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Association of glutamine synthetase polymorphisms rs2296521, rs10911021 and rs12136955 with plasma ammonia concentration in valproic acid-treated Egyptian epilepsy patients

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ABSTRACT

Introduction. The use of valproic acid (VPA) in the treatment of some psychiatric and neurological disorders such as bipolar disorder, migraines, and epilepsy is associated with hyperammonemia. However, the mechanism of this negative effect of VPA is unclear. In this study, we investigate gene *glutamate-ammonia ligase* (GLUL) polymorphisms for the glutamine synthetase (GS) enzyme, a key enzyme that catalyzes the removal of ammonia by incorporating it with glutamate to form glutamine, and we investigate whether it has a relationship with the emergence of hyperammonemia during VPA-based therapy.

Patients and methods. We enrolled 180 Egyptian epilepsy patients in this study. Patient history, general and neurological examination and blood samples from arm veins were taken. Real time TaqMan PCR polymorphism for three polymorphism SNPs (rs2296521, rs10911021 and rs12136955) of GLUL was done. We assessed the relationship between the patient features, including three GLUL polymorphisms, and the development of hyperammonemia during VPA-based therapy.

Results. We found that the ammonia levels showed a positive correlation with VPA treatment duration ($p = 0.015$) and a negative correlation with carbamazepine total dose per day ($p = 0.027$) and with WBCs count ($p = 0.026$). Also, female patients having rs2296521 SNPs with the A allele and patients having rs10911021 SNPs with the C allele were at high risk for elevated plasma ammonia levels. Moreover, patients having rs12136955 SNPs with the A allele or associated hypertension as a co-morbidity were at high risk for elevated plasma ammonia levels.

Conclusions. Female patients who have rs2296521 with the A allele, rs10911021 with the C allele, or rs12136955 with the A allele, are independent risk factors for elevated plasma ammonia levels during VPA-based therapy. Moreover, carbamazepine combined therapy may protect against the development of hyperammonemia in VPA-treated patients.

Keywords: epilepsy, glutamine synthetase, hyperammonemia, polymorphism, valproic acid, carbamazepine

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Introduction

Valproic acid (VPA) is a commonly used medicine that is approved by the US Food and Drug Administration (FDA) to treat a wide range of neurological and psychiatric disorders [1]. As many as 36% of patients taking VPA develop asymptomatic elevation in plasma ammonia concentration without deterioration of liver function tests [2]. More importantly, during VPA toxicity, hyperammonemia occurs [3] in the absence of liver failure. Hyperammonemia is associated with several neurological symptoms if it exceeds 100 μmol/L [4], and can cause severe coma and respiratory failure leading to life-threatening conditions if it exceeds 110 μmol/L [5]. Ammonia is a critical component of glutamatergic neurotransmission in the brain through the glutamate-glutamine shuttle. Within glutamatergic neurons, the phosphate-activated glutaminase (PAG) enzyme breaks glutamine (Gln) into glutamate (Glu) and ammonia (NH4), while in astrocytes, glutamine synthase enzyme (GS) sequesters Glu and NH4 into Gln which is shuttled back to the neurons [6].

Brain ammonia levels are kept at extremely low levels (150–250 μM), demonstrating how effectively the GS enzyme typically eliminates brain ammonia [7]. Hence, GS activity is the primary factor in maintaining physiological concentrations of NH4 in the brain. The role of all other NH4 metabolizing enzymes is negligible. On the other hand, because of the GS enzyme's limited capacity and the brain's incapacity to use alternate ammonia-metabolising routes, acute reductions in GS activity carry a significant danger of momentarily elevating ammonia concentration to hazardous levels. Similarly, Abulseoud et al. [8] recently discovered that cannabis suppresses GS enzyme activity in the striatum and causes a robust increase in brain and plasma, but not liver ammonia concentration. Furthermore, we have demonstrated that chronic VPA administration is associated with significant suppression of liver and brain GS activity and a marked increase in plasma and tissue NH4 concentrations [9].

The liver, brain, digestive system, and peripheral lymphocytes all have high levels of GS expression [10] which decreases with phenytoin administration [11]. Additionally, polymorphisms in the GS gene (GLUL) have been discovered. It is plausible to hypothesize that VPA-induced hyperammonemia is related, at least in part, to suppression of GS activity. In support of this hypothesis, Inoue et al. have shown in a study of Japanese patients that carriers for a specific polymorphism in the GLUL gene (GLUL rs10797771) are more likely to have an elevation in plasma ammonia concentration during VPA-based therapy [12]. However, no study has examined the GLUL gene polymorphism among Egyptian patients. Moreover, other SNPs such as rs2296521, rs10911021, and rs12136955, that are present in the upstream region of GS gene and hence affect its enzymatic activity, have yet to be investigated.

Therefore, the current study aimed to investigate the effect of VPA on ammonia concentration in the plasma as well as the effect of GLUL polymorphisms rs2296521, rs10911021, and rs12136955 on plasma ammonia concentration.

Patients and methods

Patients

This was a cross-sectional cohort study of 180 patients (99 males and 81 females, aged 22–69) with epilepsy treated with VPA alone or in combination with other anti-seizure medications. This study was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines, and was approved by our local IRB committee, the Mansoura Faculty of Medicine (Code R.21.08.1408). Study patients were recruited at the Outpatient Clinic of the Department of Neurology, Mansoura University Hospital, Egypt between September 2021 and August 2022. Following a thorough explanation of the study's goals and procedures, each subject or their legal representative provided written informed consent.

