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CYP2A6*4 allele gene high frequency associated with low-density lipoprotein cholesterol (LDL-C) among Javanese Indonesian smokers

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ABSTRACT

The *CYP2A6* gene, which codes the *CYP2A6* enzyme, has known to have ahigh polymorphism. This polymorphism could decrease, increase, or eliminate the *CYP2A6* enzyme activity. *CYP2A6*4*, an inactive allele, decreased the *CYP2A6* enzyme activity. One of the *CYP2A6* enzyme-specific substrates is nicotine. This inactive allele could decrease nicotine metabolism that causes high nicotine levels in the blood. In addition, it caused the increasing levels of Low-Density Lipoprotein Cholesterol (LDL-C) by expanding the lipolysis process. The purpose of this research was to evaluate the effect of the *CYP2A6*4* allele gene on LDL-C levels. Respondents in this study were 31 male Javanese smokers. This research is an analytic observational study with a cross-sectional design. Polymerase chain reaction (PCR) methods use to identification the *CYP2A6*4* allele gene. This study shows that a high-frequency *CYP2A6*4* alleles gene among the subject was detected, with an allele frequency is 93.55%. Furthermore, this *CYP2A6*4* allele gene did not impact LDL-C levels, with the Odd Ratio value was 1.636 (P-Value = 0.737). In conclusion, the *CYP2A6*4* allele gene does not significantly affect the LDL-C levels in Javanese Indonesian smokers.

Keywords: cardiovascular disease, *CYP2A6*, Javanese male, LDL-C, polymorphism

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INTRODUCTION

According to the (World Health Organization, 2020), more than 1.3 billion people worldwide consume cigarettes. In addition, the death rate from smoking reaches more than 8 million people each year which is more than 7 million people are active smokers and 1.2 million people are passive smokers. The Indonesia Ministry of Health (2018) has reported that Indonesia is the third country with the highest cigarette consumers in the world. The prevalence of heavy smokers in this country was 24.3%, and light smokers were 5%, while the percentage of smokers aged ≥ 15 years in males was 62.9% and in females was 4.8% (Kemenkes RI, 2018). The Tobacco Atlas in Indonesia (2020) has reported that every year diseases caused by tobacco killed more than 225 thousand Indonesian people. Still, more than 500 thousand children (10-14 years old) and 6,4 million adults (≥ 15 years old) continuing to consume tobacco every day.

Cigarette smoke consists of various toxicologically compound, including nicotine, carbon monoxide (CO), polycyclic aromatic hydrocarbons (benzopyrene), tobacco-specific nitrosamines, aldehydes (acrolein, formaldehyde), hydrogen cyanide, nitrogen oxides, benzene, toluene, phenols (phenol, cresol), and some of the inorganic compounds (arsenic, nickel, chromium, cadmium) (Alessandro et al., 2011; Breitling, 2013). The active compound in cigarette smoke is nicotine. It is inhaled not only by active smokers but also by secondhand smokers (Benowitz, 2011; Mishra et al., 2015). Nicotine contributes to influencing Low-Density Lipoprotein Cholesterol (LDL-C) levels by increasing lipolysis. High levels of nicotine in the blood will stimulate the sympathetic adrenal system and increase catecholamine secretion. It will expand lipolysis of fat tissue adipose and increasing free fatty acids. Furthermore, free fatty acids will esterify as triacylglycerols and esters cholesterol in the liver. They are secreted into the bloodstream as VLDL and will convert into LDL as the final product (Devaranavadgi et al., 2012; Sanhia et al., 2015). This compound was metabolized through the *CYP2A6* enzyme, which coded by the *CYP2A6* gene. This gene has a highly polymorphic form that could decrease, eliminate, or increase the *CYP2A6* activity enzyme (Di et al., 2010).

The active *CYP2A6* allele gene is *CYP2A6*1* (wild type), while one of the inactive alleles is *CYP2A6*4*, a deletion allele. Combining the *CYP2A6*4* allele gene with other allele genes results in decreased nicotine metabolizing enzyme activity, impacting nicotine levels in the blood and affecting LDL-C levels (Patramurti and Fenty, 2019; Tanner, A et al., 2017; Yusof and Gan, 2009). In our previous study has revealed a high frequency of these inactive alleles among the Javanese Indonesian population and identified them as slow metabolizers (Patramurti et al.,2017). Therefore, in this study, we learned the *CYP2A6*4* allele gene frequencies among the Javanese Indonesian smokers to explain the *CYP2A6*4* allele genes effect on LDL-C levels as a possible risk factor in developing cardiovascular diseases.

