies have been found between specific toxins and the grade of hepatic encephalopathy, we were especially interested in the contribution of several metabolic disturbances that have been related to the frequent complications of severe liver disease. Granulocytes or heavy alcohol consumption, massive ascites, serum zinc and serum T3 were selected by logistic regression analysis. Several complications often found in liver disease, especially renal failure, electrolyte abnormalities, acid-base disturbances and nutritional deficiences, were not selected. Although there was a significant difference in the level of ammonia,

often considered the major toxin in the pathogenesis of hepatic encephalopathy, between the two groups of patients studied, ammonia was excluded from the model by logistic regression analysis.

Logistic regression analysis is often used in epidemiology to identify cause-effect relationships. In our study it is important to realize that different mechanisms might explain a correlation between a variable and encephalopathy. In theory three explanations have to be considered (figure 1): the variable may indeed be causally related (a); the variable may be the result of encephalopathy (b); or the variable may be an epiphenomenon: i.e. it might be related to a third factor, which is more directly related to encephalopathy in liver disease (c).

The number of granulocytes contributed significantly in the prediction of the presence of encephalopathy in our study population. However, after inclusion of alcohol consumption in the logistic regression analysis, granulocyte counts were excluded from the model, suggesting a correlation between granulocytes and alcohol abuse. How can

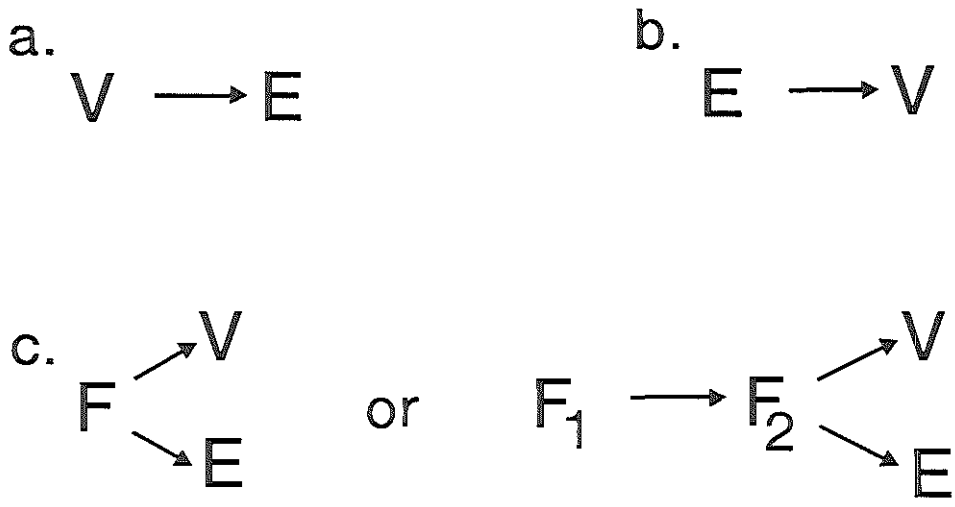

Figure 1: Theoretical mechanisms for the relation between a variable and encephalopathy; a: the variable (V) is causally related to the encephalopathy (E); b: the variable is the result of the encephalopathy; c: the variable is related to a third factor (F) which is directly related to the encephalopathy.

we explain this correlation and how can we explain its relation to encephalopathy in liver disease?

Alcohol has been reported to be a frequent precipitating factor in hepatic encephalopathy (9-10). The abuse of alcohol may facilitate endotoxemia by impairing Kupffer cell function (11), inducing bacterial overgrowth in the small intestine (12) and/or increasing the permeability of the intestinal wall (13). Previous studies have shown elevated titers of Escherichia coli antibodies in particular in alcoholic hepatitis (14) and a relation between endotoxemia and alcohol consumption in health and liver disease (15). Endotoxins may indeed cause granulocytosis. Furthermore, experimental studies have indicated that liver failure and hepatic encephalopathy can be induced by the injection of endotoxin (16-17). In clinical studies an increased incidence of endotoxemia has been found for patients with hepatic encephalopathy (18).

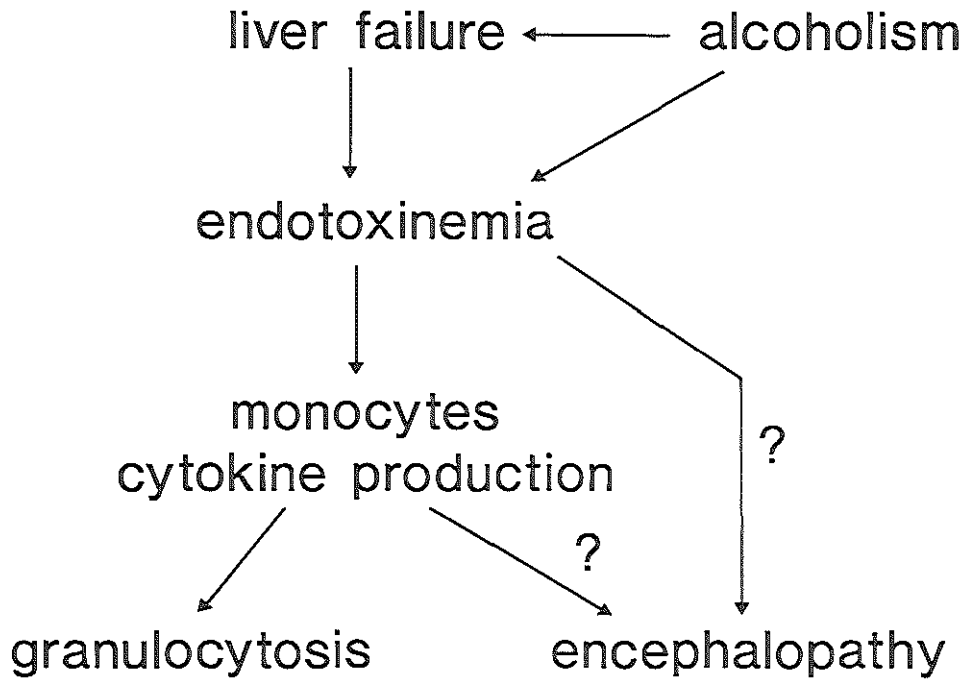

Figure 2: Hypothetical model explaining the relation between alcohol abuse, leukocytes and encephalopathy.

Alcoholic hepatitis has also been associated with the enhanced production of cytokines by monocytes, especially interleukin-1 and tumor necrosis factor (TNF) (19-20); the presence of TNF has been said to predict a poor prognosis (21). We could therefore hypothesize that alcohol induces cytokines, with or without endotoxemia, which subsequently cause granulocytosis and hepatic encephalopathy. As shown in figure 2, granulocytosis is presumed to be an epiphenomenon.

What is the relationship between the serum zinc level and hepatic encephalopathy? The total serum zinc concentration is the net result of the zinc status in the body, the serum binding proteins and serum factors that modify zinc-protein binding (22-23). Normally, a major fraction of zinc is "loosely" bound to albumin; a smaller fraction is "tightly" bound to α_2-macroglobulin. In decompensated liver disease the concentration of zinc bound to α_2-macroglobulin is increased (23). Decreased serum zinc levels in patients with encephalopathy may therefore represent either zinc deficiency or diminished albumin synthesis. Since the logistic regression analysis excluded albumin and the other variable of liver cell protein synthesis, thrombotest, from the model, serum zinc may not only represent protein binding but also zinc deficiency in encephalopathy.
The idea of zinc defiency as a causative factor in hepatic encephalopathy is supported by our recently published case report on hepatic encephalopathy (24). We described a woman with chronic recurrent portosystemic encephalopathy resistent to the standard therapy of protein restriction and lactulose. L-histidine-induced zinc loss caused an episode of hepatic encephalopathy similar to previous episodes, and chronic therapy with zinc substitution improved her quality of life. Furthermore, zinc supplements appeared to improve mild hepatic encephalopathy in a randomized clinical trial (25).

The finding of massive ascites turned out to be an important predictor of hepatic encephalopathy. Several explanations can be considered for this relation. Ascites and hepatic encephalopathy are both predictors of a poor prognosis for severe liver disease. Therefore, the correlation may not be a cause-effect relation; instead ascites and encephalopathy may be two (related) prognostic variables. The frequent use of diuretics for the treatment of ascites might also explain the correlation between ascites and encephalopathy, since diuretics have been reported to induce hepatic encephalopathy (26). However, in this study about the same numbers of patients in the two groups were treated with diuretics.

