Cardiovascular Epidemiology

Prime mover or fellow traveller: 25-hydroxy vitamin D's seasonal variation, cardiovascular disease and death in the Scottish Heart Health Extended Cohort (SHHEC)

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Abstract

Background: Theoretical links between seasonal lack of sunlight, hypovitaminosis D and excess cardiovascular disease and death prompted our adding novel to conventional cohort analyses.

Methods: We tested three postulates on 13 224 Scottish Heart Health Extended Cohort participants, assayed for 25-hydroxyvitamin D (25OHD) and followed for 22 years. (i) Endpoints enumerated by month of occurrence mirror annual seasonal oscillation in 25OHD. (ii) Endpoint seasonality is increased in people with below median 25OHD. (iii) Low 25OHD predicts endpoints independently of major risk factors.

Results: Baseline median 25OHD level was 36.4 (other quartiles 26.7, 51.7) nmol/l. The March trough was half the August peak, both well after seasonal solstices. (i) There was no demonstrable monthly variation in First Cardiovascular Event ($n = 3307$). Peaks and troughs for All Death and Cardiovascular Death ($n = 2987, 1350$) were near the solstices, earlier than extremes of 25OHD. (ii) Endpoint variability showed no difference between those above and below median 25OHD. (iii) Cox model hazard ratios (HR), by decreasing 25OHD, increased modestly and nonspecifically for all endpoints examined, with no threshold, the gradients diminishing by ~ 60% following multiple adjustment. For Cardiovascular Disease, HR, by 20 (~SD) nmol/l decrease, $= 1.224$ (1.175, 1.275) adjusted for age and sex; additionally adjusted for family history, deprivation index, smoking, systolic blood pressure, total and HDL cholesterol, $= 1.093$ (1.048, 1.139); All Deaths $= 1.238$ (1.048, 1.139) and

1.098 (1.050, 1.149). 25OHD made no independent contribution to cardiovascular discrimination and reclassification.

Conclusions: Our analyses challenge vitamin D's alleged role as major prime mover in cardiovascular disease and mortality.

Key words: : Vitamin D, causality, seasonality, cardiovascular disease, mortality, cohort study

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Key Messages

- Winter excess of cardiovascular disease and death is attributed to hypovitaminosis D through lack of sunlight.
- We added seasonality to a conventional follow-up study of a Scottish cohort.
- Despite two-to-one, August-to-March, change in population serum 25-hydroxyvitamin D (25OHD), there was no seasonal change in incident cardiovascular disease.
- Peaks in deaths appeared early in winter, therefore out-of-phase.
- There was no disproportionate seasonal susceptibility in people with low 25OHD.
- 25OHD was a modest predictor of endpoints, largely confounded by other risk factors, without an independent predictive effect for cardiovascular disease.
- Our results challenge vitamin D's alleged seasonal and causal role.

Introduction

Debate as to whether low population levels of vitamin D cause mass disease is stimulating large cohort studies, meta-analyses and trials,[1–6] and studies of mendelian randomization.[7] Earlier research produced inconclusive results[8,9] despite rapid increases in clinical testing and prescribing.[10,11] Meanwhile an existing natural experiment remains under-investigated. Population vitamin D levels are driven by exposure to sunlight.[12,13] In 1981, Scragg hypothesized that excess and winter peaks in cardiovascular disease and death in temperate countries were caused by hypovitaminosis D (analogous to childhood rickets a century earlier, see Figure 1).[14] Now embedded in popular medical culture, this seasonality hypothesis warrants critical testing, both in its own right and to pursue this debate along a novel perspective.

Midwinter daylight hours in Edinburgh are 39% of midsummer (contrast with 61% in New York and Madrid). The midwinter sun is also low in the sky, and there is a gross seasonal decline in ultraviolet radiation. After deriving three postulates from the seasonality hypothesis, we tested them on a Scottish cohort with long follow-up,[15] examining Bradford Hill criteria for causality between vitamin D, cardiovascular disease and mortality–specificity, temporality, strength of association and gradient.[16]

Methods

Rationale

Participants in the Scottish Heart Health Extended Cohort (SHHEC)[15] were recruited over much of 7 years, so

Figure 1. Childhood rickets from hypovitaminosis D, especially prevalent in Scotland in the early 1900s. (Wellcome Library, London).

archived recruitment serum could be used subsequently to show the annual cycle in vitamin D, averaged for each of the 12 calendar months, measured as 25-hydroxyvitamin D (25OHD). First cardiovascular events and deaths during follow-up were also cumulated to the 12 calendar months of occurrence to record seasonal variation. Because low vitamin D levels occur in winter and higher levels in summer, the bulk of readings swinging up and down *en masse*, 'raw' recruitment readings (25OHD^{raw}) were modified by seasonal adjustment (25OHD^{adj}). This defined the estimated ranking of vitamin D status within the cohort, regardless of season. It was used to divide the population in half for one analysis, and in descending fifths for another.

