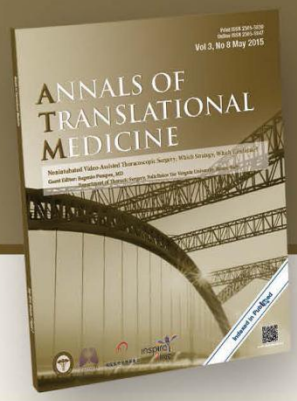
ANNALS OF TRANSLATIONAL MEDICINE



Peer Review File

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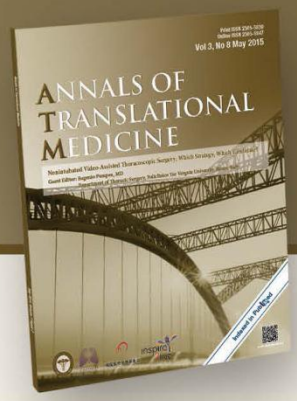
Reviewer:

Comment 1: Title. I suggest that the title should be more conclusive. The authors evaluate both, candidate genes for obesity and those for psychiatric disorders.
Reply 1: Thank you for your suggestion. We agree with you that the title should include obesity and psychiatric disorders. However, we feel that neuroendocrine disorder is more suitable than psychiatric disorder due to the reason that not all the candidate genes showed significant association with either psychiatric disorder or obesity in our population. Therefore, the revised title for this manuscript should be "Genetic polymorphisms in neuroendocrine disorder-related candidate genes associated with pre-pregnancy obesity in gestational diabetes mellitus patients by using a stratification approach" (Page 1, Line 1-3).
Changes in the text 1:
Genetic polymorphisms in neuroendocrine disorders-related candidate genes associated with pre-pregnancy obesity in gestational diabetes mellitus patients by using a stratification approach

Comment 2: Introduction. The term "sceptical" must be revised.
Reply 2: Thank you for your comment. We have removed the term "sceptical" from the sentence. The changes in the text were shown below (Page 5, Line 74-90).
Changes in the text 2:
Pre-pregnancy obesity is a major burden throughout the world, especially in developing countries (1). ~~because~~ Obesity in increasing ~~in trend~~ worldwide, especially in ~~the~~ Asia~~n~~ ~~region~~ (2) and the prevalence of obesity among women is higher than in men a~~re more than in men~~ (3). Pre-pregnancy obesity is a predictor of adverse pregnancy outcomes, with many studies reporting that pre-pregnancy obesity is associated with higher odds of having gestational diabetes mellitus [odd ratio (OR)=3.98]; gestational hypertension disorders (OR=3.68); preeclampsia (OR=3.20), macrosomia (OR=2.17) (4-6); preterm delivery [relative risk (RR)=1.35]; and caesarean section (RR=1.66) as compared to women with normal weight (6).

Studies have reported that pre-pregnancy obesity is associated with dietary preference, sedentary lifestyle and lack of awareness in metabolic management (7,8), however ~~these findings are sceptical and how~~ the underlying mechanism for these associated factors can influence metabolism in women still remains unclear. Genetic factors are now regarded as a highly plausible explanation for explaining the association between pre-pregnancy obesity and



aforementioned associated factors (9-11) as studies have shown that genetic factors had contributed e to 40% to 70% of variation in the risk of developing obesity (9-12).

Comment 3: Recently, there is increasing support for the notion that obesity is a neuroendocrine disorder in which both genetic predisposition and environmental risk factors act in concert. Why did the authors not consider the Fat Mass–and Obesity-Associated (FTO) gene analysis? In large genome-wide association studies, the strongest genetic signal for BMI has been in the FTO locus. Eighty-nine genetic variants within introns 1 and 2 of FTO have been associated with BMI. The authors should explain in a little more detail why they chose these thirteen specific single nucleotide polymorphisms (SNPs) for their study.