Inclusion criteria

1. Males and females aged 21–70 years.
2. Able and willing to understand and sign informed consent.
3. Had a documented seizure disorder diagnosis for at least six months.
4. Had been prescribed valproic acid (VPA) or one of its derivatives either as monotherapy or in combination with other anti-seizure medications.
5. Dose of VPA did not change for at least four weeks before inclusion in the study.

Exclusion criteria

Patients with a) cognitive impairment or mental retardation as determined by WASI IQ full score of 80 or above, or b) acute or chronic unstable medical conditions other than seizure disorder, or c) medical conditions known to alter ammonia levels such as in-born errors of metabolism, or d) phenylketonuria or other urea cycle abnormalities or on a ketogenic diet, or e) known genetic disorders or autoimmune disorders, or f) non-adherence to therapy as determined by patient or family report, or VPA blood levels as judged by the treating physician's discretion, were excluded from the study.

Methods

Medical records were collected to extract the following data:

- Demographic data.
- Family and medical history including history of medical problems, seizure diagnosis and course of illness.
- Most recent physical examination, neurological examination, and laboratory results.

Study visits was arranged for participants where the following procedures were performed:

- Blood pressure measurement and full neurological examination.
- Collection of data about depression, anxiety and stress exposure using the State-Trait Anger Scale (depression anxiety stress scale (DASS)) which consists of 21 items (DASS-21).
- Data about medications doses, duration of use, and last dose was collected.
- Blood sample collection: 10 mL of blood was collected from antecubital vein via venipuncture and divided into: i) 4 mL of blood was stored in two red-top (containing EDTA) tubes, one tube for genetic polymorphism testing (stored at −80°C), and the second for CBC, and ii) 6 mL of blood was centrifuged and used for rapid assay of NH_3 and stored at −80°C for further analysis of VPA blood levels and liver function tests (ALT, AST, ALP, albumin and total proteins).

Assay methods for plasma ammonia, VPA level and liver functions

Measurement of plasma ammonia level was promptly performed using commercially available colorimetric kits (AM1040, Biodiagnostics, Giza, Egypt), liver enzymes including ALT, AST, ALP, and albumin (BIOMED, Diagnostics, Cairo, Egypt), and total plasma proteins using a spectrophotometer (Robonik Prietest automated biochemistry analyser system, India, Department of Medical Physiology, Mansoura Faculty of Medicine). The VPA concentration was determined using an IMMULITE autoanalyser (Siemens, Germany).

Polymorphism study

Samples collection and DNA extraction

Two mL of peripheral venous blood was withdrawn from all subjects in an ethylene di-amine tetra-acetic acid-containing test tube and stored at −20°C until the extraction of DNA. Genomic DNA was extracted from the blood samples using the Gene JET Genomic DNA Purification Kit (Thermo Fisher Scientific, USA) (Cat. no. K0721) according to the manufacturer's instructions. A NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific) was used to determine the DNA concentration and purity. The DNA concentration range was from 20 to 30 ng/μL, and all samples had an A260/A280 ratio between 1.8 and 2.0. All DNA samples were stored at −20°C for subsequent PCR reactions. All steps of polymorphism study were done in the Department of Medical Biochemistry, Mansoura Faculty of Medicine.

Genotyping of GLUL gene SNPs

The genotypes for the three GLUL gene SNPs (rs2296521 A/G, rs10911021 C/T, and rs12136955 A/G) were determined using allelic discrimination through TaqMan fluorescence probes-based real-time polymerase chain reaction (StepOne™ Real-Time PCR System, Applied Biosystems, USA) using the pre-designed TaqMan SNP Genotyping Assays: C_9693728_20, C_1797276_20, and C_27830023_20 respectively (Thermo Fisher Applied Biosystems, Foster City, CA, USA). The primer sequence of rs2296521 SNP is forward, reverse: CACAGGCCCCAGCTACCTCGCACTG[A/G], TAAGAGTATACTGCCTTTTAAAAAC (accession #NM_001033044.3), rs10911021 SNP is forward, reverse: AGAGCAATCTTCTGCAGCCTGTTCT[C/T], ATGTGAGGACCAGAAAAGTTTCCGC (accession #NM_001009992.1) and rs12136955SNPTGAAGTACAGTGGCAAAGTTTCTAC[A/G], GGCAACCGTCCATGTTTGCCCAGCT (accession #NM_001033044.3) For each of the three GLUL gene SNPs, a separate real-time PCR was carried out in a 48-well plate with a total volume of 25 μL. Initially, 12.5 μL of the 2 x TaqMan Universal Master Mix was placed in each well, and then 1.25 μL of the 20 x TaqMan SNP genotyping assay mix specific for each GLUL gene SNP was added. Next, a 5 μL DNA sample was diluted in 5.25 μL of nuclease-free water, added to the designated wells, and mixed gently. The PCR protocol used for DNA amplification and polymorphism detection was: I. holding stage (10 minutes at 95°C); II. cycling stage (40 cycles of 15 seconds of DNA denaturation at 95°C, and a one-minute extension step at 60°C); III. post-PCR read stage (one minute reading of final fluorescence at 60°C). For allelic discrimination, the StepOne software utility included in the StepOne™ Real-Time PCR system was used to collect and analyse the data, and assign allele calls for each sample based on the dye released.

