



Association between Three Variants in the *PRKAA2* gene, rs2796498, rs9803799, and rs2746342, with 10-year ASCVD Risk on Newly Diagnosed T2DM in Yogyakarta, Indonesia

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Abstract

Edited by: Slavica Hristomanova-Mitkovska
Citation: Virginia DM, Wahyuningsih MSH, Nugrahaningsih DAA. Association between Three Variants in the *PRKAA2* gene, rs2796498, rs9803799, and rs2746342, with 10-year ASCVD Risk on Newly Diagnosed T2DM in Yogyakarta, Indonesia. Open Access Maced J Med Sci. 2021 Aug 14; 9(A): 541-547. https://doi.org/10.3889/oamjms.2021.6213

Keywords: *PRKAA2*; Genetic variation; Atherosclerosis cardiovascular disease; Type 2 diabetes mellitus; Metformin; Indonesia

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Received: 17-Apr-2021

Revised: 06-May-2021

Accepted: 04-Aug-2021

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Funding: This study was supported by the Indonesia Endowment Fund for Education and Rekognisi Tugas Akhir Grant

Competing Interests: The authors have declared that no competing interests exist.
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BACKGROUND: AMPK has pivotal roles in glucose and lipid metabolism, including AMPK α 2, which *PRKAA2* encodes. Metformin as an anti-hyperglycemia agent acts through AMPK. Poor glycemic control among patients with type 2 diabetes mellitus (T2DM) could increase atherosclerosis cardiovascular disease (ASCVD) risk. Therefore, *PRKAA2* genetic variation might contribute to 10-year ASCVD risk in patients with newly diagnosed T2DM receiving monotherapy metformin.

AIM: The study aimed to detect an association between *PRKAA2* genetic variation with 10 year-ASCVD risk among newly diagnosed T2DM patients prescribed monotherapy metformin.

METHODS: This present study was a casecontrol study involving 107 participants. Analysis of *PRKAA2* genetic variation was performed using the TaqMan assay.

RESULTS: A total of 91 participants who fulfilled our criteria enrolled in this study. Most of the participants were female, with a mean age of 54.40 \pm 7.75 years old, mean HbA1c level of 8.35 \pm 1.31% and the lipid profile indicated normal conditions. There was a significant difference in age ($p < 0.01$), HbA1c level ($p = 0.04$), sex ($p < 0.01$), and smoking status ($p < 0.01$) between low-risk and high-risk groups. The GT genotype of rs9803799 had 187.86 times higher possibility for high-risk of 10-year ASCVD risk than TT genotype (OR = 187.86, 95%CI:2.98–11863.51). The dominant model of rs9803799 showed that GT+GG had a 94.33 times higher possibility for high-risk of 10-year ASCVD risk than TT genotype (OR=94.33; 95%CI:2.32–3841.21). Other results showed that the G allele of rs980377 had 20.48 times higher possibility for high-risk of 10-year ASCVD risk than the T allele (OR = 20.48; 95%CI:1.48–283.30). These associations were found after multivariate analysis.

CONCLUSION: Our findings indicated that rs9803799 as one of the *PRKAA2* genetic variations might impact the 10-year ASCVD risk among newly diagnosed T2DM patients receiving monotherapy metformin. After considering non-genetic factors, patient assessment should include potential genetic factors in cases with hyperglycemia involving treatment affecting glucose and lipid metabolism such as monotherapy metformin.

Introduction

AMPK is an energy sensor and maintains homeostasis so it has an important role in glucose and lipid metabolism. AMPK consists of three subunits (α , β , and γ). Phosphorylated AMPK in Thr172 in the α subunit has the primary function to induce AMPK downstream activation [1]. Accordingly, AMPK is the target of the mechanism of action of metformin. Metformin is an oral antidiabetic agent with a mechanism as an insulin sensitizer and reduces gluconeogenesis by AMPK activation [2], [3]. A study in mice showed that AMPK α 2 knockout had higher glucose levels and lower insulin concentration during meals and insulin resistance compared to wild type [4]. AMPK α 2, which is encoded by *PRKAA2*, is one of the pharmacogenetic research targets, especially in metformin pharmacodynamic research. AMPK α 2 is dominant in muscles and the

liver [5], [6]. AMPK α 2 was involved in reducing left ventricle pressure and smooth muscle relaxation [7], [8]. Therefore, it might contribute to lowering atherosclerosis cardiovascular disease (ASCVD) risk.