Ascites in liver disease has been associated with a hyperdynamic circulation and reduced systemic vascular peripheral resistance (27). Diminished hepatic metabolism of a vasodilator substance generated in the splanchnic bed has been proposed as the etiologic mechanism leading to the hyperkinetic circulatory state (27-28). S. Sherlock recently discussed the possible contribution of the altered circulatory state to the development of the hepatorenal and hepatopulmonary syndrome and its possible role in cerebral edema (29). Hepatic encephalopathy has been found to be associated with elevated serum levels of substance P (30) and prostacyclin synthesis stimulating factor (31), both of which have an important vasodilatory effect. The correlation between ascites and hepatic encephalopathy might therefore be explained by a similarity in etiologic factors, i.e. substances with vasodilatory as well as cerebral actions (figure 3). Not only vasodilators but also vasoconstricting agents, especially norepinephrine and

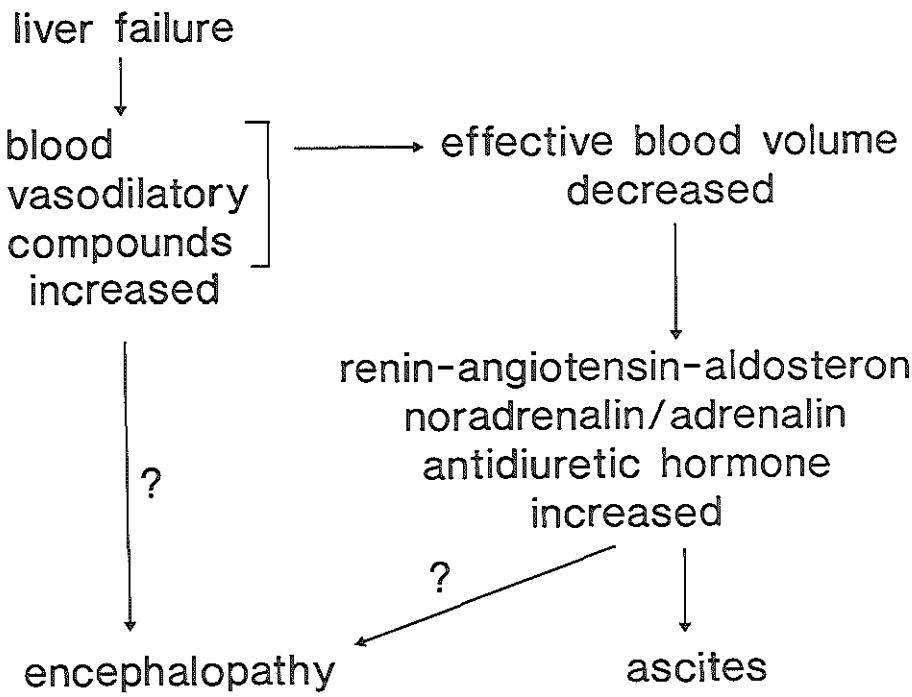

Figure 3: Hypothetical model explaining the relation between ascites and encephalopathy.

the renin-angiotensin-aldosteron system, are found in elevated concentrations in patients with ascites and liver failure (27). As far as the authors know, no reports on an association of these factors with hepatic encephalopathy have been published.

The level of T3 was strongly correlated with encephalopathy in liver disease. What causes this relationship? Serum T3 is largely protein-bound. It is generated in the liver from thyroxine, which enters the liver via specific receptors (32). Decreased T3 levels may therefore represent diminished protein binding, decreased T4 transport into the hepatocyte and diminished intracellular metabolism. The contribution of protein binding may be eliminated by using the ratio of the thyroid hormones T3 and T4, since these two hormones bind to the same serum proteins with almost similar affinities (32). Testing the difference in the ratio T3/T4 between the two study groups yielded a p-

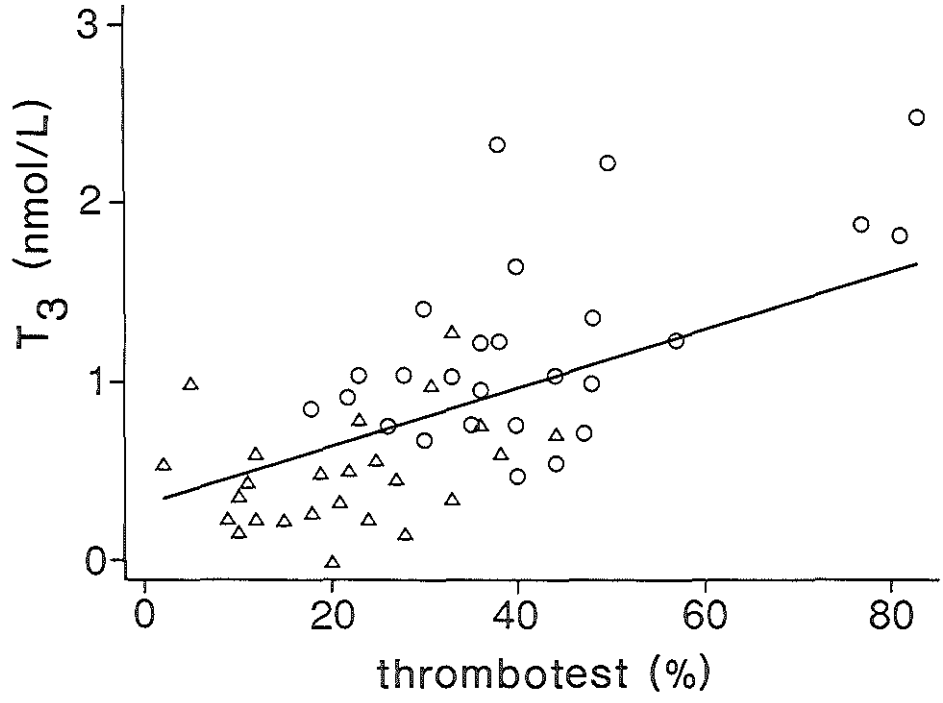

Figure 4: Relation between serum T3 levels and thrombotest as a parameter of liver function for the entire group of patients studied (▵:group 1; o:group 2; $r=0.87$, $p=0.012$).

level of significance of 0.0001 (mean±S.D., group 1 0.008±0.004; group 2 0.014±0.005). Decreased T3 levels, therefore, cannot be explained by diminished protein binding alone.

Decreased T4 transport across the cell membrane and diminished intracellular metabolism of T4 into T3 may represent liver function. Correlations between T3 levels and the parameters of liver function have indeed been reported (33). We also found a significant relationship between T3 and thrombotest in this study (figure 4). However, low thyroid hormone concentrations are also found in a variety of nonthyroidal illnesses other than liver disease (34). Correlations between the indices of thyroid function, especially T3 and T4, and markers of the severity of the underlying disease have been demonstrated in general (35-36). Therefore, what causes the relationship between T3 and encephalopathy? Is it related to liver function or the severity of disease in general, or can thyroid hormones be causally related to encephalopathy? Preliminary findings seem to indicate that liver function alone cannot explain the relationship. Substituting the ratio T3/T4 for T3 in the first backwards procedure of the logistic regression analysis, we found that thrombotest and the ratio were both selected instead of T3 alone.

The relation between T3 and encephalopathy in liver disease may represent T4 transport across the cell membrane and thus may be an indirect relationship (T3 as an epiphenomenon). A study of T4 liver plasma membrane transport seems worthwhile. In mouse neuroblastoma cells L-amino acids were shown to be competitive inhibitors of thyroxine transport across the cell membrane (37). If the thyroxine binding protein of the liver is similar to that of neurons, those L-amino acids which are increased in hepatic encephalopathy (phenylalanine, tyrosine and tryptophane) may inhibit T4 uptake in brain as well as the liver and hence contribute to the low serum T3 levels (figure 5). In that case, the role of L-amino acids in the pathogenesis of hepatic encephalopathy may have to be reconsidered.

