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## Correlation of Blood Biomarkers with Age Informs Pathomechanisms in Succinic Semialdehyde Dehydrogenase Deficiency (SSADHD), a Disorder of GABA Metabolism

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### Abstract

We hypothesized that blood levels of GABA and  $\gamma$ -hydroxybutyric acid (GHB), biomarkers of succinic semialdehyde dehydrogenase deficiency (SSADHD), would correlate with age. GABA and GHB were quantified in plasma and red blood cells (RBCs) from eighteen patients (age range 5–41 years; median 8). Both metabolites negatively correlated with age ( $P < 0.05$ ). Plasma and RBC GHB declined with age, reaching a nadir and approximate steady-state by 10 years. Declining plasma GABA achieved this approximate steady state at 30–40 years of age. These biomarker relationships may reflect further GABA- and GHB-ergic neurotransmission imbalances that correlate with the onset of adolescent/adulthood neuropsychiatric morbidity and epilepsy in SSADHD.

### Introduction

A rare disorder of GABA catabolism, SSADHD has been reported in approximately 200 patients worldwide (Malaspina et al 2016). The phenotype is non-specific, with

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- EEJ: acquisition and analysis of data
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- GSS: acquisition and analysis of data
- PLP: conception and design of study; drafting of manuscript
- JBR: conception and design of study; drafting of manuscript
- KMG: conception and design of study; data analysis; manuscript drafting

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developmental delay, hypotonia, absence of formulated speech, and the onset of neuropsychiatric morbidity (obsession-compulsion, attention-deficit, oppositional defiant disorders) in adolescence to adulthood. Autism spectrum disorder is frequently considered in the differential diagnosis (Malaspina et al 2016; Parviz et al 2014). Detection of SSADHD requires measurement of  $\gamma$ -hydroxybutyrate (GHB; the biochemical hallmark) in peripheral biofluids, although GABA is also elevated in CSF (Gibson et al 1995; 2003). Both compounds have either neurotransmitter, or neuromodulatory, roles in CNS (Maitre et al 2016; Fig. 1).

The natural history of SSADHD remains unknown, and a longitudinal study that may define natural history is lacking. Nonetheless, anecdotal evaluation of reported cases has highlighted the onset of neuropsychiatric morbidity in adolescence and adulthood (Parviz et al 2014; Pearl et al 2014). Moreover, Knerr and coworkers have identified a more severe disease course in a subset of patients, in which the epileptic disorder appears more severe (Knerr et al 2007). Importantly, some patients have expired with SUDEP (sudden unexplained death in epilepsy) in adulthood (Lapalme-Remis et al 2015; Knerr et al 2010; Gibson and Pearl, unpublished). To explore the natural history of SSADHD, we have sought to identify biomarkers in physiological fluids which may provide pathophysiological insight into disease evolution. Here, we examine the levels of both GABA and GHB in urine, plasma and RBCs to address potential age-dependent correlations. Our working hypothesis centered on the prediction that blood levels of both biomarkers would reflect biomarker disturbances in CNS, providing insight into disease progress and evolution.

## Methods

Whole blood and urine were obtained from patients with SSADHD, obligate heterozygotes (parents) and control individuals with informed consent (IRB 12678). For the physiological fluids evaluated, the number of control individuals were: eight (plasma total GABA and RBC GHB), eleven (plasma GHB), and thirteen (urine GHB). Control individuals were not age-matched to patients, although we speculate that the age-range of both cohorts overlapped. The majority of controls were adults, although some were siblings of patients (children and adolescents) whose genetic status was unknown. For control individuals, gender status was identified only for seven, and age identified only for four, making correlative analyses between metabolic findings and age/gender challenging. Patient age range was 5–41 years (median, 8 years), and ages were available for all. Clinical details were not available on patients at the time of sample shipment, and to our knowledge none of the patients was concurrently receiving vigabatrin or other antiepileptic which may have artefactually elevated plasma total GABA, as is the case for GABA in cerebrospinal fluid for patients receiving vigabatrin (Gibson et al 1995; Casado et al 2014).

