Vitamin D Deficiency and Its Association with Vitamin D Receptor (VDR) Gene Polymorphisms Among Malaysian Pregnant Women with Hypertensive Disorders in Pregnancy: Protocol for Nutrigenomics Study

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Abstract

Background: Vitamin D deficiency has been found to be associated with hypertensive disorders in pregnancy. The risk of hypertension was reported to be further augmented among those with vitamin D receptor (VDR) gene polymorphism. The genetic variants in VDR gene were inconsistent in different population.

Objective: we aim to examine the association of vitamin D status and VDR gene polymorphisms with hypertensive disorders of pregnancy (HDP) among Malaysian pregnant women.

Methods: This prospective study consists of two phases. The first phase, a cross-sectional study is based on medical records, questionnaire survey and laboratory testing for vitamin D status. The estimated sample size is 414 pregnant women of various ethnicity. Height, weight, body mass index at booking and gestational age at recruitment will be obtained from medical records. Questionnaire will be utilised to assess the risk factors for vitamin D deficiency. Status of vitamin D will be obtained from the measurement of the vitamin D 25(OH)D3 level in the blood. The second phase is a case-control study involving Malay ethnicity group with vitamin D deficiency. Participants will be divided into 2 groups with 101 subjects in each group; Group 1 (with HDP) and Group 2 (without HDP). Blood will be withdrawn for genetic analysis to determine the frequency and mutations of BsmI, ApaI, TaqI and FokI VDR genotypes. The association of these with development of HDP will be analysed.

Results: As of October 2023, we have enrolled 150 women for Phase one and 5 women for Phase two in each arm.

Conclusions: Our findings can be used to develop a policy on routine use of Vitamin D among pregnant women to reduce the risk of hypertensive disease in pregnancy Clinical Trial: This study was approved by the ethics and research committee involving human subjects of the Universiti Putra Malaysia (JKEUPM-2021-915). The registration for ClinicalTrials.gov identifier is NCT05659173.

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Title: Vitamin D Deficiency and Its Association with Vitamin D Receptor (VDR) Gene Polymorphisms Among Malaysian Pregnant Women with Hypertensive Disorders in Pregnancy: Protocol for Nutrigenomics Study

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ABSTRACT

Background: Vitamin D deficiency has been found to be associated with hypertensive disorders in pregnancy (HDP). The risk of developing hypertension in pregnancy was reported to be further augmented among those with vitamin D receptor (VDR) gene polymorphism. Meanwhile, the role of genetic variants in VDR gene in hypertensive disorders were inconsistently reported in different populations. Due to the higher incidence of vitamin D deficiency among Malaysian pregnant women and incidence of HDP, it was hypothesised that there may be associations between these and the genetic variants in VDR gene polymorphism.

Objective: The paper outlines the protocol to determine the association of vitamin D status and VDR gene polymorphisms among Malaysian pregnant women with hypertensive disorders of pregnancy (HDP).

Methods: This prospective study consists of two phases. The first phase, a cross-sectional study will entail gathering medical records, questionnaire survey and laboratory testing for vitamin D status. Questionnaire will be utilised to assess the risk factors for vitamin D deficiency. Status of vitamin D will be obtained from the measurement of the vitamin D 25(OH)D3 level in the blood. The second phase is a case-control study involving Malay ethnic group with vitamin D deficiency. Participants will be divided into two groups; Group 1 (with HDP) and Group 2 (without HDP). Genomic DNA will be extracted from the peripheral blood monocytes using Qiagen DNA blood kit, while VDR gene polymorphisms will be determined using polymerase chain reaction-High resolution melting (PCR-HRM) analysis. Sanger sequencing method will be used to sequence randomly selected samples from each variant to validate our PCR-HRM results. The association of these with development of HDP will be analysed.

Results: A total of 414 pregnant women of various ethnicity will be recruited for Phase 1, the cross-sectional study. The results obtained from the food frequency questionnaire will be analysed with the

vitamin D status of each participant to look for the factors associated with vitamin D deficiency. For the second phase of the study, 101 Malay ethnicity participants will be included in each group. The frequency and mutations of *BsmI, ApaI, TaqI* and *FokI* VDR genotypes will be analysed to look for the relationship of developing HDP.

Conclusions: A total of 414 pregnant women of various ethnicity will be recruited for Phase 1, the cross-sectional study. The results obtained from the food frequency questionnaire will be analysed with the vitamin D status of each participant to look for the factors associated with vitamin D deficiency. For the second phase of the study, 101 Malay ethnicity participants will be included in each group. The frequency and mutations of *BsmI*, *ApaI*, *TaqI* and *FokI* VDR genotypes will be analysed to look for the relationship of developing HDP.

