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Molecular Insights to Clinical Perspectives

The quality of medical care that we enjoy now is due to research done over decades, even centuries. Medical research involves the study of diseases, their causes, their prevention and control, and their medical management. The last few decades have generated an exponential increase in knowledge regarding the molecular pathogenesis of endocrine disorders brought about by clinical and research collaboration. This present issue of the Journal of the ASEAN Federation of Endocrine Societies (JAFES) features articles on bisphenol S, selenoprotein P, type 2 diabetes mellitus, and X-linked dystonia parkinsonism with discussions on developments integrating basic science to translational research. Novel insights and perspectives, covered in this issue, looking into mechanisms of interest, relations between genotype and phenotype, and efficacy of interventions have definitely narrowed our knowledge gap. All these have allowed us to move towards the improvement of health and healthcare outcomes.

Diagnosis, based on clinical and metabolic investigations, and on genetic analyses, is of major importance in the medical management of patients with endocrine disorders. The expansion of tools for identifying patients with increased risk for these medical conditions has allowed early preventive measures to delay the onset of these diseases. Furthermore, the identification of patients who are likely to respond to existing drugs or new therapies and the prediction of who will develop complications has better targeted these diseases and has improved the survival and the quality of life of affected individuals. The discovery of new causative genetic variants has also resulted in a better understanding of the physiological and pathological processes of the endocrine system.

Given the burden of these medical conditions and their complications, the additions to our current knowledge base provided by these researches will definitely lessen the impact of these life-threatening illnesses. As more research findings are published, more evidence-based guidelines to better manage patients will be developed resulting in significant advances in our healthcare system.

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JAFES

EDITORIAL

Molecular Basis of Endocrine-related Disorders

Identifying the genetic and molecular basis of disease is a crucial step towards effectively managing and treating endocrine-related disorders. In the 1980s, advances in the field of recombinant DNA technology facilitated the use of a molecular and genetic approach towards understanding the cellular, biochemical, and molecular basis of disease. In the past decade, the precipitous progress in the development of genome-editing and omics-based technologies provided tools for the rapid identification of molecular determinants that drive and contribute to endocrine-related health issues.

This special issue of JAFES includes articles that utilize molecular techniques and genome-wide association studies that broaden our understanding on: (1) the contribution of endocrine disrupting chemicals in cancer-related cellular hallmarks; (2) the effect of stress hormones on dysregulation of transcription associated with neurodegenerative disorders; (3) genetic determinants diabetes and response to therapy; and (4) molecular markers involved in signaling cascade induced by exercise.

This special issue highlights the importance of molecular and omics-based approaches in identifying the underlying molecular underpinnings of endocrine-related diseases and how this knowledge can be used for disease prevention, rapid diagnosis and effective disease management. As the appreciation for the utility and application of molecular studies continues to expand in the clinics, we hope that this will be followed by more molecular endocrinology-focused articles featured in JAFES.

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Journal of the ASEAN Federation of Endocrine Societies

ORIGINAL ARTICLE

Lower Plasma Selenoprotein P Levels in Regularly Exercising Young Adults

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Abstract

Objective. Physical exercise can provide many health benefits in humans. Exercise-induced reactive oxygen species (ROS) formation and its downstream signaling cascades are reported to induce mitochondrial biogenesis in exercising tissues. Selenoprotein P (SELENOP) is the antioxidant hepatokine whose hypersecretion is associated with various metabolic diseases. It was reported to impair exercise-induced reactive oxygen species signaling and inhibit subsequent mitochondrial biogenesis in mice. However, the relationship between selenoprotein P and mitochondrial dynamics in humans has not yet been reported. While reduction of plasma selenoprotein P becomes an attractive therapeutic target for metabolic diseases, the role of regular exercise in this regard is still unknown. This study aimed to analyze the influence of regular habitual exercise on plasma selenoprotein P levels and its association with leucocyte mitochondrial DNA copy number in healthy young adults.

Methodology. Plasma selenoprotein P levels and leucocyte mitochondrial DNA copy numbers were compared in 44 regularly exercising subjects and 44 non-exercising controls, and the correlation between the two parameters was analyzed. Plasma selenoprotein P levels were measured by Enzyme-linked Immunosorbent Assay, and leucocyte mitochondrial DNA copy numbers were measured using the qPCR method.

Results. The regular-exercise group had lower plasma selenoprotein P levels with higher leucocyte mitochondrial DNA copy numbers than the non-exercise group. There was a tendency of negative correlation between the two variables in our studied population.

Conclusion. Regular habitual exercise has a beneficial effect on reducing plasma selenoprotein P levels while raising mitochondrial DNA copy numbers.

Key words: mitochondria, physical exercise, reactive oxygen species, selenoprotein P

INTRODUCTION

It is undisputedly accepted that physical exercise can provide many health benefits in humans beyond body fitness.[1,2] During physical exercise, numerous metabolic adaptations in various tissues occur to meet the increased oxidative capacity and metabolic demands of the exercising tissues.[3-5] One major event during physical exercise is the transient induction of sub-pathological amounts of reactive oxygen species (ROS),[6] mainly generated from the mitochondrial respiratory chain as a byproduct of accelerated cellular respiration during physical exercise.[7] Researchers are gradually realizing that exercise-induced ROS plays a crucial role in proper cellular functioning by acting as intracellular messengers in various signaling cascades.[8,9]

Previous studies reported that the production of ROS in skeletal muscle during prolonged endurance exercise plays an important role in hormesis-like adaptive changes in skeletal muscle. In particular, acute exposure to ROS during physical exercise can activate the 5' adenosine monophosphate-activated protein kinase - peroxisome proliferator-activated receptor gamma (AMPK-PGC-1α) signaling cascade,[9,10] which plays a central role in mitochondrial biogenesis and mitochondrial DNA maintenance.[11,12] Furthermore, exercise-induced ROS signaling induces the expression of endogenous antioxidant enzymes including manganese superoxide dismutase (MnSOD) and glutathione peroxidase (GPx) through the activation of nuclear factor erythroid 2-related factor 2 (Nrf2), to restore cellular redox homeostasis.[13] Those adaptive changes are important for the health-promoting effects of regular exercise, supporting the fact that exercise is an antioxidant and medicine.

Selenoprotein P (SELENOP), encoded by the *SELENOP* gene in humans, is a selenium transport protein mainly

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secreted by the liver.[14] It is reported to have an antioxidant capacity through direct enzymatic action or by supplying selenium to synthesize intracellular antioxidant enzymes.[15] Notably, one recent report indicated that SELENOP deficiency in mice increased exercise-induced ROS formation and subsequent AMPK signaling cascade with higher mitochondrial DNA content in skeletal muscle.[16] In their study, they also reported over-activity of SELENOP impaired hydrogen peroxide (H_2O_2) -induced AMPK phosphorylation and mitochondrial biogenesis in the myocyte, indicating the inhibitory action of SELENOP on mitochondrial biogenesis.[16] However, the relationship between SELENOP and mitochondrial dynamic in human studies has not been reported.

Previously, hepatic overproduction of SELENOP was reported to be involved in insulin resistance and hyperglycemia in patients with type 2 diabetes,[17] hypoadiponectinemia[18] and impaired angiogenesis by vascular endothelial growth factor (VEGF) resistance.[19] Misu et al., reported that overproduction of SELENOP impairs insulin signaling and dysregulates cellular glucose metabolism by reducing insulin-stimulated insulin receptor phosphorylation and subsequent Akt phosphorylation in hepatocytes and glucose uptake into myocytes.[19] Since the reduction of plasma SELENOP has been considered the potential target for the prevention and treatment of metabolic diseases, exploring various factors which influence plasma SELENOP levels has interested many researchers. One potential candidate is regular exercise, which has been reported to have beneficial effects in preventing various metabolic diseases.[1] To our best knowledge, there have been no previous reports about the influence of regular exercise on plasma SELENOP levels in the healthy young population.

Therefore, the general objective of this study was to analyze the influence of regular exercise on plasma SELENOP levels and determine whether there is a correlation between plasma SELENOP levels and leucocyte mitochondrial DNA copy numbers (mtDNA CN) in young adults.

The specific objectives were to (1) Measure plasma SELENOP levels and mtDNA CN in regularly exercising and non-exercising healthy young adults; (2) Compare plasma SELENOP levels and mtDNA CN between regularly exercising and non-exercising healthy young adults, and (3) Assess the correlation between the two parameters in our studied population. This study measured the leucocyte mtDNA CN as an indicator of mitochondrial biogenesis and mitochondrial abundance in the cell.[20]

METHODOLOGY

Study design and participants

This study was a cross-sectional, comparative study performed on 44 male student volunteers from the University of Medicine 2 (UM2), Yangon, Myanmar, and 44 male students from the Institute of Sports and Physical Education (ISPE), Yangon, Myanmar.

Sample size was calculated based on the previous study that measured serum selenium levels in non-exercising and regularly-exercising groups,[21] as serum selenium levels were reported to have a strong and significant correlation with circulating SELENOP levels.[22,23] With 95% confidence interval and 90% power of the study, the calculated required sample size for each group was 44 and the total sample size was 88.

We defined a regularly-exercising person as one who does moderate intensity endurance and/or resistance exercises for a minimum of 300 minutes per week for more than 6 months.[24,25] Forty-four healthy subjects from ISPE, Yangon, Myanmar who met the criteria participated in the regular-exercise group. In contrast, the non-exercise control group comprised volunteer medical students from UM2, Yangon, Myanmar with no history of regular exercise within one year before the study. All the participants were non-obese, non-diabetic, normotensive, apparently healthy young males between the ages of 16 to 20 years. Individuals with a previous history of diabetes, hypertension, liver diseases or currently taking selenium or antioxidant vitamin supplementation were excluded from the study. Our study was approved by the Institutional Review Board, Department of Medical Research Myanmar (Ethics/DMR/2020/026). All participants were volunteers who provided written informed consent for participation.

Physical examination and blood sample collection

Personal data collection, history taking, anthropometric assessment and blood pressure measurement were performed before taking the fasting blood sample. Venous blood samples were put in EDTA tubes and centrifuged at 2500×g for 10 minutes for buffy coat and plasma separation. According to the manufacturer's instructions, genomic DNA isolation was performed from the buffy coat samples on the same day of sample collection using the blood DNA mini kit (Invitrogen, USA). During DNA extraction, the lysate of the buffy coat layer was treated with RNAase solution to eliminate possible RNA contamination in the purified DNA samples.

Measurement of plasma SELENOP levels

The SELENOP levels of the plasma samples were measured via the Enzyme-linked Immunosorbent Assay (ELISA) method by using the Human selenoprotein P (SELENOP) ELISA kit (Catalogue No: abx251264, Abbexa, UK) according to the manufacturer's instructions.

Measurement of leucocyte mitochondrial DNA copy number

DNA concentration of each sample was measured and diluted by nuclease-free water to prepare a 10ng/µl con-

centration. After that, mtDNA CN in each sample was determined by quantitative PCR analysis of mitochondrial *ND1* gene and normalized by simultaneous measurement of the nuclear gene, β-globulin (*HBB*) in QuantStudio™ 3 Real-time PCR system using SYBR™ Green PCR Master Mix (Thermo Fisher Scientific, USA). The forward primer 5′- CCC TAA AAC CCG CCA CAT CT-3′ and reverse primer 5′- GAG CGA TGG TGA GAG CTA AGG T-3′ were used for mitochondrial *ND1* gene analysis. For the nuclear β-globulin (*HBB*) gene, the forward- 5′- GCT CGG TGC CTT TAG TGA TG- 3′ and reverse- 5′- AAA ACA TCA AGC GTC CCA TAG AC- 3' primer set was used. PCR reaction was performed twice with two sets of forward and reverse primers for the mitochondrial *ND1* and nuclear *HBB* gene.

After denaturation at 95°C for 10 minutes, the samples were subjected to 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The threshold cycle number (Ct) values were defined as the numbers of PCR cycles required to produce a 20ng DNA product. The Ct values of the *ND1* and nuclear *HBB* gene were determined for each sample. The relative mitochondrial copy number in each sample was calculated by the Relative copy number (Rc) = $2^{-\Delta Ct}$, where ΔCt is the Ct^{HBB}- Ct^{ND1} as described previously[25]. After which, fold changes in mtDNA CN in each sample were calculated by setting the mean for the control group as 1.

Statistical analysis

Data analysis was performed using IBM SPSS Statistics for Windows, Version 20.0. Data were expressed as mean ± standard deviation. The normal distribution of each parameter was checked by the Shapiro-Wilk test in SPSS. The student's *t*-test for parameters with a normal distribution (BMI, height, weight, and plasma SELENOP) and Mann-Whitney U test for non-normal data (Age, fasting blood sugar (FBS), systolic blood pressure, diastolic blood pressure, and mtDNA CN) respectively, were used to analyze statistically significant differences between regular-exercise and non-exercise groups. A *p*-value of <0.05 was set as the level of significance. Correlations between different variables were analyzed using Pearson's correlation coefficient.

RESULTS

The comparison of biochemical parameters between the non-exercise and regular-exercise groups are described in Table 1. The non-exercise group was found to be significantly older than the regular-exercise group ($p<0.001$), whereas no significant difference was found for height, weight, body mass index (BMI), systolic, or diastolic blood pressures. Notably, the regular-exercise group was found to have significantly lower FBS levels than the non-exercise group ($p=0.001$).

Regularly exercising subjects had lower plasma SELENOP levels and higher mtDNA CN than non-exercising counterparts

As shown in Table 1, mean plasma SELENOP levels of the regular-exercise group (3.70 ± 0.80 μg/ml) was found to be significantly lower than the non-exercise group (4.63 ± 1. 30 μg/ml) ($p < 0.001$) (Table 1 and Figure 1A). Mitochondrial DNA copy number (mtDNA CN) was also found to be significantly higher in the regular-exercise group than the non-exercise group ($p < 0.001$) (Table 1 and Figure 1B).

To exclude the effect of age differences between the two groups, the subjects were initially stratified into two age groups (<18 years and ≥18 years), and the parameters were then compared in each group. As shown in Table 2,

Table 1. Comparison of biochemical parameters of participants

Parameter	Non-exercise group (N = 44)	Regular-exercise group (N= 44)	*p* value
Completed age (years)	18.27 ± 1.45	16.89 ± 0.75	< 0.001***
Height (m)	1.68 ± 0.06	1.71 + 0.05	0.017*
Weight (kg)	60. 13 ± 10. 80	61.89 ± 5.37	0.311
Body mass index (kg/m^2)	21.09 ± 2.72	21.06 ± 1.60	0.941
Systolic blood pressure (mmHg)	114.55 ± 9.51	115.57 ± 7.17	0.570
Diastolic blood pressure (mmHg)	74.66 ± 8.03	74.00 ± 7.31	0.932
Fasting blood sugar (mg/dl)	103.70 ± 9.41	93.61 ± 13.78	0.001**
Plasma SELENOP (μg/ml)	4.63 ± 1. 30	3.70 ± 0.80	< 0.001***
mtDNA CN	1 ± 0.61	2.44 ± 0.81	< 0.001***

Results are expressed as mean ± standard deviation. p value was calculated by either Student's unpaired t-test or Mann-Whitney U test for normally distributed and non-normally distributed parameters respectively. *p <0.05, **p <0.01 and ***p <0.001.
Abbreviation; mtDNA CN = mitochondrial DNA copy numbers, SELENOP = Selenoprotein P

Table 2. Comparison of plasma SELENOP and mtDNA CN between non-exercise and regular-exercise groups stratified by age

	Age <18 years			Age ≥18 years		
	Non-exercise group (N = 15)	Regular-exercise group (N = 34)	*p*	Non-exercise group (N = 29)	Regular-exercise group (N = 10)	*p*
Plasma SELENOP (ug/ml)	4.76 ± 1.36	3.60 ± 0.70	<0.001***	4.57 ± 1.28	3.67 ± 1.01	0.049*
mtDNA CN	1.00 ± 0.66	2.35 ± 0.82	<0.001***	1.00 ± 0.60	2.76 ± 0.72	<0.001***

Results are expressed as mean ± standard deviation. p-value was calculated by Student's unpaired t-test for plasma SELENOP levels and Mann-Whitney U test for mtDNA CN respectively. *p<0.05, **p<0.01 and ***p<0.001.
Abbreviation: mtDNA CN = mitochondrial DNA copy numbers, SELENOP = Selenoprotein P

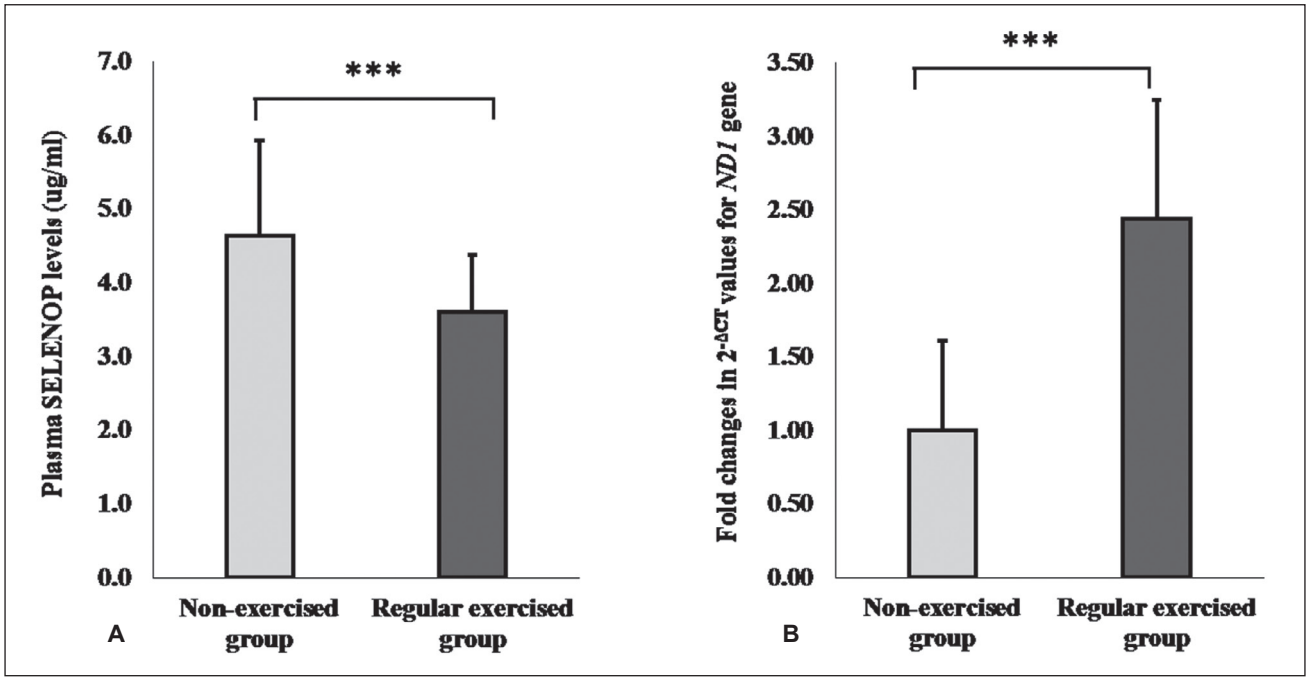

Figure 1. Differences in **(A)** plasma SELENOP levels and **(B)** mtDNA CN between non-exercise and regular-exercise groups (N= 44 for each group). mtDNA CN was calculated by $2^{-\Delta CT}$ values of mitochondrial ND1 gene copy number normalized to nuclear HBB gene. Fold changes in $2^{-\Delta CT}$ were compared between the non-exercise group and regular-exercise group. Data are presented with mean ± SD.

***p<0.001 by Student's unpaired t-test for plasma SELENOP levels and Mann-Whitney U test for mtDNA CN, respectively.

the regular-exercise group had significantly lower plasma SELENOP with higher mtDNA CN than the non-exercise controls in both age strata. Therefore, our findings confirmed that the age difference between non-exercise and regular-exercise groups did not affect the statistically significant difference in plasma SELENOP levels and mtDNA CN between the two studied groups.

Correlation between plasma SELENOP levels and mtDNA CN

We then analyzed the bivariate correlations between plasma SELENOP levels and various biochemical parameters in our subjects. As shown in Table 3, plasma SELENOP levels showed a significant, weak positive correlation with age (R=0.288, p=0.006) and BMI (R=0.273, p=0.01), while no correlation was detected between plasma SELENOP and FBS (R=0.058, p=0.59), height (R=0.016, p=0.884), systolic and diastolic blood pressures (R=0.057, p=0.599 and R=0.055, p=0.614 respectively). A weak, positive but nonsignificant correlation was found between plasma SELENOP level and weight (R=0.203, p=0.058). Notably, there was a trend of negative correlation between plasma SELENOP levels and mtDNA CN (R=- 0.203), although the p-value failed to reach a statistically significant level (p=0.059).

Table 3. Bivariate correlation between plasma SELENOP levels and various metabolic parameters in the studied population (N= 88)

	Plasma SELENOP
Fasting blood sugar	R = 0.058, p = 0.590
Age	R = 0.288, p = 0.006**
BMI	R = 0.273, p = 0.01*
Height	R = 0.016, p = 0.884
Weight	R = 0.203, p = 0.058
Systolic blood pressure	R = - 0.057, p = 0.599
Diastolic blood pressure	R = 0.055, p = 0.614
mtDNA CN	R = - 0.203, p = 0.059

Statistical method by Pearson's correlation coefficient. *p< 0.05, **p< 0.01 and ***p< 0.001. Abbreviation: BMI = Body Mass Index, mtDNA CN = mitochondrial DNA copy numbers, SELENOP = Selenoprotein P

DISCUSSION

Selenoprotein P (SELENOP) is the major selenium transport hepatokine. Its hepatic hypersecretion was previously reported to be associated with various metabolic disorders.[16-19,21,26,27] In this regard, exploring various factors which can influence circulating SELENOP has interested researchers aiming for a more comprehensive management of metabolic diseases.

To examine whether regular exercise has benefits in reducing circulating SELENOP levels, we compared plasma SELENOP levels in non-exercising controls and regularly-exercising young adults. All the participants were relatively healthy male students between the ages of 16 to 20 years. The two groups were comparable in height, weight, BMI, systolic and diastolic blood pressures. The only significant difference was that fasting blood glucose levels were lower in the regular-exercise group, consistent with the well-known beneficial effects of physical exercise on glucose metabolism.[28-30]

In this study, we also found that plasma SELENOP levels were significantly lower in regularly-exercising subjects compared to their non-exercising counterparts, independent of age.

To our best knowledge, this is the first report of the influence of long-term regular exercise on plasma SELENOP levels in healthy individuals. It was previously reported that plasma SELENOP levels did not change significantly after eight weeks of aerobic exercise training in sedentary postmenopausal women[16] or after an acute bout of 60 minutes of moderate-intensity treadmill training in obese men.[31] In contrast, the regularly exercising participants in this study were healthy young athletes who had been getting regular sports-type exercise training for approximately 20.06 ± 5.04 hours per week for more than two years. The training program included at least 3 hours per day, six days a week of endurance exercise such as running and jogging in addition to sports type-specific training for each student. Therefore, the type, duration and intensity of physical exercise may be the critical factors in exercise-mediated suppression of plasma SELENOP levels.

A previous study reported that plasma SELENOP levels were higher in patients with pulmonary arterial hypertension (PAH) compared with controls, and higher plasma SELENOP level was associated with poor outcomes in PAH patients.[32] Moreover, higher plasma SELENOP levels were reported to be positively associated with carotid intima-media thickness and increased risk of heart failure.[33,34] As our study found lower plasma SELENOP levels in regular exercise, it is possible that the cardio-protective benefits of regular exercise may at least partly be mediated by its action on the reduction of plasma SELENOP levels.

There are some potential mechanisms for lower plasma SELENOP levels in physical exercise. First, since the liver is the primary source of circulating SELENOP,[35] the lower plasma SELENOP levels in regularly exercising persons may be due to lower hepatic *SELENOP* expression. Takayama et al[36] reported that hepatic AMPK activation by metformin decreased nuclear localization and subsequent transcriptional inactivation of FoXO3, resulting in suppression of hepatic SELENOP expression. As physical exercise has been reported to enhance hepatic AMPK activities,[37,38] exercise-induced AMPK-FoXO3 activation may be responsible for suppressing hepatic *SELENOP* expression and subsequent lower plasma SELENOP levels in physical exercise. More longitudinal and in-vitro experiments are in great demand to confirm the assumption.

Second, the lower SELENOP levels in the regular-exercise group may be related to accelerated uptake and cellular utilization of SELENOP by peripheral tissues to synthesize selenium-containing antioxidant enzymes. Regarding the concept of exercise-induced hormesis, previous literature reported that the physiological amount of ROS produced during exercise can stimulate antioxidant enzyme expression in skeletal muscle[39-41] to restore intracellular redox homeo-stasis and protect from harmful oxidative damage. It was also reported that regular endurance exercise training increases GPX1 levels in skeletal muscles by 20%–177%.[40] Furthermore, a previous paper reported that serum selenium concentrations was lower in professional athletes to synthesize GPX enzymes in skeletal muscles.[23] As SELENOP is the major selenium supplier for synthesizing intracellular selenium-containing antioxidant enzymes, the cellular uptake and utilization of SELENOP may be augmented during antioxidant adaptation in chronic exercise, lowering its plasma level. In other words, our data suggest that SELENOP may have a particular crucial physiological role in the exercise-induced hormesis effect of habitual physical training. Further in-vivo and in-vitro uptake studies are recommended to address this hypothesis.

As expected, the leucocyte mtDNA CN was found to be significantly higher in the regularly-exercising than non-exercising individuals. That finding is consistent with the previous report[25] supporting the effect of physical exercise on mitochondrial biogenesis. Peroxisome-proliferator-activated receptor γ co-activator-1α (PGC-1α), the master regulator of mitochondrial biogenesis and exercise-induced H_2O_2 production in skeletal muscles, was reported to increase PGC-1α expression through AMPK activation.[42] Then PGC-1α binds to and co-activates the transcriptional function of nuclear respiratory factors 1 (NRF-1) on the promoter for mitochondrial transcription factor A (Tfam)[12] to induce mtDNA replication. Therefore, transient induction of oxidative stress during physical exercise activates mitochondrial biogenesis via the AMPK-PGC-1α signaling cascade to improve mitochondrial quantity with higher oxidative capacity and ATP production during the physiological state of increased metabolic demand.

Although it was not statistically significant, we found a trend of inverse correlation between antioxidant SELENOP levels and mtDNA CN in our population. The previous report also indicated higher mitochondrial DNA content in the skeletal muscle of trained SELENOP deficient mice than its wild-type counterparts.[16] Therefore, we assume that the relationship between SELENOP and mtDNA CN might be bi-directional depending on metabolic conditions. During physiological adaptation of regular exercise, increased mitochondrial numbers with accelerated oxygen consumption and subsequent ROS production by physical exercise may lower plasma SELENOP level due to increased utilization for compensated antioxidant enzyme synthesis. On the other hand, when SELENOP is over-expressed, the over-activity of SELENOP and its reductive stress may suppress exercise-induced mitochondrial biogenesis in skeletal muscle.[16] Failure to reach a statistically significant level in our study may be due to the fact that, contrary to the study of Misu et al,[16] the participants in our study were healthy volunteers with plasma SELENOP levels within

the physiological range. Therefore, little variations in plasma SELENOP levels among participants, as well as the small sample size, may result in failure to achieve a statistically significant correlation between the two variables. Large-scale clinical studies involving those with insulin resistance are recommended for future perspectives.

Limitations of the study

Our study had certain limitations. We could not examine the course of plasma SELENOP changes with exercise duration because of our cross-sectional study design. A longitudinal study is recommended to analyze the time-dependent changes in plasma SELENOP level and its physiological significance from acute to habitual exercise.

CONCLUSIONS

In conclusion, our study found lower plasma SELENOP levels and higher leucocyte mtDNA CN in the regular-exercise group than the non-exercise group, which remained significant after stratifying the subjects into two age groups. These findings suggest that regular exercise training is an effective measure for improving mitochondrial function while lowering circulating SELENOP levels in humans. Further longitudinal studies are recommended for a better understanding of the role of SELENOP in exercise metabolism which can provide a new therapeutic approach to enhance the benefits of physical exercise in clinical settings.

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All authors certified fulfillment of ICMJE authorship criteria.

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ORIGINAL ARTICLE

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Bisphenol S Increases Cell Number and Stimulates Migration of Endometrial Epithelial Cells

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Abstract

Objective. To determine whether bisphenol S (BPS), a common substitute for bisphenol A (BPA), induces cell proliferation and migration in human endometrial epithelial cells (Ishikawa) and adult mouse uterine tissues.

Methodology. Human endometrial Ishikawa cells were exposed to low doses of BPS (1 nM and 100 nM) for 72 hours. Cell proliferation was assessed through the viability assays MTT and CellTiter-Glo®. Wound healing assays were also used to evaluate the migration potential of the cell line. The expression of genes related to proliferation and migration was also determined. Similarly, adult mice were exposed to BPS at a dose of 30 mg/kg body weight/day for 21 days, after which, the uterus was sent for histopathologic assessment.

Results. BPS increased cell number and stimulated migration in Ishikawa cells, in association with the upregulation of estrogen receptor beta *(ESR2)* and vimentin *(VIM)*. In addition, mice exposed to BPS showed a significantly higher mean number of endometrial glands within the endometrium.

Conclusion. Overall, *in vitro* and *in vivo* results obtained in this study showed that BPS could significantly promote endometrial epithelial cell proliferation and migration, a phenotype also observed with BPA exposure. Hence, the use of BPS in BPA-free products must be reassessed, as it may pose adverse reproductive health effects to humans.

Key words: BPS, endocrine-disrupting chemicals, Ishikawa cells, uterus, hyperplasia

INTRODUCTION

Endocrine-disrupting chemicals (EDCs) are examples of environmental contaminants that alter the normal function of hormones in the body.[1] Their ability to disrupt the synthesis, secretion, transport, and binding of hormones essential in maintaining homeostasis, and in reproductive and developmental processes can lead to adverse health effects in intact organisms and their offspring.[1,2] One of the most common EDCs is bisphenol A (BPA), a chemical that is extensively used in manufacturing plastic consumer products.[3] BPA causes deleterious effects on human health by mimicking the action of estrogen and disrupting the normal endocrine pathways.[3–8] For instance, exposure to environmentally relevant doses of BPA increases susceptibility to prostate cancer[6] and promotes estrogen-related diseases, such as breast cancer and endometriosis, as observed in animal models.[9–11]

Rising concerns about the negative impact of BPA on human health have led to restrictions regarding its production. This has also resulted in the search for safer alternatives to this chemical. For example, bisphenol S (BPS) has been used as a common chemical substitute for BPA and is found in many consumer products labeled as "BPA-free." BPS was originally thought to be a safer alternative to BPA, but studies now show that BPS also exhibits endocrine disrupting activities similar to that of BPA. Indeed, subcutaneous injection of BPS in rat neonates delayed their onset of puberty and altered their estrous cycle.[12] BPS is obesogenic, cardiotoxic, neurotoxic and immunotoxic,[13–16] but its impact on cell proliferation and migration in adult endometrial cells has not yet been examined. Thus, this study determined the proliferative and migratory effects of BPS on adult human endometrial epithelial cell lines and adult mouse uteri.

