Influence of Vitamin K Epoxide Reductase Complex 1 Gene Polymorphisms on Anticoagulation with Acenocoumarol in Patients with Cerebral Venous Thrombosis

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Authors’ contributions

This work was carried out in collaboration between all authors. Author TD contributed to the conception and design of the study, acquisition and analysis of data, genetic analysis and manuscript writing. Author RC contributed to conception and design of the study, carried out overall supervision of the work, revised and approved the final version of the paper. Author DN provided the study materials and contributed to interpretation of the data.

ABSTRACT

Aims: Acenocoumarol, a commonly prescribed oral anticoagulant drug, exhibits wide inter-individual variability in response. This study aimed at evaluating the contribution of genetic variations in Vitamin K epoxide reductase complex, subunit 1 (VKORC1), to variability in the response to acenocoumarol, in patients with cerebral venous thrombosis (CVT).

Place and Duration of Study: National Institute of Mental Health and Neuro Sciences, Bangalore, India, between September 2009 and January 2013.

Methodology: 476 acenocoumarol-treated aseptic CVT patients (153 males, 323 females) were genotyped for \textit{VKORC1} -1639G>A and 1173C>T polymorphisms. Mean daily acenocoumarol dose for achieving and maintaining the optimum international normalized ratio (INR) was calculated for different genotypes.

Results: Genotype distribution of \textit{VKORC1}-1639G>A was as follows: 69.7% were wild,
25.6% heterozygous and 4.6%, mutant. Mean acenocoumarol dose required to achieve the optimum INR was lower in heterozygous (1.82±0.71mg/day) and homozygous mutants (1.75±0.69mg/day) when compared to wild type patients (2.31±0.89mg/day). Bearing the \textit{VKORC1} -1639A allele independently increased the odds of requiring a low dose (Adjusted OR 3.9; 95% CI 1.97-7.73; \textit{p}<0.0001). Significant differences in dose requirement during maintenance phase were observed in patients of different genotypes. \textit{VKORC1} -1639G>A and 1173C>T were observed to be tightly linked ($r^2=0.98$) and no difference in the genotype distributions was observed between the two polymorphisms. Factors such as age and co-medication with phenytoin were also found to influence the drug dosage.

**Conclusion:** Our findings support the use of \textit{VKORC1} genotyping during anticoagulation with acenocoumarol in patients with CVT.

**Keywords:** Cerebral venous thrombosis; acenocoumarol; vitamin K epoxide reductase complex 1 (VKORC1); polymorphism; linkage disequilibrium.

1. **INTRODUCTION**

Thrombosis of the venous channels in the brain is an atypical cause of cerebral infarction compared to arterial disease, but, it is important because it can cause substantial morbidity and mortality[1]. Management of cerebral venous thrombosis (CVT) involves treatment of the underlying condition, treatment of the clinical complications, symptomatic therapy and typically, anticoagulation therapy [2]. Initial anticoagulation with heparin is followed by treatment with oral vitamin K antagonists. Acenocoumarol, a coumarin type of oral anticoagulant commonly used in India, has a narrow therapeutic index and needs careful monitoring during initiation of therapy as well during maintenance. Numerous challenges exist in achieving effective anticoagulation during initiation of therapy, as small dose variations may result in hemorrhagic or thrombotic complications [3]. Moreover, once a suitable dosage of acenocoumarol has been established, control of therapy can be affected by diet, development of acute medical conditions (e.g., fever, diarrhea) and interactions with co-prescribed drugs [4].

Anticoagulant activity of acenocoumarol results from inhibition of hepatic vitamin K epoxide reductase (VKOR), that affects the synthesis of various coagulation factors [5]. Recent studies have shown that certain single nucleotide polymorphisms (SNPs) in the \textit{VKORC1} gene are associated with reduced efficacy of vitamin K recycling as a result of lower VKOR activity [6], and account for inter-individual variability in dose-anticoagulant effect response [7]. \textit{VKORC1} -1639G>A and 1173C>T polymorphisms have been found to be major determinants of the response to acenocoumarol in anticoagulated Caucasians [8]. While -1639G>A SNP is located in the promoter region of \textit{VKORC1} and has been associated with a significantly lower acenocoumarol dose requirement than the wild-type [9], 1173C>T polymorphism located in the intronic region was also found to influence the acenocoumarol dose [10].