In fact, ASCVD causes the highest mortality and morbidity among diabetic patients, especially in type 2 diabetes mellitus (T2DM). Consequently, ASCVD is considered in T2DM therapy management [9]. Patients with T2DM have a 2–4 fold higher possibility of developing ASCVD risk, although this risk is not equal among individuals because of the heterogeneity in the population [10]. The increased risk of ASCVD mortality is associated with younger age groups, poor glycemic control, and increased renal complications [11]. In addition, patients with newly diagnosed T2DM tend to have higher poor glycemic control [12].

Pathogenesis of ASCVD and T2DM is related to epigenetic, genetic, and cell signaling interference,

which is correlated with inflammatory and metabolic pathways [13]. High glucose levels elevate advanced glycosylation end products (AGEs) formation. AGEs deposition increases fibrosis, cardiac stiffness, and inhibits diastolic relaxation. AGEs are associated with macrophages function resulting in escalating ASCVD risk [14], [15]. Insulin resistance could increase blood lipid levels, and systemic inflammation markers including, C-reactive protein, interleukin 6, and amyloid A which could predict ASCVD complications among patients with T2DM [16].

Several ASCVD risk calculators are freely available, including the Framingham risk score, SCORE, and pooled-cohort equation (PCE). The PCE was reported to be able to estimate ASCVD risk better when the racial difference was found [17]. A previous study in Sleman showed that there was no significant difference in the results of ASCVD risk calculated using SCORE or PCE [18].

Metformin is the first choice recommended by international guidelines [9], [19]. A previous study in newly diagnosed T2DM patients in the Asian population showed that metformin could control HbA1c after 3 months [20]. The effect of metformin reducing ASCVD risk in patients with T2DM remains controversial. A previous meta-analysis study reported that metformin did not show a protective effect on ASCVD risk [21]. On the other hand, a review stated that metformin could reduce ASCVD risk effectively [22]. Recent research determined that genetic variation affects metformin efficacy, but it still requires clinical research to confirm these findings [23].

However, the effect of genetic variation in AMPK α 2, encoded by *PRKAA2*, has not been discovered yet related to ASCVD risk. Accordingly, this recent study aimed to examine the association of *PRKAA2* genetic variation and ASCVD risk among newly diagnosed T2DM patients who receive monotherapy metformin.

Methods

Study designs and participants

A casecontrol study was conducted among newly diagnosed T2DM patients who receiving monotherapy metformin from ten primary health care centers in the Sleman District of Daerah Istimewa Yogyakarta, Indonesia. A total of 107 participants were examined who were required to fast at least 10 hours for clinical laboratory measurements. In this study, the inclusion criteria were: adult participants who were aged 40–70 years old, newly diagnosed T2DM with monotherapy metformin, and without previous ASCVD history. The exclusion criteria were: patients who did not fulfill PEC requirements as outlined in the following

conditions. Using the PEC calculation requirements were: systolic blood pressure 90–200 mmHg, diastolic blood pressure 60–130 mmHg, total cholesterol 130–320 mg/dL, HDL-c 20–100 mg/dL, and LDL-c 30–300 mg/dL. The high ASCVD risk (> 5%) was categorized as the case group, and the low ASCVD risk (< 5%) was categorized as the control group.

Written informed consent was obtained and signed by all participants. The protocol of the study was reviewed and approved by the Medical and Health Research Ethics Committee (MHREC) Faculty of Medicine, Public Health, and Nursing Universitas Gadjah Mada - Dr. Sardjito General Hospital (KE/FK/0633/EC/2019).

