

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

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20 **Running title:** 25-hydroxyvitamin D and cardio-metabolic risk

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22 **Key words:** 25-hydroxyvitamin D, vitamin D, cardio-metabolic risk, adolescents

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35 **Abstract**

36

37 Evidence associating serum 25-hydroxyvitamin D (25(OH)D) concentrations and cardio-metabolic
38 risk factors is inconsistent and studies have largely been conducted in adult populations. We examined
39 the prospective associations between serum 25(OH)D concentrations and cardio-metabolic risk
40 factors from adolescence to young adulthood in the West Australian Pregnancy Cohort (Raine) Study.
41 Serum 25(OH)D concentrations, BMI, homeostatic model assessment for insulin resistance (HOMA-
42 IR), triglycerides, high-density lipoprotein-cholesterol (HDL-C) and systolic blood pressure (SBP)
43 were measured at the 17 ($n=1015$) and 20 ($n=1117$) year follow-ups. Hierarchical linear mixed models
44 with maximum likelihood estimation were used to investigate associations between serum 25(OH)D
45 concentrations and cardio-metabolic risk factors, accounting for potential confounders. In males and
46 females respectively, mean (SD) serum 25(OH)D concentrations were 73.6 (28.2) and 75.4 (25.9)
47 nmol/L at 17 years, and 70.0 (24.2) and 74.3 (26.2) nmol/L at 20 years. Deseasonalised serum
48 25(OH)D₃ concentrations were inversely associated with BMI (Coefficient=-0.01; 95%CI -0.03,-
49 0.003; $p=0.014$). No change over time was detected in the association for males; for females the
50 inverse association was stronger at 20 years compared with 17 years. Serum 25(OH)D concentrations
51 were inversely associated with log HOMA-IR (Coefficient =-0.002; 95%CI -0.003,-0.001; $p<0.001$)
52 and positively associated with log triglycerides in females (Coefficient=0.002; 95%CI 0.0008,0.004;
53 $p=0.003$). These associations did not vary over time. There were no significant associations between
54 serum 25(OH)D concentrations and HDL-C or SBP. Clinical trials in those with insufficient vitamin
55 D status may be warranted to determine any beneficial effect of vitamin D supplementation on insulin
56 resistance, while monitoring for any deleterious effect on triglycerides.

57 **Introduction**

58

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

70

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

81

82 **Methods**

83

84 ***Participants***

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

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

93

94 ***Serum 25-hydroxyvitamin D concentrations***

95 Serum 25(OH)D concentrations were determined at the 17 and 20 year follow-ups. Venous blood
96 samples were taken from an antecubital vein after an overnight fast and stored at -80°C until analysis.
97 Serum 25(OH)D₂ and 25(OH)D₃ concentrations were measured using isotope-dilution liquid
98 chromatography-tandem mass spectrometry (LC-MS/MS) (RDDT, Victoria, Australia), according to
99 published methodology⁽¹³⁾. The method for the quantification of vitamin D metabolites (25(OH)D₂
100 and 25(OH)D₃) in serum/plasma used hexa-deuterated 25(OH)D₃ as an internal standard on either the
101 API 4000 QTRAP system or the Agilent 6410 QQQ LC-MS/MS system. Serum Calibration
102 Standards containing nominal amounts of 25(OH)D₂/25(OH)D₃ from CHROMSYSTEMS were used
103 to generate calibration curves. Tri-level Quality Control samples containing 25(OH)D₂/25(OH)D₃
104 designated as Level Low, Level 1 and Level 2 were purchased from UTAK Laboratories (PM
105 Separations). At 17 years, the coefficient of variation (CV) for 25(OH)D₃ was 7.1% at 27 nmol/L,
106 5.0% at 75 nmol/L, and 5.3% at 164 nmol/L. The CV for 25(OH)D₂ was 8.8% at 23 nmol/L, 6.7% at
107 66 nmol/L, and 6.7% at 150 nmol/L. At 20 years, the CV for 25(OH)D₃ was 5.8% at 28 nmol/L, 5.2%
108 at 80 nmol/L, and 9.2% at 188 nmol/L. The CV for 25(OH)D₂ was 7.9% at 25 nmol/L, 6.6% at 75
109 nmol/L, and 10.4% at 185 nmol/L.