Postulates

Through the above rationale we developed three 'Dundee Postulates' for testing the seasonality hypothesis (see Box 1).

Scottish Heart Health Extended Cohort

This report comes from the Scottish Heart Health Extended Cohort (SHHEC),[15] the MORGAM Biomarker study (MOnica, Risk, Genetics, Archiving and Monograph) and BiomarCaRE (Biomarkers for Cardiovascular Risk Assessment in Europe).[17–19] SHHEC combines men and women of the Scottish Heart Health Study (SHHS)[15] aged 40–59 years, recruited across 23 districts of Scotland in 1984–87,with those of Scottish MONICA[20] aged 25–64 years from Edinburgh and North Glasgow 1986 and North Glasgow 1989, 1992 (–74 years) and 1995. With ethical committee approval, participants completed questionnaires, attended recruitment survey clinics, gave blood and consented to follow-up of their medical records. Each of the 23 SHHS districts was visited twice in different seasons, whereas the North Glasgow MONICA surveys involved four independent population samples in the same seasons at

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Box 1. Dundee Postulates based on the seasonality hypothesis

Seasonality Postulate 1. Endpoints, (First Cardiovascular Event, All Death and Cardiovascular Death), enumerated by calendar month of occurrence, inversely reflect seasonal fluctuation in recruitment 25-hydroxyvitamin D: 25OHD^{raw}.

Seasonality Postulate 2. This endpoint variability is accentuated in those with low vs high seasonally adjusted 25OHD^{adj}, who experience more hypovitaminosis D in winter.

Postulate 3. Low vs high 25OHD^{adj} is predictive of endpoints, independently of other risk factors.

3-year intervals. For both studies, general practitioners were recruited randomly first, and their patient lists were then subjected to second-stage random sampling.[21]

Previously diagnosed cardiovascular disease was identified by self-report and hospital discharge diagnoses: any coronary heart disease; cerebrovascular disease, including transient ischaemic attacks; heart failure; and peripheral arterial disease. Mortality and hospital inpatient episodes were followed by record linkage through 2009.[22] Cardiovascular disease morbidity was first diagnosis as above; mortality was any certified cardiovascular cause. Primary endpoints were First Cardiovascular Event, All Death and Cardiovascular Death. Secondary endpoints were Coronary Heart Disease, Cerebrovascular Disease, Non-cardiovascular Death and Cancer Death. The same SHHEC database followed through 2005 was used previously to derive the ASSIGN cardiovascular risk score.[23]

Laboratory procedures

After venepuncture, blood was left to coagulate up to 4 h. Separated serum was stored at 4°C pending laboratory testing 1–3 days later. Aliquots were biobanked long-term at -40°C[24] and assayed for 25OHD in the MORGAM/BiomarCaRE biomarker laboratory as kits became available in 2009 and 2011. The ARCHITECT system (Abbott Laboratories) uses chemiluminescent microparticle immunoassay; the intra-assay coefficient of variation (CoV) was 5.0–9.5% and inter-assay CoV 1.4–8.7%. The assay is highly sensitive to natural 25OHD$_3$, less so to synthetic 25OHD$_2$.[11,25]

Statistical methods

The main test of the correspondence of seasonality between 25OHD^{raw} and endpoint events (Postulate 1) was an ecological analysis comparing their monthly patterns. To test for increased susceptibility in winter in the 'LowD people' vs 'HighD' halves of 25OHD^{adj} (Postulate 2), their endpoint events were apportioned separately between 'Low-D' and 'High-D' seasons and the resulting two-by-two tables tested for heterogeneity using the chi square statistic. Cox proportional hazard analyses for Postulate 3 included endpoints after recruitment, regardless of season. Hazard ratios were adjusted for age and sex, then multiply adjusted for ASSIGN cardiovascular risk factors.[23] The cohort was divided into fifths in descending order of 25OHD^{adj}. The baseline hazard ratio of 1·0 was ascribed to the first fifth (100–80th centile).

For First Cardiovascular Event, discrimination, measured using a c-statistic, differences in discrimination and the net reclassification improvement (NRI),(with thresholds of 10% and 20% 10-year risk) on adding 25OHD^{adj}

to the prognostic model, over and above ASSIGN variables, were computed allowing for censoring.[26–28]

Results

Population numbers

Cross-sectional analyses included 13 224 participants assayed for 25OHD[raw]. Follow-up studies excluded 498 below age 30 and 1098 with previous cardiovascular disease. For Seasonality Postulates 1 and 2, events were followed for whole calendar years so 31 victims afflicted in the calendar year of recruitment were excluded, leaving 11 597, whereas for the Cox proportional hazards analyses (Postulate 3) the 31 were included, making 11 628.

Recruitment 25OHD[raw]

Seasonal change was as anticipated. The averaged 25OHD[raw] by calendar month of recruitment was twice as high in August (maximum) as in March (minimum), the extremes occurring weeks after the solstices. Female values were lower than in men, with a smaller summer rise (Table 1). Differences were highly significant. After adjustment for seasonality, there was little variation between

months: 25OHD[adj]. Differing monthly totals reflect cumulative recruitment activity.