MATERIALS AND METHOD

Materials

Whole blood; Forward primer for *CYP2A6*1* and *CYP2A6*4* alleles: (5 'CCT CAT CAC ACA CAA CTT CCT C 3'); reverse primer for *CYP2A6*1* alleles: (5 'CGC AGG TAC TGG GTG CTT GGT AG 3'); reverse primer for the *CYP2A6*4* allele: (5 'TGC AGG TAC TGG GTG CTT GGT AG 3'); Promega Taq Green Master Mix; FavorPrepTM Genomic DNA Mini Kit (DNA isolation kit); 10X Tris Borate EDTA (TBE) Buffer pH 8.3 (Omnipur); agarose (Genedirex); Loading Dye (SMOBiO); GelRed™ Nucleic Acid Gel Stain (SMOBiO); and AccuBand™ 100 bp+3K DNA ladder II (SMOBiO) (DNA marker).

Methods

Respondent recruitment

The sample population involved 31 adult subjects from online driver motorcycles. The respondent recruitment process is using a broadcast system on the social media group. According to

this study's inclusion and exclusion criteria, the researcher selected the respondents who register. The inclusion criteria are male active smokers with health conditions and had three generations of Javanese tribe, namely parents, grandparents, and great grandparents. For at least 20 years, the participant will be active smokers with cigarettes per day of ≥10 and willing to fill out informed consent. The exclusion criteria are patients taking regular medication or treatment for smoking cessation and an illness condition required rest of ≥10 days in the past month. In addition, patients with cardiovascular disease.

A standard questionnaire was used to obtain demographics data, smoking behavior, and medical history from each respondent. The questionnaire is only filled out by respondents eligible to participate in this study and voluntarily participate in the research. All information relating to the respondent's identity will be kept confidential. Therefore, the data collected from the respondent have managed anonymously and be known only to the researcher. The procedure utilized in this study was authorized by the Health Research Ethics Committees, Faculty of Medicine, Duta Wacana University, Yogyakarta, with ethical clearance. No. 1235/C.l6/FK/202l.

Cigarette dependence analysis

The cigarette dependence was analyzed using the Fagerstrom Test for Nicotine Dependence (FTND) instrument. This instrument's overall total score is 0-10, with a higher FTND score indicating a stronger dependence on nicotine (Heatherton et al., 1991).

Blood sampling

Respondent first examined the general condition, which measured BMI and blood pressure. The blood sample was taken as much as 3 mL from a vein with the patient fasting for 12 hours and collected into a K2 vacutainer tube containing EDTA (1.8 mg/mL blood). The blood is stored at ± 4^{o}C. The Low-Density Lipoprotein Cholesterol (LDL-C) was promptly measured using the Direct CHOD PAP method in a clinical laboratory.

DNA isolation

DNA isolation aims to obtain pure DNA and separate it from other components such as fat, carbohydrates, and protein (Murtiyaningsih, 2017). In this study, DNA isolation used the whole blood sample because blood cells contain more DNA than other samples (Pelt-Verkuil et al., 2008). The DNA isolation in this study was carried out using the FavorPrep^{TM} Genomic DNA Mini Kit. The procedure was carried out following the stated protocol with a blood sample of 200 µL. The DNA isolate purity test was carried out by electrophoresis method using 1.0% agarose gel. For about 3.0 µL of DNA, the isolate was mixed with 2.0 µL of aquabidestilata, and 1.0 µL of loading dye. 5 µL of this mixture was injected into the agarose well gel. Electrophoresis was running at 110 V for 30 minutes. The DNA isolates were stored at temperature -70^{o}C and stable for two years.