Blood samples, upon receipt in the laboratory, were fractionated into plasma, mixed polymorphonuclear cells, and packed red blood cells. Although GABA levels have been effectively quantified in platelets isolated from whole blood (Kowa et al 1992), we opted for isolation of plasma since an objective was to determine if total GABA levels might be elevated in this compartment and potentially inform GABA concentrations in whole bloodspots for eventual newborn screening of SSADHD. Moreover, since we have recently

demonstrated that both GHB and GABA are significantly elevated in brain and peripheral organs (kidney, liver) of SSADH-deficient mice (Vogel et al, in revision), we assume that peripheral GABA metabolism mimics the pathway of metabolism shown in brain. The latter underscores the utility of measuring these biomarkers in peripheral tissues (plasma, red blood cells) as a surrogate for brain, or even cerebrospinal fluid, measurements. Total GABA in plasma, and GHB in urine, plasma and RBC extracts were quantified as described (Kok et al 1993; Gibson et al 1990). Statistical analysis was performed using GraphPad Prism 6.0 (San Diego, CA).

## Results

Plasma total GABA values were: control,  $2.16 \pm 0.19$   $\mu\text{mol/L}$  (mean  $\pm$  SEM,  $n=8$ ; median, 2.06, range 1.7–3.32); obligate heterozygotes (parents),  $2.16 \pm 0.12$  ( $n=5$ ; median 2.24, range 1.86–2.42); SSADHD,  $4.16 \pm 0.25$  ( $n=16$ , median 4.04; range 2.14–5.52;  $p < 0.001$  compared to control and obligate heterozygotes, two tailed  $t$  test) (Fig. 2A). When stratified for patient age, plasma total GABA showed a significant negative correlation (Fig. 2B;  $P = 0.0082$ , Spearman coefficient, 10 male, 6 female). A negative correlation was also observed when evaluating only male patients ( $P = 0.0007$ ), but not with female patients (Fig. 3A, D).

Plasma GHB values were: control,  $1.14 \pm 0.23$   $\mu\text{mol/L}$  (mean  $\pm$  SEM,  $n=11$ ; median 0.8, range 0.5–2.9); obligate heterozygotes,  $1.14 \pm 0.23$  ( $n=5$ ; median, 1.1, range 0.5–1.9); SSADHD,  $179.1 \pm 44.1$  ( $n=18$ , median 67.6, range 25.5–533;  $p < 0.05$  compared to both groups, two-tailed  $t$  test) (Fig. 2D). Plasma GHB showed a significant negative correlation with age (Fig. 2E;  $P = 0.0017$ , Spearman coefficient, 12 male, 6 female patients). A negative correlation was also observed when evaluating only male patients ( $P = 0.0014$ ), but not with female patients (Fig. 3B, E). RBC GHB levels were: control,  $4.19 \pm 0.45$   $\mu\text{mol/L}$  (mean  $\pm$  SEM,  $n=8$ ; median 3.9, range 2.8–6.4); obligate heterozygotes,  $5.56 \pm 1.05$  ( $n=5$ ; median 5.4, range 2.9–9.3); SSADHD,  $58.0 \pm 11.5$  ( $n=16$ , median 54.2, range 9.6–139;  $p < 0.05$  compared to both groups, two-tailed  $t$  test) (Fig. 2G). RBC GHB showed a significant negative correlation (Fig. 2H;  $P = 0.0214$ , Spearman coefficient, 12 male and 6 female patients). A negative correlation was also observed when evaluating only male patients ( $P = 0.0078$ ), but not with female patients (Fig. 3C, F). Urine samples were also available from 18 patients, although age characteristics were known only for 8 patients. Urine GHB levels were: control,  $1.68 \pm 0.77$  mmol/mol creatinine (mean  $\pm$  SEM,  $n=13$ ; median, 0.4, range 0.12–10.1); obligate heterozygotes,  $1.84 \pm 1.37$  ( $n=14$ ; median 0.4, range 0.10–19.6); SSADHD,  $158.5 \pm 27.0$  ( $n=18$ , median 153, range 26.8–514;  $p < 0.0001$  compared to both groups, two-tailed  $t$  test). There was no correlation between urine GHB concentration and age (data not shown).

