**FAS -670A>G genetic polymorphism is associated with Treatment Resistant Depression**

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**Abstract**

**Background**

Hippocampal neurogenesis has been suggested as a downstream event of antidepressants (AD) mechanism of action and might explain the lag time between AD administration and the therapeutic effect. Despite the widespread use of AD in the context of Major Depressive Disorder (MDD) there are no reliable biomarkers of treatment response phenotypes, and a significant proportion of patients display Treatment Resistant Depression (TRD). Fas/FasL system is one of the best-known death-receptor mediated cell signaling systems and is recognized to regulate cell proliferation and tumor cell growth. Recently this pathway has been described to be involved in neurogenesis and neuroplasticity.

**Methods**

Since FAS -670A>G and FASL -844T>C functional polymorphisms never been evaluated in the context of depression and antidepressant therapy, we genotyped FAS -670A>G and FASL -844T>C in a subset of 80 MDD patients to evaluate their role in antidepressant treatment response phenotypes.

**Results**

We found that the presence of FAS -670G allele was associated with antidepressant bad prognosis (relapse or TRD: OR=6.200; 95% CI: [1.875–20.499]; p=0.001), and we observed that patients carrying this allele have a higher risk to develop TRD (OR=10.895; 95% CI: [1.362–87.135]; p=0.008).
Moreover, multivariate analysis adjusted to potentials confounders showed that patients carrying G allele have higher risk of early relapse (HR=3.827; 95% CI: [1.072–13.659]; p=0.039). FAS mRNA levels were down-regulated among G carriers, whose genotypes were more common in TRD patients. No association was found between FASL-844T>C genetic polymorphism and any treatment phenotypes.

**Limitations**

Small sample size. Patients used antidepressants with different mechanisms of action.

**Conclusion**

To the best of our knowledge this is the first study to evaluate the role of FAS functional polymorphism in the outcome of antidepressant therapy. This preliminary report associates FAS -670A>G genetic polymorphism with Treatment Resistant Depression and with time to relapse. The current results may possibly be given to the recent recognized role of Fas in neurogenesis and/or neuroplasticity.

**Keywords**

- Neurogenesis;
- Neuroplasticity;
- FAS;
- Antidepressants;
- Treatment Resistant Depression;
- Polymorphisms

**1. Introduction**

The Fas/FasL system, also known as CD95 or APO-1, is one of the major apoptotic pathways, mediating cell cytotoxicity, immune cell regulation and tumor cell growth (Lambert et al., 2003, Suda et al., 1993 and Trauth et al., 1989). Fas, a cell surface receptor, interacts with its natural ligand FasL (CD95L) to initiate a death signaling cascade, leading to apoptosis. Whereas Fas is ubiquitously expressed and particularly abundant in liver, heart, kidney, pancreas, brain, thymus, lymphoid tissues, and activated mature T lymphocytes, its ligand is predominantly expressed in activated T lymphocytes and NK cells, mediating immune cell clearance (Guicciardi and Gores, 2009, Oshimi et al., 1996 and Suda et al., 1995). In brain, Fas expression has
been documented in cortical, hippocampal, sensory, and motorneurons, and in all types of glial cells. Fas-mediated apoptosis is responsible for the neural and glial cells clearance upon brain injury, such as stroke and spinal cord damage (Zuliani et al., 2006). Further than apoptosis, Fas/FasL has emerged as an important regulator of a widespread of non-apoptotic functions, as cell activation, proliferation and differentiation, in several cell types (Barca et al., 2013, Lambert et al., 2003 and Wajant et al., 2003). Recent studies point out novel functions of Fas pathway in brain, such as dendritic branching of immature neurons, axonal elongation in dorsal root ganglia cells, migration of glioblastoma cells and neurogenesis in the adult brain (Corsini et al., 2009, Desbarats et al., 2003, Kleber et al., 2008 and Zuliani et al., 2006). Authors claimed that activation of death or regeneration pathways seemed to be influence by the surrounding context, such as the presence of growth factors, expression of pro-apoptotic molecules (caspase 8), pro-survival proteins (Akt) and by oxidative stress (Lambert et al., 2003).

