# Profile of PRMT-1 Gene Polymorphism in Hemodialysis Patients with Increased ADMA Levels

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## ABSTRAK

***Tujuan:*** *untuk menentukan distribusi polimorfisme genetik PRMT-1 dan kadar ADMA pada pasien hemodialisis berkelanjutan.* ***Metode:*** *genotyping polimorfisme PRMT-1 dilakukan pada 57 pasien hemodialisis di Rumah Sakit Al-Irsjad. Semua partisipan menjalani pemeriksaan fisik, mengisi kuesioner dan pengambilan darah vena 5ml. Darah kemudian diekstraksi dengan menggunakan kloroform. Pemeriksaan single nucleotide polymorphism (SNPs) dilakukan dengan polimerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Kadar ADMA diukur menggunakan ELISA dan pemeriksaan petanda kimia klinik serum.* ***Hasil:*** *penelitian ini diikuti 57 pasien yang menjalani hemodialisis, 54 (95,4%) pasien tersebut mengalami kenaikan kadar ADMA. Dilakukan sekuensing DNA pada 13 pasien dan menunjukkan dugaan polimorfisme gen PRMT-1 pada titik 5837, karena pada titik tersebut ada perbedaan genotip C dan G.* ***Kesimpulan:*** *polimorfisme gen PRMT-1 diduga menjadi salah satu penyebab peningkatan kadar ADMA.*

***Kata kunci:*** *ADMA, PRMT-1, polimorfisme, penyakit ginjal kronis.*

## ABSTRACT

***Aim:*** *to determine the distribution of PRMT-1 gene polymorphism and ADMA levels among continuing hemodialysis patients.* ***Methods:*** *genotyping of PRMT-1 polymorphism was performed in 57 hemodialysis patients at Al Irsjad Hospital. All participants were recruited for physical examination, questionnaire, and collection of 5 mL fasting venous blood. The blood was treated with phenol-chloroform extraction of genomic DNA. The candidate's single nucleotide polymorphisms (SNPs) were genotyped by using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The ADMA plasma levels was determined by ELISA and all biochemical indicators of serum were examined.* ***Results:*** *fifty-seven hemodialysis patients participated in our study, 54 (95.4%) of them had increased ADMA plasma levels. DNA sequencing analysis of 13 patients showed a suspected PRMT-1 gene polymorphism at sequence 5837 as there were different genotypes between C and G.* ***Conclusion:*** *the increased levels of ADMA might be caused by PRMT-1 gene polymorphism.*

***Key words:*** *ADMA, PRMT-1 polymorphism, chronic kidney disease.*

## INTRODUCTION

Various research evidences suggest that renal dysfunction is associated with a high risk of cardiovascular disease (CVD) complications. Chronic kidney disease (CKD) has been considered to be a risk factor for CVD. As endothelial dysfunction is an early stage of atherosclerosis in patients with hypertension, diabetes and CKD, the decline in the availability of nitric oxide (NO) is considered as an independent risk factor for CVD among those patients.[1]

Previous studies have shown that endothelial dysfunction may predict the current and future CVD.[2] Decreased availability of NO is closely related to the asymmetric dimethyl arginine (ADMA), an inhibitor of NOS.[3] ADMA is an independent risk factor for atherosclerosis progression, death due to CVD and all causes of mortality.[2] The increase of ADMA in animal studies increased blood pressure.[4] Moreover, in endothelial cell culture of patients with CKD who had increased ADMA levels, it is found that ADMA significantly inhibits NO production.[5] Several recent studies have shown that concentrations of ADMA in CKD patients, even at stage 1, has increased.[3,6,7] Moreover, decreased levels of ADMA during hemodialysis is associated with improved endothelial function in patients with end-stage kidney disease.[1]

ADMA is produced about 300 μmol per day. Dimethyl arginine was obtained from the degradation of protein methylation product and produced by the protein methyl transferase (PRMT) enzymes. Currently, there are 9 genes of PRMT enzymes, which can be divided into 2 groups: PRMT type I (1-4, 6 and 8) that produce ADMA and L-NMMA, and PRMT type II (5, 7, 9) that produce Symmetric Dimethyl Arginine (SDMA) and L-NMMA. Meanwhile, 90% ADMA degradation is known to occur through the metabolism by dimethylarginine dimethylaminohydrolase (DDAH) enzymes into citrulline and amines.[1,3,8,9] The fact shows that an increase in ADMA levels is closely associated with the risk of CVD in patients with CKD. Moreover, type I-PRMT enzymes play a major role in the production of ADMA. However, the mechanism of type I-PRMT on increased ADMA levels in patients with CKD remains unknown.

