Articles

The effect of vitamin D supplementation on hepcidin, iron status and inflammation in pregnant women in the United Kingdom.

Vickie S. Braithwaite 1\*, Sarah R. Crozier 2, Stefania D’Angelo 2, Ann Prentice 1, Cyrus Cooper 2,3,4, Nicholas C. Harvey 2,3, Kerry S. Jones 1,5, and the MAVIDOS Trial Group 6

1 MRC Nutrition and Bone Health Research Group, Clifford Allbutt Building, University of Cambridge, Cambridge, UK; [vb287@cam.ac.uk](mailto:vb287@cam.ac.uk), [ann.prentice@mrc-lmb.cam.ac.uk](mailto:ann.prentice@mrc-lmb.cam.ac.uk), [kerry.jones@mrc-epid.cam.ac.uk](mailto:kerry.jones@mrc-epid.cam.ac.uk)

2 MRC Lifecourse Epidemiology Unit, University of Southampton, Southampton General Hospital, Southampton, UK; [src@mrc.soton.ac.uk](mailto:src@mrc.soton.ac.uk), [sd@mrc.soton.ac.uk](mailto:sd@mrc.soton.ac.uk), [cc@mrc.soton.ac.uk](mailto:cc@mrc.soton.ac.uk), [nch@mrc.soton.ac.uk](mailto:nch@mrc.soton.ac.uk)

3 NIHR Southampton Biomedical Research Centre, University of Southampton and University Hospital Southampton NHS Foundation Trust, Southampton, UK

4 Oxford NIHR Biomedical Research Centre, Nuffield Department of Orthopaedics, Rheumatology and Musculoskeletal Sciences, The Botnar Research Centre, University of Oxford, Oxford, UK

5 NIHR BRC Nutritional Biomarker Laboratory, University of Cambridge, Cambridge, United Kingdom

6 MAVIDOS Trial Group: Nigel K Arden, Nicholas J Bishop, Andrew Carr, Elizabeth M Curtis, Elaine M Dennison, Richard Eastell, Robert Fraser, Saurabh V Gandhi, Keith M Godfrey, Hazel M Inskip, M Kassim Javaid, Stephen Kennedy, Rebecca J Moon, M Zulf Mughal, Aris T Papageorghiou, David M Reid, Sian M Robinson, Inez Schoenmakers

**\*** Correspondence: vb287@cam.ac.uk; Tel.: 0044 7929022806

Received: n/a; Accepted: n/a; Published: n/a

**Abstract:**

Iron and vitamin D deficiencies are common during pregnancy. Our aim was to identify whether antenatal vitamin D3 (VitD) supplementation affects iron status (via hepcidin suppression) and/or inflammation. Using a sub-set of the UK multicenter Maternal Vitamin D Osteoporosis Study (MAVIDOS); a double-blinded, randomized, placebo-controlled trial (ISRCTN82927713; EudraCT2007-001716-23) we performed a secondary laboratory analysis. Women with blood samples from early and late pregnancy (VitD (1000 IU/d from~ 14 weeks gestation n=93; placebo n=102) who gave birth in the springtime (March-May) were selected as we anticipated seeing the greatest treatment group difference in change in 25-hydroxyvitamin D (25OHD) concentration. Outcomes were hepcidin, ferritin, C-reactive protein and α1-acid glycoprotein concentration in late pregnancy (25OHD concentration was measured previously). By late pregnancy, 25OHD concentration increased by 17 nmol/L in the VitD group and decreased by 10 nmol/L in the placebo group; hepcidin, ferritin and inflammatory markers decreased but no treatment group differences were seen. In late pregnancy, positive relationships between 25OHD and hepcidin and 25OHD and ferritin in the placebo group were observed but not in the treatment group (group\*25OHD interaction, P<0.02). VitD supplementation had no effect on hepcidin, ferritin or inflammatory status suggesting no adjunctive value of VitD in reducing rates of antenatal iron-deficiency.

**Keywords:** Vitamin D; C-reactive protein; hepcidin; ferritin; inflammation; pregnancy

1. Introduction

Vitamin D deficiency and anemia frequently co-exist [1-5]. A number of mechanisms could explain this association [1] and recent data provide evidence for a direct role of vitamin D in the suppression of hepcidin, the primary regulator of systemic iron homeostasis. Hepcidin inhibits and ultimately degrades ferroportin, the transmembrane protein that transports iron, and therefore controls the amount of iron absorbed in the intestine and released from cellular storage. Hepcidin is suppressed when iron status is low to maximize dietary iron absorption and the release of iron from stores. Conversely, inflammation causes an increase in hepcidin concentration that reduces dietary iron absorption and the capacity for iron egress from cells, resulting in decreased hemoglobin [6].

