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ORIGINAL PAPER

Further evidence for the association between a polymorphism in the promoter region of SLC6A3/*DAT1* and ADHD: findings from a sample of adults

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Abstract The dopamine transporter (SLC6A3/*DAT1*) plays a key role in the regulation of dopaminergic neurotransmission and is the major site of action for methylphenidate, a first-line medication for attention deficit hyperactivity disorder (ADHD). Most genetic association studies with ADHD have investigated a 40-bp variable number of tandem repeats (VNTR) polymorphism in the 3′-untranslated region (UTR) of the *DAT1*, but these investigations have reported heterogeneous findings. The few studies focused on the 5′ region have reported promising results. Despite rs2652511 not being included, nor having any proxy SNP available in GWAS, the few candidate gene studies that analyzed it suggested an association with ADHD and schizophrenia. Here, we analyzed the −839 C/T (rs2652511) promoter variant and the 3′-UTR and intron 8 (Int8) VNTR polymorphisms in 522 adults with ADHD and 628 blood donor controls. The diagnostic procedures followed the DSM-IV criteria. A significant association was detected ($P = 0.002$) between the rs2652511 C-allele with ADHD. In addition, the 6-repeat allele of Int8 VNTR was associated with higher inattention scores ($P = 0.034$). The haplotype analysis including *DAT1* 3′-UTR and Int8 VNTR polymorphisms did not reveal associations with ADHD susceptibility or severity dimensions. These findings extend to adult samples previous findings from children samples on the role of the rs2652511 polymorphism in the promoter region of *DAT1* as a risk factor for ADHD susceptibility.

Keywords ADHD · Dopamine transporter gene · Polymorphism · Promoter

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Introduction

Attention deficit hyperactivity disorder (ADHD) is an early-onset and highly heritable neuropsychiatric disorder characterized by symptoms of inattention, hyperactivity, and impulsivity, persisting into adulthood in up to 60 % of the cases [1]. Its prevalence was estimated at 5.29 % in school-aged children [2] and 2.5 % in the adult population [3].

Dopaminergic signaling proteins are involved in ADHD pathophysiology. Dopamine transporter (*SLC6A3*, also known at *DAT1*) regulates neurotransmission by terminating dopamine signaling at the synapse through

high-affinity reuptake of dopamine into presynaptic terminals [4]. The association between *DAT1* and ADHD was suggested in linkage and association studies [5] and confirmed in meta-analyses [6, 7], showing small but significant effects on the susceptibility to ADHD. A 40-base pair (bp) variable number of tandem repeats (VNTR) polymorphism in the 3′-untranslated region (UTR) of the gene has received most attention in association studies with ADHD, and meta-analyses have suggested heterogeneity in odds ratios among the findings [6, 7]. Some studies suggest that the 10-repeat allele of the 3′-UTR VNTR might only increase ADHD risk in children in a haplotype with allele 6 in the intron 8 (Int8) VNTR [8, 9]. On the other hand, the first association study of the two *DAT1* VNTR and ADHD in adults could not confirm the relationship between the 10–6 haplotype and persistent form of the disorder [10]. In other studies, the 9–6 haplotype was associated with ADHD in adults [11, 12].

Despite both in vivo and in vitro studies investigated allele-specific expression in *DAT1* VNTR polymorphisms, a clear picture of the functional variation that might predispose to ADHD has not been achieved [8, 13]. On the other hand, some studies started to suggests the presence of independent functional variants at the 5′ end of the gene [14]. Greenwood and Kelsoe [15] observed that polymorphisms in the promoter region of the *DAT1* were involved in differential expression. Other groups have identified SNP associations with ADHD in the 5′ region of the *DAT1* gene. For example, Genro et al. [16] using a sample of children detected association between the rs2652511 C-allele in the susceptibility for ADHD. In addition, Brookes et al. [8] demonstrated that four SNPs located in the 5′ region of the *DAT1* gene were associated with ADHD in children (rs2652511, rs2550946, rs550948, rs11564750). Interestingly, linkage disequilibrium does not extend across the entire *DAT1* gene, allowing for independent effects of 5′ and 3′ regions on psychiatric disorders. It is noteworthy that independent studies of *DAT1* 5′ promoter polymorphisms of the *DAT1* gene suggested an association between rs2652511 C-allele and susceptibility for ADHD in children [8, 16, 17], but this specific finding has not been replicated in adult ADHD samples. Since this SNP is not included and does not match any proxy SNP in available GWAS arrays, it is not surprising that no previous GWAS finding presented a nominally significant association between rs2652511 and ADHD.