Statistical analysis

Data was analysed using SPSS version 27. Normality of the data was tested using a one-sample Kolmogorov-Smirnov test. Qualitative data was described using numbers and percentages. Association between categorical variables was tested using the chi-square test and odds ratio [95% confidence interval (CI)]. If at least 25% of cells had expected counts below 5, the Fisher's exact test was used as an alternative. Continuous variables were presented as mean ± standard deviation (SD) for normally distributed data and median (Q1 to Q3) for non-normally distributed data. The association between numerical variables within two groups was tested using an independent t-test and mean differences (95% CI) for normally distributed data and a Mann-Whitney U test for non-normally distributed data. The relationship between numerical variables within more than two groups was tested using the Kruskal-Wallis H test for non-normally distributed data. Spearman's correlation test was performed between ammonia levels and other continuous variables. A two-step transformation of the ammonia level was

needed to perform the multiple linear regression test. The level of significance was considered at a p-value < 0.05.

Results

Patient characteristics and demographics and distribution of GLUL polymorphism

Our study involved 180 patients; 55% were male and 45% were female. They were 22–69 years old. 85% of them had a negative history of epilepsy and 15% had a positive family history. Also, 87.2% had no associated comorbidities and 90% of them had normal arterial blood pressure. 76.1% of the patients received double doses of VPA, 64.4% received 500 mg per time, and 67.22% of them had a duration of therapy of more than 10 years. The most frequent concomitant anti-seizure drug was levetiracetam (87.78%) followed by carbamazepine (28.89%), phenytoin (8.3%), topiramate (8.3%), lamotrigine (6.11%) and lastly gabapentin and zonisamide (0.6% each). Finally, the distributions of the three investigated SNPs (rs2296521; rs10911021; rs12136955) GLUL polymorphisms showed that the rs2296521 SNPs include 18.5% AA form, 74% AG form, and 7.5% GG form, while the rs10911021 SNPs include 46.5% CC form, 43.7% CT form, and 9.8% TT form. Also, the rs12136955 SNPs include 17.6% AA form, 50% AG form, and 32.4% GG form (Tab. 1).

Correlations between ammonia level and age, drug therapy, clinical, and laboratory parameters

A plasma level of ammonia above 200 µg/dL was reported in one case only, i.e. 0.5% of the patients. The ammonia levels did not show any significant correlations with the patient's age, clinical or lab parameters. However, the plasma ammonia level showed positive correlations with the therapy duration ($p = 0.015$), total carbamazepine dose per day ($p = 0.027$), and WBCs count ($p = 0.026$) and a negative correlation with the patient sex ($p = 0.005$) (Tab. 2). Also, the DASS score did not show any significant correlation with the plasma ammonia levels. However, we found that the patients with low doses of VPA (< 2,000 mg/day) had significantly higher DASS score (median (Q1-Q3) of DASS: 23.50 (14.75–35.0) than the patients with high doses of VPA (> 2,000 mg/day) (median (Q1-Q3) of DASS:15.0 (11.0-–23.50) ($p = 0.036$).

Association between ammonia level and sociodemographic data, clinical data and gene polymorphism

The ammonia level did not show any significant associations with the sociodemographic data of the patients including family history, comorbidities, and blood pressure. However, there was an association between male sex and high plasma ammonia. Male patients had a higher ammonia level (median male vs. female 35.0 vs. 29.2 µg/dL) ($p = 0.005$).

Table 1. Demographic data or patient characteristics and distribution of glutamine synthetase gene polymorphism SNPs (rs2296521; rs10911021; rs12136955)

Parameter	No. of patients (%)/ /mean ± SD
Age (years)	
Minimum – maximum	22–69 years
Mean ± SD	39.53 ± 9.99
Sex (male/female)	Male: 99 (55%); female: 81 (45%)
Family history	
Negative	153 (84.5%)
Positive	27 (14.5%)
Comorbidity	
No	157 (87.2%)
Hypertension	9 (5%)
DM	5 (2.8%)
Both (HTN and DM)	9 (5%)
ABP	
Normal	162 (90%)
Borderline hypertension (130/85 mmHg)	5 (2.8%)
Hypertension (140/90 mmHg)	13 (7.2%)
Drug therapy	
Total dose of VPA (mg per day)	
Mean ± SD	1,311.1 ± 383.51
Doses of VPA per day	
Single dose	5 (2.8%)
Twice per day	137 (76.1%)
Three doses per day	37 (20.6%)
Four doses per day	1 (0.6%)
Duration from last dose in hours	
Median (Q1-Q3)	4 (3–11)
Duration of treatment in years	
< 1	8 (4.5%)
1–10	51 (28.3%)
10	121 (67.2%)
Concomitant drugs with VPA	
Levetiracetam	158 (87.8%)
Carbamazepine	52 (28.9%)
Phenytoin sodium	15 (8.3%)
Topiramate	15 (8.3%)
Lamotrigine	11 (6.1%)
Gabapentin	1 (0.6%)
Zonisamide	1 (0.6%)
Distribution of GLUL SNPs	
rs2296521 (146 samples)	
AA	27 (18.5%)
AG	108 (74%)
GG	11 (7.5%)
Homozygous	38 (26%)
Heterozygous	108 (74%)
rs10911021 (144 samples)	
CC	67 (46.5%)
CT	63 (43.7%)
TT	14 (9.8%)
Homozygous	81 (56.3%)
Heterozygous	63 (43.7%)
rs12136955 (142 samples)	
AA	25 (17.6%)
AG	71 (50%)
GG	46 (32.4%)
Homozygous	71 (50%)
Heterozygous	71 (50%)

Table 2. Correlation between ammonia level and age, drug therapy, clinical, and laboratory parameters

