Serum 25-hydroxyvitamin D concentrations and cardio-metabolic risk factors in adolescents and young adults

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Abstract

Evidence associating serum 25-hydroxyvitamin D (25(OH)D) concentrations and cardio-metabolic risk factors is inconsistent and studies have largely been conducted in adult populations. We examined the prospective associations between serum 25(OH)D concentrations and cardio-metabolic risk factors from adolescence to young adulthood in the West Australian Pregnancy Cohort (Raine) Study. Serum 25(OH)D concentrations, BMI, homeostatic model assessment for insulin resistance (HOMA-IR), triglycerides, high-density lipoprotein-cholesterol (HDL-C) and systolic blood pressure (SBP) were measured at the 17 (n=1015) and 20 (n=1117) year follow-ups. Hierarchical linear mixed models with maximum likelihood estimation were used to investigate associations between serum 25(OH)D concentrations and cardio-metabolic risk factors, accounting for potential confounders. In males and females respectively, mean (SD) serum 25(OH)D concentrations were 73.6 (28.2) and 75.4 (25.9) nmol/L at 17 years, and 70.0 (24.2) and 74.3 (26.2) nmol/L at 20 years. Deseasonalised serum 25(OH)D_3 concentrations were inversely associated with BMI (Coefficient=-0.01; 95%CI -0.03,-0.003; p=0.014). No change over time was detected in the association for males; for females the inverse association was stronger at 20 years compared with 17 years. Serum 25(OH)D concentrations were inversely associated with log HOMA-IR (Coefficient =-0.002; 95%CI -0.003,-0.001; p<0.001) and positively associated with log triglycerides in females (Coefficient=0.002; 95%CI 0.0008,0.004; p=0.003). These associations did not vary over time. There were no significant associations between serum 25(OH)D concentrations and HDL-C or SBP. Clinical trials in those with insufficient vitamin D status may be warranted to determine any beneficial effect of vitamin D supplementation on insulin resistance, while monitoring for any deleterious effect on triglycerides.

Introduction

Cardiovascular disease is the leading cause of deaths related to non-communicable diseases, and improving the detection and treatment of cardiovascular disease is a major goal of clinical medicine [1]. Low serum 25-hydroxyvitamin D (25(OH)D) concentrations are frequently reported in populations worldwide [2; 3; 4; 5; 6; 7] and there are a number of potential mechanisms implicating low vitamin D status in cardio-metabolic risk. There appears to be a role for vitamin D in the development of insulin resistance: vitamin D receptors are strongly expressed in pancreatic beta-cells [8] and the active form of vitamin D (1,25-dihydroxyvitamin D) may protect against insulin resistance in peripheral tissues, such as skeletal muscle [9]. Animal and *in vitro* studies suggest that 1,25-dihydroxyvitamin D is a potent negative regulator of renin gene expression and may prevent the over-stimulation of the renin-angiotensin system [10], a regulatory pathway that plays an essential role in blood pressure.

A recent review of systematic reviews and meta-analyses found that highly convincing evidence of a clear role of vitamin D does not exist for cardiovascular disease, hypertension or type 2 diabetes [11]. However, the epidemiological evidence associating serum 25(OH)D concentrations and cardio-metabolic risk has been largely cross-sectional in design and focuses on adult populations, with conflicting results. Using data from the 17 and 20 year follow-ups of the Western Australia Pregnancy Cohort (Raine) Study, we investigated the prospective associations between serum 25(OH)D concentrations and cardio-metabolic risk factors including body mass index (BMI), insulin resistance, high-density lipoprotein cholesterol (HDL-C), triglycerides and systolic blood pressure (SBP). To our knowledge, this is the first prospective study examining associations between serum 25(OH)D concentrations and cardio-metabolic risk factors in adolescents and young adults.

Methods

Participants

The Raine Study is a prospective, population-based, longitudinal study and its methodology has been described previously [12]. In brief, a total of 2900 pregnant women from the public antenatal clinic at King Edward Memorial Hospital or surrounding private clinics in Perth, Western Australia, were recruited between May 1989 and November 1991, and gave birth to 2868 live children. These children underwent assessment at birth and at ages 1, 2, 3, 5, 8, 10, 14, 17 and 20 years. Recruitment and all follow-ups were approved by the ethics committees of King Edward Memorial Hospital for Women

and the Princess Margaret Hospital for Children, Perth, Western Australia. Informed and written consent was obtained from the participant and/or their primary caregiver at all follow-ups.

Serum 25-hydroxyvitamin D concentrations

Serum 25(OH)D concentrations were determined at the 17 and 20 year follow-ups. Venous blood samples were taken from an antecubital vein after an overnight fast and stored at -80°C until analysis. Serum $25(OH)D_2$ and $25(OH)D_3$ concentrations were measured using isotope-dilution liquid chromatography-tandem mass spectrometry (LC-MS/MS) (RDDT, Victoria, Australia), according to published methodology [13]. The method for the quantification of vitamin D metabolites ($25(OH)D_2$ and $25(OH)D_3$) in serum/plasma used hexa-deuterated $25(OH)D_3$ as an internal standard on either the API 4000 QTRAP system or the Agilent 6410 QQQ LC-MS/MS system. Serum Calibration Standards containing nominal amounts of $25(OH)D_2$/$25(OH)D_3$ from CHROMSYSTEMS were used to generate calibration curves. Tri-level Quality Control samples containing $25(OH)D_2$/$25(OH)D_3$ designated as Level Low, Level 1 and Level 2 were purchased from UTAK Laboratories (PM Separations). At 17 years, the coefficient of variation (CV) for $25(OH)D_3$ was 7.1% at 27 nmol/L, 5.0% at 75 nmol/L, and 5.3% at 164 nmol/L. The CV for $25(OH)D_2$ was 8.8% at 23 nmol/L, 6.7% at 66 nmol/L, and 6.7% at 150 nmol/L. At 20 years, the CV for $25(OH)D_3$ was 5.8% at 28 nmol/L, 5.2% at 80 nmol/L, and 9.2% at 188 nmol/L. The CV for $25(OH)D_2$ was 7.9% at 25 nmol/L, 6.6% at 75 nmol/L, and 10.4% at 185 nmol/L.

