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Diet contains carcinogens and deficiency of vitamin C determines the level of oxidative DNA damage among Jakarta toll collectors.

Ela Laelasari[1*], Haryoto Kusnoputranto[2], Budiawan[3], Mustofa[4], Sitti Patimah[5]

[1]Faculty of Medicine and Health Sciences, Public Health Study Programs, UIN Syarif Hidayatullah, Ciputat, Jakarta, Indonesia

[2]Department of Environmental Health, School of Public Health, University of Indonesia, Depok, Indonesia

[3]Department of Chemistry, Faculty of Mathematics and Natural Sciences, University of Indonesia, Depok, Indonesia

[4]Department of Clinical Pharmacology, Faculty of Medicine, University of Gadjah Mada, Yogyakarta, Indonesia

[5]Nutrition Department, School of Public Health, Universitas Muslim Indonesia, Makassar, Indonesia

Abstract

Consuming meat food products that undergo high-temperature combustion is able to provide opportunities exposed to carcinogenic substances and leads to oxidative stress. Oxidative stress is also a precondition for the occurrence of DNA damage and cancer can be detected by the presence of 8-hydroxy-'2-deoxyguanosine (8OHdG). The study aimed to investigate whether the high prevalence of oxidative stress in Jakarta highway toll collector is independently or causally implicated in deficiency of vitamin C and the grilled food as the third factor modified the association. A cross-sectional study and urinary 8-OHdG was detected using the Enzyme-Link Immunosorbent Assay (ELISA) method on 161 toll gate collectors. The study found a strong relationship between grilled food and 8-OHdG (p-value 0.037); prevalence risk ratio (PRR) 2.01 (95% CI 1.044-3.890). However, relationship between variable vitamin C and the 8-OHdG stratified by grilled food founded inverse negatively, it was due to the high frequency of subjects 8-OHdG presented significantly with the deficiency of vitamin C. There was not enough intake of vitamin C among people who ate grilled food. Mantel-Haenzel formula was calculated and the estimation found the condition of independence from the test were X^2_{MH}=0.843 among the group who consumed the grilled food. The association between intake of grilled food and oxidative stress is the mere result of the effect of deficiency of vitamin C. Special attention should be given to minimize diet containing various carcinogens like the meat grilling process with a high temperature to decrease degenerative diseases and cancer risk in sub-population with high expose to air pollution.

Keywords: 8-hydroxy-2'-deoxyguanosine, Grilled food, Oxidative stress, Vitamin C.

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Introduction

Oxidative stress in consequence of the condition of imbalance between levels of antioxidants and free electrons that reside in the body cells is believed to play a role in the process of DNA damage leading to cancer [1]. Micronutrient deficiencies are widely known to have a correlation with the incidence of cancer [2,3]. This condition is due to the absence of protection against free electrons and the lack of micronutrient role as an antioxidant in the body.

However, little attention has been paid to the role of micronutrient deficiency in carcinogenesis. Some empiric epidemiological studies have discovered an inverse relationship between micronutrient deficiency and tumor growth [4,5]. It was also found to have the risk of some forms of cancer, including prostate cancer [6] and cervical cancer [7]. Several studies [8] reported a stronger relationship between people who consumed grilled food than non-grilled food consumption. Increasing the levels of 8-hydroxydeoxyguanosine (8-OHdG), a marker of reliable DNA oxidative damage, has been detected in the urine of people who eat grilled food; alternatively, patients with colon cancer [3,9].

Pyrolysis process from meat and other products of protein cooked at high temperature, or grilled food is a potential source of free electrons from carcinogenic pollutant such as poly-aromatic hydrocarbon (PAHs). Although natural sources or anthropogenic PAHs are many and varied, the food seems to be the main route of exposition among toll gate workers; high concentrations of PAHs in the food are usually found in charcoal grilled/roasted food through pyrolysis of fat and

smoke from a heat source that in some Indonesian local languages called satay.

There are more than 100 kinds of PAHs. Furthermore, 17 kinds of them are identified to be the most harmful than the other PAHs [10]. The potent toxic effect is based on the number of benzene rings. Generally, PAHs consist of a unitary aromatic ring about two to four rings; the benzene rings more than four are stable and toxic [10,11].