The relationship among hippocampal neurogenesis, depression and the antidepressants mechanism of action has generated a considerable amount of interest and controversy. Despite antidepressant drugs produce a rapid increase in neurotransmitters synaptic levels, clinical improvement usually takes 3–4 weeks to be observed (Frazer and Benmansour, 2002). This is thought to be explained by the “neuroplasticity hypothesis” of depression, where stress decreases hippocampal neurogenesis and/or synaptic plasticity in prefrontal cortex (Pilar-Cuellar et al., 2013), and antidepressant treatment stimulates neurogenesis and/or neuronal plasticity (Duman and Monteggia, 2006). It is known that the maturation of newly developed neurons requires about 3–4 weeks. Moreover, different classes of antidepressants stimulate the proliferation of neuronal progenitors (Duman, 2004, Malberg, 2004 and Malberg et al., 2000) and increase the survival of newly developed neurons (Wang et al., 2008). In addition, ablation of neurogenesis by irradiation reduces some of the antidepressants effects (Santarelli et al., 2003). Thus, intact hippocampal neurogenesis is required for at least some of the behavioral effects of antidepressants in animal models.

Despite the widespread of antidepressants available in the clinical context, a wide fraction of patients fail to remit to treatment, and some display Treatment Resistant Depression (TRD). Identifying patients at risk of treatment resistance using biomarkers could possibly have direct impact on individualized therapy. Even though this is an area of increase investigation, the studied biological markers only explain a small fraction of the therapeutic outcome and are seek for biomarkers of response, not remission, the most important clinical outcome.
Taking in consideration the link between neurogenesis and the mechanism of action of antidepressant drugs, and the recent recognized function of Fas/FasL system in neurogenesis and neuritogenesis, it may be hypothesized that the variability of AD therapeutic effect may be influenced by differential expression of Fas/FasL system. Functional polymorphisms in the promoter region of FAS and FASL genes are known to alter the transcriptional activity of these genes. One is an A to G substitution in -670 position of FAS gene, which decreases activity of FAS gene promoter and consequently reduces Fas receptor expression (Huang et al., 1997, Kanemitsu et al., 2002 and Sibley et al., 2003). Another is a thymine (T) to cytosine (C) transition at position -844 of theFASL gene, which is associated with an higher basal expression of FasL (Lima et al., 2014 and Wu et al., 2003). These functional polymorphisms have been associated with a higher risk of cancer development in various models (Zhang et al., 2009a and Zhang et al., 2009b). In neuropsychiatric diseases, one SNP and two haplotypes in FAS gene have been associated with treatment resistant schizophrenia (Jia et al., 2011). Despite the recent implications of Fas in neuroplasticity and neurogenesis, and the importance of these phenomena in depression and antidepressant treatment, FAS polymorphisms never been studied in the context of depression and antidepressant response. In this circumstance, we aim to evaluate the role of FAS -670A>G and FASL -844T>Cfunctional polymorphisms in antidepressant treatment response phenotypes.

2. Material and methods

2.1. Patients

The cohort included participants followed in a 27 months-follow-up study at Hospital Magalhães Lemos (HML) (Carvalho, 2012). Patients with the suspicion of depression were sent to the HML by their family doctor and those whose clinical interview confirmed the diagnosis of depression were submitted to further evaluation for major depression using the Structured Clinical Interview for DSM Axis I Disorders (SCID-I), and for personality disorders using the Axis II Disorders (SCID-II). Severity of the depressive symptoms was evaluated with Beck Depression Inventory (BDI), we used a cut-off of 20 as the point for entry into this study, since the goal was to include moderate to severe major depressive episodes. Subjects with more than one previous depressive episode, severe physical illness, psychiatric disorders with non-depressive psychotic symptoms, substance dependence or personality disorders were excluded from the study. From a total of 92 Portuguese MDD patients which full field the referred criteria, 80 patients agreed to participate in the genetic study.
This cohort included 21 males and 59 females, aged from 18 to 60 years, with a median age of 41.5 years old (mean age 40.48; standard deviation 11.06). Patients were treated according to the Texas Medication Algorithm (Trivedi et al., 2004). Clinical response to adequate antidepressant treatment (administered after at least 6 weeks and at adequate doses) was measured by Beck Depression Inventory (BDI) score. Patients who had a BDI score less than 10 after 6 weeks of at least one single adequate antidepressant treatment and absence of criteria of Major Depression according to SCID-I, were defined as remitters. The treatment outcome phenotype was defined as 'Treatment Resistant Depression' when the patient failed to reach a BDI score less than 10 and present criteria of Major Depression according to SCID-I, after at least two adequate antidepressant treatments with different drugs within the current episode. Relapse was defined as any depressive episode during the 24-month follow up. Bad Prognosis was considered when patient displayed Treatment Resistant Depression or relapsed. The study was approved by the ethical committee of Hospital Magalhães Lemos, and written informed consent according to “The Code of Ethics of the World Medical Association” (Declaration of Helsinki) was obtained from each individual after explanation of the study.