Correlations between plasma total GABA and GHB revealed a profile which attained a plateau at total GABA levels of  $\sim 5$   $\mu\text{mol/L}$  and GHB concentrations  $\sim 300$   $\mu\text{mol/L}$  (Fig. 2C;  $P < 0.0001$ ). Similar results were seen for plasma total GABA ( $\sim 5$   $\mu\text{mol/L}$ ) and RBC GHB of  $\sim 65$   $\mu\text{mol/L}$  ( $P = 0.0006$ ; Fig. 2I). The relationship between plasma and RBC GHB was linear (Fig. 2F;  $P < 0.0001$ , Pearson coefficient). For patients, there was no significant difference with respect to gender for any of the metabolites evaluated (Figs. 4A–C). Information on gender and age characteristics for the control cohort were, unfortunately,

limited (Fig. 4). For the few control individuals for whom gender was known, plasma and RBC GHB levels were not significantly different (Figs. 4E, F), but only 2 individuals were identified as male. For plasma total GABA, the mean value in males was significantly higher in males as compared to females (Fig. 4A), but for males the control cohort contained only 2 individuals.

## Discussion

Total GABA, as well as GHB in plasma and RBC extracts, revealed a significant negative correlation with age (Fig. 2B, E, H). Low plasma GABA has previously been associated with neuropsychiatric morbidity. Plasma GABA was negatively correlated with age and aggressiveness in subjects having a first-degree relative with primary unipolar depressive disorder (Bjork et al 2001), and positively correlated with depression and apathy and negatively correlated with age in severe Alzheimer disease (Lancot et al 2007). The phenotype of SSADHD often includes neuropsychiatric morbidity in adolescence to adulthood, including attention deficit-hyperactivity, oppositional defiant and obsessive compulsive disorders (Malaspina et al 2016). We found tapering of levels for GHB at about 10 years of age, whereas this same tapering for plasma total GABA was not evident until at least 30 years of age. The lowered levels of GHB may track with the onset of neuropsychiatric morbidity in patients (Parviz et al 2014), but further studies are needed to verify this hypothesis. Moreover, we know that a subset of patients with SSADHD has a more severe phenotype, featuring adult-onset epilepsy (Parviz et al 2014), and there have been instances of SUDEP (sudden unexplained death in epilepsy) in adulthood (Lapalme-Remis et al 2015; Knerr et al 2010; Gibson and Pearl, unpublished observation). Previously, Casado and coworkers (2014) documented that GABA levels in cerebrospinal fluid from 55 pediatric controls showed a progressive increase with age. Unfortunately, we could not assess this age-related parameter in our control cohort since information on age characteristics was limited.

Several publications have highlighted alterations in GABAergic neurotransmission in SSADHD (Pearl et al 2009; Reis et al 2012; Buzzi et al 2006), both in the orthologue mouse model and patients, and it may be reasonable to assume that high levels of GABA and GHB during ontogeny induces an altered balance between GABAergic/GHBergic neurotransmission with that of excitatory glutamatergic neurotransmission (Jansen et al 2008; Vogel et al 2016; Talaei et al 2016). Our prediction is that as levels of GABA and GHB fall with age, this balance is likely disrupted and correlates with neuropsychiatric morbidity in patients. A comprehensive longitudinal evaluation of SSADHD, correlating brain and blood levels of GABA and GHB with age and phenotype, could address this issue.

Our study represents the first identification of elevated total GABA in the plasma of patients with SSADHD. This is relevant for several reasons. First, appropriate testing for GHB in order to achieve accurate diagnosis may be overlooked due to the non-specific neurological phenotype (Lapalme-Remis et al 2015). Additionally, clinical trials for SSADHD are either underway or completed (Pearl et al 2014; [www.clinicaltrials.gov](http://www.clinicaltrials.gov)), and a therapeutic pipeline is under development using the corresponding murine model (Malaspina et al 2016). Accordingly, early detection of the disease, preferably through newborn screening, would be

desirable. This may be even more compelling since many patients with SSADHD are not diagnosed until at least 2 years of age, such that the early course of the disease is largely unknown (Malaspina et al 2016). Thus, early detection via newborn screening could begin to outline the early natural history of SSADHD, while informing symptomatic management and providing families with important predictive information. Forni and colleagues (2013) presented methodology for quantitation of GHB in newborn bloodspots. The method, however, is not readily interfaced to current newborn screening platforms, which quantifies nitrogenous species amino acids, L-carnitine analogs; (Therrell et al 2015). Accordingly, plasma total GABA may provide a more rapid path to newborn screening of SSADHD, although it is likely that other neurological conditions could result in elevated GABA levels in newborn bloodspots (e.g., neuropsychiatric conditions, GABA-transaminase deficiency, etc).