*In vitro* work has shown a 1,25-dihydroxyvitamin D (1,25(OH)2D) (the active metabolite of vitamin D)-induced, dose-dependent decrease in hepcidin expression [7, 8]. The identification of the vitamin D response element (VDRE) on the human hepcidin promoter also supports the direct effect of 1,25(OH)2D [8]. Several recent *in vivo* studies in healthy adults further support an effect of both single and longer-term high doses of vitamin D on hepcidin regulation. An increase in 25-hydroxyvitamin D concentration (25OHD; the status marker of vitamin D) of 68 to 109 nmol/L 72 h after an oral dose of 100,000 IU vitamin D2 was accompanied by a 34% decrease in hepcidin concentration (*n*=7) [8]. A further study found a 73% decrease in hepcidin concentration one week after a single dose of 250,000 IU vitamin D3 (*n=*18) [9]. In early-stage, chronic kidney disease patients, the percentage increase in 25OHD concentration after 3 months of vitamin D3 supplementation (50,000 IU vitamin D3/wk) was associated with an inverse change in hepcidin concentration (*n*=38) [7]. In addition to the effects on hepcidin concentration, vitamin D may also have immunomodulatory effects which may in turn alter hepcidin expression and iron status [10-12].

Pregnant women are at high risk of anemia [13, 14] due to the high iron demands of pregnancy, particularly in low-resource settings, and data suggest associations between low hemoglobin, low iron status and adverse birth outcomes [15]. Improvements in iron stores and hemoglobin are associated with increases in birth weight [16]. There is conflicting evidence as to whether C-reactive protein (CRP), as a marker of inflammation, rises [17] or remains the same during pregnancy [18]. However, inflammation may be relevant in pregnant individuals affected by conditions such as obesity or pre-eclampsia, known to be associated with inflammatory states [19]. Low vitamin D status may be a contributing factor to iron-deficiency anemia via direct effects on hepcidin or through potential inflammatory effects of low vitamin D status.

The effect of vitamin D supplementation on iron status has not been investigated in pregnant women. Through the mechanisms outlined above, adequate vitamin D status may be necessary for optimal hepcidin function and may help to provide protection against iron deficiency; potentially offering a complementary approach to combat anemia during pregnancy. The aim of this current study was to examine if daily vitamin D3 supplementation (cholecalciferol, 1000 IU/day) in pregnancy suppresses hepcidin concentration and/or affects iron body stores (ferritin) and inflammation (CRP and AGP) compared to placebo.

2. Materials and Methods

2.1. Study Design and Participants

The original MAVIDOS study (Maternal Vitamin D Osteoporosis Study) has been described previously in full [20, 21]. In brief, MAVIDOS was a multicenter, double-blinded, randomized, placebo-controlled trial conducted between October 2008 and February 2014 and is registered in the International Standard Randomized Control Trial registry (ISRCTN 82927713) and the European Clinical Trials Database (EudraCT 2007-001716-23). Women over the age of 18 years with a singleton pregnancy were recruited at their early pregnancy ultrasound scan. Women were screened for vitamin D status and only those with 25OHD concentrations between 25-100 nmol/L and who were not currently taking dietary supplements with more than 400 IU of vitamin D/day, were eligible. Exclusion criterion included any known metabolic or chronic disease known to interfere with bone metabolism, taking medication that might interfere with intrauterine growth, foetal anomalies at the 18-21 week ultrasound scan, and diagnosis of cancer in the last 10 years. Women were screened for eligibility at 10-17 weeks gestation and were then randomized to receive oral vitamin D3 (cholecalciferol 1000 IU/day; n=565) or matched placebo (n=569) (Merck KGaA, Darmstadt, Germany) from ~14 weeks gestation until delivery. Randomisation was by computer-generated sequence in randomly permuted blocks of ten and both participant and research team were masked to treatment allocation.

Vitamin D status (as measured by circulating 25OHD concentration) in the UK population changes markedly depending on season, reflecting the availability of UVB irradiation and capacity for endogenous vitamin D synthesis. Between around September to March/April [22] there is little capacity for endogenous synthesis and so vitamin D status relies on dietary or supplemental sources of vitamin D. Therefore, women from the MAVIDOS study who gave birth in the springtime (March-May) and with a blood sample available at both early (~15 weeks) and late (~34 weeks gestation) pregnancy (n=195) were selected for this sub-study as we anticipated seeing the greatest treatment group difference in change in 25OHD concentration from early to late pregnancy and minimal endogenous vitamin D production.

Ethical approval for the original trial was obtained by the Southampton and South West Hampshire Research Ethics Committee and written, informed consent was obtained from all participants. The additional analyses measured as part of this sub-study was within the remit of the original ethics application.

2.2 Anthropometry and Characteristics

Maternal age, self-reported ethnicity, parity and gestational age (determined by ultrasound) were recorded in early pregnancy and weight and height were recorded in early and late pregnancy. Supplement use within the last 3 months was recorded using a questionnaire in early and late pregnancy and was coded into whether or not the supplement included iron. As determined by the return of empty packs, 87% of women took at least 80% of the treatment tablets provided.

2.3 Biochemical Analysis

Venous blood samples were collected in early and late pregnancy into lithium-heparin-coated tubes and the plasma were stored at -80°C for subsequent analysis at MRC Elsie Widdowson Laboratory, Cambridge, UK. Hepcidin was measured by ELISA (ELISA, Hepcidin 25 bioactive HS, DRG Diagnostics, Marburg, Germany; inter-assay %CV between 8-13) and ferritin, CRP and α1-acid glycoprotein (AGP) was measured on the automated platform by Dimension Xpand, Siemens (ferritin and high sensitivity-CRP kits from Siemens Healthcare, Erlangen Germany, and AGP kits from Sentinel Diagnostics, Milano, Italy. Intra-assay %CV <6 for ferritin and CRP and <11 for AGP). 25OHD had been measured as part of the main trial by radioimmune assay (Liaison, RIA automated platform, Diasorin, Stillwater, MN, USA) as previously described and was DEQAS accredited [21].

