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Cyptochrome 2d6 polymorphism and tramadol use in schools in Benin

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Abstract

The use of tramadol by adolescents and young adults in schools is a public health issue. The objectives of this study was to identify students who use tramadol and to investigate the cytochrome CYP2D6 profiles of students at to investigate the potential risks that could be yielded from the usage of tramadol. To achieve this, 453 students participated in the study; R diversity 3.6.1 software in the RStudio environment was used to identify students experimenting with tramadol by calculating a score according to the ASSIST V3.0 tool. The CYP2D6 duplication allele and deletion allele were tested by PCR on DNA extracts from peripheral blood collected from these individuals. Sixty-seven students were found to be using tramadol and of these, 25 students or 37.31% required brief intervention for medical care. Similarly 85.43% of the subjects expressed the CYP2D6 gene. Of these, 7.28% had the duplication allele (CYP2D6dup) and 1.32% the deleted allele (CYP2D6*5). In addition, all subjects at moderate risk due to tramadol use expressed the CYP2D6 gene; the duplication allele (CYP2D6dup) was found in both types of subjects in the proportions of 4% (at risk) and 7.47% (not at risk) respectively. The genetic polymorphism of cytochromes P450 2D6 does not influence tramadol usage by these subjects.

Keywords: Genetic Polymorphism; Cytochrome 2D6; Teenagers; Tramadol

1. Introduction

The use of psychoactive substances (PAS) is an age-old practice that continues and is far from disappearing. The reasons for using these substances are not only diversified but have evolved considerably over time. Our society's view of young people's use of APS at the beginning of the 19th century is still perceived as problematic [1].

Indeed, adolescence, a critical transition period according to the World Health Organisation (WHO), is characterised by a significant rate of growth and changes of all kinds. It is therefore an unstable period marked by the emergence of behaviours such as risk-taking, the search for impulsiveness and novelty, likely to expose them to the use of APS [2] . In Benin, a study conducted among teenagers in schools by Kpatchavi and Adounkpè in 2016 revealed that 67.3% of students use APS (Kpatchavi and Adounkpe, 2016), including tramadol. This is a tier II analgesic drug commonly prescribed in therapy by clinicians.

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The biological activity of tramadol and drugs in general depends in part on their metabolism. This is a set of interactions with enzymes located primarily in the liver and to a lesser extent in the gut, brain, skin and adrenal glands. Metabolism plays an important role in drug elimination, which in turn may have pharmacological and toxicological implications in therapy [4]. The cytochrome P450 superfamily (CYP 450) is the most important enzyme system involved in the biotransformation of endogenous and exogenous substances. It represents the most important phase I drug metabolising enzymes that convert more than 90% of all drugs into more hydrophilic compounds. This superfamily consists of more than 50 enzymes of which cytochrome 2D6 (CYP2D6) is one of the most important enzymes [5]. Indeed, although it represents only a small percentage (< 2%) of all CYP P450s, its role in drug metabolism is largely predominant as it is involved in the metabolism of 20-25% of drugs, including tramadol [6]. Moreover, it is a highly polymorphic enzyme, a polymorphism responsible for variation in responses ranging from therapeutic ineffectiveness to serious adverse events in patients depending on the phenotype expressed for this enzyme [7]. This phenotype is notably influenced by either the presence of a duplication of the gene or by its deletion.

Notwithstanding the diversity of studies on the use of ARVs by adolescents, few studies have focused on the impact of CYP 450 genetic polymorphism on ARV use in Benin. Moreover, at present, no study has been reported on CYP2D6 polymorphism in our country, yet the consequences are not negligible. We therefore felt it was important to investigate the CYP2D6 genetic polymorphism in adolescents and young adults using tramadol in schools in the cities of Cotonou and Parakou.