110

111 ***Cardio-metabolic risk factors***

112 Participants at 17 years were weighed to the nearest 100 g using a Wedderburn Digital Chair Scale
113 and height was determined to the nearest 0.1 cm with a Holtain Stadiometer. At 20 years, weight was
114 measured to the nearest 100 g using Personal Precision scales UC-321 (A and D Company) and height
115 was measured to the nearest 0.1 cm with a wall mounted Seca 202. Body mass index (BMI) was
116 calculated as weight in kilograms divided by height in metres squared. Fasting blood samples at the
117 17 and 20 year follow-ups were analysed for serum glucose, HDL-C and triglycerides, determined
118 enzymatically on an Architect c16000 Analyser (Abbott Laboratories, Lake Forest, Illinois) (intra-
119 assay CVs were 1.0% for glucose, 2.0% for HDL-C, and 1.9% for triglycerides). Serum insulin was
120 determined on an Architect i2000SR Analyser (intra-assay CV 1.78%). The homeostatic model
121 assessment for insulin resistance (HOMA-IR) score was calculated as follows: HOMA-IR score =
122 (fasting insulin (μU/mL) x fasting glucose (mmol/L)) / 22.5⁽¹⁴⁾. Resting blood pressure at 17 and 20
123 years was obtained using an oscillometric sphygmomanometer (Dinamap ProCare 100, Soma
124 Technology, USA). After five minutes of quiet rest, six automatic recordings were taken every two

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

127

128 ***Potential confounding variables***

129 Participants were classified as Caucasian if both parents were Caucasian, or as non-Caucasian if one
130 or both parents were of an alternate ethnicity. Physical activity at the 17 year follow-up was assessed
131 using a self-reported questionnaire based on exercise outside of school hours per week, with exercise
132 defined in three categories as activity causing breathlessness or sweating (≥ 4 times per week, 1-3
133 times per week and $<$ once per week). At 20 years, physical activity was assessed using a self-reported
134 questionnaire recording time spent in moderate or vigorous physical activity (≥ 4 times per week, 1-
135 3 times per week and $<$ once per week).

136

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

142

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

152

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

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

163

164 *Addressing potential seasonal effects*

165 Models investigating HDL-C, triglycerides and HOMA-IR were not adjusted for season as these
166 biomarkers change relatively quickly over time and the blood sample for these biomarkers was taken
167 on the same day as that for the measurement of serum 25(OH)D concentrations. Similarly, SBP
168 changes relatively quickly over time and it would not be appropriate to adjust for season if serum
169 25(OH)D concentrations and SBP measurements were taken on the same day. Although 95% of
170 participants had blood for serum 25(OH)D concentrations and SBP measurements taken within 31
171 days of each other, approximately 5% of participants had measurements taken more than 31 days
172 apart. In order to determine if the time difference between measurement of serum 25(OH)D
173 concentrations and SBP influenced the association between serum 25(OH)D concentrations and SBP,
174 we deseasonalised the serum 25(OH)D₃ concentrations using sine and cosine curves⁽¹⁷⁾ and moved
175 the projected date back to match the day that SBP was measured. This technique has previously been
176 reported by Lucas and colleagues⁽¹⁸⁾. For models investigating BMI, which changes relatively slowly
177 over time, we used deseasonalised serum 25(OH)D₃ concentrations⁽¹⁷⁾.

178

179 *Statistical analyses*

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

188

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

195

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

206

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

214

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

220

221 **Results**

222

223 ***Participant characteristics***

224 Full data for outcome variables and HC were available for 1015 participants at the 17 year follow-up
225 and 1117 participants at the 20 year follow-up. A CONSORT flow diagram is shown in Figure 1, and
226 participant characteristics for males and females are shown in Table 1. Mean (SD) serum 25(OH)D
227 concentrations were 73.6 (28.2) and 75.4 (25.9) nmol/L at the 17 year follow-up in males and females,
228 respectively; and 70.0 (24.2) and 74.3 (26.2) nmol/L at the 20 year follow-up in males and females,
229 respectively. At the 17 year follow-up, only 4 participants had detectable serum 25(OH)D₂

230 concentrations, ranging from 5.44 to 8.12 nmol/L. At the 20 year follow-up, 13 participants had
231 detectable serum 25(OH)D₂ concentrations, ranging from 5.24 to 7.07 nmol/L.