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METHODOLOGY

Chemicals

BPS (Supelco 43034) reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). For the *in vitro* model, stock solutions of BPS were dissolved in dimethyl sulfoxide (DMSO). For the *in vivo* model, BPS was diluted at the desired concentration using distilled water (vehicle). The solution was sonicated for 10 minutes at room temperature and was fed to mice through oral gavage.

Cell culture maintenance

Ishikawa (ECACC 99040201) cells were obtained from the European Collection of Authenticated Cell Cultures (ECACC) and maintained in a growth medium containing Minimum Essential Medium (MEM) with 2 mM glutamine (Gibco™ 11095080), 5% fetal bovine serum (FBS) (Gibco™ 10500064), 1% non-essential amino acid (NEAA) (Gibco™ 11140050), and 1 μg/ml of gentamicin (Gibco™ 15750060). Cells were kept in a 25 cm^2 tissue culture flask at 37°C with 5% CO_2. Steroid-depleted medium containing phenol-free DMEM/F12 (Gibco 21041025) with or without charcoal-stripped FBS (CS-FBS) was used for all assays involving BPS.

Cell viability assay

Cell viability was assessed through MTT and CellTiter-Glo® assays. For the MTT assay, cells were seeded at 10,000 cells/well in 96-well plates (Corning 3596) and incubated in 100 μl steroid-depleted medium containing 2% CS-FBS at 37°C under 5% CO_2 for 48 hours. Cells were then treated with BPS (1 nM or 100 nM) or vehicle control (DMSO) in a fresh steroid-depleted medium with 0% or 2% CS-FBS for 72 hours. The spent medium was aspirated and processed for MTT assay as previously described.[17] For the CellTiter-Glo® assay, cells were also seeded and treated as above and processed following the manufacturer's instructions. Plates were read using a luminometer to obtain the relative luminescence units (RLU). Relative viability was calculated by normalizing the RLUs with the vehicle control. Experiments were performed in quadruplicates per treatment.

Wound healing assay

Cell migration was evaluated by wound healing assay. Cells were seeded at 300,000 cells/well into 6-well plates containing a growth medium. After 24 hours, wells were replaced with a steroid-depleted medium supplemented with 2% CS-FBS, and cells were allowed to grow to 80% confluency. The cell monolayer was carefully scraped using a 200 μl pipette tip to form a cell-free area. The wounded monolayer was washed thrice with the same medium to remove cell debris. Before treatment, cells were exposed to 10 μg/ml mitomycin C (MMC) for 2 hours to ensure that wound closure was due to migration and not proliferation. After exposure to MMC, cells were washed twice with 1x phosphate-buffered saline (PBS) and incubated with a freshly prepared treatment medium containing 1 or 100 nM BPS for 72 hours. Photomicrographs of cells were captured using an inverted microscope (Nikon ECLIPSE Ts2-FL). Denuded areas at 0 and 72 hours post-induction of injury were measured using the Image J Software (version 1.50i, National Institute of Health, Bethesda, MD, USA). Percent closure was determined by subtracting the final gap area from the initial gap area and dividing the difference with the initial gap area, multiplied by one hundred. Experimental results were shown as mean ± SEM percent closure. A wound healing assay was performed thrice in triplicates per trial.

Gene expression

Cells were seeded at 250,000 cells/well on a 6-well plate using a steroid-depleted medium containing 2% CS-FBS and were incubated for 48 hours. Cells were then treated with BPS (1 nM, 100 nM) or vehicle control for 72 hours with the same steroid-depleted medium. RNA was extracted from each treatment group using the phenol-chloroform extraction protocol with TRIzol reagent (Invitrogen 15596026). Complementary DNA (cDNA) synthesis from a total of 1000 ng of RNA was performed using the SensiFAST cDNA synthesis kit (BIO-65053) with a PCR condition of 25°C for 10 minutes, 42°C for 15 minutes, 48°C for 15 minutes, and 85°C for 5 minutes. Quantitative PCR (qPCR) was done using the SensiFAST SYBR Hi-ROX kit (BIO-92020) containing 400 nM forward and reverse primers and 2 μL of cDNA in RNAse/DNAse-free water. Samples were run in a StepOne Plus qPCR machine with the following PCR conditions: 95°C for 2 minutes, 40 cycles of 95°C for 5 seconds and 60°C for 15 seconds, and a melt curve step of 95°C for 15 seconds and 60°C for 1 minute. Primers for genes involved in estrogen signaling (*ESR1, ESR2*), cell proliferation (*CCND1, CCNB1, GREB1A, CMYC*), and epithelial-mesenchymal transition (EMT) (*CDH1, CDH2, ERBB2, VIM*) were used (Table 1). The housekeeping gene, beta-actin (*ACTB*), was used for normalization.

Animals

All procedures were conducted in accordance with the guidelines for the care and use of laboratory animals, following the protocols approved by the Institutional Animal Care and Use Committee, UP Diliman. Inbred wild-type C57BL/6J mice were used in this study to ensure that all mice will have a similar genetic background across treatment groups. Mice were housed in intact polysulphonate cages and maintained in a facility with a 12-hr light/dark cycle, a temperature of 21 ± 1°C, and relative humidity of 60%. A phytoestrogen-free diet (1814P; Altromin, Germany) and ultra-pure water (in glass bottles changed twice per week) were provided *ad libitum*.

Mouse treatment

The sample size for the mouse experiment was determined based on a previous study of BPA, which showed a statis-

Table 1. Primer sequences for qPCR

Gene name	Forward (5'-3')	Reverse (5'-3')
ESR1	CGACTATATGTGTCCAGCCAC	CCTCTTCGGTCTTTTCGTATCC
ESR2	CAGGTCTGGGGTTGGAGAAT	AGTAACTCAAGGGGCCAGTC
CCNB1	CATGGTGCACTTTCCTCCTT	AGGTAATGTTGTAGAGTTGGTGTCC
CCND1	GCTGTGCATCTACACCGACA	TTGAGCTTGTTCACCAGGAG
GREB1A (Greb1 Isoform a)	CTGAAGCTAGACACGGAGGC	AGAGGTTATGAACAGTGCTACTCAC
MYC (c-Myc)	GTCCTCGGATTCTCTGCTCTC	CCAGACTCTGACCTTTTGCCA
CDH1	GTAACGACGTTGCACCAACC	GGGTCAGTATCAGCCGCTTT
CDH2	GCGTCTGTAGAGGCTTCTGG	GCCACTTGCCACTTTTCCTG
ERBB2	TTTGCTGTCCTGTTCACCAC	TCATCCTCATCATCTTCACATTG
VIM	CAGATGCGTGAAATGGAAGA	TGGAAGAGGCAGAGAAATCC
ACTB	AGAGCTACGAGCTGCCTGAC	AGCACTGTGTTGGCGTACAG

tically significant increase of approximately 100% in the average number of endometrial glands after BPA exposure at a sample size of four per treatment.[18] From their study, mice exposed to BPA had a mean endometrial glands/stroma ratio of 8 mm^2 and a standard deviation of 2. A minimum sample size of 5 mice per treatment group was needed to achieve 90% power using the sample size formula for endometrial gland proliferation. Factors used to compute the sample size were as follows: 1) significance level of 5% and 2) assumed effect size of 58.7%.[19] In this study, 7-month-old C57BL/6J female mice were randomly assigned to two experimental groups with five animals per group treated with either BPS (30 mg/kg body weight per day) or vehicle (distilled water). BPS was administered daily for 21 days through oral gavage. During the 21-day treatment, mice were subjected to daily vaginal smears to monitor the estrous cycle. Mice were sacrificed at the estrus stage following the 21-day dosing periods. Mouse uteri were then collected and fixed in 10% neutral buffered formalin (NBF) for histopathological analysis.

Histopathological Analysis

Formalin fixed-left uteri were sent to the Pathology Division of Providence Hospital Quezon City for histological processing. Uteri from mice (n=5 per group) were assessed based on the mean number of endometrial glands and thickness of the uterine walls per field of view at the end of the 21-day BPS or vehicle treatment. Each uterine section was cut into ten slices at 5 μm thickness and was divided into ten fields of view with a number assigned to each field of view. Each field of view has an approximate area of 2 x 2 mm under a 10x objective. Five fields of view per slice were randomly selected using a random number generator before observation and quantification of endometrial glands. The thickness of the uterine walls (endometrium, myometrium, perimetrium) was measured in the same field of view where endometrial glands were counted. Uterine walls were also examined for the presence of edema, hyperplasia, hypertrophy and other histopathological changes.

Statistical Analysis

Quantitative data collected were described using the mean ± SEM. The comparison of two mouse groups was performed using unpaired student's t-test, while the comparison of three groups of Ishikawa cells used one-way analysis of variance (ANOVA). Two-way ANOVA was used to determine the effects of serum concentration and BPS treatment on cell viability. Tukey's test was used as a post hoc comparisons test. Fisher's exact test was used to determine the association between BPS treatment and the occurrence of uterine lesions. A 5% level of significance was used for the hypothesis testing conducted. All statistical analyses were performed using GraphPad Prism version 6.0.

RESULTS

BPS increased cell number and migration in human endometrial Ishikawa cells

Previous studies have shown that BPA promoted endometrial cell proliferation.[4,20] This study tested whether the BPA analog BPS can increase the total number of cells in human endometrial epithelial cells, such as Ishikawa cells. In human sera, bisphenol concentrations were previously detected in the picomolar to nanomolar ranges.[21–25] This study used 1 and 100 nM BPS to reflect the physiologically relevant concentration of bisphenols found in human sera. At these concentrations, BPS significantly enhanced the total number of Ishikawa cells in the absence of serum using either the metabolism-based MTT assay (Figure 1A, Appendix 1A) or the ATP-based CellTiter Glo® assay (Figure 1B, Appendix 1B). A 3- to 8-fold increase in cell proliferation was observed after BPS treatment using the MTT assay ($p<0.005$), while a slightly lower but significant increase in fold change was seen in the CTG assay ($P<0.029$). However, while adding 2% serum significantly increased the total number of viable endometrial epithelial cells, this was not enhanced further with BPS treatment ($p>0.999$) (Figure 1A-B, Appendix 1A-B). The proliferative effects of serum appear to blunt the ability of BPS to increase the total cell count of endometrial epithelial cells. It is interesting that the ability of BPS to increase the viability of endometrial Ishikawa cells was comparable to BPA both in 0% and 2% FBS, as indicated by a similar fold change using MTT and CTG assays (Figure 1A-D, Appendix 1A-D).

The wound healing assay examined the effect of BPS on *in vitro* migration in human endometrial Ishikawa cells. Before treatment, cells were exposed to 10 μg/ml MMC to ensure that gap closure was due to migration and not

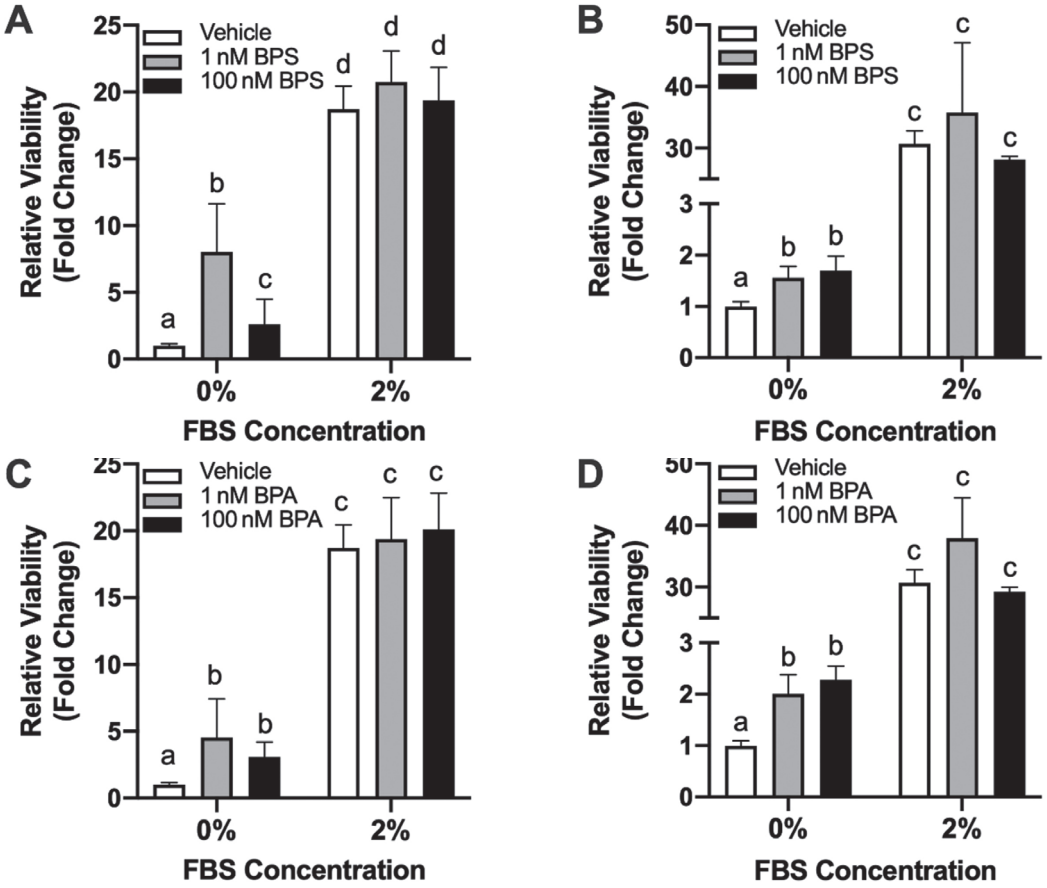

Figure 1. ***BPS increases the mean relative viability of human endometrial Ishikawa cells at low but not at high serum.*** Ishikawa cells were cultured in steroid-free media and then exposed to 1 nM and 100 nM **(A, B)** BPS and **(C, D)** BPA with 0% or 2% CS-FBS for 72 hrs before assessment of proliferation using **(A, C)** MTT and **(B, D)** CellTiter-Glo® assay. Numerical data were analyzed as mean relative viability ± SEM (n=3 and n=6 per treatment, respectively). Different letters denote significant differences at $p<0.05$ using two-way ANOVA followed by Tukey's post hoc test.

proliferation. Results showed that low dose (1 nM) but not high dose (100 nM) of BPS significantly increased the migration of human endometrial Ishikawa cells (Figure 2). A gap closure of 40% was noted in 1 nM BPS as compared to 15% gap closure in the vehicle and 19% gap closure in the 100 nM BPS groups (Figure 2B). Similarly, only 1 nM of BPA was able to significantly enhance the migration of endometrial Ishikawa cells albeit with values that are slightly lower compared to BPS. These data suggest that BPS at 1 nM concentration can promote migration of endometrial Ishikawa cells similar to BPA.

BPS significantly upregulated the expression of *ESR2* and *VIM* genes

BPS is an endocrine-disrupting chemical that is known to mimic estrogen.[12] We therefore measured whether BPS could influence the expression of estrogen receptors, *ESR1* and *ESR2*. We found that BPS increased the expression of *ESR2* but not *ESR1* (Figure 3A). We then examined the effects of BPS on the expression of genes involved in cell proliferation and migration. BPS affected the expression of proliferation -related genes (Figure 3A), but only low-dose BPS significantly increased the expression of *VIM* (Figure 3B), suggesting that BPS may induce a mesenchymal-like transition in these cells, consistent with their increased migratory phenotype at this dose. Although no gene expression analysis was carried out in cells exposed to BPA, several studies have already demonstrated that BPA can enhance the expression of genes involved in proliferation and migration.[26–28]

BPS increased the mean number of endometrial glands in the mouse uterus

To confirm whether the results in cell culture will also manifest *in vivo*, mice were fed orally with 30 mg/kg body weight per day BPS for 21 days, as this concentration is lower than the no adverse effect limit (NOAEL) (60 mg/kg body weight per day) set by the European Chemical Agency (ECHA) for BPS[29] and is the concentration of BPA which rendered significant histological effects in tissues of mice and other vertebrate species.[11,30]

We then performed a microscopic examination of the uterus from exposed and unexposed mice. Mouse uteri from the control group exhibited benign proliferative endometrium characterized by the presence of small, tubular glands

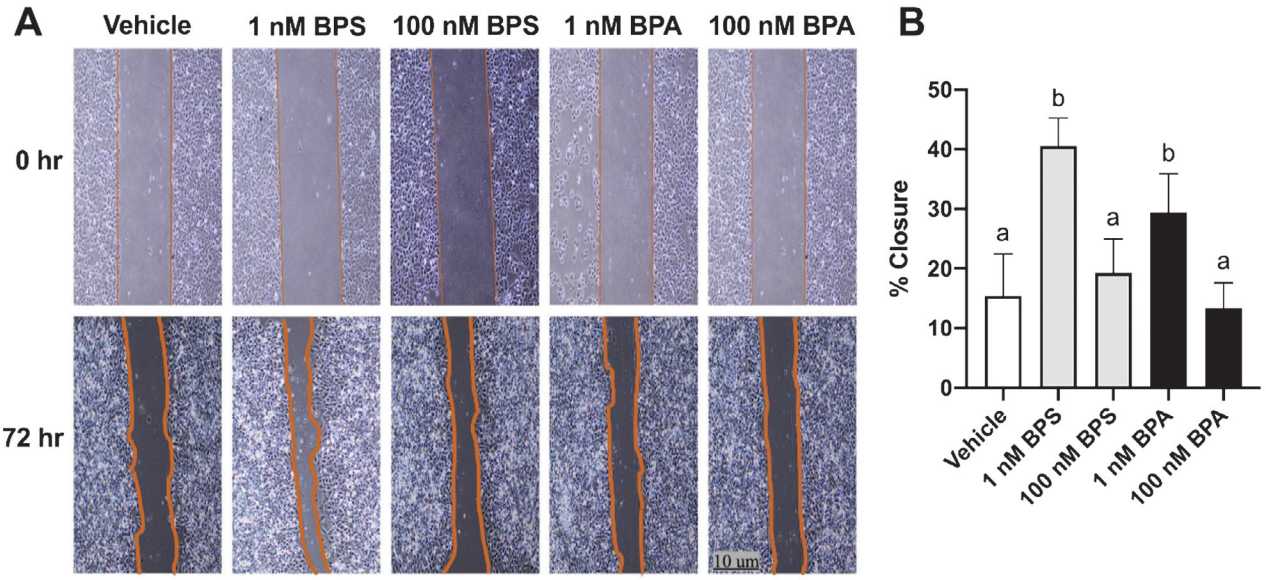

Figure 2. ***A low-dose of BPS promotes migration in human endometrial Ishikawa cells.*** **(A)** Representative photomicrographs of Ishikawa cells exposed to vehicle, 1 nM, or 100 nM BPS or BPA in steroid-depleted medium supplemented with 2% FBS taken at 0 hr and 72 hrs post induction of gap. Cells were exposed to 10 µg/mL MMC before treatment with BPS. **(B)** Percent (%) gap closure of Ishikawa cells after 72 hr exposure to BPS or vehicle. Data were analyzed as mean % closure ± SEM (n=9). Different letters denote significant differences at $p<0.05$ using one-way ANOVA followed by Tukey's post hoc test.

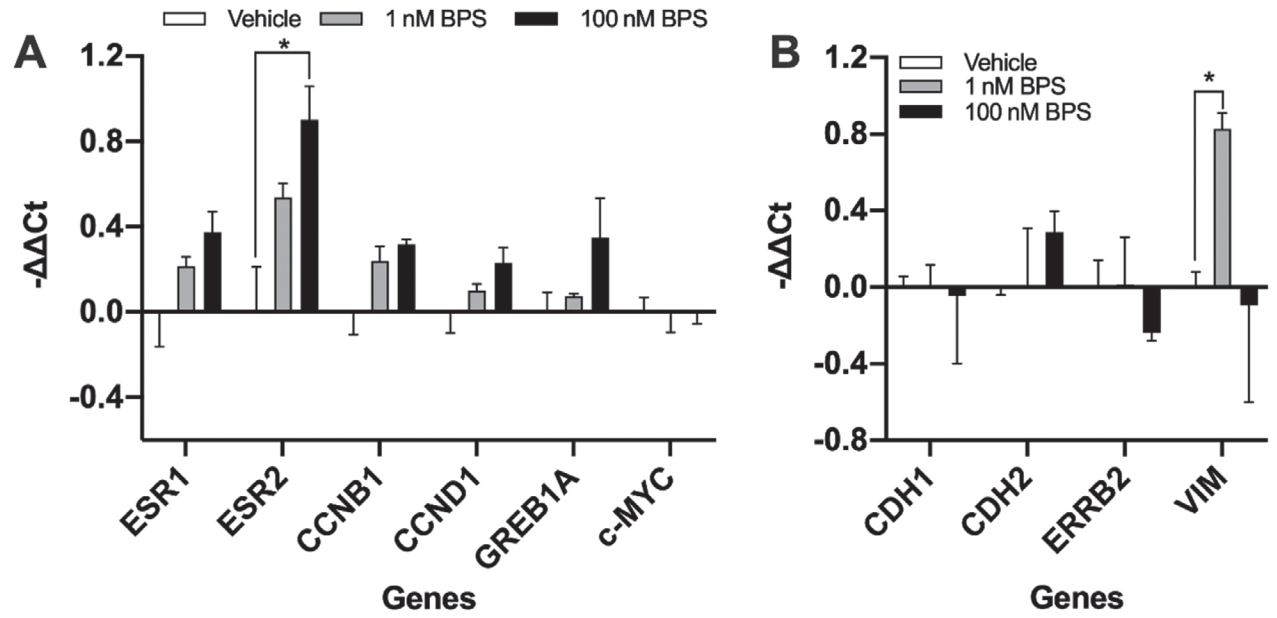

Figure 3. ***BPS significantly upregulates the expression of ESR2 and VIM genes.*** Relative $\log_2$ mRNA expression (-ΔΔCt) of **(A)** ESR and cell proliferation-related genes and **(B)** migration-associated genes in Ishikawa cells treated with BPS (1 nM, 100 nM) or vehicle (DMSO) for 72 hrs using qRT-PCR. All data were expressed as mean ± SEM (n=3 per treatment). Asterisks denote significant differences at $p<0.05$ relative to vehicle control using Kruskal–Wallis one-way ANOVA with Tukey post hoc test.

which were regularly spaced in the abundant stroma (Figure 4A). On the other hand, the uterus of mice exposed to BPS developed non-atypical hyperplasia, which is characterized by the presence of hyperproliferative, irregularly-shaped glands (Figure 4A). In addition, the mean number of glands per field of view in the BPS-treated group was 55 ± 0.42 which was significantly higher than the vehicle-treated mice which has a mean value of 22 ± 0.25 glands per field of view (Figure 4B). The incidence of non-atypical hyperplasia was also more evident in 80% of mice exposed to BPS than in the vehicle, as supported by a significant p-value of less than 0.05 using Fisher's exact test (Table 2). Hence, this provides further evidence for the proliferative effect of BPS on endometrial epithelial cells.

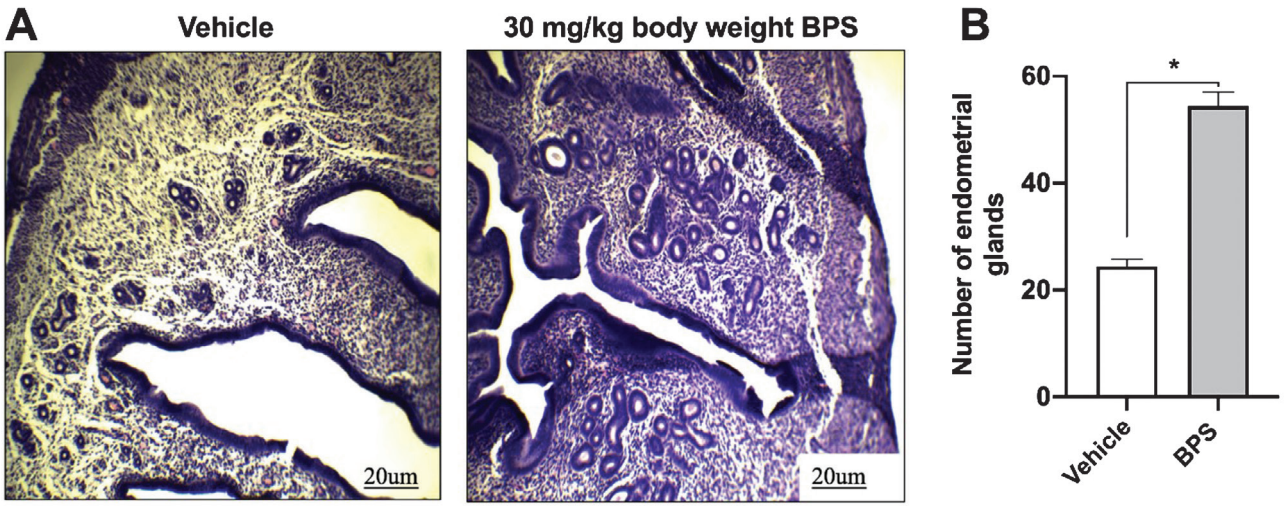

Figure 4. ***BPS increases the number of endometrial glands in the mouse uterus.*** **(A)** Representative photomicrographs of H&E-stained uteri from mice exposed orally to 30 mg/kg body weight BPS or vehicle for 21 days. **(B)** Quantification of the number of endometrial glands. Numerical data were analyzed as the mean number of glands ± SEM (n=5). Differences in letters denote significance at $p<0.05$ using Student's t-test.

Table 2. Two-sided Fisher's exact test for BPS and endometrial hyperplasia

	With endometrial hyperplasia	Without endometrial hyperplasia	Total
Vehicle	0	5	5
BPS	4	1	5
Total	4	6	10
p-value <0.05			

Table 3. Two-sided Fisher's exact test for BPS and adenomyosis-like lesion

	With Adenomyosis	Without Adenomyosis	Total
Vehicle	0	5	5
BPS	2	3	5
Total	2	8	10
p-value = 0.17			

BPS stimulated the migration of endometrial glands into the myometrium

Aside from the proliferation of endometrial glands, the migration and invasion of endometrial mucosal cells and stroma into the mouse myometrium were also apparent in two out of five mice exposed to BPS (Figure 5A), but not in any of the mice in the vehicle group, although the difference was not statistically significant (Table 3). These changes were reminiscent of adenomyosis, a gynecologic disorder wherein normal-appearing endometrial mucosa is found within the myometrium of the uterus. Adenomyosis was observed in 40% of mice exposed to BPS. Microscopic features of adenomyosis seen in mice exposed to BPS include the presence of endometrial glands and stroma within a hypertrophic smooth muscle (Figure 5A-B). The presence of histiocytes and neutrophils in the ectopic endometrial tissues was also observed (Figure 5C). In addition, uterine wall thickness of exposed and unexposed mice was measured to determine the presence of myometrial hypertrophy. Results showed a significant increase in the myometrial thickness of mice exposed to BPS relative to the vehicle group (Figure 5D). An increase in myometrial thickness was associated with adenomyosis and leiomyoma in mice exposed to BPA.[31] In humans, myometrial thickening is often brought about by myometrial hyperplasia and hypertrophy which may indicate the presence of uterine lesions such as adenomyosis.[32]

DISCUSSION

Studies on BPA have demonstrated that this chemical can increase the proliferation and migration of malignant cells, such as in colon and endometrial cancer.[3,4] However, the role of BPS on cell proliferation and migration in endometrial epithelial cells has not yet been explored. This study has revealed that BPS increased the total number of viable cells and stimulated migration in endometrial epithelial cells both *in vitro* and *in vivo*, specifically in the Ishikawa cell line.

This increase in cell number is likely associated with cell proliferation, as BPA, an analog of BPS, also induced cell proliferation in endometrial cancer cells.[20] Surprisingly, the proliferative effect of BPS on endometrial cells occurred in the absence but not in the presence of serum. The ability of serum to blunt BPS-induced proliferation is likely due to its stronger growth-stimulatory effect than BPS alone. The serum contains several growth factors, nutrients, and other elements essential for cell growth and survival.[33]

The proliferative effects of BPS were also observed in mouse uteri and were evident in the endometrial glandular epithelium—the primary site of endometrial hyperplasia. This is similar to the disordered proliferation of endometrial epithelial glands without changes in cell morphology when exposed to estradiol.[34] In BPS-treated mice, the following observations were made: the presence of higher gland-to-stroma ratios in the endometrium, irregularly-shaped endometrial glands, and highly variable density of proliferative glands. Because the dose used

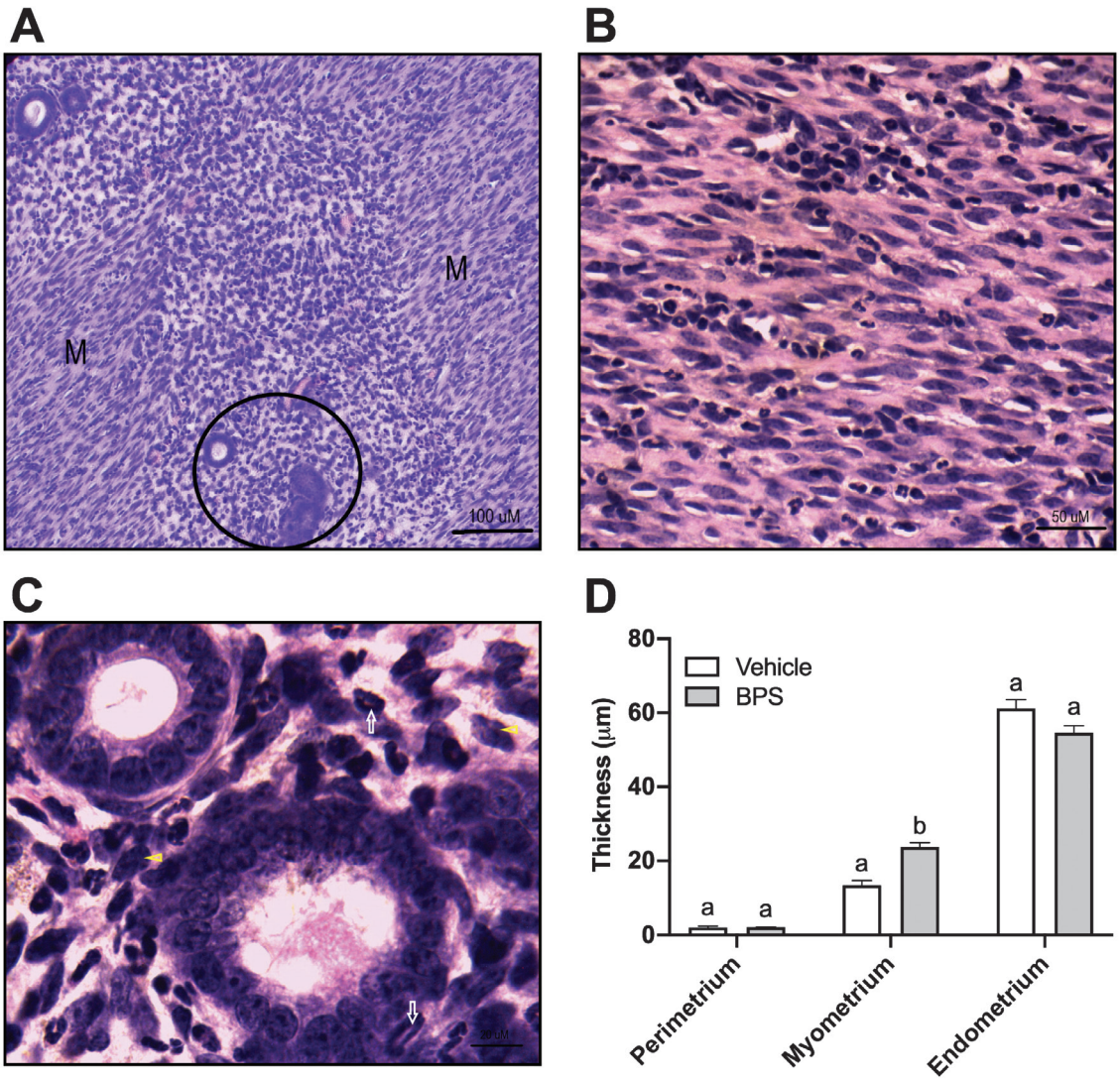

Figure 5. ***BPS promotes the migration of endometrial glands and stroma in the uterus of mice.*** **(A)** Representative photomicrographs of uteri from mice exposed orally to 30 mg/kg body weight per day BPS for 21 days showed the presence of endometrial mucosa and stroma (encircled) within the myometrium (M). **(B)** High power view of the myometrial layer showing hypertrophic smooth muscles. **(C)** High-power view of endometrial glands lined by normal-appearing epithelia within the myometrium. Histiocytes (yellow arrow) and neutrophils (white arrow) were seen in the ectopic endometrial tissues. **(D)** The thickness of uterine walls (i.e., perimetrium, myometrium, and endometrium) in mice treated with BPS or vehicle (n=5 per group). Numerical data were analyzed as the mean thickness of uterine walls ± SEM (n=5 per group). Differences in letters denote significance at $p<0.05$ using Student's t-test.

in this study is lower than the NOAEL set by ECHA for BPS[29], exposure to higher concentrations will likely lead to similar uterotrophic effects.