Cardio-metabolic risk factors

Participants at 17 years were weighed to the nearest 100 g using a Wedderburn Digital Chair Scale and height was determined to the nearest 0.1 cm with a Holtain Stadiometer. At 20 years, weight was measured to the nearest 100 g using Personal Precision scales UC-321 (A and D Company) and height was measured to the nearest 0.1 cm with a wall mounted Seca 202. Body mass index (BMI) was calculated as weight in kilograms divided by height in metres squared. Fasting blood samples at the 17 and 20 year follow-ups were analysed for serum glucose, HDL-C and triglycerides, determined enzymatically on an Architect c16000 Analyser (Abbott Laboratories, Lake Forest, Illinois) (intra-assay CVs were 1.0% for glucose, 2.0% for HDL-C, and 1.9% for triglycerides). Serum insulin was determined on an Architect i2000SR Analyser (intra-assay CV 1.78%). The homeostatic model assessment for insulin resistance (HOMA-IR) score was calculated as follows: HOMA-IR score = (fasting insulin (μU/mL) x fasting glucose (mmol/L)) / 22.5 [14]. Resting blood pressure at 17 and 20 years was obtained using an oscillometric sphygmomanometer (Dinamap ProCare 100, Soma Technology, USA). After five minutes of quiet rest, six automatic recordings were taken every two

minutes with subjects supine and with an appropriate cuff size. The averages of the last five readings of SBP were calculated.

Potential confounding variables

Participants were classified as Caucasian if both parents were Caucasian, or as non-Caucasian if one or both parents were of an alternate ethnicity. Physical activity at the 17 year follow-up was assessed using a self-reported questionnaire based on exercise outside of school hours per week, with exercise defined in three categories as activity causing breathlessness or sweating (≥ 4 times per week, 1-3 times per week and < once per week). At 20 years, physical activity was assessed using a self-reported questionnaire recording time spent in moderate or vigorous physical activity (≥ 4 times per week, 1-3 times per week and < once per week).

A baseline measure of family income was available at the 17 year follow-up, completed by the primary caregiver, with 12 categories ranging from $1-$8000 per year to ≥ $104,000 per year (Australian dollars). At the 17 and 20 year follow-ups, the participants answered the question "do you currently smoke cigarettes". At the 17 and 20 year follow-ups, the use of hormonal contraception (HC) (yes/no) was reported by the participant.

A self-reported, semi-quantitative food frequency questionnaire (FFQ) developed by the Commonwealth Scientific and Industrial Research Organisation in Adelaide, Australia [(15)] was used at the 17 year follow-up to assess alcohol intake (g/day). This 212-item FFQ assesses usual dietary intake over the previous year, collecting information on the frequency of consumption of individual foods, mixed dishes and beverages, along with information on usual serving sizes in relation to a standard serving size (in household units). Participants were asked how often they usually drank six types of alcohol (low alcohol beer, regular beer, alcoholic soda, wine/champagne, sherry/port/liqueur and spirits). Frequency was recorded as never, rarely, times per month, times per week or times per day.

At the 20 year follow-up, alcohol intake (g/day) was assessed using a self-reported semi-quantitative FFQ developed by the Anti-Cancer Council of Victoria for use in the ethnically diverse Australian population [(16)]. The questionnaire includes three questions on alcohol consumed over the last twelve months. Participants were asked to report how often they drank beer, wine and/or spirits, with six types of alcohol specified (low alcohol beer, full strength beer, red wine, white/sparkling wine, fortified wine, spirits/liqueurs) and ten frequency choices ranging from "never" to "every day". A second question asked how many glasses of beer/wine and/or spirits they usually drank on days they

were drinking (total number of glasses per day, ranging from one to ten or more). A third question asked about the maximum number of glasses of beer/wine and/or spirits they drank in 24 hours (maximum number of glasses per 24 hours ranging from 1-2 to 19 or more).

Addressing potential seasonal effects

Models investigating HDL-C, trigylcerides and HOMA-IR were not adjusted for season as these biomarkers change relatively quickly over time and the blood sample for these biomarkers was taken on the same day as that for the measurement of serum 25(OH)D concentrations. Similarly, SBP changes relatively quickly over time and it would not be appropriate to adjust for season if serum 25(OH)D concentrations and SBP measurements were taken on the same day. Although 95% of participants had blood for serum 25(OH)D concentrations and SBP measurements taken within 31 days of each other, approximately 5% of participants had measurements taken more than 31 days apart. In order to determine if the time difference between measurement of serum 25(OH)D concentrations and SBP influenced the association between serum 25(OH)D concentrations and SBP, we deseasonalised the serum $25(OH)D_3$ concentrations using sine and cosine curves [17] and moved the projected date back to match the day that SBP was measured. This technique has previously been reported by Lucas and colleagues [18]. For models investigating BMI, which changes relatively slowly over time, we used deseasonalised serum $25(OH)D_3$ concentrations [17].

Statistical analyses

Characteristics of the participants for whom full data for cardio-metabolic risk factors and HC were available were described at both the 17 and 20 year follow-ups. A three-level variable for sex (to distinguish females not using HC, females using HC, and males) was employed to assess the interactive effects of HC use on the relationship between serum 25(OH)D concentrations and all outcomes, except BMI. For BMI, there was no difference between females not using HC and females using HC. Additional power was required to demonstrate statistically significant differences between females and males; therefore, for the BMI model, we used a two-level variable for sex. HOMA-IR, triglycerides and HDL-C were not normally distributed, so log transformations were applied.