Follow-up

First Cardiovascular Event, All Death and Cardiovascular Death were enumerated by calendar month of occurrence (Table 2, columns b–d). Resulting endpoint totals were split between the two sub-populations, LowD people / HighD people, either side of the median 25OHD[adj] of 38.2 nmol/l (Table 2, columns e–j). Results are unadjusted for length of month.

Seasonal pattern of 25OHD[raw] vs occurrence of endpoints (Postulate 1)

The raw monthly totals of endpoints from Table 2 were adjusted to a monthly duration of 30.44 days. Numbers were then normalized to percentage of the corresponding monthly mean, as was also done for 25OHD[raw] (see Supplement Table W1, available as Supplementary data at *IJE* online). Results are displayed as radar plots in Figure 2, contrasting the seasonal pattern in 25OHD[raw] and the three primary endpoints.

Circular plots in colour are shown against 100% in black. Calendar months in which deviation exceeds the

Table 1. Mean monthly 25-hydroxyvitamin D: 25OHD[raw] (standard deviation) by calendar month of recruitment. Then after adjustment: 25OHD[adj]. 13 224 participants in the Scottish Heart Health Extended Cohort (SHHEC) with archived serum

Month (rank)	Number	Men 25OHD[raw] nmol/l $n = 6539$	Women 25OHD[raw] nmol/l $n = 6685$	All 25OHD[raw] nmol/l $n = 13 224$	All 25OHD[adj] nmol/l $n = 13 224$
January (3)[a]	680	33.7 (15.6)	30.9 (17.1)	32.2 (16.5)	41.6 (21.2)
February (2)[a]	689	32.3 (13.3)	29.3 (12.7)	30.6 (13.1)	40.6 (17.3)
March (1)[a]	1266	30.1 (12.3)	29.7 (13.0)	29.9 (12.6)	41.2 (17.4)
April (4)[a]	1448	34.3 (17.0)	31.6 (15.0)	32.9 (16.1)	41.6 (20.3)
May (5)[a]	1585	37.9 (15.9)	33.9 (15.0)	36.0 (15.6)	41.3 (17.9)
June (9)[b]	1543	47.0 (22.1)	40.9 (19.5)	43.7 (21.0)	43.2 (20.7)
July (11)[b]	900	60.5 (23.2)	53.1 (20.0)	56.8 (22.0)	40.5 (15.7)
August (12)[b]	1344	64.2 (27.0)	53.1 (22.1)	58.6 (25.3)	41.4 (17.8)
September (10)[b]	1060	58.1 (26.1)	51.0 (24.6)	54.6 (25.6)	41.8 (19.6)
October (8)[b]	1240	45.3 (18.4)	39.9 (18.2)	42.4 (18.5)	41.0 (17.9)
November (7)[b]	971	39.9 (16.8)	36.6 (16.6)	38.3 (16.8)	41.3 (18.1)
December (6)[a]	498	38.8 (18.7)	33.7 (16.7)	36.3 (17.9)	41.5 (20.5)
All	13224	44.0 (22.6)	39.0 (19.9)	41.5 (21.4)	41.5 (18.7)
Standard error of the mean		0.28	0.24	0.19	0.16
				March vs August $P < 0.0001$	

A general linear model was fitted to estimate the excess log 25OHD[raw] each month compared with January, adjusting for age, sex and their interaction. The excess was subtracted so that each month had standardized 'January' levels; finally all values were reweighted to achieve the observed overall mean, yielding 25OHD[adj]. The ranking of months (low to high) is based on mean monthly values for sexes combined of 25OHD[raw].

[a]Low-D season.
[b]High-D season.

Table 2. Endpoint numbers by calendar month of follow-up: overall totals, and then by sub-populations of LowD people and HighD people separated at the median (38.2 nmol/l) of 25OHD^{adj} ($n = 11\,597$ for sexes combined, LowD people $n = 5799$, HighD people $n = 5798$)

a Population endpoint month	b All First CV Event	c All All Death	d All CV Death	e LowD people First CV Event	f HighD people First CV Event	g LowD people All Death	h HighD people All Death	i LowD people CV Death	j HighD people CV Death
January	281	277	140	148	133	161	116	84	56
February	250	217	93	138	112	121	96	55	38
March	270	236	112	136	134	115	121	59	53
April	295	247	114	174	121	137	110	66	48
May	271	221	100	146	125	119	102	61	39
June	287	216	96	152	135	123	93	54	42
July	285	234	104	165	120	143	91	63	41
August	253	243	115	153	100	139	104	63	52
September	276	224	109	142	134	114	110	55	54
October	296	249	122	143	153	131	118	71	51
November	267	257	122	152	115	140	117	73	49
December	276	276	123	148	128	164	112	75	48
Total	3307	2897	1350	1797	1510	1607	1290	779	571
Mean	275·6	241·4	112·5	149·8	125·8	133·9	107·5	64·9	47·6