Previous studies showed that early neonatal exposure of rats to BPA and BPS caused long-term changes in reproductive and endocrine phenotypes, which manifested as delayed onset of the estrous cycle and dysregulated production of hormones.[12] In zebrafish, BPA also caused transgenerational aberrant expression of genes related to reproduction and gonadal differentiation up to the F2 and F3 generations.[35] In this study, we showed that even when exposure took place outside the critical window of development, BPS can still elicit immediate architectural changes in the adult mouse uterus.

Previous publications have shown that BPS has an estrogenic activity comparable to BPA.[36,37] Moreover, BPS promotes cell progression, proliferation, and migration through an ER-dependent pathway in MCF-7 breast cancer cells.[38,39] However, it remains unclear whether the ability of BPS to increase endometrial cell viability in this study is due to its estrogenic activity. Further studies are needed to determine the contribution of ER on BPS response in endometrial cells.

The expression of proliferative genes in Ishikawa cells was assessed to understand the molecular basis of the proliferative potential of BPS on endometrial epithelial cells. Although the expression of proliferative genes was slightly higher in BPS-treated cells, no significant difference was noted between the vehicle and BPS-treated

group. Surprisingly, only the expression of *ESR2* was significantly upregulated by 100 nM BPS. This is relatively new, as previous studies have shown the putative role of ESR2 as a tumor suppressor in endometrial cancer cell lines such as RL95/2 and HEC-1A cells.[40] However, elevated ESR2 has been implicated in other non-malignant proliferative diseases, such as endometriosis.[41–43] Hence, the mechanisms involved in the upregulation of ESR2 after BPS treatment are worth investigating in future studies.

Aside from proliferation, the impact of BPS on migration was also studied. *In vitro* migration of Ishikawa cells exposed to BPS followed a non-monotonous response. Compared to the 100 nM dose, 1 nM BPS significantly increased migration. Similar results were also noted in migration assays involving human non-small cell lung cancer cells (NSCLC), MCF-12A human mammary epithelial cells, and triple-negative breast cancer cells (TNBC).[3,44,45] The ability of 1 nM BPS to promote significant migration may be attributed to the activation of store-operated calcium^{2+} channels (SOCE) similar to the mechanism of action of BPA.[46,47] SOCE plays an important role in regulating cell movement at both the front and rear of migrating cells.[48] SOCE in cancers are dysregulated to promote cancer migration, invasion, and metastasis.[46] Interestingly, a recent study involving human prostate cancer (PCa) showed that only 1 nM and 10 nM BPA can modulate ion channel protein expression involved in calcium entry and cancer cell migration.[47]

Our study observed the presence of atypical endometrial mucosa within the myometrial layer of mice treated with BPS. This condition is similar to adenomyosis, which is associated with an enlarged uterus, heavy menstrual bleeding, pelvic pain and infertility in women.[49] The presence of endometrial epithelial cells and fibroblasts in the myometrium is often associated with hyperplasia of surrounding smooth muscles.[49,50] While the histology of adenomyosis is well-described in humans, the etiology of the disease remains unknown.[50] However, some studies suggest a potential relationship between environmental exposures to toxicants and the risk of adenomyosis.[50,51] Hence, it is tempting to speculate that the BPS-induced migration in endometrial epithelial cells that was observed in this study may also be observed in humans, implicating the role of BPS on the development of adenomyosis. It is important to note that the sample size used in this study was not large enough to show a statistically significant increase in the appearance of adenomyosis-like lesions after BPS versus vehicle treatment. Additional experiments are still needed to ascertain the contribution of BPS to the development of adenomyosis. Nonetheless, one study has already implicated the role of BPA in increasing the incidence of adenomyosis in mice[31], further supporting the idea that BPS-induced adenomyosis is also likely possible.

CONCLUSION

Our study demonstrated that BPS at 1 nM concentration can significantly promote cell proliferation and migration in endometrial Ishikawa cells and the phenotypic effect of BPS *in vitro* was confirmed *in vivo*. Similarly, BPS induced the proliferation of endometrial glands in the uterus and promoted the migration and invasion of endometrial mucosa in the myometrium of mice. Further studies are needed to determine the overall impact of BPS on the organism as a whole. Nonetheless, the results obtained in this study support other studies that BPS might not be an ideal substitute for BPA as it can elicit similar estrogenic effects as BPA.

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Statement of Authorship
All the authors certified fulfillment of ICMJE authorship criteria.

Authors Contribution Statement
KB, CMM, LA, and MV conceived the study. KB, CMM, MM, LA, and MV developed the methodology. KB, CMM, MM, EMR, LA, and MV validated the results and synthesized the data. KB, CMM, MM, and EMR conducted the research and investigation process and prepared the original draft of the manuscript. KB and MV provided the study materials. MV curated the data and acquired financial support for the project. LA and MV reviewed and edited the manuscript and supervised the research activity planning. CMM and MV managed the research activity planning and execution.

Author Disclosure
The authors declared no conflict of interest.

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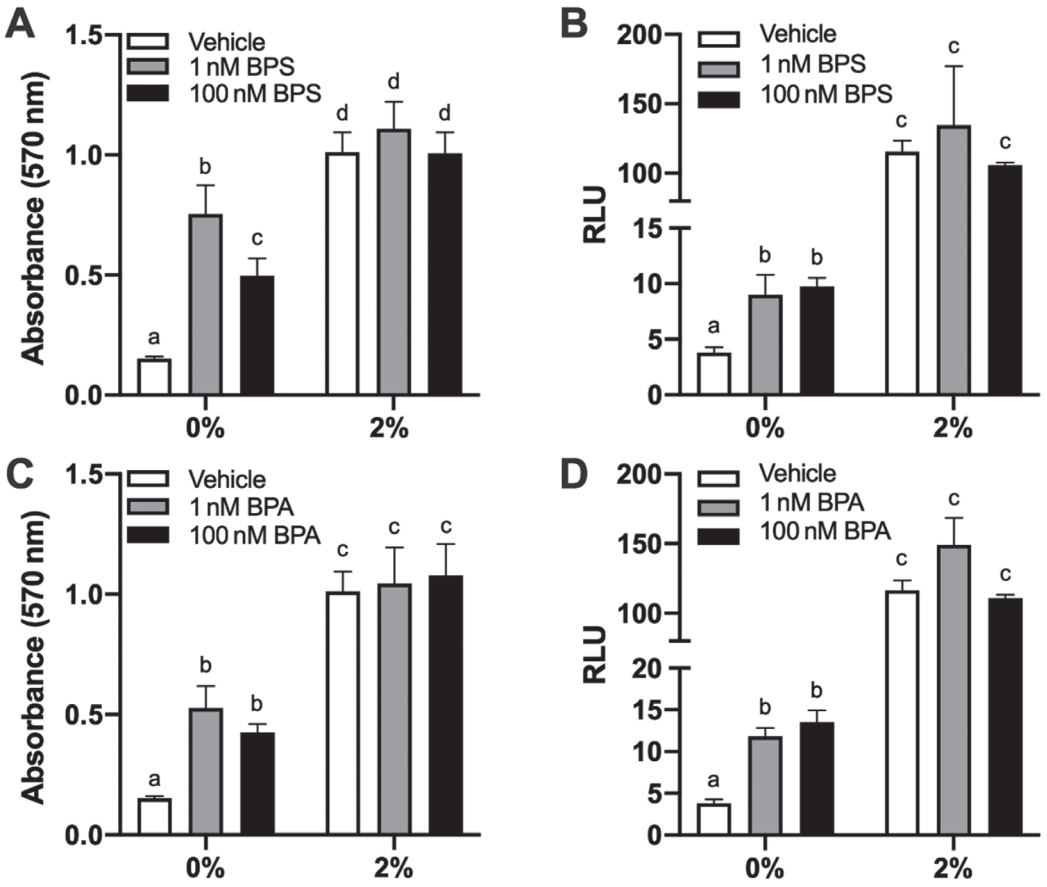
APPENDIX



Appendix 1. ***BPS increases absorbance and luminescence readings in low but not at high serum.*** Ishikawa cells were cultured in steroid-free media and then exposed to 1 nM and 100 nM **(A, B)** BPS and **(C, D)** BPA with 0% or 2% CS-FBS for 72 hrs before assessment of viability using **(A, C)** MTT and **(B, D)** CellTiter-Glo® assay. Numerical data were analyzed as mean absorbance ± SEM (n=3 and n=6 per treatment, respectively). Different letters denote significant differences at $p<0.05$ using two-way ANOVA followed by Tukey's post hoc test.

ORIGINAL ARTICLE

Journal of the ASEAN Federation of Endocrine Societies

The Effect of Glucocorticoids on *TAF1* Gene Transcription in X-linked Dystonia Parkinsonism

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Abstract

Objective. X-linked Dystonia Parkinsonism (XDP) is associated with a SINE-VNTR- Alu (SVA) retrotransposon insertion in an intron of the *TAF1* gene that alters gene transcription and splicing. In this study, we determined if the SVA insertion introduces glucocorticoid (GC)-responsive *cis*-regulatory elements that may contribute to dysregulated *TAF1* transcription and XDP disease progression.

Methodology. We performed *in silico* analysis to identify potential GC receptor (GR) binding sites within the XDP-SVA. We also conducted promoter-reporter assays on HeLa and HEK293T cells to assess the intrinsic promoter activity of three XDP-SVA variants representing different hexameric repeat lengths associated with differences in disease onset. We treated XDP fibroblast cell models with GR agonist (CORT) or antagonist (RU486), then subjected *TAF1* and the XDP-associated aberrant transcript, *TAF1-32i* to gene expression analysis.

Results. A transcription factor binding site search revealed three binding sites for GR within the XDP-SVA—two within the SINE region and one in the Alu region. Promoter-reporter assays showed induction of XDP-SVA promoter activity upon CORT treatment that was dependent on the cell line and XDP-SVA hexamer repeat length. Gene expression analysis showed that baseline *TAF1* levels differed between control and patient fibroblast cell lines, and treatment with CORT led to an increasing trend in the expression of the aberrant *TAF1-32i* transcript but did not reach statistical significance. Treatment with RU486 increased *TAF1* mRNA expression only in the control cell lines.

Conclusion. Using reporter assays, the XDP-SVA was shown to exhibit CORT-dependent transcriptional activation. Gene expression analysis also showed that GC signaling may influence *TAF1* and *TAF1-32i* expression, possibly through interaction with the XDP-SVA. Our data provide a potential link between stress and XDP progression.

Key words: XDP, glucocorticoids, stress, neurodegenerative disease, TAF1

INTRODUCTION

X-linked Dystonia Parkinsonism (XDP) is a neurodegenerative disease that primarily affects males native to the island of Panay, Philippines. It is characterized by the initial appearance of focal dystonia, followed by traits commonly associated with Parkinson's Disease, including tremors, bradykinesia, masked facies, hypomimia, and drooling.[1] At present, the prevalence rate of XDP is unknown[2] with the most recent estimate from 2010 placing it at 5.74 in 100,000 on Panay Island, and 0.31 in 100,000 in the Philippines.[3] Symptoms manifest late into adulthood, with an average age of onset at 39-40 years,[1] making early diagnosis and treatment difficult. Currently, available treatment options include oral medication, chemodenervation, neuroablative surgery, and deep brain stimulation.[1] However, these only serve to alleviate symptoms as currently there is no cure. Patient demise usually occurs due to complications in the aerodigestive tract caused by extreme motor dysfunction.[1]

As an X-linked recessive disorder, XDP patients harbor disease-specific nucleotide changes (DSCs) within the DYT3 gene locus[4] and an insertion of a SINE-VNTR-Alu (SVA)-type retrotransposon in the *TAF1* locus[5] located on the X-chromosome. The SVA insertion contains a hexameric repeat domain whose length appears to inversely correlate with the age of disease onset.[6] Furthermore, an aberrant *TAF1* transcript, known as *TAF1-32i*, was found to be exclusively transcribed in XDP patient cells.[5] The *TAF1-32i* transcript is a truncated version of the canonical *TAF1* mRNA that terminates ~700 bp before the SVA insertion point and incorporates an intronic segment from the same region. The expression level of the aberrant *TAF1-32i* transcript is inversely proportional to *TAF1* mRNA expression levels.[5] Removal of the SVA insertion via CRISPR/Cas9-mediated gene editing in patient-derived neural stem cells abolished *TAF1-32i* transcript synthesis and rescued the attenuated expression of *TAF1* mRNA,[5] suggesting that the insertion of the SVA retrotransposon may lead to the synthesis

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of aberrant transcripts and a decrease in expression of canonical *TAF1* that may consequently translate to a reduction of TAF1 protein synthesis. In promoter-luciferase assays, the XDP-SVA has been shown to exhibit intrinsic promoter activity[6] as well as *cis*-regulatory activity via *TAF1* promoter repression.[7] It has been proposed that the insertion of the SVA retrotransposon provides new binding sites within the locus, which may lead to the transcriptional interference of *TAF1* or the binding of trans-acting elements that contribute to disease progression.[6]

Several studies have also looked into the psychosocial aspect of XDP. Measurements of Quality of Life (QoL) through in-depth interviews indicate a pattern of QoL impairment which manifests not only physically but also socially and emotionally.[8] Furthermore, case studies have found that depressive symptoms are prevalent among XDP patients, the severity of which are comparable to those seen in patients with Parkinson's disease (PD), Alzheimer's disease (AD), and spinocerebellar degeneration.[9] As of 2015, the suicide rate among the population of XDP patients is 10.8% which is higher than the 2015 Philippine estimate of 3.59 in 100,000 cases.[10] As such, it is also of interest to understand how and why psychosocial factors may influence XDP progression and pathophysiology. One way in which these may be integrated is through the Hypothalamic-Pituitary-Adrenal (HPA) axis which mediates the stress response. The HPA integrates environmental and social stimuli and the neuroendocrine system. The presence of stressors activates the HPA axis that leads to the secretion of glucocorticoids (GC) which then binds to the GC receptor (GR), a member of the steroid receptor superfamily.[11] In the presence of a bound ligand, GR forms dimers that translocate to the nucleus and bind to GC response elements (GREs) of target genes to regulate GC-dependent transcription.[12] Activation of these target genes leads to the reorganization and modulation of neural circuits, which translate to the phenotypes displayed by stressed individuals.[13] One such target is Krüppel-like Factor 9 (KLF9), a transcription factor that is highly expressed in the brain and is involved in various neuronal development processes.[14]

Stress and dysregulation of the HPA axis have been shown to contribute to the physiology of neurodegenerative diseases. Patients afflicted with PD exhibit increased GC levels indicative of dysregulated GR function, leading to chronic inflammation characteristic of the disease.[15] In AD, stress has been shown to contribute to disease progression through the induction of Tau-hyperphosphorylation. Stress-associated GC secretion leads to aggregation of misfolded and hyperphosphorylated variants of the Tau protein which then causes cytoskeletal instability and neuronal damage, ultimately resulting in neurodegeneration.[16] However, less is known about the role of stress in XDP.

In this study, we determined whether a relationship between the stress response and XDP-associated dysregulated *TAF1* gene transcription exists. We hypothesized that the SVA insertion provides a binding site for GR and that GR binding to the disease-associated XDP-SVA contributes to the dysregulation of *TAF1* transcription through a decrease in expression of the canonical transcript and an increase in expression of aberrant transcripts such as *TAF1-32i*. Towards this end, we tested XDP-SVA promoter activity upon acute hydrocortisone (CORT) treatment via a luciferase reporter assay. We also employed cellular models of XDP for use in gene expression analysis upon GR agonist and antagonist treatment to identify the XDP-specific role of GR on *TAF1* and *TAF1-32i* transcription.

METHODOLOGY

In Silico Analysis

We subjected the full SVA sequence to a transcription factor binding site (TFBS) search using LASAGNA-Search tool 2.0.[17] Search parameters were adjusted to identify binding sites for NR3C1 or GR, by selecting the following TFs: vertebrates, JASPAR ID MA0113.1; Homo sapiens, TRANSFAC Accession Number T00337 and T01920; vertebrates, TRANSFAC Accession Number M00205. For the TF Model Input, the option "Use TRANSFAC TFBSs" under "LASAGNA-Aligned Models" was selected. We filtered results to include only those with associated p-values <0.001. We subsequently mapped putative binding sites onto the SVA sequence to identify the location of putative GREs.

Cell Culture

HeLa cells were cultured in Modified Eagle Medium (MEM) with 10% fetal bovine serum (FBS), 1X Penicillin-Streptomycin-Glutamine, and 2.5% Amphotericin B. HEK293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% FBS, 1X Penicillin-Streptomycin-Glutamine, and 2.5% Amphotericin B. For reporter assays, HeLa and HEK293T cells were seeded in a 96-well plate at a density of 1.0×10^4 cells/well. Upon cell attachment, media was replaced with MEM (HeLa cells) or DMEM (HEK293T) supplemented with 10% charcoal-stripped serum (CSS) to remove possible effects of steroid hormones from the media.

Immortalized fibroblast cell lines from XDP patients and ethnically matched controls with Filipino ancestry were obtained from the laboratory of Dr. Christopher Bragg of the Massachusetts General Hospital (USA). The cell lines were generated in a previous study with participants that were evaluated at Massachusetts General Hospital, or in regional clinics in Panay Island affiliated with Jose Reyes Memorial Medical Center (Manila, Philippines). Sample tissue collection and cell line derivation were done with the approval of the institutional review board at both institutions and with the informed consent of all study participants.[5] Cell lines were validated through clinical evaluation of the source patients, as well as genetic testing for the seven known haplotype markers of XDP, including the SVA insertion.[5] The patient fibroblasts used in this

study, with the identifier 35833, were sourced from an XDP patient who had an age of onset of 36 years. The XDP-SVA hexameric domain for these cells was found to have a repeat length of 50. The ethnically matched control fibroblasts had the identifier 36176.[5] Primary fibroblasts were cultured in DMEM supplemented with 20% FBS, 1X Penicillin-Streptomycin-Glutamine, and 2.5% Amphotericin B. For gene expression analysis, control and XDP fibroblasts were seeded in 6-well plates at a density of 2.0 x 10^6 cells/well three days before hormone treatment. Upon cell attachment, the media was replaced with DMEM with 20% CSS for treatment with hydrocortisone (CORT; Sigma H0888) or 20% FBS for treatment with the GR antagonist RU486 (Sigma M8046). All cell lines were incubated in 5% CO_2 at 37°C.

Reporter Gene Assays

Three XDP-SVA construct variants, bearing 25 (Hex25), 41 (Hex41), or 52 (Hex52) repeats of the hexamer were obtained from the laboratory of Dr. Christopher Bragg. The inserts were subcloned from pGL3-B vectors[6] into the pGL4.23 luciferase-reporter vector (Promega), with the XDP-SVA upstream of the luc2 reporter in the anti-sense direction. Briefly, the pGL3-B constructs were digested with *SacI* and *HindIII* enzymes and were then subjected to agarose gel electrophoresis. Bands corresponding to the XDP-SVA (~3kb) were extracted using the Wizard SV Gel and PCR Clean-up System (Promega) and ligated into linearized pGL4.23 vectors.

HeLa and HEK293T cells were co-transfected with the pGL4.23 vector constructs and the pRL-TK vector (Promega) which codes for *Renilla* luciferase under a constitutive promoter. Transfection was performed using X-tremeGENE HP DNA Transfection Reagent (Roche). Transfection with an empty vector and a vector containing a CORT-responsive enhancer sequence for *Cyb561* (upstream *Cyb561* enhancer, uCE) served as negative and positive controls, respectively. At least 16 hours post-transfection, cells were treated with either 100nM CORT or an equivalent volume of ethanol (vehicle control) for 4 hours, after which the cells were harvested. Luminescence measurements were performed using the Dual Luciferase Assay System (Promega), through the GloMax®-Multi Jr detection system (Promega) for HeLa cells, and Fluoroskan FL microplate luminometer (Thermo Fisher) for HEK293T cells. Relative light unit (RLU) measurements were obtained via normalization of luc2 luminescence against that of *Renilla* luciferase. Assays were done with four replicates per treatment per construct.

TAF1 and *TAF1-32i* Gene Expression analysis

Control and XDP fibroblasts (at least 3 replicates per treatment) were treated with 100nM CORT, 50nM RU486, or an equivalent volume of vehicle control for 24 hours before harvesting for RNA extraction using TRIzol Reagent (Invitrogen) following the manufacturer's protocol.

Table 1. Primer sequences used for RT-qPCR experiments

Target		Sequence
GAPDH	Forward	5'-GGCATGGACTGTGGTCATGAG-3
	Reverse	5'-TGCACCACCAACTGCTTAGC-3'
NR3C1	Forward	5'-TATCCTCTGCCTCCCATTCT-3'
	Reverse	5'-CACCTTCCTGTCTCCTGTTTAC-3'
KLF9	Forward	5'-TGGCTGTGGGAAAGTCTATG-3'
	Reverse	5'-GTCTGAGCGGGAGAACTTTT-3'
TAF1	Forward	5'-GGGAGAGCTTTCTGGATGATGTAAA-3'
	Reverse	5'-ACAATCTCCTGGGCAGTCTTAGTAT-3'
TAF1-32i		Custom Taqman ID AJWR28J (Thermo Fisher)

The RNA extracts were treated with DNAse I (Sigma, AMPD1) and RNAse inhibitor (Applied Biosystems), then converted to cDNA using the ABI High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, 4374967). Quantitative PCR master mixes were prepared using PowerUP SYBR Green (Applied Biosystems, A25742) for *GAPDH*, *KLF9*, *NR3C1* (GR), and *TAF1* or a custom Taqman primer-probe (Thermo Fisher, ID AJWR28J) for *TAF1-32i* (Table 1). Quantitation of *KLF9* transcript levels was used as a positive control as its transcription has been previously shown to be induced by CORT.[14] Quantitative PCR was performed using the ABI 7500 Fast sequence detector (Applied Biosystems). Relative gene expression levels across treatments were obtained using the ΔΔCt method, using *GAPDH* Ct values for normalization.

Statistical Analysis

For luciferase assays, data (in relative light units) were log-transformed and analyzed by unpaired sample *t*-test and Tukey's post-hoc test ($p<0.05$; GraphPad Prism 6). For gene expression analysis, data were analyzed using Student's *t*-test and Tukey's post-hoc test ($p < 0.05$; GraphPad Prism 6).

RESULTS

Identification of GREs within the SVA

LASAGNA-Search 2.0 was used to identify GREs within the XDP-SVA. The tool uses an alignment-based approach to match query sequences to the consensus sequence of a given TFBS. From the search, ten hits were obtained (Table 2). Removing redundant results, three unique hits were obtained. Two GREs mapped to the SINE, 271 and 400bp from the XDP-SVA insertion point, and one GRE mapped to the Alu element, 2,199 base pairs from the insertion point (Figure 1).

CORT-dependent activity of XDP-SVA luciferase reporter constructs

We performed dual luciferase assays on HeLa cells and HEK293T cells following transfection with reporter constructs of varying hexameric repeat lengths within the XDP-SVA-sequence. The XDP-SVA is inserted upstream and anti-sense to the reporter gene in the same way the SVA is inserted into the *TAF1* gene in XDP patients. In

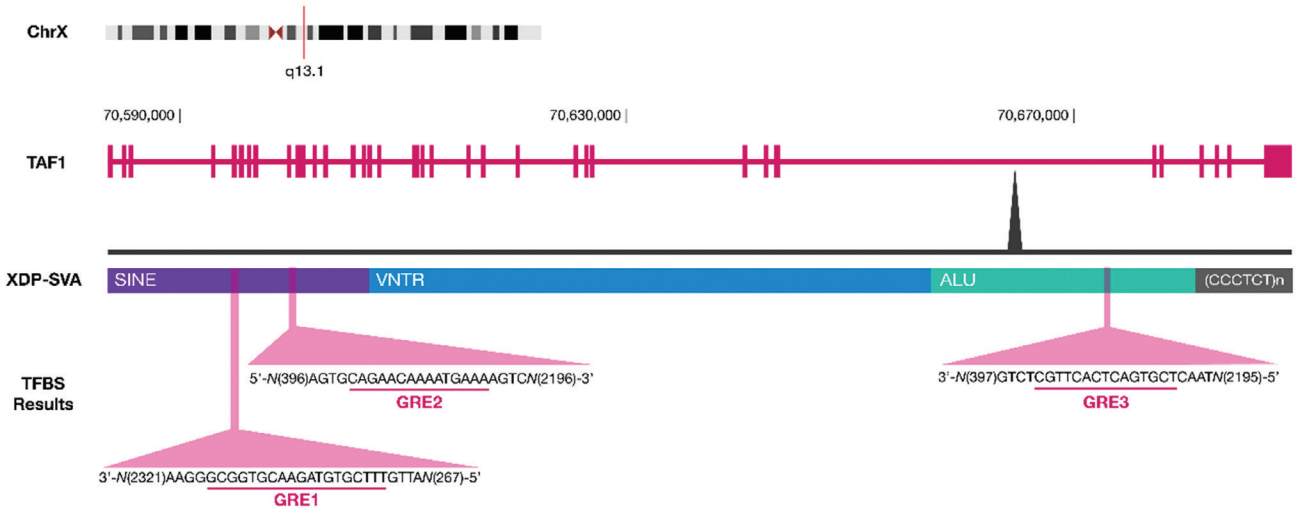

Figure 1. Identification of putative GREs within the XDP-SVA. The map shows the location of the SVA insertion within the *TAF1* locus (Xq13.1). LASAGNA-Search 2.0 identified three GREs: two within the SINE, and one within the Alu region. GRE1 is found in the negative strand and is located 271 bp from the XDP-SVA insertion point. GRE2 is found in the positive strand and is located 401 bp from the insertion point. GRE3 is found in the negative strand and is located 2199 bp from the insertion point.

Table 2. LASAGNA-search 2.0 results show potential GREs within XDP-SVA sequence

Name	Sequence	Pos	Strand	Score	*p*-value	E-value
GR-alpha (T00337)	cagaacaaaatgaaa	400	+	138.5	0.000375	0.97
GR-alpha (T00337)	cggtgcaagatgtgct	273	-	136.68	0.000425	1.10
GR-alpha (T00337)	agaacaaaatgaaaa	401	+	127.1	0.0009	2.34
GR-beta (T01920)	cagaacaaaatgaaa	400	+	138.5	0.0008	2.08
GR-beta (T01920)	cggtgcaagatgtgct	273	-	136.68	0.000825	2.15
GR (M00205)	ggtgcaagatgtgct	273	-	16.38	5.0E-5	0.130
GR (M00205)	ggtgcaagatgtgctt	272	-	12.97	0.0004	1.04
GR (M00205)	cgttcactcagtgct	2199	-	12.84	0.000425	1.10
GR (M00205)	ggtgcaagatgtgc	274	-	12.15	0.0005	1.30
GR (M00192)	gcggtgcaagatgtgcttt	271	-	8.66	0.000725	1.88

Each entry corresponds to a putative GRE. Information on the binding site sequence, position (in bp) within the SVA, and strand are given for each entry, as well as its corresponding score, p-value, and e-value as determined by the alignment algorithm.

HeLa cells, CORT treatment increased luciferase activity in the positive control ($t(6) = 3.531$, $p = 0.012$) and in XDP-SVA constructs Hex25 ($t(6) = 2.601$, $p = 0.040$) and Hex41 ($t(6) = 3.295$, $p = 0.016$) (Figure 2A). In HEK293T cells, CORT hormone treatment increased the normalized luciferase activity of cells transfected with Hex25 ($t(5) = 3.919$, $p = 0.011$) and Hex52 ($t(6) = 3.079$, $p = 0.022$) reporter constructs while cells transfected with the Hex41 construct only showed an increasing pattern of CORT-induced reporter activity that did not reach statistical significance ($t(6) = 2.241$, $p = 0.066$) (Figure 2B).

Analysis of aberrant and canonical *TAF1* transcript expression in XDP fibroblasts

XDP fibroblasts and ethnically matched controls were used to determine the effect of hormone treatment on the expression of *TAF1* and *TAF1-32i* transcripts. Before hormone treatment, we quantified *NR3C1* (GR) mRNA expression in these cell lines and found that *NR3C1* mRNA levels did not differ between control and XDP fibroblasts (Figure 3). Gene expression fold change values for three target genes, *KLF9* (positive control), *TAF1*, and *TAF1-32i* were measured in CORT-treated control and XDP fibroblasts (Figure 4). The established CORT-responsive gene *KLF9* exhibited CORT-dependent induction in XDP fibroblasts but not in control-derived cells ($t(5) = 8.315$, $p = 0.0004$) (Figure 4A). The expression level of *TAF1* mRNA was not significantly affected by CORT treatment in both control and XDP fibroblasts. However, baseline *TAF1* mRNA level is significantly reduced in XDP relative to control fibroblasts ($t(5) = 4.409$, $p = 0.007$) (Figure 4B). As expected, we did not detect *TAF1-32i* transcript expression in both vehicle- and CORT-treated control fibroblasts (data not shown). CORT treatment of XDP fibroblasts led to an increasing trend in the expression of the aberrant *TAF1-32i* transcript but did not reach statistical significance ($t(5) = 2.545$, $p = 0.0516$) (Figure 4C).

