Original Article

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The Role of *CYP2B6*6* Gene Polymorphisms in 3,5,6-Trichloro-2-pyridinol Levels as a Biomarker of Chlorpyrifos Toxicity Among Indonesian Farmers

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Objectives: One of the most widely used pesticides today is chlorpyrifos (CPF). Cytochrome P450 (CYP)2B6, the most prominent catalyst in CPF bioactivation, is highly polymorphic. The objective of our study was to evaluate the role of *CYP2B6*6*, which contains both *516G>T* and *785A>G* polymorphisms, in CPF toxicity, as represented by the concentration of 3,5,6-trichloro-2-pyridinol (TCPy), among vegetable farmers in Central Java, Indonesia, where CPF has been commonly used.

Methods: A cross-sectional study was conducted among 132 vegetable farmers. Individual socio-demographic and occupational characteristics, as determinants of TCPy levels, were obtained using a structured interviewer-administered questionnaire and subsequently used to estimate the cumulative exposure level (CEL). TCPy levels were detected with liquid chromatography-mass spectrometry. *CYP2B6*6* gene polymorphisms were analyzed using a TaqMan® SNP Genotyping Assay and Sanger sequencing. Linear regression analysis was performed to analyze the association between TCPy, as a biomarker of CPF exposure, and its determinants.

Results: The prevalence of *CYP2B6*6* polymorphisms was 31% for **1/*1*, 51% for **1/*6*, and 18% for **6/*6*. TCPy concentrations were higher among participants with *CYP2B6*1/*1* than among those with **1/*6* or **6/*6* genotypes. *CYP2B6*6* gene polymorphisms, smoking, CEL, body mass index, and spraying time were retained in the final linear regression model as determinants of TCPy.

Conclusions: The results suggest that *CYP2B6*6* gene polymorphisms may play an important role in influencing susceptibility to CPF exposure. *CYP2B6*6* gene polymorphisms together with CEL, smoking habits, body mass index, and spraying time were the determinants of urinary TCPy concentrations, as a biomarker of CPF toxicity.

Key words: Agrochemical toxicity, Biological monitoring, *CYP2B6*, Genetic susceptibility, Occupational exposure

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INTRODUCTION

Approximately 33 million Indonesians are farmers, most of whom are small-scale farmers. Among them, pesticide use is very common and occupational exposure is significant. Organophosphates (OPs), of which chlorpyrifos (CPF) accounts for 40%, are among the most commonly used pesticides [1]. Despite the extensive use of pesticides, farmers in the small-

scale agricultural sector are often not aware that they are susceptible to the health impacts of CPF exposure. The health impacts reported in previous studies include neurological symptoms, alterations in reproductive hormone levels, and metabolic and endocrine disorders [2-4]. However, the severity of exposure is also determined by several other factors, such as metabolism in the human body, the type of pesticide, and exposure concentration and duration [5,6].

Human cytochrome P450 (CYP) is known to have important effects related to its role as catalyst for numerous drugs and chemicals, including pesticides, in metabolic reactions [7,8]. Several OPs, including CPF and parathion, are metabolically activated to their oxon form through reactions catalyzed by CYP [9]. CPF in particular can undergo a desulfurization reaction activated by CYP2B6, resulting in the formation of the active metabolite CPF-oxon (CPF-O). Following the bioactivation and detoxification of CPF, a specific metabolite (3,5,6-trichloro-2-pyridinol; TCPy) excreted in urine is formed [9,10]. Therefore, TCPy, which can be used to estimate the internal uptake of CPF, has been used as a biomarker of CPF exposure in several epidemiological studies [11,12].

CYP2B6 is highly polymorphic in terms of expression and enzymatic activity due to the presence of common single-nucleotide polymorphisms (SNPs), and its genetic variants are associated with inter-individual variability [13]. *CYP2B6* is the most prominent catalyst of CPF bioactivation. In particular, *CYP2B6*6* is expressed at lower levels, thereby reducing its ability to activate CPF-O formation [14]. To date, *CYP2B6* polymorphisms have been studied in several different populations, but there are limited data on its distribution among Indonesian population. Furthermore, the role of *CYP2B6* gene polymorphisms in pesticide exposure has generally been evaluated in human liver microsomes or animal studies, whereas epidemiological studies focusing on the relationship between susceptibility and exposure biomarkers, especially those emphasizing CPF, are scarce. Therefore, our study aimed to evaluate the role of *CYP2B6* gene polymorphisms in CPF toxicity, as represented by the level of TCPy, among vegetable farmers in Central Java, Indonesia, where CPF has been commonly used. We hope that our results will provide valuable information about determinants of CPF exposure, particularly its susceptibility biomarker.

