Intron 4 VNTR A/B polymorphism of endothelial nitric oxide synthase gene in periodontitis

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Abstract. Nitric Oxide (NO) is an important mediator in the inflammatory and immune systems. The eNOS gene is one of the three isoforms of Nitric Oxide Synthase (NOS), which is responsible for synthesizing NO. Periodontitis is an inflammatory disease in periodontal tissue with genetic involvement. Polymorphism in eNOS gene changes the functional aspect of this gene and is associated with several inflammatory diseases including periodontitis. Aim: To detect Endothelial Nitric Oxide Synthase intron 4 gene polymorphism in Indonesian population with periodontitis. Analysis of the Endothelial Nitric Oxide Synthase (eNOS) intron 4 gene polymorphism was observed by carrying out PCR method followed by electrophoresis for the analysis, without the usage of restriction enzyme. The chi-square test and odds ratio were performed for statistical analysis. In this study, there were 34 samples with AA genotype, 3 samples with AB genotype, and 13 samples with BB genotype in periodontitis group. Whereas in the control group, there were 41 samples with AA genotype and 9 samples with BB genotype. AB genotype was absent in the control group. In periodontitis group, there were 71 A alleles and 29 B alleles, and in the control group, 82 A alleles and 18 B alleles were found. Polymorphic genotypes and alleles were found higher in periodontitis sample (32% and 29%) than healthy controls (18%). The polymorphism of eNOS intron 4 was found in periodontitis patients. There is no significant distribution difference was found between the periodontitis patients and the control group. ENOS intron 4 gene polymorphism does not affect the risk of periodontitis.

1. Introduction
Periodontitis is an inflammatory disease in periodontal tissue that is caused by specific microorganism, that results in progressive destruction of periodontal ligament and alveolar bone that give arise to a deep periodontal pocket pocket, gingival recession, or both of them [2]. Periodontitis is the major cause of tooth loss in the world adult population. This population is at risk of experiencing multiple tooth loss, edentulism, and masticatory dysfunction, hence affecting their nutrition, quality of life and self-esteem as well giving huge impact on socioeconomics and healthcare cost) [1]. Severe periodontitis is the 6th most common medical condition in the world, with an overall prevalence around 11.2%. Based on Riset Kesehatan Dasar (RISKESDAS) 2018, the prevalence of periodontal disease in Indonesia in all age groups is quite high, reaching 74.1%. Due to the large prevalence of periodontitis and its impact on health, it is necessary to carry out further research on this disease [3].

The occurrence of periodontitis can be caused by many factors (multifactorial). In addition to microorganisms in biofilm which are the local predisposing factor of periodontitis, there are host factors
and environmental factors that also play a role in the occurrence of this disease [2]. Although pathogenic bacteria are believed to be the main cause of periodontitis, most of the tissue damage that occurs is caused by host's response to the bacteria [4]. Each individual may have different levels of response to bacterial challenges, which then will determine their susceptibility to periodontitis [5]. It is because there are host factors of each individual that play an important role in affecting an individual's response to the disease and the severity of periodontitis. One of these host factors is genetic factors [2].

Genes and their variations (polymorphism) may affect the response of host to bacterial challenges that caused periodontitis and the progression of this disease. Genetic polymorphisms in some situations may alter the expression of genes which leads to changes in the non-specific and specific immune thereby affecting the outcome of the disease [2]. In other words, genetic polymorphism may act as a risk or protective factor of the development of periodontitis [6]. Some genetic factors that affect periodontitis are inflammatory cytokines, cell surface receptors, and several types of enzymes [2]. One of enzymes that play a role in the pathogenesis of periodontitis is Endothelial Nitric Oxide Synthase (eNOS) [8]. eNOS is in charge of synthesizing nitric oxide (NO), an important mediator of immunity and inflammation. NO is the most powerful endogenous vasodilator. NO produced by eNOS is involved in an important process relevant to the pathogenesis of the periodontal disease, which regulates osteoblast activity and inhibits platelet aggregation [9]. The alteration of the functional aspect of this gene may lead to exaggerated inflammation and destruction of periodontal tissue.