Expression of *TAF1* and *TAF1-32i* transcripts in control and XDP fibroblasts was also observed after treatment with the GR-specific antagonist RU486. Treatment with RU486 did not affect *KLF9* mRNA expression in the control and XDP fibroblasts (Figure 5A). We observed an increase in *TAF1* mRNA expression with RU486 treatment of control cells ($t(5) = 3.597$, $p = 0.007$) (Figure 5B) while *TAF1* and aberrant *TAF1-32i* transcript expression were not affected by RU486 treatment in XDP fibroblasts (Figure 5B and 5C).

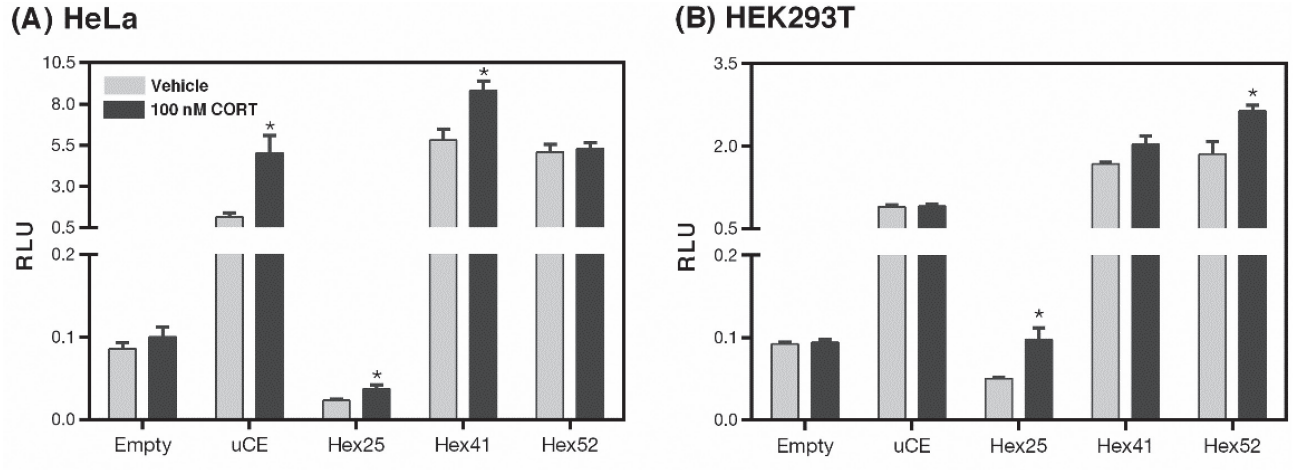

Figure 2. Luciferase reporter assay to assess CORT-dependent transactivation of XDP-SVA promoter-luciferase constructs. Luciferase reporter constructs containing three variants of the XDP-SVA representing different hexameric domain repeat lengths were transfected into **(A)** HeLa and **(B)** HEK293T cells. Induction of luciferase activity (measured in Relative Luminescence Units, RLU) following CORT treatment was observed in HeLa cells transfected with the uCE (positive control), Hex25, and Hex41 constructs, while only HEK293T cells transfected with the Hex25 and Hex52 constructs showed significant CORT-dependent induction of promoter activity. Bars represent the mean of each data set ± standard error of the mean with statistical significance determined through Student's t-test (*$p < 0.05$). Each treatment was done with four replicates each.

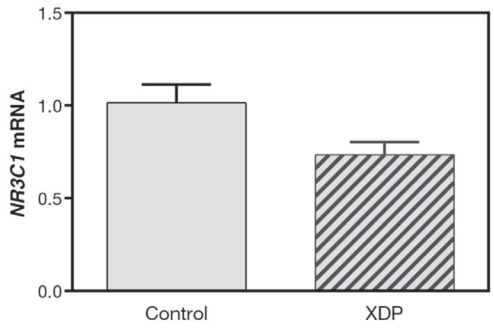

Figure 3. Baseline expression of *NR3C1* in XDP fibroblasts. Untreated control and XDP fibroblasts were harvested for analysis of *NR3C1* (GR) mRNA. The expression level of *NR3C1*, normalized to *GAPDH* mRNA, did not significantly differ across control and XDP fibroblasts (p = 0.0718, Student's *t*-test). Bars represent the fold induction ± standard error of the mean relative to control fibroblasts. Three to four replicates were used for each setup.

DISCUSSION

In this study, we aimed to establish the possible effects that the stress hormone CORT, as mediated by GR, may have on XDP. We hypothesized that this could occur via the interaction of GR with the XDP-SVA, a retrotransposon insertion previously identified to be a causal mutation of the disease. Through *in silico* analysis, three candidate GREs were identified. Indirect evidence for GR association with the SVA sequence was provided by reporter assays, which revealed CORT-responsiveness of the XDP-SVA in human cell lines. Gene expression analysis showed that *NR3C1* (GR) mRNA levels in XDP and control fibroblasts were not significantly different. Control fibroblasts treated with CORT showed a decreasing but non-significant trend in *TAF1* mRNA expression. Conversely, treatment of the control fibroblasts with the GR antagonist RU486 increased *TAF1* expression. In XDP fibroblasts, CORT treatment did not alter *TAF1* mRNA expression and led to an increasing but non-significant trend of *TAF1-32i* expression. However, treatment of the XDP fibroblasts with RU486 did not affect *TAF1-32i* transcript levels. Overall, the results suggest that GR signaling, through association with the XDP-SVA, could enhance the dysregulation of *TAF1* leading to the production of *TAF1*-32i. This in turn contributes to the disease phenotype.

GR is predicted to bind to the XDP-SVA

A representative XDP-SVA sequence was used as an input for LASAGNA-Search 2.0 to identify possible GR binding sites within the SVA sequence. For the search, all factors named 'GR' were selected. Results returned ten possible binding sites of which only three sites were non-redundant. Mapping the putative GREs to the XDP-SVA sequence revealed two unique sites in the SINE region and one in the Alu region. Sequences similar to the SINE and Alu have been found to bind other nuclear hormone receptors such as estrogen and progesterone receptors. Of these, only the Alu sequences have been tested *in vitro* for nuclear receptor binding.[18] While this type of analysis provided a starting point in establishing the association of GRs to the SVA, the results are not definitive in terms of direct GR binding to the SVA. This must be tested experimentally through electrophoretic mobility shift assays, mutational analysis of GR binding sequence, or GR chromatin immunoprecipitation assays. Nevertheless, this *in silico* analysis provides a possible association between the stress-dependent gene regulatory consequences of the

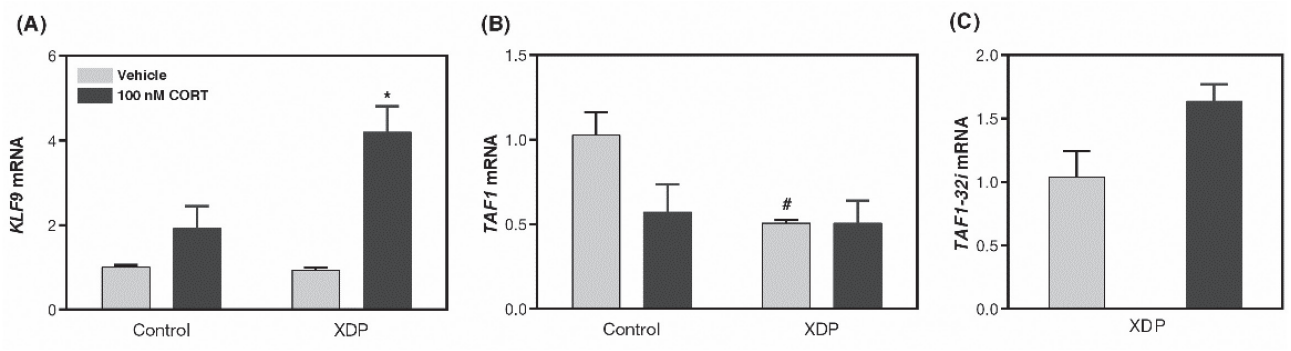

Figure 4. Analysis of *TAF1* and *TAF1-32i* expression in CORT-treated control and XDP fibroblasts. Control and XDP fibroblasts were treated with 100nM CORT or an equivalent volume of 100% ethanol (vehicle control) for 24 hours before harvesting for analysis of mRNA. Fold induction values were obtained using the ΔΔCt method with *GAPDH* as a housekeeping control. **(A)** *KLF9* mRNA levels, used as a positive control for CORT treatment, increased significantly in XDP fibroblasts ($p = 0.0004$, Student's *t*-test), but not in control fibroblasts ($p = 0.1224$). **(B)** *TAF1* transcript levels differ across vehicle-treated control and XDP fibroblasts ($p = 0.007$, Student's *t*-test). CORT treatment did not significantly change *TAF1* expression levels in either control or XDP fibroblasts (Control, $p = 0.0833$; XDP, $p = 0.7448$, Student's *t*-test). **(C)** The apparent increase in *TAF1-32i* expression levels upon CORT treatment of XDP fibroblasts approaches significance ($p = 0.0516$, Student's *t*-test). Bars represent the fold induction ± standard error of the mean relative to vehicle control with statistical significance determined through Student's t-test ($^{*}p < 0.001$ for statistically significant effects of CORT within a cell line, and $^{\#}p < 0.01$ for statistically significant effects between the same hormone treatment). All treatments were done with three to four replicates.

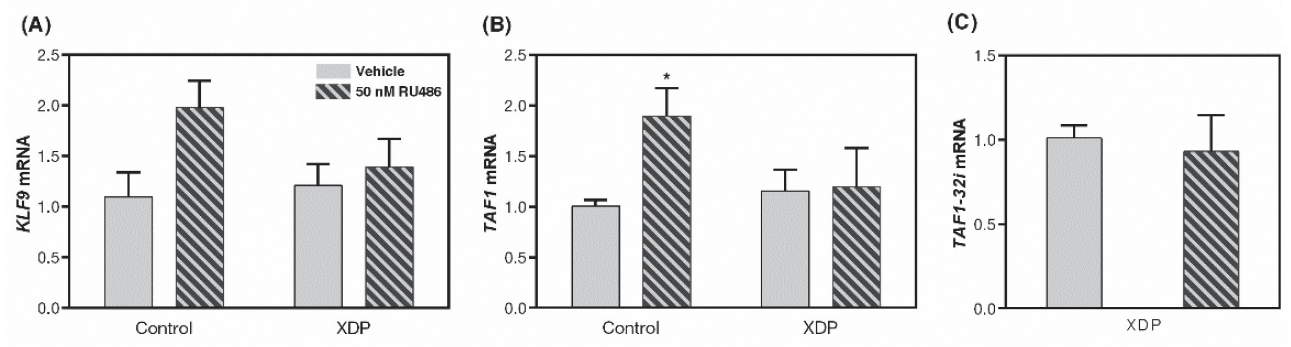

Figure 5. Analysis of *TAF1* and *TAF1-32i* expression in control and XDP fibroblast cells treated with GR-specific antagonist RU486. Control and XDP fibroblasts were treated with 50nM RU486 or an equivalent volume of vehicle control for 24 hours before harvesting for analysis of mRNA. Fold induction values were obtained using the ΔΔCt method with *GAPDH* as a housekeeping control. **(A)** No significant change in *KLF9* mRNA levels is observed upon RU486 treatment. **(B)** *TAF1* expression levels in control fibroblasts increase with RU486 treatment ($p = 0.007$). No effect of RU486 treatment is seen in XDP fibroblasts. **(C)** RU486 treatment did not alter *TAF1-32i* expression levels in XDP fibroblasts. Bars represent the mean fold induction ± standard error of the mean relative to vehicle control with statistical significance determined through Student's *t*-test ($^{*}p < 0.01$). All treatments were done with four to five replicates.

SVA insertion and the production of aberrant transcription products from the *TAF1* locus.

The XDP-SVA confers GC responsiveness in human cell lines

We performed luciferase reporter assays *in vitro* to determine the GC response in the context of the XDP-SVA. The XDP-SVA reporter constructs have proven intrinsic promoter activity.[6] We wanted to test if the XDP-SVA would be responsive to CORT treatment. We made use of different variants of the XDP-SVA to test for possible effects of hexameric repeat length on reporter activity induction. In HeLa and HEK293T cell lines used in the promoter-reporter assays, baseline promoter activity represented by the vehicle treatment showed that only constructs Hex41 and Hex52 have increased reporter activity relative to the empty vector control, consistent with previously published studies that used the same reporter constructs in human neuroblastoma (SH-SY5Y) and human osteosarcoma epithelial (U2OS) cells.[6] Similar to the results we obtained for Hex25, an XDP-SVA promoter-luciferase construct with 35 hexameric repeat length (Hex35) did not exhibit promoter activity in SH-SY5Y cells.[6] However, Hex35 exhibited promoter activity in U2OS cells, higher than what was observed for Hex41 and Hex52. They also tested promoter activity of the XDP-SVA with no hexameric repeats (ΔHex) and observed luciferase activity equal to the empty vector in SH-SY5Y cells, but higher than the empty vector in U2OS cells.[6] These results suggest that SVA insertion confers intrinsic promoters outside of the hexameric repeat domain. Moreover, the previously demonstrated lack of a consistent trend between hexameric repeat lengths and promoter activity across cell lines was

also reflected in our findings, indicating that the XDP-SVA confers promoter activity in a cell-specific manner.

Comparing reporter assay results across treatments, it appears that CORT treatment enhances the intrinsic promoter activity of XDP-SVA. This suggests that GR may associate with the SVA to mediate CORT-dependent transcriptional regulation. However, the CORT-dependent induction was only observed in two of three XDP-SVA promoter-reporter constructs used. Whether GR directly binds the identified GREs within the SVA or indirectly associates through the recruitment of TFs remains to be seen.

GCs can alter *TAF1* and *TAF1-32i* expression in an XDP cell model

Following confirmation of expression of endogenous GR in XDP and control fibroblasts, we conducted expression analysis to determine the effects of CORT and the GR-specific antagonist RU486 on *TAF1* and *TAF1-32i* transcript levels. We observed lower expression of *TAF1* mRNA in vehicle-treated XDP fibroblasts relative to control cells maintained in steroid-stripped media. This is in agreement with previous studies that found reduced expression of the functional *TAF1* transcript in XDP cell lines compared to corresponding control cells.[5] However, the reduced expression of *TAF1* mRNA in XDP versus control fibroblasts was not observed when the cells were maintained in full serum as in the RU486-treated cells, providing further evidence that steroid hormones may be involved in *TAF1* transcriptional regulation.

CORT treatment of control fibroblasts led to a decreasing but non-significant trend in *TAF1* mRNA level ($t(6) = 2.075$, $p = 0.0833$). Conversely, RU486 treatment significantly upregulated *TAF1* expression in control fibroblasts ($t(5) = 3.597$, $p = 0.007$). In addition, we also observed an increasing trend in the expression of the aberrant *TAF1-32i* transcript in CORT-treated XDP-fibroblasts; however, this increase did not reach statistical significance ($t(5) = 2.545$, $p = 0.0516$). These data suggest that in the context of the wild-type *TAF1* gene, GCs can negatively regulate the expression of the canonical *TAF1* transcript through transcriptional repressor activity of GR. In the presence of the SVA as in XDP cells, the CORT-dependent negative regulation of *TAF1* expression is lost and the effect of CORT shifts towards positive regulation of the aberrant *TAF1-32i* transcript expression, possibly through GR association with the SVA as established by the CORT-dependent transactivation assay. The SVA sequence may sequester GR from the *TAF1* *cis*-regulatory elements where it would normally bind. Concurrently, GR binding to the SVA could lead to the recruitment of trans-acting elements, resulting in faulty transcription and transcript processing, hence producing aberrant transcripts. Further experiments involving GR chromatin immunoprecipitation assays in control and XDP cells are needed to confirm this mechanism of GR-dependent regulation.

Although these results establish a possible link between stress as mediated by GR and *TAF1* dysregulation, we performed expression analysis only on one clonal population of XDP and control fibroblasts. *TAF1* and *TAF1-32i* expression in cell lines from other probands and unaffected controls should be interrogated to definitively ascribe the transcriptional effects to the SVA insertion, and not to other genetic variations. Alternatively, neural stem cells (NSCs) which may more accurately recapitulate XDP neuropathology, may be employed as *in vitro* models. Prolonged or iterative GC treatment may also be employed to better observe the effect of chronic stress on *TAF1* transcriptional regulation. Lastly, further studies should also consider how the decrease in *TAF1* expression and the emergence of aberrant transcripts as observed in XDP cell lines lead to disease neuropathology (i.e. progressive loss of neurons in the striatum) and clinical manifestations.

CONCLUSION

Overall, our results suggest that acute stress, through CORT-dependent GR transactivation of the XDP-SVA, could enhance the dysregulation of *TAF1* expression in XDP patients and enhance the production of aberrant transcripts such as *TAF1-32i*, which in turn may contribute to the disease phenotype. Our study provides a new facet for the management of XDP cases, taking into account psychological and social factors in the form of stress. It may also provide a basis for new treatment avenues, wherein GC levels may be manipulated to reduce the production of aberrant transcripts which may consequently delay disease onset.

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Statement of Authorship
Both authors certified fulfillment of ICMJE authorship criteria.

Authors Contribution Statement
SEDC and PB developed the methodology; verified results; synthesized the study data; reviewed and edited the manuscript; and prepared the data presentation. SEDC implemented computer code and supporting algorithms; conducted the research and investigation process; curated data; and prepared the original draft of the manuscript. PB formulated the research goals and aims; provided the study materials; supervised and managed the research activity planning and execution; and acquired financial support for the project.

Author Disclosure
Both authors declared no conflict of interest.

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ORIGINAL ARTICLE

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Genetic Variants Associated with Poor Responsiveness to Sulfonylureas in Filipinos with Type 2 Diabetes Mellitus

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Abstract

Introduction. Sulfonylureas (SUs) are commonly used drugs for type 2 diabetes mellitus (T2DM) in the Philippines. This study aimed to associate genetic variants with poor response to gliclazide and glimepiride among Filipinos.

Methodology. Two independent, dichotomous longitudinal substudies enrolled 139 and 113 participants in the gliclazide and glimepiride substudies, respectively. DNA from blood samples underwent customized genotyping for candidate genes using microarray. Allelic and genotypic features and clinical associations were determined using exact statistical methods.

Results. Three months after sulfonylurea monotherapy, 18 (13%) were found to be poorly responsive to gliclazide, while 7 (6%) had poor response to glimepiride. Seven genetic variants were nominally associated ($p<0.05$) with poor gliclazide response, while three variants were nominally associated with poor glimepiride response. For gliclazide response, 3 carboxypeptidase-associated variants (rs319952 and rs393994 of *AGBL4* and rs2229437 of *PRCP*) had the highest genotypic association; other variants include rs9806699, rs7119, rs6465084 and rs1234315. For glimepiride response, 2 variants were nominally associated: *CLCN6-NPPA-MTHFR* gene cluster – rs5063 and rs17367504 – and rs2299267 from the *PON2* loci.

Conclusion. Genetic variants were found to have a nominal association with sulfonylurea response among Filipinos. These findings can guide for future study directions on pharmacotherapeutic applications for sulfonylurea treatment in this population.

Key words: genetic variants, sulfonylureas, resistance, Filipino, gliclazide, glimepiride

INTRODUCTION

Despite the availability of new drugs, sulfonylureas (SU) remain one of the most prescribed drugs in the treatment of type 2 diabetes mellitus (T2DM).[1] Because of its relatively low price and availability, it is popular in low-resource countries like the Philippines, where local health centers and diabetes clubs distribute SUs through the Department of Health's Philippine Package of Essential NCD Intervention (Phil PEN) program.

However, despite patient compliance, SUs may fail to regulate high sugar levels among T2DM patients. In one study, there was a 21.3% monotherapy failure in Korean patients taking SUs.[2] Meanwhile among Filipino patients, a study showed that only 15% of those taking oral hypoglycemic agents (OHAs) achieved the American Diabetes Association (ADA) HbA1c target of <7.0%,[3] suggesting that Filipinos may have some degree of resistance towards OHAs. While this survey did not exclusively focus on SUs, given the mentioned availability

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of these drugs to Filipinos, it can be inferred that some variability may also be due to SU. Moreover, in a study that followed progressive up-titration using gliclazide modified release (gliclazide MR), 35% of the participants did not reach the desired HbA1c level.[4] On the other hand, glimepiride is usually found to be less effective than other OHAs or administered in combination with other drugs to reach the ADA standard of glucose level.[5-7]

Genetics can influence an individual's responsiveness to sulfonylureas. Variants of genes such as *TCF7L2* (transcription factor 7 like 2 gene), *ABCC8* (ATP binding cassette subfamily C member 8 gene), encoding the sulfonylurea receptor 1, *KCNJ11* (potassium inwardly rectifying channel subfamily J member 11 gene), *CYP2C9* (cytochrome P450 family 2 subfamily C member 9 gene), and *CYP2C19* (cytochrome P450 family 2 subfamily C member 19 gene) have been previously linked with gliclazide and glimepiride response.[8-11]

Nonetheless, interethnic differences may infer genetic variation in the trait of interest. For instance, *TCF7L2*, a well-known gene associated with therapeutic response to SUs exhibited varying risk alleles among German, Chinese, and Indian populations.[12,13] In another case, Japanese patients with a mutant-type allele of the *CYP2C9* gene showed a better response to glimepiride compared with the wild-type allele.[14] On the other hand, in Chinese T2DM patients, *CYP2C19* genetic polymorphisms are the more likely determinants of gliclazide response instead of *CYP2C9*.[15]

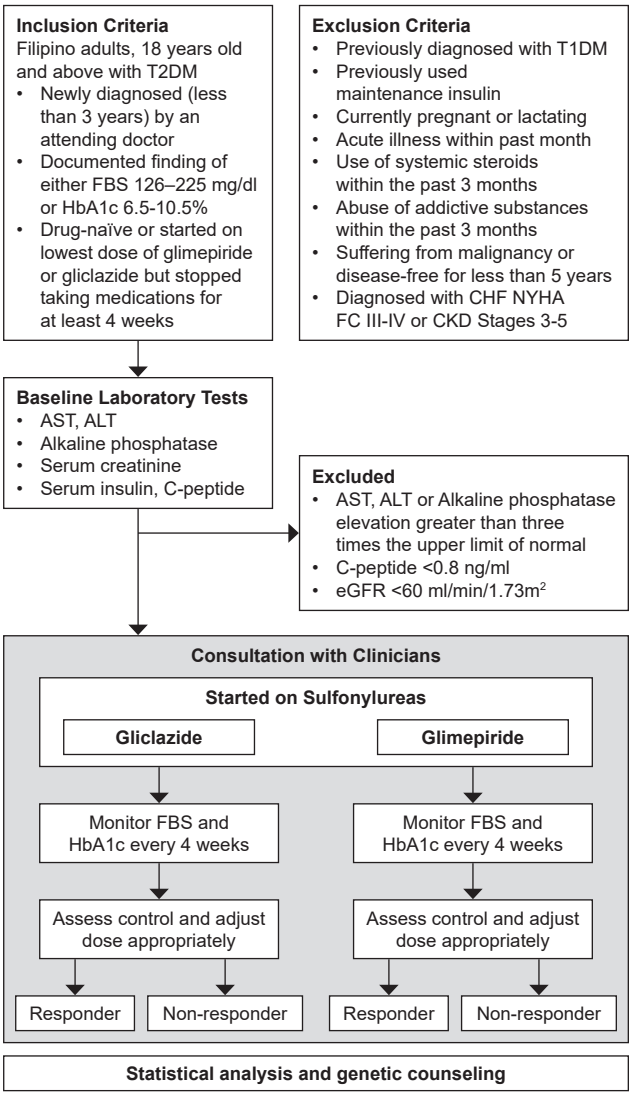

Figure 1. Flowchart of participant enrollment and follow-up.

However, there are no known studies that looked at genetic variants and their association with SU resistance among Filipinos. Although there were other variants that were associated with SU use, interethnic variability makes it pertinent to perform a separate study for Filipinos, who are underrepresented in the previous studies. Most studies were done on non-Filipino populations, mainly Caucasians, Blacks, Han Chinese, and even South Asians. We also reviewed the status of Malays, with little success. No documentation on specific targeting of Malay individuals was known to the authors. Thus, the current study investigated the association of genetic variants with treatment response to gliclazide and glimepiride. Among the SUs, the present study selected gliclazide and glimepiride because of their improved insulin release and diminished side effects such as hypoglycemic episodes and weight gain compared with older generation SUs.

The study results may aid in the creation of health policies for prescribing SUs to patients with T2DM. The findings may also serve as a first step in the development of test kits for personalized medicine to attain therapeutic targets.

METHODOLOGY

Study design and enrollment of participants

The study was implemented in compliance with the University of the Philippines Manila – Research Ethics Board (Study Protocol Code: UPMREB-2012-0187-NIH). Volunteer participants were enrolled from March 2014 to January 2019 from different institutions in the Philippines, such as Philippine General Hospital in Manila, Corazon Locsin Montelibano Memorial Regional Hospital in Bacolod City, Southern Philippines Medical Center in Davao City, and other government hospitals, health centers, and private clinics in Metro Manila and nearby provinces.

Screening of participants in this case-control study was performed following the inclusion and exclusion criteria (Figure 1). The study population is composed of adults (>18 years old) Filipinos with at least 3 generations of Filipino ascendancy. Screening involved baseline laboratory tests for fasting blood sugar (FBS), glycated hemoglobin (HbA1c), fasting serum insulin, C-peptide, alkaline phosphatase, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and serum creatinine.

Included participants were started on either gliclazide or glimepiride following the study's treatment algorithm

and administered by the attending physician. Medication adherence was assessed on the day of follow-up and computed as follows: (number of packets consumed/ number of packets prescribed) multiplied by 100%. Responders were defined as those whose HbA1c levels changed by more than or equal to 0.5% (absolute value difference) from baseline after 3 months of treatment, while poor responders were those whose HbA1c changed less than 0.5% from baseline after 3 months of treatment.[16,17]

The initial estimates for the minimum sample sizes were done by assuming a recessive model (as this model typically requires the largest sample sizes), an odds ratio of >2.5 (risk) or <0.4 (protective, an alpha of 0.05, and power of 80%), with a 1:2 case-control ratio. We recognize the limitation of alpha errors in the setting of multiple testing. To overcome this limitation, the conservative Bonferroni adjusted *p*-value <0.05 correction was initially considered. However, the study did not reach the estimated sample sizes due to the few prospective participants passing the screening criteria. The study screened more than 17,000 participants to come up with the present numbers. In particular, there were challenges in recruiting drug naive T2DM cases and those who were not on medication for the past 3 months.

Thus, in this study, the practical *q* value for all allelic and genotypic results for all variants is 1, and thus we are not able to reject the likelihood that the results are false positives. Nonetheless, we expect results using both nominal statistical inference and sensible biological insights, although with caution and reservation. Note that the assumptions are considered liberal as we considered the largest minimum based on the recessive model. Thus, when we analyzed the results, many of the variants found had a much lower alpha that became nominally significant at smaller sample sizes. This is true for additive models and the dominant models which require fewer sample sizes than the recessive model.

Besides the statistical inference, the significance of these findings can be enhanced in other ways, particularly, biological relevance/plausibility, multiplicity in results, and literature replicability. However, we will cite select findings with caution, especially if the findings are not supported by such information; nonetheless, these minor findings should still be considered as preliminary findings that need verification.

The actual sizes per subgroup were set at 62 cases and 124 controls to assume a power of 80% at alpha error <0.05 using the recessive genetic model. However, because of the lower-than-expected number of cases, further importance to the enrichment of variants per gene and observed biological theme/s was given.

DNA extraction and quantification

DNA extraction from whole blood samples was performed using QIAamp DNA Blood Mini Kit (QIAGEN, Victoria, Australia) following manufacturer instructions. Eluted DNA was quantified using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, MA, USA) at 260 nm. DNA samples with an $A_{260/280}$ of between 1.7 to 2.0, and a minimum concentration of 50 ng/ul were stored at -20°C until microarray genotyping.

Genotyping

The customized bead chips included 2,842 variants that were associated with various conditions, treatment responses, and adverse effects of various drugs, including those related to T2DM and SUs. These variants were researched extensively from different sources, such as Pharmacogenetics Knowledgebase (PharmGKB) (Thorn et al., 2013),[18] National Human Genome Research Institute Genome-wide Association Study (NHGRI GWAS Catalog),[19] PubMed, and selected patent databases such as Patentscope and Espacenet Variants with odds ratios (ORs) greater than 2.5 or less than 0.40 were preferentially included because of their perceived clinical relevance; variants with less established ORs were also included to assess their frequency in the local population. The selected SNPs were submitted to Illumina, Inc. for scoring to estimate their specificity and determine if the variants will be able to discriminate between responders and poor responders.

The variants were interrogated using Illumina iSelect Infinium Beadchip customized genotyping microarray (Illumina, CA, USA) using the manufacturer's prescribed procedures. Beadchips were scanned using an Illumina HiScan microarray scanner.

GenomeStudio v2.0 and gPlink v2.05.10 were used to evaluate the quality of sample data and for quality control. Variants with call frequencies of more than or equal to 95% were included in the study. Participants with an individual missingness rate (MIND) of more than 5% were excluded from further analysis. Other tests performed to exclude SNPs include frequency tests (minor allele frequency (MAF) <1%), genotype missingness rate (GENO) >5%, and Hardy-Weinberg equilibrium test (significant among controls >0.001).

Statistical analyses

For the clinical data, comparison of categorical variables used chi-square tests or Fisher exact test, as appropriate. For comparison between 2 quantitative variables, an independent t-test was performed.

Description of variants and associated genes were taken from *http://genome.ucsc.edu* (accessed April 20, 2022).[20]

Allelic and genotypic characteristics were assessed using gPlink v2.05.10. To test for allelic and genotypic association in a small sample set, the non-parametric Fisher-Irwin exact test was used. Correcting for multiple testing was done via the computation of *q* values.

To address the concern regarding the stability of the findings, p-values were used as the main determinants of association. The CI would be secondary, as it serves as a rough guide as to the directionality of the effect. In place of the *q* values, we focused on other parameters, such as biological relevance/plausibility, multiplicity in results, and literature replicability to increase confidence in the results, while interpreting the associations with adequate caution. As the sample sizes were small, the genetic association used Fisher-Irwin exact test for categorical variables.