Trial registration: ClinicalTrial.gov NCT05659173; https://clinicaltrials.gov/study/NCT05659173

Keywords: gestational hypertension, pre-eclampsia, vitamin D deficiency, vitamin D receptor gene polymorphism, Malaysia

Introduction

Background

Vitamin D deficiency is a global health issue, affecting people across all ethnicities and age groups in the world. It was estimated that more than 1 billion people are vitamin D deficient worldwide[1]. It has become a pandemic despite the availability of sunlight in Asia, Africa, the Middle East and Latin America. The most vulnerable populations at risk of vitamin D deficiency are pregnant mothers and their foetuses. Several studies have linked low vitamin D status with a higher risk of adverse short and long-term health outcomes. Besides, risk factors such as dietary habits, cultural and religious practices such as wearing dark veils covering nearly all body parts that discourage sun exposure and lack of government regulations for vitamin D fortification of foods further worsen the condition [2], [3]

Hypertensive disorders in pregnancy (HDP) account for approximately 14% of maternal mortality globally. Its spectrum includes gestational hypertension (GH), pre-eclampsia (PE), eclampsia and HELLP (Hemolysis, Elevated Liver enzymes and Low Platelets) syndrome. PE complicates 2-8% of all pregnancies worldwide with 16% maternal deaths globally and 9% mortality rates in Asia and Africa with 1.6-2.5% in Malaysia [4-5]. HDP are among the disorders associated with vitamin D deficiency [6]. Vitamin D exerts its effect through the nuclear Vitamin D receptor (VDR) and several single nucleotide polymorphisms (SNPs) occur on the VDR gene such as *BsmI, ApaI, TaqI and FokI* single nucleotide polymorphisms (SNPs). BsmI and FokI VDR variants have been reported to affect vitamin D binding and are associated with the risk of HDP [7].

In pregnant mothers, maternal 25-hydroxyvitamin D (25(OH)D) can freely cross the human placenta where the placenta expresses VDR and enzyme CYP27B1 which converts 25(OH)D to its biologically active form, 1,25-dihydroxycholecalciferol ($1,25(OH)_2D$) [8]. VDR is a protein comprising two functional domains (N-terminal dual zinc finger DNA binding domain and C-terminal ligand-binding activity domain) and linking region [9-10]. The gene encoding VDR is sited on chromosome 12 (12q12-14) [11-12]. Several SNPs in the VDR gene that are associated with metabolic disorders and vitamin D deficiency include rs1544410 (*BsmI*), rs7975232 (*ApaI*), and rs731236 (*TaqI*) [12].

Genetic variants in the VDR gene are associated with the dysregulation of metabolic biomarkers such as anthropometric parameters related to insulin resistance, obesity, cardiovascular diseases and atherogenic lipid abnormalities in different populations[12-13]. This later translates to complications in pregnancy with adverse health consequences for both mother and foetus. Vitamin D exerts its effect through the nuclear VDR and several single nucleotide polymorphisms (SNPs) occur on the VDR gene such as *BsmI, ApaI, TaqI* and *FokI* SNPs. *TaqI* gene variant which is located at the 3′ untranslated region (UTR) of the VDR gene, has been shown to affect messenger RiboNucleic acid (mRNA) stability and VDR expression in tissues. On the other hand, *FokI* SNP located near the

promoter region develops an altered VDR activity due to the change in the amino acid sequence of the protein [14]. *FokI* polymorphism of the VDR gene is also associated with upregulation of angiotensin II type I receptor and renin gene transcription leading to hypertension [15]. *BsmI* mutated allele influences VDR mRNA stability resulting in a reduction of VDR protein amount in tissues [16].