Since the discovery of SSADHD in the early 1980s (Jakobs et al 1981), GHB has been employed as the biochemical hallmark of the disease, and its identification in physiological fluids has enabled accurate diagnosis. Later studies on SSADHD also revealed elevations of GABA in the CNS, using both cerebrospinal fluid analyses as well as magnetic resonance spectroscopy (Gibson et al 1995; 2003; Novotny et al 2003). We now demonstrate, for the first time, that measurement of GABA and GHB in peripheral biofluids may inform about the natural history of the disorder, and thereby provide a mechanism by which to predict the sequence and timing of the onset of symptoms and complications with development.

## Acknowledgments

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## Abbreviations

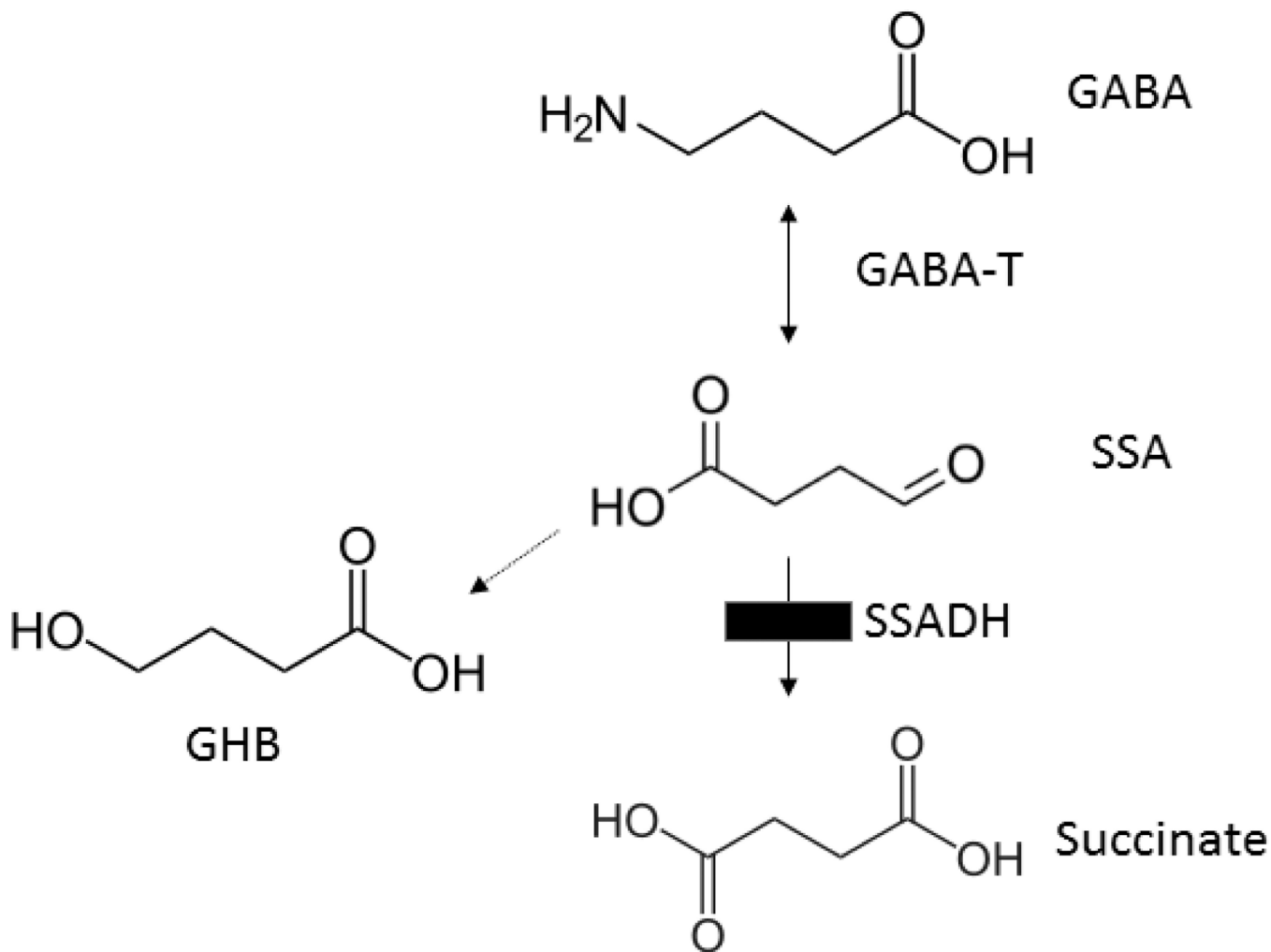
<b>GABA</b>	4-aminobutyrate
<b>GABA-T</b>	GABA-transaminase
<b>SSADH</b>	succinic semialdehyde dehydrogenase
<b>SSADHD</b>	succinic semialdehyde dehydrogenase deficiency
<b>GHB</b>	$\gamma$ -hydroxybutyric acid
<b>NBS</b>	newborn screening