		Ammonia level during valproic acid-based therapy (μg/dL)	
	Mean ± SD/ Median (Q1–Q3)	r	p-value
Age [years]	39.53 ± 9.99	0.088	0.272
Sex		–0.224	0.005
Family history		–0.038	0.677
Comorbidity		–0.129	0.106
ABP		–0.045	0.571
VPA therapy			
Dose [mg]/time	609.72 ± 181.48	–0.078	0.326
Total dose of VPA (mg) per day	1,311.1 ± 383.51	–0.114	0.114
Number of doses per day	2 (2–2)	–0.039	0.624
Hours since last dose	4 (3–11)	–0.130	0.106
Duration of VPA therapy in years	15 (8–24.25)	0.201	0.015
Valproic acid level (μg/mL)	49.4 (32.60–70.95)	–0.096	0.231
Concomitant drug therapy			
Levetiracetam total dose (mg/day)	1,593.5 ± 689.50	–0.119	0.275
Carbamazepine total dose (mg/day)	827.27 ± 281.49	–0.346	0.027
Phenytoin sodium total dose (mg/day)	264.28 ± 63.33	0.322	0.284
Topiramate total dose (mg/day)	100 (100–100)	2	NA
Lamotrigine total dose (mg/day)	100 (100–200)	0.162	0.419
Gabapentin total dose (mg/day)	300 (300–525)	–0.86	0.333
Zonisamide total dose (mg/day)	200 (200–200)	1.0	NA
DASS score	22 (14–33)	–0.119	0.135
RBCs count (millions/mm^3)	4.64 ± 0.55	–0.049	0.542
Hb level (g/dL)	12.87 ± 1.54	–0.120	0.132
WBCs count (thousands/mm^3)	6.56 ± 1.81	–0.177	0.026
PLT count (thousands/mm^3)	185.89 ± 56.04	0.006	0.940
AST (U/L)	18.07 (14.56–24.60)	–0.024	0.761
ALT (U/L)	10.59 (7.53–14.32)	0.086	0.284
ALP (U/L)	85.36 ± 24.80	0.060	0.454
Albumin (g/dL)	4.57 ± 0.43	0.078	0.333
Total plasma protein (g/dL)	7.04 ± 0.54	0.036	0.656

Pearson's correlation for parametric data and Spearman's correlation for non-parametric data

Moreover, there was no statistically significant difference in plasma ammonia level between the low dose of VPA group (< 2,000 mg/day) (median of ammonia: 33.05 μg/dL) and the high dose of VPA group (> 2,000 mg/day) (mean of ammonia: 34.0 μg/dL) (p = 0.645). Also, there was no statistically significant association between ammonia levels and the different studied SNPs in the current study or with patients having specific alleles in the different studied SNPs (Tab. 3). Moreover, a multivariate logistic regression analysis performed utilizing the stepwise method was applied to sex, WBCs, therapy duration and carbamazepine total dose. This demonstrated that carbamazepine total dose significantly reduced the risk of developing high plasma ammonia levels during VPA-based therapy (p = 0.020) (Tab. 4).

Association between SNPs and sociodemographic data, clinical data and lab data

Female patients having rs2296521 SNPs with the A allele (95% confidence interval 2.014 (0.601 to 6.754), with low Hb levels in the blood (p = 0.034), ALT (p = 0.047) and receiving lamotrigine (p = 0.027) were at high risk for elevated plasma ammonia levels (Tab. 5). On the other hand, female patients having rs10911021 SNPs with the C allele (95% confidence interval 1.534 (0.940 to 2.503), receiving lamotrigine (p = 0.041) and with low VPA plasma levels (p = 0.009) (95% CI 18.163 (4.575 to 31.752) were at high risk of elevated plasma ammonia levels (Tab. 6). Moreover, patients

Table 3. Association between ammonia level and sociodemographic, clinical data and genotypes

	Ammonia level during valproic acid-based therapy (ug/dL)		
	Median (Q1 to Q3)	Test of significance	p-value
Sex			
Male (n = 79)	35.0 (28.0–48.9)	Z = 2.811	p = 0.005
Female (n = 59)	29.2 (22.0–44.0)		
Family history			
No (n = 121)	33.2 (27.4–48.4)	Z = 0.419	p = 0.675
Yes (n = 17)	32.3 (25.3–44.9)		
Comorbidities			
None (n = 117)	33.2 (27.5–48.6)	ZH = 2.657	p = 0.448
Hypertension (n = 9)	28.0 (24.5–43.0)		
DM (n = 5)	28.0 (22.2–42.3)		
Both (n = 7)	31.0 (20.0–47.2)		
Blood pressure categories			
Normal (n = 122)	33.1 (27.5–48.0)	ZH = 0.528	p = 0.768
Borderline (n = 3)	40.1 (23.8–47.7)		
Hypertension (n = 13)	28.0 (24.5–50.0)		
VPA dose (low vs. high dose)			
Low (< 2,000 mg/day) (n = 159)	33.05 (27.348.1)	Z = –0.460	p = 0.645
High (> 2,000 mg/day) (n = 21)	34.0 (26.3–42.0)		
GLUL genotyping			
rs2296521			
AA (n = 27)	32.6 (25.6–42.4)	ZH = 1.040	p = 0.594
AG (n = 108)	34.2 (27.5–48.7)		
GG (n = 11)	30.0 (19.0–48.0)		
rs2296521H			
Homozygous (n = 38)	31.9 (25.3–42.8)	Z = 0.988	p = 0.323
Heterozygous (n = 108)	34.2 (27.5–48.7)		
rs2296521			
Having A allele (n = 135)	33.2 (27.3–47.8)	Z = 0.697	p = 0.4860
Not having A allele (n = 11)	30.0 (19.0–48.0)		
rs10911021			
CC (n = 67)	35.0 (27.3–47.0)	ZH = 0.792	p = 0.793
CT (n = 63)	33.1 (27.0–48.0)		
TT (n = 14)	30.8 (27.5–54.5)		
rs10911021H			
Homozygous (n = 81)	34.4 (27.5–48.0)	Z = 0.681	p = 0.496
Heterozygous (n = 63)	33.2 (27.0–47.8)		
rs10911021			
Having C allele (n = 130)	33.6 (27.2–47.9)	Z = 0.165	p = 0.869
Not having C allele (n = 14)	30.8 (27.5–54.5)		
rs12136955			
AA (n = 25)	40.4 (30.1–49.0)	ZH = 3.337	p = 0.189
AG (n = 71)	33.2 (27.0–47.8)		
GG (n = 46)	30.4 (26.6–48.3)		
rs 12136955 H			
Homozygous (n = 71)	34.4 (27.5–48.0)	Z = –0.241	p = 0.810
Heterozygous (n = 71)	33.2 (27.0–47.8)		
rs 12136955			
Having A allele (n = 96)	35.8 (27.4–48.0)	Z = –1.140	p = 0.254
Not having A allele (n = 46)	30.4 (26.6–48.31)		