2.4 Statistical Analysis

Statistical analysis was performed using Stata 14.1 (Stata Statistics and Data Analysis, Texas, USA) on an intention to treat basis. Variables were assessed for normality. Normally distributed data are presented as mean (standard deviation (SD)) and non-normally distributed data are presented as median (interquartile range (IQR)). Treatment group differences were assessed using 2-sample t-tests for normally distributed variables, Mann-Whitney U-tests for non-normally distributed variables and chi-squared tests for categorical variables. Proportions of iron deficient women were compared between treatment groups using chi-squared tests. Changes in variables over time were tested within each treatment group using paired t-tests for normally distributed, Wilcoxon signed-rank tests for non-normally distributed and McNemar’s tests for categorical variables. Differences between treatment groups in changes in characteristics were tested for using regression models with late pregnancy value as the outcome and early pregnancy value and treatment group as predictors, to account for regression to the mean. Linear regression models were fitted with 25OHD concentration as the predictor and hepcidin, ferritin, CRP and AGP concentrations as outcomes. Hepcidin, ferritin and CRP were transformed to normality using Fisher-Yates transformation [23] and are presented in SD units. The presented coefficients are those associated with a 10 nmol/L change in 25OHD concentration. Percentage changes in hepcidin and 25OHD concentrations from early to late pregnancy were calculated and transformed to normality using the Fisher-Yates transformation. Percentage change in 25OHD concentration was examined as a predictor of percentage change in hepcidin concentration using a linear regression model. CRP >5 mg/L [24] and/or AGP>500 mg/L were considered indicative of inflammation. There were no AGP concentrations >1000 mg/L so the lower cut-off of 500 mg/L was used [24]. Ferritin concentrations <15 μg/L were considered indicative of iron deficiency [25]. Thresholds for 25OHD of <25 nmol/L and <50 nmol/L were used to assess vitamin D status [26].

3. Results

3.1 Early Pregnancy (pre-supplementation)

Women giving birth in Spring were mean (SD) 30 (5) years old, predominantly white, 47% nulliparous and were seen at a mean (SD) of 15.7 (1.0) weeks gestation (Table 1). 92% of women reported the use of supplements over the previous 3 months and 60% of these women reported taking supplements that contained iron. 10% of women were iron deficient (ferritin <15 µg/L), 12% had 25OHD concentrations <25 nmol/L and 66% <50 nmol/L and there were no treatment group differences. The inclusion of women with 25OHD < 25 nmol/l is explained by the time between the screening blood sample and early pregnancy blood sample. Of women who had 25OHD < 25 nmol/L, 17% were also iron deficient compared to 9% in women with 25OHD > 25 nmol/L (P=0.19). Similarly, using a 25OHD concentration < or > than 50 nmol/L, 11% and 8% of women respectively also were iron deficient (P=0.44).

**Table 1.** Maternal Early Pregnancy Outcomes1

|  |  |  |  |
| --- | --- | --- | --- |
| Measures in Early Pregnancy | All | Placebo | Vitamin D3 |
|  | **n = (195)** | **n = (102)** | **n = (93)** |
| White ethnicity, *n (%)* | 182 (94%) | 95 (93%) | 87 (95%) |
| Previous children, *median (IQR)* | 1 (0, 1) | 1 (0, 1) | 1 (0, 1) |
| Nulliparous, *n (%)* | 91 (47%) | 49 (49%) | 42 (46%) |
| Age (years), *mean (SD)* | 30.4 (5.2) | 30.8 (5.2) | 29.9 (5.2) |
| Gestation (weeks), *mean (SD)* | 15.7 (1.0) | 15.8 (1.0) | 15.6 (1.0) |
| Weight (kg), *median (IQR)* | 69.6 (61.5, 80.0) | 70.5 (62.4, 79.6) | 68.0 (60.7, 81.0) |
| Height (m), *mean (SD)* | 1.66 (0.07) | 1.66 (0.07) | 1.66 (0.07) |
| BMI (kg/m2), *median (IQR)* | 24.7 (22.1, 28.7) | 24.9 (22.0, 28.8) | 24.6 (22.1, 28.6) |
| Supplement use, *n (%)* | 178 (92%) | 92 (90%) | 86 (93%) |
| Iron supplement use, *n (% of those using supplements)* | 106 (60%) | 49 (53%) | 57 (66%) |
| CRP (mg/L), *median (IQR)* | 5.4 (3.1, 8.3) | 4.2 (2.9, 7.8) | 6.2 (3.6, 11.5) |
| CRP > 5 mg/L, *n (%)* | 103 (53%) | 48 (47%) | 55 (60%) |
| AGP (mg/L), *mean (SD)* | 506 (115) | 500 (109) | 512 (122) |
| AGP > 500 mg/L, *n (%)* | 96 (49%) | 50 (49%) | 46 (49%) |
| Hepcidin (μg/L), *median (IQR)* | 7.3 (3.0, 16.7) | 7.4 (2.5, 16.5) | 6.9 (3.3, 16.8) |
| Ferritin (μg/L), *median (IQR)* | 38 (25, 60) | 43 (26, 62) | 35 (25, 55) |
| Ferritin < 15 μg/L, *n (%)* | 19 (9.7%) | 11 (10.8%) | 8 (8.6%) |
| 25-hydroxyvitamin D (nmol/L), *mean (SD)* | 44.1 (16.0) | 42.5 (15.8) | 45.7 (16.2) |
| 25-hydroxyvitamin D < 25 nmol/L, *n (%)* | 23 (12%) | 15 (15%) | 8 (9%) |
| 25-hydroxyvitamin D < 50 nmol/L, *n (%)* | 128 (66%) | 70 (69%) | 58 (62%) |

1Normally distributed variables are presented as mean (SD), non-normally distributed variables as median (IQR) and categorical variables as *n* (%). There were no significant differences between groups (P>0.05).