232
233 ***Deseasonalised serum 25-hydroxyvitamin D₃ concentrations and BMI***

234 In a univariate analysis, deseasonalised serum 25(OH)D₃ concentrations were inversely associated
235 with BMI (Coefficient=-0.02; 95%CI -0.03,-0.02; $p<0.001$) and the association persisted after
236 adjusting for sex (Table 2). A significant three way interaction between time, sex and serum
237 25(OH)D₃ concentrations indicated that the relationship between serum 25(OH)D₃ concentrations
238 and BMI changed over time differently in males compared to females ($p=0.026$). No change over
239 time was detected in the association for males, whereas for females the inverse association was
240 stronger at 20 years compared with 17 years (Figure 2). The model estimates that a one standard
241 deviation increase in serum 25(OH)D₃ concentrations (approximately 25 nmol/L) was associated with
242 a reduction in BMI of 0.4 kg/m² in females at 17 years; 0.6 kg/m² in females at 20 years; 0.6 kg/m²
243 in males at 17 years; and 0.5 kg/m² in males at 20 years.

244
245 ***Serum 25-hydroxyvitamin D concentrations and insulin resistance***

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

252
253 ***Serum 25-hydroxyvitamin D concentrations and triglycerides***

254 In a univariate model, serum 25(OH)D concentrations were positively associated with log
255 triglycerides (Coefficient=0.001; 95%CI 0.0004, 0.002; $p=0.03$). A significant interaction between
256 serum 25(OH)D concentrations and sex indicated the relationship between serum 25(OH)D
257 concentrations and log triglycerides differed in males when compared to females ($p=0.016$). There
258 was a positive association in females not using HC after adjusting for BMI (Coefficient=0.0023;
259 95%CI 0.0008, 0.0038; $p=0.003$) (Table 4), and a similar effect in females using HC. Coefficients
260 from the model estimate that a one standard deviation increase in serum 25(OH)D concentrations was
261 associated with a 6% increase in triglycerides in females. There was no significant association
262 between serum 25(OH)D concentrations and triglycerides in males ($p=0.738$).

263
264 ***Serum 25-hydroxyvitamin D concentrations and HDL-C***

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

270

271 *Serum 25-hydroxyvitamin D concentrations and SBP*

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

279

280 **Discussion**

281

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

286

287 The inverse association between deseasonalised serum 25(OH)D₃ concentrations and BMI was
288 consistent between 17 and 20 years in males, but increased over time in females. Similarly, an inverse
289 association between serum 25(OH)D concentrations and waist circumference was identified by
290 Gagnon and colleagues in a prospective study of 4164 adults participating in The Australian Diabetes,
291 Obesity and Lifestyle (AusDiab) Study⁽¹⁹⁾, and by Ganji and colleagues in a cross-sectional study of
292 5867 adolescents participating in the United States' National Health and Nutrition Examination
293 Survey (NHANES)⁽²⁰⁾.

294

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

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

306
307 There may be associations between vitamin D responsiveness, vitamin D-receptor (VDR) gene
308 polymorphisms and insulin resistance. Associations between single nucleotide polymorphisms
309 (SNPs) and responses in insulin sensitivity to vitamin D supplementation of 4000 IU per day were
310 determined in 81 South Asian women⁽⁴²⁾. The improvement in insulin sensitivity was significantly
311 greater in women with the *FokI* Ff genotype compared with women with the *FokI* FF genotype.
312 Therefore, differences in the VDR gene may explain differences in response of insulin sensitivity to
313 vitamin D supplementation. Further explorations of the influence of SNPs on responsiveness of
314 cardio-metabolic risk factors to vitamin D intervention are warranted.