Z — Mann-Whitney U test; ZH — Kruskal-Wallis test

Table 4. Multiple linear regression analysis of significant independent predictors of ammonia level during valproic acid-based therapy

	Ammonia level*			
Predictors	**β**	**Adjusted R^2**	**t**	**p-value**
Carbamazepine total dose per day	–0.031	0.118	–2.52	0.020
Constant		80.916		
Model F, significance		F = 5.966, p = 0.020		
Total R^2		0.142		

*data has been transformed into normally distributed data; β — regression coefficient; F — ANOVA model; R2 — R-squared; Significant predictors: sex, WBCs count, duration treatment, carbamazepine total doses per day

having rs12136955 SNPs with the A allele and associated with hypertension as a comorbidity (95% confidence interval 1.175 (0.218 to 6.325) and with a combined levetiracetam drug dose (p = 0.022) were at high risk for elevated plasma ammonia levels (Tab. 7).

Discussion

This the first study, to the best of our knowledge, to investigate in Egyptian patients VPA-induced hyperammonemia and GLUL polymorphism (rs2296521, rs10911021, rs12136955). Only one patient, or 0.5% of the total, had a plasma ammonia level above 200 µg/dL with VPA-based therapy in the current study, which is significantly less than the 2.3%, 6.3%, and 9.9% of patients described in other trials [12,15]. In agreement with our results, Hoekstra et al. [16] failed to report an elevation of plasma ammonia levels above 170 µg/dL during VAP-based therapy. It has been demonstrated that the neurological and clinical symptoms of hyperammonemia are linked to 170 µg/dL ammonia levels [16]. Moreover, Tseng et al. [17] reported that 44 patients had plasma ammonia levels of more than 93 µg/dL from VPA-based therapy, and six of them developed consciousness disturbance, ataxia, nausea, and fatigue.

In the current study, we examined the depression anxiety score (DASS) and found a non-significant correlation between ammonia levels and the DASS score. Moreover, we found higher DASS scores in patients with low doses of VPA (< 2,000 mg/day) compared to patients with high doses of VPA (> 2,000 mg/ /day). On the other hand, Inoue et al. [12] did not thoroughly evaluate the clinical symptoms linked to the onset of hyperammonemia, since those symptoms were not detailed in electronic medical records.

In the current study, there was an association between male gender and high plasma ammonia levels during VPA-based therapy. Male patients had a higher mean VPA plasma level and higher mean ammonia levels. This finding is inconsistent with those reported by Inoue et al. [12], who found that the mean VPA daily dose was considerably higher in female patients than in male patients.

Although earlier research found a link between the dose of VPA and higher plasma ammonia levels, our study found no link between the dose of VPA and higher plasma ammonia levels in Egyptian patients. Also, we did not find any significant difference in plasma ammonia level between the low dose of VPA group (< 2,000 mg/day) and the high dose of VPA group. This contradicts the findings reported by Sharma et al. [18], who reported higher plasma ammonia levels in a high-VPA-dosage group. Additionally, in the univariate analysis conducted by Inoue et al. [12], there was no association between the daily dose of VPA and the emergence of hyperammonemia, a finding in line with our findings in the current study. Also, Pleym et al. [19] suggested that males have more UDP-glucuronosyl transferase activity than females, indicating that females have a greater need for cytochrome P450-derived metabolites. So, the variations in VPA metabolism and ammonia levels are probably due to gender-based changes in UDP-glucuronosyl transferase activity.

Glutamate synthetase becomes an important enzyme in ammonia clearance when liver failure causes an increase in plasma ammonia levels. We believe it is important to look into any potential relationships between the emergence of hyperammonemia and GS activity, as well as the implications of this or other GLUL polymorphisms. Previous studies demonstrated that the GS activity in a mouse's brain was significantly reduced by anti-seizure medications such as VPA, carbamazepine and phenytoin [9, 11]. Additionally, Inoue et al. [12] investigated the effect of some SNPs of GLUL gene such as rs10911070, rs10797771, and rs10911021) [12] on GS activity in Japanese patients.