Can thyroid hormones be causally related to encephalopathy? T3 instead of T4 was selected as a predictor for the presence of encephalopathy. However, in contrast to peripheral tissues, the cells of the central nervous system do not obtain most of their T3 by direct cellular uptake but from de-iodination of T4 (38). Decreased availability of T3 in the neuronal cell due to low serum levels therefore seems unlikely but diminished T4 uptake in neuronal cells may induce deficient intracellular T3 levels. Furthermore, the non-metabolic roles for thyroid hormones in the central nervous system are as yet

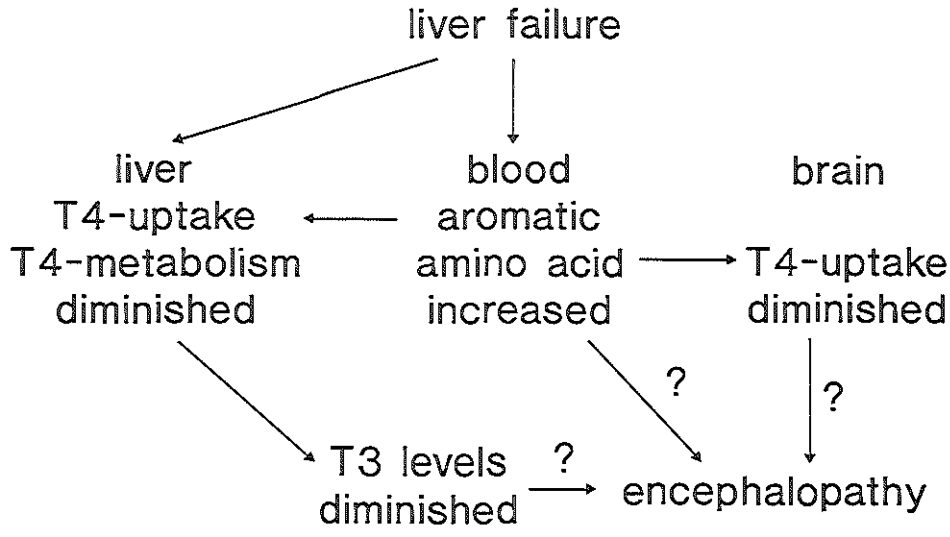

Figure 5: Hypotheses for the relation between serum T3 and encephalopathy. In a, T3 is a causal factor of encephalopathy; in b and c T3 is an epiphenomenon.

poorly defined, but several studies have demonstrated modulatory effects on neurotransmission, especially glutamate and benzodiazepine receptors (39-41). Since correlations between thyroid hormones and encephalopathy without liver disease have also been reported (Glasgow Coma Scale ratings for traumatic brain injury) (42), further studies on the role of T3 and T4 in central nervous system functioning are needed.

Of the variables excluded during logistic regression analysis, ammonia is the one most often related to hepatic encephalopathy. As mentioned earlier, our study did not interfere with the therapeutic strategy for these patients. Patients with hepatic encephalopathy were indeed treated with lactulose and/or neomycin more often than the controls. Therefore, we cannot conclude from this study that ammonia does not induce hepatic encephalopathy in general. Furthermore, previous studies have already demonstrated that the correlation between hepatic encephalopathy and ammonia levels is better immediately after admittance than later during hospitalization (43). The possibility of a role for ammonia is further substantiated by the close correlation

between glutamine levels in cerebrospinal fluid and encephalopathy (44-45).

In conclusion, our case-control study yielded the variables granulocytes and alcohol, zinc, massive ascites and T3 as predictors for the presence of encephalopathy in liver disease. A case-control study, however, cannot be the ultimate proof for theories on pathogenesis. Further prospective studies are needed to investigate the selected variables in new groups of patients. Furthermore, the effect of active interference with the suggested factors has to be studied.

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Chapter 9

OVERT HEPATIC ENCEPHALOPATHY PRECIPITATED BY ZINC DEFICIENCY. A CASE REPORT

The contents of this chapter have been published in Gastroenterology (1991;100:1114-1118) under the same title with the following authors: C.C.D. van der Rijt, S.W. Schalm, H. Schat, K. Foeken, G. de Jong.

ABSTRACT

Encephalopathy in liver disease may be unresponsive to protein restriction, lactulose and neomycin. Zinc supplements have been reported to improve psychometric performance in liver cirrhosis but the importance of zinc deficiency in overt hepatic encephalopathy has not yet been clearly established. We studied a patient with severe recurrent hepatic encephalopathy to determine the relation between her signs of encephalopathy and zinc deficiency. The study included a period in which zinc deficiency was artificially induced by oral histidine. An episode of overt encephalopathy occurred that was identical to earlier episodes and responded to oral zinc. The study showed an association between encephalopathy and zinc deficiency by successive zinc depletion and supplementation regimens. Long term zinc supplementation improved severe recurrent hepatic encephalopathy and thereby the quality of life.

INTRODUCTION

Encephalopathy is a complication of acute and chronic liver failure. Its pathogenesis is still unresolved but the accumulation of gut-derived nitrogenous substances due to impaired hepatic clearance is generally thought to be a major mechanism (1). Protein restriction, lactulose and neomycin, given to reduce the production of nitrogenous toxins, are effective in some but not all patients (1).

In patients without liver disease zinc deficiency may induce neurological and psychiatric symptoms: ataxia, lethargy, depression and hallucinations (2-4). In liver disease the concentrations of zinc in serum (5), leukocytes (6) and liver (7) are decreased. The importance of zinc deficiency in hepatic encephalopathy has not yet been clearly established, although zinc supplements have been reported to improve mild chronic hepatic encephalopathy, as measured by psychometric testing (8-10), as well as some episodes of hepatic encephalopathy following gastro-intestinal bleeding (11).

We have studied a patient with incapacitating recurrent hepatic encephalopathy to determine the relation between her signs of encephalopathy and markers of zinc deficiency.

CASE REPORT

A 54-year-old woman with congenital liver fibrosis was admitted for evaluation of the need for liver transplantation because of recurrent episodes of hepatic encephalopathy. Fifteen years previously she had undergone a side-to-side porto-caval shunt operation for recurrent severe bleeding from esophageal varices. After surgery she remained well until 5 years ago when the first episode of encephalopathy occurred. Since then episodes of encephalopathy have recurred with increasing frequency (6 episodes in the 6 months prior to admission) despite a low protein diet, lactulose and neomycin. According to her husband a typical episode was preceded by a short period in which she vigorously cleaned the house. She then complained of a tremor around the mouth, nausea and vomiting, and subsequently slept for one or more days. During this latter period she did not eat or drink and was unable to speak or walk. Handling her caused aggressive resistance. She was sometimes incontinent. Upon awakening she again felt nauseated. Afterwards she had a total amnesia for the episode.

Upon admission to our hospital mental signs of hepatic encephalopathy were not obvious although a slowness of speech was noted. Neurological examination revealed a mild high-frequency tremor (but not a flapping tremor) and impaired heel-toe walking without other abnormalities. Electroencephalography demonstrated an alpha rhythm of 8c/s; the mean dominant frequency (MDF) as measured by spectral analysis of the EEG was 8.5 Hz. Biochemical analysis was consistent with a diagnosis of chronic stable liver disease (bilirubin 36 μmol/l, alkaline phosphatase 259 U/l, gamma-GT 173 U/l, ASAT 55 U/l, ALAT 55 U/l, albumin 29 g/l and prothrombin time 21 sec, which is 3 sec above normal). The arterial plasma ammonia level was 108 μmol/l. A search for encephalopathy-inducing metabolic or structural disturbances revealed a depressed serum zinc level: 34 μg/dl (normal 80-150 μg/dl). She was observed for 6 weeks without changing her treatment with protein restriction (60 grams/day), lactulose (60 ml/day) and neomycin (4 g/day). An episode of encephalopathy did not occur, despite spontaneous fluctuations of the arterial ammonia level between 83 and 165 μmol/l. To examine her protein tolerance the protein intake was increased to 100 grams/day for one week. After 3 days her speech became slurred and her gait wide-based. However, no deterioration could be detected in the electroencephalogram. The symptoms resolved spontaneously, despite continuation of the 100-gram protein diet. She was then given zinc acetate (600 mg/day orally for 3 weeks)

to correct the possible zinc deficiency and hopefully to prevent new episodes of encephalopathy after discharge from the hospital. During this period serum zinc rose from 46 to 91 μg/l and the activity of the serum zinc-dependent enzyme alkaline phosphatase increased from 257 to 404 U/l (normal $<$100 U/l).

For 2 months she felt very well and had no new episodes of encephalopathy. However, later on her condition again deteriorated: upon readmission (4 months after discharge) she was lethargic and her speech was slurred. This time slowing of the electroencephalogram was obvious: the MDF was 3.8 Hz. Neurological examination revealed a cerebellar syndrome: saccadic eye movements, dysdiadochokinesia, impaired coordination and an ataxic gait. A CT-scan of the brain did not reveal any abnormalities. Precipitating causes of hepatic encephalopathy, such as infections, changes in diet, electrolyte abnormalities, obstipation, alcohol intake and use of sedatives or diuretics, could not be identified. The serum zinc level and the activity of serum alkaline phosphatase had both dropped once again (58 μg/100ml and 274 U/l, respectively).