METHODS

Study Population

We conducted a cross-sectional study, the participants of which came from vegetable farming areas in Central Java, Indonesia. The main products of this area are garlic, shallots, potatoes, chilies, and cabbage. The recruitment process lasted from July to October 2020. This time was chosen as optimal for the assessment of actual exposure, as farmers carry out routine pesticide application during this period. The minimum sample size of our study was determined to be 124 participants, as we used the standard deviation (SD) for urinary TCPy from a previous study (2.97 μg/g creatinine) [15], to achieve 80% power and a 5% significance level (2-tailed), for detecting a significant difference of 1.5 μg/g creatinine in mean TCPy levels between groups. The eligibility criteria were vegetable farmers, male or female, aged 18-65 who have actively used CPF for at least 1 year. A small remuneration was given to the participants for their participation. As the sample frame, there were 195 vegetable farmers who met the eligibility criteria and gave written consent to participate in the study. We decided to take the total sample consecutively.

The study consisted of 2 phases. In the first phase, we obtained participants' socio-demographic and occupational characteristics through a structured interviewer-administered questionnaire. Twenty participants were considered to have withdrawn from the study because they did not attend the second phase. Among those who attended the second phase and were informed of their general health condition, 24 participants who did not undergo blood sampling for the *CYP2B6* genotyping assay or completed the health questionnaire were excluded, leaving only 151 participants. Finally, there were 132 participants whose urine samples were available to test for urinary TCPy (corrected with urinary creatinine) and analyzed in this study.

Occupational Characteristics and Cumulative Exposure Level

Occupational characteristics consisted of several variables related to agricultural activities. The participants used almost no modern technology except for motorized knapsack sprayers during the insecticide application. All pesticide handling and farming activities were done manually.

A validated quantitative approach was used to estimate the cumulative exposure level (CEL) [16]. In brief, pesticide han-

dling activities, personal protective equipment (PPE) utilization, personal hygiene, and spill management practices were identified, then a score was given for each parameter and further used to estimate the daily exposure intensity level (IL). The IL, combined with frequency of annual spraying days and duration (lifetime years) of pesticide use, was used to estimate CEL. For example, the estimated CEL of a participant with a daily IL of 20/day for an average of 100 application day/y (frequency) over 20 years (duration) would be 40 000. The median value was used to classify participants into high-exposure and low-exposure groups, as described previously [17].

*CYP2B6*6* Genotyping (*516G>T–rs3745274* and *785A>G–rs2279343*)

*CYP2B6*6* contains both *516G>T* and *785A>G* polymorphisms. Therefore, the *CYP2B6*6* genotype was classified as follows: **1/*1* (GG/AA); **1/*6* (GT/AG); and **6/*6* (TT/GG).

Genotyping was performed by Prodia Clinical Lab (Jakarta, Indonesia). Whole blood samples were drawn from each participant using 3-mL ethylenediaminetetraacetic acid anticoagulant tubes (Vacuette®). Samples were transported to Prodia Clinical Lab and stored at -20°C prior to analysis. Genomic DNA extraction was performed using the spin column method according to the manufacturer's protocol (Genomic DNA Mini Kit; Geneaid Biotech Ltd., New Taipei City, Taiwan). A NanoDrop™ One spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used to analyze DNA purity and concentrations. The extracted DNA was stored at -20°C until further use.

The *CYP2B6–516G>T* polymorphism was analyzed using the TaqMan® SNP Genotyping Assay C___7817765_60 (Applied Biosystems, Foster City, CA, USA) as the primers and probe on a Rotor-Gene Q (Qiagen GmbH, Hilden, Germany) thermocycler. TaqMan GTXpress Master Mix (Applied Biosystems) was used as the master mix of the polymerase chain reaction (PCR) reagent. The laboratory kit was intended for research use only. Genotypes were determined by an allelic discrimination plot using fluorescence signals (FAM and VIC from the TaqMan probe) according to the manufacturer's instructions (Supplemental Material 1). In addition, 6 samples underwent DNA sequencing to validate the above *CYP2B6 516G>T* allelic discrimination assay. The PCR product of 533 bp was used for sequencing (Supplemental Material 2). The *CYP2B6–785A>G* genotype was analyzed using Sanger sequencing. Samples were amplified by PCR with primers as described by Zakeri et al. [18], which produced a 640 bp PCR product for sequencing (Supplemental Material 3). All steps in sequencing were performed by the 1st Base DNA Sequencing Division (Apical Scientific Sdn Bhd, Selangor, Malaysia). Examples of the DNA sequencing results for *CYP2B6 516G>T* and *785A>G* are presented in Supplemental Materials 4 and 5.