Hierarchical linear mixed models with maximum likelihood estimation (MLE) were used to investigate associations between serum 25(OH)D concentrations and cardio-metabolic risk factors (BMI, HOMA-IR, triglycerides, HDL-C, SBP) over time. All models included adjustment for sex and BMI (excluding the model with BMI as an outcome) and investigated possible interactions between time, serum 25(OH)D concentrations and each of sex or BMI. The hierarchical structure accounted for potential correlation between a small number of siblings.

Additional covariates (race, physical activity, family income, smoking, alcohol intake and month of birth) were then included individually to determine if a significant association with the outcome was detected or if the covariate influenced any relationship between serum 25(OH)D concentrations and the outcome. Sample sizes were held fixed for these comparisons based on the sample without missing data for each covariate in turn. Due to considerable missing data for some covariates, models utilising MLE for covariates (structural equation models in Stata) were also employed to investigate possible bias the loss of sample may have introduced. These models were not longitudinal models but rather approximations achieved by identifying repeated measures on the subject as a per person cluster, resulting in an adjusted, robust variance. The sibling adjustment was not made in these models. These models were used to determine if multiple imputation was necessary.

While some of the additional covariates were significantly associated with the outcomes, the analysis determined that these associations were independent of the relationship with serum 25(OH)D concentrations. The significance of the relationship between serum 25(OH)D concentrations and the outcome was unaltered by the inclusion of the additional covariates, and variations in the magnitude of the relationships (the coefficient) were considered to be minimal (Supplementary Tables 1 and 2). Hence, it was determined that multiple imputation to facilitate inclusion of variables that had no effect on the relationship of interest was unnecessary.

Analyses were performed using IBM SPSS Statistics Release Version 19.9.9.1 (IBM SPSS Inc., 2010, Chicago, IL) and StataCorp 2011 *Stata Statistical Software: Release 12* (College Station, TX: StataCorp LP). Statistical significance was defined as two-tailed $p < 0.05$. The regression tables are reported per 1 nmol/L change in serum 25(OH)D concentrations and as log transformations of HOMA-IR, triglycerides and HDL-C.

Results

Participant characteristics

Full data for outcome variables and HC were available for 1015 participants at the 17 year follow-up and 1117 participants at the 20 year follow-up. A CONSORT flow diagram is shown in Figure 1, and participant characteristics for males and females are shown in Table 1. Mean (SD) serum 25(OH)D concentrations were 73.6 (28.2) and 75.4 (25.9) nmol/L at the 17 year follow-up in males and females, respectively; and 70.0 (24.2) and 74.3 (26.2) nmol/L at the 20 year follow-up in males and females, respectively. At the 17 year follow-up, only 4 participants had detectable serum 25(OH)D_2

concentrations, ranging from 5.44 to 8.12 nmol/L. At the 20 year follow-up, 13 participants had detectable serum 25(OH)D_2 concentrations, ranging from 5.24 to 7.07 nmol/L.

Deseasonalised serum 25-hydroxyvitamin D_3 concentrations and BMI

In a univariate analysis, deseasonalised serum 25(OH)D_3 concentrations were inversely associated with BMI (Coefficient=-0.02; 95%CI -0.03,-0.02; p<0.001) and the association persisted after adjusting for sex (Table 2). A significant three way interaction between time, sex and serum 25(OH)D_3 concentrations indicated that the relationship between serum 25(OH)D_3 concentrations and BMI changed over time differently in males compared to females (p=0.026). No change over time was detected in the association for males, whereas for females the inverse association was stronger at 20 years compared with 17 years (Figure 2). The model estimates that a one standard deviation increase in serum 25(OH)D_3 concentrations (approximately 25 nmol/L) was associated with a reduction in BMI of 0.4 kg/m^2 in females at 17 years; 0.6 kg/m^2 in females at 20 years; 0.6 kg/m^2 in males at 17 years; and 0.5 kg/m^2 in males at 20 years.

Serum 25-hydroxyvitamin D concentrations and insulin resistance

In a univariate model, serum 25(OH)D concentrations were inversely associated with log HOMA-IR (Coefficient=-0.003; 95%CI -0.005, -0.002; p<0.001) and the inverse association was maintained after adjusting for BMI (Coefficient=-0.002; 95%CI -0.003, -0.001; p<0.001) (Table 3). No significant interactions were found between time, serum 25(OH)D concentrations and each of sex or BMI. The model estimates that a one standard deviation increase in serum 25(OH)D concentrations was associated with a 5% decrease in HOMA-IR.

Serum 25-hydroxyvitamin D concentrations and triglycerides

In a univariate model, serum 25(OH)D concentrations were positively associated with log triglycerides (Coefficient=0.001; 95%CI 0.0004, 0.002; p=0.03). A significant interaction between serum 25(OH)D concentrations and sex indicated the relationship between serum 25(OH)D concentrations and log triglycerides differed in males when compared to females (p=0.016). There was a positive association in females not using HC after adjusting for BMI (Coefficient=0.0023; 95%CI 0.0008, 0.0038; p=0.003) (Table 4), and a similar effect in females using HC. Coefficients from the model estimate that a one standard deviation increase in serum 25(OH)D concentrations was associated with a 6% increase in triglycerides in females. There was no significant association between serum 25(OH)D concentrations and triglycerides in males (p=0.738).

Serum 25-hydroxyvitamin D concentrations and HDL-C

Serum 25(OH)D concentrations were positively associated with log HDL-C in a univariate model (Coefficient=0.0005; 95%CI 0.0002, 0.0008; p=0.004). However, there was no association between serum 25(OH)D concentrations and HDL-C after adjusting for BMI (Supplementary Table 3) and no significant interactions were found between time, serum 25(OH)D concentrations and each of sex or BMI.