To investigate the possibility of a relation between serum zinc levels and symptoms, she was again treated with zinc acetate for a period of 3 weeks. This was followed by a zinc depletion regimen under continuous control of her clinical state, quantitative EEG-analysis and assessment of serum zinc levels. During the zinc treatment she felt well. Her serum zinc level rose to 90 μg/100ml and alkaline phosphatase to 400 U/l. One week after stopping zinc administration, zinc loss was induced by L-histidine (8 grams the first day, increased incrementally to 32 grams/day on day 7) (5). Urinary zinc levels rose from 1600 to 8500 μg/24hrs and serum zinc levels declined (figure 1). On day 8 she complained of tremor around the mouth and in the hands. On days 9 and 10 anorexia and nausea were present and on day 10 she became drowsy and felt depressed. In the evening she wandered around aimlessly, disorientated in time and place, and that night she became stuporous. The clinical picture that evolved was recognized by her husband as being similar to the episodes at home. She no longer responded to questions and became aggressive during (medical) handling. On neurological examination she opened her eyes when spoken to loudly, but neither spoke nor followed commands. She actively resisted physical examination. Appropriate localizing responses followed the application of a painful stimulus. When the limb tendon reflexes were tested both the ipsilateral and contralateral extremities responded, in itself not an indication of neurological abnormalities. On mental status

examination an organic brain syndrome was diagnosed with severe clouding of consciousness, disorientation and mutism. Electroencephalography demonstrated long trains of triphasic waves as objective evidence of severe encephalopathy. Serum zinc had declined to 46 μg/100ml, serum alkaline phosphatase to 235 U/l. The arterial ammonia levels had been below 100 μmol/l during the study period, except on day 10 when it was 168 μmol/l. On the morning of the stuporous state it was 79 μmol/l.
L-histidine was discontinued and zinc acetate given. Lactulose and neomycin dosages remained unchanged during the entire study period. A gradual recovery was observed in 3 days. After 2 days she was cooperative again. Consciousness was still impaired with disorientation in time and place, and her memory and cognitive functions were still poor. She was very depressed and cried during examination. The following day consciousness was normal, her mood had improved markedly and cognitive functions were unimpaired. Serum zinc reached normal values within one day of supplementation: 114 μg/dl. Serum alkaline phosphatase increased more gradually to 449 U/l after 10 days. According to her husband full recovery was achieved more rapid than at home.

The patient was discharged from the hospital on zinc acetate therapy, in a dose of 600 mg/day which was decreased to 200 mg/day in the course of 2.5 months. For a period of almost 2 years just one episode of hepatic encephalopathy occurred. At that time the patient was obstipated.

DISCUSSION:

We have described a patient with chronic liver disease in whom a relation between recurrent episodes of encephalopathy and zinc deficiency was demonstrated. At the time of admission for recurrent episodes of encephalopathy, her serum zinc level was markedly decreased. During an observation period of about 2 months the serum zinc level rose spontaneously from 34 to 46 μg/dl but no encephalopathy occurred, despite fluctuating hyperammonemia and a 100-gram daily protein intake for one week. After zinc supplementation for 3 weeks, the serum serum zinc level rose to 91 μg/dl (normal) and she felt very well subjectively for about two months. Then mental and neurological deterioration developed; the serum zinc level had again dropped to 51 μg/dl. After zinc supplementation and complete recovery, histidine-induced zinc loss resulted in an episode

of encephalopathy that was identical to earlier episodes. During maintenance therapy with zinc acetate, just one period of mental confusion reappeared, this one precipitated by obstipation.

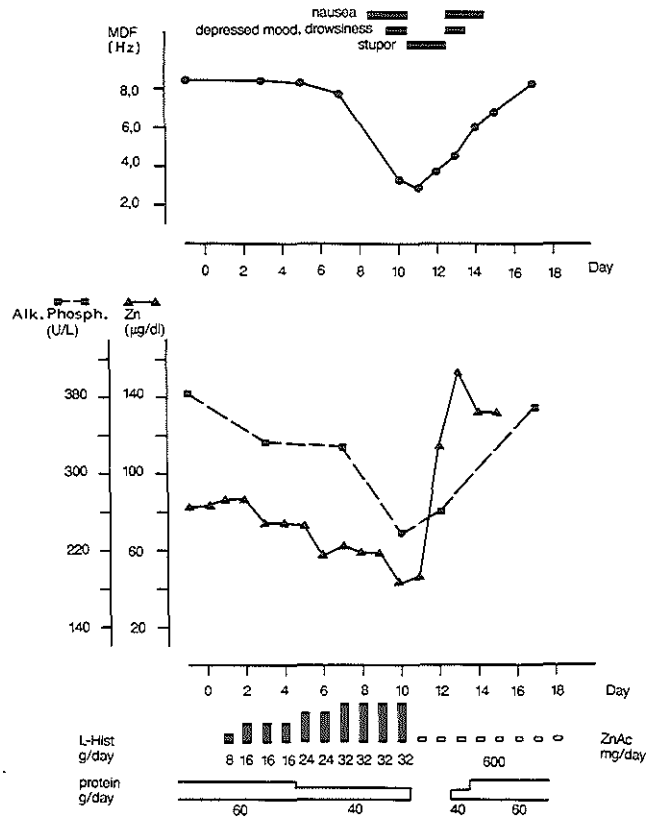

Figure 1: Clinical course during L-histidine-induced zinc loss and supplementation. Upper panel: the symptoms and the course of the mean dominant frequency (MDF) of the EEG; middle panel: the course of serum zinc levels and the activity of alkaline phosphatase in serum; lower panel: the dose of L-histidine during the depletion period, the zinc acetate dosage during supplementation and protein intake during both periods. (Alk. Phosph. alkaline phosphatase, L-Hist L-histidine, MDF mean dominant frequency, Zn zinc, ZnAc zinc acetate)

The psychiatric and neurological signs and symptoms that were observed have all been described as clinical manifestations of hepatic encephalopathy: slowness of speech, inappropriate behavior, anger, depression, impaired consciousness, tremor and ataxia (1, 12). Indeed, the picture of recurrent episodes of encephalopathy in a patient with chronic liver disease without neurological or non-hepatic metabolic diseases is consistent with the diagnosis of hepatic encephalopathy.

A syndrome of acute zinc loss has been described in patients with progressive systemic sclerosis who have been treated with high doses of L-histidine (5). Neurological symptoms consisted of tremor and ataxia; psychiatric symptoms included sleepiness, depressive mood and paranoid ideation (5). In our patient the impairment of consciousness seemed to be more severe, but the overall clinical picture was almost identical. Acute zinc deficiency has also been described during total parenteral nutrition (6-7, 13-15). Disturbances of mental function were less prominent in these studies, although depression and mental confusion were reported (6-7). Moreover chronic zinc deficiency in children with acrodermatitis enteropathica is often characterized by moody depression (16). The symptomatology of our patient, therefore, closely resembles that of hepatic encephalopathy but is also compatible with the zinc deficiency syndrome.

The pathogenesis of hepatic encephalopathy is still poorly understood. The most popular theories focus on the role of ammonia, an imbalance between cyclic and branched chain amino acids or GABA (1). All three factors are presumed to influence neurotransmission in the central nervous system. Zinc is a major trace element in the human body and is found in several enzymes (17). Encephalopathy due to zinc deficiency in patients with an underlying liver disease may be attributable to further disturbance of the metabolism of specific toxins. Indeed, in healthy volunteers zinc deficiency caused a rise in plasma ammonia levels (18) and patients with liver cirrhosis exhibited a stimulated synthesis of urea during zinc therapy (8). We observed a high arterial ammonia level just one day before our patient became stuporous but did not find a clear relation between zinc and ammonia levels in our patient. Furthermore, the plasma ammonia concentrations before and during the episode of encephalopathy that followed L-histidine-induced zinc loss were similar to or even lower than the values found during the first observation period when encephalopathy did not occur. The effect of zinc deficiency also could not be explained by a decreased ratio of branched chain to aromatic amino acids (1.05 during encephalopathy

and 1.13 after recovery). Therefore, instead of stimulating the accumulation of toxins, zinc deficiency may affect central nervous system functioning by itself and may thus make the brain more susceptible to the effects of toxins. Correction of the zinc deficiency may then improve the underlying neurone dysfunction and protect against elevated concentrations of toxins. As encephalopathy did not occur during the first observation period when zinc levels were low but rose slightly, decreasing zinc levels may be especially harmful to the brain.

An effect of zinc on central nervous system functioning is supported by in vitro studies that seem to relate zinc to neurotransmission: uptake into synaptosomes (19), depolarization-induced release from brain tissue (20-21) and post-synaptic modulation of excitatory neurotransmission (22-23). In addition to neurotransmission, electrophysiologic membrane function might also be affected by membrane-associated zinc (24).