However, human cells have special mechanism to prevent themselves from external exposure. The element of micronutrient such as vitamin B12, folic acid, vitamin C, E, A, zinc and iron contained in the daily diet is very effective for the prevention of diseases, not just limited to infectious diseases but also degenerative diseases caused by DNA damage [2,12].

Vitamin C is one of the micronutrients with the same purpose. Thus vitamin C deficiency hypothesis to represent the host susceptibility to cancers is associated with the consumption of this grilled food. However, controversy continues regarding the causal role of Vitamin C deficiency in carcinogenesis because of potential bias in epidemiological studies [13]. For example, the effect of the decrease in micronutrient vitamin C cannot be completely ruled out in the condition of DNA damage. There is a contribution from other endogen factors. Nevertheless, the grilled food consumption is associated with vitamin C deficiencies [14-16], an inverse relationship between vitamin C deficiency and cancer risk may simply reflect micronutrient vitamin C deficiency caused by the consumption of grilled food.

However, the relationship between vitamin C deficiencies and levels of urinary 8-OHdG has been inconsistent. Some studies [7,17] had reported an inverse relationship between vitamin C and some diseases. Also, there is limited evidence to show the effect of modifying the consumption of grilled food on micro-nutrient deficiencies and levels of 8-OHdG [18]. Therefore, this study investigated whether vitamin C intake has modified the effect of oxidative stress due to grilled food intake among healthy employees using the ELISA test.

Materials and Methods

Sampling

Subjects were obtained from the calculation of sample size on a cross-sectional study for a sample population with precision in this study using the alpha 5% and 10% precision. Based on information from Kelsey, a large proportion of the sample was calculated by taking into account the prevalence of the study by Lemm; obtained a minimum sample totalled 81 subjects [19,20]. However, to avoid a loss to follow-up of participants, this study has increased the number of samples of at least 10% as a correction value. So the analysis of large sample measurement for the proportion of the population exposure in the count of at least 134 subjects. The determination of the location of the sampling point by multistage cluster sampling probability and probability sampling is random sampling using a sampling frame in the form of a list of employees of the toll collectors, to obtain 25% of quota sampling from 9 different toll gates in Jakarta, Indonesia.

Health-related lifestyle like smoking and diet were confirmed using a detailed questioner. Smoking habits were fixed based on questioner for smoking history on the value of Brinkman index. There were two groups of the smoker, the participants with Brinkman index $\geq$ 600 classified into heavy smoking, <600 as light smoking [21]. The categorization on smoking habit was calculated by the interviewer as the number of cigarettes inhaled since the first time of smoking by the participants.

Statistical analysis

Analytical statistics of this study performed using SPSS v.22. The association between eating grill food habit and the 8-OHdG level was assessed with Man-Whitney. We determined the dominant factor correlating to the 8-OHdG level using multivariate logistic regression. The p-Wald under 0.25 was counted in the analysis. All statistical analysis was two-tailed and was considered to be statistically significant at the 0.05 level according to Alfa 5%. The consuming statement of the variable was eating grill food with protein material-based foods or including all products in the form of food or food material such as fish, meat, and chicken. The cooking process was done by passing the food above a glowing ember.

We combined the technique with a graph of 3D and Prevalence ratio (PRR) [22] to investigate the condition of oxidative stress, whether the grilled food intake and the level of vitamin C implicated the oxidative stress.

We calculated the effect of modifications through the analysis stratified through the Mantel-Haenzel formula [22,23]. Estimate of the risk of the effects of exposure to outcomes adjusted by the rate of DNA damage was compared to the end. Having obtained the value, Odds Ratio (ORMH) is determined by thresholds, 1. Provisions relationship role of food consumption, fuel exposure and outcome micronutrient deficiencies of vitamin C are in control by 8-OHdG which were tested by the Breslow-Day test resulted in the estimated value of the relationship. The result was compared with the value of homogeneity and if $p>0.05$ it indicates homogeneity.

Nutrisurvey

Grilled food and micronutrient status of vitamin C was gained from pooled analysis of dietary data. Micronutrient information about nutrition obtained from questionnaires was related to typical Indonesian food. The recall had collected data in a range of 3 d and frequency of consumption. Food model was shown to recall the shape and amount of dose of food consumed. Vitamin C derived from a different type of food were vegetables, fruit, vegetable salad with nut sauces (Gado-gado), oranges, apples, bananas, tomatoes, green/red peppers and other relevant sources. Grilled food represents high protein of animal product produced by cooking at high temperature. They are identified as red meat, fish, chicken, and other animal

products containing protein that is cooked using charcoal at high temperature or deep frying.