Anthropometric examination, clinical laboratory measurements and ASCVD score calculation

Anthropometric measurements including weight, height, and waist circumference were conducted by a nutritionist. Blood pressure was measured by well-trained nurses. Age, gender, family history of diabetes, smoking status, meal routine, and physical activity intensity were collected by a questionnaire. Hypertension therapy, statin user, and aspirin consumption were obtained from interviews and then were confirmed through medical records.

A professional analyst collected a blood sample after overnight fasting, lasting approximately 10–12 h. Prodia did all clinical laboratory examinations as an accredited laboratory. Fasting blood glucose was measured using the hexokinase method, and HbA1c was assessed using high-performance liquid chromatography D-10. T2DM patients were defined by HbA1c > 6.5%. Lipid profiles, including total cholesterol, triglycerides, and high-density lipoprotein cholesterol (HDL-c), were assayed using Cobas C-311. Low-density lipoprotein cholesterol (LDL-c) was calculated using the Friedewald equation (total cholesterol – HDL-c - Triglycerides/5 in mg/dL, but triglycerides should be <400 mg/dL). Blood pressure >140/>90 mmHg was classified as hypertension. This study adopted the formulation developed by ACC/AHA pooled cohort equation (<http://tools.acc.org/ASCVD-Risk-Estimator-Plus/#!/calculate/estimate/>) to calculate the 10-year ASCVD risk score [24]. The 10-year ASCVD risk score < 5% was defined as the low-risk (control) group and the risk score > 5% was defined as the high-risk (case) group.

DNA isolation and PRKAA2 genotyping analysis

DNA was isolated from blood samples which was done by adding EDTA using the Geneaid[®] Blood DNA Mini Kit and deposited at –20°C until the genotyping procedure. Genotyping was performed

using the TaqMan[®] genotyping assay and Applied Biosystems[®] qPCR 7500 Fast Real-Time PCR System to identify rs2796498, rs9803799, and rs2746342. Genotype identification of rs2796498, rs9803799, and rs2746342 was performed using specifically designed TaqMan primer sequences as follows:

rs2796498: CTGTAACAGTGTTAGTGATTTAAAC[A/G]GAGAGAGCAACCTTACCCTTTTCAGT

rs9803799: TAAATACAGGGTTTATATCCCCACA[G/T]TCAATGTAAATTCCTTTTTTTAAAA

rs2746342: AGAGAGGCTAAGATGCAGGCTGTAC[G/T]CTGGGTAGCCATGTACTCAGTTGTA

The total volume of amplification mixture used in the real-time PCR was 10 mL, including 5 mL TaqMan GTXpress mix, 2.5 mL nucleotide-free water, and a 0.5 mL TaqMan SNP genotyping assay, and 2 mL of genomic DNA sample. The amplicons were set up according to the following program: forty cycles at hold 95°C for the 20s, at denaturing 95°C for 3s, and then annealing 60°C for 30s.

Statistical analysis

We stratified these data by ASCVD risk category and then analyzed the differences using independent t-tests for numeric variables and chi-squared tests for categorical variables. We involved the dominant and recessive models in detecting an association with 10-year ASCVD risk. The association between the three studied variants and 10-year ASCVD risk was analyzed using logistic regression analysis. Confounder variables, including sex, age, HDL-c, total cholesterol, triglycerides, high-intensity physical activity, the routine of lunch, dinner, and snack that had a significant association to 10-year ASCVD risk were introduced into multivariate analysis. All statistical analysis were performed using SPSS version 25.0, and $p < 0.05$ was adopted as statistically significant.

Results

Of the 107 participants who were newly diagnosed with T2DM and only received monotherapy metformin for three months, 16 were excluded because they did not fulfill PCE requirements. For our sample of 91 participants, the average age was 54.40 ± 7.75 years old, 74.7% were female, BMI and waist circumference tended toward overweight status, mean blood pressure was in the pre-hypertension category, the mean of HbA1c level was $8.35 \pm 1.31\%$, and lipid profile was in the normal conditions. Only 36.3% of participants had a family history of T2DM, and only 16.5% were active or former smokers. High ASCVD risk as the case group

had 34 patients and low ASCVD risk as the control group had 57 patients.