The diagnosis of zinc deficiency is difficult and cannot be based on serum levels only (25). In liver disease the diagnosis of zinc deficiency is particularly difficult because of the decrease in albumin levels and alterations in the distribution of serum zinc between albumin ("loosely-bound" zinc) and alpha-2 macroglobulin ("tightly-bound" zinc) (26). Measurement of zinc levels in tissues such as hair, leukocytes and erythrocytes has been suggested as indicator for the zinc status (27). However, the rapid changes in zinc status as observed during our zinc depletion and supplementation periods would not have been reflected properly in tissue levels, as tissue turnover is slow (27). On the other hand, rapid increases in serum alkaline phosphatase have been reported after treatment of zinc deficiency (28). Therefore, we felt justified to use serum zinc levels together with levels of the zinc dependent enzyme alkaline phosphatase as a simple method for monitoring zinc status. Changing levels of serum zinc in a single patient in whom intercurrent infections and fluctuations in albumin levels are absent, seem to indicate changes in the zinc status. The close relation between serum zinc levels and the activity of the zinc metalloenzyme alkaline phosphatase in serum supports a true state of zinc deficiency.

The diagnosis of zinc deficiency is further substantiated by analysis of the zinc content in the diet and the urine. The zinc content in the diet our patient used at home was rather low: 3.8-5.6 mg/day; a higher concentration, 7.0-8.8 mg/day, was found in the hospital diet. The difference in zinc content in the 2 diets might explain that the serum zinc level

rose from 34 to 46 μg/dl during the first observation period. On readmission, urinary excretion of zinc was increased, 2700 μg/day. Urinary zinc concentrations were also monitored during histidine-induced zinc loss. The already increased urinary output of 1600 μg/24hrs just before the use of L-histidine rose to 8500 μg/24hrs at the end of the depletion period. Because intestinal absorption of zinc from food is far less than 50% (25), urinary zinc loss may account for a rapid induction of zinc deficiency during the experimental depletion period.

Although our observations on the relation between zinc deficiency and manifestations of encephalopathy in chronic liver disease suggest a contribution of zinc deficiency to the pathogenesis of hepatic encephalopathy, Schölmerich et al failed to find a correlation between serum zinc levels and the presence of hepatic encephalopathy in liver cirrhosis (29). However, the estimation of zinc deficiency by serum levels only in a group of patients may be much more hazardous than in a single patient. Furthermore, the beneficial effects of zinc substitution on mild hepatic encephalopathy, as demonstrated in several studies (8-11), further support a pathogenetic role for zinc in hepatic encephalopathy.

In conclusion, our observations suggest that overt hepatic encephalopathy can be precipitated by zinc deficiency. Further studies are needed to support this finding and to evaluate the consequences of zinc substitution for treatment of the various manifestations of hepatic encephalopathy.

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Chapter 10

DISCUSSION AND CONCLUSIONS

The pathogenesis of hepatic encephalopathy is still unknown. Ammonia has been suggested as the major etiologic toxin. Medical therapy, consisting of oral protein restriction, lactulose or newer disaccharides and/or antibiotics, is aimed at decreasing elevated plasma ammonia levels. This strategy, however, is not always successful. Especially patients with a severely impaired liver function often respond poorly.

New theories on the pathogenesis of hepatic encephalopathy were developed, the most important being the false neurotransmitter hypothesis based on the imbalance between aromatic and branched chain amino acids and the GABA-benzodiazepine receptor theory. These theories were rather attracive, because they tried to explain the neuropsychological disorder at the neuronal level, i.e. as a disorder of neurotransmission. More specific therapies seemed possible. Several clinical trials on therapy with branched chain amino acids for hepatic encephalopathy were instituted but most of them could not demonstrate a beneficial effect. Direct intervention with L-Dopa or bromocriptine to restore catecholaminergic neurotransmission is possibly effective in a few carefully selected patients. The application of the false neurotransmitter theory to explain hepatic encephalopathy therefore seems limited (for references, see Chapter 1).

The GABA theory for the pathogenesis of hepatic encephalopathy can be discarded in view of the controversial findings on the number of GABA receptors, plasma GABA concentrations and blood-brain barrier permeability for GABA. Later, the concept of endogenous benzodiazepine ligands in hepatic encephalopathy was introduced as a result of several observations on the beneficial effect of the benzodiazepine antagonist flumazenil in experimental and clinical hepatic encephalopathy. Our negative findings in a controlled experimental study and a double-blind controlled clinical trial on flumazenil for hepatic encephalopathy do not support this concept.

Since the start of our double-blind clinical trial on the effect of flumazenil in hepatic encephalopathy, evidence has accumulated on the presence of "endogenous" benzodiazepine agonists in hepatic encephalopathy (1-3). After the detection of benzodiazepine-like compounds by radioreceptor assays (3-4), elevated concentrations of well-known benzodiazepines, including diazepam and N-desmethyldiazepam, have recently been demonstrated by HPLC and mass spectroscopy in brain extracts from rats

with hepatic encephalopathy (5-6). Measurements in cerebrospinal fluid from humans with hepatic encephalopathy also indicated elevated concentrations of N-desmethyldiazepam (6). Diazepam and its metabolite N-desmethyldiazepam have indeed been identified as naturally occurring compounds in animals and humans (7-10). Therefore, the relevant question now is, what is the significance of naturally occurring benzodiazepines in the pathogenesis of hepatic encephalopathy and how can we explain our negative findings?

Table 1: Benzodiazepine activities in human and animal body fluids.

	DZP (ng/ml)	DM-DZP (ng/ml)	DZP-E (ng/ml)	author, ref nr
human serum/plasma				
HE			3-12	Olasmaa, 6
10 mg DZP i.m., peak level	±140	±40		Kanto, 13
after 3 hrs	±	±80		
controls			<1.41	Olasmaa, 6
			0.3-3	Wildmann, 7
human CSF				
HE	±1.4*	±0.5*	0.9-14	Olasmaa, 6
			±54	Mullen, 4
10 mg DZP i.m., peak level	±4			Kanto, 13
after 3hrs		±2		
controls	±1.8*	±0.05*	<0.8	Olasmaa, 6
			±11	Mullen, 4
rat brain				
HE	11-26	7-9	60-85	Basile, 5
	±21*	±9*		Olasmaa, 6
5 mg/kg DZP i.p., after 15min	±860			Mennini, 14
1.3 mg/kg DZP i.p., after 15 min	±170	±50		Mennini, 14
controls	2-4	1-3	±15	Basile, 5
	±1*	±5*	±26	Olasmaa, 6
	2-5	2-5	10-20	Wildmann, 8

DZP diazepam; DM-DZP N-desmethyldiazepam; DZP-E diazepam-equivalents as measured by radio-receptor assay; *: diazepam and N-desmethyldiazepam were not measured directly but by a radio-receptor assay of a particular peak yielded by HPLC with retention times similar to diazepam and N-desmethyldiazepam, respectively.

Our study has been criticized because we could have excluded potential responders from the clinical flumazenil trial by including a benzodiazepine screening assay in the selection procedure (4). The precipitation of hepatic encephalopathy by synthetic

benzodiazepines is however rather common (11). Twelve of the 15 patients excluded from our controlled trial because of benzodiazepine use had already been identified by history and the study of medical reports; only one patient was excluded on the basis of a positive screening test only. Moreover, flumazenil did not affect the grade 2 encephalopathy when the patient participated in the open part of the study. Furthermore, the three responders in the open study had indeed previously been treated with benzodiazepines. Our negative findings in the clinical study cannot therefore be explained simply by wrong exclusion criteria. Furthermore, a French controlled study on flumazenil for hepatic encephalopathy, recently reported in preliminary form, also did not demonstrate an effect of flumazenil (12).

Although benzodiazepine ligands indeed seem to be elevated in plasma, CSF and brain tissue from humans and animals with hepatic encephalopathy, hypnotic doses of synthetic benzodiazepines induce considerably higher tissue levels (table 1) (13-14). Furthermore, the presence of compounds with effects opposite to those of the classic benzodiazepines, the so-called inverse agonists, must also be considered. Two of these agents, DBI (diazepam-bound inhibitor) and ODN (octadecaneuropeptide), have been found to be elevated in experimental and human hepatic encephalopathy (15-16). The balance between these opposing agents may be more important than the absolute cerebral levels of the benzodiazepines alone. On the basis of the results of our study with a benzodiazepine antagonist, which directly interfere at the receptor level, we cannot support the concept of endogenous benzodiazepines in hepatic encephalopathy. Nevertheless, we studied a small group of patients and larger controlled studies are needed for definitive conclusions.