The influence of \textit{VKORC1} genotypes on acenocoumarol dose requirement has been determined in studies of patients with deep vein thrombosis, pulmonary thromboembolism and valve replacement surgery [11,12], the pathophysiology of which are different to that of CVT [13]. While deep vein thrombosis and pulmonary embolism are frequently associated with trauma, immobilization or surgery, CVT has been found to be triggered due to hypercoagulable state, and is less frequently associated with surgery and immobilization or
No study has investigated the influence of VKORC1 variants on the effective dosing of acenocoumarol in patients with CVT; a unique thrombotic condition necessitating long-term, low-intensity anticoagulation therapy, in addition to long-term medication with anti-epileptic drugs, in a majority of cases. The aim of this study was to evaluate the contribution of VKORC1 gene -1639G>A and 1173C>T polymorphisms to the acenocoumarol dose required for induction and maintenance of stable, effective anticoagulation in a large cohort of aseptic CVT patients.

2. MATERIALS AND METHODS

2.1 Study Group

The study was approved by the Ethics Committee of National Institute of Mental Health and Neuro Sciences (NIMHANS), a tertiary care centre for neurological disorders located in Bangalore, India. Written informed consent was obtained from all participants. We recruited 476 first-ever aseptic CVT patients who were prescribed acenocoumarol for a period of 6 months or more. Diagnosis of CVT was confirmed by magnetic resonance imaging (MRI)/MR venography. Patients were excluded when they were (i) diagnosed with thyroid disease, severe congestive cardiac failure, renal or liver dysfunction (ii) CVT was secondary to head trauma, invasive procedures, sepsis, neuroinfection or malignancy or any other terminal illnesses (iii) treated with other CYP2C9 inducers like rifampin, or inhibitors like valproic acid. The base-line demographic data, history of conventional vascular risk factors and family history of vascular events were recorded. Puerperal CVT was diagnosed when CVT occurred during the first four weeks after childbirth. Women were considered to be on oral contraceptives (OCP) if they had taken them until a week or less before the thrombotic event.

Blood was collected to measure anticoagulation before and after institution of oral acenocoumarol. Anticoagulation was measured by International Normalised Ratio (INR). According to World Health Organization (WHO), different INR ranges have been recommended in various clinical states [14]. Long-term, low-intensity anticoagulation with an INR range of 1.5-2.0 is preferred for the treatment of CVT, as it reduces the risk of recurrent thrombosis [15].

Acenocoumarol dosing was separated into initial and maintenance phases. During the initiation or induction phase, the mean daily dose of acenocoumarol required for induction of anticoagulation was calculated from the sum of the drug administered and the time (in days) taken to achieve the stable therapeutic INR of 1.5-2.0, at three consecutive determinations. After induction of anticoagulation, patients were monitored and the INR was maintained by dose titration for a minimum period of six months. During the maintenance phase, the daily acenocoumarol dose, defined as 3 consecutive follow-up visits having INR measurements within the therapeutic range at the same mean daily dose, was recorded.

2.2 Estimation of INR

Plasma prothrombin time was estimated by the Quick method using liquid thromboplastin reagent with an ISI (International Sensitivity Index) of 1.60 in a semi-automated coagulation analyzer (ST-4, Stago, France). INR was calculated as INR = (PT patient/PT normal)\(^{10}\).
2.3 Analysis of VKORC1 Polymorphism

Genomic DNA was extracted from blood using the conventional phenol-chloroform extraction method and quantified by using NanoDrop 2000 (Thermo Fisher Scientific, MA, and USA). VKORC1 1173C>T (rs 9934438) PCR was done using the following forward and reverse primers: sense primer 5′-AGAGACTTTACTTAAAGGTCTA-3′; anti-sense primer 5′-TTCCAAAGGCCACCTGGGC-3′ [16]. PCR products of 200 bps were then digested with StyI at 37°C for 16 h and were electrophoretically separated on a 2% agarose gel. The 1173C allele is characterized by diagnostic StyI restriction fragments of 144 and 56 bps. The gel pattern for individuals with 1173CC homozygous wild type showed 144 and 56 bps bands whereas homozygous mutant genotype (1173TT) did not undergo digestion by StyI. Individuals with 1173 CT heterozygous genotype showed both intact PCR band of 200bps and two cleaved bands of 144 and 56bps.