Serum 25-hydroxyvitamin D concentrations and SBP

There was no difference in the relationship between serum 25(OH)D concentrations and SBP when we moved the projected date of blood measurement back to match the day that SBP was measured. Therefore, we did not deseasonalise serum 25(OH)D concentrations or adjust for season in models with SBP as the outcome measure. An inverse association between serum 25(OH)D concentrations and SBP (Coefficient=-0.02; 95%CI -0.04, -0.003; p=0.023) in a univariate analysis was not significant after adjusting for BMI (Supplementary Table 4). No significant interactions were found between time, serum 25(OH)D concentrations and each of sex or BMI.

Discussion

This study has shown that serum 25(OH)D concentrations were inversely associated with BMI and HOMA-IR in an adolescent and young adult population. Serum 25(OH)D concentrations were positively associated with triglycerides in females only. There were no significant associations between serum 25(OH)D concentrations and HDL-C or SBP.

The inverse association between deseasonalised serum 25(OH)D_3 concentrations and BMI was consistent between 17 and 20 years in males, but increased over time in females. Similarly, an inverse association between serum 25(OH)D concentrations and waist circumference was identified by Gagnon and colleagues in a prospective study of 4164 adults participating in The Australian Diabetes, Obesity and Lifestyle (AusDiab) Study [19], and by Ganji and colleagues in a cross-sectional study of 5867 adolescents participating in the United States' National Health and Nutrition Examination Survey (NHANES) [20].

We found a significant inverse association between serum 25(OH)D concentrations and HOMA-IR, a result which is supported by a number of other studies in children and adolescents [20; 21; 22; 23; 24; 25; 26], including the study by Ganji and colleagues [20]. The prospective study by Gagnon and colleagues also found that serum 25(OH)D concentrations were inversely associated with HOMA-IR in adults [19]. However, Nam and colleagues found no significant associations between serum 25(OH)D

concentrations and insulin resistance or fasting glucose in a cross-sectional study of 1504 children and adolescents participating in the Korean NHANES, after adjusting for age, sex, physical activity, alcohol consumption and supplement use [27]. Although a six-month trial supplementing 4000 IU/day of vitamin D in 35 obese adolescents found significant improvements in HOMA-IR compared with placebo [28], vitamin D supplementation trials in adults have shown mixed results on glycaemic status, insulin resistance and insulin sensitivity [29; 30; 31; 32; 33; 34; 35; 36; 37; 38; 39; 40; 41].

There may be associations between vitamin D responsiveness, vitamin D-receptor (VDR) gene polymorphisms and insulin resistance. Associations between single nucleotide polymorphisms (SNPs) and responses in insulin sensitivity to vitamin D supplementation of 4000 IU per day were determined in 81 South Asian women [42]. The improvement in insulin sensitivity was significantly greater in women with the F*ok*I Ff genotype compared with women with the F*ok*I FF genotype. Therefore, differences in the VDR gene may explain differences in response of insulin sensitivity to vitamin D supplementation. Further explorations of the influence of SNPs on responsiveness of cardio-metabolic risk factors to vitamin D intervention are warranted.

We found a positive association between serum 25(OH)D concentrations and triglycerides in females. Other observational studies have shown an inverse association [19; 43], or no association [27], between circulating 25(OH)D concentrations and triglycerides. However, our finding was supported by a recent randomised controlled trial in 200 hypertensive adults with low serum 25(OH)D concentrations: Pilz and colleagues showed that vitamin D supplementation significantly increased triglycerides compared with placebo [44]. Although the authors hypothesised that this result was a chance finding, they noted that further validation from additional studies was warranted. In contrast, a trial in 200 healthy overweight subjects participating in a 12 month weight-reduction programme found that, compared with the placebo group, vitamin D supplementation lowered triglycerides but increased LDL-C [45]. We found no statistically significant associations between serum 25(OH)D concentrations and HDL-C, which is supported by the studies by Nam and colleagues, and Gagnon and colleagues [19; 27]. A recent review of vitamin D supplementation and lipid profile found that most randomised controlled trials showed no effects, or even adverse effects, on serum lipids [46].

We found no independent association between serum 25(OH)D concentrations and SBP. This finding conflicts with a systematic review and meta-analysis of 22 cross-sectional studies and eight prospective studies (largely in adult populations), which showed that circulating 25(OH)D concentrations were inversely associated with risk of hypertension [47]. However, the results of vitamin D supplement trials and SBP are equivocal. A meta-analysis of 16 randomised trials of

vitamin D supplementation on blood pressure in adults showed an overall non-significant reduction in both SBP and diastolic blood pressure [48]. The trial by Pilz and colleagues showed that vitamin D supplementation had no significant effect on SBP [44].

There is growing interest in the health effects of sun exposure beyond the synthesis of vitamin D, and it is plausible that serum 25(OH)D concentrations are merely a biomarker of previous sun exposure. Recent *in vivo* experiments indicate that chronic skin exposure to low dose ultraviolet radiation (UVR) limits the development of obesity and signs of metabolic syndrome in mice fed a high fat diet [49]. Skin exposure to UVR induces several immune effector molecules, and low-dose UVR irradiation of the skin has been shown to lower blood pressure in healthy volunteers independently of vitamin D, through modulation of nitric oxide bioavailability [50]. Further investigations to elucidate the vitamin D-independent effects of low-dose exposure to UVR on cardio-metabolic risk factors are warranted.

Strengths of our study include the young age of the cohort, and the prospective design based on a longitudinal cohort. We were able to investigate confounders that potentially impact upon serum 25(OH)D concentrations and cardio-metabolic risk factors, including race, month of birth, BMI, physical activity, family income, hormonal contraceptive use, smoking and alcohol intake. However, despite our best efforts to adjust for confounders, we cannot rule out the possibility of residual confounding. Weaknesses of our study include the measurement of physical activity, which was not based on a validated questionnaire and may be subject to self-reporting bias. Furthermore, our assessment of physical activity did not differentiate between indoor and outdoor activity. The non-significance of physical activity in relation to cardio-metabolic risk factors may reflect the limitations of the measurement, rather than the true absence of an association between physical activity and the outcome. In light of the evidence associating genetic factors with responses in insulin resistance to vitamin D supplementation [42], it is possible that genetic variation may modify the association between serum 25(OH)D concentrations and cardio-metabolic risk factors. In our study, no data were available for genetic factors relating to vitamin D; further studies in this area would benefit from investigating factors such as the F*ok*I Ff genotype.