Software is calculating the daily average of the food consumed by the workers, and then compares it with the Recommended Dietary Allowance (RDA); the average daily level of food intake meets the requirements of adequate nutrition of nearly all (>90%) healthy people prevailing in the country of Indonesia through food modelling, to remind the participants what they consume for several days. Nutrisurvey analysis based on the Ministry of Health of the Republic of Indonesia in 2013 establishes RDA of vitamin C for 30-49 years of age, sex-male and female to be 90 mg and 75 mg respectively [24].

Enzyme-linked immunosorbent assay (ELISA)

Urinary 8-OHdG analysis was done with urine samples stored at -80°C temperature before laboratory analysis. ELISA kit (Oxis Health Products, Inc. Portland, USA) was used as a method for measuring 8-OHdG. Quality control laboratory analysis was determined by the Clinical Chemistry Laboratory assay Inter taking into account the value of the Coefficients Variation (CV%) in the ELISA test. 12 urine samples were obtained from the administration and finance workers corporate headquarters toll road. This value is needed to monitor the precision of the workmanship of the ELISA technique in the laboratory before the research. This study gained CV by 9%. The execution of the ELISA technique is done two times (duplicate) for each urine sample.

Frozen urine samples placed at a room temperature was then centrifuged at 2500 rpm for 10 min. Under conditions of room temperature, 50 ul of 8-OHdG monoclonal anti-body and 50 ul of each sample of urine or standard solution are loaded into a microplate coated with 8-OHdG, and then incubated at 37°C for 1 h and determination of standard analytical range from 0.5 to 200 ng/ml was done. 8-OHdG levels were adjusted to the value in milligrams per decilitre of urine creatinine. The method of creatinine analysis used creatininase (creatinine test kit ADVIA, Siemens Diagnostics); the absorption of each sample of urine creatinine was assessed by spectrophotometry at a wavelength of 596 nm.

We provided two documents of informed consent for different purposes. One is a statement of willingness to be interviewed and another form to do the laboratory biomarker analysis. The entire study protocol had been approved by the ethics committee of the Faculty of Public Health, University of Indonesia (FKM UI) in Depok, Indonesia (53/H2.F10/PPM. 00/2011).

Results

The demographic profile was obtained from 162 subjects in the form of an average age of workers in the age range 38.0 (± 5.14). Largely the sexes of all participants were male (74.1%) and status as permanent workers. Most of participants' education levels were high school (76.5%) (Table 1). Investigated according to diet, we found food pattern and intake of vitamin C based on RDA prevailing in Indonesia 2014. Diets contain various carcinogens determined as grilled food consumption. From all participants, we found that most of them (56.2%) had not eaten grilled food. The median of vitamin C of both sexes (male and female) was found to be 12.28.

Table 1. *Subject's characteristics.*

Variable	n	%
Age (y)		
Mean	38.77	
Median	38	
SD	5.14	
Sex		
Male	120	74.1
Female	42	25.9
Education		
High school	124	76.5
Diploma	11	6.8
University	27	16.7
Smoking (Index Brinkman)		
>600 tobacco years	74	46
<600 tobacco years	87	54
Use PPE mask		
Yes (<3 in 5 d)	119	73.9
No (≥ 3 in 5 d)	42	26.1
Grilled food consumption		
Yes	71	43.8
No	91	56.2
Vitamin C both sexes (RDA)		
Median	12.28	
Mean	22.29	
SD	29.3	
Urinary 8-OHdG (ng/mg creatinine)		
Median	10.3	
Mean	11.5	
SD	-6.86	

Description of oxidative stress performed by urinary 8-OHdG has median and mean average of 10.3 ng/mg creatinine, and 11.5 ng/mg creatinine respectively. The results obtained show that the continuous data distribution was not normal in the spread. Those were urinary 8-OHdG, smoking with Brinkman index and variable measurement micronutrient vitamin C and Zinc. The relationship between an independent variable of the

eating habits of grilled food and the dependent variable of DNA damage represented by 8-OHdG has obtained significant results.