Reply 3: Thank you for your comments. First, we agree with you that FTO locus has many significant genes associated with BMI as supported by large genome-wide association studies, therefore to yield a significant association using genes in FTO locus among GDM mother with obesity would within our expectation. Second, the reason why we choose thirteen specific SNP related to psychiatric disorders was because study had shown that maternal obesity increase maternal leptin, insulin, glucose, triglycerides, and inflammatory cytokines which leads to alterations in serotonergic system, dopaminergic system and hypothalamic pituitary adrenal axis. Therefore, it increased risk of behavioral and mental health disorders. However, there is lack of study examine association between genetic polymorphisms in psychiatric disorders-related candidate genes associated with pre-pregnancy obesity in gestational diabetes mellitus patients. We have now added explanations on the reasons why we chose these thirteen specific single nucleotide polymorphisms (SNPs) for our study in Introduction section as follow (Page 6, Line 101-115).

Changes in the text 3:

This custom SNPs provides excellent coverage of many previously tested neuroendocrine disorder-related candidate genes for obesity, including BDNF (16,17), FKBP5 (18), NPY5R (19), EPHX2 (20) and TPH2 (21). In contrast, genetic association studies of obesity with the following neuroendocrine disorder-related candidate genes, such as ANO2 (22), HTR2C (23), LHPP (24), NRG1 (25), OXTR (26), RORA (27), SDK2 (22), TEX51 (22) and PLEKHG1(22) have not been evaluated. It is well known that obesity is closely related to ~~with~~ psychiatry symptoms, since a large proportion of individuals with psychiatric symptoms such as depression or anxiety also tend to be obese (28-30); Similarly, those who are obese are at higher risk of developing depression or anxiety symptoms (28,31,32). In addition, there is increasing support for the notion that obesity is a neuroendocrine disorder in which increased leptin, insulin, glucose, triglycerides, and inflammatory cytokines lead to alterations in hypothalamic pituitary adrenal axis, serotonergic and dopaminergic system, increasing the risk of behavioural and mental health disorders (33-35). Thus, the relevance of neuroendocrine disorders-related candidate genes in ~~as~~ predisposa~~ling~~ for pre-pregnancy obesity is worth investigating.

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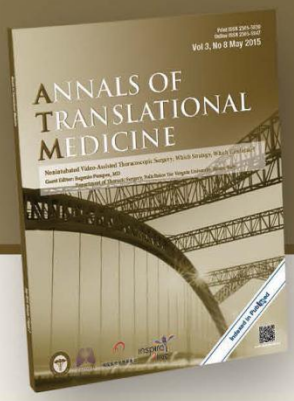


Comment 4: Materials and methods, authors should better describe how they conducted the anthropometric evaluation. It is not specified the time of sampling. It is not described the basis for the sample size population. There is no information about physical activity

Reply4: Thank you for your comments. We have now added sentences on how anthropometric data were obtained as well as specific time of sampling and sample size calculation. However, we didn't capture information of physical activity of respondents in this study. We have addressed this in study limitation.

Changes in the text 4: Materials and Methods. The revised text for this comment is shown below (Page 7-11, Line 125-244).

2 Materials and Methods

2.1 Study population

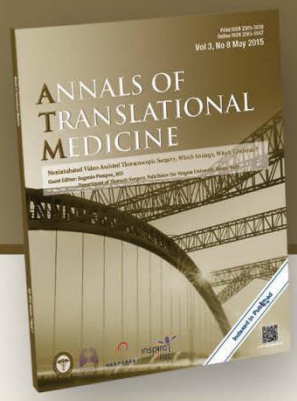
We performed a post-hoc case-control analysis of a cross-sectional study among GDM women (n=312) to check for candidate SNPs that may be associated with obesity in this particular population according to the Asian and International criteria-based BMI.

The study participants were women with GDM who were enrolled for a cross-sectional study (37). All participants were native Malaysian with GDM and residents of surrounding areas. They were ~~and~~ recruited during ~~at~~ second or third trimester care at two tertiary hospitals in Klang Valley, Malaysia between 1st June 2018 and 31st October 2018. The inclusion criteria were previously described in the study by Lee et al., 2019 (37). In brief, the participant must be a Malaysian woman , pregnant, ~~aged ≥~~ 18 years of age or older ~~old~~ and with a diagnosis of GDM according to Malaysian Clinical Practice Guidelines (38,39).