Numbers are not corrected for length of month, but see Supplementary Table W1 (available as Supplementary data at *IJE* online). For ranking of months and Low-D/ High-D season see Table 1.
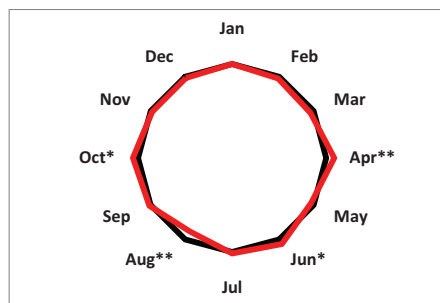
First CV Event, First Cardiovascular Event; All Death, deaths from all causes; CV Death, death from cardiovascular causes.
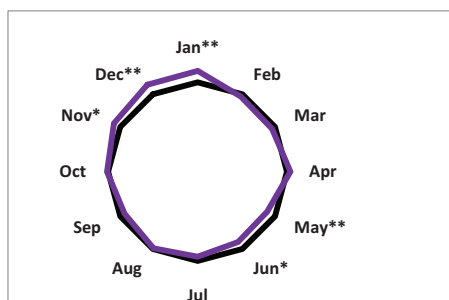
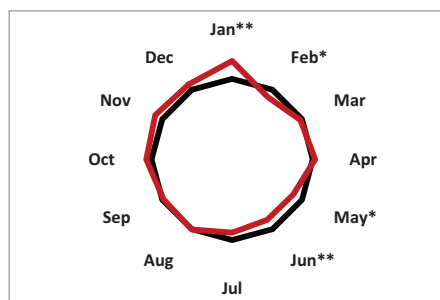a–j are for convenience of reference in the main text.
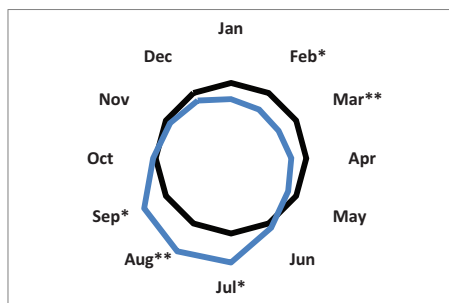
Figure 2. Radar plots from the SHHEC population of monthly variation in: A, recruitment 25-hydroxyvitamin D (25OHD^{raw}); B, First Cardiovascular Event; C, All Death; and D, Cardiovascular Death, testing Seasonality Postulate 1. Sexes combined, $n = 11\,597$, 22-year follow-up. Mean for all months (100%) shown in black. (Plotted from Supplementary Table W1, available as Supplementary data at *IJE* online).
*Outside coefficient of variation, **Highest and lowest monthly values (see text).

coefficient of variation (CoV) are asterisked; highest and lowest months have double asterisks.

Monthly values of $25OHD^{raw}$ (Figure 2A) show a cyclical annual doubling and halving, peak and trough each lasting several months, centred on late summer (August) and late winter (March), respectively. The seasonality hypothesis, Postulate 1, predicts the converse pattern for endpoints.

For First Cardiovascular Event (Figure 2B), the plot shows no deviation from circularity ($P = 0.84$). Between-months CoV is small at 5.0%; no deviation persists. Huge seasonal changes in $25OHD^{raw}$ appear to leave the monthly incidence of First Cardiovascular Events unperturbed.

Patterns for All Death (Figure 2C) and Cardiovascular Death (Figure 2D) are ovoid and consistent with seasonal variation in deaths in national statistics. CoVs are larger than for First Cardiovascular Events at 7.8% and 10.7%. For both, the peak is in early winter; the trough in early summer. With smaller numbers, Cardiovascular Death appears more subject to random deviation. For neither fatal endpoint do February or March plots, the nadir in $25OHD^{raw}$, show the peak predicted by the seasonality hypothesis.[14] The reciprocals of the monthly vitamin D values (Supplement Table W1, available as Supplementary data at *IJE* online) are 127% in February and 130% in March, but no endpoint value exceeds 100%.

In contrast to $25OHD^{raw}$ there is no seasonal pattern for First Cardiovascular Event. What there is for All Death and Cardiovascular Death precedes rather than follows the late seasonal swings in $25OHD^{raw}$, failing Bradford Hill's criterion of temporality.[16]

Increased Low-D season susceptibility to endpoints in those with low $25OHD^{adj}$ (Postulate 2)

The null hypothesis for Postulate 2 involved testing for heterogeneity in endpoint occurrences. The cohort is split in half at the median $25OHD^{adj}$ value of 38.2 nmol/l into LowD people and HighD people and the year into two 6-month periods, Low-D season and High-D season, at the 39.3 nmol/l monthly median for $25OHD^{raw}$. After the lowest 6 months against the highest, this was repeated for the lowest vs highest four, and then lowest three by highest, identifiable in Table 1. The seasons were not conventional summer and winter but reflected the delayed response of blood levels of $25OHD^{raw}$ to seasonal sunlight. Aggregate endpoint totals were sums of appropriate monthly numbers, see Table 2, columns e–j.