More evidence suggests that genetic variability involving gene polymorphisms and mutations of specific maternal susceptibility genes such as the VDR gene plays a vital role in the pathogenesis of HDP [6]. *BsmI, ApaI, TaqI* and *FokI* VDR variants have been reported to affect vitamin D binding and are associated with the risk of hypertension [7]. FokI polymorphism of VDR is also associated with upregulation of angiotensin II type I receptor and renin gene transcription leading to hypertension. Unfavourable VDR genetic background can significantly decrease the effectiveness of vitamin D action thereby contributing to the development of HDP [6, 17]

Moreover, the presence of genetic polymorphism in the VDR gene constitutes an important factor of individual susceptibility to the biological effects of vitamin D. Perhaps, HDP is apparent in some pregnant women, whereas others do not present with a more serious sequela. Considering the influence of genetic variability and the role of *BsmI*, *ApaI, TaqI* and *FokI* polymorphisms in the aetiopathogenesis of HDP, we therefore, aim to explore the possible association between the frequencies of these polymorphisms with HDP in Malaysian pregnant mothers. With the likely high prevalence of vitamin D deficiency among Malaysian pregnant women, we hypothesised that there are polymorphisms and mutations in the *BsmI* and *FokI* VDR gene fragments of vitamin D deficient-HDP Malaysian pregnant mothers.

Methods

Objectives

This prospective study is divided into two phases. Phase one (1) is a cross-sectional study involving

Malaysian pregnant mothers to achieve objective number 1. The second phase is a case-control study among Malay pregnant women to achieve the second, third and fourth objectives. The objectives include:

1. To determine the prevalence of vitamin D deficiency and associated risk factors among Malaysian pregnant mothers.
2. To determine the frequency of *BsmI, ApaI, TaqI* and *FokI* VDR genotypes among vitamin D deficient Malay pregnant women with HDP.
3. To understand and associate distributions of VDR genotype and allele frequency with vitamin D deficiency among Malay pregnant mothers.
4. To identify mutations in the *BsmI, ApaI, TaqI* and *FokI* VDR gene fragments among Malay pregnant women with HDP.

Settings

This study will be carried out at Hospital Sultan Abdul Aziz Shah (HSAAS), a tertiary university hospital in Selangor, Malaysia. The hospital provides a range of healthcare services to the neighbouring community and also serves as the teaching Hospital for the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia(UPM). All pregnant women attending Obstetrics and Gynaecology Department of HSAAS who fulfilled the inclusion and exclusion criteria will be recruited between 1st November 2022 and 31st March 2024. A systematic random sampling method will be used to recruit the study participants for both the cross-sectional and case-control studies.

Design

1) Cross-sectional study (Phase 1)

To achieve objective 1, Malaysian pregnant women, at any gestation, with singleton viable pregnancy at the time of recruitment and literate in English or Malay language will be recruited.

Women with multiple pregnancy and on vitamin D supplementation are excluded. The gestation will be determined from the first day of the last menstrual cycle or measurement of fetal crown-rump length on an ultrasound scan. Those on vitamin D supplements or known chronic diseases that can affect vitamin D levels such as autoimmune disease and cancer are excluded from the study.

Information on clinical, socio-demographic, dietary intake and anthropometric data (height, weight and body mass index) will be collected using a proforma. The pre-pregnancy body mass index (BMI) will be calculated from pre-pregnancy body weight recall or obtained from the woman's antenatal booking record and the measured height. The BMI (kg/m^2) will be calculated by dividing the weight (kg) by the square of the height (m^2). Participants will then be interviewed by a single trained interviewer to answer a validated questionnaire to determine their risk factors associated with vitamin D deficiency.

Validation of questionnaires

The questionnaire was adapted from studies by Humayun et al 2012, Jamil et.al 2019 and Syed Nor et.al 2022 [18,20]. Permission was obtained from all authors to adopt their questionnaires and translate them into Malay language. The translation will be done by two English-Malay-English translators before the validation test. A pilot test will be carried out among 40 pregnant women (about 10% of calculated sample size) to assess the questionnaire's clarity of meaning, appropriateness of the words used and cultural acceptance of the questionnaire. Participants recruited for the pilot testing will not be included in the study. The reliability will be measured by calculating the internal consistency, and assessing how well each question varies together. Cronbach's alpha value of at least 0.7 indicates good internal consistency of the questionnaire [21].

In addition, the content validity index (CVI) will be calculated to measure the validity of the questionnaire where the relevancy of questions will be assessed by three experts in the field. Item-level CVI will be calculated by assessing each question's validity, a score of > 0.78 is considered

acceptable [22]. The overall validity of the questionnaire will also be measured by calculating scale-level CVI (S-CVI) and a score >0.9 is considered good [22].

Sampling process

About 5mls of blood will be withdrawn and dispensed into plain blood bottle for the measurement of vitamin D ($25(OH)D_3$). $25(OH)D_3$ will be analysed using the Electrochemiluminescence immuno assay (Elecsys) technique. The Elecsys Vitamin D total III assay employs a vitamin D binding protein labelled with a ruthenium complex as capture protein to bind 25-hydroxyvitamin D_3 and 25hydroxyvitamin D_2. Cross-reactivity to 24,25-dihydroxyvitamin D is blocked by a specific monoclonal antibody.