Genotyping for VKORC1 -1639G>A polymorphism (rs 9923231) was done using sense primer 5′-GCCAGCAGGAGGGAAATA-3′; anti-sense primer 5′-AGTTTGGACTACAGGTGCCT -3′ primers A [17]. PCR products of 290bp were then digested with MspI at 37°C for 16 h and were electrophoretically separated on a 2% agarose gel. The 1639G allele is characterized by diagnostic MspI restriction fragments of 168 and 122 bps. Thus the gel pattern for individuals with 1639GG homozygous wild type showed 168 and 122bp bands whereas homozygous mutant genotype (1639AA) did not undergo digestion by MspI. Individuals with 1639 GA heterozygous genotype showed an intact PCR band of 290 bps and two cleaved bands of 168 and 122bp. The results of genotyping were confirmed by sequencing of the PCR products.

2.4 Statistical Analysis

Statistical analysis was performed using SPSS v.16.0 (Corporation, NY, USA) and GraphPad prism version 5.0.1 (Graph Pad Software, Inc. La Jolla, USA). Genotype frequencies were determined using standard frequency analysis and deviations of allelic frequencies from Hardy-Weinberg equilibrium was evaluated by $\chi^2$ test. Differences in baseline characteristics between different subgroups were assessed by the $\chi^2$ test for categorical variables and Mann-Whitney test for continuous parameters. Initial and maintenance dose, when analyzed as a continuous variable, was expressed as the mean ± standard deviation (S.D.) and pair-wise comparison between different genotypes was done using one-way analysis of variance (ANOVA). Dose was also treated as an ordinal variable [low (<2mg/day), moderate (≥2-<4mg/day), and high (≥4mg/day)] and the relation between the ordinal variable dose and allelic variants was established by univariate odds ratio (OR) at 95% confidence interval (CI). A multinomial logistic regression was used to assess the influence of certain parameters (age, alcohol, puerperium, oral contraceptive use (OCP) and phenytoin) on the probability that a patient would receive a low or a high dose of acenocoumarol. pvalue <0.05 was considered statistically significant. Using the power and sample size calculation software G*Power, version 3.1.7 (Heinrich Heine University Düsseldorf, Germany), for a power of 95% and a significance level of 5%, the sample size was calculated to be a total of 150 for the study.
3. RESULTS AND DISCUSSION

3.1 Characteristics of the Study Group

The study group comprised of 476 (153 males and 323 females) CVT patients with a mean age of 29.94±10.12 years. Follow up information for a period of six months could be obtained only for 300 patients (111 males and 189 females), as some of the patients did not report for follow-up till this period. Demographic and clinical characteristics of patients are summarized in Table 1. 78% patients were less than 40 years age, 26% consumed alcohol, 35.9% of female patients were diagnosed with puerperal CVT and 10.6% of the female patients were on oral contraceptives till the occurrence of thrombotic event. 76% of patients had seizures and were administered with phenytoin.

Table 1. General characteristics of the study group and acenocoumarol dose requirement during induction of anticoagulation

| Characteristics            | No. of patients n(%) | Acenocoumarol dose |   |   |   |
|----------------------------|-----------------------|--------------------|---|---|---|
|                            |                       | Low (<2mg/day) n(%)| Medium (≥2-<4mg/day) n(%) | High (≥4mg/day) n(%) | p Value |
| Age <40 years              | 234 (78)              | 50 (21.3)          | 166 (71)                     | 18 (7.7)             | <.001* |
| Age ≥ 40 years             | 66 (22)               | 30 (45.5)          | 36 (54.5)                    | -                    | .41   |
| Male                       | 111 (37)              | 35 (31.5)          | 74 (66.7)                    | 2 (1.8)              | .52   |
| Female                     | 189 (63)              | 45 (23.8)          | 128 (67.7)                   | 16 (8.5)             | .93   |
| Alcohol consumption        | 75 (26)               | 21 (28)            | 52 (69.3)                    | 2 (2.7)              | .16   |
| Without alcohol            | 225 (74)              | 59 (26.2)          | 150 (66.7)                   | 16 (7.1)             | .005* |
| Puerperium                 | 68 (35.9)             | 13 (19.1)          | 49 (72.1)                    | 6 (8.8)              |        |
| Non puerperium             | 121 (64.1)            | 32 (26.4)          | 79 (65.3)                    | 10 (8.3)             |        |
| OCP use                    | 20 (10.6)             | 2 (10)             | 15 (75)                      | 3 (15)               |        |
| Without OCP               | 169 (89.4)            | 43 (25.4)          | 113 (66.9)                   | 13 (7.7)             |        |
| On phenytoin              | 228 (76)              | 69 (30.2)          | 146 (64)                     | 13 (5.8)             |        |
| Without phenytoin         | 72 (24)               | 11 (15.3)          | 56 (77.8)                    | 5 (6.9)              |        |