2.2. DNA extraction and FAS -670G>A and FASL -844T>C polymorphism analysis

Peripheral blood samples were collected following standard venipuncture technique in EDTA containing tubes, and the genomic DNA was extracted from the whole blood with a commercial kit (E.Z.N.A. – Omega Bio-tek, Norcross, USA), according to the manufacturer's instructions and stored at −20 °C. The FAS -670G>A (rs1800682) and FASL -844T>C (rs763110) polymorphisms were determined using a Sequenom MassARRAY system (San Diego, CA, USA). The genotyping was undertaken using the SequenomiPLEX platform, according to the manufacturer's instructions (Sequenom, San Diego, CA, USA). The detection of SNP was carried out by analyzing the primer extension products generated from previously amplified genomic DNA using a Sequenom chip-based matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry platform. Sequenom Assay Design 3.1 software was used to design the primers for polymerase chain reaction (PCR) amplification and single base extension assays (Sequenom, San Diego, CA, USA) according to the manufacturer's instructions. Genotyping data was read blindly to the study endpoint. For quality control, 10% of the samples were randomly selected for a second analysis and 100% of concordance was observed.
2.3. RNA extraction and FASL mRNA expression analysis

FAS mRNA expression was analyzed in peripheral blood samples collected from a subset of 38 patients at the end of the study. The subset of patients included 30 remitters and 8 patients with TRD. Patients were similar regarding age, gender and the last psychotropic drugs prescribed prior to blood draw (p>0.05). Whole blood was recovered from patients between 8.30 and 12.30 a.m. RNA was isolated from blood leukocytes using “Tripure” reagent (Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's instructions. The quality and quantity of the RNA were estimated by measuring OD at 260/280 nm and 260 nm respectively, using the “Nanodrop” (ND1000, Nano Drop Technologies Inc. Wilmington, DE, USA). Up to 2 µg of total RNA was reverse transcribed with random primers, using the “High Capacity cDNA Reverse Transcription Kit” (Applied Biosystems, Foster City, CA). Real-time PCR amplification of cDNA samples was performed in a StepOne™ Real-Time PCR System (Applied Biosystems) using TaqMan® Gene Expression Master Mix, primers and probes provided by Applied Biosystems. Expression of FAS was measured with TaqMan expression assay (ID: Hs00236330_m1) from Applied Biosystems. GAPDH gene was selected for normalization from a set of 4 housekeeping genes, ACTB, GAPDH, HPRT and 18S, since it presents higher stability among the MDD samples (data not shown). Samples were subdivided into groups according to their FAS-670A>G genotypes to compare FAS expression levels within them. Relative quantification of the target transcript is based on the mean cycle threshold deviation of AA and AG+GG groups, normalized by reference transcripts. This relative expression analysis was performed using the REST 2009 Software, ver. 2.0.13 (http://www.qiagen.com/Products/REST2009Software.aspx?r=8042). The efficiency of the amplification reaction for each primer-probe is above 95% (as determined by the manufacturer).