Urinary 3,5,6-Trichloro-2-pyridinol

Spot urine samples were collected using a sterile urine container. We obtained the last spraying date to be deducted against the urine collection date to calculate the number of post-spraying days, which was defined as the number of days between the last spraying day and urine collection. Samples were stored in a -20°C freezer until analysis and then analyzed for TCPy levels by Prodia Industrial Toxicology Laboratory. All solvents used in these methods were analytical grade for high-performance liquid chromatography (HPLC) or liquid chromatography tandem mass spectrometry: the analytical standard was 3,5,6-trichloro-2-pyridinol (product No. 33972; batch: BCBZ8746; Sigma-Aldrich, St. Louis, MO, USA); the internal standard was 3,5,6-trichloro-2-pyridinol-4,5,6-$^{13}C_3$-^{15}N (Sigma-Aldrich); and the other materials used were hydrogen chloride, sodium chloride, and acetonitrile (Merck KGaA, Darmstadt, Germany); methanol (Tedia, Fairfield, OH, USA); and formic acid (Kanto Chemical Co., Tokyo, Japan).

Briefly, the extraction procedure was adopted from Smith et al. [19], and the separation and detection procedure was adopted with small adjustments from the CDC 6103.03 laboratory method [20]. Chromatographic separation was performed using Agilent HPLC (Infinity 1260; Santa Clara, CA, USA). Agilent Ultivo triple quadrupole mass spectrometer (Agilent) with Masshunter software program was used for data acquisition and data analysis. The within-run precision for TCPy analysis at 1 ppb was excellent, with a 4.77% relative SD and a standard curve correlation coefficient of 0.998.

Detectable TCPy levels were adjusted by urinary creatinine to reduce intra-individual and inter-individual variability [21,22]. Urinary creatinine was analyzed in a Proline R-910 system using a commercial kit (Proline Creatinine PAP FS; Proline, West Java, Indonesia) for quantitative determination in accordance with standard clinical laboratory methods. The TCPy values were expressed as μg/g creatinine.

Statistical Analysis

The analysis was performed using SPSS version 20 (IBM Corp., Armonk, NY, USA). The study population characteristics were

summarized with the frequency distribution for categorical variables, while continuous variables were described using mean±SD or median (minimum-maximum). The chi-square test was used to evaluate the significance of differences in genotype frequencies according to sex and CEL. Allele and genotype frequencies were calculated directly. The Mann-Whitney U test and the Kruskal Wallis test were used to evaluate the significance of differences in TCPy levels. All *p*-values were two-sided, and $p<0.05$ was considered the threshold of statistical significance. Age and sex, as individual predictors, together with *CYP2B6*6* and CEL, as important occupational factors, were included in the multivariate model together with the variables associated with TCPy at a significance level of $p\leq0.20$ in the simple regression analysis. All determinants associated with TCPy at a significance level of 0.05 were retained in the final stepwise model. Several assumptions were met for the multiple linear regression analysis. There was no multicollinearity in this data, as the variance inflation factor scores were well below 10, and the tolerance scores were above 0.2. The Durbin-Watson statistic showed that the values of the residuals were independent, as the obtained value was very close to 2 in the linear regression model. We also calculated the Cook's distance values for each participant, and since no values over 1 were found, we suggest that there were no residual outliers biasing our model.

Table 1. Characteristics of the study population (n=132)

Characteristics	Description
Age (y)	49.9±9.5
Body mass index (kg/m^2)	22.9±2.9
Male	119 (90.2)
Low level of education	114 (86.3)
Smoking	65 (49.2)
Member of farmers' society	123 (93.2)
Intensity level	11.8 (1.0-23.0)
Lifetime years of pesticide use (y)	25 (1-45)
No. of days spraying per year (day)	104 (37-364)
Cumulative exposure level (×10^3)	25.95 (1.28-136.58)
Post-spraying days (day)	1 (1-10)
Arable land area (acres)	0.20 (0.01-0.70)
Daily work duration (hr)	6 (3-10)
Duration of spraying pesticide (hr/day)	0.43 (0.04-2.25)
Volume of the mixture applied (L/day)	19.7 (2.3-85.0)

Values are presented as mean±standard deviation or number (%) or median (minimum–maximum).