OCP- oral contraceptives; * p Value <0.05 is statistically significant

3.2 Association of Clinical Characteristics with Acenocoumarol Dose

Patients were divided into 3 groups based on the mean daily acenocoumarol dose required to achieve the optimum INR during the initiation phase: low (<2mg/day), medium (≥2-<4mg/day), and high (≥4mg/day). Majority of patients aged ≥40 years required either a moderate (54.5%) or low dose (45.5%) of acenocoumarol compared to those aged <40 years who required either a high (7.7%) or medium dose (71%) (p=0.0001). Patients aged ≥40 years had 3-fold increased odds of requiring low dose of acenocoumarol (Adjusted OR, 3.04; 95%CI, 1.59-5.81; p=0.001), compared to those < 40 years, when adjusted to other clinical covariates influencing drug dosage. Alcohol consumption was reported only by male patients. Puerperal state, OCP use, alcohol consumption and gender did not show any association with variations in acenocoumarol dosage.

Significant differences (p=0.005) in dose requirement were observed between patients treated with phenytoin and patients who were not on phenytoin. While 30.2% of phenytoin-treated patients required lower acenocoumarol dose, only 15.3% of non-phenytoin treated
patients required a low dose to achieve optimum anticoagulation. Co-administration of phenytoin increased the odds of requiring a lower dose, 2.89-fold (Adjusted OR, 2.89; 95% CI, 1.38-6.09; *p* =0.005).

### 3.3 Prevalence of VKORC1 Genotypes

The genotype distributions were determined for all the 476 patients. Allelic frequencies for both VKORC1 1173C>T and -1639G>A were in Hardy–Weinberg equilibrium. The genotype distribution of VKORC1 -1639G>A was as follows: 69.7% of patients had the wild (GG) genotype, 25.6% were heterozygous (GA) and 4.6% had homozygous mutant AA genotype. (Table 2). There was no difference in the distribution of GG, GA and AA genotypes of VKORC1 -1639G>A, and CC, CT, TT genotypes of VKORC1 1173C>T. Complete linkage disequilibrium between these polymorphisms was observed (*r*² = 0.98). Patients with -1639 heterozygous AG genotype were found to have 1173 heterozygous CT genotype and patients with the homozygous -1639 GG were found to have 1173 homozygous CC genotype.

| VKORC1     | Genotype frequency (N=476) | Allele frequency |
|------------|----------------------------|------------------|
| 1639G>A    | GG 332 (69.7)               | G=0.83           |
|            | GA 122 (25.6)               | A=0.17           |
|            | AA 22 (4.6)                 |                  |
| 1173C>T    | CC 332 (69.7)               | C=0.83           |
|            | CT 122 (25.6)               | T=0.17           |
|            | TT 22 (4.6)                 |                  |
| VKORC1     | 1173C>T                    | D' 106.31        |
| 1639G>A    |                            | LOD 1            |
|            |                            | *r*² 0.98        |

* *p* Value <0.05 is statistically significant

### 3.4 Association of VKORC1 Polymorphism with Acenocoumarol Dose

The mean daily acenocoumarol initiation dose was found to be significantly lower (*p*<0.0001) in patients with VKORC1 -1639 heterozygous (1.82±0.71 mg/day) and homozygous mutant genotype (1.75±0.69 mg/day) when compared to patients with the wild genotype (2.31±0.89 mg/day). Significant differences in the daily dose requirement were observed during the maintenance phase between patients of different genotypes (Table 2). Wild type patients required 3.39±1.36 mg/day to maintain stable anticoagulation while heterozygous patients required 2.58±0.79 mg/day. Homozygous mutant patients showed similar dose requirement (2.58±0.73 mg/day) as heterozygous patients.