For the genotypic association tests, the mode of inheritance or the effect of the genotypes was inferred based on the distribution of the genotypes among the case and control participants identified using gPlink v2.05.10. Crude ORs were used to infer the impact of an allele or a genotype on the phenotypic outcome. As with classical epidemiology, an OR greater than 1.0 denotes susceptibility or risk, and an OR less than 1.0 denotes protection. The ORs were computed using exact logistic regression; in this case, those with $p<0.05$ are considered to have a nominal association.

Because of the small sample sizes, we deferred doing multiple regression analyses and limited the interpretations to univariate analyses.

RESULTS

Gliclazide

Originally, 139 patients were enrolled in the gliclazide substudy (Figure 2A). Three participants (2 cases and 1 control) were removed due to a low genotyping rate (MIND >0.05). After screening, 136 participants remained after data quality control of which 18 were non-responders and 118 were responders.

Table 1 summarizes the clinical characteristics between cases and controls in the gliclazide substudy. Age was comparable between both groups. Although not significant, there is a noticeable trend of more males and smokers among the non-responders. Notably, both HbA1c and FBS were lower at baseline among non-responders compared with the responders. Mean HbA1c significantly decreased from baseline to the third month among gliclazide responders by 22% (8.55% to 6.63%, $p < 0.05$).

Among the 2,842 candidate variants investigated, 1,262 variants were excluded based on significant Hardy-Weinberg disequilibrium, genotypic missingness, and minor allele threshold test results (Figure 2). Seven variants were nominally associated with poor gliclazide response: rs2229437, rs319952, rs393994, rs9806699, rs1234315, rs7119, and rs6465084. However, there was no significant genotypic and allelic association observed after adjustment for multiple testing (Bonferroni-adjusted $\alpha = 3.2 \times 10^{-5}$).

Table 2 presents allelic features of nominally significant associated genes, while Table 3 presents the genotypic features of nominally significant associated genes.

Two variants, rs319952 and rs393994, were particularly interesting as they are both intronic polymorphisms of the AGBL carboxypeptidase 4 (*AGBL4)* gene. Both are intronic variants exhibiting similar recessive mode of inheritance. Both variants confer almost similar genotypic ORs of 6-7 increasing the confidence of common effects. Both have A as their risk alleles.

The variant rs2229437 had the lowest p-value. Remarkably, this variant is a missense SNP in another carboxypeptidase gene, the prolylcarboxypeptidase *(PRCP)* gene. Sorting Intolerant from Tolerant algorithm predicted a deleterious

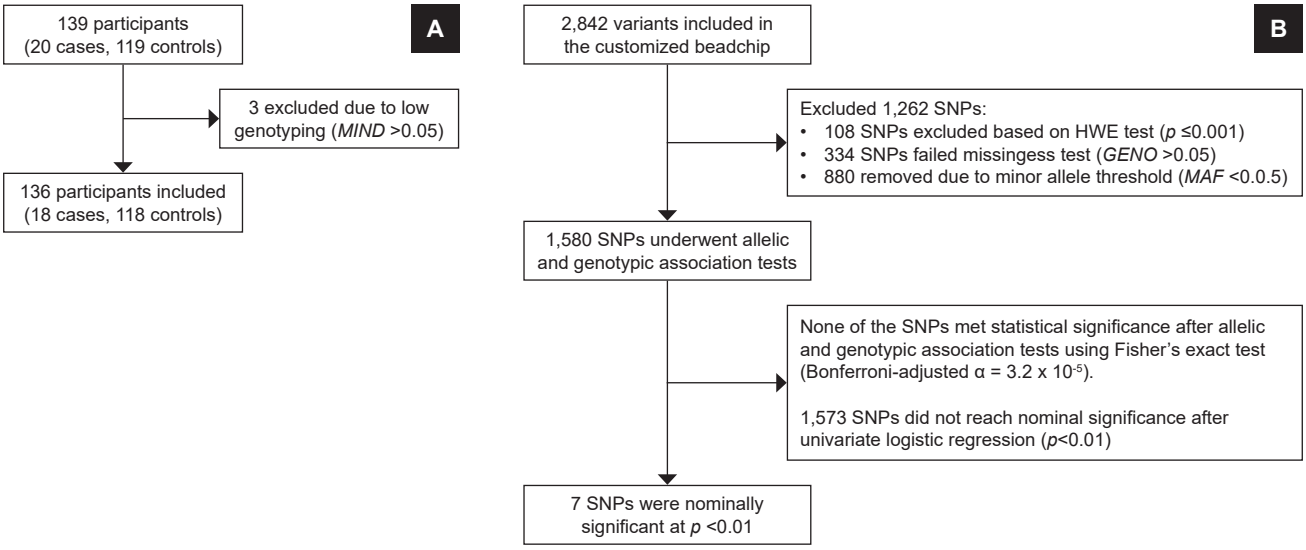

Figure 2. Schematic diagram of data processing and analysis for the gliclazide group. A total of 139 participants **(A)** and 2,842 SNPs **(B)** were analyzed to determine the association of genetic variants with poor gliclazide response.

Abbreviations: mind – individual missingness; SNP – single nucleotide polymorphism; HWE – Hardy-Weinberg equilibrium; geno – genotypic missingness; MAF – minor allele frequency.

Table 1. Clinical characteristics of participants in gliclazide substudy

Characteristics	Gliclazide poor responders (n = 18)	Gliclazide responders (n = 118)	*p*-value*
Age, years, mean (SD)	55.56 (12.77)	53.09 (10.12)	0.354
Male, %	44.44	26.27	0.056
Hypertension, %	55.56	47.46	0.261
Ever smoked, %	27.78	14.41	0.076
Alcohol use, %	16.67	32.20	0.090
BMI, kg/m^2, mean (SD)	26.64 (3.71)	26.02 (3.80)	0.519
Waist circumference, cm, mean (SD)	93.25 (9.37)	91.17 (10.16)	0.416
Baseline			
FBS, mg/dL, mean (SD)	143.94 (19.05)	167.07 (32.67)	0.004
HbA1c, %, mean (SD)	7.22 (0.66)	8.55 (1.15)	<0.001
Creatinine, mg/dL, mean (SD)	0.80 (0.29)	0.73 (0.23)	0.248
3rd month**			
FBS, mg/dL, mean (SD)	128.00 (35.33)	116.29 (22.91)**	0.065
HbA1c, %, mean (SD)	7.26 (0.79)	6.63 (0.85)**	0.003

Abbrev: BMI, body mass index; FBS, fasting blood sugar; HbA1c, glycosylated hemoglobin A1c; HDL, high-density lipoprotein; LDL, low-density lipoprotein; SD, standard deviation
*Significant at $p<0.05$ using Student's t-test or Fisher's exact test
**3rd-month values are significantly different compared with baseline values at $p< 0.05$ using paired t-test

Table 2. Allelic characterization of variants associated with poor gliclazide response

SNP	Implicated gene	Risk allele	Frequency		p-value*	q-value*	Predicted effect	Predicted Impact	
			Cases	Controls				SIFT	PolyPhen
rs2229437	*PRCP*	G	0.4444	0.2076	0.003247	1	Missense (E/D)	0.02 (deleterious)	0.009 (benign)
rs319952	*AGBL4*	A	0.8824	0.6504	0.005649	1	Intron variant	n/a	
rs393994	*AGBL4*	A	0.8611	0.6525	0.012310	1	Intron variant	n/a	
rs9806699	*ENSG00000259354// C15ORF48*	G	0.6389	0.4025	0.010840	1	5-upstream variant	n/a	
rs7119	*HMG20A*	A	0.5	0.2585	0.005146	1	3' untranslated region	n/a	
rs6465084	*GRM3*	A	1	0.8475	0.006797	1	Intron variant	n/a	

Abbrev: *PRCP*, prolylcarboxypeptidase; *AGBL4*, ATP/GTP binding protein like 4; *TNFSF4*, TNF superfamily member 4; *HMG20A*, high mobility group 20A; *GRM3*, glutamate metabotropic receptor 3; E/D, glutamic acid (E) to aspartic acid (D) mutation; SIFT, Sorting Intolerant Form Tolerant; Polyphen, Polymorphism Phenotyping.
*Variants are nominally significant at $p<0.05$.

Table 3. Genotypic characterization of variants associated with poor gliclazide response

SNP	Chrom No.	Model	Genotypes	Frequency		Crude OR	*p*-value*
				Cases	Controls		
rs2229437	11	DOM	GG and TG vs TT	77.78	36.44	6.02 (1.75, 26.73)	0.002
rs319952	1	REC	AA vs AG and GG	82.35	39.82	6.95 (1.80, 39.85)	0.002
rs393994	1	REC	AA vs AG and GG	77.78	38.98	5.41 (1.57, 23.98)	0.004
rs9806699	15	REC	GG vs AG and AA	44.44	12.71	5.39 (1.59, 18.14)	0.006
rs1234315	1	GENO	TC vs CC	5.56	45.76	0.08 (0.001, 0.60)	0.006
rs7119	15	ALLELIC	TT vs CC	38.89	18.64	1.33 (0.37, 4.52)	0.803
			AG vs GG	55.56	44.92	2.85 (0.77, 13.21)	0.138
rs6465084	7	REC	AA vs GG	22.22	3.39	14.13 (1.92, 114.88)	0.007

Abbrev: ADD, additive; DOM, dominant; DOMDEV, dominant deviant; REC, recessive.
*Variants are nominally significant at $p<0.05$

effect on its protein (SIFT = 0.02), although Polymorphism Phenotyping v2 (Polyphen v2) algorithm indicates that the resulting amino acid change from glutamic to aspartic acid has an otherwise benign impact (PolyPhen = 0.009). Upon univariate logistic regression analysis, the presence of the G allele resulted in an OR of 6.02 than the TT genotype (dominant model: 95% CI 1.75, 26.73; $p = 0.002$).

Other variants that were nominally associated with poor gliclazide response were: rs71119 (*HMG20A*), rs9806699 (*C15ORF48*), rs1234315 (*TNFSF4,* TNF superfamily member 4 geneTNF), and rs6465084 (*GRM3*, glutamate metabotropic receptor 3 gene).

Glimepiride

Among the 113 participants in the glimepiride substudy, five (1 case and 4 controls) were excluded due to a low genotyping rate (MIND >0.05), the control majority being expected due to the high control: case ratio. Thus, 7 non-responders and 101 responders were retained (Figure 3A). The clinical characteristics of the participants for this arm of the study are found in Table 4.

Table 5 presents the allelic features of nominally significant associated genes, while Table 6 presents the genotypic features of nominally significant associated genes.

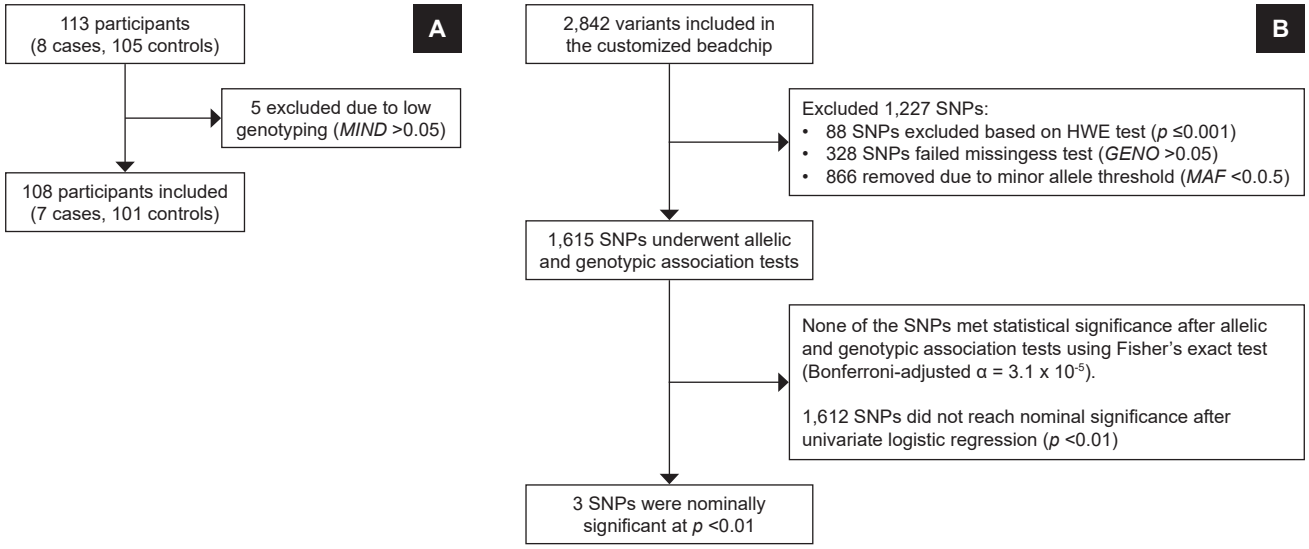

Figure 3. Schematic diagram of data processing and analysis for glimepiride group. A total of 139 participants **(A)** and 2,842 SNPs **(B)** were analyzed to determine the association of genetic variants with poor glimepiride response.

Abbreviations: mind – individual missingness; SNP – single nucleotide polymorphism; HWE – Hardy-Weinberg equilibrium; geno – genotypic missingness; MAF – minor allele frequency.

Table 4. Clinical characteristics of participants in glimepiride substudy

Characteristics	Glimepiride poor responders (n = 7)	Glimepiride responders (n = 101)	*p*-value*
Age, years, mean (SD)	46.14 (7.73)	52.16 (9.86)	0.117
Male, %	14.29	32.67	0.118
Hypertension, %	71.43	49.50	0.131
Ever smoked, %	28.57	24.75	0.411
Alcohol use, %	14.29	38.61	0.099
BMI, kg/m^2, mean (SD)	27.42 (3.69)	24.94 (3.25)	0.056
Waist circumference, cm, mean (SD)	92.64 (9.94)	88.01 (7.36)	0.119
Baseline			
FBS, mg/dL, mean (SD)	183.77 (40.62)	174.58 (31.03)	0.459
HbA1c, %, mean (SD)	8.54 (1.15)	8.86 (1.07)	0.448
Creatinine, mg/dL, mean (SD)	0.56 (0.13)	0.75 (0.23)	0.033
3rd month**			
FBS, mg/dL, mean (SD)	157.03 (57.46)	122.07 (25.95)**	0.002
HbA1c, %, mean (SD)	8.60 (1.33)	6.86 (0.85)**	0.001

Abbrev: BMI, body mass index; FBS, fasting blood sugar; HbA1c, glycosylated hemoglobin A1c; HDL, high-density lipoprotein; LDL, low-density lipoprotein; SD, standard deviation
*Significant at $p< 0.05$
**3rd-month values are significantly different compared with baseline values at $p<0.05$ using paired t-test

Table 5. Allelic characterization of variants associated with poor glimepiride response

SNP	Implicated gene	Risk allele	Frequency		*p*-value*	*q*-value*	Predicted effect	Predicted Impact	
			Cases	Controls				SIFT	PolyPhen
rs5063	*NPPA / near CLCN6*	T	0.5	0.2475	0.05627	1	Missense (V/M)	0.27 (tolerated)	0.58 (benign)
rs2299267	*PON2*	G	0.4286	0.1436	0.01347	1	Intron variant	n/a	
rs17367504	*MTHFR/ near CLCN6*	G	0.5	0.2673	0.07160	1	Intron variant	n/a	

Abbrev: *CLCN6,* chloride voltage-gated channel 6; *NPPA,* natriuretic peptide A; *PON2,* paraoxonase 2; *MTHFR,* methylenetetrahydrofolate reductase; V/M, valine (V) to methionine (M) mutation; SIFT, Sorting Intolerant From Tolerant; Polyphen, Polymorphism Phenotyping.
*Variants are nominally significant at p<0.05.

Table 6. Genotypic characterization of variants associated with poor glimepiride response

SNP	Chrom No.	Model	Genotypes	Frequency		Crude OR	*p*-value*
				Cases	Controls		
rs5063	1	DOM	TT and TC vs CC	100	41.58	13.04 (1.88, inf)	0.006
rs2299267	7	DOM	GG and AG vs AA	85.71	27.72	15.22 (1.73, 729.02)	0.008
rs17367504	1	DOM	GG and AG vs AA	100	43.56	12.04 (1.74, inf)	0.008

Abbrev: DOM, dominant. Variants are nominally significant at $p< 0.05$; exact logistic regression was done to compute the crude odds ratio.
*Variants are nominally significant at $p<0.05$

The age distribution is comparable between groups. As opposed to the gliclazide substudy, there are more males and alcohol users among responders of the glimepiride group. Mean HbA1c significantly decreased from baseline to the third month among glimepiride responders by 23% (8.86% to 6.86%, p <0.05).

Among the 2,842 variants selected for the study, 88 SNPs failed the HWE test (p ≤0.001), 328 variants failed the missingness test (GENO >0.05), while 866 were further removed due to low minor allele frequency (MAF <0.01).

It is interesting that although rs5063 is mainly considered as a missense variant in the *natriuretic peptide A (NPPA*) gene, resulting in a valine to methionine substitution, SIFT and PolyPhen v2 predicted tolerated and benign effects, and GTEx Portal indicated the variant as an Expression Quantitative Trait Loci (eQTL) for the nearby *CLCN6* and methyltetrahydrofolate reductase (*MTHFR*) genes.

Thus, 2 of the 3 nominally associated variants belong to a set that appears to influence both *CLCN6* and *MTHFR* genes (Table 5). The variants seem to have a similar dominant model with a high-risk effect. The presence of the rs5063 T allele conferred an OR of 13.04 towards poor glimepiride response than the CC genotype (95% CI: 1.88, inf; p = 0.006) (Table 6). Meanwhile, the presence of the G allele conferred an OR of 12.04 compared with the AA genotype (model: 95%, CI:1.74, inf; p = 0.008) in the variant, rs17367504 found in the same gene.

Another variant of interest would be rs2299267, an intronic variant of the paraoxonase 2 (*PON2*) gene. Seemingly acting in a dominant model, the G allele in the *PON2* gene variant conferred an OR of 15.22 times poor glimepiride response (Table 6) than the AA genotype (dominant model: 95% CI: 1.73, 729.02; p = 0.008).

DISCUSSION

Type 2 diabetes mellitus is commonly treated with SUs in the Philippines. However, some patients fail to meet treatment targets despite compliance. Several studies pointed out that genetics contribute to the variable response, and these genetic associations differ across various ethnicities. This study investigated such association among Filipinos using a candidate gene approach. Seven variants had been nominally associated with poor response to gliclazide and three variants with poor response to glimepiride.

Gliclazide

The three variants with the lowest p-values – rs2229437, rs319952, and rs393994 – are all found near genes that code for carboxypeptidases that are related to metabolic processes.

Two of the variants of the *ABGL4* gene, rs319952, and rs393994, have higher odds of poor gliclazide response. *ABGL4* codes for cytosolic carboxypeptidase 6, a metallocarboxypeptidase that mediates deglutamylation of target proteins to form tubulins like microtubules.[16,17] Microtubules negatively regulate insulin secretion in pancreatic beta cells, and their depolymerization is necessary for glucose-stimulated insulin secretion. High glucose levels destabilize microtubules and are balanced by new microtubule formation, which likely prevents glucose over-secretion. As a result, microtubule density is greater in dysfunctional beta cells of diabetic mice. Few studies have explored the connection between *ABGL4* and diabetes, more so sulfonylureas. Of these, one study identified *AGBL4* as one of the down-regulated genes using differential gene expression between T2DM patients and healthy controls.[21] Such downregulation may be explained by the destabilization of microtubules in patients with high glucose levels. Further studies are required to understand the contribution of these *AGBL4* variants to gliclazide nonresponse.

Curiously, another carboxypeptidase gene variant, rs2229437 in *PRCP*, a gene coding for prolylcarboxypeptidase was found to be highly, albeit nominally, associated with gliclazide response. It was previously found to play a role in appetite suppression and weight gain.[22] Pharmacological inhibition studies on *PRCP*-knockout mice showed that lower levels of *PRCP* activity decreased appetite and were resistant to diet-induced obesity.[23,24] Plasma *PRCP* concentrations were also found to be higher among diabetic fatty rats fed with a high-fat diet compared to their lean controls.[25] In the current study, participants with GG and TG genotypes were more likely to be poorly responsive to gliclazide compared with those with the TT genotype in a dominant model. The GG genotype is associated with a higher expression of the *PRCP* gene in subcutaneous adipose tissue,[26] which may result in higher levels of circulating *PRCP* and an increased likelihood of diabetes. Interestingly, the administration of metformin among rats and humans with high *PRCP* levels reversed this elevation.[25] In case the hypothesis is found to be correct in subsequent functional studies, it may be advisable to avoid giving patients gliclazide and prescribe metformin instead.

Of relative relevance would be rs1234315, which is within 1000 bp upstream of *SLC30A4-ASI*. This variant was previously reported to affect several drugs like statins.[27,28] The possible mechanism of the variant on how exactly it affects SUs is yet to be elucidated. Its function as a catalyst of pancreatic carboxypeptidases and zinc transporters may play a role in such a mechanism.

In retrospect, the exact role of the carboxypeptidases in the dynamics of the SUs was scarcely investigated. This paper thus suggests that such thematic association may provide clues to the mechanistic importance of carboxypeptidases in SU response.

Another interesting theme to entertain is glutamate metabolism. In addition to the polyglutamate-acting carboxy-

peptidases, another variant, rs6465084, implicated the involvement of *GRM3* (*glutamate metabotropic receptor 3*). The G-protein receptor is linked with cyclic adenosine monophosphate (AMP) signaling and has been implicated to influence insulin secretion in beta-cells through an α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid or AMPA-regulated mechanism in the pancreas.[29] Thus, the possibility of glutamate processing affecting the secretagogue function of SUs becomes plausible.

Other nominally associated variants include rs7119, an intron variant of *HMG20A*, which is responsible for regulating the metabolism-insulin secretion coupling genes and functional maturity of β-cells. Single-tissue eQTLs show that individuals with the T allele have higher expression levels of *HMG20A*.[30] Knockdown of the gene resulted in reduced glucose-induced insulin secretion.[31] While there is no literature linking the variant to gliclazide response, gliclazide's mechanism of action involves the stimulation of insulin secretion through binding to the β-cell SU receptor (SUR1).[32] The altered expression of the *HMG20A* gene may affect the functional maturity of β-cells, which may consequently contribute to poor response to gliclazide. As genotypic associations are compared pairwise, the association of the variants to the trait is set as a group. The significance of the *HMG20A* variant, rs7119, mainly relies on the AG vs GG comparison. Thus, even if the other comparisons lack significance, the variant itself is associated with the trait.

Other associated variants are rs9806699 and rs1234315 upstream of *C15ORF48* and *TNFSF4*, respectively. These were scarcely studied and no previous data defines their function. Moreover, these variants were not previously linked to diabetes or response to oral hypoglycemic agents.

Glimepiride

Three variants were nominally associated with poor glimepiride response (p<0.01), two are found near the *CLCN6-NPPA-MTHFR* gene cluster and one from the *PON2 loci.* All three variants were not previously linked to poor sulfonylurea response.

Two associated variants were noted to be near the gene cluster *CLCN6-NPPA-MTHFR,* rs5063 and rs17367504, each resulting in more than ten times higher odds of poor glimepiride response. Although the SNPs are 50kb apart from each other, with different hypothetical effects - 5063 is an intronic variant of the *NPPA* gene, while rs17367504 is an intronic variant of *MTHFR*, both variants seem to influence the expression of *MTHFR*, *CLCN6*, and *NPPA* and *NPPA* in various tissues, including the *NPPA* antisense RNA.[33] The variants in the *MTHFR-CLCN6-NPPA-NPPA* gene cluster were investigated by a previous study providing novel insights into the mechanisms of cardiac dysfunction.[34,35] Moreover, the *MTHFR* gene is implicated in T2DM susceptibility.[36] These findings are interesting considering that the *MTHFR* gene is widely implicated in drug metabolism, such as with methotrexate, by acting through 1-carbon transfer.[37] However, such speculation and questions on the role of other implicated genes remain to be investigated.

Another variant that conferred a high OR is rs2299267, an intronic region of the *PON2* gene and upstream from the *PON1* and *PON3* genes. In a study differentiating the effect of SUs, specifically glimepiride and glibenclamide, it has been found that the SUs increased *PON1* hepatic activity.[38] As this finding may imply an influence on hepatic metabolism, studies that aim to understand the contribution of *PON* variants to glimepiride response are worth exploring in the future.

Nonetheless, the authors recognize the obvious limitation of the study's sample size. The lack of association in multiple testing resulting in false positives is possible.

Studies of similar candidate approaches with small sample sizes have been published. For instance, one study that shows the correlation of *KCNQ1* polymorphism with glycemic parameters only had 91 subjects (44 cases and 47 controls).[39] In addition, a longitudinal study on various metformin/sulfonylurea combinations of up to 6 months involved 88 individuals comprising of 17 cases and 71 controls.[40] Both demonstrated associations of genotypes with glycemic parameters. The main difference in approach is they utilized continuous glycemic variables. In contrast, our study used categorical glycemic parameters as these are real-life clinical parameters and to determine pharmacogenetic markers likely foreseen. Negative results were seen in several studies. For instance, a lack of association was found in assessing the use of GLP-1 analogue exenatide to control blood sugar that enrolled only 36 patients,[41] or in associating *CYP2C9 rs1067910* to glycemic responses to pooled sulfonylureas that had only 30 samples.[42] Both failed to demonstrate treatment effects.

We underscore that validation research using the markers of interest may be conducted in a larger-scale study of patients with T2DM. Alternatively, as some findings suggest thematic enrichment, functional studies can be conducted. The preliminary results of this study may provide impetus to evaluate the clinical relevance of the identified SNPs on SU treatment.

CONCLUSION

Interethnic variations compel the conduct of pharmacogenetic studies in scarcely studied populations, such as Filipinos. In this context, as sulfonylureas such as gliclazide and glimepiride are used on a national scale to treat T2DM, we observed several variants to be nominally associated with sulfonylurea response among Filipinos. With this data, new possibilities on the pharmacodynamics and pharmacokinetics of sulfonylureas are suggested, and the results of the current study may guide future directions in SU research.

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Statement of Authorship
All authors certified fulfillment of ICMJE authorship criteria.

Authors Contribution Statement
EPP and JBN conceived the study; developed the methodology; provided study materials; validated data; acquired financial support for the study.

EMC conceived the study; developed the methodology; provided study materials; validated data; acquired financial support for the study; reviewed and edited the manuscript; supervised and managed the research activity planning.

GJJ conceived the study; developed the methodology; provided study materials; validated data; reviewed and edited the manuscript; supervised the research.

EPP, JBN and AYCA synthesized the data; conducted the research; curated the data; prepared the original draft; reviewed and edited the manuscript; supervised and managed the research activity planning.

ELAR and MGF applied the statistical techniques; collected and curated the data; helped in the preparation of the original draft; reviewed and edited the manuscript; supervised and coordinated the research activity planning.

MVG and MUN validated data; collected and curated the data; reviewed and edited the manuscript; supervised and coordinated the research activity planning.

CAC, MDM, CVJ, PND, APM developed the methodology; collected data; reviewed and edited the manuscript.

AUC, JMQ, AML, DCB, NMM collected and curated the data; reviewed and edited the manuscript; supervised and coordinated the research activity planning.

VDR, KJAC and JPF supervised and coordinated the research activity planning.

NMM, VSR, KJAC, JPF, JB, JM, CDD and CEP synthesized the data; conducted the investigation process; curated the data; prepared the original draft; reviewed and edited the manuscript.

Author Disclosure
EPP is the Editor-in-Chief of the JAFES. EPP, JBN, EMCDP, AYCA have patent application at UP Manila. GJJ served as CME/ Speaker's Bureau of the following companies: MSD, J& J, Astra Zeneca, Boehringer-Ingelheim, Sanofi Aventis, Novo Nordisk, Abbott Nutrition, LRI-Therapharma, UMed, Woerwag. CAC represented the Philippine College of Endocrinology, Diabetes and Metabolism (PCEDM) as the primary investigator and was given a research grant by Servier Laboratories through the PCEDM; received compensation as lecturer for the following companies: AstraZeneca,Getz Pharma, LRI-Therapharma, MSD, Natrapharm, Novo Nordisk, Sanofi, Servier and Zuellig-Lilly; received compensation for module development/presentations for Natrapharm and received support for registration to scientific meetings/ conventions from MSD, Natrapharm, Sanofi and Servier; received medical equipment and PPEs from BioFemme, LRI-Therapharma, Natrapharm and UAP; serves as the head of the Reproductive Endocrinology and Transgender Health Council of the PCEDM. PND received honoraria for lectures.

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ORIGINAL ARTICLE

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Single Nucleotide Polymorphism at rs7903146 of Transcription Factor 7-like 2 gene Among Subjects with Type 2 Diabetes Mellitus in Myanmar

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Abstract

Objectives. To investigate the association between the single nucleotide polymorphism (SNP) rs7903146 in the transcription factor 7-like 2 (TCF7L2) gene and type 2 diabetes mellitus (T2DM) and to examine the impact of this variant on pancreatic beta-cell function in the Myanmar population.

Methodology. A case-control study was undertaken in 100 subjects with T2DM and 113 controls. The SNP rs7903146 was genotyped using the allele-specific polymerase chain reaction method. Plasma glucose and serum insulin levels were determined using the enzymatic colorimetric method and ELISA respectively. Beta-cell function was calculated by the HOMA-β formula.

Results. The frequencies of carrier genotypes (CT and TT) were higher in subjects with T2DM than in controls. The minor T alleles of rs7903146 were found to statistically increase type 2 diabetes risk than the C allele with an allelic odds ratio of 2.07 (95% CI 1.39-3.09, p=0.0004). The mean HOMA-β level of the group with non-carrier genotype (CC) was significantly higher than that of the groups with carrier genotypes (CT and TT) in subjects with T2DM and controls with a p-value of 0.0003 and less than 0.0001, respectively.

Conclusion. The rs7903146 variant of the TCF7L2 gene was found to be associated with T2DM and low β-cell function among Myanmar subjects.

Key words: type 2 diabetes, risk variants, bioinformatics, whole exome sequencing, Pashtun population

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a complex disorder of carbohydrate metabolism and is characterized by abnormal glucose homeostasis leading to hyperglycemia.[1] Environment and lifestyle changes are believed to play a primary role in the current epidemic of T2DM, but the inherent susceptibility to the disease is widely attributed to complex genetic factors. There is compelling evidence that genetic susceptibility to the disease is polygenic, and genome-wide association studies have identified over 400 loci associated with T2DM risk.[2] The TCF7L2 gene was found to be associated with T2DM. Five SNPs in intron 3 of the TCF7L2 gene were identified and the association of each SNP with T2DM was studied in different populations. Frequencies of the risk allele (T) were found to be high among the Japanese, Chinese, and African-American populations but were low in the Finnish population.[3-5] Data among the Myanmar population are yet to be available. In the present study, we investigated the SNP rs7903146 genotypes in 100 subjects with T2DM and 113 controls in Myanmar. The impact of these variants on beta-cell function was also studied in these two groups.