However, in the current study, we investigated three other SNPs in the GLUL gene, rs2296521 and rs10911021, and rs12136955 in Egyptian patients. We did not find any association between the GLUL gene polymorphisms (SNPs rs2296521, rs10911021, and rs12136955) and elevated plasma ammonia levels in Egyptian epilepsy patients. These findings are opposite to those reported by Inoue et al. [12] in Japanese patients, who found a significant association between elevated plasma ammonia levels and GLUL rs10797771 polymorphism, which was increased by coadministration of phenytoin. This disagreement between results has been reported by previous studies

Table 5. Association of genotyping rs2296521 and sociodemographic, therapy, laboratory parameters

	Having A allele n = 135	Not having A allele n = 11	Test of significance	p-value	OR (95% CI)
Sociodemographic data					
Sex					
Female/male	56/79	7/4	Fisher's exact test	0.21	2.47 (0.69 to 8.84)
Age					
Median (Q1–Q3)	38.0 (32.0–46.0)	41.0 (37.0–44.0)	Z = –0.95	0.34	
Family history					
Yes/No	16/119	2/9	Fisher's exact test	0.63	1.046 (0.88 to 1.24)
Comorbidity					
Hypertension/no	6/116	1/10	Fisher's exact test	0.46	0.52 (0.06 to 4.73)
DM/no	5/116	0/10		> 0.99	NA
Both/no	8/116	0/10		> 0.99	NA
Blood pressure categories					
Hypertension/normal	10/121	1/10	Fisher's exact test	> 0.99	0.83 (0.10 to 7.13)
Borderline/normal	4/121	0/10		> 0.99	NA
DASS	21 (13 to 31)	22 (9 to 31)	Z = 0.07	0.95	
VPA therapy					
VPA dose per time (mg)	500.0 (500.0 to 750.0)	500.0 (500.0 to 1,000.0)	Z = 0.06	0.95	
Total dose per day (mg/day)	1,500 (1,000-15,000)	1,500 (1,000–2,000)	Z = –0.35	0.54	
Number of doses / days	2 (2 to 2)	2 (2 to 3)	Z = –0.60	0.55	
Hours since last dose	4 (3 to 12)	5 (2 to 12)	Z = 0.39	0.70	
Therapy duration in years	15 (7.8 to 25)	20 (8.8 to 25.8)	Z = 0.42	0.67	
Combined therapy					
Levetiracetam total dose (mg/day)	1,500.0 (1,000.0 to 2,000.0)	2,000.0 (1,000.0 to 2,000.0)	Z = 0.61	0.54	
Carbamazepine total dose (mg/day)	800.0 (600.0 to 1,200.0)	800.0 (800.0 to 1,200.0)	Z = 0.71	0.48	
Phenytoin sodium total dose (mg/day)	300.0 (200.0 to 300.0)	300.0 (300.0 to 300.0)	Z = 0.66	0.51	
Topiramate total dose (mg/day)	1,000.0 (1,000.0 to 1,000.0)	1,000.0 (1,000.0 to 1,000.0)	NA	NA	
Lamotrigine total dose (mg/day)	100.0 (62.5 to 187.5)	200.0 (200.0 to 200.0)	Z = 2.21	0.03	
Gabapentin total dose (mg/day)	300.0 (300.0 to 600.0)	NA	NA	NA	
Zonisamide total dose (mg/day)	200 (200 to 200)	NA	NA	NA	
Laboratory data					
Valproic acid level(µg/mL)	50.8 ± 25.1	65.0 ± 2 2.8	t = 1.81	0.07	14.13 (–1.34 to 29.60)
Plasma ammonia level (ug/dL)	33.2 (27.3 to 47.8)	30.0 (19.0 to 48.0)	Z = 0.70	0.49	
RBCs count (million/mm^3)	4.6 ± 0.6	4.5 ± 0.4	t = 0.79	0.43	–0.13 (–0.47 to 0.20)
Hb level (g/dL)	13.1 (11.7 to 14.0)	11.7 (10.7 to 13.5)	Z = 2.12	0.03	
WBCs count (thousand/mm^3)	6.4 (5.3 to 7.6)	6.6 (5.7 to 7.2)	Z = 0.61	0.55	
Platelet count (thousand/mm^3)	184.8 ± 55.7	190.7 ± 54.6	t = 0.34	0.74	5.90 (–28.58 to 40.38)
AST (U/L)	17.7 (14.1 to 24.2)	17.8 (13.0 to 22.7)	Z = 0.54	0.59	
ALT (U/L)	11.0 (7.7 to 16.4)	9.3 (5.5 to 11.4)	Z = 1.99	0.047	
ALP (U/L)	81.5 (72,5 to 94.9)	80.5 (69.4 to 96.3)	Z = 0.48	0.64	
Albumin (g/dL)	4.6 (4.4 to 4.9)	4.7 (4.2 to 4.9)	Z = 0.26	0.80	
Protein (g/dL)	7.1 ± 0.5	7.0 ± 0.5	t = 0.69	0.49	–0.11 (–0.48 to 0.26)

Z — Mann-Whitney U test; t — independent t -test; NA— not applicable

Table 6. Association of genotyping rs10911021 and sociodemographic, therapy, laboratory parameters