The "new therapies" proposed for hepatic encephalopathy thus have not changed our approach to clinical hepatology. Therapies aimed at reducing the plasma ammonia concentration still seem to be the most appropriate but are insufficient for patients with chronic portosystemic encephalopathy and encephalopathy due to a markedly reduced liver cell function. What makes the treatment of hepatic encephalopathy so difficult? Two considerations seem worthwhile:

1. ammonia is a major toxin but current therapies are unable to achieve a sufficient reduction in level,
2. liver failure induces accompanying complications.

Currently, ammonia is again considered to be a major toxin responsible for the induction of hepatic encephalopathy (17). Although near normal plasma ammonia levels have been reported in severe encephalopathy, a close correlation between ammonia and encephalopathy was found in a group of patients studied immediately after hospitalization and before the institution of therapy (18). Since ammonia in plasma has a half-life expressed in seconds (19), plasma levels may not reflect the induced metabolic or physiological alterations with a longer half-life. Indeed, glutamine levels in CSF, reflecting ammonia detoxification in the central nervous system, exhibit a highly significant correlation with the depth of hepatic encephalopathy (20). Furthermore, since the detoxification system for ammonia surrounding cerebral capillaries is already nearly saturated at physiological ammonia concentrations, just a small increase may be sufficient to induce pathological ammonia levels in the central nervous system (21-22). The mechanisms of ammonia toxicity in the brain may be altered glutamatergic neurotransmission, possibly glutamate neurotoxicity, (23) or inhibition of GABA-induced hyperpolarization of the neuronal membrane (24). Once an understanding of the underlying mechanism of ammonia toxicity has been achieved, new drugs that interfere directly at the neuronal level can be developed. Like branched chain amino acids, L-Dopa and flumazenil, these drugs will be the ultimate test of the relevance of current concepts on the role of ammonia in the pathogenesis of hepatic encephalopathy.

Complications often associated with severe liver disease may also explain problems encountered in the treatment of hepatic encephalopathy. Our case-control study was performed to identify factors that might contribute to the encephalopathy. The case report on zinc deficiency highlighted the importance of screening for modulating factors, especially deficiencies. Treatment of zinc deficiency may be especially important if the current hypothesis on ammonia-induced glutamate neurotoxicity is confirmed, since zinc seems to block the binding of glutamate to its receptor, thereby diminishing a possible neurotoxic effect (25). Univariate analysis of the data from our case-control study indicated that hepatic encephalopathy was indeed associated with the failure of multiple organs. Severe complications such as cerebral hemorrhage (26) and hyponatriemia (27) have been reported in the literature but, because of their low prevalence, cannot be detected in a case-control study. The specific correlations, determined by logistic regression analysis, between encephalopathy and alcohol (or

granulocytes), ascites and 3,3',5-triiodothyronine (T3) have to be further investigated. In the event of a role for endotoxins and lymphokines in the precipitation of encephalopathy, as suggested by the correlation with alcohol and granulocytes, future studies should investigate the effect of antibodies against endotoxins and lymphokines. Should the relation between T3 and encephalopathy be causal, substitution therapy could be tried.

On the basis of these considerations with regard to the pathogenesis and treatment of hepatic encephalopathy, I propose a strategy for the diagnosis and treatment of patients with hepatic encephalopathy in clinical practice:

1. Non-metabolic neurological disorders have to be excluded by thorough neurological examination and further tests, when indicated.
2. Laboratory screening should exclude electrolyte abnormalities, oxygen deficiency, acid-base disturbances and disorders of glucose metabolism.
3. Flumazenil will antagonize benzodiazepines taken recently-whether known or unknown.
4. Dietary protein restriction, lactulose and/or neomycin will lower plasma ammonia levels and is preferred over newer therapies.
5. Precipitating causes of hepatic encephalpathy (GASCAPAPI: gastro-intestinal hemorrhage, azotemia..., infection) should be treated.
6. When chronic portosystemic encephalopathy does not react to conventional therapy, zinc and bromocriptine may be tried.

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Chapter 11

SUMMARY
SAMENVATTING

SUMMARY

The pathogenesis of hepatic encephalopathy is still unsolved. Therapy, therefore, is often insufficient. For the development of effective, new therapies insight into the disease-inducing substrates and the mechanisms of its toxic actions in the central nervous system are required. For both studies on pathogenesis and therapy of hepatic encephalopathy, methods for the quantitation of its severity are needed.

For the measurement of hepatic encephalopathy clinical grading, conventional electroencephalography and psychometric tests are mostly used. The former two methods are characterized by considerable intra- and interobserver error; the last one can only be used in mild encephalopathy. Quantitative EEG analysis has been shown to be usefull in longitudinal studies but its value for the classification of the whole spectrum of severities of encephalopathy had not been investigated.

In studies on the pathogenesis of hepatic encephalopathy ammonia was considered as a major toxin from the beginning. However, its importance was rejected by many hepatologists because the correlation between plasma concentrations and encephalopathy is poor. New theories, especially the false neurotransmitter theory and the GABA-benzodiazepine receptor hypothesis, tried to explain cerebral dysfunction directly at the neuronal level. The GABA-benzodiazepine receptor theory as originally proposed by Schäfer and Jones resulted in the use of flumazenil, a benzodiazepine receptor antagonist, in hepatic encephalopathy. Uncontrolled clinical studies seemed to indicate remarkable therapeutic activity and therefore supported the presence of endogenous benzodiazepines.

Besides specific toxins little attention has been given to the role of nonspecific metabolic factors, often present in liver failure, in the precipitation of encephalopathy in liver disease.

With regard to these considerations the aims of this thesis were: 1. the development of an objective method, spectral analysis, for the measurement of hepatic encephalopathy; 2. to study the effect of flumazenil in hepatic encephalopathy; and 3. to study the role of aspecific factors in the induction of hepatic encephalopathy.

In chapter 2 we evaluated the use of automated EEG analysis to quantitate the depth of hepatic encephalopathy objectively in a group of 66 patients with cirrhosis of the liver and 51 controls. The mean dominant frequency (MDF) could be used to discriminate

between the grades 0-1 ($\geq$6.4 Hz) and 2-4 ($<$6.4) hepatic encephalopathy. Further identification of grade 1 hepatic encephalopathy was possible by the use of the relative power of theta activity ($\geq$35%), whereas a relative delta activity $\geq$70% seemed to identify the grades 3 and 4 encephalopathy. A separate study revealed the value of the total power of the EEG for the discrimination between the grades 3 and 4 encephalopathy ($\geq$250 μV^2 vs $<$250 μV^2 for grade 3 and 4 respectively). Validation of the clinical value of objectively grading hepatic encephalopathy was achieved in the study described in chapter 3. A highly significant correlation was found between the grade of hepatic encephalopathy as measured by spectral analysis of the EEG and survival in a group of 60 patients with cirrhosis of the liver and 10 patients with acute hepatic failure.
In recent years several modality evoked potentials have been advocated for the detection of hepatic encephalopathy. According to chapter 4, no direct evidence to support their use over quantitative EEG analysis is yet available.

The effect of the benzodiazepine antagonist flumazenil on hepatic encephalopathy was studied in a rabbit model of acute ischemic liver failure (chapter 5) and a controlled double-blind clinical trial (chapter 6). In the animal experiment flumazenil or the placebo was administered at two easily recognizable time points in the course of the development of hepatic encephalopathy in two groups of 8 rabbits respectively. The response of flumazenil did not differ from that of the placebo as measured by clinical evaluation of the severity of the encephalopathy, spectral analysis of the EEG and survival analysis.

In the clinical study eighteen patients with hepatic encephalopathy resistant to conventional therapy were studied for the effect of flumazenil and the placebo following a cross-over design. Great care was taken not to include patients who had used synthetic benzodiazepines by using a plasma screening test. Cross-over analysis for the immediate effect of 1.0 mg of flumazenil could be performed in 16 patients. There was a trend toward a decrease in the clinical grade of hepatic encephalopathy 15 minutes after the injection of flumazenil ($p=0.06$), but neither conventional EEG-grading nor spectral analysis changed significantly. In 8 patients cross-over analysis could be performed for the effect during a 3-day infusion period (0.25 mg/hr). No significant difference between flumazenil and the placebo was found for the clinical

grade, the EEG grade and the MDF. In a separate open study 13 patients who were excluded from the double-blind study largely because of renal failure or evidence for recent benzodiazepine use were studied for the immediate effect of 1.0 mg of flumazenil. The MDF increased significantly in the whole group of patients but clinical and/or EEG grades improved only in the patients who had used benzodiazepines.