The sample will first be incubated with pre-treatment reagent 1 and 2, where bound 25-hydroxyvitamin D is released from the vitamin D binding protein (VDBP). Subsequently, the pre-treated sample is incubated with the ruthenium labelled vitamin D binding protein, a complex between the 25-hydroxyvitamin D and the ruthenylated VDBP. A specific unlabelled antibody binds to 24,25-dihydroxyvitamin D present in the sample and inhibits cross-reactivity to this vitamin D metabolite. During the third incubation, streptavidin coated microparticles and 25-hydroxyvitamin D labelled with biotin are added. Unbound ruthenylated labelled vitamin D binding proteins become occupied. A complex consisting of the ruthenylated vitamin D binding protein and the biotinylated 25(OH)D is formed and becomes bound to the solid phase via interaction of biotin and streptavidin.

The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell/ProCell M. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier. Results are determined via a calibration curve which is instrument specifically generated by a 2-point calibration and a master curve provided via the reagent barcode or e-barcode.

Quality control

Per-run control sera (deficient, insufficient, and normal) were used for the sample analysis. All laboratory analytical protocols will be performed with strict adherence to the quality control measures.

Classification of vitamin D status

The results will be further classified using the guidelines of the Institute of Medicine (IOM), (2011) classification of vitamin D status, as vitamin D deficient (<30 nmol/L), insufficient (30– 50 nmol/L), and normal (≥ 50 nmol/L) [23].

2) Case-control study (Phase 2)

For the second phase of the study, we choose to study the Malay ethnic group, who contributes to the majority of Malaysian population. Malay ethnic pregnant women who were found to have vitamin D deficient from the first phase will be recruited for the second phase of the study. Those with non-viable pregnancy or known to have chronic hypertension diagnosed prior to pregnancy will be excluded. They will then be divided into 2 groups; Group 1-Those with HDP (Gestational hypertension, pre-eclampsia, eclampsia, HELLP syndrome), Group 2-Those with normal blood pressure.

Blood collection and Deoxyribonucleic acid (DNA) Extraction

About 5.0 millilitres (ml) of blood will be collected into the ethylene diamine tetraacetic acid (EDTA) container for genetic analysis. The blood samples will be transported to the laboratory under cold chain. Immediately after collection, the genomic DNA will be extracted using the QIAamp DNA blood kit (Qiagen, Hilden, Germany) procured from the Analisa Sdn Bhd Malaysia. The

extracted DNA will be checked for purity using the UV spectrophotometer and the integrity will be checked by running the genomic DNA on 2% agarose gel using Triacetate EDTA (TAE) buffer and DNA ladders all procured from Vivantis Sdn. Bhd. Malaysia. The genomic DNA with high quality will be stored at -20 degrees pending genetic analysis.

VDR Genotyping

The polymerase chain reaction-High Resolution Melting (PCR-HRM) technique will be used to amplify the VDR gene by using specific primers. The amplified region of the VDR gene (amplicon) will be kept in a PCR reaction tube. Sets of primers spanning 5'-3' will be synthesised based on published sequences [24] and will be procured from Apical Scientific and Biotechnology Sdn. Bhd. Malaysia. These primers will be used to run PCR-HRM analysis to detect the polymorphisms of the VDR variants. The extracted DNA will be subjected to PCR-HRM following the manufacturer's protocol using the cycling conditions based on previously published paper [24].

Principle

PCR-HRM analysis is one of the sensitive and specific technique for the detection of mutation on double-stranded (ds) DNA samples (amplicon). Firstly, the region of interest on the DNA is amplified using specific primers in real-time prior to the HRM melt phase. The HRM process begins with slow denaturation of the dsDNA at a temperature between 50–95 °C in conjunction with an intercalating fluorescent dye, SYBR Green (Roche, Germany) procured from RNZ Sdn. Bhd. Malaysia. When the melting temperature of the dsDNA is reached, the two strands 'melt' apart. The midpoint of the melt curve is described as the point when 50% of the DNA is double stranded and 50% is single stranded. The shape of the curve is dependent upon the characteristics of the dsDNA, which relate to whether it is homozygous wild-type, homozygous mutant or heterozygous wild-type and mutant. When the two strands 'melt' apart, the fluorescence level drops. As the HRM is

monitored in real-time, this curve gives a real time picture of the characteristics of the DNA being tested.