3.2 Late Pregnancy

Women were seen at a mean (SD) of 34.7 (0.8) weeks gestation in late pregnancy (Table 2). Reported supplement use decreased in both treatment groups to 62% in total. Of those taking supplements, 98% in the vitamin D3 group contained iron, compared to 89% in the placebo group (P=0.03). Hepcidin, ferritin, CRP and AGP concentrations decreased in both groups by late pregnancy (Table 2 & 3). By late pregnancy, 67% of women were iron deficient (P=0.44). Mean 25OHD concentration increased by 17 nmol/L in the vitamin D3 group and decreased by 10 nmol/L in the placebo group (Table 3). Correspondingly, the percentage of women with 25OHD concentration <25 and <50 nmol/L increased to 50 and 83% (P<0.001) respectively in the placebo group (Table 2) and decreased in the vitamin D3 group to 11 and 24% (P<0.0001) respectively. Of the 61 women who had 25OHD <25 nmol/L, 79% were also iron deficient compared to 61% of the 132 women with 25OHD >25 nmol/L (P=0.02). Using a 25OHD concentration < or > than 50 nmol/L, 76% and 56% of women respectively were iron deficient (P=0.004).

**Table 2.** Maternal Late Pregnancy Outcomes2

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Measures in Late Pregnancy | All | Placebo | Vitamin D3 | P-value |
|  | **n = (195)** | **n = (102)** | **n = (93)** |  |
| Age (years), *mean (SD)* | 30.6 (5.3) | 31.2 (5.2)d | 30.0 (5.3)d | 0.13 |
| Gestation (weeks), *mean (SD)* | 34.7 (0.8) | 34.6 (0.6)d | 34.8 (0.9)d | 0.06 |
| Weight (kg), *median (IQR)* | 78.8 (71.1, 90.4) | 78.5 (73.2, 90.0)d | 79.2 (69.9, 92.8)d | 0.69 |
| BMI (kg/m2), *median (IQR)* | 28.4 (25.5, 32.9) | 28.6 (25.5, 32.5)d | 28.3 (25.4, 32.9)d | 0.71 |
| Supplement use, *n (%)* | 119 (62%) | 61 (61%)d | 58 (63%)d | 0.77 |
| Iron supplement use, *n (% of those using supplements)* | 111 (93%) | 54 (89%)a | 57 (98%)b | 0.03 |
| CRP (mg/L), *median (IQR)* | 3.9 (2.5, 7.3) | 3.8 (2.3, 6.4)c | 4.1 (2.6, 8.1)c | 0.16 |
| CRP > 5 mg/L, *n (%)* | 69 (38%) | 31 (32%)b | 38 (43%)b | 0.13 |
| AGP (mg/L), *mean (SD)* | 452 (115) | 442 (93)d | 463 (135)b | 0.20 |
| AGP > 500 mg/L, *n (%)* | 57 (29%) | 28 (27%)c | 29 (32%)b | 0.53 |
| Hepcidin (μg/L), *median (IQR)* | 0.97 (0.79, 1.99) | 0.93 (0.74, 1.57)d | 0.99 (0.84, 2.30)d | 0.19 |
| Ferritin (μg/L), *median (IQR)* | 10 (7, 18) | 10 (7, 17)d | 10 (8, 18)d | 0.94 |
| Ferritin < 15 μg/L, *n (%)* | 129 (67%) | 65 (64%)d | 64 (70%)d | 0.44 |
| 25-hydroxyvitamin D (nmol/L), *median (IQR)* | 47.1 (21.4, 66.8) | 25.2 (16.9, 45.8)d | 64.6 (52.0, 75.7)d | 0.0001 |
| 25-hydroxyvitamin D < 25 nmol/L, *n (%)* | 61 (31%) | 51 (50%)d | 10 (11%) | 0.001 |
| 25-hydroxyvitamin D < 50 nmol/L, *n (%)* | 107 (55%) | 85 (83%)b | 22 (24%)d | 0.001 |

2Normally distributed variables are presented as mean (SD), non-normally distributed as median (IQR) and categorical variables as *n* (%). P-value obtained from 2-sample t-test for normally distributed variables, Mann-Whitney U-tests for non-normally distributed variables and chi-squared tests for categorical variables.

a, b, c, d indicates that late pregnancy variable is significantly different than early pregnancy variable by *P<0.05, 0.01, 0.001* and *0.0001* respectively. This is calculated from paired t-tests for normally distributed, Wilcoxon signed-rank tests for non-normally distributed and McNemar’s tests for categorical variables.