Moreover, we compared the participant's characteristics according to ASCVD risk classification. We found age, HbA1c level, sex, and smoking status were significantly different between those two groups ($p < 0.05$). The high-risk group tended to have older participants than the low-risk group ($p < 0.01$). Notably, the HbA1c level was higher in the low-risk group ($8.57 \pm 1.40\%$) than the high-risk group ($7.99 \pm 1.06\%$) ($p = 0.04$). Female patients were found more common in the low-risk (94.7%) than high-risk group (41.2%) ($p < 0.01$). Smoking status was found higher significantly ($p < 0.01$) in the high-risk group (38.2%) than in the low-risk group (3.5%). Demographic characteristics are presented in Table 1.

Table 1: Characteristics of participants with different 10-year ASCVD risk

Variable	Newly diagnosed T2DM patients using monotherapy metformin			p-value
	Total (n = 91)	Low-risk (n = 57)	High-risk (n = 34)	
Age (years old)	54.40 ± 7.75	50.98 ± 6.20	60.12 ± 6.70	0.00**
BMI (kg/m ²)	25.05 ± 3.96	25.47 ± 4.26	24.35 ± 3.33	0.19
Waist circumference (cm)	87.23 ± 8.66	87.67 ± 8.44	86.50 ± 9.09	0.54
Systolic blood pressure (mmHg)	125.73 ± 11.19	124.82 ± 11.90	127.24 ± 9.87	0.32
Diastolic blood pressure (mmHg)	80.70 ± 6.95	79.68 ± 6.26	82.41 ± 7.77	0.09
HbA1c (%)	8.35 ± 1.31	8.57 ± 1.40	7.99 ± 1.06	0.04*
Fasting blood glucose (mg/dL)	136.95 ± 29.99	137.02 ± 29.44	136.82 ± 31.36	0.98
Total cholesterol (mg/dL)	184.70 ± 27.23	182.16 ± 26.21	188.97 ± 28.60	0.25
Triglyceride (mg/dL)	138.05 ± 57.68	133.70 ± 59.76	145.35 ± 54.10	0.35
HDL-c (mg/dL)	47.48 ± 8.34	48.61 ± 8.11	45.59 ± 8.51	0.09
LDL-c (mg/dL)	109.58 ± 22.02	106.77 ± 21.13	114.29 ± 22.98	0.12
Sex, female	68 (74.7)	54 (94.7)	14 (41.2)	0.00**
Family history of diabetes, yes	33 (36.3)	22 (38.6)	11 (32.4)	0.47
Smoking status, yes	15 (16.5)	2 (3.5)	13 (38.2)	0.00**
Meal routine				
Breakfast, yes	60 (65.9)	36 (63.2)	24 (70.6)	0.47
Lunch, yes	80 (87.9)	48 (84.2)	32 (94.1)	0.16
Diner, yes	65 (71.4)	37 (64.9)	28 (82.4)	0.08
Snack, yes	44 (48.4)	31 (54.4)	13 (38.2)	0.14
Physical activity intensity				
High, yes	13 (14.3)	6 (10.5)	7 (20.6)	0.19
Intermediate, yes	67 (73.6)	41 (71.9)	26 (76.5)	0.63
Low, yes	90 (98.9)	57 (100)	33 (97.1)	0.37

Data are presented in mean ± SD or n (%). *p-value<0.05, **p-value<0.01. BMI: Body mass index, HDL-c: High density lipoprotein cholesterol, LDL-c: Low density lipoprotein cholesterol

There was no significant difference in the proportion of 10-year ASCVD risk between genotypes in each SNP of *PRKAA2* in this study ($p > 0.05$). Mostly, all of the genotypes of rs2796498, rs9803799, and rs2746342 had a low proportion of high-risk groups. However, it was found that only GT of rs9803799 had a higher proportion of high-risk ASCVD than other genotypes (Figure 1).