Sequencing using Barcode tagged (BT-Seq) Analysis

BTSeq analysis will be used to verify the single nucleotide polymorphisms (SNPs) of the VDR gene variant(s) among vitamin D deficient Malay pregnant mothers with HDP obtained from the PCR-HRM results. BT-Seq services will be sought from the TreeCode Sdn. Bhd. Malaysia. This part of the experiment will involve 10 amplicon samples each from the VDR variants which will be selected as representative samples to act as the reference genotype for all SNPs detected by the PCR-HRM analysis. The analysis will be done on each VDR variant amplicon that will be synthesised based on published primers spanning the four VDR SNPs (*BsmI, ApaI, TaqI* and *FokI*) using PCR [24]. The amplicons will be run on agarose gel and cut purified from the gel before sending for sequencing (BT-Seq). Discrimination of the three possible genotypes of each polymorphism (common homozygotes, heterozygotes, and rare homozygotes) in 4 distinct groups will be obtained from 180 samples by PCR-HRM analysis. Validation of the polymorphisms obtained from the PCR-HRM analysis will be done on 10 representative samples by NGS based BT-Seq analysis. A T→C transition in introns 8 and 9 will reflect the presence of the SNPs in intron 8 and 9 for BsmI and TaqI, respectively. A C→T transition at the junction of intron 1 and exon 2 reflects the *FokI* variant, and the *ApaI* variant is reflected by a T→G transition in intron 8. Distribution of vitamin D status and the genotype frequency with Hardy-Weinberg equilibrium will be obtained for each SNP.

Sample Size Estimation

A total of 414 pregnant women will be recruited for the Cross-sectional study (Phase 1) and will be used to determine the prevalence of vitamin D deficiency and associated risk factors (objective 1).For Phase 2, which is the case control study, total of 101 Malaysian pregnant women with HDP

(case) and a total of 101 normotensive vitamin D deficient pregnant women will be recruited as a control.

Sample size calculation for Cross-sectional study (Phase 1)

The sample size for the study will be calculated using the following formula [25].

$$n = \frac{Z^2 pq}{d^2}$$

Where:

n = Minimum number of sample size

Z = Level of significance at 95% confidence interval (1.96)

p = Prevalence rate

q = 1- p

d = Tolerable margin of error (5%) = 0.05

According to Woon *et al.*,2019, the prevalence of vitamin D deficiency among Malaysian pregnant women is 42.6% [26].

$$n = \frac{Z^2 pq}{d^2}$$

$$= \frac{(1.96)^2 \times 0.426\ (1-0.426)}{(0.05)^2}$$

$$= \frac{3.8416 \times 0.426 \times 0.574}{0.0025}$$

$$= \boldsymbol{376}$$

Therefore, the minimum sample size required for the cross-sectional study after addition of 10%

attrition rate is approximately 414 Malaysian pregnant women.

Sample size Determination for Case-Control study (Phase 2)

The minimum number of subjects who will participate in the study will be determined by the formula below [27].

$$n = \frac{r+1}{r} \frac{P^*(1-P^*)(Z_\beta + Z_{\alpha/2})^2}{(P_1 - P_2)^2}$$

According to a study by Caccamo *et al.*, 2020, the prevalence of vitamin D among women with GH and pregnant women without hypertension is 21% and 11% respectively [6].

Z_β = Power (80%) = 0.84

$Z_{\alpha/2}$ = for 0.05 significance level, for 95%= 1.96

P_1 = Proportion exposed in the cases=21%= 0.21

P_2 = Proportion exposed in the controls=11%= 0.11

Effect size (P*) = $P_1 - P_2$

= 0.21- 0.11

= 0.1

$$n = \frac{(1+1)}{1} \frac{(0.1)(1-0.1)(0.84+1.96)^2}{(0.1)^2}$$

= 81.846

Considering drop-out or missing samples, 10% attrition is added to the calculated sample size. Hence, our minimum number of participants in each group (case and control) will be 101.

Ethical Considerations

Ethical consideration and safeguards for the manuscripts to be produced includes the following:

- The study has obtained ethical review and approval by the Ethics Committee for Research

Involving Human Subjects of Universiti Putra Malaysia.

- Informed consent and capacity to consent: All suitable participants will be given a patient information sheet consisting of information about the study. Those agreed will be asked to sign a written consent form (available in both Malay and English language).
- Privacy and confidentiality protection: To preserve the privacy of the participants, any personal details will be removed at publications. Only anonymipsed and de-identified information will be made available in future manuscript. A database consisting of all the data of the study will be kept in a password protected database and a password protected desktop computer at the host organisation and only accessible by the named researchers.
- Compensation: As this is an observational cross-sectional and case-control study, there will be no compensation given to the participants as it entails to be low risk.