Although we used the same laboratory for measurement of serum 25(OH)D concentrations using LC-MS/MS methods at both follow-ups, this laboratory was not participating in an external quality control scheme at the time of analysis. Laboratories using LC-MS/MS methods have been shown to give higher results than those certified to the international standard reference method developed by the National Institute of Standards and Technology and Ghent University [51], which may have partly

contributed to the relatively high serum 25(OH)D concentrations in our population. Given that our population of adolescents/young adults has relatively high vitamin D status in comparison with those in other countries [52], our findings may not be generalisable to all populations. Furthermore, although the availability of data from two cohort follow-ups in adolescence and young adulthood enabled us to examine changes over time, we cannot infer causality in the relationship between serum 25(OH)D concentrations and cardio-metabolic risk factors.

Our results show that serum 25(OH)D concentrations are independently associated with a number of cardio-metabolic risk factors in adolescents and young adults, with differential effects on BMI with time between sexes. In particular, the finding that serum 25(OH)D concentrations were inversely associated with BMI and insulin resistance would suggest a cardio-protective benefit, but this may be offset by the positive association between serum 25(OH)D concentrations and triglycerides. Well-designed clinical trials in those with insufficient vitamin D status may be warranted to determine any causal, beneficial effect of vitamin D supplementation on insulin resistance in adolescents and young adults, while monitoring for any deleterious effect on triglycerides.

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Table 1. Characteristics of the Raine Study participants for whom full data for outcome variables and hormonal contraceptive use were available

	17 year follow-up (*n* =1015)				20 year follow-up (*n* =1117)			
	Male		Female		Male		Female	
	n		*n*		*n*		*n*	
Race (% Caucasian)	*453*	85	*407*	85	*505*	84	*447*	86
Serum 25(OH)D [nmol/L, mean (SD)]	*534*	73.6 (28.2)	*481*	75.4 (25.9)	*598*	70.0 (24.2)	*519*	74.3 (26.2)
HOMA-IR [median (IQR)]	*534*	1.5 (1.3)	*481*	1.6 (1.3)	*598*	0.5 (0.7)	*519*	0.6 (0.9)
Total cholesterol [mmol/L, mean (SD)]	*534*	3.9 (0.7)	*481*	4.3 (0.7)	*598*	4.2 (0.8)	*519*	4.5 (0.8)
LDL-C [mmol/L, mean (SD)]	*534*	2.2 (0.7)	*481*	2.4 (0.6)	*598*	2.4 (0.7)	*519*	2.6 (0.6)
HDL-C [mmol/L, median (IQR)]	*534*	1.2 (0.3)	*481*	1.4 (0.4)	*598*	1.2 (0.3)	*519*	1.4 (0.4)
Triglycerides [mmol/L, median (IQR)]	*534*	0.9 (0.5)	*481*	0.9 (0.5)	*598*	1.0 (0.6)	*519*	1.0 (0.6)
SBP [mm/Hg, mean (SD)]	*534*	120.2 (10.2)	*481*	110.1 (9.6)	*598*	123.4 (12.6)	*519*	111.4 (11.2)
BMI [kg/m^2, mean (SD)]	*534*	22.8 (4.3)	*481*	23.2 (4.6)	*598*	24.5 (2.5)	*519*	24.3 (5.4)
Physical activity (%)								
≥ 4 times per week	*143*	33	*77*	18	*329*	73	*218*	47
1-3 times per week	*273*	54	*231*	53	*75*	17	*141*	30
< once per week	*59*	13	*125*	29	*47*	10	*107*	23
Family income at 17 years (%)								
≤ $40,000 per year	*119*	25	*125*	28	NR	NR	NR	NR
$40,001-78,000 per year	*176*	37	*172*	38	NR	NR	NR	NR
> $78,000 per year	*185*	39	*151*	34	NR	NR	NR	NR
Hormonal contraceptive use (% Current user)	NA	NA	*155*	32	NA	NA	*308*	59
Smoking (% Current smoker)	*96*	19	*111*	23	*73*	16	*62*	13
Alcohol consumers (%)	*201*	59	*201*	56	*511*	92	*469*	94
Alcohol intake in consumers [g/day, median (IQR)]		6.0 (12.3)		5.4 (8.9)		15.9 (30.0)		8.4 (16.4)

25(OH)D, 25-hydroxyvitamin D; HOMA-IR, homeostatic model assessment for insulin resistance; LDL-C, low-density lipoprotein; HDL-C, high-density lipoprotein; SBP, systolic blood pressure; BMI, body mass index; NA, not applicable; NR, not reported.

Table 2. Adjusted associations between deseasonalised serum 25(OH)D_3 concentrations and body mass index

Variable	Coefficient (95% CI)[1]	*p*
Deseasonalised 25(OH)D_3 (nmol/L)	-0.01 (-0.03, -0.003)[2]	0.014
Time		
20 year follow-up	1.96 (1.08, 2.85)	<0.001
Sex		
Male	0.33 (-0.94, 1.61)	0.610
Time*25(OH)D_3		
20 year follow-up	-0.01 (-0.02, -0.0002)	0.047
Time*Sex		
20 year, Male	-0.82 (-2.02, 0.39)	0.182
Sex*25(OH)D_3		
Male	-0.01 (-0.03, 0.005)	0.190
Time*Sex*25(OH)D_3		
20 year, Male	0.02 (0.002, 0.03)	0.026
Constant	24.47 (23.52, 25.42)	<0.001