3. Statistical analysis

Data preparation and analysis was carried out using the computer software IBM Statistical Package for Social Sciences – SPSS for Windows (version 22.0) and Epi Info (version 6.04a). A 5% level of significance was used in the Chi-square (χ²) analysis to compare the categorical variables. Odds ratio (OR) and 95% confidence interval (CI) were calculated as a measure of association between genotypes and the evaluated phenotypes development risk. Fisher's Exact Test was used for tables containing cells where values are less than 5 individuals. Kaplan-Meier survival curves were used to evaluate correlation between
genotypes and time to relapse and were compared by Breslow statistical test. Further, multiple Cox regression analysis was used to assess the effect of the evaluated polymorphisms on the time to relapse after AD treatment and to adjust for potential confounders. Pair Wise Fixed Reallocation Randomization Test ©, calculated by REST software, was used to determined the relative gene expression between genotype groups. This tool integrates randomization and bootstrapping methods that test the statistical significance of calculated expression ratios by the hypothesis test \( p(H1) \) representing the probability of the alternate hypothesis that the difference between groups is due only to chance (Pfaffl et al., 2002). A posteriori power analysis was performed using Quanto software (version 1.0; http://hydra.usc.edu/gxe). The size of our sample is sufficient to detected association effects for the studied SNPs of at least 2.8 OR, with a statistical power of 80% and a maximal experimental-wise alpha error of 5%.

4. Results

4.1. Antidepressant response phenotypes

The clinical and socio-demographic variables are presented in Table 1. Remission was reported in 61 patients (76.25%), from this 16 remitters (20.0%) presented relapse. Nineteen patients (23,8%) were unable to respond to two trials of different drugs and presented Treatment Resistant Depression. It was considered that patients presenting relapse or TRD, belong to a Bad Prognosis group, which represented 43.8% (35 patients).
4.2. Genotype frequencies of FAS and FASL polymorphisms and treatment outcome

The genotype distribution of \textit{FAS} -670G>A and \textit{FASL} -844T>C polymorphisms were evaluated among the different treatment response phenotypes (Table 2).

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Patients ((n=80))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years mean (SD) [min-max](^a)</td>
<td>40.48 (11.06) [18.0–60.0]</td>
</tr>
<tr>
<td>Gender, female (n) (%)</td>
<td>59 (73.8)</td>
</tr>
<tr>
<td>Ethnicity, Caucasian (n) (%)</td>
<td>80 (100.0)</td>
</tr>
<tr>
<td>School attendance, years mean (SD) [min-max]</td>
<td>9.63 (3.90) [4–20]</td>
</tr>
<tr>
<td>Economic status (n) (%)</td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>30 (37.5)</td>
</tr>
<tr>
<td>Medium</td>
<td>41 (51.3)</td>
</tr>
<tr>
<td>High</td>
<td>6 (7.5)</td>
</tr>
<tr>
<td>Student</td>
<td>3 (3.6)</td>
</tr>
<tr>
<td>Civil Status (n) (%)</td>
<td></td>
</tr>
<tr>
<td>Single</td>
<td>13 (16.3)</td>
</tr>
<tr>
<td>Married</td>
<td>61 (76.3)</td>
</tr>
<tr>
<td>Divorced</td>
<td>14 (17.5)</td>
</tr>
<tr>
<td>Widow</td>
<td>2 (2.5)</td>
</tr>
<tr>
<td>Baseline BDI Score, mean (SD), [min-max]</td>
<td>27.81 (7.40) [20.0–48.0]</td>
</tr>
<tr>
<td>Total time in the study, weeks, mean (SD), [min-max]</td>
<td>62.26 (29.83) [15.0–135.0]</td>
</tr>
<tr>
<td>Remission, (n) (%)</td>
<td>61 (76.26)</td>
</tr>
<tr>
<td>Treatment Resistant Depression, (n) (%)</td>
<td>19 (23.8)</td>
</tr>
<tr>
<td>Relapse, (n) (%)</td>
<td>16 (20.0)</td>
</tr>
<tr>
<td>Bad prognosis(^b), (n) (%)</td>
<td>35 (43.6)</td>
</tr>
<tr>
<td>Last treatment prescribed, (n) (%)</td>
<td></td>
</tr>
<tr>
<td>SSRI(^\text{a})</td>
<td>43 (53.8)</td>
</tr>
<tr>
<td>SNRI(^\text{a})</td>
<td>8 (10.0)</td>
</tr>
<tr>
<td>Tricyclic drugs</td>
<td>36 (45.0)</td>
</tr>
<tr>
<td>Benzodiazepines</td>
<td>25 (31.3)</td>
</tr>
<tr>
<td>Mood stabilizers</td>
<td>9 (11.3)</td>
</tr>
<tr>
<td>Other drugs</td>
<td>9 (11.3)</td>
</tr>
</tbody>
</table>

SSRIs – Selective serotonin recapture inhibitors. SNRIs – Serotonin and norepinephrine reuptake inhibitors.