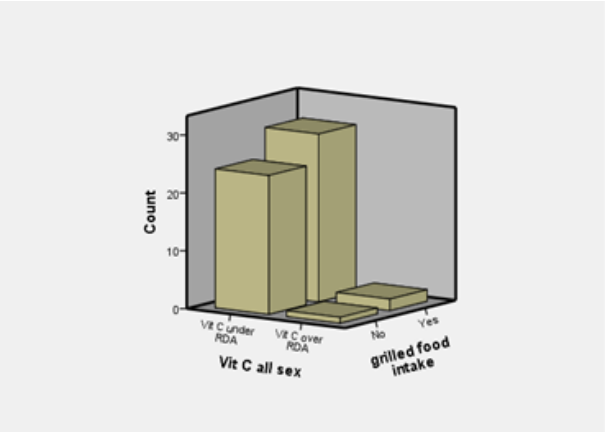

Figure 1. *Oxidative stress cases stratified by vitamin C status and grilled food intake.*

This paper summarized for bivariate and multivariate statistical analysis for identified significant association between independent variables as ROS sources based on theory and the level of 8-OHdG. The correlation of using mask as Personal Protection Equipment (PPE), vitamin C status, and smoking found no statistical significance (P-value>0.05).

Furthermore, the association between eating grilled food with a level of 8-OHdG showed high correlation (P-value=0.028) through Man-Whitney analysis. We investigated dominant factor from three candidate variables; those were vitamin C intake, eating grilled food, personal protective equipment (PPE), and smoking, and then calculated by multivariate logistical analysis. The analysis found eating grilled food was the only one factor associated with the higher level of 8-OHdG; p-value 0.037; PRR 2.01 (95% CI 1.044-3.890).

The level of urinary 8-OHdG was categorized by mean average of 11.9 ng/mg Creatinine for some individuals who ate grilled food with an intake of vitamin C (Figure 1). From whole participants, there were highest cases of elevated oxidative stress performed from group of people who consumed grilled food, however stratified analysis gives different description, the effect is further increased among people who were deficient of vitamin C.

Table 2. *Number of workers with higher level of 8-OHdG according to intake of vitamin C and eating grilled food categories.*

Vitamin C	Eaten grilled food			Not eaten grilled food			Crude		
	8-OHdG (mean 11.9 ng/mg creatinine			8-OHdG (mean 11.9 ng/mg creatinine			8-OHdG (mean 11.9 ng/mg creatinine		
	Higher	Lower	Total	Higher	Lower	Total	Higher	Lower	Total
Lower	29	40	69	24	61	65	53	101	154
Higher	2	0	2	1	4	5	3	4	7
Total	31	40	71	25	65	90	56	105	161
Stratum-specific	PRR=0.42			PRR=1.42			Crude PRR	PRR=0.803	
	(95% CI 0.319-0.554)		(95% CI 0.237-8.418)			(95% CI 0.332-1.941)			

The Mantel-Haenzel determined the risk estimate generated from two groups of workers. All participants were divided into two groups based on country; Indonesian grilled food dietary intake, then categorized into the eaten or not eaten grilled food. Further, we investigated the association between vitamin C level based on Indonesian-specific food database and the level of urinary 8-OHdG. The proportion of high level of 8-OHdG from all participants in lower and higher vitamin C intake were 34.4% *vs.* 75%; p<0.05 (Table 2). The ratio between lower and higher vitamin C intake was PRR 0.803 (95% CI 0.332-1.941).

Analysis of data was stratified by variable grilled food consumption; the difference between group of workers who had deficiency of vitamin C according to the eating grilled food and 8-OHdG creatinine categories were PRR 0.420 (95% CI 0.319-0.554) and 1.421 (95% CI 0.237-8.418) respectively.

We assessed the effect of modification among two groups; we calculated Breslow-Day test. The analysis found X^2=0.123. This result indicated that there is homogeny due to X^2=0.123, p-value>0.05. Furthermore, we calculated the Mantel-Haenzel estimate. The estimation of Mantel-Haenzel test showed Odds-Ratio (ORMH) as 0.591. We compared the ORMH to 1 for zero hypotheses (Ho); there is no association between variable vitamin C and outcome urinary 8-OHdG, adjusted for confounder's exposure of the grilled food. The paper explored the Mantel-Haenzel test for the condition of independence from the test and it was found to be X^2_{MH}=0.843.