Identification of CYP2A6*1 and *4

The *CYP2A6*1* and **4* alleles gene in isolated DNA were analyzed using the PCR method. *CYP2A6*4* alleles gene has a whole gene deletion due to unequal crossover junctions (Fukami et al., 2007). A specific primer used for *CYP2A6*1* and **4* alleles gene identification using the PCR method. The primer using in this study would amplify the nucleotide sequence of the gene from 10643 to 10993. There are six variations in the nucleotide sequence between the *CYP2A6*1* and *CYP2A6*4* genes among this amplified sequence. The primers forward (5'-CCT CAT CAC ACA CAA CTT CCT C-3') used in this study will anneal to both *CYP2A6*1* and *CYP2A6*4* alleles. We used two types of reverse primers for the *CYP2A6*1* and *CYP2A6*4* alleles are 5'-CGC AGG TAC TGG GTG CTT GGT AG-3' and 5'-TGC AGG TAC TGG GTG CTT GGT AG-3'. These primers will produce a PCR product with a length size of 350 bp. As the two primer reverse has a one difference nucleotide base

located on the first sequence (C>T), each reverse primer will anneal to a specific allele. In addition, we also adopted the PCR conditions using by Gitaningtyas (2018).

The PCR reaction consists of 12.50 µL Taq Green Master Mix Promega, 1.25 µL forward primer, 1.25 µL reverse primer, 5.0 µL DNA isolate, and 5.0 µL free water nuclease with a final mixture volume of 25.0 µL. The PCR was performed under the following conditions: initial denaturation at 95°C for 5 minutes; followed by denaturation at 98°C for 20 seconds; annealing at 64°C for 15 seconds and extension at 72°C for 30 seconds. The amplification cycle was carried out 30 times and then ended with a final extension at 72°C for 5 minutes.

PCR product analysis

The PCR products were analyzed using the electrophoresis method using 1.5% agarose. Each well was consisting 5 µL of PCR products. Electrophoresis was carried out with a voltage condition of 110V and running for 30 minutes. The results obtained were viewed using a UV transilluminator and documented using a DSLR camera. The *CYP2A6*1* and *CYP2A6*4* allele PCR products detected on the electrophoresis method have the same band length of 350 bp.

Data Analysis

The *CYP2A6*1* and *CYP2A6*4* allele was analyzed using the PCR product detected on the electrophoresis method with a band length of 350 bp. The allele frequencies were calculated using the equation (1) (2). In addition, the Odds Ratio (OR) value was calculated with SPSS software version 25.0 using Mantel-Haenszel Common Odds Ratio Estimate (95% CI) to describe the effect of the *CYP2A6*4* allele gene on Low-Density Lipoprotein Cholesterol (LDL-C)

$$frequency = \frac{Number\ of\ CYP2A6*1\ alleles\ obtained}{sum\ of\ all\ alleles\ (31)} x\ 100\% \quad (1)$$

$$frequency = \frac{Number\ of\ CYP2A6*4\ alleles\ obtained}{sum\ of\ all\ alleles\ (31)} x\ 100\% \quad (2)$$

RESULT AND DISCUSSION

Participants in this study were 31 male active smokers with health conditions and had three generations of Javanese tribe, namely parents, grandparents, and great grandparents. The participant for at least 20 years to be active smokers with cigarettes per day of ≥10. In this study, the characteristics and health observation data of respondents as shown in Table 1.

Table 1. Characteristics and health observation data of respondents

Respondents Characteristics Profile			
Characteristics	**n**	**Mean ± SD**	**Range**
Age (Years)	31	39.7±7.7	27-62
First smoking (years)	31	15.7±4.2	7-30
Cigarettes per day (Sticks)	31	13.5±4.4	10-21
Smoking duration (Years)	31	23.2±4.8	20-38
Respondents Health Observation			
Characteristics	**n**	**Mean ± SD**	**Range**
BMI	31	25.1±4.8	17-36
Waist circumference (Cm)	31	89.5±11.3	65-112
Systolic blood pressure (mmHg)	31	128.4 ±24.1	90-195
Diastolic blood pressure (mmHg)	31	82.6±9.4	65-100

As described in Table 1, all respondents in this study were adults with the age range of 27-62 years old. Most of them worked as online motorcycle taxis. All of the respondents have smoked for more than 20 years. As shown in Table 1, some respondents have smoked before 15 years. The Health Ministry of Indonesia has reported that more than 30% of Indonesian children have smoked a cigarette before the age of 10 years (Kemenkes RI, 2018). They are some factors that encourages the children to smoke including TV advertisements, smoking environment, and sponsorships on activities in which teenagers are involved the most. In addition, based on cigarettes consume per day and the duration of smoking, this study indicated that the respondents are in the moderate-heavy category (Patramurti and Fenty, 2019; Watanabe et al., 2011).