25(OH)D_3, 25-hydroxyvitamin D_3

[1]Estimated difference in body mass index from the reference category of categorical variables or per 1 unit increase of continuous variables

[2]Coefficient for females, 17 year follow-up

Females, 20 year follow-up: Coefficient= -0.03; 95%CI -0.04, -0.02; p<0.001

Males, 17 year follow-up: Coefficient= -0.03; 95%CI -0.04, -0.01; p<0.001

Males, 20 year follow-up: Coefficient= -0.02; 95%CI -0.03, -0.01; p=0.001

Table 3. Adjusted associations between serum 25(OH)D concentrations and log homeostatic model assessment for insulin resistance

Variable	Coefficient (95% CI)[1]	*p*
25(OH)D (nmol/L)	-0.002 (-0.003, -0.001)	<0.001
Time		
17 year follow-up	Reference	
20 year follow-up	-0.86 (-0.91, -0.81)	<0.001
Sex		
Female	Reference	
Female using HC	0.18 (0.10, 0.26)	<0.001
Male	0.01 (-0.06, 0.08)	0.814
Body mass index (kg/m^2)	0.06 (0.06, 0.07)	<0.001
Constant	-0.93 (-1.11, -0.74)	<0.001

HC, hormonal contraception; 25(OH)D, 25-hydroxyvitamin D

[1]Estimated difference in log homeostatic model assessment for insulin resistance from the reference category of categorical variables or per 1 unit increase of continuous variables

417 Table 4. Adjusted associations between serum 25(OH)D concentrations and log triglycerides

Variable	Coefficient (95% CI)[1]	*p*
25(OH)D (nmol/L)	0.0023 (0.0008, 0.0038)[2]	0.003
Time		
20 year follow-up	-0.12 (-0.17, -0.06)	<0.001
Sex		
Female using HC	0.24 (0.08, 0.40)	0.003
Male	0.25 0.12, 0.39)	<0.001
Time*Sex		
20 year, Female using HC	0.08 (-0.01, 0.17)	0.070
20 year, Male	0.11 (0.04, 0.18)	0.001
Sex*25(OH)D		
Female using HC	-0.0003 (-0.0021, 0.0016)	0.779
Male	-0.0021 (-0.0038, -0.0004)	0.016
Body mass index (kg/m^2)	0.03 (0.02, 0.03)	<0.001
Constant	-0.95 (-1.11, -0.80)	<0.001

418

419 HC, hormonal contraception; 25(OH)D, 25-hydroxyvitamin D

420 [1]Estimated difference in log triglycerides from the reference category of categorical variables

421 or per 1 unit increase of continuous variables

422 [2]Coefficient for females not using HC

423 Males: Coefficient=0.00015; 95%CI -0.0008, 0.001; *p*=0.738

424

The authors have no potential conflicts of interest.

TAM, LJBeilin and WHO designed the research; TAM, LJBeilin and WHO conducted the research; PGH provided essential reagents or provided essential materials; SB and LJBlack analysed data; LJBlack, SB and TAM wrote the paper; CEM, R-C H, WCS P-D, RML and PHH provided critical revision of the manuscript for important intellectual content; TAM had primary responsibility for final content. All authors read and approved the final manuscript.