**Table 3.** Maternal change in characteristics over pregnancy (late - early pregnancy)3

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Change in Characteristic | All | Placebo | Vitamin D3 | P-value |
|  | **n = (195)** | **n = (102)** | **n = (93)** |  |
| Maternal pregnancy weight (kg), mean (SD) | 9.8 (3.6) | 9.7 (3.5) | 9.8 (3.8) | 0.65 |
| Maternal pregnancy BMI (kg/m2), *mean (SD)* | 3.6 (1.3) | 3.6 (1.2) | 3.5 (1.4) | 0.44 |
| CRP (mg/L), *mean (SD)* | -1.6 (10.3) | -1.8 (9.4) | -1.4 (11.2) | 0.12 |
| AGP (mg/L), *mean (SD)* | -54 (126) | -58 (101) | -50 (149) | 0.76 |
| Hepcidin (μg/L), *median (IQR)* | -5.1 (-14.1, -1.3) | -5.6 (-14.9, -1.1) | -4.3 (-11.0, -1.6) | 0.10 |
| Ferritin (μg/L), *mean (SD)* | -31.9 (58.5) | -36.5 (41.2) | -26.9 (72.9) | 0.67 |
| 25-hydroxyvitamin D (nmol/L), *mean (SD)* | 2.6 (27.1) | -10.7 (17.9) | 17.1 (28.0) | <0.0001 |

**3**P-value obtained from regression models with late pregnancy value as the outcome and early pregnancy value and treatment group as predictors, to account for regression to the mean.

3.3 Regression Analysis

In early pregnancy, 25OHD concentration was not significantly associated with hepcidin or ferritin concentrations and there was no difference in these relationships by treatment group (Table 4). In late pregnancy, 25OHD concentration was significantly positively associated with hepcidin concentration. However, there was a significant interaction between treatment group and 25OHD concentration (P=0.02), such that there was a positive association between 25OHD concentration and hepcidin in the placebo group (Beta Coefficient (95% Confidence Interval) per 10 nmol/L change in 25OHD): 0.14 (0.04 to 0.24) hepcidin SDs, P=0.006) but no association in the vitamin D3 group (-0.02 (-0.11 to 0.07), P=0.63) (Figure 1 & Table 4). A similar interaction (P=0.003) was found between late pregnancy 25OHD concentration and ferritin in the placebo group (0.25 (0.17, 0.33) ferritin SDs, P<0.001) but not in the vitamin D3 group (0.06 (-0.03, 0.15), P=0.19) (Figure 1 & Table 4). There was no significant treatment group interaction between 25OHD and CRP or AGP in early or late pregnancy (Figure 1 & Table 4). When the groups were analyzed together, there was no significant relationship between percentage change in hepcidin and percentage change in 25OHD (beta (95% CI) SD per SD = 0.06 (-0.09 to 0.20), P=0.44) and no significant treatment group by 25OHD concentration interaction (P=0.32).

**Table 4.** Early pregnancy 25-hydroxyvitamin D (per 10 nmol/L) as a predictor of concentrations in early and late pregnancy in all women4

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **All Women (n=195)** | **Beta (95% CI)** | **P-value** | **n** | **Interaction P-value\*** |
| Early pregnancy: hepcidin (SDs) | 0.02 (-0.07, 0.11) | 0.67 | 195 | 0.99 |
| Late pregnancy: hepcidin (SDs) | 0.06 (0.01, 0.11) | 0.03 | 195 | 0.02 |
| Early pregnancy: ferritin (SDs) | 0.06 (-0.02, 0.15) | 0.16 | 195 | 0.85 |
| Late pregnancy: ferritin (SDs) | 0.09 (0.04, 0.15) | < 0.001 | 193 | 0.003 |
| Early pregnancy: CRP (SDs) | -0.10 (-0.19, -0.02) | 0.02 | 193 | 0.23 |
| Late pregnancy: CRP (SDs) | -0.01 (-0.06, 0.05) | 0.85 | 184 | 0.32 |
| Early pregnancy: AGP (mg/L) | -5.3 (-15.4, 4.9) | 0.31 | 195 | 0.06 |
| Late pregnancy: AGP (mg/L) | -0.1 (6.4, 6.2) | 0.97 | 194 | 0.66 |

4Regression models with 25-hydroxyvitamin D concentrations (per 10 nmol/L) as the exposure and biochemical measures as the outcomes (in 8 separate models). \*P-value for interaction between 25-hydroxyvitamin D concentration and treatment group; P<0.05 indicates a significant difference in slope between the two groups. Hepcidin, ferritin and CRP concentrations were transformed to normality using Fisher-Yates transformation and are presented in SD units.

**Figure 1.** Scatterplot of 25-hydroxyvitamin D against markers of iron and inflammation in late pregnancy by treatment group



Hepcidin

Ferritin

CRP

AGP

(a)

(b)

(c)

(d)

Scatterplots of 25OHD concentration in late pregnancy with hepcidin (a), ferritin (b), CRP (c) and AGP (d). Vitamin D3 supplemented group open circles, dashed line; placebo group filled circles, solid line. Regression analysis found a significant 25-hydroxyvitamin D\*group interaction for hepcidin (P=0.02) and ferritin (P=0.003) but not for CRP (P=0.32) and AGP (P=0.66). For hepcidin (SDs) the placebo group beta coefficient (95% CI) was 0.14 (0.04 to 0.24) nmol/L, P=0.006 and -0.02 (-0.11 to 0.07) nmol/L, P=0.63 in the vitamin D3 group. For ferritin (SDs) the placebo group beta coefficient (95%) was 0.25 (0.17 to 0.33) nmol/L, P<0.0001) and 0.06 (-0.03 to 0.15), P=0.19 for the vitamin D3 group.