We have also done the health observations among the participants as described in Table 1. Based on BMI data and waist circumference, all of the respondents are in the overweight category. This condition is related to their job as online driver motorcycle, including the low level of physical activity category because most of the activities carried out on a seated vehicle. In addition, the systolic and diastolic blood pressure all of the respondents are at normal blood pressure (Perhimpunan Dokter Hipertensi Indonesia, 2019).

The PCR products were identified by the electrophoresis method using 1,5% agarose gel. Figures 1 describe the *CYP2A6*1* and the *CYP2A6*4* PCR product. As shown in these figures, the specific primers used in this study would produce the PCR product with the same size (350bp). It is due to the only one nucleotide difference base using to amplify at the reverse primer.

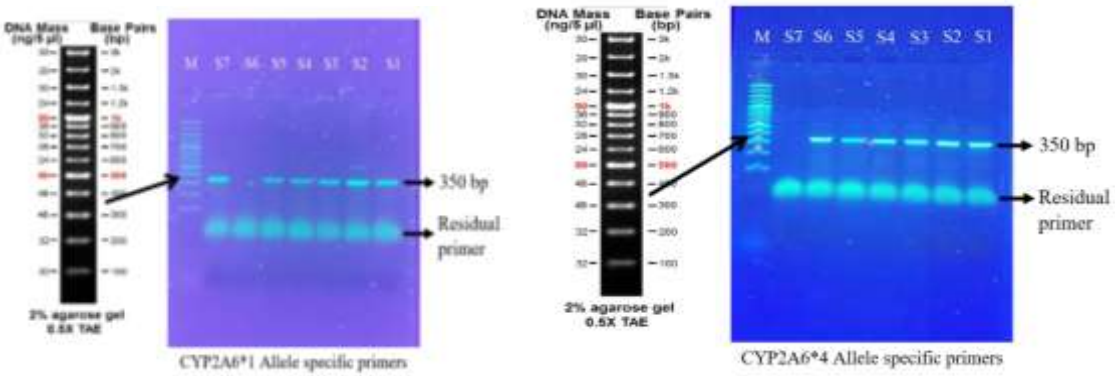

Figure 1. Electropherogram PCR products with *CYP2A6*1* and*4* allele-specific primers

M	: 100 bp + 3K bp DNA ladder
S1-5 and 7 (Primer *CYP2A6*1*)	: *CYP2A6*1* allele PCR products (Normal allele)
S1- 6 (Primer *CYP2A6*4*)	: *CYP2A6*4* allele PCR products (Inactive allele)
Electrophoretic conditions	: 1.5% agarose stationary phase; mobile phase TBE 1X solution; 110V voltage with running time 30 minutes; injection volume 5 µL

We have amplified all the DNA isolated samples. Based on our result in Table 2, both *CYP2A6*1* and *CYP2A6*4* alleles have the same allele frequencies, with a value are 93,55%. These results were in line with another study, in which CYP2A6*4 alleles were found a high frequency in the Javanese Indonesian population (Patramurti et al., 2017). In this study, we have also identified the genotype belonging to the respondent base on their allele. They are *CYP2A6*1/*1* (normal metabolizer) 6.45%; *CYP2A6*1/*4* (slow metabolizer) 87.10%; and *CYP2A6*4/*4* (poor metabolizer) 6.45%. This result was in line with the previous study, which is among the Javanese Indonesia population, the percentage of slow metabolizer was high (Patramurti and Fenty, 2017; Patramurti et al., 2015).

Table 2. *CYP2A6 * 1* and *CYP2A6 * 4* alleles, genotypes, and phenotypes

Allele			Frequency
*CYP2A6*1*			93,55%
*CYP2A6*4*			93,55%
Genotype (Phenotype)			**Frequency**
*CYP2A6*1/*	*CYP2A6*1*	(normal metabolizer)	6,45%
*CYP2A6*1/*	*CYP2A6*4*	(slow metabolizer)	87,10%
*CYP2A6*4/*	*CYP2A6*4*	(poor metabolizer)	6,45%

The presence of the *CYP2A6*4* allele significantly affects cigarette dependence. Individuals with the *CYP2A6*4* allele had a lower potential for smoking dependence than individuals with the *CYP2A6*1* allele (Akrodou, 2015; Liu et al., 2011; Minematsu et al., 2006). Base on their genotype and their cigarette dependence, we have identified the genotype effect on cigarette dependence among the respondents. The cigarette dependence was obtained based on the assessment of the Fagerstrom Test for Nicotine Dependence (FTND) questionnaire (Heatherton et al., 1991).

