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Relationship between methylation status of vitamin D-related genes, vitamin D levels, and methyl-donor biochemistry

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ABSTRACT

Vitamin D is known for its role in the regulation of gene expression via the vitamin D receptor, a nuclear transcription factor. More recently, a role for vitamin D in regulating DNA methylation has been identified as an additional mechanism of modulation of gene expression. How methylation status influences vitamin D metabolism and response pathways is not yet clear. Therefore, we aimed to assess the relationship between plasma 25-hydroxycholecalciferol (25(OH)D) and the methylation status of vitamin D metabolism enzyme genes (*CYP2R1, CYP27B1* and *CYP24A1*) and the vitamin D receptor gene (*VDR*). This analysis was conducted in the context of dietary vitamin D, and background methyl donor related biochemistry, with adjustment for several dietary and lifestyle variables. Percentage methylation at CpG sites was assessed in peripheral blood cells using methylation sensitive and dependent enzymes and qPCR. Standard analytical techniques were used to determine plasma 25(OH)D and homocysteine, and serum folate and B12, with the relationship to methylation status assessed using multi-variable regression analysis. *CYP2R1* and *VDR* methylation were found to be independent predictors of plasma 25(OH)D, when adjusted for vitamin D intake and other lifestyle variables. *CYP24A1* was related to plasma 25(OH)D directly, but not in the context of vitamin D intake. Methyl-group donor biochemistry was associated with the methylation status of some genes, but did not alter the relationship between methylation and plasma 25(OH)D. Modulation of methylation status of *CYP2R1, CYP24A1* and *VDR* in response to plasma 25(OH)D may be part of feedback loops involved in maintaining vitamin D homeostasis, and may explain a portion of the variance in plasma 25(OH)D levels in response to intake and sun exposure. Methyl-group donor biochemistry, while a potential independent modulator, did not alter this effect.

1. Introduction

The active vitamin D metabolite, 1,25-dihydroxycholecalciferol (1,25(OH)D; calcitriol), is well known for its influence on gene regulation via its action on the vitamin D receptor (VDR), which acts as a nuclear transcription factor [1]. However, the potential for vitamin D to modulate gene expression indirectly, through modulation of epigenetic marks is now also being investigated [2]. A better understanding of the relationship between vitamin D status and modulation of DNA methylation may help to explain some of

Abbreviations: 1,25(OH)D, 1,25 dihydroxycholecalciferol; 25(OH)D, 25-hydroxycholecalciferol; PBCs, peripheral blood cells; TOMS, Total Ozone Mapping Spectrometer; VDR, vitamin D receptor.

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the variance that exists in vitamin D metabolism [3–5] and the influence of vitamin D status on disease risk. This may be particularly important in elderly populations, in which vitamin D deficiency is common and who may be at higher risk of diseases linked to suboptimal vitamin D status including osteoporosis [6], diabetes [7], cardiovascular disease [8], autoimmune disease [9] and some cancers [10–13].

The metabolism of vitamin D is a multistep process involving several enzymes. Ergocalciferol (vitamin D2; from dietary sources) and cholecalciferol (vitamin D3; from dietary sources and endogenous synthesis in the skin) are converted by calciol-25-hydroxylase (coded for by the gene *CYP2R1*) into 25-hydroxycholecaliferol (25(OH)D; calcidiol), which is the circulating storage form of the vitamin. Calcidiol-1α-hydroxylase (*CYP27B1* gene) then converts 25(OH)D into 1,25(OH)D, the biologically active ligand for the VDR [14,15]. A 24-hydroxylase enzyme (*CYP24A1* gene) is responsible for the inactivation of both 25(OH)D and 1,25(OH)D via hydroxylation [15] (Fig. 1).