\(^a\) Remitters: 41.02 (10.98) [18.0–60.0]; Relapse: 41.13 (10.87) [23.0–55.0]; TRD: 38.63 (11.79) [19.0–60.0].

\(^b\) Includes patients with Treatment Resistant Depression plus patients that presented relapse.
No significant deviations from the Hardy–Weinberg equilibrium proportions were observed for both SNPs (p>0.05).

Our results revealed that patients carrying FAS -670 AG or GG genotypes are more prone to have poor prognosis (relapse or TRD: OR=6.200; 95% CI: [1.875–20.499]; p=0.001; Table 2). We also observed that patients carrying these genotypes (AG or GG) have a higher risk to develop TRD (OR=10.895; 95% CI: [1.362–87.135]; p=0.008; Table 2) when compared with remitters. Moreover when compared with patients exhibiting remission without relapse, patients carrying AG and GG genotypes present a 14-fold increased risk of developing TRD (OR=14.40, 95% CI:[1.76–117.4]; p=0.003).

Despite not influencing the risk of relapse, FAS -670A>G polymorphism seems to influence time to relapse after AD treatment. Kaplan–Meier analysis (Fig. 1) revealed that carriers of the FAS -670 AG or GG genotypes seem to relapse after 88 weeks, while patients carrying AA genotype relapse after 100 weeks (p=0.032, Breslow test). Moreover, multivariate analysis adjusted to potentials confounders (gender, age, school years and marital status) showed that patients carrying AG or GG genotypes have higher risk to early relapse (HR=3.827; 95% CI: [1.072–13.659]; p=0.039).
Regarding *FASL* -844T>C genetic polymorphism no association was found between genotypes and any of the studied treatment phenotypes.

### 4.3. Peripheral Fas mRNA expression

To verify whether the different *FAS* -670A>G genotypes modulate the expression of FAS transcripts, we evaluated mRNA expression by quantitative real-time PCR in the peripheral blood of MDD patients whose RNA samples were available (38 individuals). Results demonstrated a decrease in FAS expression among AG+GG carriers, whose genotypes were more common in TRD patients (Fig. 2). In this group of patients, FAS mRNA was down-regulated (in comparison to carriers of AA genotype) by a fold of change of 0.628 ($p=0.032$; 95% CI: [0.070–5.732]).
5. Discussion

Our data suggests an association of FAS -670A>G genetic polymorphism with resistance to antidepressant treatment and poor prognosis in depressed patients. Moreover, we also observed an association of this polymorphism and time to relapse. Additionally, we detected that Fas mRNA levels were downregulated in patients carrying AG+GG genotype configuration. These results point towards a putative connection between decreased FAS activity and impaired antidepressant treatment response.

Genetic polymorphisms in FAS gene have been associated with treatment resistant schizophrenia (Jia et al., 2011), Alzheimer disease (Erten-Lyons et al., 2010) and cancer (Han et al., 2013), but never have been studied in depression and antidepressant response. In this context, we evaluated the role of FAS and FASL polymorphisms in antidepressant treatment response in a cohort of depressed patients with a 27 month follow up.

Our results revealed that patients carrying FAS -670 AG or GG genotypes are more prone to have poor prognosis and have a higher risk to develop TRD. Functional studies of FAS gene showed that the -670A to G transition in the promoter region of FAS disrupt a STAT1 transcription factor binding site, which diminish the promoter activity and consequently down-regulate gene expression (Huang et al., 1997 and Sibley et al., 2003). These evidences suggest that the lack of remission may be associated with a reduced Fas receptor expression, originated from decreased activity of FAS gene promoter. So, how can a low expression of Fas, a molecule naturally involved in apoptotic cascades, could be a factor associated with Treatment Resistant Depression and poor prognosis?