2.2 Socio-demographic background and clinical characteristics

Socio-demographic backgrounds and clinical characteristics were recorded at enrollment to obtain information related to maternal profile, past-obstetrics history, concurrent medical problems, family history and psychiatric symptoms (including depression, anxiety and stress). These data were obtained from the self-administered questionnaire and ~~also~~ medical records.

2.3 Measurement of pre-pregnancy obesity



The anthropometric data of participants were obtained from each mother's health records. Pre-pregnancy weight and height were self-reported by the pregnant mothers and recorded by a medical assistant during the first antenatal booking. Pre-pregnancy obesity is defined as women ~~who have~~ with a BMI ≥ 30 kg/m^2 before the pregnancy visit by using the international BMI classification (40). It is calculated by dividing weight at pre-pregnancy weight in kilograms (Kg) by height in meters squared (m^2)(41). Body mass index (BMI) ~~is a ratio of weight to height and~~ it is used to estimate the total body fat and assesses the risk for diseases related to increased body fat. The WHO criteria for International criteria-based BMI classifies a BMI of <18.5kg/m^2 as underweight; 18.5-24.9kg/m^2 (as normal); 25.0-29.9kg/m^2 (overweight); and >30kg/m^2 as obese (42-44).

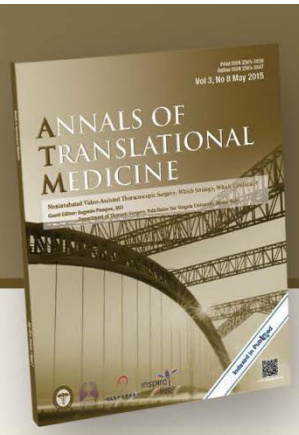
Studies have showed that Asian people may have increased health risks at a lower BMI compared to Caucasians; therefore, the Asian criteria-based BMI was modified specifically for Asian adults. ~~, in which the~~ Its cut-off points are lower than those defined for International criteria. For instance, WHO recommended cut-points for Asian criteria-based BMI categories as follows: <18.5kg/m^2 (underweight); 18.5-22.9kg/m^2(normal); 23.0-27.4kg/m^2(overweight) and ≥ 27.5 (obesity) (45,46). ~~in which these~~ This categorizing scheme ~~is same with~~ follows National Institute for Health and Care Excellence (NICE) ~~guidelines~~ recommendations for Asians (47,48).

2.4 Participants

Regarding patients and controls, we analyzed the association between candidate genes and obesity using two different criteria-based BMI categories which are the Asian and International criteria based BMI categories. Participants in control group were those patients with normal weight and those overweight as defined using BMI value, while participants in the patient group were those defined as being obese. Upon completion of sample collection and analysis, data for baseline BMI and polymorphisms of candidate genes were readily available for a total of 312 participants.

2.5 Study outcomes, predictors and potential confounders

The study outcomes were association between genetic polymorphism in neuroendocrine disorder-related candidate genes and pre-pregnancy obesity. The association was presented in crude odd ratio and adjusted odd ratio (95% Confidence interval). The predictors in this study were neuroendocrine disorder-related candidate genes. The potential confounders were socio-demographic background and clinical characteristics.



2.6 Blood sample collection, DNA extraction and Mass-array genotyping

~~The~~ Detailed blood sampling and DNA extraction methods have been previously described (49). In brief, 5 mL of blood samples of participants were collected by a phlebotomist and genomic DNA was isolated by using the QIAamp Blood DNA Mini Kit (QIAGEN, Hilden, Germany). The genotyping analysis for ~~of~~ candidate genes polymorphism was conducted using the Agene ® MassARRAY platform. SNP analysis performed using a ~~was done with~~ Typer Analyzer.

2.7 Bias

We performed Bonferroni correction for multiple statistical significance tests to minimize bias arising from multiple testing errors.