	Having C allele n = 130	Not having C allele n = 14	Test of significance	p-value	OR (95% CI)
Sociodemographic data					
Sex					
Female/male	58/72	4/10	Fisher's exact test	0.395	0.497 (0.15 to 1.66)
Age					
Median (Q1 to Q3)	38.0 (31.8 to 46.3)	37.5 (32.8 to 40.8)	Z = - 0.64	0.52	
Family history					
Yes/no	17/113	0/14	Fisher's exact test	0.38	NA
Comorbidity					
Hypertension/no	6/112	1/12	Fisher's exact test	0.53	0.62 (0.07 to 5.80)
DM/no	4/112	1/12		0.42	0.43 (0.04 to 4.15)
Both/no	8/112	0/12		> 0.99	NA
Blood pressure categories					
Hypertension/normal	10/116	1/13	Fisher's exact test	> 0.99	1.12 (0.13 to 9.47)
Borderline/normal	4/116	0/13		> 0.99	NA
DASS	21.0 (13.0 to 31.0)	16.5 (9.8 to 36.0)	Z = 0.74	0.46	
VPA therapy					
VPA dose per time (mg)	500.0 (500.0 to 750.0)	500.0 (500.0 to 750.0)	Z = 0.40	0.69	
Total dose per day (mg/day)	1,500 (1,000–15,000)	1,500 (1,000-2,000)	Z = –0.31	0.71	
Number of doses per day	2 (2 to 2)	2 (2 to 2.3)	Z = 0.19	0.85	
Hours since last dose	4 (3 to 12)	6 (1.5 to 14.0)	Z = 0.44	0.66	
Therapy duration in years	15.0 (7.0 to 25.0)	15.0 (11.3 to 24.3)	Z = 0.54	0.59	
Combined therapy					
Levetiracetam total dose (mg/day)	1,500.0 (1,000.0 to 2,000.0)	1,000.0 (1,000.0 to 1,500.0)	Z = 1.71	0.09	
Carbamazepine total dose (mg/day)	800.0 (600.0 to 1,200.0)	800.0 (650.0 to 800.0)	Z = 0.57	0.56	
Phenytoin sodium total dose (mg/day)	300.0 (200.0 to 300.0)	0	NA	NA	
Topiramate total dose (mg/day)	100.0 (100.0 to 100.0)	0	NA	NA	
Lamotrigine total dose (mg/day)	100.0 (100.0 to 200.0)	50.0 (25.0 to 100.0)	Z = 2.04	0.04	
Gabapentin total dose (mg/day)	300.0 (300.0 to 600.0)	0	NA	NA	
Zonisamide total dose (mg/day)	200.0 (200.0 to 200.0)	0	NA	NA	
Laboratory data					
Valproic acid level (µg/mL)	50.4 ± 24.2	68.6 ± 26.8	t = 2.64	0.009	18.16 (4.58 to 31.75)
Plasma ammonia level (ug/dL)	33.6 (27.2 to 47.9)	30.8 (27.5 to 54.5)	Z = 0.17	0.87	
RBCs count (million/mm^3)	4.6 ± 0.5	4.6 ± 0.5	t = 0.40	0.69	–0.06 (–0.36 to 0.24)
Hb level (g/dL)	12.83 ± 1.59	12.75 ± 1.26	t = 0.18	0.86	–0.077 (–0.79 to 0.94)
WBCs count (thousand/mm^3)	6.5 (5.3 to 7.7)	6.1 (4.8 to 6.7)	Z = 1.61	0.11	
Platelet count (thousand/mm^3)	187.1 ± 56.3	160.8 ± 40.9	t = 0.26	0.09	–26.32 (–56.94 to 4.296)
AST (U/L)	17.3 (14.1 to 22.7)	22.6 (12.9 to 26.3)	Z = 0.96	0.34	
ALT (U/L)	10.6 (7.5 to 14.6)	12.4 (6.5 to 22.1)	Z = 0.199	0.84	
ALP (U/L)	81.2 (72.2 to 94.2)	85.4 (66.0 to 101.2)	Z = 0.56	0.57	
Albumin (g/dL)	4.59 ± 0.40	4.73 ± 0.29	t = 0.25	0.23	–0.13 (0.08 to 0.35)
Protein (g/dL)	7.0 ± 0.5	7.2 ± 0.6	t = 1.037	0.42	0.15 (–0.13 to 0.42)

Z — Mann-Whitney U test; t — independent t-test; c2 — chi-square test; NA — not applicable

Table 7. Association of genotyping rs12136955 and sociodemographic, DASS, therapy, laboratory parameters