2. Material and methods

Our work took place in two cities in Benin; Cotonou in the south and Parakou in the north. These are two cosmopolitan cities where habits of consumption of psychoactive substances are developing. This study obtained the favourable opinion of the Research Ethics Committee of the Institute of Applied Biomedical Sciences (ISBA) of Cotonou (N°125 of 11/02/2020) and the authorization of the authorities in charge of secondary education in these two cities.

2.1. Type and period of study

This was a descriptive cross-sectional study with an analytical focus on the use of tramadol by adolescents and young adults in schools during the period from March 2020 to June 2020. These students were regularly enrolled in public and private secondary schools of general, technical and vocational education in the cities of Cotonou and Parakou.

2.2. Study population

Ten secondary schools were selected in each of the aforementioned cities using stratified sampling techniques. The colleges were randomly selected. Then, in each of the selected colleges, simple random sampling was used to select subjects based on the total number of students enrolled in each city. The sample size N was calculated using the Schwartz formula

$N = k.\varepsilon^2\ p\ q\ /\ i^2$ (ε = Reduced deviation for a risk equal to 5% = 1.96; p = 50%; q = Opposite event to p; then q = 1 - p = 1-0.5 = 0.5; i = Margin of sampling error; this is the expected error. Here we take i = 0.05 (5%). K = cluster effect)

By numerical application of the Schwartz formula, we have: N= (1.96)2 x 0.5 x 0.5/(0.05)2 = 384. For k = 2, this is N= 768.

The total number W of adolescent and youth pupils to be surveyed per city was calculated taking into account the total number of pupils registered this school year per city according to the formula: W= (NxT)/E, where N = Sample size calculated earlier (total adolescent pupils to be surveyed in this research), T = Total number of pupils registered this school year per city, and E = Sum of the total number of pupils registered this school year in both cities. Knowing that the number of pupils registered this school year per city is 84,092 and 40,427 respectively in Cotonou and Parakou. The total number of subjects to be surveyed will be 519 pupils in Cotonou and 249 pupils in Parakou. However, 627 (384 Cotonou and 243 Parakou) were finally included in our study.

2.3. Inclusion criteria

All adolescents and young adults in grades 4 to 12 who are regularly enrolled in a secondary school in Cotonou and Parakou, from 12 to 24 years old and who have given their free and informed consent to participate in the study are included in this study.

2.4. Data collection

2.4.1. Data collection tools and techniques.

The WHO ASSIST V3.0 tool adapted to our context was used. It consists of two sections, one providing information on socio-demographic characteristics and the other assessing patterns of tramadol use. This tool makes it possible to objectify and quantify subjects' experience with tramadol.

A pre-test of this tool was conducted in two colleges (public and private). These two colleges are excluded from our final sample. The ASSIST tool proposes, at the end of the score, the continuation of medical care ranging from: 0 to 3 points no intervention; 4 to 26 brief intervention; ≥27 more intensive treatment [8]. The score obtained for a substance is used to determine the level of risk associated with use and the type of therapeutic intervention required.

Of the 627 subjects who responded to the questionnaire, 453 gave their consent for blood sampling.

2.4.2. Laboratory process.

DNA extraction

DNA was extracted from 5 ml of peripheral blood collected from each participant on EDTA tubes. DNA extraction was performed using the organic phenol-chloroform method [9, 10]. Quantification of DNA was performed using a UV-visible spectrophotometer (Thermo Scientific Evolution 60S).

Search for the duplication allele of the CYP2D6 gene

For the determination of CYP2D6 duplicated gene carrier subjects, a Multiplex PCR was performed to search according to the method described by Pedro and colleagues [11]. The sense primer (2D6dupl-F) is specific for the 3′ flanking sequences of CYP2D6 and the antisense primer (2D6dupl-R) is specific for the flanking sequences of CYP2D7. In addition, in the same PCR, the entire CYP2D6 gene was amplified using sense (DPKup) and antisense (DPKlow) primers (Table 1). This Multiplex PCR was performed in a final volume of 25 µL containing 50-100 ng/µL of human genomic DNA, 0.2 mM dNTPs, 1X Dream Taq Buffer with 20mM MgCl2, 0.1 µM of each primer (sense and antisense) (Table 1) and 0.08 units of Dream Taq. The reaction mixture was subjected to the amplification program shown in Table 2. The reaction was amplified using a thermal cycler (Applied Biosystems Simp Amp®).