When the patients were classified according to their dosage requirement into low (<2mg/day), moderate (≥2-<4mg/day), and high (≥4mg/day) dose groups, majority of the patients with wild and heterozygous genotype required a moderate dose of acenocoumarol, during both initial and maintenance phases (Table 3). However, patients with the mutant genotype required low dose (60%) during initiation but moderate dose (85%) during maintenance of anticoagulation. Carriers of the mutant allele showed increased odds of requiring a low dose (Adjusted OR, 3.9; 95% CI, 1.97-7.73) during the initiation phase
whereas during the maintenance phase the difference in dose requirement was not significant (Adjusted OR, 1.71; 95% CI, 0.66-4.45).

Table 3. Association of VKORC1 variants with acenocoumarol dosage in CVT patients and odds of requiring a low dose

| VKORC1 -1639G>A Genotype | Patients followed up (N=300) | Mean dose±SD (mg/day) | p Value | Acenocoumarol dose (mg/day) |
|---------------------------|-----------------------------|-----------------------|---------|-----------------------------|
|                           |                             |                       |         | Low (<2) n(%)               | Medium (≥2-<4) n(%) | High (≥4) n(%) |
| Initiation phase          |                             |                       |         |                             |                     |               |
| GG                        | 200                         | 2.31±0.89             | -       | 40 (20)                     | 145 (72.5)          | 15 (7.5)      |
| GA                        | 80                          | 1.82±0.71             | <0.0001 | 28 (35)                     | 50 (62.5)           | 2 (2.5)       |
| AA                        | 20                          | 1.75±0.69             | <0.0001 | 12 (60)                     | 8 (40)              | -             |
| Maintenance phase         |                             |                       |         |                             |                     |               |
| GG                        | 200                         | 3.39±1.36             | -       | 16 (8)                      | 108 (54)            | 76 (38)       |
| GA                        | 80                          | 2.58±0.79             | <0.0001 | 10 (12.5)                   | 67 (83.75)          | 3 (3.75)      |
| AA                        | 20                          | 2.58±0.73             | <0.0001 | 3 (15)                      | 17 (85)             | -             |
| VKORC1 1639GG Vs GA+AA    |                             |                       |         | Crude OR at 95% CI          | Adjusted OR, 95% CI | p Value      |
| Initiation phase          | 2.5 (1.47-4.26)             | 3.9 (1.97-7.73)       | .27     |                             |                     |               |
| Maintenance phase         | 1.05 (0.48-2.29)            | 1.71 (0.66-4.45)      |         |                             |                     |               |

* p Value <0.05 is statistically significant
§ adjusted for age, OCP use, puerperium and phenytoin

The etiology of CVT is multi-factorial, involving acquired and genetic factors and diverse conditions have been recognized as risk factors for this condition [18]. The primary treatment of CVT involves long-term, low-intensity anticoagulation therapy, as recurrence of CVT is potentially fatal [15]. Oral anticoagulation therapy is recommended for 6–12 months in patients with idiopathic CVT and in those with an underlying prothrombotic state [19], and for lifelong in patients with multiple persistent risk factors [20]. In addition, prolonged medication with antiepileptic drugs are prescribed for patients with seizures [21]. Acenocoumarol is a frequently used anticoagulant drug, and individualization of acenocoumarol therapy based on genetic factors is one of the most promising clinical applications of pharmacogenetics. Because of its low therapeutic index and a large variability in dose-dependent anticoagulant effect response, patients on treatment with this drug require constant medical supervision in order to maintain the target INR. Lack of careful monitoring may lead to significant clinical consequences like inadequate or excessive pharmacologic effects which could in turn lead to an increased risk of thrombo-embolic or hemorrhagic events. This study aimed to establish the association between the polymorphisms of the acenocoumarol target gene (VKORC1) and the effective doses required to achieve and maintain the target INR.

We observed the VKORC1 -1639G to be the most prevalent (0.83) allele in our population; a result that is dissimilar to findings in Chinese [22], Japanese [23] and Caucasian populations [24], but consistent with previously reported studies from India [25]. In our patients we also found that VKORC1 -1639G>A and 1173C>T polymorphisms were in strong linkage disequilibrium ($r^2=0.98$), a finding which is consistent with previous reports suggesting that both the polymorphisms were equally informative on the genetic influence of VKORC1 on warfarin dose within different racial groups [26]. In an earlier study, D’Andrea et al. observed no alternative mRNA splicing mechanism associated with VKORC1 1173C>T polymorphism
and concluded that the polymorphism might be in linkage disequilibrium with other variants that modify the VKOR activity [7]. Another study by Bodin et al. detected complete linkage disequilibrium between \textit{VKORC1} -1639G>A and 1173C>T polymorphisms [8]. Hence our results are consistent with these reports.