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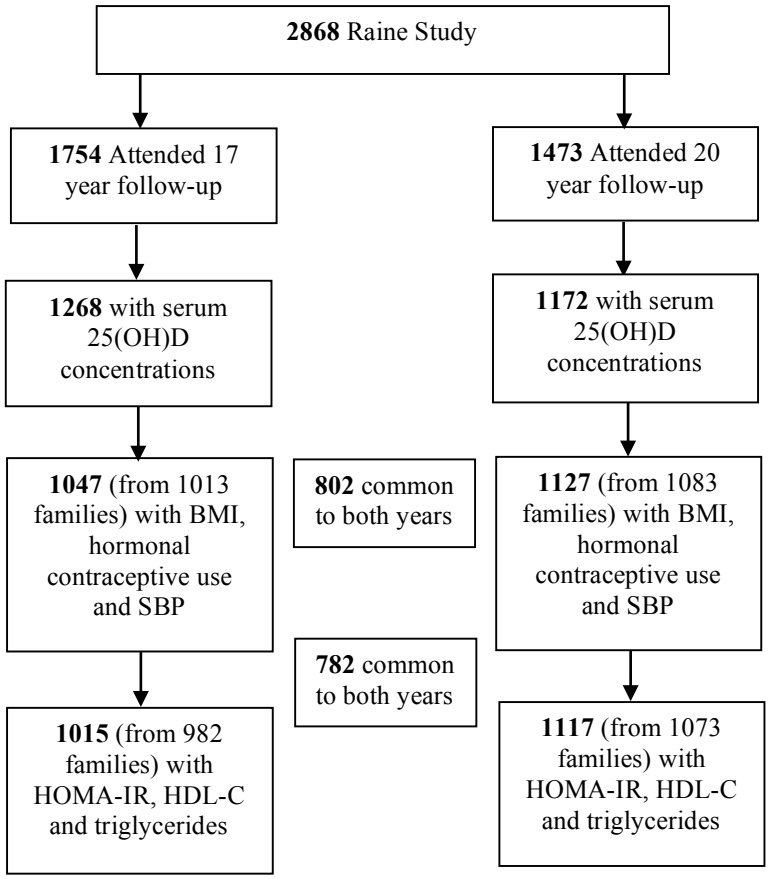
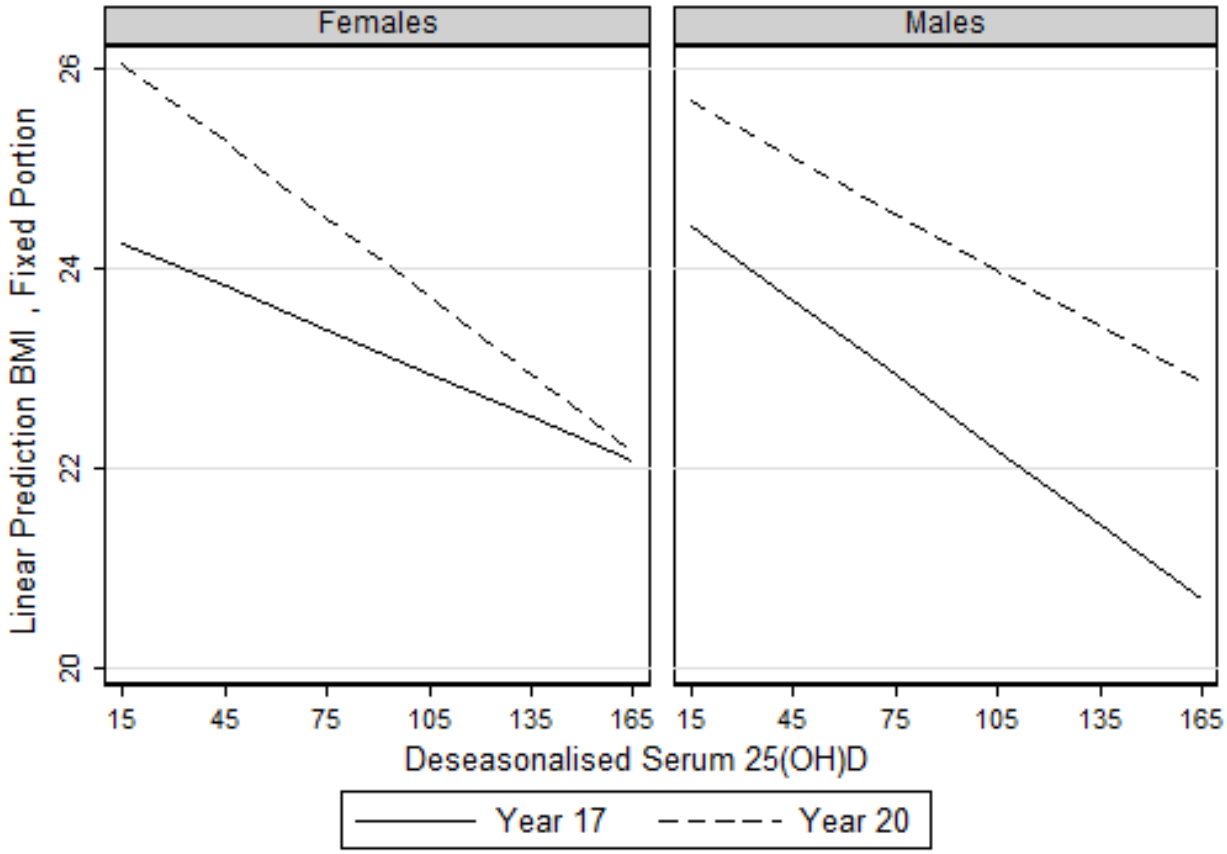


Figure 1. CONSORT Flow Diagram



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583 Figure 2. A graphical representation of the three-way interaction in the BMI model showing an
584 inverse association between deseasonalised serum 25-hydoxyvitamin D_3 concentrations and BMI
585 that does not change over time in males, but is stronger in females at 20 years compared with 17
586 years.
587 25(OH)D, 25-hydoxyvitamin D

Supplementary Table 1. Effect of covariates, missingness and modelling technique on estimated serum 25(OH)D coefficient for models with SBP, log HDL-C and log Homa-IR as outcomes

Outcome	Covariate	Covariate p[1]	(A) Final reported Hierarchical LMM	p	(B) Hierarchical LMM unadjusted reduced sample	p	(C) Hierarchical LMM adjusted reduced sample	p	(D) Clustered analysis adjusted reduced sample	p	(E) Clustered analysis adjusted with MLE	p
			Serum 25(OH)D coefficient and p values									
SBP			0.00225	0.806								
	Alcohol	0.006			0.00848	0.41	0.00448	0.67	0.00565	0.60	0.00305	0.74
	Family income	0.23			-0.00189	0.87	-0.00346	0.77	-0.00227	0.85	0.00459	0.62
	Smoking	0.998			-0.00151	0.87	-0.00151	0.87	0.00149	0.88	0.00676	0.47
	Physical activity	0.82			0.00159	0.87	0.00277	0.78	0.00510	0.62	0.00828	0.39
	Race[2]	0.38					0.00082	0.93	0.00451	0.64		
log HDL-C			0.00010	0.528								
	Alcohol	<0.001			0.00006	0.76	-0.00004	0.81	0.00006	0.76	0.00009	0.65
	Family income	0.56			-0.00004	0.87	-0.00005	0.81	0.00001	0.98	0.00020	0.30
	Smoking	0.34			0.00011	0.53	0.00011	0.52	0.00020	0.32	0.00022	0.23
	Physical activity	0.18			0.00009	0.60	0.00003	0.85	0.00006	0.79	0.00013	0.51
	Race[2]	0.034					0.00016	0.34	0.00031	0.10		
log Homa-IR			-0.00216	<0.001								
	Alcohol	0.84			-0.00223	<0.001	-0.00222	<0.001	-0.00231	<0.001	-0.00223	<0.001
	Family income	0.64			-0.00226	0.002	-0.00219	0.002	-0.00237	0.003	-0.00222	<0.001
	Smoking	0.85			-0.00209	<0.001	-0.00208	<0.001	-0.00220	<0.001	-0.00226	<0.001
	Physical activity	0.12			-0.00230	<0.001	-0.00208	0.001	-0.00214	0.001	-0.00201	0.001
	Race[2]	0.68					-0.00221	<0.001	-0.00235	<0.001		

(A): Hierarchical LMM adjusting for sex and BMI, reported as final model

(B): Hierarchical LMM based on sample with covariate data (reduced due to missing data on covariate), without covariate included in the model

(C): Hierarchical LMM (B) with covariate included in the model

(D): Clustered analysis of model with covariate included without MLE

(E): Same as (D) with MLE for missing covariate data

The difference between Models C and D illustrates the difference between Hierarchical LMM and clustered analysis, which tends to result in a loss of power.