315
316 We found a positive association between serum 25(OH)D concentrations and triglycerides in females.
317 Other observational studies have shown an inverse association^(19; 43), or no association⁽²⁷⁾, between
318 circulating 25(OH)D concentrations and triglycerides. However, our finding was supported by a
319 recent randomised controlled trial in 200 hypertensive adults with low serum 25(OH)D
320 concentrations: Pilz and colleagues showed that vitamin D supplementation significantly increased
321 triglycerides compared with placebo⁽⁴⁴⁾. Although the authors hypothesised that this result was a
322 chance finding, they noted that further validation from additional studies was warranted. In contrast,
323 a trial in 200 healthy overweight subjects participating in a 12 month weight-reduction programme
324 found that, compared with the placebo group, vitamin D supplementation lowered triglycerides but
325 increased LDL-C⁽⁴⁵⁾. We found no statistically significant associations between serum 25(OH)D
326 concentrations and HDL-C, which is supported by the studies by Nam and colleagues, and Gagnon
327 and colleagues^(19; 27). A recent review of vitamin D supplementation and lipid profile found that most
328 randomised controlled trials showed no effects, or even adverse effects, on serum lipids⁽⁴⁶⁾.

329
330 We found no independent association between serum 25(OH)D concentrations and SBP. This finding
331 conflicts with a systematic review and meta-analysis of 22 cross-sectional studies and eight
332 prospective studies (largely in adult populations), which showed that circulating 25(OH)D
333 concentrations were inversely associated with risk of hypertension⁽⁴⁷⁾. However, the results of
334 vitamin D supplement trials and SBP are equivocal. A meta-analysis of 16 randomised trials of

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

338

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

348

349 Strengths of our study include the young age of the cohort, and the prospective design based on a
350 longitudinal cohort. We were able to investigate confounders that potentially impact upon serum
351 25(OH)D concentrations and cardio-metabolic risk factors, including race, month of birth, BMI,
352 physical activity, family income, hormonal contraceptive use, smoking and alcohol intake. However,
353 despite our best efforts to adjust for confounders, we cannot rule out the possibility of residual
354 confounding. Weaknesses of our study include the measurement of physical activity, which was not
355 based on a validated questionnaire and may be subject to self-reporting bias. Furthermore, our
356 assessment of physical activity did not differentiate between indoor and outdoor activity. The non-
357 significance of physical activity in relation to cardio-metabolic risk factors may reflect the limitations
358 of the measurement, rather than the true absence of an association between physical activity and the
359 outcome. In light of the evidence associating genetic factors with responses in insulin resistance to
360 vitamin D supplementation ⁽⁴²⁾, it is possible that genetic variation may modify the association
361 between serum 25(OH)D concentrations and cardio-metabolic risk factors. In our study, no data were
362 available for genetic factors relating to vitamin D; further studies in this area would benefit from
363 investigating factors such as the *FokI* Ff genotype.

364

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

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

376
377 Our results show that serum 25(OH)D concentrations are independently associated with a number of
378 cardio-metabolic risk factors in adolescents and young adults, with differential effects on BMI with
379 time between sexes. In particular, the finding that serum 25(OH)D concentrations were inversely
380 associated with BMI and insulin resistance would suggest a cardio-protective benefit, but this may be
381 offset by the positive association between serum 25(OH)D concentrations and triglycerides. Well-
382 designed clinical trials in those with insufficient vitamin D status may be warranted to determine any
383 causal, beneficial effect of vitamin D supplementation on insulin resistance in adolescents and young
384 adults, while monitoring for any deleterious effect on triglycerides.

385

386 **Acknowledgements**

387 The authors gratefully acknowledge the Raine Study participants and their families. We thank the
388 Raine Study Team for cohort coordination and data collection. Core funding for the Raine Study is
389 provided by the University of Western Australia; the Faculty of Medicine, Dentistry and Health
390 Sciences at the University of Western Australia; the Telethon Kids Institute; the Women and Infants
391 Research Foundation; Curtin University; and the Raine Medical Research Foundation. Data
392 collection and biological specimens at the 17-year follow-up were funded by the National Health and
393 Medical Research Council (Programme Grant ID 353514 and Project Grant ID 403981) and the Ada
394 Bartholomew Medical Research Trust. Data collection for the 20-year follow-up was funded by the
395 National Health and Medical Research Council Project ID 1022134.