4. Discussion

Antenatal supplementation with vitamin D3 (cholecalciferol, 1000 IU/day) in women due to give birth in March-May in the UK, increased 25OHD concentrations in the supplement group from early to late pregnancy by 17 nmol/L while the placebo group experienced a significant seasonal decrease in 25OHD concentrations (-11 nmol/L). Despite a 40 nmol/L difference between the two groups by late pregnancy, no group differences were seen in hepcidin, ferritin or markers of inflammation.

Rates of iron deficiency (ferritin < 15 µg/L) increased significantly in both groups to ~70% by late pregnancy while hepcidin, CRP and AGP concentrations decreased significantly. This decrease in ferritin is highly likely to indicate a corresponding decrease in hemoglobin concentrations and an increase in anemia prevalence [17, 27], although this was not verified due to a lack of stored whole blood samples. In addition, the decrease in hepcidin is likely to reflect the increased requirement for iron mobilization from stores and iron intestinal absorption as iron demands increase across pregnancy.

As with other studies, this study highlights the finding that iron deficiency is often associated with lower vitamin D concentration [2, 3, 5]. This was seen in late pregnancy where 79% of women with 25OHD <25 nmol/L were also iron deficient compared to 61% of women with iron deficiency and 25OHD >25 nmol/L.

Smith et al. randomized 28 healthy individuals in Atlanta, Georgia, USA, to receive one oral bolus of vitamin D3 (250,000 IU) or placebo [9]. Similar to our study, 75% of participants had 25OHD concentrations < 50 nmol/L (20 ng/mL) at baseline and the bolus dose increased 25OHD concentrations by ~21 nmol/L in the treatment group at 1 week follow-up. Unlike our study, however, Smith et al. found that those in the treatment group had a ~73% decrease in hepcidin concentration with no significant change detected in the placebo group and no change in ferritin or inflammatory markers in either group 1 week post treatment. *HAMP*, the gene encoding hepcidin, is known to contain the vitamin D response element in its promoter region [8]. Smith et al., speculated that the vitamin D bolus dose resulted in a direct suppression of *HAMP* gene expression and therefore a reduction in hepcidin concentration rather than indirectly acting through inflammation [9].

The major differences in study design between the study by Smith et al. and the current study were: 1) population (non-pregnant versus pregnant) 2) mode of vitamin D supplementation (250,000 IU bolus versus 1000 IU/day) 3) follow-up time (1 week versus 19 weeks) and 4) Smith et al. observed no changes in 25OHD concentration, markers of iron status or markers of inflammation in the placebo group over time. The metabolic demands of pregnancy and pregnancy-related changes in vitamin D and iron metabolism (and their binding proteins) [28, 29] may influence vitamin D-hepcidin-iron interactions. In addition, the kinetics and metabolism of bolus versus frequent, lower doses of vitamin D are different [30]. Therefore, while the overall change in 25OHD concentration in the treatment arm of the two studies was similar at follow-up, it is possible that the effect of vitamin D supplementation on hepcidin depends on the dose and/or frequency of vitamin D given.

No group difference was found in hepcidin or ferritin concentrations in late pregnancy or in change in hepcidin or ferritin between early and late pregnancy. However, there was a significant group interaction between 25OHD concentration and hepcidin and between 25OHD and ferritin in late pregnancy. Interestingly, in late pregnancy, the positive relationships between 25OHD and hepcidin and between 25OHD and ferritin that were seen in the placebo group were not observed in the vitamin D3 group suggesting an effect of supplementation on this relationship. Therefore, there may be a relationship between 25OHD concentration and iron metabolism when 25OHD concentrations are lowest and not at higher concentrations. This finding differs from that of Thomas et al. who reported no relationship between 25OHD and hepcidin concentrations in non-supplemented adolescent pregnant women from the USA at ~26 weeks gestation and at delivery [1]. However, whilst vitamin D status in early pregnancy and in the supplemented group in late pregnancy were similar to the Thomas study, late pregnancy vitamin D status in the placebo group was notably lower, and further supports a concentration-dependent relationship between vitamin D and iron metabolism. Other non-hepcidin mediated mechanisms may explain associations observed between vitamin D and iron status. Thomas et al. found that the relationship between 25(OH)D and hemoglobin concentration was mediated by erythropoietin (EPO), with an inverse relationship between 25(OH)D and EPO in mid-gestation and at delivery [1].

The current study found that CRP decreased as pregnancy progressed, in contrast to some studies that have suggested an increase [17] or no change in CRP throughout pregnancy [18]. The impact of inflammation on vitamin D status [26] and the potential of vitamin D to mediate the immune response and temper inflammation is of interest [10, 11]. However, we found no effect of vitamin D supplementation on CRP or AGP, suggesting that vitamin D3 supplementation did not impact inflammatory pathways.