Pharmacokinetics of flumazenil as studied in four healthy volunteers and 7 patients with acute or chronic liver disease were reported in chapter 7. Elimination half-lives in liver disease (2.0-6.6 hr) were 2-6 times the values found for controls (0.85-1.2 hr); the plasma clearance rates were decreased 3-5 times (120-320 ml/min and 700-850 ml/min for patients and controls, respectively). This study was important for the evaluation of the duration of the wash-out period in the clinical cross-over study on flumazenil. Since the duration of the wash-out period, one day, was about four times the longest half-live time, this duration seems appropriate.

Chapter 8 reports on a study using logistic regression analysis to find which metabolic disturbances often found in severe liver disease could be of importance in hepatic encephalopathy. Twenty-six cases with encephalopathy and chronic or acute liver disease together with 26 cases with liver disease but without encephalopathy were selected. Univariate analysis indicated that the group of patients with encephalopathy indeed differed from those without encephalopathy with respect to several variables representing liver function (liver cell protein synthesis, detoxification function and liver cell damage), infection and endocrinologic function and levels of zinc, glucose and vitamins. Furthermore, patients with encephalopathy had more severe ascites and consumed more alcohol. Using logistic regression analysis heavy drinking, granulocytosis, severe ascites and low serum levels of T3 and zink were selected as highly predictive for the presence of hepatic encephalopathy in the patients studied. Hypotheses on the relation of these variables to encephalopathy are described.

The importance of zinc deficiency in clinical hepatic encephalopathy is further strenghtened by the case report described in chapter 9. We report on a patient with chronic recurrent hepatic encephalopathy resistant to protein restriction and treatment with lactulose and neomycin. During substitution therapy for possible zinc deficiency the patient improved subjectively but deteriorated after stopping zinc acetate. After a

new substitution period, zinc deficiency was artificially induced by L-histidine induced urine zinc loss. Severe stupor occurred, the episode being similar to previous ones. Chronic substitution therapy followed, without recurrence of severe deterioration of consciousness.

In chapter 10 a discussion on the findings in this thesis is given. The findings do not support a beneficial effect of flumazenil on hepatic encephalopathy and therefore cannot support the clinical significance of endogenously occurring benzodiazepines in the pathogenesis of hepatic encephalopathy. Renewed interest in the role of ammonia in the pathogenesis of hepatic encephalopathy is found. Other factors as endotoxins and zinc deficiency may contribute to the occurrence of encephalopathy. Direct interventions at the neuronal level as described for the GABA-benzodiazepine receptor complex have to be developed and tested, especially for ammonia-induced alterations in the central nervous sytem.

In summary, quantitative EEG analysis has been introduced and validated as an objective method to measure hepatic encephalopathy. Clinical and experimental studies could not support an effect of the benzodiazepine antagonist flumazenil for hepatic encephalopathy, and therefore question the role of endogenous benzodiazepines in its pathogenesis. New hypotheses were developed following the idea that aspecific factors might be important.

SAMENVATTING

Omdat de pathogenese van hepatische encephalopathie nog steeds onduidelijk is, heeft behandeling vaak onvoldoende effect. Voor de ontwikkeling van nieuwe, effectieve therapieën, is inzicht in het ziekte-inducerend substraat met zijn werkingsmechanismen in het centrale zenuwstelsel van belang. Voor zowel studies naar pathogenese als naar therapie, zijn methoden nodig om de ernst van hepatische encephalopathie te quantificeren.

Voor het meten van hepatische encephalopathie wordt meestal gebruik gemaakt van klinische gradering, conventionele electroencephalografie en psychometrische testen. De eerste twee methoden worden gekenmerkt door behoorlijke intra- en interobserver error; de laatste kan alleen voor milde encephalopathie worden gebruikt. In longitudinale studies is quantitatieve EEG analyze waardevol gebleken, maar zijn waarde voor de classificatie van het hele spectrum van gradaties was nog niet bestudeerd voor de start van deze studie.

In studies naar de pathogenese van hepatische encephalopathie is ammoniak van begin af aan beschouwd als een belangrijk toxine. Dit idee werd echter verworpen door verscheidene auteurs vanwege een slechte correlatie tussen plasma concentraties en encephalopathie. Nieuwe theorieën, speciaal de false neurotransmitter theorie en de GABA-benzodiazepine hypothese, probeerden de slechte cerebrale functie direct op neuronaal niveau te verklaren. De GABA-benzodiazepine receptor theorie, zoals aanvankelijk gepostuleerd door Schäfer en Jones, leidde tot het gebruik van flumazenil, een benzodiazepine receptor antagonist, in hepatische encephalopathie. Niet-gecontroleerde studies leken op het bestaan van endogene benzodiazepines te wijzen, met belangrijke therapeutische consequenties.

Behalve voor specifieke toxines is er altijd weinig aandacht geweest voor een rol van aspecifieke metabole verstoringen, die vaak aanwezig zijn bij leverfalen, in het ontstaan van encephalopathie bij leverziekte.

Uitgaande van deze overwegingen was het doel van dit proefschrift: 1. de ontwikkeling van een objectieve methode, spectraal analyse, voor het meten van hepatische encephalopathie; 2. bestudering van het effect van flumazenil op de hepatische encephalopathie; en 3. bestudering van de rol van aspecifieke factoren in het ontstaan van hepatische encephalopathie.

Hoofdstuk 2 beschrijft een studie naar het objectief meten van hepatische encephalopathie gebruik makend van geautomatiseerde EEG analyse in een groep van 66 patienten met levercirrose en 51 controles. De graden 0-1 en 2-4 hepatische encephalopathie konden van elkaar worden onderscheiden met behulp van de mean dominant frequency (MDF) ($\geq$6,4 Hz voor graad 0-1 en $<$6,4 Hz voor graad 2-4 HE). Graad 1 hepatische encephalopatie werd verder gekenmerkt door een verhoogd percentage theta activiteit in het power spectrum ($\geq$35 %), terwijl een relatieve delta activiteit $\geq$70% gevonden werd bij de graden 3 en 4 encephalopathie. Een aparte studie toonde aan, dat een verder onderscheid tussen graad 3 en 4 mogelijk was met behulp van de totale power van het EEG: $\geq$250 μV^2 voor graad 3 en $<$250 μV^2 voor graad 4). Wij onderzochten de klinische waarde van het objectief meten van hepatische encephalopathie in de studie beschreven in hoofdstuk 3. Wij vonden een sterk significante correlatie tussen de graad van hepatische encephalopathie, zoals gemeten met spectraal analyze, en de overleving in een groep bestaande uit 60 patienten met levercirrose en 10 patienten met acuut leverfalen.
Sinds enkele jaren worden evoked potentials (BAEP's, VEP's en SSEP's) gepropageerd om hepatische encephalopathie te detecteren en te quantificeren. Zoals aangegeven in hoofdstuk 4, bestaan er vooralsnog geen directe bewijzen voor hun superioriteit boven spectraal analyze.

Het effect op de hepatische encephalopathie van de benzodiazepine-antagonist flumazenil werd bestudeerd in een konijnen model voor acuut ischemisch leverfalen (hoofdstuk 5) en een gecontroleerde dubbelblinde klinische trial (hoofdstuk 6). In de dier-experimentele studie werden flumazenil en placebo toegediend aan twee groepen van 8 konijnen; het middel werd op 2 goed herkenbare punten in het beloop van de encephalopathie gegeven. Het effect van flumazenil verschilde niet van dat van de placebo wat betreft de klinische beoordeling van de ernst van de encephalopathie, spectraal analyze van het EEG en de overleving.

In de klinische studie werden 18 patienten met hepatische encephalopathie die onvoldoende reageerde op conventionele therapie behandeld met flumazenil en placebo volgens een cross-over studie-design. Om patienten met recent gebruik van synthetische benzodiazepinen uit sluiten van de studie, werd een analyze van het plasma hierop verricht. Cross-over analyze voor de beoordeling van het directe effect,

15 minuten na toediening van 1,0 mg flumazenil, was mogelijk voor 16 patienten. Deze toonde een trend van verbetering na flumazenil wat betreft de klinische graad van encephalopathie ($p=0.06$), maar conventionele EEG gradering en spectraal analyze toonden geen significante veranderingen. Voor 8 patienten was cross-over analyze mogelijk wat betreft het effect van flumazenil tijdens een 3 dagen durende infusie van 0,25 mg/uur. Wij vonden geen significante verschillen tussen flumazenil en placebo wat betreft klinische graad, EEG graad en de MDF. Dertien andere patienten die waren uitgesloten van de dubbel-blinde studie, voornamelijk wegens nierfalen en recent benzodiazepine gebruik, werden opgenomen in een open studie naar het directe effect van 1,0 mg flumazenil. De MDF steeg significant voor de gehele groep patienten, maar de klinische en EEG graad van encephalopathie verbeterden alleen voor de patienten die recent benzodiazepinen gebruikt hadden.