Associations between *PRKAA2* genetic variation and 10-year ASCVD risk, both in bivariate analysis and in multivariate analysis, are listed in Table 2. Bivariate analysis failed to find any association between *PRKAA2* genetic variation and 10-year ASCVD risk. After adjusting for sex, age, lipid profiles, physical activities high intensity, daily routine of lunch, dinner, and snack, using multivariate analysis, only rs9803799 had a significant association with 10-year ASCVD risk. Patients with GT genotype had 187.86 times higher possibility for high-risk of 10-year ASCVD risk than TT genotype (OR = 187.86, 95%CI: 2.98–11863.51).

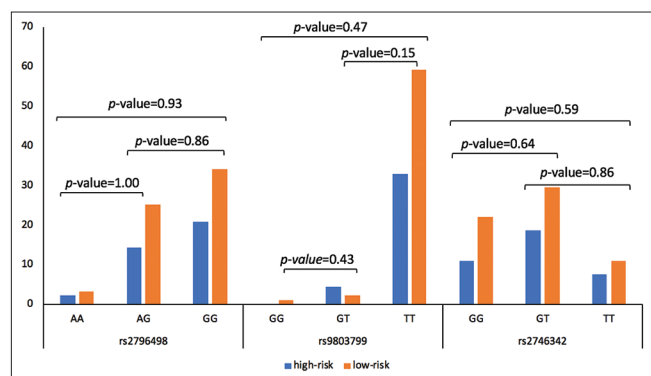


Figure 1: The 10-year ASCVD risk based on PRKAA2 genetic variation, in percentage

The dominant model also showed that GT+GG had 94.33 times higher possibility for high-risk 10-year

Table 2: The association between PRKAA2 genetic variation and 10-year ASCVD risk

SNP	Genotype	Newly diagnosed T2DM patients using monotherapy metformin (n = 91)					
		high-risk (n = 34) n (%)	low-risk (n = 57) n (%)	OR (95% CI)	p-value	AOR ^a (95%CI)	p-value ^a
PRKAA2 rs2796498	AA	2 (5.9)	3 (5.2)	1.09 (0.17–7.12)	0.93	2.51 (0.13–47.96)	0.54
	AG	13 (38.2)	23 (40.4)	0.92 (0.38–2.24)	0.86	2.87 (0.54–15.20)	0.22
	GG	19 (55.9)	31 (54.4)	0.94 (0.40–2.21)	1.00 (reference)	2.80 (0.58–13.62)	0.20
Dominant model	AA+AG	15 (44.1)	26 (45.6)	0.94 (0.40–2.21)	0.89	2.80 (0.58–13.62)	0.20
	GG	19 (55.9)	31 (54.4)	-	-	-	-
Recessive model	AA	2 (5.9)	3 (5.3)	1.13 (0.18–7.10)	0.90	1.70 (0.11–26.60)	0.71
	AG+GG	32 (94.1)	54 (94.7)	0.98 (0.49–1.95)	0.95	1.92 (0.62–5.94)	0.26
PRKAA2 rs9803799	G allele	51 (75.0)	85 (74.6)	-	-	-	-
	GG	0 (0)	1 (1.8)	-	-	-	-
	GT	4 (11.8)	2 (3.5)	3.60 (0.62–20.82)	0.15	187.86 (2.98–11863.51)	0.013*
Dominant model	TT	30 (88.2)	54 (94.7)	2.40 (0.50–11.45)	0.27	94.33 (2.32–841.21)	0.016*
	GG+GT	4 (11.8)	3 (5.3)	-	-	-	-
Recessive model	TT	30 (88.2)	54 (94.7)	-	-	-	-
	GG	0 (0)	1 (1.8)	-	-	-	-
Allele model	GT+TT	34 (100)	56 (98.2)	1.72 (0.42–7.11)	0.46	20.48 (1.48–283.30)	0.024*
	G allele	4 (5.9)	4 (3.5)	-	-	-	-
PRKAA2 rs2746342	T allele	64 (94.1)	110 (96.5)	1.40 (0.41–4.78)	0.59	3.69 (0.23–60.37)	0.36
	TT	7 (20.6)	10 (17.5)	1.26 (0.48–3.33)	0.64	3.36 (0.58–19.47)	0.18
	GT	17 (50.0)	27 (47.4)	1.26 (0.48–3.33)	0.64	3.36 (0.58–19.47)	0.18
Dominant model	GG	10 (29.4)	20 (35.1)	1.30 (0.52–3.24)	0.58	3.41 (0.62–18.68)	0.16
	TT+GT	24 (70.6)	37 (64.9)	-	-	-	-
Recessive model	GG	10 (29.4)	20 (35.1)	1.22 (0.42–3.57)	0.72	1.79 (0.15–21.27)	0.64
	TT	7 (20.6)	10 (17.5)	-	-	-	-
Allele model	GG+GT	27 (79.4)	47 (82.5)	1.19 (0.65–2.19)	0.57	1.89 (0.65–5.47)	0.24
	T allele	31 (45.6)	47 (41.2)	-	-	-	-
Allele model	G allele	37 (54.4)	67 (58.8)	-	-	-	-
	T allele	31 (45.6)	47 (41.2)	-	-	-	-