In the current study, we attempted to replicate the association of the rs2652511 C-allele in a sample of adults with ADHD. Our analysis also addressed the role of the two most studied polymorphisms (Int8 and 3′-UTR VNTR polymorphisms) in *DAT1*.

Materials and methods

Subjects

The ADHD sample included 522 adult patients that were recruited consecutively in the ADHD outpatient program of the Hospital de Clínicas de Porto Alegre (HCPA). The average age of the sample was 34 years (SD = 11.1). The inclusion criteria were (a) native Brazilian of European descent, (b) aged 18 years or older, and (c) fulfillment of DSM-IV diagnostic criteria for ADHD [1], both currently and during childhood. The exclusion criteria were (a) evidence of clinically significant neurological diseases that might affect cognition (e.g., delirium, dementia, epilepsy, head trauma, multiple sclerosis), (b) current or past history of psychosis, and (c) intelligence quotient (IQ) $\leq$70 [18]. Data on symptom dimensions were available for 420 patients.

The control sample was composed by 628 blood donors recruited at the blood bank of the same hospital. The inclusion criteria were being both native Brazilians of European descent and at least 18 years old. The exclusion criteria for controls were the same used for the patients and the lack of DSM-IV ADHD diagnosis. Both samples have been characterized elsewhere [19, 20].

Regarding ethnicity, the population from the southernmost state of Brazil is mainly of European descent [21], and no significant population structure was found in the European-derived population of Rio Grande do Sul [22]. The inter-ethnic admixture estimated that individuals from southern Brazil present a predominantly European ancestry (94 %) [23], making population stratification unlikely to occur in this situation [24]. Moreover, we used morphological classification based on skin color and morphological traits combined with self-classification for ethnicity. Thus, cases and controls were included in the study if, in addition to morphological characteristics of European ancestry, they informed to have grandparents solely of European origin.

The project was carried out in accordance with the Declaration of Helsinki and approved by the human ethics committee of the HCPA. All patients signed an informed consent.

Diagnostic procedures

The presence of ADHD and comorbid diagnoses in cases and controls were evaluated through the following semi-structured interviews: (a) Schedule for Affective Disorders and Schizophrenia for School-Age Children-Epidemiologic Version (K-SADS-E), adapted to adults, for ADHD and oppositional defiant disorder (ODD) [25, 26]; (b) Structured Clinical Interview for DSM-IV (SCID-IV-R) for the

Axis I psychiatric comorbidities [27] (cases) and Structured Clinical Interview for DSM-IV screening module—SCID-I/P for the Axis I psychiatric disorders [28] (controls); and (c) Mini-International Psychiatric Interview (M.I.N.I) for the diagnoses of conduct and anti-social personality disorder [29]. The estimated IQ scores were obtained from the vocabulary and block design subtests of the Wechsler Adult Intelligence Scale—Revised (WAIS-R) [30] administrated by a trained psychologist.

The Portuguese version of the Swanson, Nolan and Pelham Rating Scale-version IV (SNAP-IV) addressed the severity of current ADHD and ODD symptoms [31]. SNAP-IV includes items from the DSM-IV criteria for ADHD and ODD. The scale is based on 0–3 ratings: not at all, just a little, quite a bit, and very much. SNAP-IV scores are computed by summing the scores in items from each dimension (inattention, hyperactivity/impulsivity, and oppositional defiant), divided by the number of items in the dimension. A more detailed description of the ascertainment methods and psychiatric profile of the ADHD sample are described elsewhere [32].