De farmacokinetiek van flumazenil, zoals bestudeerd in 4 gezonde vrijwilligers en 7 patienten met acute en chronische leverziekte is beschreven in hoofdstuk 7. De eliminatie halfwaarde tijd was 2-6 keer verlengd in de leverpatienten (2,0-6,6 uur) ten opzichte van de controles (0,85-1,2 uur); de plasma klaring was 3-5 keer verlaagd (120-320 ml/min en 700-850 ml/min voor patienten, respectievelijk controles). Deze studie was belangrijk voor de beoordeling van de wash-out periode in de klinische flumazenil cross-over studie. Omdat de duur van de wash-out periode, 1 dag, ongeveer vier maal zo lang was als de grootste halfwaarde tijd, lijkt deze duur adequaat.

In hoofdstuk 8 wordt een onderzoek beschreven waarin logistische regressie analyze toegepast werd om frequent voorkomende, metabole ontregelingen bij patienten met een ernstige leverziekte te correleren aan de aanwezigheid van encephalopathie. Zesentwintig patienten met encephalopathie en chronische of acute leverziekte en 26 patienten met leverziekte zonder encephalopathie werden geselecteerd. Univariate analyze toonde aan, dat de 2 groepen patienten van elkaar verschilden met betrekking tot velerlei variabelen betreffende leverfunctie (eiwitsynthese door de lever, ontgiftiging en levercelverval), infectie en endocriene functie, en plasma/serum concentraties van zink, glucose en vitamines. Patienten met encephalopathie hadden tevens ascites in ernstiger mate en zij gebruikten meer alcohol. Met behulp van logistische regressie analyse werden de volgende factoren geselecteerd als meest voorspellend voor de aanwezigheid van encephalopathie: het gebruik van veel alcohol, granulocytose,

ernstige ascites en lage serum concentraties van zink en T3. In het hoofdstuk worden hypothesen genoemd met betrekking tot de relatie tussen deze factoren en encephalopathie.

Het belang van zink deficientie bij de patient met hepatische encephalopathie wordt verder gesteund door de case report in hoofdstuk 9. Wij beschrijven een patient met chronisch recidiverende hepatische encephalopathie ondanks eiwitbeperking en behandeling met lactulose en neomycine. Tijdens zinksubstitutie op verdenking van zinkdeficientie trad een subjectieve verbetering op, maar de patient verslechterde opnieuw na staken hiervan. Na een nieuwe substitutie periode werd zinkdeficientie gecontroleerd geinduceerd met behulp van L-histidine, wat leidde tot zinkverlies met de urine. Er ontstond een stupor die vergelijkbaar was met eerder doorgemaakte episoden van encephalopathie. Tijdens onderhoudsbehandeling met zinksubstitutie deden zich soortgelijke episoden niet meer voor.

Hoofdstuk 10 bespreekt de conclusies van dit proefschrift. De resultaten geven geen steun voor een therapeutisch effect van flumazenil op hepatische encephalopathie en daarom ook niet voor de klinische relevantie van endogene benzodiazepinen in de pathogenese van hepatische encephalopathie. Opnieuw is interesse ontstaan naar de rol van ammoniak in de pathogenese. Andere factoren zoals endotoxinen en zinkdeficientie kunnen mogelijk een rol spelen in het ontstaan van encephalopathie. Directe interventies op neuronaal niveau, zoals beschreven voor het GABA-benzodiazepine receptor complex, moeten ontwikkeld en getest worden, speciaal met betrekking tot ammoniak-geinduceerde veranderingen in het centrale zenuwstelsel.

Samenvattend is quantitatieve EEG analyse geintroduceerd en gevalideerd als objectieve meetmethode bij hepatische encephalopathie. Klinische en dierexperimentele studies gaven geen steun voor een effect van de benzodiazepine-antagonist flumazenil op hepatische encephalopathie, en daarom ook niet voor een rol van endogene benzodiazepinen in de pathogenese. Naar aanleiding van de hypothese dat aspecifieke factoren van belang zouden zijn, werden nieuwe ideeën ontwikkeld.

DANKWOORD

Het doen van wetenschappelijk onderzoek en speciaal het werken aan een proefschrift lukt niet zonder medewerking van vele mensen. Naast een morele steun, met name van naaste familieleden, is er vooral ook veel praktische hulp nodig.
Mijn promotor Solko Schalm wil ik bedanken voor het vertrouwen wat hij in mij stelde ook in tijden dat de uitvoering van het onderzoek moeizaam verliep. Steeds wist hij mij opnieuw te motiveren en, door het plaatsen van kritische opmerkingen, te prikkelen tot het ontwikkelen van nieuwe ideeën. De samenwerking met Rob de Knegt was behalve gezellig, ook wetenschappelijk gezien heel vruchtbaar door vele discussies. Daarnaast was hij bereid series ammoniakbepalingen te verrichten voor het welslagen van de experimentele studies.

Met name de dierexperimenten verliepen lange tijd moeizaam, maar konden na suggesties van Prof. Terpstra en Dr. Groenland bevredigend worden afgerond. Het personeel van het chirurgisch laboratorium, en heel speciaal Enno Collij, heeft daar met veel doorzettingsvermogen aan bijgedragen. De mensen van het centraal proefdierenbedrijf dank ik voor hun hulp aan een recent experiment.

Het klinische onderzoek had natuurlijk niet uitgevoerd kunnen worden zonder de bereidheid van de patienten hieraan deel te willen nemen. Mirjam Roesdi heeft mij op de meest vreemde tijdstippen geholpen met de uitvoering van de klinische trial. Ciska Verploeg was steeds enthousiast bloedafnames te verzorgen en schuwde daarbij niet naar andere afdelingen te gaan. De laboranten van de afdeling klinische neurophysiologie hielpen zo ver mogelijk met het aanbrengen van EEG electroden, terwijl de technici een mobiele computeropstelling ontworpen en altijd insprongen bij technische problemen. De HPLC analyzes in plasma naar benzodiazepines werden allen verricht door Ruben Klepperts. In de case-control studie heb ik veel hulp ontvangen van Gosse de Jong, die alle patienten neurologisch onderzocht om eventuele focaal neurologische afwijkingen op te sporen of uit te sluiten. De proefpersonen in de flumazenil farmacokinetiek studie dank ik voor hun medewerking.

Het grootste deel van mijn tijd tijdens het onderzoek heb ik doorgebracht op het laboratorium interne geneeskunde II. Hier kon ik opnieuw moed putten na een mislukt

experiment door de steun die ik dan van velen kreeg. Deze kreeg ik tevens op het secretariaat van de hepatologie. Jan Boot gaf mij waardevolle adviezen wanneer ik problemen had met het gebruik van de computer. Willeke Beukman en Marianne van Noord bewerkten mijn manuscripten en hielpen met de voorbereidingen voor dit proefschrift. Mijn beheersing van de Engelse taal werd verbeterd door de lessen die ik kreeg van Hansje v.d. Grient, terwijl mevr. Bieger de wetenschappelijke manuscripten corrigeerde.

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CURRICULUM VITAE

De auteur van dit proefschrift werd geboren op 22 januari 1960 te Roosendaal en Nispen. Zij volgde het Atheneum-B aan het Sint Gertrudis Lyceum in Roosendaal en behaalde het diploma in 1978. Van 1978 tot 1979 was zij werkzaam als leerling operatie-assistente in het Diakonessenhuis Refaja te Dordrecht. Vanaf 1979 tot aan het behalen van het artsdiploma in maart 1986 studeerde zij geneeskunde aan de Erasmus Universiteit te Rotterdam. Tijdens haar studie was zij gedurende één jaar, 1980-1981, werkzaam als verpleeghulp in een studententeam en werkte zij sinds 1982 als student-assistent op de afdeling Inwendige Geneeskunde II (hoofd Prof. J.H.P. Wilson) van het Academisch Ziekenhuis Dijkzigt Rotterdam. Onder leiding van Prof. S.W. Schalm werd toen een begin gemaakt aan haar onderzoek op het gebied van de hepatische encephalopathie, wat zij vervolgens na haar artsexamen, van april 1976 tot aan juli 1979 op dezelfde afdeling uitvoerde. Het proefschrift is gebaseerd op dit onderzoek. Sinds juli 1979 volgt zij de opleiding tot internist in het Franciscus Gasthuis te Rotterdam (opleider Dr. H.S.L.M. Tjen).