The results in Table 3 show no evidence of heterogeneity by season in endpoints between LowD and HighD sub-populations. There is no increased susceptibility by season in the former when tested on a six-by-six, four-by-four or three-by-three basis. The second seasonality postulate is thus not supported in the SHHEC data.

Further analysis by rearrangement of two-by-two results is shown in Supplement Table W2 (available as Supplementary data at *IJE* online). The Low-D season overall does not show excess of First Cardiovascular Events compared with High-D, but LowD people do have more endpoint events than HighD people. The latter finding is further explored in population fifths, using the Cox proportional hazards model.

Table 3. Testing the null hypothesis for Dundee Seasonality Postulate 2 (greater seasonal susceptibility in LowD people) using 2×2 tables. Endpoints are partitioned by LowD people/HighD people at risk, and by Low-D season/High-D season of occurrence. Comparisons are of lowest and highest months for $25OHD^{raw}$, 6 months vs 6, 4 vs 4 and 3 vs 3 (see Table 1)

	High-D vs Low-D months compared	Endpoint numbers in LowD people, Low-D season $n = 5799$	Endpoint numbers in LowD people, High-D season $n = 5799$	Endpoint numbers in HighD people, Low-D season $n = 5798$	Endpoint numbers in HighD people, High-D season $n = 5798$	chi square for two-by-two table	P-value (Yates)
First CV Event	6 vs 6	890	907	753	757	0.03	0.86
	4 vs 4	596	612	500	489	0.28	0.60
	3 vs 3	422	460	379	354	2.23	0.14
All Death	6 vs 6	817	790	657	633	0.00	1.00
	4 vs 4	534	519	443	398	0.65	0.42
	3 vs 3	397	396	333	305	0.56	0.45
CV Death	6 vs 6	400	379	282	289	0.43	0.51
	4 vs 4	264	235	195	189	0.31	0.58
	3 vs 3	198	181	147	147	0.25	0.62

Half-populations and half-years are almost identical in size. Endpoints from each population are being compared in two different time periods, so there is no need to adjust for other factors. Correction for trivial differences both in numbers at risk and in cumulated numbers of days in comparison months had no effect on chi square or P-values. Event numbers from Table 2. Further ratios explored in Supplement Table W2 (available as Supplementary data at *IJE* online). Numbers are not corrected for length of month–but see Supplementary Table W1 (available as Supplementary data at *IJE* online). For ranking of months and Low-D/ High-D season see Table 1.

Table 4. Hazard ratios for primary endpoints, Cox proportional hazards model by fifths of descending 25OHD[adj] values, and by 20-nmol/l change (HRD20), age and sex adjusted; and then after multiple adjustment (ma) for ASSIGN variables, sexes combined and separately.[a] All $n = 11\,628$, men $n = 5658$, women $n = 5970$

Fifths All[b]	Events / At risk	2 2327	3 2323	4 2325	5 2326	95% CL for 5	HRD20	95% CL for HRD20
First CV Event	3332	**1.26**	**1.48**	**1.59**	**1.70**	1.52,1.90	**1.224**	1.175,1.275
ma		**1.13**	**1.27**	**1.24**	**1.25**	1.11,1.40	**1.093**	1.048,1.139
All death	2928	**1.25**	**1.40**	**1.65**	**1.67**	1.48,1.89	**1.238**	1.185,1.294
ma		1.13	**1.17**	**1.28**	**1.22**	1.08,1.39	**1.098**	1.050,1.149
CV Death	1368	**1.48**	**1.86**	**2.11**	**2.21**	1.83,2.66	**1.363**	1.275,1.458
ma		**1.27**	**1.51**	**1.55**	**1.48**	1.21,1.79	**1.168**	1.090,1.252
Men[b]	Events / At risk	1131	1132	1132	1131			
First CV Event	1936	**1.33**	**1.62**	**1.60**	**1.84**	1.58,2.13	**1.238**	1.176,1.304
ma		**1.24**	**1.44**	**1.32**	**1.42**	1.21,1.65	**1.123**	1.065,1.184
All Death	1715	**1.36**	**1.55**	**1.62**	**1.89**	1.62,2.22	**1.255**	1.188,1.327
ma		**1.25**	**1.35**	**1.29**	**1.39**	1.18,1.64	**1.116**	1.055,1.180
CV Death	823	**1.66**	**2.11**	**2.09**	**2.57**	2.01,3.27	**1.394**	1.282,1.516
ma		**1.49**	**1.82**	**1.63**	**1.80**	1.40, 2.33	**1.212**	1.112,1.323
Women[b]	Events / At risk	1195	1192	1197	1193			
First CV Event	1396	1.05	**1.21**	**1.54**	**1.43**	1.21,1.70	**1.187**	1.111,1.269
ma		0.99	1.01	1.17	1.08	0.90,1.30	1.046	0.976,1.121
All Death	1213	**1.12**	**1.14**	**1.59**	**1.45**	1.21,1.75	**1.207**	1.123,1.298
ma		1.05	0.98	1.20	1.13	0.93,1.37	1.067	0.991,1.149
CV Death	545	1.23	**1.37**	**1.95**	**1.74**	1.30,2.33	**1.295**	1.157,1.450
ma		1.10	1.15	1.33	1.25	0.92,1.69	1.094	0.974,1.228

Where 95% confidence limits >1.0, hazard ratios are printed bold. HRD20 is per 20-nmol/l decrease in 25OHD[adj] ($\sim$ one standard deviation).