	Having A allele n = 96	Not having A allele n = 46	Test of significance	p-value	OR (95% CI)
Sociodemographic data					
Sex					
Female/male	42/54	19/27	c2 = 0.08	0.78	0.91 (0.44 to 0.84)
Age					
Median [Q1 to Q3]	38.0 (32.0 to 46.0)	37.5 (30.8 to 44.0)	Z = 0.58	0.56	
Family history					
Yes/no	7/89	8/38	Fisher's exact test	0.08	1.50 (0.86 to 2.61)
Comorbidity					
Hypertension/no	5/83	2/39	Fisher's exact test	> 0.99	1.18 (0.218 to 6.325)
DM/no	3/83	2/39		0.66	0.71 (0.11 to 4.39)
Both/no	5/83	3/39		0.71	0.78 (0.18 to 3.44)
Blood pressure categories					
Hypertension/normal	6/86	5/41	Fisher's exact test	0.51	0.57 (0.17 to 1.98)
Borderline/normal	4/86	0/41		0.31	NA
DASS	20.5 (13.0 to 30.0)	21.0 (12.0 to 33.5)	Z = 0.69	0.49	
VPA therapy					
Valproic acid dose per time [mg]	500.0 (500.0 to 750.0)	500.0 (500.0 to 750.0)	Z = 0.48	0.63	
Total dose per day [mg]	1,000 (1,000-1,500)	1,500.0 (1,000.0 to 1,500.0)	Z = 0.59	0.56	
No. of doses per day	2 (2 to 2)	2 (2 to 2)	Z = 0.21	0.83	
Hours since last dose	4 (3 to 12)	4 (2 to 11)	Z = 1.28	0.20	
Therapy duration in years	15.0 (8.0 to 20.8)	20.0 (5.3 to 28.0)	Z = 1.11	0.27	
Combined therapy					
Levetiracetam total dose [mg/day]	1,500.0 (1,000.0 to 2,000.0)	1,000.0 (1,000.0 to 1,500.0)	Z = 2.28	0.022	
Carbamazepine total dose [mg/day]	800.0 (400.0 to 1,200.0)	800.0 (550.0 to 1,050.0)	Z = 0.63	0.53	
Phenytoin sodium total dose [mg/day]	300.0 (200.0 to 300.0)	300.0 (200.0 to 300.0)	Z = 0.36	0.72	
Topiramate total dose [mg/day]	–	1,000.0 (1,000.0 to 1,000.0)	NA	NA	
Lamotrigine total dose [mg/day]	100 (100 to 200)	125.0 (43.8-200)	Z = 0.18	0.85	
Gabapentin total dose [mg/day]	300 (300 to 600)	NA (no cases)	NA	NA	
Zonisamide total dose [mg/day]	200 (200 to 200)	NA (no cases)			
Laboratory data					
Valproic acid level [µg/mL]	49.5 ± 26.0	57.0 ± 23.8	T = 1.64	0.102	7.47 (—1.51 to 16.44)
Plasma ammonia level [µg/dL]	35.8 (27.4 to 48.0)	30.4 (26.6 to 48.3)	Z = 1.14	0.25	
RBCs count [million/mm^3]	4.6 ± 0.6	4.6 ± 0.5	t = 0.096	0.92	0.009 (—0.18 to 0.20)
Hb level [g/dL]	12.8 ± 1.5	12.8 ± 1.6	t = 0.01	0.99	0.003 (—0.55 to 0.56)
WBCs count [thousand/mm^3]	6.59 ± 1.9	6.62 ± 1.5	t = 0.07	0.94	0.024 (-0.64 to 0.69)
Platelet count [thousand/mm^3]	184.8 ± 53.7	181.9 ± 59.6	t = 0.29	0.77	–2.89 (–22.64 to 16.86)
AST [U/L]	18.0 (14.2 to 24.0)	17.4 (14.1 to 23.3)	Z = 0.36	0.72	
ALT [U/L]	10.5 (7.7 to 16.4)	10.6 (6.7 to 15.8)	Z = 0.78	0.44	
ALP [U/L]	81.8 ± 18.29	83.9 ± 20.45	t = 0.61	0.55	2.067 (–4.67 to 8.81)
Albumin [g/dL]	4.6 ± 0.4	4.6 ± 0.4	t = 0.31	0.76	0.022 (–0.12 to 0.16)
Protein [g/dL]	7.1 ± 0.5	7.1 ± 0.5	t = 0.23	0.82	0.021 (–0.155 to 0.19)

Z — Mann-Whitney U test; t — independent t-test; c2 — chi-square test; NA — not applicable

that examined the polymorphism in the carbamoyl-phosphate synthase-1 (*CPS1*4217C > A) gene, a key regulatory enzyme for the urea cycle and the removal of ammonia from the liver in Japanese patients [15,20]. Although Yagi et al. [15] found a significant association between elevated plasma ammonia levels and CPS14217C > A gene polymorphism, Inoue et al. [20] did not find any such significant association.

It has been claimed that one of the variables leading to the elevation of the plasma ammonia level during VPA-based therapy is the concurrent use of anti-seizure medications [21]. In the current study, 6.11% of the patients received VPA monotherapy which is lower than in previous studies [12]. Inoue et al. [12] found that the coadministration of phenytoin with the GLUL rs10797771 polymorphism increased the likelihood of developing severe hyperammonemia. Also, Inoue et al. [20] and Yamamoto et al. [10] reported that the highest risk factor for the development of hyperammonemia was phenytoin coadministration. We found a positive association between plasma ammonia levels, treatment duration and WBCs count, and a negative association between plasma ammonia levels and carbamazepine total dose per day, suggesting that adding carbamazepine as a combined anti-seizure therapy might protect against the development of hyperammonemia. Moreover, multilinear regression revealed a significant negative association between plasma ammonia level and carbamazepine total dose per day, suggesting that carbamazepine might negatively influence VPA-induced hyperammonemia. On the other hand, coadministration of levetiracetam and other drugs as anti-seizure medications did not influence the risk for development of hyperammonemia. In line with this finding, Patsalos [22] demonstrated that levetiracetam at a concentration of five times its therapeutic level and its primary metabolite L057 did not affect 11 different drug-metabolising enzymes, and failed to stimulate CYP activity in rat hepatocyte primary cultures. Moreover, we found that female patients having the A allele in rs2296521 SNPs and patients having the A allele with a high Hb level and treated with lamotrigine were at risk for elevated plasma ammonia levels. Also, we demonstrated that female patients having C alleles in the rs10911021 SNPs and patients having C alleles in these SNPs with a low VPA plasma level and treated with lamotrigine were at risk for elevated ammonia plasma levels. Moreover, we found that patients having the A allele in rs12136955 SNPs with hypertension as a comorbidity and treated with levetiracetam dose were at high risk for developing high plasma ammonia levels.

Although the current work is the first study to examine the effect of GLUL gene polymorphism on VPA-induced hyperammonemia among Egyptian patients, it carries some limitations, such as low sample size, absence of a control group, no examination of other SNPs of GS enzyme gene, as well as not testing the gene polymorphism of other enzymes involved in the metabolism of NH4, such as CPS enzyme genes.

Conclusions

The rs2296521, rs10911021, and rs12136955 GLUL polymorphisms did not show any correlation or association with VPA-induced hyperammonemia among Egyptian patients. However, female patients who have rs2296521 with the A allele and rs10911021 with the C allele might be independent risk factors for elevated plasma ammonia levels during VPA-based therapy. Also, patients having rs12136955 SNPs with the A allele or associated hypertension as a comorbidity are at high risk for elevated plasma ammonia levels. Moreover, carbamazepine combined therapy may protect against the development of hyperammonia in VPA-treated patients.

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