\textit{VKORC1} -1639G>A polymorphism has been reported to be associated with decreased enzyme activity and thus lower dose requirement in the carriers of the mutant alleles [27]. A study on Romanian population showed a higher odds of requiring a low dose in subjects with \textit{VKORC1} mutation (-1639GA genotype= OR, 6.5; 95%CI, 1.38–30.5; \(p=0.01\); AA genotype= OR, 11.6; 95%CI: 2.26-59.58; \(p=0.003\)) [28]. Our study confirmed the association of this polymorphism with low dosage of acenocoumarol (\(p<0.0001\)), and showed approximately 4-fold increased odds of requiring a low acenocoumarol dose for induction of anticoagulation, when adjusted with age, smoking, OCP use, puerperium and phenytoin (Adjusted OR, 3.9; 95% CI,1.97-7.73). However, the influence on acenocoumarol dose was confined to only the first month of anticoagulation treatment. Our findings are supported by other studies [24,25,29] which observed the influence of this polymorphism on warfarin response only during the first month of anticoagulation therapy.

The present study showed that clinical factors such as age, puerperium, oral contraceptive use and co-medication with other drugs like phenytoin modulate acenocoumarol dosage. Previous studies have documented an inverse correlation between acenocoumarol dosage and age of the patients [30]. Tassies et al., reported a 3.7-fold increased odds of needing a lower acenocoumarol dose in patients aged more than 70 years [31]. We observed 3-fold increased odds of requiring low dose of acenocoumarol in patients aged \(\geq\)40 years compared to those <40 years, when adjusted to other clinical covariates influencing drug dosage. The mechanism for increased sensitivity to acenocoumarol with aging is not well-understood. Factors such as hypoalbuminemia (leading to a reduced volume of distribution), decreased dietary vitamin K intake (resulting in a decreased capacity to synthesize functional clotting factors), reduced absorption of vitamin K, and polypharmacy (producing drug-drug interactions that potentiate acenocoumarol) may play a role.

Phenytoin is a widely used anti-epileptic drug for the treatment of seizures in CVT patients. Majority of patients in our study (76%) presented with seizures and were co-administered with phenytoin. Both acenocoumarol and phenytoin share a common major metabolic enzyme, Cytochrome P4502C9 (CYP2C9) [32]. Panegyres et al. [33] described a fatal interaction of phenytoin and warfarin causing spontaneous retroperitoneal hemorrhage in a patient with right occipital infarction, and later death due to cardiac arrest. We observed significant alteration in acenocoumarol dose requirement in patients treated with phenytoin. A 2.89-fold increased risk of requiring lower acenocoumarol dose during the initiation period of anticoagulation was detected in patients treated with phenytoin compared to patients not on phenytoin.

Patients treated with coumarin-based oral anticoagulants are susceptible to numerous other drug interactions. Drugs like clopidogrel, nonsteroidal anti-inflammatory drugs and antibiotics, increase the risk of major hemorrhage, as a consequence of interactions with coumarinic oral anticoagulants [34]. Thus, careful monitoring of the effects of these drugs is required when co-medicating CVT patients on anticoagulation treatment. Moreover, further studies on the effect of co-administration of these drugs in patients in CVT are warranted.
4. CONCLUSION

In conclusion, the study highlights the need for clinicians to consider genetic as well as non-genetic factors when medicating CVT patients with acenocoumarol. Genotype-guided dosing can decrease inter-individual variability in acenocoumarol response in patients with CVT, and minimize adverse drug effects, thus improving clinical outcomes and a patient’s quality of life. Further studies to identify other genes and to elucidate their effect on acenocoumarol response are needed.

CONSENT

All authors declare that written informed consent was obtained from the patient (or other approved parties) for undertaking this study.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the Ethics Committee of the National Institute of Mental Health and Neuro Sciences, Bangalore, India, and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki

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COMPETING INTERESTS

The authors have no competing interest.

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