Researches on polymorphisms in the eNOS gene show that this gene plays a role as a risk factor of periodontitis [8,16]. Polymorphism in this gene acts as the risk factor of periodontitis by increasing NO levels resulting excessive inflammation and destruction of the alveolar bone [10,17]. The presence of B allele is associated with increased susceptibility of periodontitis. The eNOS gene polymorphism is also associated with other diseases related with periodontitis, such as osteoporosis and obesity. This study is aimed to determine the association between eNOS intron 4 gene polymorphism and the development of periodontitis.

2. Material And methods

2.1 Sample collection

This study used a cross sectional study design. The variables used were the eNOS gene polymorphism as the independent variable, periodontitis as the dependent variable and Non Periodontitis DNA as control. The samples of this study used 50 samples of Periodontitis DNA and 50 control (Non Periodontitis DNA) samples for each variable. DNA samples were extracted from blood serum stored in the refrigerator at the Laboratory of Oral Biology, Faculty of Dentistry, University of Indonesia at -20°C. In addition, other materials used include: Double distilled water (ddH2O), Primers gen eNOS intron 4 VNTR (F: 5’-AGG CCC TAT GGT AGT GCC TTT-3’ and R: 5’-TCT CTT AGT GCT GTG GTC AC-3’), 70% alcohol, TAE buffer, My Taq HS Red Mix 2x, agarose powder, 10,000x in Water Red Nucleic Acid Gel Stain, and 100bp DNA ladder.

| Sample    | Frequency | Percentage |
|-----------|-----------|------------|
| Peridontitis | 50        | 50%        |
| Control   | 50        | 50%        |
| Total     | 100       | 50%        |

2.2 DNA amplification using PCR

DNA amplification was initiated by mixing 10 μL MyTaq HS Red mix, 0.5 μL forward primer, 0.5 μL reverse primer, 0.2 μL template DNA, and 8.8 μL ddH2O in a PCR tube to produce 20 μL PCR mix. The PCR mix was vortexed using a vortexer and spun down using a microcentrifuge. The PCR mix is then fed into the PCR machine with temperature and optimization.
For the eNOS gene, starting with initial denaturation at 95°C for 4 minutes, followed by final denaturation at 94°C for 1 minute, annealing at 57.4°C for 30 seconds, initial extension at 72°C for 1 minute 30 seconds, and a final extension at 72°C for 1 minute. In the final denaturation to the initial extension, 35 cycles were carried out.

2.3 Electrophoresis
The electrophoresis of PCR products was carried out using agarose gel with a concentration of 2.5%. The agarose gel was made by mixing 2 grams of agarose powder with 80 mL of TAE buffer into the tube. The tube is then put in the microwave for 4 minutes. Then, the tube is placed on the orbital shaker to cool down for 5 minutes at a rotating speed of 100 rpm and 1 μL of red gel is added. And then the tube is lifted and poured into a gel mold that has been attached to the gel comb. Then wait the agarose gel until it solidifies. After hardening, the gel comb is removed to form a well according to the gel comb used.

The hardened agarose gel was then transferred to an electrophoresis tube containing a buffered TAE solution. The agarose gel well then filled with PCR products, each 5 μL. One of the agarose gel well was filled with a 100 bp marker, which was made by mixing 1 μL of DNA ladder, 1 μL of loading dye, and 4 μL of ddH2O on paraffin paper. After all wells are filled, electrophoresis starts with a voltage of 70 volts and a current of 400 amperes for 50 minutes. DNA bands and markers will travel from the negative pole to the positive pole. After the electrophoresis is complete, transfer the agarose gel to the Gel doc machine connected to the computer for visualization. On the computer screen, you will see a white base pair band according to the product target.

2.4 Processing and analysis of data
The data obtained from the research on the distribution of the eNOS VNTR gene polymorphism (intron 4 A / B) in the periodontitis and non-periodontitis (control) groups were obtained and then processed with the Statistical Program for Social Science (SPSS) computer program version 23 using statistical tests with the Continuity Correction test for Looking at the differences in the distribution of polymorphisms in the periodontitis patient group and the control group, the Chi-square test used to see if the population is in the Hardy-Weinberg equilibrium, and the calculation of the odds ratio to see whether the eNOS VNTR gene polymorphism (intron 4 A / B) affects the level of risk of periodontitis.

3. Results
The visualized PCR products show 393 bp fragment length for A alleles or/and 420 bp fragment length for B alleles (Figure 1).