Discussion

The habit of eating meat and another protein product produced by cooking through the flames and high temperatures had provided opportunities exposed to PAH as carcinogen substances and the risk of cancer in a group of toll gate workers in Jakarta, Indonesia. On the other hand, the source of pollutants such as cigarette smoke and outdoor air pollution also contributed to the occurrence of an imbalance between antioxidants and free electrons in the body.

There are more than 100 kinds of PAHs. Furthermore, 17 kinds of them are identified the most harmful than the other PAHs

[10]. The potent toxic effect was based on the number of benzene rings. Generally, PAHs consist of a unitary aromatic ring about 2 to 4 rings, benzene rings more than four are more stable and toxic [10,11]. Poly-aromatic hydrocarbons from pyrolysis of food entering the human body by further series of genes play a role in the biotransformation to produce the epoxide that is more dangerous than the original form and also releases ROS.

This paper explores the mechanism by which sources of ROS from daily habits represent the food. The foods with overproduction of ROS give many contributions to elevated 8-OHdG as a marker of oxidative stress. The migration path of the pollutants produces free electrons and ROS after exposure to tobacco smoke and grilled food. Free radicals can form an unbalanced condition between the level of oxidants and antioxidants in the body which then affects the DNA damage. The Figure 1 showed in each eaten grilled food category the frequency of oxidative stress increase according to deficiency of vitamin C, while in each vitamin C level did not affect the frequency of oxidative stress. Thus, the crude association between intake of grilled food and oxidative stress is the mere result of the effect of deficiency of vitamin C.

ROS has reactive characteristics by inducing oxidative stress against proteins [25], fats [26] and DNA [27]. Excessive ROS production may cause DNA cross-link, DNA strand break, and chromosomal rearrangement. The occurrence of mutations in DNA takes place with a marked increase in the levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG) [1,18,25,28]. Chemical analogue of 8-OHdG is 8-hydroxyguanosine and 8-hydroxyguanine, both associated with degenerative diseases, such as cancer and cardiovascular disorders. As a biomarker to repair DNA damage, 8-OHdG is formed by the reaction of hydroxyl radical interaction with guanine at C-8 of deoxyguanosine form guanosine (8-hydroxy-2'-deoxyguanosine). Increased excretion of 8-hydroxy-2'-deoxyguanosine (8-OHdG) has been detected in smokers [29] and baked goods [30].

Disturbances in the DNA repair system (repair system) and cytotoxic effects are other effects of oxidative stress [31]. The term disorder in single or double stranded, as well as several types of DNA damage caused by ROS, had played a significant role in carcinogenesis [1]. Other studies have found a correlation with other degenerative diseases such as diabetes mellitus and cardiovascular disorders [32].

There is no exposure to play alone in carcinogenesis. Oxidative DNA damage is a condition due to multifactorial effects or exposure [33]. There may be another factor that is not recorded in this study which has also given a contribution to 8-OHdG. The higher level of 8-OHdG among two groups seems to be an uncertain condition according to the imbalance of antioxidant level and free electron in the body. The paper was applied to investigate whether the oxidative stress causally implicated in the high frequency of deficiency of vitamin C independently of the confounding effect of the grilled food intake (Table 2). From estimates of Mantel-Haenzel analysis, we determined the absence of an association between exposure and outcome controlled by grilled food consumption as the third variable. Tests conditional ORMH was shown by X^2_{MH}, to determine that the conditional independence with initial assumption was not significant between exposure and outcome adjusted for the third variable as a confounder. The estimate of ORMH seems smaller than the result of Mantel-Haenzel test (X^2_{MH}). We had to reject Ho. It indicated there was the modifier effect from grilled food (X^2=0.123, p-value>0.05), furthermore intake of vitamin C engendered positive modifier because it determined the different estimate of risk from eating grilled food associated with the oxidative stress.