Table 3. Effect of Allele gene *CYP2A6*4* on cigarette dependence

Dependency Level Cigarettes	Number of Respondents		
	CYP2A6*1/*1 **n(%)**	***CYP2A6*1/*4*** **n(%)**	***CYP2A6*4/*4*** **n(%)**
Very low	- (0)	3 (9.7)	- (0)
Low	1 (3.2)	12 (38.7)	- (0)
Medium	1 (3.2)	7 (22.6)	2 (6.45)
High	- (0)	4 (12.9)	- (0)
Very high	- (0)	1 (3.2)	- (0)

Table 3 above describes that account 48.4% of the respondents have a very low and low dependency. These due to the *CPY2A6*4* allele was dominating among the respondents and causing a decrease in cigarette dependence than individuals with *CYP2A6*1* allele (Akrodou, 2015; Liu et al., 2011; Minematsu et al., 2006). Otherwise, Table 3 shows that the cigarette dependence on medium, high, and very high is 45.15%. The other factor that influences smoking behavior is environmental factors. All of the respondents have worked as online driver motorcycle they usually could not avoid smoking when the other drivers are smoke (Akrodou, 2015).

CYP2A6 is an enzyme responsible for metabolizing 70-90% nicotine and converting it into an inactive form by oxidizing nicotine to nicotine ions Δ1‟(5‟)-iminium. Furthermore, nicotine iminium ions are converted to cotinine by the enzyme aldehyde oxidase. This metabolite, cotinine, will also be metabolized by *CYP2A6* into several hydroxylation metabolites, namely 3-hydroxycotinine, 5-hydroxycotinine, and nor cotinine, excreted from the body (Benowitz, L et al., 2016). Smokers with *CYP2A6*4* tend to have to decrease in nicotine metabolism. Therefore, this condition causes high nicotine levels in the blood, increasing LDL-C levels by stimulating the sympathetic adrenal system and increasing catecholamine secretion. The impact is expanding lipolysis of adipose tissue fat and increasing free fatty acids. In the liver, free fatty acids esterify as triacylglycerols and esters cholesterol. They are secreted into the bloodstream as VLDL and will convert into LDL, which circulates in the blood (Devaranavadgi et al., 2012). To evaluate the *CYP2A6*4* allele on LDL-C levels among smokers, we used the odds ratio value as shown in Table 4.

Table 4. The *CYP2A6*4* allele gene effect on LDL-C levels

Allele	LDL-C Levels		Sum n(%)	P-*Value*	OR (95%CI)
	≥130 mg/dL n(%)	<130 mg/dL n(%)			
***CYP2A6*4* allele present**	18 (58.1)	11 (35.5)	29 (93.5)		1.636
No *CYP2A6*4* allele present	1 (3.2)	1 (3.2)	2 (6.5)	0.737*	(0.093-28.904)
Sum n(%)	19 (61.3)	12 (38.7)	31 (100)		

*****P-*Value* >0,05 showed that the data were not significantly different between groups
P-Value is calculated using Mantel-Haenszel Common Odds Ratio Estimate (95% CI)

In Table 4 show that 58.1% of respondents with the *CYP2A6*4* allele have LDL-C levels ≥ of 130 mg/dL. Furthermore, the calculation of the Odd Ratio shows that individuals with the *CYP2A6*4* allele have a 1.636-fold greater risk of increasing LDL-C levels than individuals without the *CYP2A6*4* allele. Therefore, based on this odds ratio value, this result was in line with another study that smokers with the *CYP2A6*4* allele have on decreasing the activity of the *CYP2A6* enzyme, which causes high nicotine levels in the blood, thereby increasing LDL-C levels and reducing HDL-C levels (Rao and Subash, 2013).

As described in Table 4, the P-Value obtained is > 0.05, indicates that the data is not significantly different between *CYP2A6*4* allele present or without *CYP2A6*4* allele present. This result can occur because the allele influence on nicotine metabolism is not only due to the *CYP2A6*4* allele. According to Mwenifumbo (2008) and Schoedel's (2004), other alleles such as the *CYP2A6*7* and *CYP2A6*9* alleles can affect nicotine metabolism impact LDL-C levels. Furthermore, genotypes and phenotypes can be formed from the present other allele, namely normal metabolizers (*CYP2A6*1/*1*); intermediate metabolizer (*CYP2A6*1/*7; CYP2A6*1/*9*); slow metabolizer (*CYP2A6*1/*4; CYP2A6*1/*7/*4; CYP2A6*1/*9/*4; CYP2A6*7/*7; CYP2A6*9/*9*); and poor metabolizers (*CYP2A6*4/*4; CYP2A6*4/*7; CYP2A6*4/*9*) (Bajaj, 2012; Messner and Bernhard, 2014; Mwenifumbo et al., 2008; Mwenifumbo and Tyndale, 2007; Nakajima et al., 2006; Schoedel et al., 2004).