![Figure 1](image_url)
Subjects were divided into polymorphic for genotypes that had B alleles, namely AB and BB and non-polymorphic for genotypes that did not have B alleles, namely AA. The wildtype genotype in this polymorphism is AA. Using the SPSS v.23 program, the Continuity Correction test was used in this study to see if there were differences in the distribution of polymorphisms between samples of periodontitis sufferers and control samples. The significance value obtained was $p = 0.106$ for the genotype and $p$-value = 0.095 for the allele, so it cannot be concluded that there is a significant difference or significant difference in the distribution of polymorphism status between the periodontist patient sample group and the control sample group.

### Table 2. Frequency distribution of the eNOS VNTR gene polymorphisms (Intron 4 A/B)

|                | Periodontitis (n=50) | Control (n=50) | $p$ value |
|----------------|----------------------|----------------|-----------|
| **Genotype**   |                      |                |           |
| Non-polymorphic| 34 (68%)             | 41 (82%)       | 0.106     |
| Polymorphic    | 16 (32%)             | 9 (18%)        |           |
| **Allele**     |                      |                |           |
| Non-polymorphic| 71 (71%)             | 82 (82%)       | 0.095     |
| Polymorphic    | 29 (29%)             | 18 (18%)       |           |

### Table 3. Calculation of Odds Ratio and Confidence Interval in the Research Population

| Exposure | Periodontitis | Non Periodontitis | OR       | 95% CI      |
|----------|---------------|-------------------|----------|-------------|
| Allele A | 71            | 82                | 1.861    | 0.954 – 3.631 |
| Allele B | 29            | 18                |          |             |

The level of risk affected by the eNOS VNTR gene polymorphism (intron 4 A / B) was calculated using odds ratios. The results obtained were that people who had the B allele were 1.8 times more likely to experience periodontitis. However, a 95% CI was obtained (0.954 - 3.631). The confidence interval in this study includes number 1, so it can be concluded that there is no difference in the risk of periodontitis in individuals who have both the A allele and the B allele.
4. Discussion

Endothelial nitric oxide synthase (eNOS) is the main enzyme responsible for regulating the production of nitric oxide (NO) in blood vessels. NO is a free radical compound associated with various physiological functions, including modulation of cardiovascular tone and integrity, regulation of platelet aggregation, neurological transmission, and strong oxidative activity that contributes to kill pathogenic microorganisms. Products produced by pathogenic microorganisms stimulate the synthesis of NO [11]. Then, NO will play a role as a host response to pathogenic microorganisms. As the host response to pathogenic microorganisms, NO plays a role in vasodilation of blood vessels, increases in vascular permeability, regulates cell adhesion to the endothelium, and regulates osteoclast and osteoblast activity [9].

Polymorphisms that occur in Intron 4 eNOS VNTR gene have 2 common alleles, namely A with 4 repetitions and B with 5 repetitions. The B allele is associated with susceptibility to periodontitis because it causes an increase in NO levels produced [8,11]. Increased NO levels can cause inflammation and excessive periodontal tissue destruction in various ways: namely mediating the pathological effects of LPS, TNF, IL-1, and other cytokines; dilates blood vessels and increases vascular permeability resulting in swelling and bleeding of the gingiva; inhibits platelet adhesion; and increasing osteoclast activity [12]. From these mechanisms, it can be said that the eNOS VNTR gene polymorphism (intron 4 A / B) can affect the susceptibility of individuals to experience periodontitis.

This study was a descriptive study with laboratory analysis to determine the distribution of genotypes and polymorphism alleles of the eNOS VNTR gene (Intron 4 A / B) in the periodontitis group and healthy individuals (controls) in Indonesia. To determine the presence or absence of polymorphisms in the sample, the Polymerase Chain Reaction (PCR) technique was used. PCR is a technique used to amplify DNA. After going through the stages of denaturation, annealing, and elongation, followed by electrophoresis of PCR products on agarose gel 2.5% and then visualized using Gel doc. The 27 bp variable number of tandem repeats (VNTR) polymorphism in the intron 4 eNOS gene has two types of alleles, namely the A allele (393 bp) and the B allele (420 bp) which is visible as a white band on the results of the electrophoresis visualization of PCR products. In this study, these alleles formed 3 types of genotypes, namely AA (homozygote wild type), AB (heterozygote variant), and BB (homozygote variant).