Our study was conducted to determine whether there was any gene polymorphism of type I-PRMT enzyme that may lead to increased ADMA levels in CKD patients. We expected that the results of study may be used to elaborate the molecular mechanisms of increased ADMA levels. If there is genetic polymorphism of type-I PRMT enzyme, which plays a major role in ADMA production; then the definitive management therapy can be determined to reduce ADMA levels. The study also tried to disclose molecular mechanisms underlying increase of ADMA in patients with end-stage kidney disease. Since the ADMA synthesis is primarily carried out by type I-PRMT enzymes, the authors hypothesized that there was a polymorphism of type I PRMT gene that may lead to increased ADMA levels in patients with CKD. To test the hypothesis, in addition to performing PCR on blood samples of patients with CKD, we also examined ADMA levels in the same patients; therefore, we can identify the association between elevated ADMA levels and type I-PRMT enzyme gene polymorphism.

## METHODS

We conducted a cross-sectional study to determine the genetic polymorphism of PRMT-I enzyme and ADMA levels in hemodialysis (HD) patients. The study took place at Al-Irsyad Hospital and Institute of Tropical Disease, Airlangga University, Surabaya. The study population was patients undergoing regular HD in Al-Irsyad Hospital and Institute of Tropical Disease, Airlangga University Surabaya. The samples were patients who met the inclusion criteria. Sample size was all populations that met the inclusion criteria during the study or total population sampling. Inclusion criteria were men/women aged 18-65 years, who were undergoing regular/sustainable HD for three months/more, stable, having Hb >8g/dL and albumin >3 mg/dL, not taking drugs containing antioxidants in the last one month period, and have agreed and completed the informed consent form. Exclusion criterion was a blood transfusion prior to sampling.

To determine PRMT-1 gene polymorphism,

we performed blood sampling, DNA extraction, DNA amplification with PCR, and detection of PCR products by electrophoresis. Two percent agarose gel that had been made previously containing ethidium bromide was placed in a gel electrophoresis apparatus, added with TBE buffer 1 x until the gel was completely submerged. A marker, as much as 10 µl, was mixed by pipetting gently with 10x loading buffer of 2 µl that had been dripped on parafilm. From the first round PCR products, as much as 7 µl was taken and mixed by gently pipetting with 10x loading buffer of 2 µl that had been dripped on parafilm. Each of PCR product mixture was inserted into the gel slot. Gel electrophoresis apparatus was closed and run with 100 volts for about 30 minutes. Subsequently, it was observed under short-wave ultraviolet light. The observed results were documented with gel doc.

### DNA Isolation and Purification with Low Melting Agarose

DNA bands appeared white on electrophoresis with 2% agarose S gel. Moreover, electrophoresis was repeated using low melting agarose gel (L). Two percent agarose L gel containing ethidium bromide was prepared, then a mixture of 15 µLDNA and 10 x 2 µl loading buffer was made. This mixture was applied to the gel wells with intervals without the use of markers and subsequently, the electrophoresis instrument was run. Electrophoresis results were observed with long-wave ultraviolet light. Gel containing DNA was isolated by cutting using a cutter that was washed each time of cutting. Gel slices were put into a sterile Eppendorf tube 1.5 µL. Then, we carried out DNA purification of PCR products from agarose L using QiaQuick Gel extraction Kit (Qiagen, Inc.. Cat. no. 28704).

### PCR for Sequencing Preparation

Labeling with PCR for sequencing was performed by using the ready reaction cycle sequencing kit (ABI prism big dye terminator VII, applied biosystems). Sequencing reaction with a total volume of 20 µL was made in a sterile microcentrifuge tube with composition of: DNA from PCR results of 5 µl, sense or antisense primer of 4 pmol/µL for HBV; 1.5 µl (PRMT primer), 2 µl Ready reaction mixture Big Dye Terminator, 7 µl Buffer Big Dye, 4.5 µl DW, mixed by gently pipetting. Subsequently, they were poured into PCR machine that had been set to a temperature of 96°C for 3 min. PCR was performed for 25 cycles. Each cycle consisted of four stages: denaturation for 10 seconds in a temperature of 96°C, annealing for 5 seconds at 50°C, extension for 4 minutes at 60°C. Once the cycle was complete, the temperature was cooled to 4°C.