Ethics Statement

The study protocol was approved by the Ethical Committee of the Faculty of Medicine Universitas Indonesia on March 23, 2020 (No. KET-339/UN2.F1/ETIK/PPM.00.02/2020).

RESULTS

The characteristics of the 132 participants included in our study are outlined in Table 1. Our study population was vegetable farmers with a mean age of 49.9 years, consisting of 90.2% male and 86.3% with a low educational level (≤9 years of formal education). Our participants had been using pesticides for about 25 years, with a median frequency of spraying of 104 days per year. The median (minimum-maximum) CEL was 25.95 (1.28-136.58), and 48% of the participants were categorized as belonging to the high-CEL group. The urinary creatinine-adjusted TCPy levels were 2.31 (0.17-49.12) μg/g creatinine.

*CYP2B6*6* gene polymorphisms were common in our study population, with a distribution of 31.1% for the **1/*1* genotype, 50.8% for **1/*6*, and 18.2% for **6/*6*, with no significant difference based on sex or CEL groups (Table 2). The minor allele frequencies of *CYP2B6 785A>G* and *516G>T* were the same, at 43.9%.

The median TCPy level was significantly associated with *CYP2B6*, smoking habits, spraying time, and the use of additional

Table 2. Distribution of *CYP2B6* genotype according to the sex group and CEL

Genotype	All (n=132)	Sex			CEL		
		Male (n=119)	Female (n=13)	*p*-value[1]	High (n=63)	Low (n=69)	*p*-value[1]
*CYP2B6*6*				0.882			0.328
**1/*1*	41 (31.1)	37 (31.1)	4 (30.8)		21 (33.3)	20 (29.0)	
**1/*6*	67 (50.8)	61 (51.3)	6 (46.2)		28 (44.4)	39 (56.5)	
**6/*6*	24 (18.2)	21 (17.6)	3 (13.0)		14 (22.2)	10 (14.5)	

Values are presented as number (%).
CEL, cumulative exposure level (low: ≤25.9; high: >25.9).
[1]From chi-square test.

Table 3. Comparison of TCPy concentrations of the study population grouped according to the *CYP2B6*6* genotype, socio-demographic, and occupational characteristics

Variables	n	TCPy[1]	*p*-value[2]
*CYP2B6*6*			
**1/*1*	41	4.53 (0.39-49.12)	0.005[3,4]
**1/*6*	67	2.21 (0.53-22.22)	
**6/*6*	24	1.66 (0.17-20.74)	
Sex			
Female	13	2.08 (0.56-30.31)	0.601
Male	119	2.35 (0.17-49.12)	
Smoking status			
Smoking	65	1.63 (0.17-42.64)	<0.001
Not smoking	67	3.68 (0.39-49.12)	
Spraying time			
Other than morning	47	4.97 (0.17-42.64)	<0.001
Morning time	85	1.78 (0.39-49.12)	
Type of knapsack sprayer			
Manual pressurized	30	2.34 (0.17-49.12)	0.942
Motorized	102	2.31 (0.39-30.61)	
Additional pesticides to CPF			
>2 pesticides	35	4.28 (0.17-42.64)	0.005
≤2 pesticides	97	1.79 (0.39-49.12)	
Direct contact with pesticides			
Frequent	95	2.35 (0.39-49.12)	0.897
Rare/never	37	2.12 (0.17-42.64)	
Cumulative exposure level			
High	63	2.63 (0.53-49.12)	0.343
Low	69	2.12 (0.17-42.64)	

TCPy, 3,5,6-trichloro-2-pyridinol; CYP, cytochrome P450; CPF, chlorpyrifos.
[1]Median (minimum-maximum) in μg/g creatinine.
[2]From Mann-Whitney test.
[3]From Kruskal Wallis test.
[4]Post-hoc testing with the Mann-Whitney U test between **1/*1* to **1/*6* and **6/*6* showed significant differences, at $p=0.039$ and $p=0.002$, respectively.

pesticides to CPF, as described in Table 3.