*p < 0.05. ^aAdjusted odd ratio for sex, age, HDL-c, total cholesterol, triglyceride, high-intensity physical activity routine of lunch, dinner, and snack

ASCVD risk than the TT genotype (OR = 94.33; 95%CI: 2.32–3841.21). Those findings indicated that the G allele had 20.48 times higher possibility for high-risk 10-year ASCVD risk than the T allele (OR = 20.48; 95%CI: 1.48–283.30).

Remarkably, AA as wildtype and the AG genotype in rs2796498 showed higher odds of high-risk 10-year ASCVD risk in the multivariate analysis than bivariate analysis. However, it did not become significant statistically (p > 0.05). Mutant genotype in rs2746342 contributed a higher odds to gain high-risk of 10-year ASCVD risk than wildtype genotype, but there was no significant association (p > 0.05), even after multivariate analysis.

Discussion

Our results emphasized the findings from a previous study in Sleman District which found that 10-year ASCVD risk among participants was categorized as low-risk. However, they used the Framingham risk score to measure ASCVD risk [25]. This study found that the group with high-risk of 10-year ASCVD risk had more older participants than the low-risk group. The findings of various studies have reported that age significantly contributed to ASCVD risk as an independent risk factor [26], [27]. Notably, the HbA1c level was higher in the low-risk group than the high-risk group. The literature has discovered the relationship between HbA1c level and ASCVD risk. Our finding was the opposite of studies stating that increasing HbA1c level is a major risk factor of ASCVD outcomes [28], [29]. Our results align with the previous findings that females tend to have lower ASCVD risk, especially before menopause. Menopause could reduce the sex hormones that could affect ASCVD risk through changes in vasculature, cardiac muscle, metabolism, and coagulation [30], [31], [32]. Smoking is one of the substantial factors involved in escalating ASCVD events, and the risk is doubled [33]. Our results confirmed that report, where the proportion of smokers was higher in the high-risk group than the low-risk group.

The proportion of low-risk 10-year ASCVD risk was dominant in almost all genotypes, except GT of rs9803799. It seemed that both the heterozygote and the homozygote genotype, could have a role in affecting disease risk [34]. However, our findings could not explain any significant difference in ASCVD risk proportion between each genotype.