Testing for the deleted CYP2D6 allele (CYP2D6*5)

To determine whether individuals were gene carriers of the CYP2D6*5 allele, a multiplex PCR was performed. The primers 5′2D6*5 and 3′2D6*5 are specific for identifying the presence of the CYP2D6*5 allele (3.5 kb). Furthermore, in the same PCR, the entire CYP2D6 gene (5.1 kb) was amplified as an internal control of the reaction using the DPKup sense and DPKlow antisense primers. (Table 1). The amplification reactions, amplification conditions and analysis of the PCR products were the same as those used for the determining the duplication of the CYP2D6 gene.

Table 1 Sequence of primers

Primers	Sequences	References
DPKup	5’-GTTATCCCAGAAGGCTTTGCAGGCTTCA-3’	Y3980C07
DPKlow	5’-GCCGACTGAGCCCTGGGAGGTAGGTA-3’	Y3980C08
2D6dupl-F	5’-CCTGGGAAGGCCCCATGGAAG-3’	Y3980C09
2D6dupl-R	5’-CAGTTACGGCAGTGGTCAGCT-3’	Y3980C10
5’2D6*5	5’-CACCAGGCACCTGTACTCCTC-3’	Y3980D01
3’2D6*5	5’-CAGGCATGAGCTAAGGCACCCAGAC-3’	Y3980D02

Table 2 PCR amplification programme

Gene	Amplification programme					
CYP2D6	**Denaturation**	**Denaturation**	**Hybridization**	**Denaturation**	**Hybridation**	**Elongation**
	94 °c(2 min)	95 °c(20 s)	68 °c(4 min)	95 °c(20 s)	68 °c(4 min)	68 °c(7 min)
	10X			20X augmentation de10s/cycle		

The amplification products were separated at 135 volts for 25 minutes on a 0.6% agarose gel stained with Ethidium Bromide and visualised using a UV Transilluminator (Vilber Lourmat) equipped with a camera and LCD screen. For the PCR to determine the duplication allele of the CYP2D6 gene, the expected fragment sizes are 5.1 kb for the CYP2D6 gene and 3.5 kb for the duplication gene. For the PCR to detect the deleted CYP2D6 allele (CYP2D6*5), the expected fragment sizes are 5.1 kb for the CYP2D6 gene and 3.5 kb for the deleted gene (CYP2D6*5).

2.5. Statistical analysis

All data collected were entered into EpiData Entry version 3.1 and analysed using R 3.6.1 with the RStudio environment. Dichotomous (or categorical) categorical variables were described in terms of number and frequency and quantitative variables were described in terms of mean, standard deviation, and rank (minimum, maximum). Comparisons of proportions were made using Pearson's chi-square test, and in case of invalidity of this test, by the two-tailed Fischer's exact test. The significance level was 5%.

3. Results

3.1. Socio-demographic characteristics of participants.

In this study, 58.5% of the respondents were male, with a sex ratio of 1.41 in favour of men. The average age was 17±2 years with extremes of 12 and 23 years, the most represented group being 15 to 19 years old (71.3%). The most represented ethnic group was Fon at a rate of 42.9%.

3.2. Distribution of subjects according to risk related to tramadol experimentation

Using R diversity 3.6.1 software with the RStudio environment, we found 67 students using tramadol by calculating scores according to the ASSIST V3.0 tool. Among them, after calculation of the scores, 25 students or 37.31% were at moderate risk and therefore required a brief intervention for management. Table 3 shows the risks and types of intervention after calculation of the scores.