According to the National Center for Biotechnology Information, the TCF7L2 gene is located on the long arm of chromosome 10 and has 14 exons and 13 introns.[6] In 2006, Grant et al. identified that a common microsatellite in the TCF7L2 gene region (DG10S478) in intron 3 of TCF7L2 gene was associated with T2DM in an Icelandic case-control sample. This association was supported in two further populations, a Danish female cohort, and an European-American cohort. There are five SNPs found to have the strongest correlation to DG10S478 in HapMap samples; rs12255372; rs7903146; rs7901695; rs11196205; rs7895340. All five SNPs were shown to be associated in various

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degrees with T2DM. Among all five SNPs, rs12255372 and rs7903146 were found to be in strong linkage disequilibrium with DG 10S478 and have a higher strength of association with T2DM than other SNPs.[7] Peng et al., published a meta-analysis in which significant associations were found between T2DM and rs7903146, rs12255372, rs11196205, rs7901695, and rs7895340 with summary odds ratios (95% CI) of 1.39 (1.34–1.45), 1.33 (1.27–1.40), 1.20 (1.14–1.26), 1.32 (1.25–1.39) and 1.21 (1.13–1.29), respectively.[8] In this study, we focused on the rs7903146 variant of the TCF7L2 gene which showed the highest odds ratio in the study of Peng et al.[8] The risk T allele is associated with increased risk of T2DM due to impaired insulin secretion, impaired incretin effects, as well as enhanced rate of hepatic glucose production.[9] Cropana et al., conducted a study in which genetic variations in the TCF7L2 gene may increase the risk of T2DM in obese adolescents. They found that the rs7903146 variant in the TCF7L2 affects functional beta-cell capacity with impaired proinsulin processing and impaired hepatic insulin sensitivity.[10]

The TCF7L2 gene has recently been implicated in the pathogenesis of T2DM through the regulation of pancreatic beta-cell insulin secretion.[11] It encodes for an enteroendocrine transcription factor that has a role in the wingless-type integration site family, member 1(WNT) signaling pathway, which seems to be critical to pancreatic islet development and adipogenesis.[12] WNT signaling is also required for islet beta-cell proliferation.[13] TCF7L2 forms heterodimers with β-catenin to induce the expression of various genes such as glucagon-like peptide 1, the insulin gene and genes that encode proteins involved in processing and exocytosis of insulin granules.[14]

GLP-1 produced in intestinal endocrine L cells lowers the blood glucose levels through stimulation of insulin secretion and biosynthesis in pancreatic cells. Variants of the TCF7L2 gene could influence the susceptibility to T2DM by altering the level of GLP-1.[15] However, some studies have shown that risk allele carriers of TCF7L2 have normal concentrations of GLP-1 but have impaired insulin secretion in response to GLP-1 infusion, which indicated that the effect lies at the level of GLP-1 action on pancreatic beta-cells rather than secretion of GLP-1.[16] Ferreira et al., found that after treatment with exenatide, only carriers of the T allele (CT/TT) showed significantly decreased postprandial plasma insulin levels compared to the non-carrier CC genotype groups.[17] In concert with TCF7L2, the WNT signaling pathway and GLP-1 exert a critical effect on blood glucose homeostasis by stimulating insulin production by pancreatic beta-cells.

METHODOLOGY

A case-control study was conducted in the present study. After exclusion of patients with malignancy, chronic liver diseases, chronic renal failure and those who were taking insulin, 100 subjects with T2DM were recruited from the out-patient department and in-patient care of the Medical Ward of North Okkalapa General Hospital by simple random sampling method. According to WHO criteria 2006, individuals with fasting plasma glucose concentration more than or equal to 7.0 mmol/l or 126 mg/dl or 2-hour postprandial glucose level more than or equal to 11.1 mmol/l or 200 mg/dl were considered as having T2DM.[18] Inclusion criteria were subjects diagnosed with T2DM, above 35 years of age regardless of gender. One hundred and thirteen healthy persons without T2DM from the population of Quarter B, North Okkalapa Township were selected by simple random sampling to serve as non-diabetic controls. The control subjects had fasting plasma glucose levels less than 6.1 mmol/l or less than 110 mg/dl (based on WHO 2006 criteria).[18]

The estimated sample size for each study group was calculated using the following formula:

P_1 = Proportion of T allele in type 2 diabetes = 0.13 (13%)[19]
P_2 = Proportion of T allele in controls = 0.06 (6%)[19]
$N_1 = N_2$ = Number of each group

$$N_1 = N_2 = \frac{2Z\alpha^2\, P(1-P)}{(P_1-P_2)^2}$$ [20]

$$P = \frac{P_1+P_2}{2} = \frac{0.13+0.06}{2} = 0.095$$

Zα = 1.64 (standard normal deviation for α = 0.1 = Zα = 1.64)

$$N1 = N2 = \frac{2\times(1.64)^2\times 0.095(1-0.095)}{(0.07)^2}$$

$$= 94.38$$

= (Number for each group - T2DM and controls)

So, a total of 200 subjects were selected for this study.

A total of 5 ml of fasting venous blood was collected for the determination of fasting plasma glucose, fasting serum insulin, and for genotyping. Fasting insulin and glucose levels were used to estimate insulin secretion using homeostatic model assessment (HOMA-β). Plasma glucose level was determined in duplicate by a glucose oxidase method adapted to an autoanalyzer (Human, Germany). Serum insulin concentrations were determined by Enzyme-linked Immunosorbent Assay (DRG International, Inc, USA).

Genomic DNA was extracted by the salting-out method and rs7903146 genotype assays were conducted by allele-specific polymerase chain reaction method and agarose gel electrophoresis.

Table 1. Primers used for rs7903146 genotyping by allele-specific PCR.[21]

Primer	Sequence 5'–3'
rs7903146 C	Forward primer specific for allele C detection GAACAATTAGAGAGCTAAGCACTTTTTAGAAAC
rs7903146 T	Forward primer specific for allele T detection GAACAATTAGAGAGCTAAGCACTTTTTAGAGAT
rs7903146 R	Common reverse primer AGATGAAATGTAGCAGTGAAGTGC

Statistical analyses

Descriptive statistics were used to describe the profile of study participants. Quantitative variables were described using the mean and standard deviation. Meanwhile, qualitative variables were summarized as frequencies and percentages. Comparison of mean HOMA-β levels among TCF7L2 gene carriers and non-carriers were performed among diabetic and non-diabetic study participants separately. Hardy-Weinberg equilibrium and the association between disease status and the genetic variants were tested by Pearson's Chi-square test. Odds ratio, 95% confidence intervals, and all statistical tests were carried out using SPSS software version 16.0. A *p-value* <0.05 was considered statistically significant.

Ethical consideration

This research is approved by the Ethical Research Committee of the University of Medicine 2, Yangon.

RESULTS

A total of 250 participants were recruited for this study, 213 subjects had complete data and were completely analyzed for the TCF7L2 gene polymorphism, serum insulin and glucose levels. The results were presented for 213 subjects, 100 with T2DM and 113 without T2DM.

Representative genotyping of rs7903146(C/T) by allele-specific PCR is shown in Figure 1. The clinical and biochemical characteristics of subjects with and without T2DM are presented in Table 2.

Genotype distribution and analysis of the association of rs903146 of the TCF7L2 gene in subjects with and without T2DM are shown in Table 3. The CC, CT, and TT genotype frequencies were 17%, 76%, and 7% in subjects with T2DM and 46%, 51%, and 3%, respectively in the control subjects. The CT and TT genotypes are more frequent in subjects with T2DM than in controls. The CC genotype is more frequent in controls than in subjects with T2DM. The risk of T2DM is higher in the homozygous (TT) genotype group with an odds ratio of 7.14 (95% CI 1.66 – 30.71, p=0.008) than in the heterozygous (CT) genotype group with an odds ratio of 4.00 (95% CI 2.10 - 7.64, p <0.0001).

In Table 4, the C and T allele frequencies are 55% and 45% in subjects with T2DM and 72% and 28% in controls. The T alleles of rs7903146 statistically increase T2DM risk

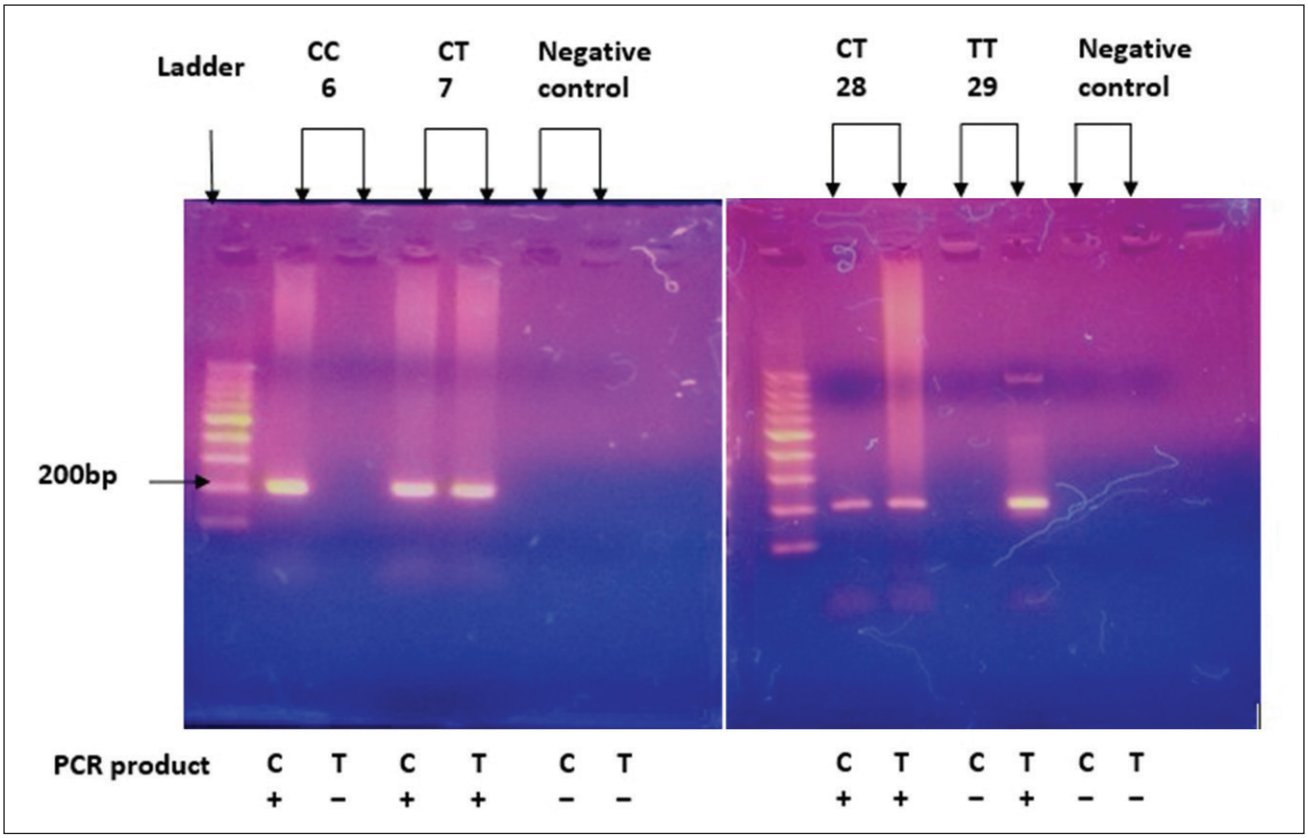

Figure 1. Genotyping of rs7903146 of TCF7L2 gene in agarose gel. This figure showed representative genotyping of rs7903146(C/T) by allele specific PCR. For each sample, two PCR reactions were performed, one with primers rs7903146 C and rs7903146 R (PCR C) and a second with primers rs7903146 T and rs7903146 R (PCR T). PCR products for C and T were identified after separation on an agarose gel. A 205 base pair (bp) band indicated the presence of the allele and amplification failure indicated the absence of the allele. Since products for C and T were placed side by side on the agarose gel, if 205 bp bands were seen on PCR C and PCR T, it indicates CT genotype. In the CC genotype, 205bp band was seen only on PCR C and absent in PCR T. In the TT genotype, 205 bp band was seen only on PCR T and absent in PCR C.

Table 2. Clinical and biochemical characteristics of subjects with and without T2DM

Parameters	Subjects with T2DM (n=100)	Subjects without T2DM (n=113)	*p*-value
Age (years)	57.47±10.71	47.05±8.94	<0.001
BMI (kg/m^2)	26.85±4.71	22.78±4.31	<0.001
Fasting plasma glucose (mg/dl)	136.02±15.76	92.02±11.03	<0.001
Fasting serum insulin (uIU/ml)	16.19±6.18	13.05±5.27	<0.001
HOMA-β	79.69±24.94	174.02±61.39	<0.001

Results were shown in Mean±SD

Table 3. Genotype distribution and analysis of the association of rs7903146 of TCF7L2 gene in subjects with and without T2DM

Genotype	Subjects with T2DM (n=100)	Subjects without T2DM (n=113)	OR	95% CI	*p*-value
CC (%)	17 (17%)	52 (46%)		Reference	
CT (%)	76 (76%)	58 (51%)	4.00	2.10-7.64	<0.0001
TT (%)	7 (7%)	3 (3%)	7.14	1.66-30.71	0.008

$\chi 2 = 21.06$, $p<0.0001$, OR = odds ratio, 95% CI = 95% confidence interval

Table 4. Allele frequencies and analysis of SNP at rs7903146 of TCF7L2 gene in subjects with and without T2DM

Allele(2N)^2	Subjects with T2DM (n=100)	Subjects without T2DM (n=113)	OR (95% CI)	*p*-value	Adjusted OR (95% CI)	*p*-value
C allele	110 (55%)	162 (72%)	2.07	0.0004	1.98	0.002
T allele	90 (45%)	64 (28 %)	(1.39-3.09)		(1.27– 3.08)	

$\chi 2 = 12.79$, $p<0.0001$, OR = odds ratio, 95% CI = 95% confidence interval, adjusted odds ratio and p-values were obtained from logistic regression analyses adjusting for BMI

Table 5. Comparison of HOMA-β level in non-carrier and carrier risk allele of Subjects with and without T2DM

Groups	Genotypes		*p*-value
	Non-carrier (CC)	Carrier (CT and TT)	
Subjects with T2DM	99.18±13.78 (n=17)	75.70±24.89 (n=83)	0.0003
Subjects without T2DM	210.12±53.77 (n=52)	143.25±49.86 (n=61)	<0.0001

Results were shown in Mean±SD

compared to the C allele with an allelic odds ratio of 2.07 (95% CI 1.39-3.09, p=0.0004, and significantly increase T2DM risk even after adjusting for BMI.

The mean HOMA-β levels of the non-carrier (CC) genotype group are significantly higher than that of carrier genotype groups (CT and TT) with the $p< 0.0001$ in controls as well as in subjects with T2DM with a $p<0.0003$ as shown in Table 5.

DISCUSSION

This study found a significant association in samples from a Myanmar population between the variants rs7903146 of TCF7L2 and T2DM and this is similar to that previously reported in samples from European-origin populations.[7,22,23] The data from the present study shows that the CT and TT genotypes are more frequent in patients with T2DM with a frequency of 76% and 7% respectively, compared to a frequency of 51% of CT genotype and 3% of TT genotype in controls.

On the other hand, the CC genotype is more frequent in controls with a frequency of 46% compared to a frequency of 17% in subjects with T2DM as shown in Table 3. Genotype distribution of SNP at rs7903146 did not conform to the Hardy-Weinberg equilibrium in both subjects with T2DM (χ^2= 28.66, df=1, $p<0.001$) and in the control group (χ^2=7.89, df=1, p=0.005) (data not shown). It is also noted that deviation from Hardy-Weinberg equilibrium at rs7903146 SNP of TCF7L2 gene was also reported in the study of Marquezine et al.[24]

The risk of T2DM was higher in the risk allele carrier homozygous genotype (TT) group than the heterozygous genotype (CT) group compared to the non-carrier homozygous genotype (CC) group as shown in Table 3. In the present study, the frequency of the T allele was significantly higher in subjects with T2DM (45%) compared to controls (28%). The T allele of rs7903146 increases the risk of type 2 diabetes than the C allele with an allelic odds ratio of 2.07 (95% CI 1.39 - 3.09) with a p=0.004 as shown in Table 4. Chandak et al., showed that the T allele was more frequent in subjects with T2DM (37%) than healthy control subjects (29%) in the Indian population with an odds ratio of 1.46 (95% CI 1.22–1.75, $p=3 \times 10^{-5}$).[25] Moreover, Bahaaeldin et al., also found that the frequency of the T allele was higher in subjects with T2DM (32.9%) compared to controls (26.7%) in an Egyptian study with an odds ratio of 1.35 (95% CI 0.68-2.6), but this was statistically insignificant ($p>0.05$).[26] In agreement with the current work, the T allele was more frequent in subjects with diabetes (45.3%) than healthy controls (34.5%) and is associated with a high risk of diabetes with an odds ratio of 2.13, (95% CI 1.12-7.31, p =0.005) in a Moroccan population.[27]

The odds ratio of rs7903146 of the TCF7L2 polymorphism in this study appears to be higher than that of other studies. However, the odds ratio of rs7903146 of the TCF7L2 polymorphism in the present study was comparable to the studies of Dabelea et al.,[5] and Ren et al.[4] A lack of association between the TCF7L2 rs7903146 variant and T2DM was reported in the study among Hong Kong Chinese[28] and in the Iran study of Pourahmadi et al.[29] Mandour et al., reported that the frequency of the TT genotype was significantly lower among subjects with diabetes compared to healthy controls. Moreover, the study found that the T allele of TCF7L2 rs7903146 was associated with a lower risk of T2DM.[30] These disparate results may be due to differences in ethnicity.

The frequency of risk allele T of rs77903146 was more prevalent in the European, American, and African populations than the Asian population. The higher frequencies of the T allele in the present study suggest that the genetic background and environmental conditions of Myanmar Asians may be different from that of other Asian populations. This difference might be due to many factors such as ethnic stratification, variation in study design, sample size, variation in methods for SNP detection, or gene-gene and gene-environment interaction.

In this study, risks of T2DM were consistently higher for the homozygous (TT) genotype (odds ratio 7.14, 95% CI 1.66 –30.71, p=0.008) than for the heterozygous (CT) genotype of rs7903146 SNP, (odds ratio 4.00, 95% CI 2.10-7.64, p<0.0001). It may indicate that subjects carrying both risk alleles (homozygous carrier, TT) have about a sevenfold higher risk of T2DM compared to those without the risk allele (homozygous non-carrier, CC). Subjects carrying one risk allele (heterozygous carrier, CT) may have a fourfold risk of developing T2DM compared to those without any risk allele (homozygous non-carrier, CC). Barra and colleagues found that the odds ratio for the homozygous (TT) genotype was 4.04 (95% CI 1.48-11.0, p=0.004) and that of the heterozygous (CT) genotype was 1.06 (95% CI 0.63-1.80, p=0.81).[14] Our study showed the odds ratios for the homozygous and heterozygous genotypes were higher than other studies. This may be due to genetic variation among different ethnicities in the different studies. Based on the data from the present study, it shows that the polymorphism rs7903146 of the TCF7L2 gene is associated with increased susceptibility to T2DM in the population.

Although insulin resistance is the leading factor in the pathogenesis of T2DM, there is evidence that pancreatic beta-cells also play an important role in the development of T2DM. Many genes associated with T2DM have been linked to beta-cell mass and function. Decreased beta-cell mass and impaired insulin secretion have been reported in patients with T2DM in numerous studies. Gene variants of beta-cell loss which are associated with decreased beta-cell function are also regulated as risk factors for T2DM.[31]

In this study, the mean HOMA-β levels of non-carrier genotypes (CC) was significantly higher than carrier genotypes (CT, TT) in control subjects as well as subjects with T2DM as shown in Table 5. This indicates that risk allele T carrier genotype groups have impaired beta-cell function compared to non-carrier groups. The trend of reduced HOMA-β between different genotypes was similar to the study of Loos et al.[32] Therefore, the risk allele T of rs7903146 is strongly associated with reduced HOMA-β levels and results in diabetes by affecting the function of pancreatic beta-cells. The impaired beta-cell function of risk allele T carrier groups could be due to reduced beta-cell mass through defects in cell differentiation and maturation. This finding was supported by the findings of Papadopoulou and Edlund, in which TCF7L2 activates many genes downstream of the WNT signaling pathway, which is required for the development of the pancreas and islets during embryonic growth.[33]

Similarly, Takamoto et al., demonstrated that newborn mice expression of TCF7L2 dominant-negative form showed a reduction in the area of beta-cells and pancreatic insulin content leading to impaired glucose tolerance with decreased insulin secretion. They also reported that TCF7L2 in pancreatic beta-cells plays a crucial role in the metabolism of glucose through the regulation of beta-cell mass during development.[34] These findings suggest that individuals with at-risk T allele carrier (CT, TT) genotypes of rs7903146 of the TCF7L2 gene exhibit a reduction in HOMA–β levels compared to the non-carrier genotype (CC) group and this may be due to reduced beta-cell mass and subsequent impaired beta-cell function.

In addition, TCF7L2 has been involved in the expression of GLP-1 and gastric inhibitory peptide receptors in beta-cells which mediate the effects of the corresponding incretin hormones to promote beta-cell proliferation.[7] Shu and coworkers found that there was a decrease in TCF7L2 protein level and decreased expression of GLP-1 receptors on pancreatic islet cells of subjects with diabetes. This downregulation of GLP-1 receptors may be the underlying etiology for impaired GLP-1-induced insulin secretion by pancreatic beta-cells in T2DM.[35] Moreover, TCF7L2 is also involved in the expression of beta-cell genes which are required for insulin secretory granule fusion. The number of morphologically docked vesicles was unchanged by TCF7L2 suppression, however, secretory granule movement and capacitance decreased, indicating defective vesicle fusion. This defect in insulin exocytosis may increase diabetes incidence in the carrier of TCF7L2 allele.[36]

CONCLUSION

This study found a significant association between the rs7903146 TCF7L2 variant and T2DM. It also showed higher frequencies of T allele carrier genotypes compared to other studies. Moreover, it provides evidence that variants in TCF7L2 rs7903146 may play a crucial role in the pathogenesis of T2DM by reducing insulin secretion.

Therefore, this study revealed that TCF7L2 is an important gene for determining susceptibility to T2DM in the studied population in Myanmar.

Limitations of study

The limitations of this study are that GLP-1 level was not measured and only a small sample size was included.

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None.

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ORIGINAL ARTICLE

Evaluation of Type 2 Diabetes Risk Variants (Alleles) in the Pashtun Ethnic Population of Pakistan

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Abstract

Objective. To evaluate the Type 2 Diabetes (T2D) risk variants in the Pashtun ethnic population of Khyber Pakhtunkhwa using nascent whole-exome sequencing (WES) to better understand the pathogenesis of this complex polygenic disorder.

Methodology. A total of 100 confirmed patients with T2D of Pashtun ethnicity were included in the study, DNA was extracted from whole blood samples, and paired-end libraries were prepared using the Illumina Nextera XT DNA library kit carefully following the manufacturer's instructions. Illumina HiSeq 2000 was used to obtain sequences of the prepared libraries followed by bioinformatics data analysis.

Results. A total of n=11 pathogenic/likely pathogenic variants were reported in the CAP10, PAX4, IRS-2, NEUROD1, CDKL1 and WFS1. Among the reported variants CAP10/rs55878652 (c.1990-7T>C; p.Leu446Pro) and CAP10/rs2975766 (c.1996A>G; p.Ile666Val) identified were novel, and have not yet been reported for any disease in the database.

The variants CAP10/rs7607759 (c.1510A>G, p.Thr504Ala), PAX4/rs712701 (c.962A>C; p.His321Pro), PAX4/rs772936097 (c.748-3delT; p.Arg325Trp), IRS-2/rs1805097 (c.3170G>A; p.Gly1057Asp), NEUROD1/rs1801262 (c.133A>G; p.Thr45Ala), CDKL1/rs77152992 (c.1226C>T; p.Pro409Leu), WFS1/rs1801212 (c.997G>A; p.Val333Ile), WFS1/rs1801208 (c.1367G>A; p.Arg456His), and WFS1/rs734312 (c.1832G>A; p.Arg611His) are previously identified in other ethnic populations. Our study reconfirms the associations of these variants with T2D in the Pakistani Pashtun population.

Conclusion. In-silico analysis of exome sequencing data suggests a statistically substantial association of all (n=11) identified variants with T2D in the Pashtun ethnic population. This study may serve as a foundation for performing future molecular studies aimed at unraveling T2D associated genes.

Key words: type 2 diabetes, risk variants, bioinformatics, whole exome sequencing, Pashtun population

INTRODUCTION

Diabetes Mellitus (DM) is a metabolic disease with its major hallmark being increased blood glucose level that affects around 6% of the world population.[1,2] It is classified into two major types namely Type 1 Diabetes (T1D) and Type 2 Diabetes (T2D), the latter accounting for 90-95% of total cases globally.[3] It is among the four lethal non-communicable diseases (NCDs) namely chronic respiratory diseases, cancer, cardiovascular diseases, and diabetes mellitus causing 1.5 million global deaths each year.[4] According to the most recent data from the International Diabetes Federation, there were approximately 463 million people living with diabetes in 2019. This number is expected to increase to 578 million by 2030 and 700 million by 2045.[5] Eighty percent (80%) of cases belong to developing nations. India, China, Pakistan, Bangladesh, Maldives, and Sri Lanka are the South Asian nations with the highest prevalence of T2D.[6-9] Pakistan, the sixth most populous nation of the world, presently occupies the seventh position in the list of nations with cases of DM, which might move up to be in the fourth position if the current circumstances continue.[4,10,11] The Pakistani population includes five major ethnic groups, namely Punjabis, Pashtuns, Sindhis, Baluchis and Refuges. Pashtuns constitute the major population of Khyber Pakhtunkhwa, where T2D is more common in urban areas as compared to rural areas of the province.[12]

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Many contributing factors have been identified including lifestyle, environmental and genetic susceptibility.[9] Although environmental and lifestyle factors contribute to the development of T2D, they do not completely explain its increased prevalence among South Asians and genetic makeup may have a significant role.[13]

Over the past decades, much work has been done on the genetic architecture of T2D to understand its pathogenesis. Currently, many risk variants in T2D associated genes have been reported in multiple ethnic populations.[14,15] These genes regulate the normal function of beta cells of the pancreas, insulin secretion, blood glucose homeostasis, insulin receptor functioning and differentiation of insulin-producing b-cells -mutations in these genes lead to the development of T2D. Genes responsible for T2D include CDKAL1, HLA-B, TCF7L2 SLC30A8, HHEX, IGF2BP2, CDKN2A/B, EXT2, FTO, NOTCH2, WFS1, IRS1, CAPN10, KCNQ1, HNF4A, TCF-2/ HNF1B and IRS-2.[16-22]

However, the expression of these genes varies in people due to the single nucleotide polymorphism (SNP) that results in differences in the occurrence of DM. Certain individuals are more prone to T2D due to the presence of susceptibility alleles resulting in different gene expressions.[10] It is hypothesized that the genetic mutation spectrum of T2D in the Pakistani population is different from others. Genome-wide association studies (GWAS) largely conducted among European descendants have identified a number of loci predisposing to T2D risk, namely CAPN10, PAX4, NEUROD1, IRS-2, CDKAL1 and WFS1 which were found to have significant association with the incidence of T2D. These genes of interest will be further investigated for T2D risk variants in Pakistani Pashtun population, using advanced Next-Generation Sequencing (NGS). For fast and accurate results, flexible multiplexing, high coverage, rapid preparation of paired-end libraries and quality sequencing Illumina Nextera XT DNA library kit was specifically employed. The Pakistani Pashtun population was selected for this study due to the limited genomic research/studies in this population. Secondly, this ethnic group has a unique life style, social values and behaviors that make it a suitable population for such studies.

METHODOLOGY

Subject description

A total of 100 confirmed T2D patients of Pashtun ethnicity were prospectively included in this pilot study. Selection of sample size with sufficient statistical power (>80%) is a critical step in carrying out genetic association studies. We computed the effective sample size using the Genetic Power Calculator developed by Purcell et al.[23] The following parameters were used for power and sample size estimation: RAF=0.10%, K=0.52%, Aa=1.5%, A&A=1.0%, d=0.9 in arriving with n=100 in our study. Prior to sample collection, patients' consent and thorough demographics were taken on a carefully designed Proforma. Inclusion criteria for study subjects were (i) diabetes diagnosed according to World Health Organization protocols, i.e., fasting blood glucose (FBS) level >126 mg/dL and random blood glucose (RBS) level >200 m mg/dL) and (ii) patients with a 30-80-year age range. The exclusion criteria were (i) presence of any chronic and infectious disease, (ii) study subjects with age not in the range of 30-80 years, and (iii) patients belonging to other nationality.

Ethical approval for the study

Ethical approval for the study was obtained from the Ethical Committee of the Department of Pharmacy of the University of Peshawar. All procedures and experiments were carried as per Helsinki declaration.

Blood samplings

Blood samples were collected from T2D patients admitted to the endocrinology department of the aforementioned hospitals in EDTA tubes (properly labeled), thoroughly mixed, and stored at -10°C in the freezer.

DNA extraction and quantification

DNA was extracted from 200 µl whole blood samples from patients with type 2 diabetes using the WizPrep DNA extraction kit (WizPrep no. W54100). DNA quantification was carried out with the help of the Qubit™ dsDNA HS Assay kit (Catalog No.Q32851) using Invitrogen Qubit™3 and the concentration is adjusted to 5 ng/µL.

DNA samples pooling

DNA samples of all 100 patients with T2D were pooled according to previously described protocols.[24-26] Pooling of DNA samples simplify the sequencing process and reduce cost and time. The constructed DNA pool containing an equimolar amount of DNA (100ng) from each individual was then subjected to further for libraries preparation and sequencing.[27]

Library preparation

Libraries were prepared following well-established library preparation protocols.[28] The Illumina Nextera XT DNA library kit (Cat. No. FC-142-1123) was used to prepare paired-end libraries (2×101-bp) carefully following the manufacturer's instructions.[29] Libraries preparation by Illumina Nextera XT DNA library kit involves initial fragmentation of genomic DNA by Transposome (genetically engineered enzymes) into randomly size DNA fragments adding known adopter sequence added to 5 prime and 3 prime ends in the process,[30] followed by a cleanup step to remove transposomes attached to DNA fragments to avoid interference in the subsequent steps and DNA amplification using 12 cycles of thermal PCR.[31] Once the PCR-amplification completed fragments

of size less than 150-200 bp (unamplified) were removed using paramagnetic beads.[32]

Next, following the capture approach strategy, the exome amplified fragments of DNA (pre-selected genomic regions of interest) were kept while nonspecified DNA fragments were removed using biotinylated probes.[33,34] Libraries were quantified to confirm final DNA concentration using Agilent 2100 Bioanalyzer (Agilent 228 Technologies). Finally, Illumina HiSeq 2000 was used to accomplish sequences of the prepared libraries. Illumina HiSeq 2000 generated sequence data was stored in FASTQ format.[35-37] WES and bioinformatics were carried out at the Center for Genomics, Rehman Institute of Medical Sciences, Hayatabad, Peshawar.