Simple linear regression was performed to analyze the associations between TCPy levels and contributing factors (Supplemental Material 6). Following the stepwise procedure, all determinants associated with TCPy at a significance level of 0.05 (i.e., *CYP2B6*6*, smoking habits, body mass index (BMI), CEL and spraying time) were retained (Table 4). TCPy levels were higher among participants with the *CYP2B6*1/*1* genotype ($p=0.002$) and high CEL ($p=0.012$), as well as among those who sprayed at other times than the morning ($p=0.014$). In contrast, smokers and those with a high BMI had lower TCPy levels.

Table 4. Multiple linear regression analysis of the association between TCPy and contributing factors[1]

Variables[2]	B	SE (B)	Beta	95% CI for B		*p*-value
				LL	UL	
Constant	22.61	4.82	-	13.08	32.15	<0.001
*CYP2B6*6*	-2.66	0.86	-0.24	-4.36	-0.95	0.002
Smoking	3.61	1.24	0.24	1.17	6.05	0.004
BMI	-0.57	0.21	-0.22	-0.98	-0.16	0.006
CEL	-3.11	1.22	-0.20	-5.52	-0.70	0.012
Spraying time	-3.21	1.28	-0.20	-5.74	-0.67	0.014

TCPy, 3,5,6-trichloro-2-pyridinol; B, parameter estimate; SE (B), standard error for B; CI, confidence interval; LL, lower limit; UL, upper limit; CYP, cytochrome P450; BMI, body mass index; CEL, cumulative exposure level.
[1]$R^2=0.243$; Adjusted $R^2=0.213$.
[2]*CYP2B6*: **1/*1* (reference) or **1/*6* or **6/*6*; Smoking status: smoking (reference) or not smoking; BMI in kg/m^2 (continuous variable); CEL: high (reference) or low; Spraying time: other than morning (reference) or morning time.

DISCUSSION

The present study focused on determining the associations of the most prevalent and important *CYP2B6* variants with CPF exposure. The most clinically relevant polymorphism of *CYP2B6* was *CYP2B6*6*, with a co-occurrence of *CYP2B6 785A>G* and *516G>T*) [23]. The *CYP2B6*6* variant is common due to the strong linkage disequilibrium between the *516G>T* and the *785A>G* variants [24]. Our findings indicate that *CYP2B6*6* gene polymorphisms were present in two-thirds of our study population, and the observed frequency of the *CYP2B6*6* genotype was in Hardy-Weinberg equilibrium. The minor allele frequencies of *CYP2B6 516G>T* and the *785A>G* among our participants were higher than those reported in Egyptian [25], Turkish [26], and Han Chinese [27] populations.

CPF is eliminated from the body primarily in the urine, with a relatively short biological half-life of approximately 27 hours [9]. The detection rate of urinary TCPy, a specific metabolite of CPF, in our study was 100%. Although measurement of urinary TCPy as a biomarker of CPF exposure is an established method to study CPF exposure and reflects all exposure pathways, finding a measurable amount of urinary TCPy does not necessarily mean it will cause adverse health effects, as urinary TCPy levels provide only limited evidence of exposure [28]. The urinary TCPy levels in our study populations were similar to those in previous studies in the general population [29,30]. Nevertheless, compared to other studies among populations that were extensively exposed to CPF, our results are far lower [11,31]. We suggest that differences in the nature of exposure (i.e.,

pesticide concentrations, application methods, or climate conditions) may contribute to these results.

We observed in the present study that farmers who used >2 additional pesticides had higher TCPy levels. We assumed that this practice might be associated with a tendency to use CPF beyond the recommended dose, thereby increasing the chemical uptake and resulting in higher TCPy levels. The practice of using multiple pesticides also raises concerns regarding the possibility that interactions of CPF with other pesticides may increase the potential for toxicity [32]. We found that *CYP2B6*6*, smoking status, BMI, CEL, and spraying time were associated with urinary TCPy levels. CYP2B6 is known as a prominent catalyst in CPF bioactivation; thus, this result indicates significantly higher CPF-O formation [33]. In particular, CYP2B6*6 has similar kinetic activity to that of CYP2B6*1, but it is expressed at lower levels due to the aberrant splicing [34], thereby reducing its ability to activate CPF-O formation [14]. The presence of genetic variations in human *CYP* may influence (favorably or unfavorably) the susceptibility to potential health impacts on those exposed to xenobiotics [7,35]. Since *CYP2B6* is one of the most important enzymes in CPF metabolism, individuals with higher *CYP2B6* expression (i.e., *CYP2B6*1/*1*) are more susceptible to exposure due to the higher formation of CPF-O, as further indicated by higher TCPy levels [33]. This finding is supported by the fact that the **1/*6* group also had higher TCPy levels than the **6/*6* group. In addition, there were no significant differences in genotype distribution according to sex or CEL, suggesting that the members of both groups shared similar genetic susceptibility to CPF exposure.