Table 3 Risks and types of intervention after calculation of scores

Types of intervention	Risks	Number	Percentage
No intervention	Low	42	62.68
Brief intervention	Moderate	25	37.31
	Total	67	100

3.3. Genetic polymorphism of CYP2D6 in respondents

As shown in Figure 1, the fragment sizes are 5.1 kb for the CYP2D6 gene and 3.5 kb for the duplication gene or the deleted CYP2D6 allele (CYP2D6*5).

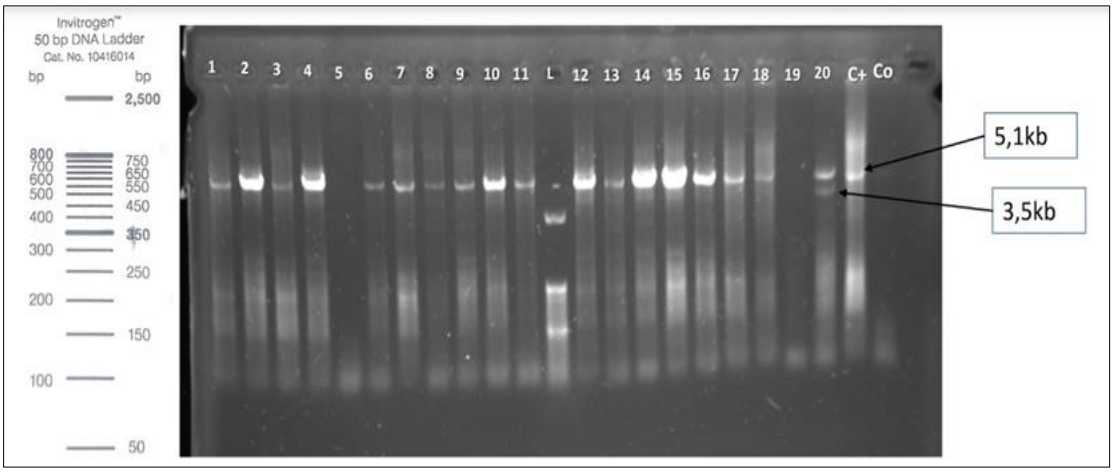

Figure 1 Visualisation of fragments on 0.6% agarose gel

*3.3.1. Frequency of the CYP2D6 gene, CYP2D6 duplication allele, and CYP2D6*5 deletion allele in the respondents*

Our results show that 85.43% of the subjects possess the CYP2D6 gene. Of these subjects with the CYP2D6 gene, 7.28% have the duplication allele and 1.32% have the deletion allele of CYP2D6. Figure 2 shows the frequencies of CYP2D6, the duplication allele of CYP2D6 and the deleted allele of CYP2D6 (CYP2D6*5) in the respondents.