Bioinformatic analysis

We used a custom-built in-house NGS bioinformatics pipeline to move from raw sequencing data to final variant calls. FASTQ files produced by the Illumina HiSeq were filtered for low-quality reads (Q>30) using Trimmomatic software tool.[38] The filtered reads were then aligned to reference genome (hg19/GRCh37) using the Burrows Wheel Aligner (BWA).[39] Polymerase chain reaction (PCR) duplicated reads generated in the library preparation step were removed using Picard software tool. Variant calling was performed using Genome Analysis Toolkit (GATK) and SAMtools. GATK was also used to base quality control recalibration.[40] For the annotation of variants, the ANNOVER software tool was used.[41] The resulting annotated variant list generated by ANNOVER was stored in the form of a Comma-Separated Values (CSV) file having a separate column for each annotation. The CSV file was then loaded into an excel file for easy filtering, viewing and interpretation of data. All T2D risk variants identified by WES were further confirmed by Sanger Sequencing.

RESULTS

Demographic and clinical profile of participants

A total of 100 confirmed T2D patients (68 males and 32 females) aged between 30 and 80 years were included in this study. Seventy-six percent (76%) were married and 20% were single. A large proportion of study subjects were illiterate and belonged to a lower socio-economic family background. Nearly all patients (95%) showed a positive family history of T2D. Increased incidence of T2D was observed in patients who were obese and with sedentary lifestyle. Similarly, the incidence of T2D was more evident in patients from urban areas compared to rural areas from Khyber Pakhtunkhwa. For details on demographics, please see Table 1. The prevalence of comorbidities (i.e., hypertension, nephropathy, cardiovascular disease, retinopathy and foot ulcer) in study subjects is described in Table 2. The average blood pressure of patients with T2D was normal 120/80 mmHg; however, patients with co-morbidities showed elevated blood pressure 140/80 mmHg.

Table 1. Sociodemographic characteristics of the study subjects

Variables	Frequency (%)
Gender	
Male	68 (68%)
Female	32 (32%)
Age (years)	
31-40	6 (6%)
41-50	39 (39%)
51-60	34 (34%)
61-70	19 (19%)
71-80	2 (2%)
Marital status	
Single	20 (20%)
Married	76 (76%)
Widow	4 (4%)
Occupation	
Labour	25 (25%)
Government servant	10 (10%)
Farmer	21 (21%)
Driver	9 (9%)
Housewife	32 (32%)
Businessman	3 (3%)
Level of education	
Un-educated	65 (65%)
Primary	5 (5%)
Secondary	23 (23%)
University	7 (7%)
Family history of T2D	
Yes	95 (95%)
No	5 (5%)
Smoking	
Non-smoker	35 (35%)
Snuff	44 (44%)
Cigarette	21 (21%)
Exercise	
Yes	15 (15%)
No	85 (85%)
Economic status	
Good	10 (10%)
Average	35 (35%)
Below average	55 (55%)
Obesity	
Yes	69 (69%)
No	31 (31%)
Location	
Urban	75 (75%)
Rural	35 (35%)

Table 2. Prevalence of comorbidities in study subjects

Disorder	Frequency (%)
Hypertension	39%
Nephropathy	2%
Cardiovascular disease	11%
Foot Ulcer	9%
Hypocholesteraemia	21%

Variants reported in the study population

Moving beyond traditional linkage analysis and genetic association studies, we performed WES to investigate T2D risk variants in the Pashtun ethnic population of Khyber Pakhtunkhwa. Sequencing metrics (Summary of variants/SNPs identified by WES) are shown in Table 3.

Table 3. Summary of Variants/SNPs reported by WES

Total variants	Homozygous variants/SNPs	Heterozygous variants/SNPs	Deletions	Insertions	Missense SNPs	SNPs expressed in pancreas	Damaging SNPs
1048575	607572	441003	99392	74390	7710	1797	570

Table 4. Risk variants in the IRS-2, CDKAL1, PAX4, WFS1 and CAPN10 gene reported in the study subjects

Genes	Identifier	Variant	Allelic frequency	Chr.pos[1]	Genotype	Mutation	SIFT score	PolyPhen score	HGVSc
CAPN10	rs55878652	T>T/C	12.04	Chr2:240598644	Het	Missense-Variant	Deleterious (0.02)	Probably damaging (0.997)	c.1990-7T>C
CAPN10	rs2975766	A>G/G	98.98	Chr2:240598657	Hom[3]	Missense-Variant	Deleterious (0.05)	Possibly damaging (0.52)	c.1996A>G
CAPN10	rs7607759	A>A/G	11.68	Chr2:240596709	Het	Missense-Variant	Deleterious (0.04)	Possibly damaging (0.83)	c.1510A>G
PAX4	rs712701	T>T/G	67.05	Chr7:127611134	Het	Missense-Variant	Deleterious (0.02)	Possibly damaging (0.597)	c.962A>C
PAX4	rs772936097	TA>TA/T	56.01	Chr7:127611134	Het	Splice-Variant	Deleterious (0.05)	Probably damaging (0.997)	c.748-3delT
IRS-2	rs1805097	C>C/T	28.33	Chr13:109782884	Het[2]	Missense-Variant	Deleterious (0.04)	Probably damaging (0.95)	c.3170G>A
NEUROD1	rs1801262	T>C/C	77.08	Chr2:181678728	Hom	Missense-Variant	Deleterious (0)	Probably damaging (0.997)	c.133A>G
CDKAL1	rs77152992	C>C/T	8.61	Chr6:21065218	Het	Missense-Variant	Deleterious (0.02)	Probably damaging (0.997)	c.1226C>T
WFS1	rs1801212	G>G/A	88.44	Chr4: 6300792	Het	Missense-Variant	Deleterious (0.02)	Probably damaging (0.997)	c.997G>A
WFS1	rs1801208	G>G/A	6.03	Chr4: 6301162	Het	Missense-Variant	Deleterious (0)	Possibly damaging (0.671)	c.1367G>A
WFS1	rs734312	G>G/A	46.92	Chr4: 6301627	Het	Missense-Variant	Deleterious (0)	Probably damaging (0.972)	c.1832G>A

[1]Chromosome position; [2]Heterozygous; [3]Homozygous

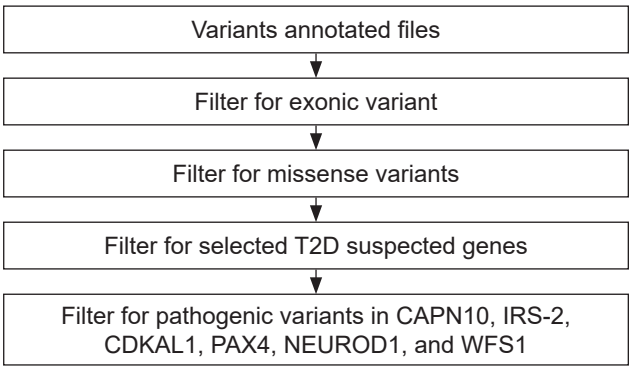

Figure 1. Variant filtration and prioritization pipeline.

The WES generates a huge amount of data that need to be filtered for easy downstream analysis. WES data was first filtered for the selected T2D associated genes (Supplementary 1) and then filtered for pathogenic variants in CAPN10, IRS-2, CDKAL1, PAX4, NEUROD1, and WFS1. The filtration pipeline is shown in Figure 1. We reported two novel missense variants/SNPs rs55878652 (c.1990-7T>C; p.Leu446Pro), rs2975766 (c.1996A>G; p.Ile666Val) and one previously reported variant rs7607759 (c.1510A>G, p.Thr504Ala) in the CAPN10 (NM_023083.3). In the IRS-2 a pathogenic missense rs1805097 (c. 3170G>A, p.Gly1057Asp) in the protein-coding region (NM_003749.2) was reported.

We reported a missense variant rs77152992 (c. 1226C>T, p.Pro409Leu) in the protein-coding region of the CDKAL1 (NM_017774.3). The reported variant was found pathogenic according to the In-Silico variants pathogenicity detection software (SIFT, PolyPhen). Similarly, two missense variants, rs712701 (c.962A>C, p.His321Pro) and rs772936097 (c.748-3delT, p.Ser424Pro) in the PAX4 (NM_006193.2) were reported. Reported variants are in the protein-coding region of the gene, heterogeneous and pathogenic. A missense variant rs1801262 (c.133A>G, p.Thr45Ala) was reported in NEUROD1 (NM_002500.4). SIFT and PolyPhen labeled this variant as pathogenic. Three heterogeneous missense variants rs1801212 (c.997G>A, p.Val333Ile), rs1801208 (c.1367G>A, p.Arg456His) and rs734312 (c.1832G>A, p.Arg611His) in the WFS1 (NM_006005.3). Reportedly, all three variants are found to be deleterious according to SIFT score (Table 4).

Validation of WES results by Sanger sequencing

The T2D risk variants identified by WES were cross-checked and validated by Sanger sequencing. The primers were designed using Primer3 version 4.0 available at (https://primer3.ut.ee/). Post-PCR amplified products were sequenced directly using Applied Biosystems' 3730XL DNA analyzer (catalog-3070XL).

DISCUSSION

T2D is a complex multifactorial disorder that is caused by a complex interaction of genetic and environmental factors.[42] Traditional linkage analysis, earlier candidate gene analysis/studies and GWAS have better explained the genetic architecture of complex polygenic disorder; however, genomic research is further revolutionized

Table 5. Risk variants identified by exome sequencing and confirmed by Sanger sequencing

SNP	Sanger Sequencing			WES			
	Chromosome position	Reference base	Variation	Chromosome position	Call	Variant frequency	Read depth
rs1805097	109782884	C	T	109782884	C>T	28.33	145
rs77152992	21065218	C	T	21065218	C>T	8.61	518
rs712701	127611134	T	G	127611134	T>G	67.05	632
rs772936097	127611134	TA	T	127611134	TA>T	56.01	228
rs1801262	181678728	T	C	181678728	T>C	77.08	497
rs1801212	6300792	G	A	6300792	G>A	88.44	512
rs1801208	6301162	G	A	6301162	G>A	6.03	124
rs734312	6301627	G	A	6301627	G>A	46.92	376
rs7607759	240596709	A	G	240596709	A>G	11.68	36
rs55878652	240598644	T	C	240598644	T>C	12.04	171
rs2975766	240598657	A	G	240598657	A>G	98.98	193

☐ Light yellow: Common bases between Sanger and Exome sequencing analysis; ☐ Light green: Same identified variant position on chromosome

by the introduction of or massively parallel or deep sequencing.[43] The NGS is one of the most important tools in identifying risk variants for T2D that have escaped detection by GWAS.[44] The most frequently implicated genes in T2D are IRS-2,[45] CDKAL1,[19] PAX4,[46] NEUROD1,[47] WFS1,[48] CAPN10,[49] ADCY5, PROX1, GCK, GCKR, DGKB,[50] TCF7L2,[51] PPARG, DUSP9, ADCY5, ARAP1, HMGA2, HNF1A, ST6GAL1, MTNR1B, HMG20A, FTO and HNF4A.[52-54] However, most of these genes associated with T2D are recognized in the European population and very few genetic studies are present in the Pakistani population. Here, we present for the first time the whole-exome sequencing in the Pakistani Pashtun population to identify possible core genes associated with T2D in this particular ethnic group.

The present study evaluated the genetic association of CAP10, PAX4, IRS-2, NEUROD1, CDKL1 and WFS1 genes with Type 2 Diabetes in the Pashtun ethnic population of KP, Pakistan**.** We reported three deleterious mutations A>A/G (rs7607759, c.1510A>G), T>T/C (rs55878652, c.1990-7T>C) and A>G/G (rs2975766, c.1996A>G) in CAPN10 gene; reported variants possibly affect the glucose haemostasis leading to T2D in the studied population. Among the reported variants, two variants namely T>T/C (rs55878652, c.1990-7T>C) and A>G/G (rs2975766, c.1996A>G) were novel mutations not reported previously in any study. The third CAPN10 variant A>A/G (rs7607759, c.1510A>G) identified in our study was previously reported in several studies carried out in different ethnic populations. One study carried out in a Tunisian population suggests strong association of rs7607759 (A>G) with T2D.[49] Two variants TA>TA/T (rs772936097, c.748-3delT) and T>T/G (rs712701, c.962A>C) in the coding region of PAX4 were reported. A large-scale meta-analysis in the Thai cohort investigated the weak association of rs712701 with T2D.[55] Genetic variations in PAX4 that has a key role in beta cells differentiation and development causes a decline in beta cells function, glucose intolerance thus act as a predisposing gene for T2D in the studied population.

Our study reported apathogenic T2D risk variant rs1805097 (also known as Gly1057Asp) in the protein-coding region of the IRS-2 gene previously reported in a study conducted in the Kurdish Iranian population. Hence, our findings support a study conducted in the Kurdish Iranian population and suggest an association of IRS-2 Gly1057Asp polymorphisms with T2D in the Pakistani Pashtun population. The IRS-2 encodes a protein the 'insulin receptor substrate 2' that mediates effects of insulin. Mutations in IRS-2 impairs normal insulin function and leads to T2D.[56] However, it is not necessary that in all ethnic populations rs1805097 of IRS-2 may increase the risk for T2D. For example, a study from Fujian, China reveals that the Insulin receptor substrate-2 (IRS-2) rs1805097, G>A polymorphism is strongly associated with colorectal cancer.[57]

Another variant of T2D susceptibility variant T>C/C (rs1801262, c.133A>G) was identified in NEUROD1a gene involved in the embryonic development of pancreatic beta cells. Mutations in the NEUROD1 leads to improper islet formation and marked reduction in beta cells number. The aforementioned mutation was already reported in a population-based genetic study in Finland.[58] Similarly, we report a heterogeneous missense mutation in the coding region of CDKL1 (C>C/T, c.1226C>T) in the studied population. This gene encodes a protein 'regulatory subunit-associated protein 1' and is broadly expressed in the beta cell and neuronal cells. Mutations in CDKL1 causes loss of pancreatic beta cell's function. More than a dozen studies conducted in a diverse population confirm that CDKL1 mutation increases susceptibility to T2D.[59,60] To our knowledge, this study is the first of its kind in the Pashtun ethnic population reporting a missense variant in CDKL1 responsible for T2D.

Furthermore, we reported three missense variants G>G/A (rs1801212, c.997G>A), G>G/A (rs1801208, c.1367G>A) and G>G/A (rs734312, c.1832G>A) in WFS1 a gene that encodes a transmembrane protein, primarily located in the endo-plasmic reticulum and highly expressed in pancreas, brain, heart and kidneys. Mutations in WFS1 are associated with T2D according to various studies conducted in different ethnic populations. All studies reveal a strong association of the aforementioned mutations with T2D.[48,61-63] Thus, our present study is an agreement with previous studies.

CONCLUSION

The present study identified a total of 11 mutations, 2 novel and 9 previously reported mutations associated with T2D in the Pashtun ethnic population using the nascent WES technology. The identified exonic mutations possibly alter the amino acid sequence and protein expression contributing to the pathogenesis of T2D. It is recommended that similar projects should be designed on a large scale to screen individuals who are genetically susceptible to T2D. Awareness campaigns on genetic and environmental risk factors should be initiated in the general public. This will help reduce/control the prevalence of the disease.

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Statement of Authorship
All authors certified fulfillment of ICMJE authorship criteria.

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Supplemental Data
Supplemental data are available upon request from the corresponding author.

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ORIGINAL ARTICLE

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Validation of Genome-Wide Association Studies (GWAS)-Identified Type 2 Diabetes Mellitus Risk Variants in Pakistani Pashtun Population

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Abstract

Objective. Recent GWAS largely conducted in European populations have successfully identified multiple genetic risk variants associated with Type 2 Diabetes Mellitus (T2DM). However, the effects conferred by these variants in the Pakistani population have not yet been fully elucidated. The objective of this study was to examine European GWAS-identified T2DM risk variants in the Pakistani Pashtun population to better understand the shared genetic basis of T2DM in the European and Pakistani cohorts.

Methodology. A total of 100 T2DM patients and 100 healthy volunteers of Pashtun ethnicity were enrolled in this study. Both groups were genotyped for 8 selected single nucleotide polymorphisms (SNPs) using the Sequenom MassARRAY® platform. The association between selected SNPs and T2DM was determined by using appropriate statistical tests.

Results. Of the 8 studied SNPs, 5 SNPs, *SLC30A8*/ rs13266634 (p=0.031, OR=2.13), *IGF2BP2*/ rs4402960 (p=0.001, OR=3.01), *KCNJ11*/ rs5219 (p=0.042, OR=1.78), *PPARG*/ rs1801282 (p=0.042, OR=2.81) and *TCF7L2*/ rs7903146 (p=0.00006, 3.41) had a significant association with T2DM. SNP *GLIS3*/ rs7041847 (p=0.051, OR=2.01) showed no sufficient evidence of association. SNPs *KCNQ1*/ rs2237892 (p=0.140, OR=1.61) and *HHEX/IDE*/ s1111875 (p=0.112, OR=1.31) showed opposite allelic effects and were not validated for T2DM risk in the study population. Among the studied SNPs, *TCF7L2*/ rs7903146 showed the most significant association.

Conclusion. Our study finding indicates that selected genome-wide significant T2DM risk variants previously identified in European descent also increase the risk of developing T2DM in the Pakistani Pashtun population.

Key words: type 2 diabetes mellitus, European GWAS, SNPs validation, replication study, Pashtun population

INTRODUCTION

Diabetes mellitus (DM) is a complex metabolic disorder with hyperglycemia as a hallmark. DM is one of the serious and common diseases of our time, causing disabling and life-threatening complications.[1,2] According to the American Diabetes Association (ADA), DM is classified into two broad forms: Type 1 Diabetes Mellitus (T1DM) and Type 2 Diabetes Mellitus (T2DM). T2DM is the most frequent subtype of DM accounting for 90% of all diabetes cases.[3] It results from the combination of defective insulin secretion and peripheral insulin resistance.[4] However, these defects or alterations are not enough to explain the complex pathophysiology of this multifaceted metabolic disorder. Other factors like obesity,[5] physical inactivity,[6] environmental,[7] and genetic factors[8] are believed to have a key role in the development of T2DM. Identification of T2DM-causing genetic and non-genetic risk factors greatly helps in the assessment and prevention of this fatal and costly disease.

In recent decades, the global burden of diabetes has significantly increased.[9] Increased prevalence of diabetes caused unprecedented health[10] and economic challenges[11] worldwide. According to the latest epidemiological data from 10th edition of the International Diabetes Federation (IDF) Diabetes Atlas, approximately 537 million people (ages 20-79 years) are living with diabetes. This number is projected to soar to 643 million by 2030 and 784 million by 2045.[12] Around 81% of people with diabetes are living in low and middle-income countries.[13] The prevalence of diabetes is not similar among different ethnicities. South Asians (people living in China, India, Pakistan, Bhutan, Nepal, Sri Lanka, and the Maldives) are at high risk of developing diabetes compared to other ancestral groups.[14,15] In terms of diabetes prevalence, Pakistan ranks 3rd behind China and India.[12] The factors for an increased prevalence of diabetes in Pakistan include rapid transition in lifestyle and a high degree of urbanization.[16,17]

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The risk of developing T2DM is strongly heritable.[18] The chances of diabetes escalate by 40% if one parent has diabetes and by 70% if both parents have diabetes.[19] Genetic studies hold great promise in predicting an individual's disease risk, exploring disease molecular pathways, and selecting treatment therapy as per individual-specific biology.[20] To date, genetic studies have provided plentiful information on the pathogenesis of T2DM.[21] GWAS identified over 400 genetic signatures associated with T2DM.[22] However, most of these large-scale genetic studies disproportionately focused on individuals of European ancestry.[23] Currently few genetic studies reporting T2DM-associated risk variants are available in South Asians.[24] T2DM is a major public health care issue in Pakistan.[25] Since genetic susceptibility plays a key role in T2DM etiology, it is important to carry out genetic studies to assess the genetic risk of T2DM in the Pakistani population. Pakistan is a developing country with limited resources for comprehensive genomic research.[26] Despite limited resources, GWAS replication studies on previously reported risk loci will help to enhance our under-standing of the genetics of T2DM in the Pakistani population. In this replication study, we attempted to investigate the 8 T2DM-associated SNPs previously identified by European GWAS in the Pashtun ethnic population of Pakistan to better understand the shared genetic basis of T2DM between Pakistanis and Europeans. The Pakistani Pashtun population was selected for this study because no comprehensive genomic research in this study group has been done. Secondly, this ethnic group has unique social values, traditions, lifestyles, and strict religious beliefs.

METHODOLOGY

Subject recruitment

A total of 200 unrelated individuals (persons with diabetes = 100 and without diabetes = 100) of Pashtun ethnicity belonging from different districts (Peshawar, Charsadda, Swat, Bannu, Kohat, Mardan, and Dir) of Khyber Pakhtunkhwa, Pakistan were included in the study. Persons with diabetes were recruited from endocrinology units of Hayatabad Medical Complex (HMC) Peshawar, Mardan Medical Complex (MMC) Mardan, and Khyber Teaching Hospital (KTH) Peshawar. Healthy volunteers that served as controls were recruited from free medical camps organized by Rehman Medical Institute (RMI) Peshawar and Khyber Medical College (KMC) Peshawar. Consent forms were taken from all the participants. Thorough demographic data, family history of diabetes, and clinical profile of all the participants were noted on a carefully designed proforma. In the case of patients and volunteers who were illiterate and/or did not understand the English language, the consent forms were read and properly explained in the local Pashtu language and then signed on their behalf by their relatives. Inclusion and exclusion of study participants were according to the previously defined criteria used for Asian populations.[27]

Inclusion criteria were (i) T2DM diagnosed as per Interna-tional Diabetes Federation-imposed etiologic classification, (ii) age above 30 years, and (iii) study subjects must be Pakistani & Pashtun ethnicity. Patients with chronic disorders like cancer and recent infections were excluded. Similarly, previously defined criteria were followed for control participants.[28] Controls were healthy volunteers from the general population with fasting blood sugars in the normal ranges. The ethical approval was obtained from the Ethical Committee of the Department of Pharmacy, University of Peshawar (Approval No. 907/PHAR). All procedures and experiments were carried out in conformity with the Helsinki declaration.

Blood sampling

Three milliliters (ml) of venous blood from the mean cubital vein was collected from each study participant with the help of a trained nurse. The blood was placed in a properly labeled ethylenediaminetetraacetic acid (EDTA) tube and was stored at -10°C.

DNA extraction

DNA was isolated from 200 μl whole blood samples using an imported WizPrep DNA extraction kit (Product code No. W54100) which gives trusted and reliable results. Quantification of DNA was conducted with the help of the Qubit™ dsDNA HS Assay kit (Catalog No. Q32851) using Introgen Qubit™, and the final DNA concentration was normalized to 10 ng/μL for genotyping.

SNPs selection

Eight T2DM-associated SNPs previously identified in different GWAS were selected to be investigated in the present study population. GWAS Catalog[29,30] which contains a high-quality curated collection of previously published large-scale genomic studies was used in the selection of SNPs. Table 1 shows detailed information on the selected SNPs

Table 1. Summary of selected T2DM risk variants curated from GWAS catalog

Variant	Allelic change	Variant description	Chromosomal position	Cytogenetic location	Mapped gene	Trait
rs13266634	C/T	Exonic	8:117172544	8q24.11	SLC30A8	T2DM
rs4402960	G/T	Intronic	3:185793899	3q27.2	IGF2BP2	T2DM
rs5219	C/T	Exonic	11:17388025	11p15.1	KCNJ11	T2DM
rs1801282	C/G	Exonic	3:12351626	3p25.2	PPARG	T2DM
rs7903146	C/T	Intronic	10:112998590	10q25.2	TCF7L2	T2DM
rs2237892	C/T	Intronic	11:2818521	11p15.4	KCNQ1	T2DM
rs1111875	T/C	Intergenic	10:94452862	10q23.33	HHEX/IDE	T2DM
rs7041847	A/G	Intronic	9:4287466	9p24.2	GLIS3	T2DM

Table 2. Socio-demographic features of cases and controls

Variables	Case n(f)	Control n(f)	*p*-value
Gender			0.061
Male	65 (65.0%)	77 (77.0%)	
Female	35 (35.0%)	23 (23.0%)	
Mean age (years)	58±12.40	56±13.43	0.951
Mean weight (kg)	62.64±6.07	59.55±8.32	0.104
Occupation			
Labourer	20 (20.0%)	14 (14.0%)	0.112
Government employee	13 (13.0%)	21 (21.0%)	
Businessman	16 (16.0%)	18 (18.0%)	
Farmer	7 (7.00%)	16 (16.0%)	
Housewife	34 (34.0%)	23 (23.0%)	
Driver	10 (10.0%)	8 (8.00%)	
Geographical area (District)			0.145
Peshawar	53 (53.0%)	19 (19.0%)	
Charsadda	13 (13.0%)	53 (53.0%)	
Swat	8 (8.00%)	7 (7.00%)	
Dir	5 (4.00%)	5 (5.00%)	
Mardan	12 (12.0%)	10 (10.0%)	
Kohat	6 (6.00%)	3 (3.00%)	
Bannu	3 (3.00%)	3 (3.00%)	
Family history of T2DM			0.012
Yes	94 (94.0%)	0 (0.0%)	
No	6 (6.00%)	100 (100%)	
Exercise			0.016
Non-exercising	85 (85.0%)	89 (89.0%)	
Walking	14 (14.0%)	4 (4.00%)	
Jogging	1 (1.00%)	5 (5.00%)	
Gym/Sports	0 (0.0%)	2 (2.00%)	
Smoking status			0.178
Cigarette smoker	19 (19.0%)	10 (10.0%)	
Snuff (smokeless tobacco)	21 (21.0%)	26 (26.0%)	
Non-smoker	60 (60.0%)	64 (64.0%)	
Diet control/compliance			0.031
Yes	50 (50.0%)	90 (90.0%)	
No	50 (50.0%)	10 (10.0%)	

kg: kilogram; T2DM: Type 2 Diabetes Mellitus; n(f): number (frequency)

retrieved from the GWAS Catalog. Selection of SNPs was based on their subsequent association with T2DM in many populations across the globe. For genotype distribution of selected SNPs in the study population, the co-dominant (additive) genetic model of inheritance was applied.

Genotyping

Sequenom MassARRAY® platform (Agena Bioscience, San Diego, CA) available at the Centre of Genomics Rehman Medical Institute, Peshawar was used for genotyping carefully following the previously described protocols.[31] SNP-Genotyping by Agena MassARRAY® platform is not only cost-effective but also generates fast, sensitive, and highly accurate results. SNPs with genotyping call rates above 90% were considered for forward analysis.

Table 3. Prevalence rates of co-morbidities among study subjects

Disease	Frequency		*p*-value
	Cases	Controls	
Hypertension	34.00%	10.00%	0.003
Ischemic heart disease	14.00%	0.00%	<0.001
Renal failure	5.00%	0.00%	<0.001
Retinopathy	61.00%	0.00%	<0.001
Hypercholesterolemia	6.00%	3.01%	0.005
Hepatitis C virus	1.00%	0.00%	<0.001
Hepatitis B virus	0.00%	0.00%	<0.001

Statistical analysis

IBM SPSS (Statistical Package for Social Sciences version 26) was used for statistical analysis. The difference in the distribution of allelic and genotypic frequencies between cases and controls was analyzed using Chi-square ($\chi 2$) test. Each SNP was tested for Hardy-Weinberg Equilibrium (HWE) using $\chi 2$ test. Odds radio (unadjusted) and 95% confidence interval were determined using logistic regression. The association analysis was performed using the Armitage trend test.[32] A *p*-value of <0.05 was considered statistically significant.

RESULTS

Subject description

The prevalence ratios of the study participants' demographics, general characteristics, and co-morbidities are described in Tables 2 and 3.[33] A greater ratio of co-morbid conditions like hypertension, renal failure, ischemic heart disease, hypercholesterolemia, nephropathy and retinopathy were observed in diabetic cases compared to controls. The majority of patients were obese and physically inactive. Most lived in the urban areas of Khyber Pakhtunkhwa. Diet compliance was recorded poor in the study subjects.