Ethical approval

The research protocol has been approved by the Ethics Committee for Research Involving Human Subjects of the Universiti Putra Malaysia (JKEUPM), with reference number of JKEUPM-2021-915. The study will be conducted in accordance with the standards of human experimentation in the Declaration of Helsinki and Malaysian Good Clinical Practice Guideline. This study protocol was also registered with C*linicaltrials.gov* with ID of NCT05659173. Written informed consent will be obtained from all eligible participants. Participation is voluntary and participants have the right to withdraw at any time without giving reason. Any amendment to the protocol or documents will be submitted for review to the ethics committee.

Data Analysis Plan

Data obtained from the study will be analysed using SPSS version 27.0 (IBM, Chicago, IL). Data entered will be checked for missing or suspicious values. These will later be verified with the

participants or omitted as missing values. Descriptive analysis will be used to summarise the sociodemographic data, anthropometric data, clinical related data, vitamin D dietary intake, sun exposure, physical activity. The prevalence of vitamin D deficiency will be expressed in frequency and percentage. The association with socio-demographic data will be analysed using the Chi-square test, independent t-test and ANOVA where applicable.

We will report comparative prevalence of *BsmI, ApaI, TaqI* and *FokI* VDR genotypes among vitamin D deficient Malay pregnant women with and without HDP. Similarly, the distributions of VDR genotype and allele frequency with vitamin D deficiency among Malay pregnant mothers will be reported as descriptive analysis. Any mutations found in the *BsmI*, *ApaI*, *TaqI* and *FokI* VDR gene fragments among Malay pregnant women with HDP will also be described. The association between vitamin D status, associated risk factors and VDR gene polymorphisms with HDP will be determined using multivariate logistic regression analysis. The level of significance will be considered at $p \leq 0.05$ at 95% confidence interval.

RESULTS

This study will provide data on the current prevalence of vitamin D deficiency, associated modifiable risk factors and its connection with sociodemographic and obstetrics outcomes. This will provide evidence for the clinicians and public health officials on the current burden of vitamin D deficiency in Malaysia, to allow policy related to vitamin D supplementation programme to be established.

The outcome of the study will identify the non-modifiable genetic component contributing to the developing vitamin D deficiency leading to HDP. The outcome will enable better understanding of the genetic variability role as a contributor to the development of HDP, thus providing more evidence to a need of a customised vitamin D supplementation in anticipating for individual variability to the response of vitamin D intake.

DISCUSSION

The study will provide information on the current prevalence and modifiable risk factors of vitamin D deficiency. The prospective associations of vitamin D deficiency with the study variables will be of immense benefit to physicians and public health experts in formulating policies related to vitamin D supplementation. The outcome will greatly advance our current understanding of vitamin D deficiency in Malaysia and at mechanistic level could be adopted by other low- and middle-income countries to tackle the widespread problem of vitamin D deficiency.

Additionally, the present study holds promise for the influence of unchangeable genetic component that may impact vitamin D deficiency and its correlation with HDP. This will constitute a significant new development in the field. The association of the genetic variants will enable further understanding of the roles of the non-modifiable component contributing to the risk of vitamin D deficiency thereby leading to the development of HDP.

Strength and limitation

The sample size we use is one of the highest among similar studies conducted in the study area. Secondly, the assessment of both modifiable and non-modifiable risk factors of vitamin D deficiency will allow deeper understanding of cause and effect as it relates not only to vitamin D deficiency but involves genetic variants and risk of developing HDP.

Potential limitations include bias in recall to answer the vitamin D food frequency components of the questionnaire as it accepts self-reporting of participants. However, efforts have

been made to ensure that these challenges are significantly tackled by conducting pre-enrolment interview with pregnant women for eligibility before they were included in the study. As the study will be conducted in a tertiary hospital setting and convenience sampling strategy is used, only subjects of interest that suit our inclusion criteria will be recruited. Thus, the results of this study may be influenced by the recruited participants group which may not necessarily replicate the whole Malaysia population. Future studies should adopt a systematic random sampling strategy with a larger sample size and multi-centred to capture pregnant women including those in rural areas.