Fas has been known to be broadly expressed in the brain, particularly in the hippocampus and cerebral cortex (Beier and Schulz, 2009). Besides regulating
apoptosis, recent evidences implicate Fas in neurogenesis activation, both in healthy and injured brain, and in neuronal branching of mature neurons (Corsini et al., 2009 and Zuliani et al., 2006). Studies of Zuliani et al. (2006) have shown that differential trigger of Fas neurite remodeling cascade or Fas apoptosis cascade is related with the surrounding milieu. These authors argued that factors present in the injured CNS, such as reactive oxygen species, increased lactate or decreased ATP levels might sensitize the cell to Fas-induced apoptosis (Zuliani et al., 2006). Alternatively, under the influence of other extracellular factors, such as neurotrophins, slits, ephrins and integrins, neurons may undergo synaptic reorganization in the presence of FasL (Zuliani et al., 2006). In this context, low expression of Fas can be responsible for impaired neuroplasticity, characterized by cognitive deficits, pyramidal neuron dendrites atrophy, reduced dendritic branches and delayed neurite regeneration, which has been observed in FAS deficient mice (lpr) (Lambert et al., 2003 and Zuliani et al., 2006).

These observations are consistent with the downregulation of FAS expression found among AG+GG carriers, whose genotypes were more common in TRD patients. A paper from Banzato, et al., (2013) also found lower FAS mRNA levels among GG genotype carriers. Further evidence for a functional role of Fas in this context was reported elsewhere describing an increase expression in Fas plasma membrane of 31% upon antidepressant treatment (Xia et al., 1996).

Besides being linked with antidepressant treatment resistance, we also observed that individuals carrying FAS -670 AG or GG genotypes develop treatment relapse earlier than the one that present FAS -670AA genotype. Patients carrying these genotypes also have higher risk to early relapse (HR=3.827). This result reinforces the impact of FAS gene polymorphism in treatment outcome, specifically the putative impact of Fas in neuronal remodeling.

With these evidences and our results we were able to think that Fas/FasL system seems to be involved in Treatment Resistant Depression, and this condition could hypothetically result of an incomplete reestablishment of neurogenesis/synaptic plasticity network, due to the reduced levels of Fas expression promoted by FAS -670G allele. Our hypothesis is in accordance with what is proposed by others authors, which have reported several neuroplasticity and proliferation-related intracellular pathways to be involved in antidepressant mechanism of action, among these Brain-derived neurotrophic factor (BDNF), b-catenin and mammalian TOR (Pilar-Cuellar et al., 2013).
The present study has some obvious limitations. First limitation is related with the use of drugs with different mechanisms of action, which difficult the interpretation of the effect of studied polymorphisms on a specific drug. However patients were treated according to the Texas Medication Algorithm and this approach is more similar to the real word, since most of the time patients are treated with different drugs and still display treatment resistance. Other limitation is related with the sample size issue. However, this study might be, to the best of our knowledge, the largest one studding the influence of genetic variants in antidepressant response in Portuguese population. Nevertheless, and despite sample size, the calculated statistical power is still sufficient to detect the magnitude of the effect reported. In order to overcome this limitation, further studies with larger sample size are required to reinforce the evidences we presented. The third limitation is related with the fact that we correlated FAS genotypes with peripheral levels of FAS transcripts, not with brain transcript levels. However, as far as we know, there are no evidences linking Fas expression in the brain upon antidepressant treatment. FASmRNA expression in hippocampus and pre-frontal cortex of postmortem tissue of depressed patients, undergoing antidepressant treatment, could be used to confirm the evidences presented herein. 

To the best of our knowledge, this is the first study to evaluate the role of FAS functional polymorphism in the outcome of antidepressant therapy. Our results demonstrate that FAS -670A>G genetic polymorphism has a role in antidepressant treatment response in our subset of MDD patients. This result might be explained by the putative influence of Fas in neurogenesis and/or neuroplasticity network, whose reestablishment is known to be essential for AD therapeutic effect. Due to growing progresses in this area, this marker should to be integrated with others to detect the genetic susceptibility of complex conditions, such as Treatment Resistant Depression.

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