### Purification and Precipitation of PCR Products for DNA Sequencing Preparation with Sodium Acetate-ethanol

Tube containing 20 µl sequencing reaction was removed from the PCR machine. Subsequently, it was aspirated and transferred into a 1.5 ml sterile Eppendorf tube. Then, 2.5 µl sodium acetate and 50 µl absolute ethanol were added to 1.5 ml sterile Eppendorf tubes, and mixed by flicking the tube. The mixture was incubated at room temperature for 5 minutes. Then, it was centrifuged at 15,000 rpm for 15 min at 4°C. The existing supernatant was carefully aspirated and discarded into a container. As much as 100 µl of 70% ethanol was added into the 1.5 mL Eppendorf tube. It was centrifuged once more at 15,000 rpm for 5 min at 4°C. Again, the supernatant was carefully aspirated and discarded into containers. Open tubes containing DNA pellets were wrapped in a plastic wrap and dried using a vacuum pump for 15 minutes. Thereafter, the dried DNA pellet were stored at a temperature of 4°C and to avoid the direct rays it was closed tightly with aluminum foil.

### DNA Sequencing

DNA sequencing was performed using ABI prism 310 genetic analyzer (applied biosystems). DNA mixture had been prepared before sequencing. HiDiformamide (applied biosystems) as much as 25 µL was added to tubes containing dried DNA pellet. It was vortexed and incubated at a temperature of 95°C for 2 min. Then, it was incubated in ice for about 3 minutes and subsequently spinned-down. The tube remained stored in ice until it was ready to be analyzed. Moreover, it was aspirated and transferred into a sterile microtube specifically for sequencing. The tube was inserted into a

sequencer machine and run on a ABI prism 310 genetic analyzer.

## RESULTS

In this study, samples were taken from Hemodialysis Unit, Al-Irsyad Hospital, Surabaya. The study was conducted between May and September 2013. There were 57 samples that met the inclusion criteria.

**Table 1.** Subject characteristics

| Variables | n (%) |
|---|---|
| Sex | |
| - Male | 39 (68.4%) |
| - Female | 18 (31.6%) |
| CKD Stage | |
| - Stage IV | 14 (24.6%) |
| - Stage V | 43 (75.4%) |
| Mean of blood pressure | |
| - Systolic | 160±2.5 mmHg |
| - Diastolic | 100±5.3 mmHg |
| Mean of age | 55.4 |

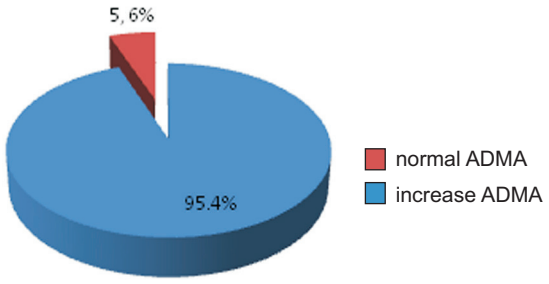


**Figure 1.** Distribution of ADMA levels in CKD-hemodialysis patients

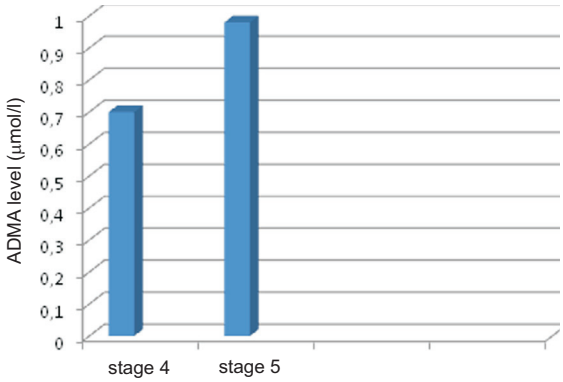


**Figure 2.** Mean difference of ADMA level in each stage

The first stage was the measurement of ADMA levels using ELISA. Of the 57 samples, there were 3 samples (5.6%) with normal range of ADMA levels (0.24 to 0.58 µmol/l) and 54 (95.4%) samples with increased ADMA levels.