Allele/genotype frequencies of the selected SNPs

All the selected 8 SNPs were successfully genotyped (call rate >90) and were found consistent with HWE (p>0.05) (Table 4). Allelic and genotypic frequency distribution

Table 4. Genotype call rate and HWE *p*-value information of the selected T2DM risk variants

Variant	Allelic change	Mapped gene	HWE p-value		Call rate
			Cases	Controls	
rs13266634	C/T	SLC30A8	1.00	0.24	98.2
rs4402960	G/T	GF2BP2	0.50	0.2	98.5
rs5219	C/T	KCNJ11	0.78	1.00	98.6
rs1801282	C/G	PPARG	1.00	0.81	99.1
rs7903146	C/T	TCF7L2	1.00	1.00	98.6
rs2237892	C/T	KCNQ1	0.71	0.47	99.3
rs1111875	T/C	HHEX/IDE	1.00	0.82	98.5
rs7041847	A/G	GLIS3	1.00	0.66	98.5

Table 5. Allele/genotype distribution and association results of eight selected SNPs in the Pakistani case-control sample

Gene/SNP	Allele/Genotype	T2DM patients	Healthy controls	Odds Ratio (95% CI)	*p*-value	Armitage trend test OR (*p*-value)
SLC30A8/ rs13266634	C	147(73.5%)	159(79.5%)	1.73(1.04-2.86)	0.052	2.13 (0.031)
	T	53 (26.55)	41 (20.5%)			
	CC	50 (50%)	66 (66%)	Reference	—	
	CT	38 (38%)	23 (23%)	2.04 (1.11- 4.21)	0.011	
	TT	12 (12%)	11 (11%)	1.30 (0.09- 67.41)	0.771	
IGF2BP2/ rs4402960	G	95 (47.5%)	137 (68.5%)	1.92 (1.93- 5.01)	0.010	3.01 (0.001)
	T	105 (52.5%)	63 (31.5%)			
	GG	17 (17%)	39 (39%)	Reference	—	
	GT	56 (56%)	41 (41%)	3.21 (2.64-12.89)	0.008	
	TT	27 (27%)	20 (20%)	2.23 (3.71-11.41)	0.042	
KCNJ11/ rs5219	C	140 (70%)	161 (80.5%)	1.08 (0.48-0.27)	0.054	1.78 (0.042)
	T	60 (60%)	39 (19.5%)			
	CC	52 (52%)	63 (63%)	Reference	—	
	CT	34 (34%)	32 (32%)	1.43 (0.72-2.01)	0.320	
	TT	41 (41%)	05(05%)	4.48 (1.26-16.7)	0.012	
PPARG/ rs1801282	C	132 (66%)	158 (79%)	1.12 (1.02–1.71)	0.013	2.81(0.002)
	G	68 (34%)	42 (21%)			
	CC	47 (47%)	58 (58%)	Reference	—	
	CG	53 (53%)	42 (42%)	1.61 (0.36-2.91)	0.022	
	GG	0.0 (0.0%)	0.0 (0.0%)	1.01(0.02-54.91)	0.991	
TCF7L2/ rs7903146	C	88 (44%)	139 (69.5)	2.88 (1.92-4.45)	0.001	3.41 (0.00006)
	T	112 (56%)	61 (30.5)			
	CC	11 (11%)	50 (50%)	Reference	—	
	CT	66 (66%)	39 (39%)	8.01 (2.64-16.51)	0.00001	
	TT	23 (23%)	11 (11%)	9.12 (3.71-26.61)	0.00002	
KCNQ1/ rs2237892	C	147 (73.5%)	158 (79%)	1.32 (0.95-2.21)	0.192	1.61 (0.140)
	T	53 (26.5%)	42 (21%)			
	CC	48 (48%)	58 (58%)	Reference	—	
	CT	52 (52%)	42 (42%)	1.52 (0.77-2.71)	0.141	
	TT	0 (0.0%)	0 (0.0%)	1.24 (0.04-62.11)	0.980	
HHEX/IDE/ s1111875	T	139 (69.5%)	129 (64.5%)	1.41 (0.85-2.01)	0.294	1.31 (0.112)
	C	61 (30.5%)	71 (35.5%)			
	TT	26 (26%)	29 (29%)	Reference	—	
	TC	41 (41%)	50 (50%)	0.72 (0.34-1.09)	0.313	
	CC	33 (33%)	21 (21%)	1.44 (1.05-3.11)	0.181	
GLIS3/ rs7041847	A	195 (97.5%)	188 (94%)	5.12 (1.12-23.1)	0.032	2.01 (0.051)
	G	05 (2.5%)	12 (6%)			
	AA	96 (96%)	88 (88%)	Reference	—	
	AG	04 (4%)	12 (12%)	0.19 (0.05-0.96)	0.014	
	GG	0 (0.01%	0 (0.0%)	0.82 (0.02-47.21)	0.981	

between diabetic cases and healthy controls, odds ratios (OD), 95% confidence intervals (95%Cl), crude *p*-values, and Armitage trend test *p*-values of the selected SNPs are summarized in Table 5. Among the tested SNPs, five SNPs namely *SLC30A8*/ rs13266634, *IGF2BP2*/ rs4402960, *KCNJ11*/ rs5219, *PPARG*/ rs1801282 and *TCF7L2*/ rs7903146 met the significant threshold (trend's test *p*-value <0.05) and showed strong association with T2DM in the study population. SNP *GLIS3*/ rs7041847 showed no sufficient evidence of association with a *p*-value of 0.051. The remaining two SNPs (*KCNQ1*/ rs2237892 and *HHEX*/ *IDE*/ s1111875) showed opposite allelic effects and were not validated for T2DM risk in the study population. The top three significant variants in our study were *TCF7L2*/ rs7903146 (*p*=0.00006, OR=3.01) followed by *IGF2BP2*/ rs4402960 (*p*=0.001, OR=3.01) and *PPARG*/ rs1801282 (*p*=0.002, OR=2.81). *TCF7L2*/ rs7903146 apart from our study was reported as a significant risk factor for T2DM by two previous replication studies in the Pakistani sub-population.[34-36]

DISCUSSION

T2DM is a 21st-century epidemic.[37] It results from a complex interaction of genetic and environmental factors.[38] The genetic component of T2DM is well explored in European compared to other ancestral populations.[39] Despite significant progress in the genetics of T2DM around the world, limited genetic studies have been conducted in the Pakistani population where the incidence of T2DM is rapidly increasing.[34,40] To fill the gap of deficient genetic studies in Pakistan, this replication study examined European-originated genome-wide significant T2DM risk variants in the Pakistani Pashtun population to shed some light on the shared genetic basis of T2DM in European and Pakistani cohorts.

In this study, we tested the association of 8 previously GWAS implicated T2DM risk variants in the Pakistani Pashtun population and confirmed the association of 6 genetic risk variants with T2DM in the study population.

SNPs *SLC30A8*/ rs13266634, *GF2BP2*/ rs4402960, *KCNJ11*/ rs5219, *PPARG*/ rs1801282 and *TCF7L2*/ rs7903146 showed significant association (p<0.05) whereas the SNP, *GLIS3*/ rs7041847 showed no sufficient evidence of association (p=0.051). The aforementioned SNPs (*SLC30A8*/ rs13266634, *IGF2BP2*/ rs4402960, *KCNJ11*/ rs5219, *PPARG*/ rs1801282 and *TCF7L2*/ rs7903146) showed the same directional effects as reported in the earlier GWAS studies.[41-44] Two SNPs, *KCNQ1*/ rs2237892 and *HHEX/IDE*/ s1111875, showed opposite allelic effects and were not validated (p>0.05) for T2DM in the present study despite their well-documented role in the pathogenesis of T2DM in previous studies.[45-49] Conflicting results in our study population reflect the possible genetic heterogeneity that exists among different populations.

The *TCF7L2*/ rs7903146, a well-documented risk variant for T2DM[44,50], showed a significant association with T2DM in the present Pashtun population. The same was reported by two earlier studies in the Punjabi population of Pakistan.[34,35] According to Lyssenko et al.,[51] the variant *TCF7L2*/ rs7903146 increases the risk for T2DM by enhancing hepatic glucose production and impaired secretion of insulin. *TCF7L2* is a transcription factor and an important component of the Wnt signaling pathway which has a key role in blood glucose regulation involving a complex mechanism. Genetic variations in *TCF7L2* disrupt the Wnt signaling pathway and impair glucose homeostasis leading to T2DM.[52,53] Of the studied variants, *IGF2BP2*/ rs4402960 also showed notable genetic association with T2DM in our investigated population. Significant association of *IGF2BP2*/ rs4402960 with T2DM has been reported in other populations.[54,55] According to the research finding of Chistiakov and colleagues, the genetic variants in *IGF2BP2* contribute to insulin resistance in persons with diabetes.[56] Among the studied SNPs, *PPARG*/ rs1801282 was the top third hit SNP validated for T2DM association in the present study population. Consistent with our study finding, the significant association of this variant has been documented in other populations.[57,58] Cumulative evidence suggests that variants in *PPARG* have an important role in glucose and lipid metabolism.[59,60] Among them, variant rs1801282 (also known as Pro12Ala) has been extensively reviewed in different studies and repeatedly documented for T2DM susceptibility.[61,62]

Likewise, the *SLC30A8*/ rs13266634 and *KCNJ11*/ rs5219 showed a strong association with T2DM in the present Pakistani Pashtun population. Both SNPs were previously reported in East Asian, South Asian, Caucasian, and European populations.[42,63-65] The *SLC30A8* located on chromosome 8q24.11 encodes Zinc transporter 8 (ZnT-8) which is highly expressed in beta cells of the pancreas. ZnT-8 carries zinc from the cytoplasm to insulin secretory vesicles. Non-synonymous variant *SLC30A8*/ rs13266634 disrupts insulin secretion from the vesicles and increases T2DM susceptibility.[66] The *KCNJ11* gene encodes Kir6.2 protein in the ATP-sensitive potassium (KATP) channel. The KATP channels are expressed throughout the body including beta cells of the pancreas where they help in the release of insulin in response to increased blood glucose levels. The *KCNJ11*/ rs5219 polymorphism alters the normal function of KATP channels and increases the risk of developing T2DM.[67,68] Last but not least, variant *GLIS3*/ rs7041847 showed no sufficient evidence of association in our study population. *GLIS3*/ rs7041847 was previously marked as a risk factor for T2DM in the Chinese population.[69] Similar to our finding, one recent study[36] also replicated variant *GLIS3*/ rs7041847 in the Pakistani population.

CONCLUSION

In conclusion, we investigated 8 selected T2DM-associated SNPs previously identified by European GWAS in the Pakistani Pashtun population. Of the selected variants, 6 genetic variants were replicated in the study population. Among the 6 replicated variants, 5 variants showed statistically significant association (p<0.05), 1 variant showed no sufficient evidence of association (p=0.051), while the remaining 2 variants showed no association with T2DM.

The strength of the present study is that it was conducted in an understudied and genetically unique Pakistani Pashtun population. The weakness of this study is the relatively small sample size. Future large-scale genomic studies are strongly suggested in Pakistani sub-populations to unmask the genetic architecture of T2DM and develop better management strategies for the control of this fatal and costly disease.

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Statement of Authorship
All authors certified fulfillment of ICMJE authorship criteria.

Author Contributions
AJ conceived the study, designed the methodology, analyzed the data and interpreted the results. Z wrote helped in designing the methodology, programmed and developed the software and supervised the research activity planning and execution. FK conducted the investigation process, provided the resources and administered the research. RA curated the data, wrote the initial draft, finalized results, did wet lab work and prepared the data presentation.

Author Disclosure
The authors declared no conflict of interest.

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To improve and standardize reporting of findings depending on the study type, authors should ensure compliance with and submit the appropriate accomplished EQUATOR (Enhancing the QUAlity and Transparency of Research) Network Guidelines. These guidelines are freely available at: http://equator-network.org.

1. CONSORT (2010) Checklist for Reporting Clinical Trials
2. CARE (2013) Checklist for Reporting Case Reports
3. COREQ (2007) Checklist for Reporting Qualitative Research
4. PRISMA (2009) Checklist for Reporting Systematic Reviews and Meta-Analyses
5. STROBE (2007) Checklist for Reporting Observational Studies
6. STARD (2015) Checklist for Reporting Diagnostic Accuracy Studies
7. CHEERS (2013) Checklist for Reporting Economic Evaluation of Health Interventions
8. SQUIRE (2015) Checklist for Quality Improvement Reporting in Healthcare
9. ARRIVE (2013) Guidelines for Reporting Animal Research

ICMJE FORM FOR DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

In order to ensure scientific objectivity and independence, the JAFES requires all authors to make a full disclosure of areas of potential conflict of interest. Such disclosure will indicate whether the person and/or his/her immediate family has any financial relationship with pharmaceutical companies, medical equip-ment manufacturers, biomedical device manufacturers, or any companies with significant involvement in the field of health care.

Examples of disclosures include but not limited to: ownership, employment, research support (including provision of equipment or materials), involvement as speaker, consultant, or any other financial relationship or arrangement with manufacturers, companies or suppliers. With respect to any relationships identified, author(s) must provide sufficiently detailed information to permit assessment of the significance of the potential conflict of interest (for example, the amount of money involved and/or the identification of any value of goods and services).

The form is also downloadable at http://www.icmje.org/conflicts-of-interest/.

ETHICS REVIEW APPROVAL

For Original Articles, authors are required to submit a scanned soft copy of the Ethics Review Approval of their research. For manuscripts reporting data from studies involving animals, authors are required to submit a scanned copy of the Institutional Animal Care and Use Committee approval.

INFORMED CONSENT

For Case Reports, Images in Endocrinology and Clinical Case Seminars, authors are required to submit scanned soft copy of signed informed consent for publication from the involved subject/s ("Patient Consent Form"). In case the involved subject/s can no longer be contacted (i.e., retrospective studies, no contact information, et cetera) to obtain consent, the author must seek ethical clearance from the institutional board to publish the information about the subject/s.

GENERAL GUIDELINES

1. The manuscript should be encoded using Microsoft Word, double-spaced throughout with 1¼ cm (½ inch) paragraph indentation, with 3-cm margins (1¼ inch) all around on A4 size paper. The preferred font style and size is Times New Roman 12.
2. The manuscript should be arranged in sequence as follows: (1) Title Page, (2) Abstract, (3) Text, (4) References, (5) Tables, and (6) Figures & Illustrations.
3. References should pertain directly to the work being reported.
4. All the sheets of the manuscript should be labelled with the family name of the main author (all in capital letters) and page number (in Arabic Numerals) printed on the upper right corner.
5. All manuscripts not complying with the above shall be promptly returned for correction and resubmission.

Title Page

1. The title should be as concise as possible.
2. Only the full names of the authors directly affiliated with the work should be included (First name, Middle initial and Last name). There are 4 criteria for authorship (ICMJE recommendations):
   - 2.1. Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND
   - 2.2. Drafting the work or revising it critically for important intellectual content; AND
   - 2.3. Final approval of the version to be published; AND
   - 2.4. Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.
3. The highest educational attainment or title of the authors should be included as an attachment whenever appropriate.
4. Name and location of no more than one (1) institutional affiliation per author may be included.
5. If the paper has been presented in a scientific forum or convention, a note should be provided indicating the name, location and date of its presentation.

Abstract

For original articles, the abstract should contain no more than 200 words with a structured format consisting of the objective/s, methodology, results and conclusion. For feature articles, case reports, interhospital grand rounds, and brief communications, the abstract should be from 50 to 75 words and need not be structured.

Keywords

At least 3 keywords but no more than 6, preferably using terms from the Medical Subject Headings (MeSH) list of Index Medicus, should be listed horizontally under the abstract for cross-indexing of the article.

Text

1. Generally, the text should be organized consecutively as follows: Introduction, Methodology, Results and Discussion, and Conclusion (IMRAD format).
2. All references, tables, figures and illustrations should be cited in the text, in numerical order.
3. All abbreviations should be spelled out once (the first time they are mentioned in the text) followed by the abbreviation enclosed in parentheses. The same abbreviation may then be used subsequently instead of the long names.
4. All measurements and weights should preferably be in System International (SI) units.
5. If appropriate, information should be provided on institutional review board/ethics committee approval.
6. Acknowledgments to individuals/groups of persons, or institution/s should be included at the end of the text just before the references. Grants and subsidies from government or private institutions should also be acknowledged.

References

1. References in the text should be identified by Arabic Numerals in superscript on the same line as the preceding sentence.
2. References should be typed double-spaced on a separate sheet. They should be numbered consecutively in the order by which they are mentioned in the text. They should not be alphabetized.
3. All references should provide inclusive page numbers.
4. Journal abbreviations should conform to those used in PubMed. Include PMID, PMCID and DOI of the references.
5. A maximum of six authors per article can be cited; beyond that, name the first three and add "et al."
6. The style/punctuation approved by JAFES conforms to that recommended by the International Committee of Medical Journal Editors (ICMJE) available at http://www.icmje.org. Follow the format of the examples shown below:

   Journal Article

   Padua FR, Paspe MG. Antinuclear antibody in the rheumatic and non-rheumatic diseases among Filipinos. Acta Med Philipp. 1990; 26(2):81-5.

   One to Six Authors (Commentary, Online)

   Krause RM. The origin of plagues: Old and new. Science. 1992;257:1073-8. PMID: 1509258. https://doi.org/10.1126/science.257.5073.1073.

Barry JM. The site of origin of the 1918 influenza pandemic and its public health implications. [Commentary]. J Translational Med. January 20, 2004;2(3):1-4. http://www.translational-medicine.com/content/2/1/3. Accessed November 18, 2005.

Mokdad AH, Bowman BA, Ford ES, Vinicor F, Marks JS, Koplan JP. The continuing epidemics of obesity and diabetes in the US. JAMA. 2001;286(10):1195-200. PMID: 11559264. https://doi.org/10.1001/jama.286.10.1195.

More than Six Authors

McGlynn EA, M.Asch S, Adams J, et al. The quality of health care delivered to adults in the United States. N Engl J Med. 2003;348(26):2635-45. PMID: 12826639. https://doi.org/10.1056/NEJMsa022615.

Jasul Jr. GV, Paz-Pacheco E, Jimeno CA, et al. AFES A.S.-O.N.E.: ASEAN Survey Of Needs in Endocrinology in the time of the COVID-19 pandemic. J AFES Fed Endocr Soc.2020;35(1):5-13. PMID:33790494. PMCID: PMC7992306. https://doi.org/10/15605/jafes.035.01.10.

Authors Representing a Group

Moher D, Schulz KF, Altman D; for the CONSORT Group. The CONSORT statement: Revised recommendations for improving the quality of reports of parallel-group randomized trials. JAMA. 2001;285(15):1987-91. PMID: 11308435. https://doi.org/jama.285.15.1987.

Book

Byrne, DW. Publishing your medical research paper: What they don't teach in medical school. Baltimore: Williams & Wilkins, 1998.

World Wide Web

The key and critical objectives of JAMA. http://jama.ama-assn.org/misc/aboutjama.dtl. Accessed April 4, 2007.

Tables

1. Cite all tables consecutively in the text and number them accordingly.
2. Create tables preferably using Microsoft Excel with one table per worksheet.
3. Tables should not be saved as image files.
4. The content of tables should include a table number (Arabic) and title in capital letters above the table, and explanatory notes and legends as well as definitions of abbreviations used below.
5. Font should be Arial Narrow size 8.
6. Each table must be self-explanatory, being a supplement rather than a duplicate of information in the text.
5. Up to a maximum of five (5) tables are allowed.

Figures and Graphs

1. Figures or graphs should be identified by Arabic Numeral/s with titles and explanations underneath.
2. The numbers should correspond to the order in which the figures/graphs occur in the text. It is recommended that figures/graphs also be submitted as image files (preferably as .tif, .jpeg, or .png files) of high resolution (at least 300 dpi).
3. Editable figures or graphs can also be created using Microsoft Word.
4. Provide a title and brief caption for each figure or graph. Caption should not be longer than 15-20 words.
5. All identifying data of the subject/s or patient/s under study such as name or case numbers should be removed.
6. Up to a maximum of five (5) figures and graphs are allowed.

Illustrations and Photographs UPDATE

1. Where appropriate, all illustrations/photographic images should be at least 600 dpi and submitted as image files (preferably as .tif, .jpeg, or .png files). All images should be checked for resolution. Right click on the image and click properties to see the resolution.
   1.1 There should be minimal processing of digital images submitted with a manuscript for review to the JAFES. A certain degree of image processing (lighting, color, contrast, size, orientation, cropping, placement of identifying markers and labels) is deemed acceptable only if the final image correctly and accurately represents the original information or data. Thus, JAFES requires all unprocessed, unaltered, and raw image files to be submitted with the manuscript to facilitate evaluation and review. These shall serve also as JAFES' records for issues that may arise after publication of the manuscript.
   1.2 Adjustments in brightness, color balance, or contrast should be applied equally to the whole image and should not result in the exclusion, hiding, obscuring, or deletion of any information that is present in the original image, enhancement of any particular portion of the image. Manipulations such as grouping of images for comparison should be indicated with image margins or clear demarcations, and must be described in the caption. Other types of manipulation such as copying and pasting of images and passing them off as multiple figures is not acceptable. Appropriate re-orientation of the whole image, as well as superimposition of arrows, markers, or other figures and labels is acceptable.
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   2.1 All image adjustment and processing tools/software used should be disclosed in the methodology section of original articles or described in the caption or description if in article types without a separate section on methods.
3. Computer-generated illustrations which are not suited for reproduction should be professionally redrawn and digitized (preferably as .tif, .jpeg, or.png files) at least 600 dpi. All letterings for illustrations should be done professionally and be of adequate size to remain readable ever after size reduction during layout.
4. All letterings for illustration should be done professionally and should be of adequate size to retain even after size reduction.
5. Figure legends should be numbered sequentially, typed double-spaced on a separate sheet of paper. Give the meaning of all symbols and abbreviations used in the figure.
6. Up to a maximum of five (5) illustrations/photographs are allowed.

N.B.: For tables, figures, graphs, illustrations, and photographs that have been previously published in another journal or book, a note must be placed on the specific item stating that such has been adapted or lifted from the original publication and referenced in the **References** portion. Appropriate copyrights and permissions should be secured from the original author/publisher.

PROCESS

1. Upon receipt of the manuscript, the Editor shall review the submission, check if it has met aforementioned criteria and consult with members of the Editorial Board to decide whether it shall be considered for publication or not.
2. Within one (1) week of submission, authors shall be notified through e-mail that their manuscript either (a) has been sent to referees for peer-review or (b) has been declined without review.
3. The JAFES implements a strict double blind peer review policy. Each manuscript is referred to two (2) peer reviewers who are deemed as subject experts. A third reviewer may be needed in case there is discordance in the peer reviewer recommendation. The manuscript is routinely referred to the JAFES in-house statistician to check appropriateness and validity of data analysis and conclusions. In addition, the manuscript is also referred to the JAFES in-house radiologist or pathologist for review if there are diagnostic imaging studies or microscopic images, respectively. The JAFES Editor-in-Chief makes the final decision.
4. For manuscripts that are reviewed, authors can expect an initial decision within forty five (45) days after submission. There may be instances when decisions can take longer than 45 days, in such cases, the editorial assistant shall inform the authors. The editorial decision for such manuscripts shall be one of the following: (a) acceptance without further revision, (b) acceptance with minor revisions, (c) major manuscript revision and resubmission, or (d) not accepted for publication.
5. Accepted manuscripts are subject to editorial modifications to bring them in conformity with the style of the journal.

EDITORIAL OFFICE CONTACT INFORMATION:

Journal of the ASEAN Federation of Endocrine Societies
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Website: https://www.asean-endocrinejournal.org

ARTICLE TYPES

Original Articles
The abstract should contain no more than 200 words with a structured format consisting of the objective/s, methodology, results and conclusion. A manuscript for original articles should not exceed 25 typewritten pages (including tables, figures, illustrations and references) or 6000 words.

Reviews
Review articles provide information on the "state of the art." JAFES encourages that reviews not only summarize current understanding of a particular topic but also describe significant gaps in the research, and current debates. The abstract should be from 50 to 75 words and should not be structured. A manuscript for reviews should not exceed 15 typewritten pages (including tables, figures, illustrations and references) or 4000 words.

Case Reports / Case Series
The abstract should be from 50 to 75 words and should not be structured. A manuscript for case reports or case series should not exceed 10 typewritten pages (including tables, figures, illustrations and references) or 3000 words.

Feature Articles
JAFES may feature articles, either as part of an issue theme, such as Summary Clinical Practice Guidelines on endocrinology from each AFES country society, or a special topic on endocrinology by an international expert or authority. The abstract should be from 50 to 75 words and should not be structured. A manuscript for feature articles should not exceed 25 typewritten pages (including tables, figures, illustrations and references) or 6000 words.

Endocrine Perspectives
JAFES may invite topic experts to publish viewpoints, opinions, and commentaries on relevant topics. A manuscript for endocrine perspectives should not exceed 10 typewritten pages (including tables, figures, illustrations and references) or 3000 words. *Not peer reviewed.

Interhospital Grand Rounds
JAFES encourages submission of special articles that summarize and document the proceedings of endocrinology grand rounds, which includes presentation of medical problems of a particular patient, evaluation and work-up, treatment and clinical course, discussion of key diagnostic and management points, and commentaries by specialty experts. JAFES recognizes the importance of this type of article as an educational tool for physicians and health practitioners. The abstract should be from 50 to 75 words and should not be structured. A manuscript for grand rounds should not exceed 25 typewritten pages (including tables, figures, illustrations and references) or 6000 words.

Brief Communications
Brief Communications are short reports intended to either extend or expound on previously published research OR present new and significant findings which may have a major impact in current practice. If the former, authors must acknowledge and cite the research which they are building upon. The abstract should be from 50 to 75 words and should not be structured. A manuscript for brief communications should not exceed 5 typewritten pages (including tables, figures, illustrations and references) or 1500 words.

Images in Endocrinology
Images may include photographs of clinical cases encountered and documented during practice. They may also include diagnostic images (e.g., photomicrographs of histopathologic diagnosis, radiographs) or special studies performed (e.g., spectral karyotype imaging, fluorescent microscope images, immunostains) that aided in diagnosis. A 250-word text should accompany the images. Submissions to this category should comply with the journal's image integrity guidelines.

Editorials
Articles that represent the scientific opinion and views of an author. Every issue of JAFES includes an Editorial by the Editor-in-Chief and may include one or two additional editorials from experts from the scientific community commenting on a particular field or issue on endocrinology. No abstract or keywords necessary.

Letters to the Editor
JAFES welcomes feedback and comments on previously published articles in the form of Letters to the Editor. No abstract or keywords necessary. A Letter to the Editor must not exceed 2 typewritten pages or 500 words.

Special Announcements
Special announcements may include upcoming conventions, seminars or conferences relevant to endocrinology and metabolism. The Editors shall deliberate and decide on acceptance and publication of special announcements. Please coordinate with the Editorial Coordinator for any request for special announcements.

Checklist Guide for Submission of Manuscripts to JAFES	
Instructions to Authors	☐ Review manuscript submission guidelines
Cover Letter	☐ Include cover letter as an attachment ☐ Indicate in the letter the title of the work ☐ Indicate all the authors (complete names, affiliations, ORCID iD, specific role/s in writing the manuscript and e-mail address) ☐ Indicate in the letter the Corresponding author: and provide complete contact information (post address, telephone, fax number, e-mail address)
EQUATOR Network Guidelines	☐ Review manuscript if compliant with appropriate EQUATOR Network Guidelines and submit checklist (e.g., CONSORT for clinical trials, CARE for case reports)
Author Form	☐ Ensure all authors have read and agreed to the following: (1) the Authorship Certification, (2) the Author Declarations, (3) the Author Contribution Disclosure, and (4) the Author Publishing Agreement, and (5) the Conversion to Visual Abstract (*optional for original articles) ☐ Submit a scanned copy of the fully accomplished form
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Patient Consent Form (if applicable)	☐ For Case Reports, Images in Endocrinology and Clinical Case Seminars, submit a scanned copy of the fully accomplished form; otherwise, obtain appropriate ethical clearance from the institutional review board.
Title Page	☐ Full names of the authors directly affiliated with the work (First name and Last name), highest educational attainment ☐ Name and location of 1 institutional affiliation per author ☐ If presented in a scientific forum or conference, provide a footnote should be provided indicating the name, location and date of presentation
Abstract	☐ Provide an abstract conforming with the format ☐ Structured for Original Articles: Objective/s, Methodology, Results, Conclusion ☐ Unstructured for Case Reports and Feature Articles
Keywords	☐ Provide 3-5 keywords (listed in MeSH)
Content	☐ Provide text/content in IMRAD format (Introduction, Methodology, Results and Discussion, Conclusion) ☐ Make sure all abbreviations are spelled out once (the first time they are mentioned in the text) followed by the abbreviation enclosed in parentheses; the same abbreviation may then be used subsequently ☐ Make sure all measurements and weights are in SI units ☐ If appropriate, provide information on institutional review board/ethics review committee approval ☐ Acknowledgments to individuals/groups of persons, or institution/s should be included at the end of the text just before the references; grants and subsidies from government or private institutions should also be acknowledged
References	☐ All references should be cited in the text, in numerical order. Use Arabic numerals ☐ Ensure all references follow the prescribed format
Tables, Figures, Illustrations and Photographs	☐ All tables, figures, illustrations and photographs should be cited in the text, in numerical order per type ☐ Provide separate files for tables, figures and illustrations ☐ Provide a title and legend (if appropriate) for each table ☐ Provide a title, legend (if appropriate), and caption for each figure and illustration (caption should be no longer than 15-20 words) ☐ If table, figure, or illustration is adapted, state so and include the reference.

Journal of the
ASEAN Federation of
Endocrine Societies

Cover Letter

(Date)

To: **The Editor-in-Chief**
Journal of the ASEAN Federation of Endocrine Societies (JAFES)

Subject: **SUBMISSION OF MANUSCRIPT FOR PUBLICATION**

We intend to publish the manuscript/, entitled "_____," under the Section *[Original Article, Review Article, Feature Article, Case Report, Case Series, Interhospital Grand Rounds, Brief Communications, Letter-to-the-Editor, Special Announcements]* in the Journal of the ASEAN Federation of Endocrine Societies.

LIST OF AUTHORS

Complete Name	Position/ Designation	Institutional Affiliation	Role in writing the manuscript	Email address	ORCID iD

On behalf of all the authors, I shall act as the corresponding author with the journal from this point onward.

Attached herewith are the following: the completely accomplished **Author Form with author contribution disclosure** and **author publishing agreement**, in which all the authors certified authorship criteria was satisfactorily met and the specific contributions of the authors are listed and the author copyright is retained granting publishing and distribution rights to the JAFES; the **Author Declaration** that the work is original and is not under simultaneous consideration in other journals and the **ICMJE Disclosure forms** of ALL the authors (*where all conflicts of interest have been declared/there are no conflicts of interest*).

For original articles, we submit a scanned copy of our Ethics Review Approval/registration in trial registries (as appropriate) and the appropriate EQUATOR Network checklist used in writing the manuscript.

For case reports/series, patient consent forms have been secured for the publication of information.

For animal studies, a scanned copy of the Institutional Animal Care and Use Committee approval was obtained.

Furthermore, we respectfully suggest the following **reviewer**(s) for our manuscript.

Name and Salutation (e.g., Prof., Dr., etc)	Position/Designation	Institutional Affiliation and specialization	Email address

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Author Form

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Based on International Committee of Medical Journal Editors (ICMJE) Criteria for Authorship.

In consideration of our submission to the Journal of the ASEAN Federation of Endocrine Societies (JAFES), the undersigned author(s) of the manuscript hereby certify, that all of us have actively and sufficiently participated in:

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3. AUTHOR CONTRIBUTION DISCLOSURE (Place an "x" mark where an author made the contribution)
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Specific Contributor role	Author 1	Author 2	Author 3	Author 4	Author 5	Author 6	Author 7	Author 8	Author 9	Author 10
Conceptualization Ideas; formulation or evolution of overarching research goals and aims.										
Methodology Development or design of methodology; creation of models										
Software Programming, software development; designing computer programs; implementation of the computer code and supporting algorithms; testing of existing code components										
Validation Verification, whether as a part of the activity or separate, of the overall replication/reproducibility of results/experiments and other research outputs										
Formal analysis Application of statistical, mathematical, computational, or other formal techniques to analyze or synthesize study data										
Investigation Conducting a research and investigation process, specifically performing the experiments, or data/evidence collection										
Resources Provision of study materials, reagents, materials, patients, laboratory samples, animals, instrumentation, computing resources, or other analysis tools										
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Visualization Preparation, creation and/or presentation of the published work, specifically visualization/data presentation										
Supervision Oversight and leadership responsibility for the research activity planning and execution, including mentorship external to the core team										
Project administration Management and coordination responsibility for the research activity planning and execution										
Funding acquisition Acquisition of the financial support for the project leading to this publication										

For submissions to the JAFES to be accepted, all authors must read and accomplish the JAFES Author Forms consisting of: (1) the Authorship Certification, (2) the Author Declarations, (3) the Author Contribution Disclosure, and (4) the Author Publishing Agreement. The completely accomplished JAFES Author Forms shall be scanned and submitted along with the manuscript. No manuscript shall be received without the completely accomplished JAFES Author Forms.

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1. Zaccardi F et al. *Diabetes Obes Metab.* 2020 Aug 5. Doi:10.1111/dom.14169

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