396 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 (<i>n</i> =1015)				20 year follow-up (<i>n</i> =1117)			
	Male		Female		Male		Female	
	<i>n</i>		<i>n</i>		<i>n</i>		<i>n</i>	
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
397 Alcohol intake in consumers [g/day, median (IQR)]		6.0 (12.3)		5.4 (8.9)		15.9 (30.0)		8.4 (16.4)

398 25(OH)D, 25-hydroxyvitamin D; HOMA-IR, homeostatic model assessment for insulin resistance; LDL-C, low-density lipoprotein; HDL-C, high-
399 density lipoprotein; SBP, systolic blood pressure; BMI, body mass index; NA, not applicable; NR, not reported.

400 Table 2. Adjusted associations between deseasonalised serum 25(OH)D₃ concentrations and
 401 body mass index

Variable	Coefficient (95% CI) ¹	<i>p</i>
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

402
 403 25(OH)D₃, 25-hydroxyvitamin D₃

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

406 ²Coefficient for females, 17 year follow-up

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

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

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

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

Variable	Coefficient (95% CI) ¹	<i>p</i>
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

412

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

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

416

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

Variable	Coefficient (95% CI) ¹	<i>p</i>
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 ¹Estimated difference in log triglycerides from the reference category of categorical variables

421 or per 1 unit increase of continuous variables

422 ²Coefficient for females not using HC

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

424

425 The authors have no potential conflicts of interest.

426

427 TAM, LJBeilin and WHO designed the research; TAM, LJBeilin and WHO conducted the

428 research; PGH provided essential reagents or provided essential materials; SB and LJBlack

429 analysed data; LJBlack, SB and TAM wrote the paper; CEM, R-C H, WCS P-D, RML and

430 PHH provided critical revision of the manuscript for important intellectual content; TAM had

431 primary responsibility for final content. All authors read and approved the final manuscript.