Each of the genes encoding enzymes involved in the vitamin D metabolism pathway is potentially regulated by DNA methylation. DNA methylation occurs at CpG sites (cytosine residues followed by guanine residues), and clusters of CpGs are referred to as CpG islands. Differential methylation of these islands in promotor regions of genes may modulate gene expression, with hypermethylation often associated with decreased expression, and hypo-methylation associated with increased expression [16]. *VDR, CYP2R1* and *CYP24A1* each have CpG islands spanning their promotor regions, and *CYP27B1* has a CpG island in the gene body (http://genome.ucsc.edu/) [2,17]. Vitamin D stimulation has also been shown to influence global methylation markers [18], and to alter the methylation status of multiple genes in multiple pathways, including those related to cell cycle regulation [19–22]. Therefore, DNA methylation and vitamin D metabolism may have a complex and bidirectional relationship.

In a small (22 African American male youths) genome-wide association study using DNA from blood leukocytes Zhu and colleagues identified a small differential methylation in *CYP2R1* and *CYP24A1* between those with severe vitamin D deficiency, compared to vitamin D sufficient individuals [19]. However, it should be noted that the statistical significance of these results did not survive corrections for multiple testing. Vitamin D deficient participants had higher methylation of *CYP2R1* and lower methylation of *CYP24A1*. This study was purely observational, and the direction of effect cannot be determined, however, following the standard paradigm of increased methylation leading to reduced gene expression, this pattern of methylation may lead to a situation where methylation status contributes to vitamin D deficiency, by decreasing conversion into the active metabolite, and increasing inactivation [19]. However an important limitation of this study was the lack of adjustment for sun exposure or vitamin D intake.

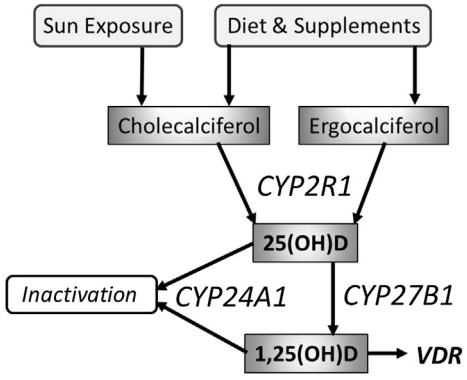

Fig. 1. Simplified flow chart of vitamin D metabolism. CYP2R1 is the gene for the enzyme calciol-25-hydroxylase; CYP27B1 is the gene for the enzyme Calcidiol-1α-hydroxylase; CYP24A1 is the gene for the 24-hydroxylase enzyme; VDR is the gene for the vitamin D receptor.

More recently, Zhou and colleagues, in a study of vitamin D supplementation in post-menopausal women, investigated methylation status of *CYP2R1*, *CYP24A1* and *CYP27B1* DNA isolated from serum [23]. In a preliminary binary analysis of 36 subjects, methylation status of responders (those who had the highest rise in serum 25(OH)D in response to supplementation) were compared to non-responders (those with a limited increase in serum 25(OH)D in response to supplementation), using methyl specific PCR. Supporting the observations of Zhu et al., non-responders had higher methylation of *CYP2R1* at baseline and after supplementation, and this may explain the reduced response to supplementation. Following supplementation both responders and non-responders exhibited reduced methylation levels of *CYP24A1*, suggesting higher *CYP24A1* expression may be a response to degrade the additional supplemental vitamin D and maintain homeostasis [23]. This suggests that there is modification of methylation status, and that this may be both a cause and a response phenomenon, depending on the situation. In the same study no differential methylation was found for *CYP27B1*. In a validation study treating plasma 25(OH)D levels as a continuous variable in independent samples (n = 117 and 145), and using pyrosequencing to interrogate individual CpGs, only a limited number of CpG sites upheld the preliminary results following corrections for multiple testing. Importantly, the validation cohort excluded the participants of the two extremes (previously defined as responders and non-responders) used in the pilot study [23]. Additionally, relevance of the methylation status at individual CpG sites is not yet fully clear, compared to the broader methylation status across the CpG island.