This present study investigated the association between three variants in the PRKAA2 gene, rs2796498, rs9803799, and rs2746342, with 10-year ASCVD risk in newly diagnosed T2DM patients who have been prescribed metformin for three months consecutively. Our

findings indicated that only rs9803799 had an association with 10-year ASCVD risk. GT genotype and dominant model (GT+GG) had a significant association to have higher risk of 10-year ASCVD risk than TT genotype, whereas TT genotype is the mutant genotype of rs9803799. The result showed that those with the G allele also have a higher chance of getting high-risk ASCVD than the T allele. In fact, there has not been a study that examines *PRKAA2* genetic variation and 10-year ASCVD risk. SNP rs9803799 is located in a 3' prime UTR responsible for controlling transcription, initiating or inhibiting translation, and localizing in the cytoplasm [35]. Previous findings indicated that rs9803799 had a significant association with metformin efficacy [36]. In line with that study's results, the rs9803799 variant might have a pivotal role in AMPK expression; then, it could influence metformin efficacy and ASCVD risk. Undoubtedly, genetic variation could not stand alone to predict ASCVD risk. In this case, we could discover a significant association after adjusting for nongenetic factors in multivariate analysis. Several studies have confirmed that genetic and environmental factors interact and that interrelationship affects the development of ASCVD [37], [38], [39].

Furthermore, a study in Caucasian populations reported that rs2796498 and rs2746342 were associated with lipid profiles. SNP rs2796498 impacted HDL-c, LDL-c, and total cholesterol, while rs2746342 affected LDL-c and total cholesterol [40]. Lipid profiles are the dominant factor which have considerable weight in the 10-year ASCVD risk calculation. Nevertheless, this study could not identify any significant association between rs2796498 and rs2746342 and ASCVD risk.

Review literature declared that *PRKAA2*, which affected the metformin effectiveness, impacted ASCVD risk [41], [42]. A study applying the Mendelian randomization model which used AMPK as metformin pharmacologic target reported that there was genetic evidence in AMPK variance that could provide ASCVD protection [43].

Theoretically, AMPK has a crucial role in the metformin mechanism for reducing ASCVD risk. Metformin can activate AMPK, either by way of the direct pathway or indirect pathway, by inhibiting mitochondrial respiratory chain complex, thus altering AMP/ATP level [44], [45]. Several molecular studies investigating the pleiotropic effect of metformin mediated by AMPK proclaimed that AMPK and metformin could reduce ASCVD risk through the various pathways. Those explanations involved: (1) increasing fatty acid oxidation through acetyl-CoA carboxylase activation, (2) enhancing glucose uptake over GLUT4 induction, thus increase catabolism, (3) elevating endothelial nitric acid synthase (eNOS) level as a consequence, it decreases reactive oxygen species and increases endothelial function, (4) activating PGC-1 α who supports left ventricle function, (5) inhibiting sterol regulatory element-binding protein 2 (SREBP-2) maturation for low-density

lipoprotein receptor (LDLR) binding, (6) suppressing Toll-like receptor 4 (TLR4) thus inhibiting the inflammatory mediator, and (7) inhibiting apoptosis through platelet-derived growth factor receptor (PDGFR) suppression [46], [47], [48], [49], [50].

This present study has several limitations. First, lipid profiles that contribute to 10-year ASCVD risk calculation were only examined after metformin therapy was initiated. Second, we only detected diet aspects from a daily meal routine, whereas diet significantly impacts ASCVD. Third, we only found one participant who had GG genotype of rs9803799. Finally, though the participants were selected from a representative sample of patients with newly diagnosed T2DM who have been prescribed only metformin, the results would be more convincing in a bigger sample size.

Conclusions

In summary, our finding confirmed that *PRKAA2* genetic variation might impact the 10-year ASCVD risk among newly diagnosed T2DM patients receiving monotherapy metformin, especially in the rs9803799. Patient assessment should consider the combination of genetic and environmental factors to determine 10-year ASCVD risk.

Acknowledgments

We thank all the participants for their participation and the engaged health practitioners in primary health care in Sleman Districts. The authors thank Prof. Iwan Dwiprahasto, M.Med.Sc., Ph.D. for supporting the study in the beginning. This study was supported by the Indonesia Endowment Fund for Education (LPDP) number: 201812220413569 and Rekognisi Tugas Akhir grant number: RTA 2488/UN1.P.III/DIT-LIT/PT/2020.

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