There were a number of limitations to this analysis. Firstly, whilst ferritin concentration is the standard World Health Organization measure of iron deficiency [24, 25], its interpretation, in common with other markers of iron status, can be affected by inflammation and pregnancy-related hemodilution. Whilst there is uncertainty over ferritin cut-offs for iron deficiency in pregnancy, as a marker of change in iron status, ferritin remains a sensitive biomarker for body iron stores [31, 32]. Hepcidin has been suggested an alternative marker of iron status in pregnant women [17] but additional confirmatory research is required. Secondly, other biomarkers of iron status, erythropoiesis and inflammation were not measured and so alternative mechanisms were not explored. Thirdly, increases in plasma volume as well as metabolic and hormonal changes during pregnancy may confound interpretation of biomarkers of nutritional status. However, the placebo controlled nature of the study should have accounted for most of these changes. Fourthly, we observed a difference between groups in the proportion of women who took iron-containing supplements in late pregnancy which was higher in the vitamin D3 group. However, the difference was small (n=3) and the method used to record supplement use was simple; whilst the brand of the supplement was recorded and coded for the presence or absence of iron in its formulation, the frequency and length or supplement use was not recorded and therefore it was not possible to determine the amount of iron in the supplement nor the amount of supplement consumed. Finally, 94% of women in this sub-study were of white, self-reported ethnicity and the participants were screened for low and high 25OHD concentration before inclusion in the trial. It is possible that a larger effect on hepcidin would be detected at lower 25OHD concentrations.

5. Conclusions

This study is the first analysis of data from an RCT investigating the effect of vitamin D supplementation on iron and inflammation in pregnancy. The study indicated that 1000 IU/day of vitamin D3 (cholecalciferol) in pregnancy had no effect on hepcidin or other markers of iron status and inflammation compared to placebo and suggests that 1000 IU/day of vitamin D would not be beneficial in ameliorating iron deficiency in pregnancy.

**Author Contributions:** Conceptualization, V.S.B., K.S.J., A.P., S.R.C., S.D., C.C. and N.C.H.; formal analysis, V.S.B, K.S.J., S.R.C. and S.D.; investigation, V.S.B. and K.S.J.; resources, A.P.; writing—original draft preparation, V.S.B. and K.S.J.; writing—review and editing, all authors, including those of the MAVIDOS Trial Group.

**Funding:** The MAVIDOS trial (ISRCTN82927713 and EudraCT2007-001716-23) was supported by grants from Arthritis Research UK, Medical Research Council (MRC) [4050502589 (MRC LEU)], Bupa Foundation, National Institute for Health Research (NIHR) Southampton Biomedical Research Centre, University of Southampton and University Hospital Southampton NHS Foundation Trust, and NIHR Oxford Biomedical Research Centre, University of Oxford. VSB, KSJ AP were funded by the MRC (programme code U105960371). The work leading to these results was supported by the European Union’s Seventh Framework Programme (FP7/2007–2013), projects EarlyNutrition and ODIN under grant agreements numbers 289346 and 613977, and by the BBSRC (HDHL-Biomarkers, BB/P028179/1), as part of the ALPHABET project, supported by an award made through the ERA-Net on Biomarkers for Nutrition and Health (ERA HDHL), Horizon 2020 grant agreement number 696295.

**Acknowledgments:** We should like to acknowledge the MAVIDOS participants and the original members of the MAVIDOS study group for participating/conducting the original trial and providing access to the data. We should also like to thank Ms Kate Guberg and Ms Lorna Cox from MRC Elsie Widdowson laboratory, Cambridge for conducting the laboratory analysis.

**Conflicts of Interest:** VSB, SRC, SD, AP and KSJ have no conflicts of interest. CC reports personal fees, consultancy, lecture fees, and honoraria from Alliance for Better Bone Health, Amgen, Eli Lilly, GlaxoSmithKline, Medtronic, Merck, Novartis, Pfizer, Roche, Servier, and Takeda, outside the submitted work. NCH reports personal fees, consultancy, lecture fees, and honoraria from Alliance for Better Bone Health, AMGen.

References

1. Thomas, C.E., et al., *Vitamin D status is inversely associated with anemia and serum erythropoietin during pregnancy.* Am J Clin Nutr, 2015. **102**(5): p. 1088-95.

2. Nikooyeh, B. and T.R. Neyestani, *Poor vitamin D status increases the risk of anemia in school children: National Food and Nutrition Surveillance.* Nutrition, 2018. **47**: p. 69-74.

3. Frelut, M.L., et al., *Impact of obesity on biomarkers of iron and vitamin D status in children and adolescents: The risk of misinterpretation.* Arch Pediatr, 2018. **25**(1): p. 3-5.

4. Michalski, E.S., et al., *Serum 25-hydroxyvitamin D but not dietary vitamin D intake is associated with hemoglobin in women of reproductive age in rural northern Vietnam.* J Clin Transl Endocrinol, 2017. **8**: p. 41-48.

5. Malczewska-Lenczowska, J., et al., *The Association between Iron and Vitamin D Status in Female Elite Athletes.* Nutrients, 2018. **10**(2).

6. Ganz, T. and E. Nemeth, *Hepcidin and iron homeostasis.* Biochim Biophys Acta, 2012. **1823**(9): p. 1434-1443.

7. Zughaier, S.M., et al., *The role of vitamin D in regulating the iron-hepcidin-ferroportin axis in monocytes.* J Clin Transl Endocrinol, 2014. **1**(1): p. 19-25.

8. Bacchetta, J., et al., *Suppression of iron-regulatory hepcidin by vitamin D.* J Am Soc Nephrol, 2014. **25**(3): p. 564-72.