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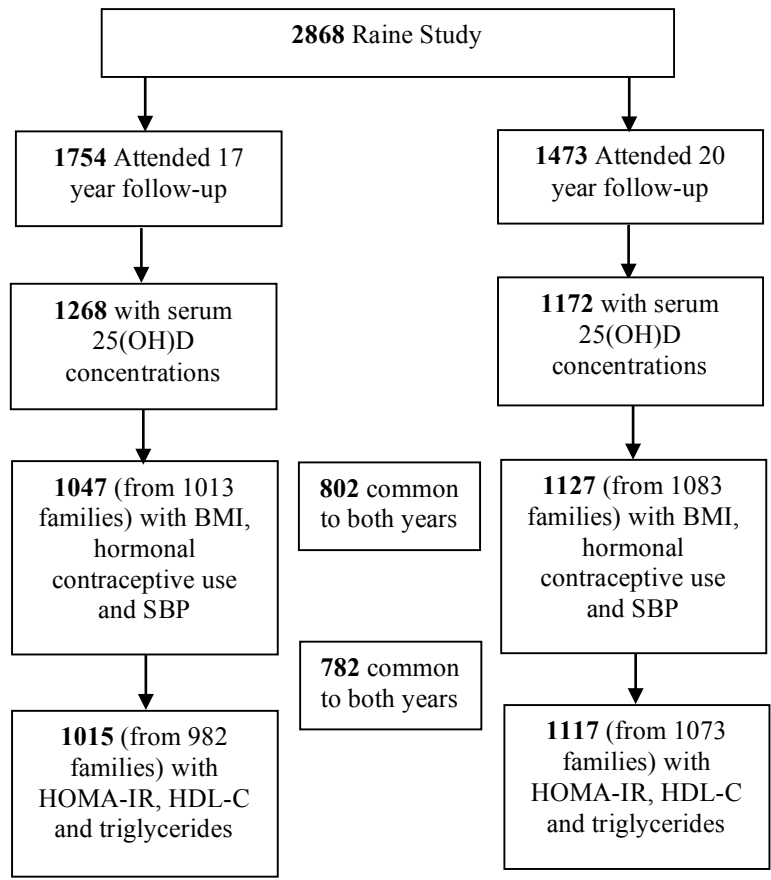
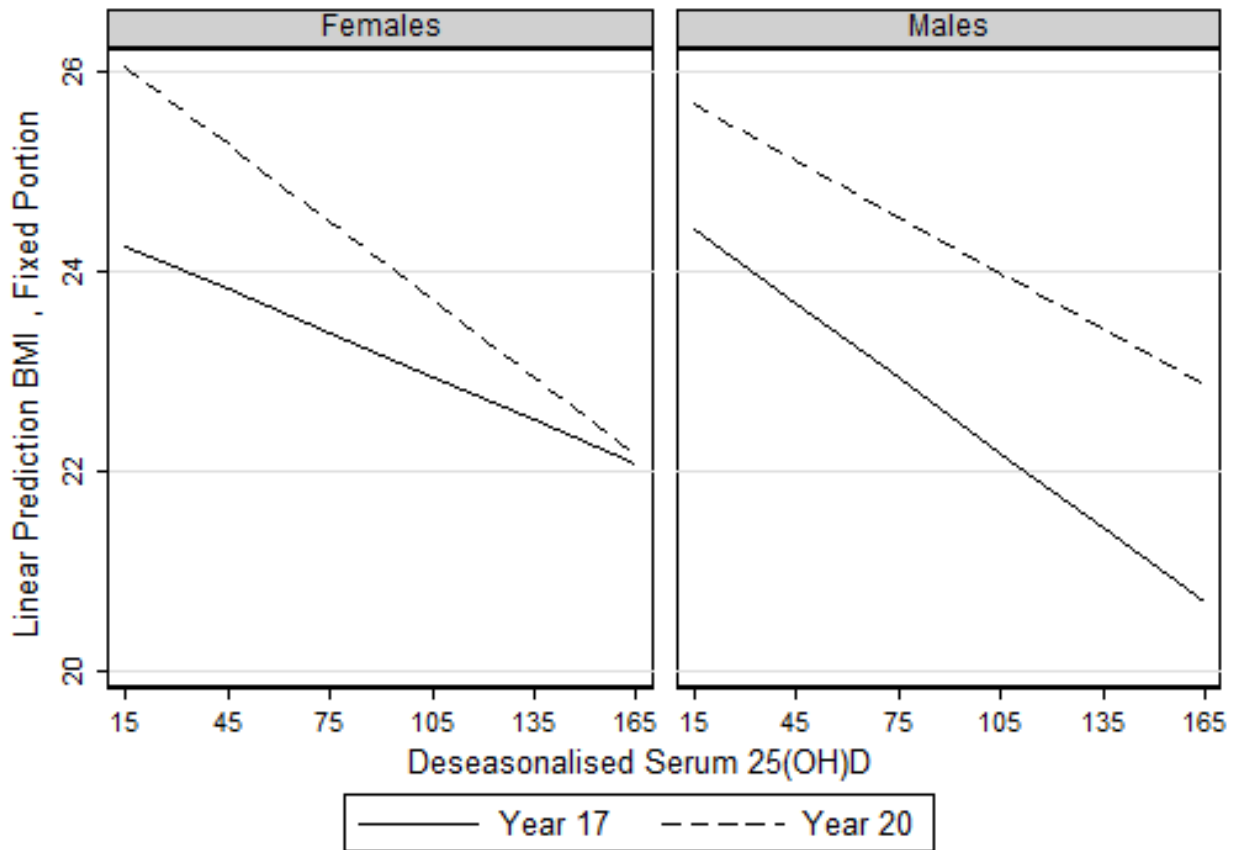


Figure 1. CONSORT Flow Diagram



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582

583 Figure 2. A graphical representation of the three-way interaction in the BMI model showing an
 584 inverse association between deseasonalised serum 25-hydroxyvitamin D₃ 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-hydroxyvitamin 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	Serum 25(OH)D coefficient and p values									
			(A) Final reported Hierarchical LMM		(B) Hierarchical LMM unadjusted reduced sample		(C) Hierarchical LMM adjusted reduced sample		(D) Clustered analysis adjusted reduced sample		(E) Clustered analysis adjusted with MLE	
				p		p		p		p		p
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.

¹In Hierarchical LMM in reduced sample

²No 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

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.

¹In Hierarchical LMM in reduced sample

²No 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	<i>p</i>
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

¹Estimated 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	<i>p</i>
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

¹Estimated 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