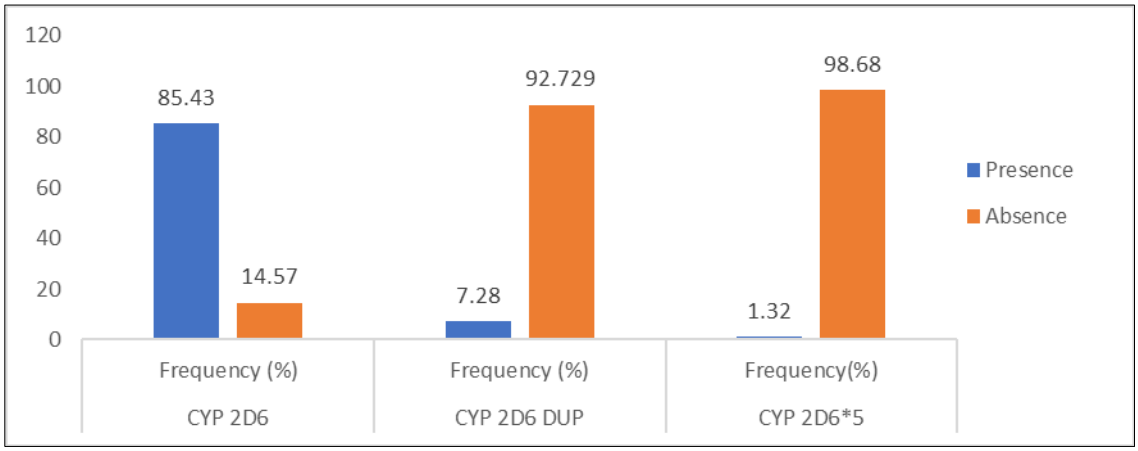

Figure 2 Frequency of the CYP2D6 gene, CYP2D6 duplication allele, and CYP2D6*5 deletion allele in subjects

3.3.2. CYP2D6 genetic polymorphism and tramadol experimentation

In subjects at moderate risk

All 25 subjects requiring brief intervention in relation to tramadol usage expressed the CYP2D6 gene. The duplication allele was present in 01 of these subjects (4%); the CYP2D6*5 allele was completely absent in these subjects. The significance levels were 0.041 and 0.2 for the CYP2D6 gene and the duplication allele respectively.

Figure 3 shows the frequency of the CYP2D6 gene, the CYP2D6 duplication allele and the CYP2D6*5 deletion allele in subjects at moderate risk due to tramadol usage.

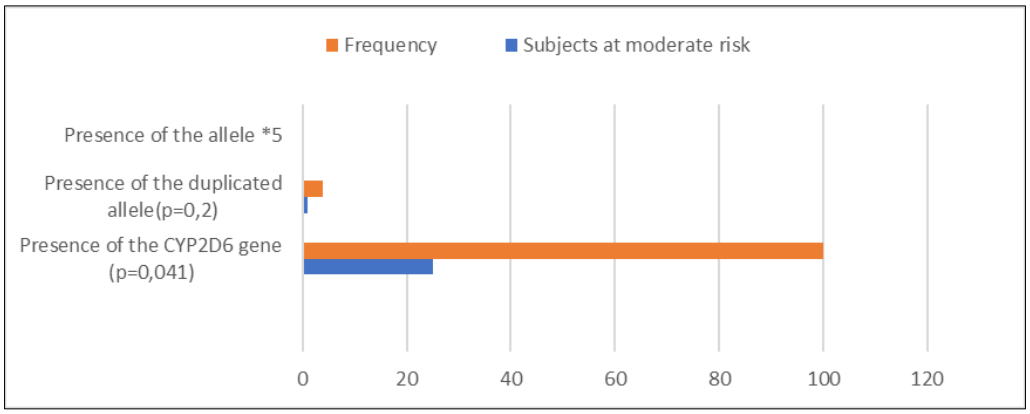

Figure 3 Frequency of CYP2D6 gene, CYP2D6 duplication allele and CYP2D6*5 deletion allele in subjects at moderate risk

In the No Risk Subjects

Among the risk free subjects despite the use of tramadol, 84.57% had the CYP2D6 gene. Of these, 7.47% had the CYP2D6 duplication allele and 1.4% had the CYP2D6*5 deletion allele.

Figure 4 Shows the frequency of the CYP2D6 gene, the CYP2D6 duplication allele and the CYP2D6*5 deletion allele in non-risk subjects.