This paper found inverse condition shown from the proportion of high level of 8-OHdG from all participants in lower, and higher vitamin C intake were different (7.6%), those who eat much vitamin C from daily food tend to have a higher level of 8-OHdG (Table 2). Eating grilled food has a bigger effect on risk of oxidative stress, especially among people who have deficiency of vitamin C. The prevalence risk ratio across strata, un-stratified from the whole participants versus groups of stratified by grilled food, found the effect estimate differ more than 10% (Table 2). It indicated the firm association between grilled food consumption and level of urinary 8-OHdG that emerged from deficiency of vitamin C. Consumption of grilled food gave an effect of modification to the group with deficiency of vitamin C. This indicates that there was not enough intake of vitamin C among people who eat grilled food.

The mechanism by which micronutrients improve people, who eat grilled food habits on DNA oxidative stress, is unclear. Individuals with micronutrient deficiencies who consume grilled foods have shown low levels of vitamin C. The imbalance between antioxidant from vitamin C, and the free electrons from grilled food may both have impact on the level of urinary 8-OHdG. However, increased levels of urinary 8-OHdG are not limited to subjects with very low micronutrient status. The level of 8-OHdG continues to decline as micronutrient levels increase until it reaches the level of RDA of vitamin C between those who had eaten grilled food and the other group. This condition describes that deficiency of vitamin C may have an impact on increasing the level of oxidative stress. This study is relevant to other studies that informed the lower vitamin C status, higher the risk of cancer [14,34].

Therefore, deficiency of antioxidants like vitamin C is not a plausible explanation. Oxidative stress mechanism of the pyrolysis process of grilled food may involve not only the presence of ROS and ROS-generating compounds in the smoke but also an increase in metabolic rate with increased mitochondrial production of ROS in the cells [29,33]. Thus, micronutrient deficiency may be associated with one or both of these ROS-producing pathways, leading to enhancement of oxidative stress caused grilled food consumption.

The lack of a clear relationship between vitamin C status and 8-OHdG levels among grilled food shows that micronutrient deficiency alone may not have a significant impact on oxidative DNA damage in the absence of exposure to carcinogenic pollutants. However, the 8-OHdG levels in a

group of subjects who consume grilled food with deficiency of vitamin C slightly elevated were compared with those in the non-deficient micronutrient who were not a group consuming grilled food. We do not deny the possibility of deficiency of vitamin C implicated to the rising level of oxidative DNA damage in people who eat grilled food (Figure 1). Moreover, the genetic profile of biotransformation carcinogens from grilled meat may play a role in activation and produce free electrons and elevated 8-OHdG [20,35,36]. Thus, reactive free electrons deplete vitamin C.

The paper has studied limitation. First, our study has used the design of the cross-section [37]. The results are not available to give further explanation between exposure and outcome. Second, another laboratory method using high technology like column chromatography LC-MS/MS may produce detailed results [25]. However, the ratio of deviation standard to the average value of ELISA method equal to 9% (<10%) indicates the quality of laboratory analysis. Therefore, we have still explained the positive role of vitamin C in preventing the oxidative stress among the subjects and relative cost effectiveness for Indonesian situation.

Conclusion

Micronutrient deficiencies itself or factors related to micronutrient deficiency modulated the carcinogenic effects of the consumption of grilled food. The increase in urinary 8-OHdG levels had given negative effect significantly to people who consume grilled food in the condition of micronutrient deficiencies. It provides mechanistic insight into the epidemiological findings of an increased risk of cancer associated with diet containing carcinogenic substances and deficiency of vitamin C. Micronutrient deficiency may represent a decline of biological functions against oxidative stress. In these cases, deficiency of vitamin C becomes the third factor that modified the relationship between grilled food consumption and oxidative stress with an impact of elevated cases of DNA damage.

The 8-OHdG represent the DNA damage due to exposure to the free electrons from poly-aromatic hydrocarbon (PAHs) during pyrolysis process and thus able to be used as a biomarker of host susceptibility to cancers associated with the habit of eating grilled food. Since switching to non-grilled food menu or stopping to consume it may decrease the level of 8-OHdG, we expected that people who are regularly eating grilled food in the condition of micronutrient deficiency may have health benefits if they minimize diet containing various carcinogens like the meat grilling process under high temperature.

Present study suggested further research, including the genetic profile of the biotransformation of the carcinogenic pollutants or explanation the process of DNA damage repair system.

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*Correspondence to

Ela Laelasari

Public Health Study Programs

Faculty of Medicine and Health Sciences

UIN Syarif Hidayatullah

Ciputat

Jakarta

Indonesia