Sequencing was performed in 13 samples with elevated ADMA levels. The sequencing results showed that there was a suspected occurrence of PRMT-1 gene polymorphism, which was at sequence 5837 as we found

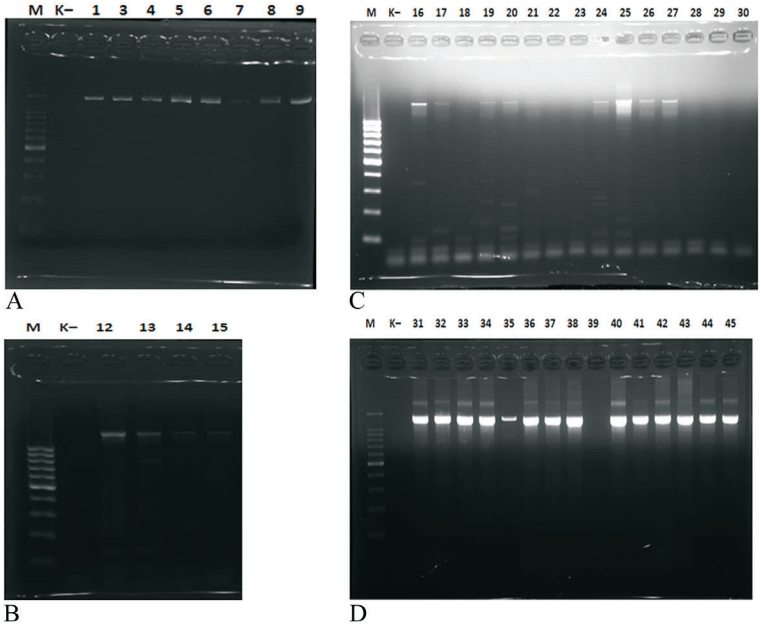


**Figure 3.** A-D. Results on detection of PCR products by electrophoresis

differences between genotype C and G at the sequence (**Figure 5**). Therefore, we performed DNA sequencing in the other 44 samples in order to obtain clearer profile about the location of PRMT-1 gene polymorphisms.

## DISCUSSION

Asymmetric dimethyl arginine (ADMA) is an endogenous molecule that can be detected in human blood and urine. ADMA blood samples are stable for 24 hours at 2°-8°C or for 24 months if it is frozen at -20°C. In addition to being stable, ADMA is easily measured. It also reflects the concentration of NO and pathobiologically and directly contributes to the incidence of endothelial dysfunction. ADMA levels in vascular endothelium is ten times higher than those in plasma and the highest levels are found in kidney and spleen. Normal ADMA levels in humans is 0.24 to 0.58 µmol/l or 80-150 ng/ml. Measurement of serum ADMA is performed quantitatively using enzyme immunoassay ADMA®-ELISA.

It has been observed that ADMA has an important role in endothelial dysfunction and it may increase in the early stages of CKD.[6] CKD