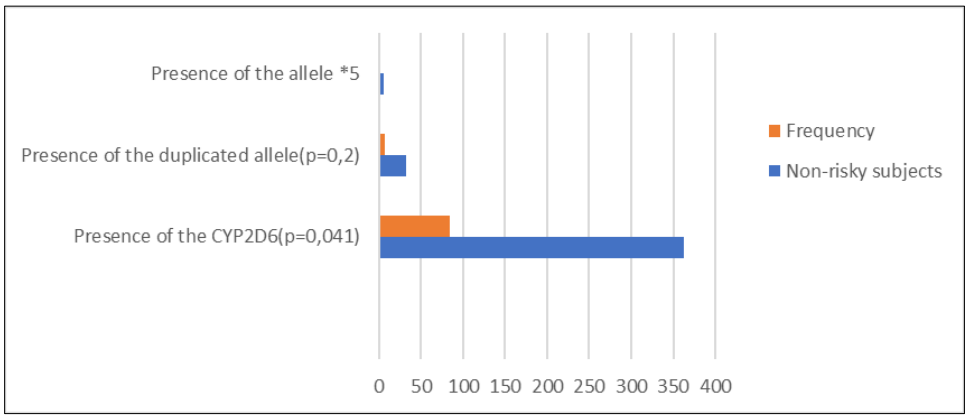

Figure 4 Frequency of CYP2D6 gene, CYP2D6 duplication allele and CYP2D6*5 deletion allele in risk-free subjects

4. Discussion

In this work, we determined the CYP2D6 profile of adolescents and young adults in schools experimenting with tramadol in the city of Cotonou and Parakou in 2020. Our work showed that male adolescents used more psychoactive substances than female adolescents, which is in line with the results of Kpozehouen et al. [12]. In Nigeria, Famuyiwa's 2011 and Adekeye's 2015 work on psychoactive substances found about 71% boys compared to 39% girls [13, 14]. This same male predominance was reported by the results of Zarrouq et al. [15] in northern Morocco in 2016 who found 53% boys against 47% girls. This can be explained by the fact that in these different countries access to school education is easier for boys than for girls, as some parents are still reluctant to send them.

Our genotyping results show that 85.43% of the subjects have the CYP2D6 gene of which 7.28% have the duplication allele (CYP2D6dup) and 1.32% have the deleted allele (CYP2D6*5). In addition, all subjects at moderate risk for tramadol use expressed the CYP2D6 gene; the duplication allele (CYP2D6dup) was found in both types of subjects in the proportions of 4% (at risk) and 7.47% (not at risk) respectively.

Our results are contrary to the work of Bradford and Tredici et al. [16, 17]. The study by Bradford [16] in black African populations (Ghanaians, Gabonese, Zimbabweans, Tanzanians and Ethiopians), revealed that non-functional CYP2D6

alleles in these populations were present at relatively low frequencies; the average frequency for the CYP2D6*5 allele was 3.9% for all populations studied with the exception of Ghanaians where less than 1% expressed this allele. These results are corroborated by the work of Tredici et al. in 2018 [17], who concluded that the CYP2D6*5 allele is the most common functionless allele. These data on CYP2D6*5 expression in a population are confirmed by a previous meta-analysis by Gaedigk et al. in 2017. The latter assumed frequencies between 1% and 7% for CYP2D6 deletions [18].

Furthermore, CYP2D6 duplications occur with frequencies of 1-2% in Europeans and Asians, but are more frequent in some African populations, in which their frequency can reach 29% as reported in previous work [18–20]. These data are in line with our results where the percentage of the duplicated CYP2D6 allele is 7.28% and therefore between 2% and 29%.

The CYP2D6 allele frequency database available online on the PharmGKB platform [21], provides information on the population-based allele frequencies reported in the literature. The ethnicities/places mentioned in the articles are mapped into seven geographically defined groups (American, Central/South Asian, East Asian, European, Near East, Oceania and Sub-Saharan Africa) and two mixed groups (African-American/Afro-Caribbean and Latino) using the biogeographical clustering system developed by PharmGKB. This database reveals that less than 2% of the sub-Saharan African population is a CYP2D6 gene duplication carrier. This is comparatively lower than the proportion found in the present study (7.28%).

CYP2D6 is the most complex and well known cytochrome locus with a large number of distinctly different common haplotypes with important clinical implications and the differences in proportions observed for both the CYP2D6*5 allele and the duplication allele are evidence of CYP2D6 polymorphism.