Conclusion

The outcome of this prospective study will determine both modifiable and non-modifiable factors for vitamin D deficiency and will provide evidence to support targeted vitamin D supplementation programmes. Furthermore, the study will further enhance our understanding of the association of genetic variability in VDR gene with the risk of developing HDP. This could bring together relevant stakeholders in government health authorities, food and drug administration and food manufacturing industries to contribute to the establishment of national intervention schemes for screening, prevention and treatment of vitamin D deficiency and customised it among susceptible child-bearing age women.

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Data Availability

All data obtained and analysed during the study will not be publicly available due to privacy issue but will be available on reasonable request through the corresponding author.

Authors' Contributions

NIB, AAMJ, NN, YI contributed to the study planning and design of the study. The laboratory design was further developed by NN and YI. YI drafted the manuscript with assistant from NIB and AAMJ with input from various stages from NN. All authors critically reviewed the manuscript and approved the final version as submitted.

Conflicts of interest

None declared.

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Abbreviations

$1,25(OH)_2D$	1,25-dihydroxycholecalciferol
25(OH)D	25-hydroxyvitamin D
BMI	body mass index
CVI	content validity index
DNA	Deoxyribonucleic acid
ds	double-stranded
dsDNA	double-stranded Deoxyribonucleic acid
EDTA	ethylene diamine tetraacetic acid
GH	gestational hypertension
HDP	hypertensive disorders of pregnancy
HELLP	Hemolysis, Elevated Liver enzymes and Low Platelets
HSAAS	Hospital Sultan Abdul Aziz Shah
IOM	Institute of Medicine
mRNA	messenger RiboNucleic acid
NGS	next generation sequencing
PCR-HRM	polymerase chain reaction-High Resolution Melting
PE	pre-eclampsia
S-CVI	scale-level content validity index
SNPs	single nucleotide polymorphisms
TAE	Triacetate ethylene
UPM	Universiti Putra Malaysia
UTR	untranslated region
VDBP	vitamin D binding protein
VDR	Vitamin D receptor

Appendix 1: QUESTIONNAIRE

Participant Code: ……	Date: ……	
BIO-DATA		
Name: ……	MRN: …….....	Date of Birth (Age): ……

Mobile Phone No.: ……		I/C No.: ……
SOCIO-DEMOGRAPHIC DATA		
Educational status	Primary [ ] Secondary [ ] Diploma [ ] Tertiary [ ]	
Household Income Per month	[ ] B40 (<RM4850) [ ] M40 (between RM4851 to RM10970) [ ] T20 (>RM10971)	
Work status/Occupation	[ ] Working [ ] Unemployed [ ] Retired [ ] Student [ ] House wife	
Marital status	[ ] Married [ ] Single [ ] Divorced [ ] Widow	
ANTHROPOMETRIC MESUREMENTS		
Height (cm) ……	Booking weight (Kg) …… Current Pregnancy weight (Kg) …….. Booking BMI (Kg/m²) …….	
CLINICAL MEASUREMENTS/DATA		
Blood Pressure at recruitment	Systolic BP (mmHg) …….. Diastolic BP (mmHg) ……..	
Last menstrual period (LMP) Estimated date of delivery (EDD)	Date of LMP [……..] Date of EDD [……..]	
Gestation Age (weeks)	[…….]	
Gravidity and Parity	[…….]	
Family History of chronic diseases	[ ] Hypertension [ ] Diabetes mellitus [ ] Liver Disease [ ] Kidney	
Past pregnancy complications	[ ] Disease [ ] Cancer [ ] Heart Disease [ ] Autoimmune Disease [ ] Obesity	

Current pregnancy problem	Others…….. [ ] Gestational hypertension [ ] Preeclampsia [ ] Eclampsia [ ] Gestational diabetes Others……
PREGNANCY PHYSICAL ACTIVITY QUESTIONS	
Preparing meals (cook, setting table, washing dishes)	[ ] None [ ] Less than 30 minutes [ ] 30 minutes – 1 hour [ ] 1 hour – 2 hours [ ] 2 hours – 3 hours
Carrying children.	[ ] None [ ] Less than 30 minutes [ ] 30 minutes – 1 hour [ ] 1 hour – 2 hours [ ] 2 hours – 3 hours
Sitting and reading, talking, or on the phone, while not at work.	[ ] None [ ] Less than 30 minutes [ ] 30 minutes – 1 hour [ ] 1 hour – 2 hours [ ] 2 hours – 3 hours
Shopping (for food,clothes, or other items)	[ ] None [ ] Less than 30 minutes [ ] 30 minutes – 1 hour [ ] 1 hour – 2 hours [ ] 2 hours – 3 hours
Light cleaning (make beds, laundry, iron)	[ ] None [ ] Less than 30 minutes [ ] 30 minutes – 1 hour [ ] 1 hour – 2 hours [ ] 2 hours – 3 hours
Heavier cleaning (vacuum,mop,sweep,wash windows)	[ ] None [ ] Less than 30 minutes [ ] 30 minutes – 1 hour [ ] 1 hour – 2 hours [ ] 2 hours – 3 hours
Mowing lawn while on a riding mower	[ ] None [ ] Less than 30 minutes [ ] 30 minutes – 1 hour [ ] 1 hour – 2 hours [ ] 2 hours – 3 hours