HRD, hazard ratio decrease; CL, confidence limits.

[a]Age, sex, family history of coronary heart disease, Scottish Index of Multiple Deprivation, tobacco smoking dose, total cholesterol and HDL cholesterol. BMI did not contribute significantly to the ASSIGN score, nor did adding it in here make any difference.

[b]First fifth(1) omitted as HR = 1.0. For sexes combined, $n = 2327$, men $n = 1132$, women $n = 1193$. Cutpoints for centiles 80, 60, 40, 20 sexes combined are: 53.84, 42.25, 34.54, 27.12 nmol/l, respectively; men: 56.53, 45.16, 36.66, 28.92 nmol/l, respectively; women: 50.64, 39.66, 32.73, 25.79 nmol/l, respectively. For full ranges and means see Supplement Table W3, available as Supplementary data at *IJE* online.

Cox proportional hazards by declining fifths of 25OHD[adj] (testing Postulate 3)

Table 4 shows the Cox proportional hazards model for primary endpoints, sexes combined and separately. Results are by declining fifths of 25OHD[adj], corrected for age and sex, and then multiply adjusted (ma) for factors in the ASSIGN score: age, sex, family history of coronary heart disease, Scottish Index of Multiple Deprivation, tobacco smoking dose, total cholesterol and high-density lipoprotein (HDL) cholesterol. Note that BMI (body mass index) did not contribute significantly to the ASSIGN score, nor did adding it in here.[22] Ranges and mean values by fifths for the sexes together and separately for 25OHD[adj] are shown in Supplement Table W3 (available as Supplementary data at *IJE* online).

There was some interaction by sex, significant for cardiovascular deaths. The gradient of risk for women is shallower than for men despite lower 25OHD[adj] values. The modest increase in risk, in results adjusted for age and sex alone, is attenuated by $\sim 60\%$ after multiple adjustment, losing statistical significance in women despite good

numbers. There was no evidence of a threshold, confirmed by spline analyses (not shown). There was no increase in risk associated with the highest 25OHD[adj] values (see Supplement Table W4, available as Supplementary data at *IJE* online). Linearity was thus modelled by analyses of hazard ratios for a 20-nmol/l decrease (HRD20) in 25OHD[adj] (the standard deviation was 18.7 nmol/l). Very similar hazard ratios for secondary endpoints (see Supplement Table W5, available as Supplementary data at *IJE* online) suggest lack of specificity in the apparent vitamin D effect. These effects were, again, strongly attenuated following multiple adjustment.

Most ASSIGN risk factors showed a significant linear relationship with 25OHD[adj]. The ASSIGN score for cardiovascular risk was 25% higher in the final fifth of 25OHD[adj] than in the first (see Supplement Table W6, available as Supplementary data at *IJE* online), consistent with the impact of ASSIGN factors in multiple adjustment.

In analyses of discrimination and net reclassification improvement for First Cardiovascular Event, sexes combined and separately, changes in the c statistic and the NRI were

small, with confidence intervals straddling zero (Supplement Table W7, available as Supplementary data at *IJE* online).

Vitamin D, measured as 25OHD^{adj}, appears to be a modest, non-specific predictor of many endpoints. Its effect is largely attenuated by competing risk factors. It has no independent effect in predicting First Cardiovascular Events beyond that of established ASSIGN risk factors, and would not therefore contribute significantly to cardiovascular risk assessment in a multifactorial risk score.

Discussion

Neglect of the hypothesized link of seasonal sunlight to cardiovascular disease and death is overdue for correction. It opens a new window on the vitamin D debate. Mendelian randomization studies[7] examine fixed differences in levels. Clinical trials raise levels, often by medication, in compliant volunteers.[3–6] Cohort studies accumulate follow-up data (perhaps to be examined differently after this).[1,2] Meanwhile a huge natural experiment, whose 'protocol' is dictated by Earth's 23.4° axis deviation and speed of angular rotation, annually raises blood levels but then lowers them to levels research ethicists might veto. Our novel analyses and results raise questions for answering, as follows.