The farmers in this study seemed to have had a long history of pesticide use, as they had spent most of their lives in the profession of farming and their agricultural practices had been applied over many years. This finding explains why the high CEL group in our study was characterized by a low frequency of PPE utilization and poor work practices [17], and had higher TCPy levels. Urinary TCPy levels were significantly lower in smokers than in non-smokers; a similar pattern was found in previous research [36,37]. Though the actual mechanism remains unclear, tobacco smoking is thought to modify the physiological transformation and metabolism of xenobiotics, including OP pesticides [38]. The highest CPF concentrations are present in fat or adipose tissue, leading to the speculation that lipid storage may play an important role in the rate of CPF elimination. CPF that is temporarily bound to fat tissue will be released and undergo bioactivation, potentially resulting in a longer-lasting effect [39]. The spraying time refers to the time of application or the time when farmers spray pesticides on their crops. In the morning, the air is more likely to be calm than at other times of day, reducing the risk of chemicals being accidentally inhaled due to high winds. Furthermore, the lower temperatures and relatively high humidity in the morning may mean that the sprayed pesticides do not evaporate, reducing the potential for spray drift that could lead to unnecessary inhalation exposure; thus, spraying in the morning may be useful for personal protection [40].

Farmers, particularly those in the small-scale sector, with limited knowledge of proper pesticide handling practices may not be aware that they are susceptible to the health impacts of CPF exposure [17]. Deleterious effects of pesticides are not determined solely by genetic susceptibility; therefore, exposure control through comprehensive preventive measures (i.e., providing proper knowledge of the potential health impacts of pesticide exposure and training on pesticide handling and utilization of proper PPE) must be considered.

Our study had some limitations. The exact amount of CPF and the composition of the mixtures used were not directly measured, and since all information regarding agricultural activities was self-reported, the exposure estimates may have been misclassified randomly. Therefore, to limit the possibility of misclassification, we randomly asked several important questions to determine the consistency of the answers. Exposure to CPF may also arise from consuming contaminated food or drink; however, information on dietary intake was not collected. In addition, although the participants were asked to attend the second phase within the specified time (1 day after spraying activities), in some cases they attended at different times according to their availability, which could affect the detected metabolites.

To the best of our knowledge, this is the first epidemiological study to report the frequency distribution of *CYP2B6*6* gene polymorphisms in association with TCPy and its role in CPF toxicity in an Indonesian agricultural population. Despite its limitations, the results suggest that *CYP2B6*6* may play an important role in reducing the susceptibility to CPF exposure. We found that *CYP2B6*6* gene polymorphisms, together with CEL, smoking, BMI, and spraying time were determinants of urinary TCPy levels, as a biomarker of CPF toxicity. The *CYP2B6*6* genotype is a potential biomarker of susceptibility to CPF exposure; thus, it will be useful in preventive measures or exposure management strategies among susceptible farming popula-

tions. Our results may warrant further investigation; in particular, a longitudinal study is needed to evaluate the influence of the *CYP2B6*6* gene polymorphism on CPF metabolism, particularly among the agricultural population in Indonesia, to reduce potential health impacts.

SUPPLEMENTAL MATERIALS

Supplemental materials are available at https://doi.org/10.3961/jpmph.21.641.

CONFLICT OF INTEREST

The authors have no conflicts of interest associated with the material presented in this paper.

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AUTHOR CONTRIBUTIONS

Conceptualization: Liem JF, Suryandari DA, Malik SG, Mansyur M, Soemarko DS, Kekalih A, Subekti I, Suyatna FD, Pangaribuan B. Data curation: Liem JF. Formal analysis: Liem JF, Mansyur M. Funding acquisition: Liem JF, Suryandari DA. Methodology: Liem JF, Suryandari DA, Malik SG, Malik SG, Mansyur M, Soemarko DS, Kekalih A, Subekti I, Suyatna FD, Pangaribuan B. Writing – original draft: Liem JF, Suryandari DA. Writing – review and editing: Suryandari DA, Malik SG, Mansyur M, Soemarko DS, Kekalih A, Subekti I, Suyatna FD, Pangaribuan B.

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