Laboratory methods

DNA was extracted from peripheral blood by a salting out procedure [33]. Polymerase chain reaction (PCR) amplification and genotyping of *DAT1* 3′-UTR VNTR and −839 C/T (rs2652511) polymorphisms were performed in a protocol described by Roman et al. [34] and Rubie et al. [14], respectively. The Int8 VNTR genotyping was performed by PCR with 10 pmol of each primer (F: 5′GCATG TGGATGTGTTCTTGC3′ and R: 5′GCAGAAACAAGGA GGAGCAG3′), 1U *Taq* polymerase, 200 µmol each of four dNTPs, 1.5 mM of $MgCl_2$, and 2.5 µl of PCR buffer. After an initial denaturation at 94 °C, followed by 35 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 5 min, the products were electrophoresed on 2.5 % agarose gel.

Statistical analyses

The genotype frequencies between patients with ADHD and controls were compared by logistic regression. Odds ratios (ORs) and 95 % confidence intervals (CIs) were obtained using the Plink (version 1.07) software (http://pngu.mgh.harvard.edu/purcell/plink/) [35]. *DAT1* haplotypes of the 3′-UTR and Int8 VNTR were estimated using Plink. Only the most common genotypes (i.e., those with a frequency of at least 5 %) were taken into account in the haplotype analysis and the tests of association with ADHD. Before haplotype estimation, the VNTR were recoded, excluding all individuals with rare alleles, so that only three genotypes for the 3′-UTR VNTR (i.e., the 9-repeat and 10-repeat alleles), and three genotypes for the Int8 VNTR (i.e., the 5-repeat and 6-repeat alleles) were considered in the analyses. The Haploview program (version 3.32) was used to estimate linkage disequilibrium (D') as a measure of pairwise LD [36]. The analyses were corrected for age and gender. *P* value was adjusted by 10,000 permutations for the significant results.

An analysis of covariance (ANCOVA) was used to test the influence of the *DAT1* polymorphisms on the ADHD severity dimensions. Sex, age, socioeconomic status, educational level, and frequent comorbidities (major depressive disorder, bipolar disorder, generalized anxiety disorder, obsessive–compulsive disorder, anti-social personality disorder, nicotine dependence, alcohol dependence, and drug dependence) were considered for inclusion as covariates using a statistical definition (association with both the study factor and outcome for a *P* value <0.20). A *P* value <0.05 was considered statistically significant.

Results

Psychiatric and socio-demographic characteristics of the sample are shown in Table 1, while genotype distributions are in Table 2. All three polymorphisms were in Hardy–Weinberg equilibrium ($P > 0.05$) and similar to those previously reported for other European or European-derived samples [16, 34]. We found a significant

Table 1 Demographic and comorbidity profiles in adult with ADHD and controls

	ADHD ($n = 522$) Mean (SD)	Controls ($n = 628$) Mean (SD)	*P* value
Age (years)	33.83 (11.1)	29.16 (8.7)	<0.01
	n (%)	*n* (%)	
Gender			
Male	269 (51.5)	311 (49.5)	0.477
Female	253 (48.5)	317 (50.5)	
Comorbidities			
Generalized anxiety disorder	109 (21.0)	64 (10.2)	<0.01
Major depressive disorder	190 (36.7)	173 (27.6)	<0.01
Oppositional defiant disorder	189 (41.9)	18 (3.0)	<0.01
Bipolar disorder	83 (16.0)	16 (2.5)	<0.01
Nicotine dependence	224 (43.0)	113 (18.0)	<0.01
Alcohol dependence	42 (8.1)	5 (0.7)	<0.01
Drug dependence	46 (8.8)	3 (0.4)	<0.01

Age (years) data are present as mean (SD). Gender and comorbidities data are present as *n* (%)

ADHD Attention deficit hyperactivity disorder; *SD* standard deviation

Table 2 Genotype analyses of the *DAT1* polymorphisms 3′-UTR VNTR, Int8 VNTR, and −839 C > T (rs2652511) and haplotype analysis for VNTR polymorphisms on susceptibility to ADHD