Baseline recruitment 25OHD^{raw}

Serial single assays across the 13 224 participants were used to describe the presumed monthly cycle of change over both the years of recruitment and following decades. Results are consistent with others, including the delays from the solstices to peak and trough values; but, with Scotland's high latitude, the two-to-one oscillation may be unusually large.[12,13] Similarity of those recruited at different times is shown by consolidated results of the two contrasting seasons in the Scottish districts. These show trivial differences in mean risk factor levels, excepting an obvious separation in mean 25OHD^{raw} which predictably closes with seasonal adjustment to 25OHD^{adj}.

Seasonally adjusted 25OHD^{adj}

As have others, we adjusted seasonally unstable 25OHD^{raw} readings to an estimated longer-term ranking within the SHHEC cohort: 25OHD^{adj}. Without adjustment, the seasonal variation is extreme (see Supplement Figure 1 showing winter and summer values, available as Supplementary data at *IJE* online). The 50th centile reading for men in January–March of 28.8 nmol/l is equal to the 6th centile in July–September. Contrast the 'LowD' half of the population of 25OHD^{adj} coming very evenly from different recruitment calendar months, with 25OHD^{raw} at the same cutpoint:

52% of participants from January–March were LowD for 25OHD^{adj} but 75% for 25OHD^{raw}; whereas for July—September, the figures were 50% and 20%, respectively. Unadjusted readings are confounded by date of sampling (also true for uncorrected clinical samples). Because the seasonal swing in values is unlikely to be uniform and will vary with the amount of sunshine in a given year and with personal exposure, 25OHD^{adj} can only be an estimate of ranking. Increased precision would necessitate multiple measurements, and even a long-term tendency would be affected by changing lifestyle, for example from inability to venture outdoors.

Single measurements as a long-term predictor: tracking

That 25OHD appears so unstable but nonetheless appears predictive suggests long-term stability. Both 25OHD^{raw} and 25OHD^{adj} are predictive in Cox proportional hazards analyses. The latter is a better predictor, but the difference is not great. External literature confirms 'tracking' over time.[4,29] Fortuitously, independent random sampling in North Glasgow for MONICA surveys at 3-year intervals sampled 35 people twice. Both 25OHD^{raw} and 25OHD^{adj} values were more repeatable than other risk factors, excluding height (see the scatter plot, Supplement Figure 2, available as Supplementary data at *IJE* online). The value of 25OHD^{adj} as a predictor is further demonstrated in Supplement Tables (available as Supplementary data at *IJE* online). Table W8 shows that the LowD vs HighD event ratio during follow-up appears similarly predictive whether originally derived from Low-D season or High-D season measurements, and Table W9 that the LowD vs HighD event ratio persists during successive 5-year periods of follow-up.

Long-term prediction from single measurements is a challenge to many cohort studies, not unique to 25OHD in SHHEC. What is unusual is that the measurement is unstable over a period of months, but must go back on track again despite these seasonal oscillations. Our current analyses exploit both phenomena. No other risk factor shows such extraordinary two-to-one seasonal variation.

Pre-analytical and laboratory processing

Serum specimens analysed for 25OHD appear robust to handling.[24] Our archived frozen specimens, kept for decades for analysis, were run off in two batches between August 2009 and February 2012 with a 17-month gap. The two (3511 and 9713) had similar descriptors and no evidence of laboratory drift. By defining population halves and fifths both separately and together, we found negligible effects on risk calculations. Use of multivitamin tablets by SHHEC recruits was uncommon (see Supplement Addendum Table

A4, available as Supplementary data at *IJE* online), so the ARCHITECT assay's comparative lack of sensitivity to 25OHD$_2$ was immaterial. Official laboratory lower limits did however cut across the SHHEC cohort, with 9.9% below the European standard of 20 nmol/l and 40.7% below the more stringent US Food and Drug Administration (FDA) limit of 32.5 nmol/l, higher percentages of 25OHD^{raw} occurring in winter.[11,25] Our Supplement Figure 2, the analyses themselves and listing of the centiles for the assays in Supplement Table W10 (available as Supplementary data at *IJE* online), raise no questions about the validity of these low readings for our epidemiological purposes. The 9.9% would involve part only of the bottom population fifth (numbered 5 in the Cox model tables), the reference fifth, number 1, HR 1·0, being the highest 20%. Readings below laboratory limits do not necessarily imply disease.

Missing data

The 13 224 SHHEC participants were those with serum available in 2009 when the new assay kits arrived at the Mainz laboratory. Recruitment in 1984–95 had numbered 18 107, a 64% response rate.[21] Virtually complete data on 16 000 were sent to Helsinki for the MORGAM cohort collaboration in 2000. In 2007, 14 751 serum specimens were sent to Mainz for the biomarker substudy.[17–19] Sequential losses were predominantly from non-availability of serum—first refusal or failure of venepuncture at recruitment (during an HIV needle scare) and later depletion in laboratory studies. Completeness of data in the 13 224 exceeds 99%, the exception being HDL-cholesterol at 97.3%. Record linkage depends on a death or hospital record and cumulated during follow-up to 91.2%; no link means either loss to follow-up or continuing good health.[22] The 13 224 with 25OHD measurements were comparable to the 4883 without in age, sex, social deprivation, risk factors and endpoint risk. (STROBE statement is in Supplement Table W11, available as Supplementary data at *IJE* online.)