Some other factors are influencing the LDL-C levels, namely smoking behavior (duration of smoking, cigarettes per day, and types of cigarettes used) and environmental factors (socioeconomic, lifestyle, level of education, and culture) (Akrodou, 2015; Minarti et al., 2014; Singh et al.,2016). According to the characteristic data shown in Table 1, related to the average number of cigarettes per day (13.5±4.4 sticks) and smoking duration (23.2±4.8 years) of the respondents, all of the respondents are associated as addictive smokers affecting the increase in LDL-C levels. So, it is in line with another study that the chronic effect of smoking behavior would arise when individuals consume more than 20 cigarettes per day and 25 years minimum smoking duration (Patramurti and Fenty, 2020).

The increasing LDL-C levels in the blood could impact a high accumulation of LDL-C on the artery walls. Furthermore, it causes atherosclerosis in smokers, especially in individuals with the *CYP2A6*4* allele makes them at greater risk of getting cardiovascular disease (Rao and Subash, 2013). Some others risk factors can affect cardiovascular disease, including BMI, waist circumference, blood pressure, age, and ethnicity. Based on data in Table 1, the BMI (25.1±4.8), waist circumference (89.5±11.3 cm), systolic blood pressure (128.4±24.1 mmHg), and diastolic blood pressure (82.6±9.4 mmHg), among the respondents, have a higher risk. It indicated that respondents participating in this study were at a higher risk of cardiovascular disease (Murtiyaningsih, 2017; World Health Organization, 2017). Therefore, prevention of the incidence of cardiovascular disease needs to be done as early as possible, especially by controlling modifiable risk factors for cardiovascular disease, including smoking behavior, blood pressure, BMI, and lipid profile (World Health Organization, 2017).

According to (World Health Organization, 2014), the cardiovascular disease caused 37% of mortality in Indonesia. Smoking behavior is the main factor for getting cardiovascular disease. Indonesia is ranking as the 3rd largest cigarette market in the world; indicating that the smoking cessation program does not effectively reduce smoking rates in Indonesia (Kemenkes RI, 2018). The current study shows high frequency of these inactive alleles among the Javanese Indonesian population. The effect of present inactive allele gene *CYP2A6*4* causing a decrease in cigarette dependence than individuals with *CYP2A6*1* allele. Based on our results, it will provide the necessary drive for tobacco control efforts of all related parties, especially the Indonesian Government. They must be all together engaged in suppressing smoking rates through collaboration and effective strategies towards a tobacco-free. Thereby the incidence rate of cardiovascular disease in Indonesia will be decreased.

As state in The Regulations Guidelines Number 188/Menkes/Pb/I/2011 and Number 7 (2011), The Indonesian Government has issued regulations about implementing No-Smoking Areas to prevent passive smoking exposure to cigarette smoke. In addition, Daerah Istimewa Yogyakarta (DIY) Province is one of the provinces that has implemented a no-smoking area through DIY Government Regulation Number 42 (2009). Furthermore, the DIY Government has implemented promotive and preventive efforts related to the impact of smoking behavior for active smokers and passive smokers. The regional program and project initiatives in tobacco control, together with various levels of society, have been crucial areas of collaboration and cooperation that addressed the health risks associated with tobacco consumption in DIY.

Study Limitations

The sample size is part of the limitation of the current study. In addition, this study only identifying the *CYP2A6*1* and *CYP2A6*4* alleles. Therefore, it was necessary required to identify other alleles that affect LDL-C levels.

CONCLUSION

Based on our study, we found the *CYP2A6*1* and *CYP2A6*4* allele genes among Javanese Indonesian smokers with their respective frequencies of 93.55%. Furthermore, the *CYP2A6*4* allele gene did not impact LDL-C levels, with the Odd Ratio value was 1.636 (P-Value = 0,737).

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