Tramadol is a relatively new drug and different subjects metabolise this drug differently because it is metabolised by a polymorphic gene. Therefore, Asians may require lower average doses of tramadol than Caucasians and Africans to achieve similar steady-state serum concentrations [22]. Both tramadol and codeine are bioactivated by CYP2D6, which can affect both efficacy and toxicity. Codeine needs to be activated to morphine by CYP2D6 via O-demethylation for its analgesic effect; similarly, tramadol needs to be activated at M1 for its opioid analgesic effect [23, 24].

By influencing the pharmacokinetics of tramadol, CYP2D6 activity has a major role in the analgesic effect of tramadol [22] as confirmed by experimental and clinical studies.

Our genotyping results also show that 7.28% of the subjects are fast metabolizers of tramadol and 1.32% of the latter are slow metabolizers. For tramadol experimentation, 4% of subjects with moderate risk for tramadol use and 7.47% of non-users are fast metabolizers. We cannot say whether the genotypes of these test subjects are consistent with their phenotypes. However, subjects with multiple copies of the CYP2D6 gene metabolise drugs more rapidly and therapeutic plasma levels will not be reached at regular drug doses [25]. Also, the study by Stamer et al. in 2003 [26] on 300 patients evaluated the impact of CYP2D6 genotype on the response to tramadol in postoperative analgesia after abdominal surgery. Nonresponders were twice as likely to be slow metabolizers (46.7%) compared to extensive metabolizers (21.6%), slow metabolizers were twice as likely to require rescue analgesics (tramadol + piritramide) in the recovery room (43% vs. 21%), and tramadol use by self-controlled intravenous pump injection was increased in slow metabolizers (26.7% vs. 11.6%) ; Tramadol consumption in slow metabolizers was also 30% higher than in extensive metabolizers [26]. Although controversial, slow metabolizers may be protected against opioid dependence for some molecules [27]. In their 1997 study, Tyndale et al. [28] found no PM by genotyping or phenotyping in a group of opiate-dependent patients, unlike patients with no previous substance dependence (4%) or dependence on many other substances (alcohol, cocaine, amphetamines; 6.5%). This under-representation of slow metabolisers in opiate-dependent patients suggests that they have pharmacogenetic protection against oral opiate dependence (odds ratio > 7). Studies by Kathiraramalainathan et al. [29] demonstrate that inhibition of CYP2D6 results in a decrease in codeine abuse. This study further supports the hypothesis that individuals who are slow metabolizers (genetically or through enzyme inhibition) are less likely to become dependent on opiates due to their inability to activate the parent molecule into more active metabolites. This hypothesis was supported by Tyndale et al. [30] in 1997 who suggested that inhibition of CYP2D6, by blocking the production of morphine or M1, could modulate the risk of addiction. However, the first clinical trials did not confirm this hypothesis according to Fernandes et al. [31] in 2002.

In this study, we did not fully genotype CYP2D6; however, the fact that the proportion of moderate-risk individuals expressing CYP2D6 was significantly higher than that of risk freeindividuals expressing CYP2D6 suggests to us, as in previous studies, that CYP2D6 pharmacogenetics may have an impact on tramadol use.

5. Conclusion

Adolescents and young adults in school in the cities of Cotonou and Parakou using tramadol in the school setting were predominantly aged 15-19 years and were male. All subjects presenting a moderate risk for tramadol consumption expressed the CYP2D6 gene, the duplication allele (CYP2D6dup) was found in both types of subjects in the proportions of 4% (subjects at risk) and 7.47% (without risk). The genetic polymorphism of cytochromes P450 2D6 does not influence tramadol experimentation in these subjects. Therefore, we suggest that CYP2D6 genotyping is of great importance in individualizing tramadol drug therapy given the wide variability among patients within ethnic populations.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

Statement of informed consent

After obtaining the favorable opinion of the Research Ethics Committee of the Institute of Applied Biomedical Sciences (ISBA) of Cotonou and the authorization of the authorities in charge of secondary education, assent and consent was obtained respectively from the adolescents and young adults after an informed explanation of the study.

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