Winter morbidity and mortality, other explanations

Excess winter mortality has declined since 1981. Related to factors other than vitamin D—home heating, influenza, respiratory disease and social conditions—they do not necessarily exclude it.[30] Short-term meteorological and air pollution precipitants of cardiovascular disease contribute.[31,32] With excess mortality in northern countries beginning in December, Healy[33] found the least excess among 14 European countries was in Finland; winter excess correlated negatively with latitude—contrary to the seasonality hypothesis (r = -0.68, P < 0.01).[14,33] Data for deaths (all

ages and causes) registered by calendar month and year in Scotland over the years 1989–2009 confirm that the winter peak each year is in December or January, therefore preceding the SHHEC trough of 25OHD in March. The summer low trough in deaths varied from May to September[34] (Supplement Table W12, available as Supplementary data at *IJE* online). SHHEC data on monthly incidence of cardiovascular events and deaths need replication from studies elsewhere.

Conclusion

Our conventional Cox proportional hazard model analyses show 25OHD^{adj} to be a modest non-specific predictor of many primary and secondary endpoints, the effect being strongly attenuated by adjustment for risk factors in the ASSIGN cardiovascular risk score.[23] Strength and gradient are not marked: findings could result from residual confounding, or alternatively from over-adjustment. It does not improve prediction of First Cardiovascular Event over and above ASSIGN variables, an obligatory question for the MORGAM Biomarker and BiomarCaRE studies.[17–19]

Seasonality analyses show that a two-to-one annual oscillation in population 25OHD has no apparent impact on incidence of First Cardiovascular Events, and follows rather than precedes the peak in All Deaths and Cardiovascular Deaths. Winter appears to influence fatality rather than incidence. There was no disproportionate susceptibility to endpoint events in the low-vitamin-D months in those ranked as having low vitamin D, as predicted by the concept of hypovitaminosis D. These results were consistent with the absence of a demonstrable 25OHD threshold both in spline analyses and in the quasi-linear gradient of increasing risk with declining levels in the Cox model fifths. Our 25OHD readings were low, many below laboratory limits; but with no visible threshold for disease in SHHEC, we cannot estimate the prevalence of 'hypovitaminosis D' in the Scottish population, a concept originating with rickets and osteomalacia. Others may do so.[13,14]

In this study, 25OHD failed two out of three of our postulate testings and was borderline for the third. Bradford Hill criteria for causality were found wanting for specificity, temporality, strength and gradient.[16] Although not excluding causation completely—there could be a modest delayed effect integrated over years—our results question the seasonal hypothesis of vitamin D, and therefore challenge its alleged causal role. They need replication in other studies.

Supplementary data

Further tables and figures are available as specified in the text. The Supplement also includes an addendum of descriptive epidemiology of

25OHD in SHHEC covering means by age and sex (Table A1), by year of recruitment (Table A2), in relation to lifestyle factors (Table A3) and diet habits from a food frequency questionnaire (Table A4).

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Commentary: Vitamin D: prime mover or fellow traveller?

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There is little doubt that vitamin D deficiency causes rickets and the physiological roles of vitamin D are widespread. However, though low vitamin D status has been associated with many other chronic diseases including cardiovascular diseases, respiratory diseases and cancers, whether such associations are causal is still debated.[1]

Skin manufacture in the presence of sunlight is the major source of vitamin D, and the hypothesis that the marked seasonal variations in cardiovascular diseases could be explained by seasonal variations in vitamin D status was first proposed by Robert Scragg.[2] He further reported, in a case control study, an inverse association between plasma 25-hydroxyvitamin D_3 levels and myocardial infarction.[3]

The Scottish Heart Health Extended Cohort (SHHEC), which comprises the Scottish Heart Health study and participants in several MONICA surveys between 1984 and 1995, aimed to examine three postulates relating to vitamin D and cardiovascular disease (CVD), using two different study designs.[4] Their main focus was on the seasonality hypothesis which they explored by using an ecological approach. They postulated that the occurrence of cardiovascular events enumerated by month of occurrence over the years of follow-up should mirror annual seasonal oscillation in serum 25-hydroxyvitamin D (25OHD), using monthly means of the 25OHD concentrations observed at baseline in the cohort. Their conclusion using this approach was that the seasonal highs and lows in CVD deaths occurred before the estimated seasonal peaks and troughs in 25OHD status observed in the baseline measurements, and that first CVD events showed no seasonal pattern. They also reported that there was no evidence that there was greater seasonal variability in cardiovascular disease occurrence among those with lower compared with higher 25OHD concentrations, their second postulate. Then, in a prospective analysis of the cohort, they reported significant associations between lower 25OHD concentrations at baseline with subsequent first CVD events, CVD deaths and all deaths, before and after multiple adjustments for other factors.

The authors' conclusions were: that 25OHD failed two out of three of their postulated testings and was borderline