Watching TV or a video	[ ] None [ ] Less than 30 minutes [ ] 30 minutes – 1 hour [ ] 1 hour – 2 hours [ ] 2 hours – 3 hours
Walking slowly for fun or exercise	[ ] None [ ] Less than 30 minutes [ ] 30 minutes – 1 hour [ ] 1 hour – 2 hours [ ] 2 hours – 3 hours
Walking more quickly for fun or exercise	[ ] None [ ] Less than 30 minutes [ ] 30 minutes – 1 hour [ ] 1 hour – 2 hours [ ] 2 hours – 3 hours
Walking quickly up hills for fun or exercise	[ ] None [ ] Less than 30 minutes [ ] 30 minutes – 1 hour [ ] 1 hour – 2 hours [ ] 2 hours – 3 hours
Jogging	[ ] None [ ] Less than 30 minutes [ ] 30 minutes – 1 hour [ ] 1 hour – 2 hours [ ] 2 hours – 3 hours
Dancing	[ ] None [ ] Less than 30 minutes [ ] 30 minutes – 1 hour [ ] 1 hour – 2 hours [ ] 2 hours – 3 hours
Prenatal exercise class	[ ] None [ ] Less than 30 minutes [ ] 30 minutes – 1 hour [ ] 1 hour – 2 hours [ ] 2 hours – 3 hours
Swimming	[ ] None [ ] Less than 30 minutes [ ] 30 minutes – 1 hour [ ] 1 hour – 2 hours [ ] 2 hours – 3 hours
Sitting at working or in class	[ ] None [ ] Less than 30 minutes

	[ ] 30 minutes – 1 hour [ ] 1 hour – 2 hours [ ] 2 hours – 3 hours
Walking quickly at work while carrying things (heavier than 4 litres jug of milk)	[ ] None [ ] Less than 30 minutes [ ] 30 minutes – 1 hour [ ] 1 hour – 2 hours [ ] 2 hours – 3 hours
Standing or slowly walking at work while carrying things (heavier than 4 litres jug of milk)	[ ] None [ ] Less than 30 minutes [ ] 30 minutes – 1 hour [ ] 1 hour – 2 hours [ ] 2 hours – 3 hours
Walking quickly at work not carrying anything	[ ] None [ ] Less than 30 minutes [ ] 30 minutes – 1 hour [ ] 1 hour – 2 hours [ ] 2 hours – 3 hours
Standing or slowly walking at work not carrying anything	[ ] None [ ] Less than 30 minutes [ ] 30 minutes – 1 hour [ ] 1 hour – 2 hours [ ] 2 hours – 3 hours
SUN EXPOSURE	
Type of dressing / body cover	[ ] None [ ] Half body cover [ ] Partial body cover [ ] Full body cover
VITAMIN D DIETARY INTAKE/FOOD FREQUENCY	
Fish (Salmon, Tuna, mackerel or sardine, etc)	[ ] 0 - 50g per serving [ ] 50 - 100g per serving [ ] 100 and above per serving [ ] None Frequency of intake (number of times per day: ……

Dairy products (milk, butter and cheese or yoghurt)	[ ] 0 - 50g per serving [ ] 50 - 100g per serving [ ] 100 and above per serving [ ] None Frequency of intake (number of times per day: ……
Vegetables (Mushrooms, oyster, Potatoes, mashed etc)	[ ] 0 - 50g per serving [ ] 50 - 100g per serving [ ] 100 and above per serving [ ] None Frequency of intake (number of times per day: ……

Supplementary Files

Multimedia Appendixes

Peer Review 1.
URL: http://asset.jmir.pub/assets/6b958f8ef4f9dc88fb13d9d8c41abf28.pdf

Untitled.
URL: http://asset.jmir.pub/assets/f6a6ccce571a87b2b5bf45b1a04c4692.pdf