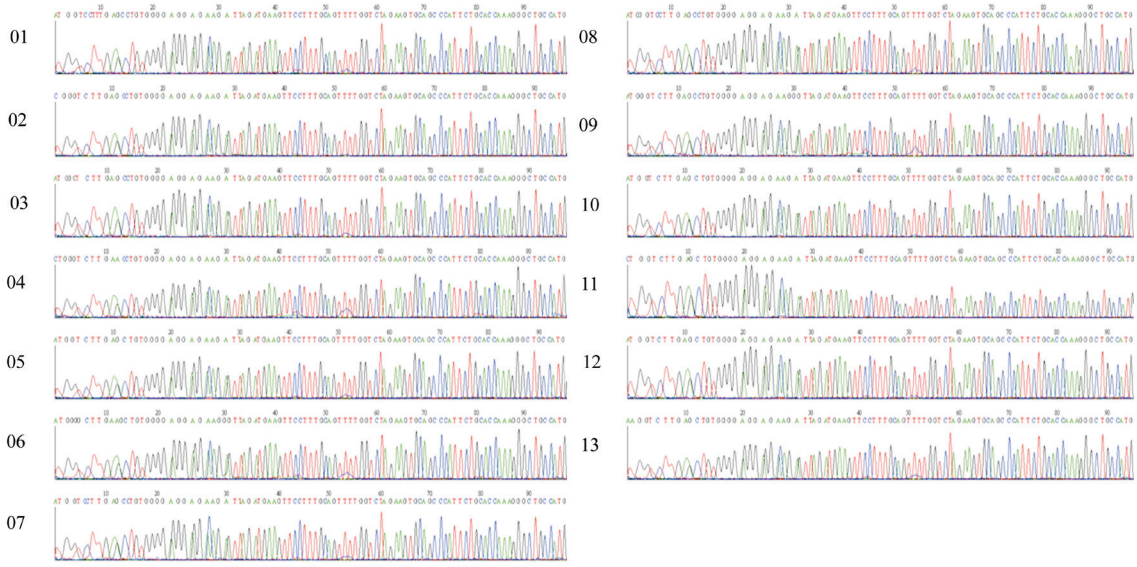


**Figure 4.** Results of DNA sequences


**Figure 5.** Suspected location of polymorphism

has become one of risk factors of CVD[10] and ADMA has been considered as the link between CKD and CVD.[1] The association between ADMA with CVD has been widely investigated in several studies. It has been recognized that ADMA infusion in healthy subjects decreases cardiac output and increases vascular resistance and blood pressure. Miyazaki et al. reported a significant positive correlation between ADMA plasma levels with the thickness of carotid artery intima, which can serve as a marker of arteriosclerosis. ADMA can also lower heart rate and cardiac output. It can inhibit angiogenesis, and increase atherogenesis. An in vitro study demonstrates that it may also accelerate the aging process of endothelial dysfunction. High concentration of ADMA can inhibit the movement of endothelial progenitor cell (EPC) from the bone marrow to damaged endothelial area. Furthermore, ADMA inhibition can increase the expression of vascular endothelial growth factor (VEGF) in endothelial cells and increase tube formation.[3,7,8,11]

Intensive research on molecular and cellular mechanisms that lead to atherogenesis has produced an understanding that vascular endothelium plays an important role to functional changes in the early stages of the vascular wall, which in turn initiates and exacerbates atherogenic process.[12] It has been found that the main cause of NOS pathway disorder is the presence of endogenous NOS inhibitors, the ADMA and N mono methyl arginine (NMA). ADMA plasma levels is ten times greater than MMA levels.[8,9] ADMA levels on vascular endothelium is ten times greater than the levels in plasma. Moreover, the highest levels are found in kidney and spleen.[8]

ADMA is synthesized in the cytoplasm of the cell and subsequently released into extracellular compartment and blood plasma. Dimethyl arginine is formed during proteolysis of methylated proteins. Protein methylation is a mechanism of post-translational modification of proteins contained in the body's cells. This process causes a change in the tertiary structure and the function of proteins. Proteolysis is catalyzed by the enzyme S-adenoylmethionine protein N-methyltransferases (protein arginine methyltransferases/PMRT). Protein arginine methyltransferases I and II remove one or more methyl groups from the methyl donor S-adenosilmethionine to L-arginine residues in the protein or polypeptide. ADMA is formed through the activity of PMRT I, depending on the number of removed methyl groups; while the Symmetric Dimethyl Arginine (SDMA) is formed through the activity of PMRT II.[3,8]

There are several theories regarding the suspected cause of increased ADMA levels. Some experimental animal studies suggest that there may be a decrease of DDAH enzyme expression. Moreover, it was found that PRMT-I enzyme inhibition in animals had lowered the ADMA levels.[1,8] However, the exact molecular mechanism on increased ADMA levels in CKD patients remains unknown.

The results obtained from this study were in accordance with previous findings, i.e. we found increased ADMA levels in CKD patients. In CKD, asymmetric increase of dimethyl arginine (ADMA) may occur due to increased production of ADMA by protein arginine methyltransferases (PRMT-1) or through the inhibition of ADMA elimination by either dimethylaminohidrolasedimethylarginine (DDAH) or kidneys. In chronic kidney disease (CKD), ADMA levels is found to be increased by about 30-40% and there is no correlation with creatinine serum levels and the stage of CKD. Other studies revealed that the higher the CKD stages, the higher the ADMA level. In LDL oxidation, the release of ADMA has also been increased significantly. Oxidative stress, a condition that can occur in patients with CKD, is associated with ADMA synthesis via the stimulation of enzymes PRMT1.[3,8,9]

## CONCLUSION

Of 57 hemodialysis patients with stage IV-V, there were as many as 54 patients (95.4%) who had increased ADMA levels; while three patients (5.6%) had ADMA levels between the normal range. PRMT-1 gene polymorphism may occur at sequence 5837. Further research should be carried out on the sequencing process with higher number of samples in order to obtain a better profile about the site of PRMT-1 gene polymorphisms and

to determine the association of PRMT-1 gene polymorphism and ADMA level.

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