The difference between Models D and F illustrates the potential impact of missing data.

[1]In Hierarchical LMM in reduced sample

[2]No missing data

No data supplied for BMI outcome as no covariates were significantly associated with outcome.

25(OH)D, 25-hydroxyvitamin D; BMI, body mass index; HC, hormonal contraception; HDL-C, high-density lipoprotein cholesterol; HOMA, homeostatic model assessment for insulin resistance; LMM, linear mixed model; MLE, maximum likelihood estimation; SBP, systolic blood pressure

Supplementary Table 2. Effect of covariates, missingness and modelling technique on estimated serum 25(OH)D coefficient for models with log triglycerides as outcome

Covariate	Covariate p[1]	(A) Final reported Hierarchical LMM	p	(B) Hierarchical LMM unadjusted reduced sample	p	(C) Hierarchical LMM adjusted reduced sample	p	(D) Clustered analysis adjusted reduced sample	p	(E) Clustered analysis adjusted with MLE	p
		Serum 25(OH)D coefficient and p values									
25(OH)D		0.00228	0.003								
Sex*25(OH)D											
Females using HC		-0.00026	0.78								
Males		-0.00212	0.016								
Alcohol	0.002										
25(OH)D				0.00212	0.012	0.00204	0.015	0.00155	0.067	0.00170	0.023
Sex*25(OH)D											
Females using HC				-0.00006	0.96	-0.00008	0.94	-0.00022	0.84	-0.00069	0.49
Males				-0.00211	0.033	-0.00225	0.022	-0.00176	0.093	-0.00151	0.11
Family income	0.851										
25(OH)D				0.00276	0.005	0.00275	0.005	0.00203	0.02	0.00175	0.02
Sex*25(OH)D											
Females using HC				-0.00023	0.84	-0.00021	0.85	-0.00061	0.58	-0.00064	0.52
Males				-0.00238	0.035	-0.00237	0.035	-0.00127	0.29	-0.00135	0.15
Smoking	0.003										
25(OH)D				0.00224	0.003	0.00217	0.004	0.00180	0.02	0.00171	0.022
Sex*25(OH)D											
Females using HC				-0.00023	0.81	-0.00009	0.92	-0.00054	0.6	-0.00050	0.62
Males				-0.00227	0.011	-0.00227	0.011	-0.00166	0.085	-0.00142	0.13
Physical activity	0.122										
25(OH)D				0.00230	0.004	0.00247	0.002	0.00217	0.01	0.00201	0.008
Sex*25(OH)D											

Females using HC		-0.00022	0.83	-0.00027	0.79	-0.00069	0.52	-0.00078	0.43
Males		-0.00241	0.011	-0.00243	0.01	-0.00187	0.07	-0.00144	0.12
Race[2]	0.276								
25(OH)D				0.00237	0.002	0.00186	0.014		
Sex*25(OH)D									
Females using HC				-0.00030	0.75	-0.00070	0.48		
Males				-0.00215	0.015	-0.00140	0.14		

(A): Hierarchical LMM adjusting for sex and BMI, reported as final model

(B): Hierarchical LMM based on sample with covariate data (reduced due to missing data on covariate), without covariate included in the model

(C): Hierarchical LMM (B) with covariate included in the model

(D): Clustered analysis of model with covariate included without MLE

(E): Same as (D) with MLE for missing covariate data

The difference between Models C and D illustrates the difference between Hierarchical LMM and clustered analysis, which tends to result in a loss of power.

The difference between Models D and F illustrates the potential impact of missing data.

[1]In Hierarchical LMM in reduced sample

[2]No missing data

25(OH)D, 25-hydroxyvitamin D; BMI, body mass index; HC, hormonal contraception; HDL-C, high-density lipoprotein cholesterol; HOMA, homeostatic model assessment for insulin resistance; LMM, linear mixed model; MLE, maximum likelihood estimation; SBP, systolic blood pressure

Supplementary Table 3. Associations between serum 25-hydroxyvitamin D and log HDL-C

Variable	Coefficient (95% CI)[1]	*p*
25(OH)D (nmol/L)	0.0001 (-0.0002, 0.0004)	0.528
Time		
17 year follow-up	Reference	
20 year follow-up	0.04 (0.03, 0.06)	<0.001
Sex		
Female not using HC	Reference	
Female using HC	-0.01 (-0.03, 0.01)	0.441
Male	-0.15 (-0.17, -0.13)	<0.001
Body mass index (kg/m^2)	-0.01 (-0.02, -0.01)	<0.001
Constant	0.65 (0.59, 0.71)	<0.001

[1]Estimated difference in log high-density lipoprotein from the reference category of categorical variables or per 1 unit increase of continuous variables

25(OH)D, 25-hydroxyvitamin D; HC, hormonal contraception

Supplementary Table 4. Associations between serum 25-hydroxyvitamin D and SBP

Variable	Coefficient (95% CI)[1]	*p*
25(OH)D (nmol/L)	0.002 (-0.02, 0.02)	0.820
Time		
17 year follow-up	Reference	
20 year follow-up	1.10 (0.34, 1.86)	0.005
Sex		
Female not using HC	Reference	
Female using HC	2.02 (0.71, 3.33)	0.003
Male	12.20 (11.04, -13.35)	<0.001
Body mass index (kg/m^2)	0.74 (0.64, 0.841)	<0.001
Constant	91.47 (88.42, 94.52)	<0.001

[1]Estimated difference in systolic blood pressure from the reference category of categorical variables or per 1 unit increase of continuous variables

25(OH)D, 25-hydroxyvitamin D; HC, hormonal contraception