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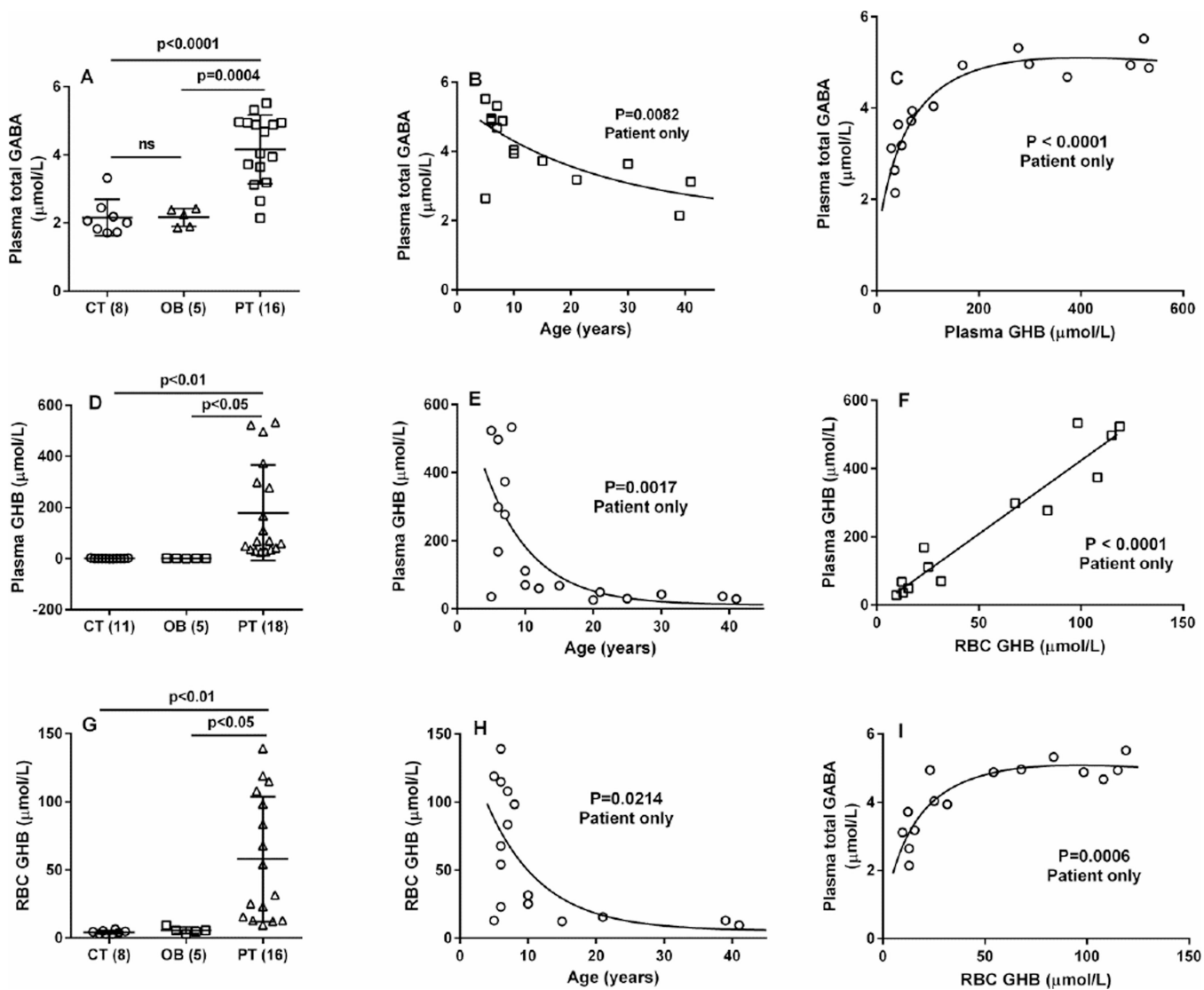
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**Fig. 1. GABA metabolism**

Abbreviations: GABA,  $\gamma$ -aminobutyric acid; GABA-T, GABA-transaminase; SSADH, succinic semialdehyde dehydrogenase; SSA, succinic semialdehyde; GHB,  $\gamma$ -hydroxybutyric acid. The site of the block in patients with SSADHD is indicated by the filled black box.