2.8 Sample size calculation

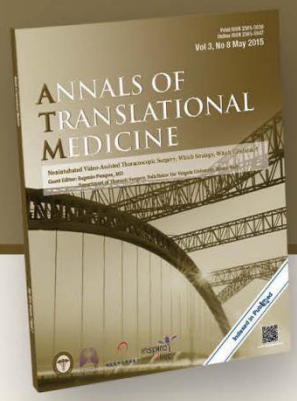
The sample size was calculated using the following formula: $n = Z_{\alpha/2}^2 p\hat{}(1 - p\hat{}) / e^2$ (50).
Let $p\hat{}$ = population proportion of class of interest, here $p\hat{} = 0.237$ (16); $Z_{\alpha/2}$ = population distribution for one sided test; and E = maximum error allow, say 0.07 (50).
$n = Z_{\alpha/2}^2 p\hat{}(1 - p\hat{}) / e^2$
If $Z_{\alpha/2}(0.95) = 1.96$; $p\hat{} = 0.237$ and $e = 0.07$, then the sample size is:
$n = (1.96)^2(0.237)(0.763) / (0.07)^2$
$n = 3.8416(0.1808) / 0.005$
$n = 0.6946 / 0.005$
n = 139. Thus, around 139 obese GDM women to estimate p with 95% CI was needed.

2.9 Quantitative variables

Data on socio-demographic background, clinical characteristics and candidate genes are presented in term of N (%). Dependent variables were categorized into two groups: normal or overweight group and obese group. Data on age and monthly family income are presented in mean ± standard deviation.

2.10 Statistical analysis

We used IBM SPSS Statistics version 21.0 to perform the data analysis. A ~~The~~ chi-square goodness-of-fit test was performed to assess the agreement of the genotype distribution among candidate genes using Hardy–Weinberg equilibrium, in which if the p-value for chi-square goodness-of-fit tests is significant ($p<0.05$), the population is not in Hardy-Weinberg



equilibrium. If the genotype distribution of candidate genes does not fit ~~to~~ Hardy-Weinberg equilibrium based on equal distribution, the expected values for genotype distribution will be adjusted according to the global population. Univariate analysis was used to analyse the association between candidate genes and obesity among the GDM mothers. Significant difference is set at a p-value < 0.05. In addition, we tested the candidate gene polymorphism associations with obesity and any polymorphism adjusted for socio-demographical and clinical moderator effects. Variables with a p-value of less than 0.25 in univariate analysis ~~were subjected for a~~ underwent Bonferroni correction for multiple statistical significance tests. Variables with p-value of less than 0.25 after a ~~an~~ Bonferroni adjustment were entered into the multiple logistic regression analysis (51), adjusting for the fact that a rigidly set p-value at <0.05 may miss many clinically important variables (52,53). A backward stepwise regression method was used (54). All analyses were made with a 95% CI, and the level of significance was set at $p < 0.05$.

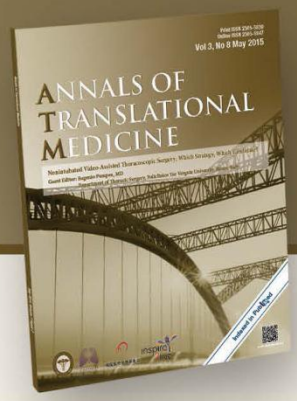
2.11 Ethical consideration

The study was approved by The Medical Research Ethics Committee, Ministry of Health Malaysia (No.: NMRR-17-2264-37814) and informed consent was taken from all the patients.

Changes in the text 4: Discussion. The revised text for this comment is shown below (Page 16, Line 344-348).

Limitations may also be present in ~~exist with~~ our study. The association between candidate genes and pre-pregnancy obesity traits could be modulated by the gene-diet-lifestyle interactions; however, information on diet intake, lifestyle factors and physical activity was not captured in this study. Therefore, the association between candidate genes and pre-pregnancy obesity as shown in this study should be interpreted cautiously.