Based on the research conducted, it was found that there were BB genotypes in 18% of the control group or as many as 9 out of 50 control samples. Meanwhile, there was no AB genotype found in the control group. In the periodontitis sample population, AB and BB genotypes were found in 32% of the periodontitis case group or as many as 16 of the 50 periodontitis case groups. At the allele level, the B allele was found in 18% of the control group and 29% of the periodontitis case group. Polymorphism can be categorized if more than 1% of the population experiences variations in the gene. Thus, it can be concluded that there is a genotype distribution and polymorphism allele distribution of the eNOS VNTR gene (Intron 4 A / B) in the periodontitis case group and the control group. This finding is consistent with the findings of previous studies on the polymorphism of the eNOS VNTR gene (intron 4 A / B) in various diseases in various populations in the world, where the distribution of genotypes and polymorphism alleles in both the case and control groups was also found [8,13,14].

In Indonesian population, the AA genotype and the A allele seemed to dominate in both sample populations, both in the disease sample and in the control sample, which equal to 68% and 71% of the periodontitis case group had genotype AA and allele A and 82% of the control group had genotype AA and allele A. This is consistent with the results of a study conducted by Erciyas et al in the Turkish population, which found that 61.5% and 69.2% of the chronic periodontitis case group had genotype AA and allele A; 65.2% and 78% of the aggressive periodontitis cases group had the AA genotype and the A allele; and 72% and 85% of the control group had the AA genotype and the A allele [8]. However, these findings differed from the results of the study conducted by Liu et al. in the Chinese population and Nasr et al. in the Tunisian population, where both BB and B allele genotypes were more dominant [13,14]. The differences in the distribution of alleles and genotypes in these studies could be influenced by differences in race and ethnicity in each study population [15]. However, in the four studies it was
found that the genotype of BB and B allele was more in the case group than in the control group. Therefore, the presence of the B allele is associated with the risk of developing periodontitis, osteoporosis, and obesity.

Based on the results of the Hardy-Weinberg equilibrium analysis, a p-value = 0.01 (p-value > 0.05) was obtained, which indicates that the study population was significantly different from the normal population according to the Hardy-Weinberg principle. Statistical tests were performed using the SPSS v 23 program (IBM, NY, USA). The Chi-square continuity correction test was used in this study to see whether there was a significant difference in the distribution of polymorphisms between the periodontitis case group and the control group. Significance values obtained, namely p-value = 0.106 for genotype and p value = 0.095 for alleles (Table 5.2). From these results, it can be concluded that there was no significant difference in the distribution of genotypes and polymorphism alleles of the eNOS VNTR gene (intron 4 A / B) between the periodontitis case group and the control group (p-value > 0.05). The insignificant results on the distribution of genotypes and polymorphism alleles between the case and control groups found in this study are the same as the results obtained in the study conducted by Erciyas et al. Against aggressive periodontitis in a population in Turkey, however, it differs from the results obtained in a study that was conducted on chronic periodontitis where when compared to the case group with the control group, there was a significant difference [8]. This study also differs from the study conducted by Liu et al. against osteoporosis in a population in China and a study conducted by Nasr et al. on obesity in the population in Tunisia [13,14]. The differences in the results of this study were found to be due to several reasons, namely differences in race and ethnicity and the number of samples used in each study. In addition, there are other factors that influence periodontitis besides genetic polymorphisms. Periodontitis can also be influenced by smoking habits, age, psychological and systemic diseases.

Based on the calculation of the odds ratio to determine whether the polymorphism of the eNOS VNTR gene (intron 4 A / B) could affect the risk level for periodontitis, the value was 1.861 with 95% CI (0.954 - 3.631). The confidence interval number of 0.954 - 3.631 includes number 1. Thus, it can be concluded that in this population, there is no difference in the risk of periodontitis in individuals who have both the A allele and the B allele.

5. Conclusion
The polymorphism of eNOS intron VNTR 4 A/B was found in periodontitis patients and the frequency is higher in periodontitis group than in the control group. However, the trend was not statistically significant for the tested sample size. The polymorphism of the eNOS VNTR gene (intron 4 A / B) did not affect the risk level for periodontitis.

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