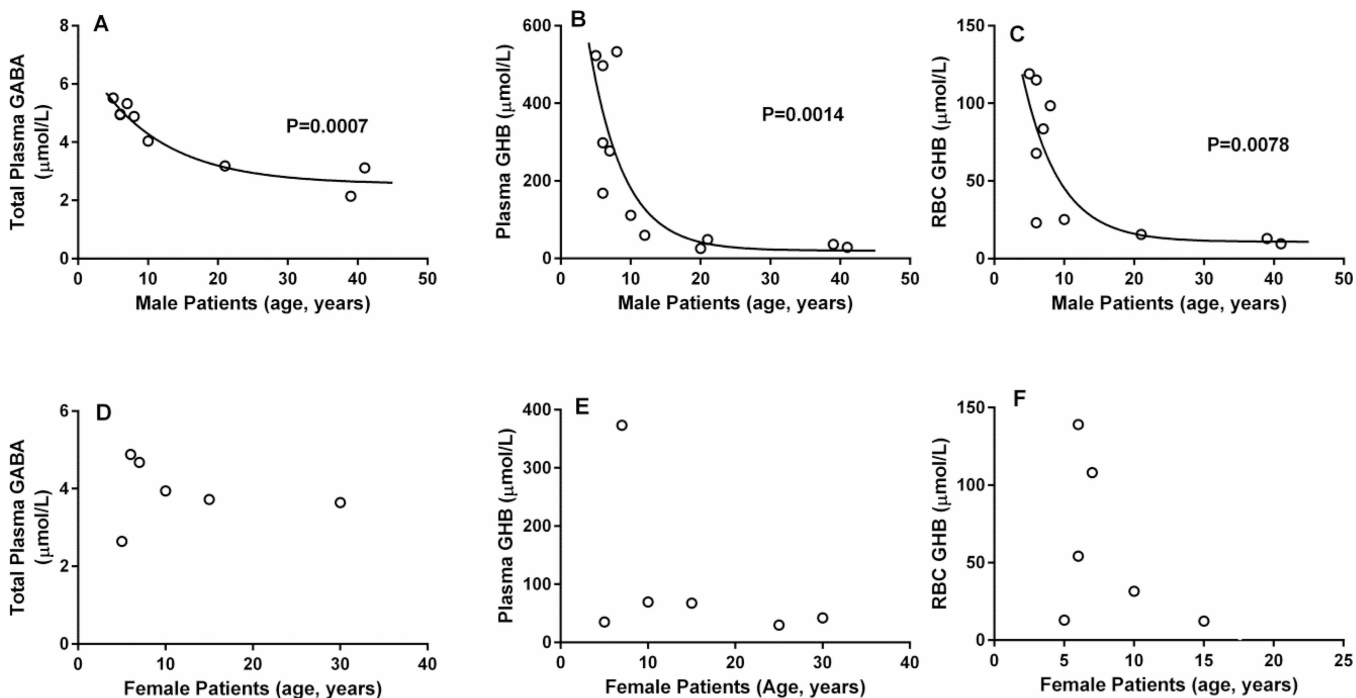




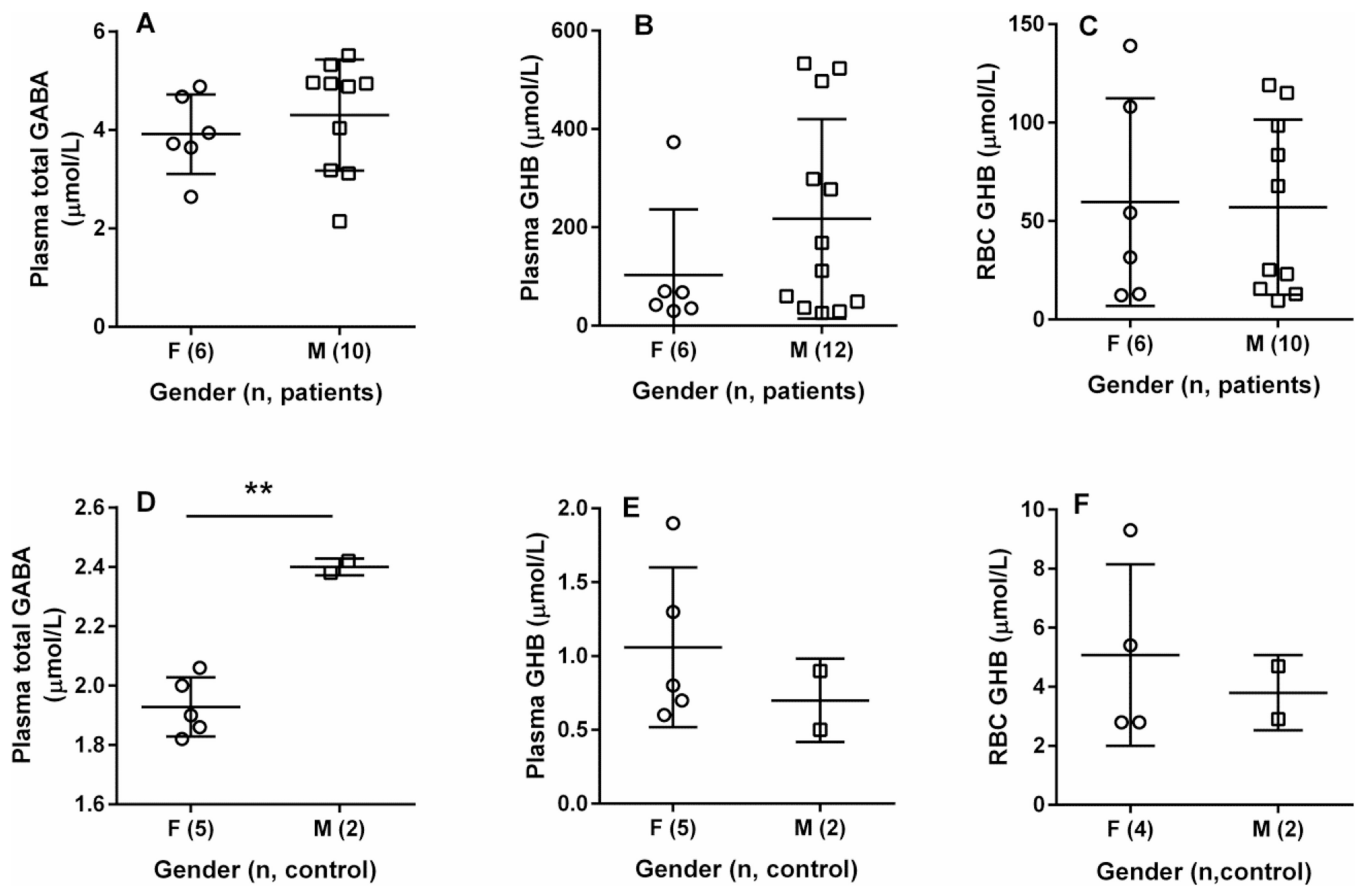
**Fig. 2. Metabolic correlations as a function of age, and between metabolites, in patients with SSADH deficiency**

(A, D, G) Graphic representation of metabolites level as a function of genotype.

Abbreviations employed: CT, control (number of individuals in parentheses); OB, obligate heterozygotes (parents); PT, patients with SSADH deficiency. Data shown as mean with SEM. Statistical analysis employed a two-way *t* test. (B, E, H) Correlation of metabolites with age (patients only). Statistical analysis employed the Spearman coefficient. (C–I) Metabolite interrelationships, in which the Spearman coefficient was employed for 2C and 2I, and the Pearson coefficient for Fig. 2F.



**Fig. 3. Metabolic correlations for patients with SSADH deficiency as a function of gender** (A–C) male patients; (D–F) female patients. For male patients, statistical analysis employed the Spearman coefficient. There was no statistically significant correlation for female patients, but the low number of participants may have impacted this analysis.



**Fig. 4. Metabolic comparisons within SSADH-deficient patient and control cohorts as a function of gender**

(A–C) gender comparisons for patients with SSADH deficiency (f, female; m, male, with parenthetical values representing the number of subjects studied). Statistical analysis employed a two-way *t* test, and no significant differences were observed for any metabolite. (D–F) gender comparisons within the control cohort. Gender characteristics were available only on a limited number of individuals. For plasma total GABA, a significantly higher mean value was observed in males vs. females (Fig. 4D; two way *t* test), but only 2 males populated this group.