Genotypes[a]	Frequency (%)		Wald *P* value[b]	OR (95 % CI)
DAT1 3′-UTR VNTR	ADHD ($n = 476$)	Controls ($n = 587$)		
10/10	243 (51.1)	303 (51.6)		1
10/9	194 (40.8)	222 (37.8)	0.618	1.06 (0.82–1.39)
9/9	39 (8.2)	62 (10.6)	0.335	0.81 (0.52–1.26)
DAT1 Int8 VNTR	($n = 497$)	($n = 596$)		
6/6	294 (59.2)	348 (58.4)		1
5/6	168 (33.8)	208 (34.9)	0.498	0.91 (0.70–1.18)
5/5	35 (7.0)	40 (6.7)	0.812	1.06 (0.64–1.73)
DAT1 −839 C > T	($n = 501$)	($n = 569$)		
TT	100 (20.0)	145 (25.5)		1
TC	241 (48.1)	302 (53.1)	0.224	1.21 (0.88–1.66)
CC	160 (31.9)	122 (21.4)	<0.001[c]	2.05 (1.43–2.94)
Haplotypes[d]	Frequency (%)		*P* value[b,e]	OR
	ADHD ($n = 479$)	Controls ($n = 570$)		
10–6	64.84	64.90	0.883	1.01
9–5	17.24	17.45	0.790	0.96
9–6	11.25	11.85	0.699	0.94
10–5	6.67	6.49	0.531	1.13

[a] Only the most common genotypes were taken into account in the tests of association with ADHD, and for this reason, sample sizes are smaller than the original 522 adult patients and 628 controls. *DAT1* VNTR: the rate of genotypes with rare alleles did not differ between ADHD patients and controls (Fisher's exact test): 3′-UTR VNTR (2, 3, 6, 7, 8, 11, 12, 13, and 14-repeat) −0.038 and 0.040, respectively ($P = 0.878$); Int8 VNTR: (4, 7, 13, and 14-repeat) −0.010 and 0.024, respectively ($P = 0.074$). Considering the rs2652511 SNP, 4 % of patients and 9 % of controls had missing data due to genotype failure

[b] Adjusted by age and gender

[c] *P* value after 10,000 permutations ($P = 0.002$)

[d] Only the 3′-UTR VNTR and Int8 VNTR polymorphisms were considered in the haplotype analysis, considering the linkage disequilibrium. Four haplotypes were found at frequencies above 5 %, representing 90.3 % of all haplotypes in the sample

[e] Global *P* value = 0.905

association between the rs2652511 C-allele with ADHD ($P = 0.002$, Table 2). The VNTR polymorphisms, however, were not associated with the disorder ($P = 0.335$ for the 3′-UTR VNTR and $P = 0.812$ for Int8 VNTR).

The haplotype frequencies in cases and in controls are shown in Table 2. Only individuals with the common genotypes for both VNTR polymorphisms were included. Four haplotypes were found at frequencies above 5 %, representing 90.3 % of all haplotypes in the total sample. The overall haplotype distributions were not statistically different between cases and controls (haplo.score global $P > 0.05$). There is moderate LD only between the 3′-UTR and Int8 polymorphisms ($D' = 0.63$; $r^2 = 0.30$), but the 5′ polymorphism is not in LD with the 3′-UTR ($D' = 0.01$; $r^2 = 0$) or with Int8 VNTR ($D' = 0.02$; $r^2 = 0$). The haplotype structure of *DAT1* in our sample is similar to the structure described by Genro et al. [17] in children with ADHD.

The results for dimensional analyses were based only on the ADHD sample and are presented in Table 3. The ANCOVA results indicated that the 6-repeat allele in Int8 was associated with higher SNAP-IV scores of inattention ($P = 0.034$), but not with hyperactivity ($P = 0.670$), ODD ($P = 0.158$), or total sum of symptoms ($P = 0.326$). The *DAT1* 3′-UTR VNTR and rs2652511 polymorphisms were not associated with any SNAP-IV dimensions.

Table 3 Influences of *DAT1* polymorphisms 3′-UTR VNTR, Int8 VNTR, and −839 C > T (rs2652511) on the SNAP-IV scores (only ADHD patients)