9. Smith, E.M., et al., *High-dose vitamin D3 reduces circulating hepcidin concentrations: A pilot, randomized, double-blind, placebo-controlled trial in healthy adults.* Clin Nutr, 2017. **36**(4): p. 980-985.

10. Laird, E., et al., *Vitamin D Deficiency Is Associated With Inflammation in Older Irish Adults.* J Clin Endocrinol Metab, 2014. **99**(5): p. 1807-1815.

11. Calton, E.K., et al., *The impact of cholecalciferol supplementation on the systemic inflammatory profile: a systematic review and meta-analysis of high-quality randomized controlled trials.* Eur J Clin Nutr, 2017. **71**(8): p. 931-943.

12. Vanherwegen, A.-S., C. Gysemans, and C. Mathieu, *Vitamin D endocrinology on the cross-road between immunity and metabolism.* Mol Cell Endocrinol, 2017. **453**: p. 52-67.

13. WHO, *The Global Prevalence of Anaemia 2011*. 2015.

14. Stevens, G.A., et al., *Global, regional, and national trends in haemoglobin concentration and prevalence of total and severe anaemia in children and pregnant and non-pregnant women for 1995&#x2013;2011: a systematic analysis of population-representative data.* Lancet Glob Health, 2013. **1**(1): p. e16-e25.

15. Allen, L.H., *Anemia and iron deficiency: effects on pregnancy outcome.* Am J Clin Nutr, 2000. **71**(5): p. 1280s-1284s.

16. Haider, B.A., et al., *Anaemia, prenatal iron use, and risk of adverse pregnancy outcomes: systematic review and meta-analysis.* Br Med J, 2013. **346**.

17. Bah, A., et al., *Serum Hepcidin Concentrations Decline during Pregnancy and May Identify Iron Deficiency: Analysis of a Longitudinal Pregnancy Cohort in The Gambia.* J Nutr, 2017. **147**(6): p. 1131-1137.

18. Watts, D.H., et al., *C-reactive protein in normal pregnancy.* Obstet Gynecol, 1991. **77**(2): p. 176-80.

19. Koenig, M.D., et al., *Hepcidin and iron homeostasis during pregnancy.* Nutrients, 2014. **6**(8): p. 3062-83.

20. Harvey, N.C., et al., *MAVIDOS Maternal Vitamin D Osteoporosis Study: study protocol for a randomized controlled trial. The MAVIDOS Study Group.* Trials, 2012. **13**: p. 13.

21. Cooper, C., et al., *Maternal gestational vitamin D supplementation and offspring bone health (MAVIDOS): a multicentre, double-blind, randomised placebo-controlled trial.* Lancet Diabetes Endocrinol, 2016. **4**(5): p. 393-402.

22. Webb, A.R., et al., *Colour Counts: Sunlight and Skin Type as Drivers of Vitamin D Deficiency at UK Latitudes.* Nutrients, 2018. **10**(4).

23. Armitage, P. and G. Berry, *Statistical Methods in Medical Research*. Third Edition ed. 2002, Oxford, United Kingdom: Blackwell Science Ltd.

24. Namaste, S.M.L., et al., *Adjusting ferritin concentrations for inflammation: Biomarkers Reflecting Inflammation and Nutritional Determinants of Anemia (BRINDA) project.* Am J Clin Nutr, 2017. **106**(Suppl 1): p. 359S-371S.

25. WHO. *Serum ferritin concentrations for the assessment of iron status and iron deficiency in populations*. Vitamin and Mineral Nutrition Information System 2011 [cited 2017 24/10/2017]; Available from: <http://www.who.int/vmnis/indicators/serum_ferritin>.

26. Roth, D.E., et al., *Global prevalence & disease burden of vitamin D deficiency: a roadmap for action in low- and middle-income countries.* Ann N Y Acad Sci, 2018.

27. Mwangi, M.N., et al., *Effect of Daily Antenatal Iron Supplementation on Plasmodium Infection in Kenyan Women: A Randomized Clinical Trial.* Jama, 2015. **314**(10): p. 1009-20.

28. Cao, C. and K.O. O'Brien, *Pregnancy and iron homeostasis: an update.* Nutr Rev, 2013. **71**(1): p. 35-51.

29. Jones, K.S., et al., *Vitamin D expenditure is not altered in pregnancy and lactation despite changes in vitamin D metabolite concentrations.* Sci Rep, 2016. **6**: p. 26795.

30. Schoenmakers, I. and K.S. Jones, *Chapter 37 - Pharmacology and Pharmacokinetics*, in *Vitamin D (Fourth Edition)*, D. Feldman, Editor. 2018, Academic Press. p. 635-661.

31. Byg, K.E., et al., *Serum ferritin is a reliable, non-invasive test for iron status in pregnancy: comparison of ferritin with other iron status markers in a longitudinal study on healthy pregnant women; erythropoiesis.* Hematology, 2000. **5**(4): p. 319-325.

32. Daru, J., et al., *Serum ferritin as an indicator of iron status: what do we need to know?* Am J Clin Nutr, 2017. **106**(Suppl 6): p. 1634s-1639s.

H:\documents\layout\new template June 2014\figures\CC-BY logo original v1.wmf© 2018 by the authors. Submitted for possible open access publication under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).