Comment 5: Discussion. If authors aimed to investigate candidate genes for psychiatry symptoms as predisposing for obesity in this population a more detailed analysis should be performed. The manuscript does not address psychiatric disorders such as depression or anxiety.
Reply 5: Thank you for your comment. We have now performed univariate analysis between pre-pregnancy obesity and psychiatric symptoms, the results were added to the end of Table 1. The analysis showed that there was no association between them. Furthermore, candidate genes BDNF rs6265 remain the only gene that statistically significance for pre-pregnancy obesity even though we included depression, anxiety and stress symptoms in multiple regression analysis. Meanwhile, depression, anxiety and stress symptoms did not show statistically significant association with pre-pregnancy obesity in both univariate and multiple regression analysis. In



addition, we also performed additional association analysis between candidate genes BDNF rs6265 and psychiatric symptoms. Likewise, the result (Table 5) showed there was not statistically significance. Hence, we confirmed that BDNF rs6265 is statistically associated with pre-pregnancy obesity and there is no interaction with depression, anxiety and stress symptoms among Malaysian GDM women.

Changes in the text 5: Result. The additional text was added to result section (Page 13-14, Line 292-296).

Table 5: Univariate analysis of the BNDF rs6265 for psychiatric symptoms among women with gestational diabetes using International criteria based BMI classifications

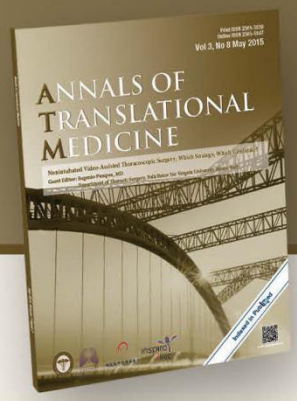
Psychiatric symptoms		Genotype GG	Genotype GA or AA	p-value
Depressive symptoms	Normal	95 (32.1)	201 (67.9)	0.180
	Mild-extremely severe	19 (42.2)	26 (57.8)	
Anxiety symptoms	Normal	62 (31.5)	135 (68.5)	0.370
	Mild-extremely severe	52 (36.1)	92 (63.9)	
Stress symptoms	Normal	96 (31.9)	205 (68.1)	0.099
	Mild-extremely severe	18 (45.0)	22 (55.0)	

We performed additional analysis to determine the association, if any between candidate gene BDNF (G > A in rs6265) and psychiatric symptoms (depression, anxiety and stress symptoms). The results are presented in Table 5. The analysis showed that there was no statistically significant association between BDNF (G > A in rs6265) and psychiatric symptoms among Malaysian women with GDM.

We also add Table 5 to support our finding to show there was no interaction between gene BNDF rs6265 and psychiatric symptoms.

Comment 6: Why did the authors only find an association between BDNF rs6265 and obesity using International criteria based BMI?

Reply 6: Thank you for your question. In univariate analysis on socio-demographic, clinical characteristics and candidate genes, different parameters and candidate genes with a p-value of less than 0.25 were observed between Asian and International criteria based BMI. For example, there were 5 candidate genes (NRG1, OXTR, BDNF, FKBP5 and PLEKHG1) and 4 clinical characteristics (parity, asthma, heart disease, anaemia) were entered multiple regression for Asian criteria-based BMI association analysis; Whereas, 5 candidates genes (NRG1, BDNF, FKBP5, RORA and HTR2C) and 7 socio-demographic and clinical characteristics (parity, asthma, heart disease, anaemia, education, smoking habit and monthly family income) were entered in multiple regression for International criteria-based BMI association analysis. Due to



the selected parameters were different between Asian and International criteria-based BMI, it is therefore reasonable to expect that BNDF rs6265 would have different degree of significance in association with pre-pregnancy obesity in light of multiple socio-demographic and clinical characteristics were taken into consideration.

Comment 7: References. The references should be listed according to instructions to authors
Reply 7: Thank you for your kind reminder. We have downloaded the referencing style endnote file from Author instruction and the Vancouver system of referencing was now used in this manuscript.

Comment 8: Table 3 is confusing
Reply 8: We have split up the multiple regression analysis between genotypes of candidate genes for obesity into Table 3 for Asian criteria-based BMI classification and Table 4 for International criteria-based BMI classification.

For editor: The idea of looking at the relationship between genetic polymorphisms in candidate genes associated with pre-pregnancy obesity in gestational diabetes mellitus is suitable. However, the study has severe drawbacks and concerns. The manuscript needs to be reviewed to clear up some language errors.