Polymorphisms	Genotype (*n*)[e]	Hyperactivity		Inattention		Total ADHD		ODD	
		SNAP-IV Mean (SD)	*P* value[a]	SNAP-IV Mean (SD)	*P* value[b]	SNAP-IV Mean (SD)	*P* value[c]	SNAP-IV Mean (SD)	*P* value[d]
DAT1 3′-UTR VNTR									
(*n* = 468)	9/9 + 9/10 (228)	1.487 (0.05)	0.968	1.818 (0.04)	0.734	0.911 (0.04)	0.749	1.412 (0.04)	0.970
	10/10 (240)	1.483 (0.06)		1.797 (0.04)		0.899 (0.05)		1.414 (0.04)	
DAT1 Int8 VNTR									
(*n* = 484)	5/5 + 5/6 (197)	1.467 (0.06)	0.670	1.741 (0.05)	0.034	0.921 (0.04)	0.326	1.374 (0.04)	0.158
	6/6 (287)	1.503 (0.05)		1.874 (0.04)		0.857 (0.05)		1.452 (0.03)	
DAT1 −839C > T									
(*n* = 494)	CC + CT (395)	1.513 (0.04)	0.529	1.857 (0.03)	0.143	0.936 (0.03)	0.185	1.454 (0.02)	0.166
	TT (99)	1.457 (0.08)		1.758 (0.06)		0.842 (0.06)		1.372 (0.05)	

Analyses restricted to the ADHD sample. *P* value calculated by ANCOVA

SNAP-IV Swanson, Nolan and Pelham Scale-version IV; *SD* standard deviation

Potential confounders considered in analyses: [a] hyperactivity: bipolar disorder, nicotine dependence, alcohol dependence; [b] inattention: bipolar disorder, nicotine dependence, alcohol dependence, and drug dependence; [c] total ADHD: bipolar disorder, nicotine dependence, drug dependence, and generalized anxiety disorder; [d] ODD: bipolar disorder, nicotine dependence, alcohol dependence, drug dependence, and generalized anxiety disorder; [e] only the most common genotypes were taken into account in the ANCOVA

Discussion

Even though *DAT1* is the best-studied gene in childhood ADHD, only few studies have analyzed this gene in the context of adult ADHD. In addition, most studies focused only on the 3′-UTR VNTR polymorphism, despite growing evidence on the role of other variants. To our knowledge, this is the first study to include rs2652511 in a candidate gene association analysis with persistent ADHD, and rs2652511 is not present and has no proxy in existing GWAS arrays. The main finding was a significant association between the rs2652511 C-allele and ADHD. The results of this study represent the first evidence among adults that support previous findings on a role of rs2652511 in ADHD susceptibility in children [8, 16, 17].

The 5′-UTR marker studied here is located in an important region that modulates the transcriptional activity of the *DAT1* gene. There are several studies demonstrating that the *DAT1* 5′ region contains multiple recognition sites for several transcription factors that modulate tissue-specific expression via promoter domain, including *NR4A2/Nurr1* [37, 38] and leader binding protein 1 (*LBP-1*) [14]. A functional in silico study involving the *DAT1* 5′-UTR region suggested that the rs2652511 T-allele introduces a binding site for LBP-1. Therefore, this 5′-UTR SNP may determine differences in transcriptional activation and brain-specific expression of the *DAT1* product [14]. If a LBP-1-like transcription factor binds around the rs2652511 T-allele repressing its expression, carriers of rs2652511 C-allele would express higher *DAT1* mRNA levels than their counterparts with a T-allele. The observations based on gene expression are parallel with the discovery by imaging studies that the *DAT1* 5′-UTR markers (i.e., rs2652511 SNP) might contribute to the dynamic process that regulates DAT density in the brain. For example, Drgon et al. [39] reported that the DAT availability in the ventral striatum measured with [^{11}C] cocaine PET was higher for the haplotype containing the alleles C (rs2652511) and G (rs2937639) in ADHD patients. The association between this *DAT1* 5′-UTR haplotype and higher DAT1 expression levels in ventral striatum was additionally in postmortem brain samples from 51 individuals using [^{3}H] carboxyflurotropane ([^{3}H] CFT) saturation radioligand binding. Overall, the functional evidences available are consistent with genetic association findings involving the same rs2652511 C-allele not only with children [8, 16, 17] and adult ADHD but also with other psychiatric disorders, such as schizophrenia [40, 41] and Parkinson's disease [42]. Previous results regarding the possible individual variant located in the promoter domain independently affect the *DAT1* transcriptional activity and DAT availability. Based on findings of PET and SPECT approaches demonstrating the changes in levels of striatal DAT availability in ADHD patients are, in part, an ADHD trait that appears to be influenced by age and sex [43] and methylphenidate treatment [44]; more studies are needed to disclose the exact functional mechanism of this variant in brain areas related to ADHD.

Despite the positive association involving the 6-repeat allele in Int8 with inattention scores, our haplotype analyses of the two VNTR polymorphisms failed to replicate the report of association between the 9–6 haplotype and the persistent form of ADHD demonstrated by Franke et al. [11]. The lack of association of *DAT1* VNTR haplotypes and ADHD in this study might be explained from different causes. Considering the DAT availability, Shumay et al. [43] demonstrated that a higher *DAT1* expression seems to be associated with the presence of Int8 VNTR 5-repeat allele, thus underscoring a potential functional effect of the Int8 variant. Since DAT density decreases during life [43, 45], there are differences between the genotype-based subgroups in age-DAT availability relationships [45] and might reflect changing requirements on the dopaminergic architecture during life. It was demonstrated that the decline of *DAT1* expression with age is less noticeable in the group of 6/6 (Int8) and 9/10 (3′-UTR) allele carriers [45]. Taking into account that our sample of ADHD is composed by adults with an average age of 33.8, the less noticeable decline of *DAT1* expression with age in the 6/6 patients may be the factor that allowed us to find an association of this allele with higher inattention scores. It is also noteworthy the fact that the male-to-female ratio changes throughout the life span, from 10 to 3:1 in childhood [46] to 1.1:1 among adults [47].

Our results should be understood in the context of some limitations. Statistical power is certainly an issue in our study, as usual in this field, considering either categorical or dimensional approaches [48]. Taking as a parameter the strongest OR in meta-analysis in genetic studies among children with ADHD −1.33 for the exon 3 VNTR of *DRD4* [6], power for our sample size would still be low (50 %). Indeed, our sample size would allow for a power of 80 % only with an OR of 1.48. However, it is more difficult to predict the statistical power for a dimensional analysis. Lack of statistical power might explain, for example, the lack of association between 3′-UTR VNTR and rs2652511 markers with dimensional analysis. In addition, several disorders are relatively frequent in our control group, making the study more conservative. The control group was designed to be representative of the lifetime population prevalence of psychiatric disorders, except for ADHD. The *DAT1* polymorphisms studied do not cover completely the gene region. Therefore, other markers in the 5′ flanking region may also be relevant. However, few studies analyzed *DAT1* markers in samples of adults, and most of them included only the 3′-UTR VNTR. Regarding rs2652511 SNP, it is not represented even in the available GWAS. Another limitation is the fact that the modulation of the *DAT1* is influenced by many genetic and environmental factors [43, 45], not evaluated in this study. Indeed, considering the clinical heterogeneity and relevance of the relationships between dopamine-related genes and ADHD in different ages, certainly more studies are needed, especially with a longitudinal design. The present data should be relevant for future meta-analytic studies to evaluate the relationship between *DAT1* variants and ADHD in adults.

In summary, our analysis yielded further evidence on the genetic contribution of the *DAT1* 5′ promoter region in ADHD. Our positive association between rs2652511 C-allele and ADHD replicates previous findings in children with ADHD [8, 16, 17]. The role of the promoter region variation in ADHD susceptibility could possibly be related to inconsistent results in association studies, even in meta-analyses, given that these studies looked only for association with the 3′-UTR VNTR polymorphism. Future longitudinal studies coupled with neuroimaging approaches could bring us a step closer to getting a clearer picture on the role of *DAT1* variations on ADHD across the life span.

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Conflict of interest The ADHD Program received unrestricted educational and research support from the following pharmaceutical companies in the last 3 years: Abbott, Bristol-Myers Squibb, Eli Lilly, Janssen-Cilag, Novartis, and Shire. Dr Belmonte-de-Abreu is on the speakers' bureau or is a consultant for Janssen-Cilag and Bristol-Myers Squibb. Dr Rohde was on the speakers' bureau and/or acted as consultant for Eli Lilly, Janssen-Cilag, Novartis, and Shire in the last 3 years. He also received travel awards (air tickets + hotel) for taking part in psychiatric meetings from Novartis and Janssen-Cilag in 2010 and authorship royalties from Oxford Press and ArtMed.

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