A limitation of these studies is minimal correction for other dietary and lifestyle variables in the former study [19], and the use of DNA found in serum in the latter [23]. Both cohorts are also of limited size [19,23]. Vitamin D status may be modulated by a number of variables including BMI, age, sex, and calcium intake in addition to vitamin D intake and sun exposure [24]. Furthermore DNA methylation status may be sensitive to cigarette smoking status, alcohol intake, age, sex, and sun exposure [25,26]. Another important consideration is that methylation potential may be related to the availability of methyl donors resulting from the one carbon metabolism cycle, and as such vitamin D metabolism may be influenced by levels of methyl donor nutrients, such as folate [27]. Therefore, in the current study we examined the relationship between methylation status of vitamin D metabolism genes (*CYP2R1, CYP27B1* and *CYP24A1*) and *VDR*, plasma 25(OH)D status and a number of dietary and lifestyle variables, including folate status, in a mixed gender elderly cohort.

2. Methods

2.1. Subjects and sample collection

Participants for this study were 80 subjects drawn from a completed cross-sectional study of 831 elderly participants (aged 65 years or more, 58.5% female) living on the Central Coast of NSW, Australia (the Retirement Health and Lifestyle Study, RHLS). Details of the selection and randomisation process are included in the supplementary methods.

Whole blood was collected from participants and stored at −20 °C. Serum and plasma were stored at −80 °C. DNA was isolated from peripheral blood cells (PBCs) using Qiagen QIAmp

DNA mini-kit as per the manufacturer's protocol for blood, including RNAse treatment. Written informed consent was obtained from all participants. Approval was obtained from the University of Newcastle Human Research Ethics Committee (H-2008-0431).

2.2. DNA methylation

Percentage DNA methylation was assessed at CpG sites across CpG islands in *CYP2R1*, *CYP27B1*, *CYP24A1*, and *VDR* genes using Qiagen EpiTect II methylation enzyme kits and PCR assays at the concentrations and cycle conditions recommended by the manufacturer [28,29]. Details of the CpG island size, location and CpG content are described in the supplementary methods (Supplementary Table 1). This system uses selective digestion of sample DNA with methylation-sensitive and methylation-dependent restriction enzymes, followed by quantification of the remaining DNA by real-time PCR [28]. Details of the reaction conditions and CpG sites assayed are included in the supplementary methods.

2.3. Plasma 25(OH)D and homocysteine levels, and serum folate and B12 levels

Plasma 25(OH)D levels were measured using a 25(OH)D vitamin D ELISA kit for serum and plasma (Enzo Life Sciences, NY, USA), according to the manufacturer's instructions [30]. Serum folate and B12 levels were assessed by the Hunter Area Pathology Service using standardised chemiluminescent laboratory protocols. A selective fluorescence assay and OP-162 homocysteine reader (Jei Daniel (JD) Biotech Corp, Taipei, Taiwan) [31] was used to measure plasma homocysteine [31,32].

2.4. Dietary and supplementary intake of vitamin D, calcium and alcohol

Daily intake of vitamin D, calcium and alcohol were estimated using food frequency questionnaires. Subjects also provided details of all supplements and their frequency and dose of intake. Dietary intake data were analysed using Foodworks™ (Version 2.10.146, Xyris Software, Brisbane, QLD, Australia) [33–35].

2.5. Sun exposure: cumulative irradiance

Sample collection for this study was distributed over approximately a 10 month period from July 2010 to April 2011. As the influence of seasonal differences in UV exposure on plasma 25(OH)D levels is known to exhibit a 6–8 week lag [36,37], information on the cumulative solar irradiance over the 6 weeks prior to sample collection was collected [30] using NASA's Total Ozone Mapping Spectrometer (TOMS) program via NASA's Aura OMI level 3 atmospheric portal (http://gdata1.sci.gsfc.nasa.gov/daac-bin/G3/gui.cgi?instance_id=omi). Values ($mW/m^2/nm \times 10^3$) were collected for the location of the clinics for a wavelength of 305 nm (the approximate wavelength of UVB responsible for the synthesis of vitamin D in the skin) [38]. Where TOMS data for a specific date was invalid or unavailable, irradiance was estimated by taking the mean of the data taken for the time period a day before and after the date [30].

2.6. Statistics

Statistical analyses were performed using JMP (Version 11, SAS Institute Inc., Cary, NC, USA). Descriptive statistics (means, 95% confidence intervals, and range) were calculated and presented as appropriate. Where necessary, variables were transformed to normalise distributions prior to regression analysis. Log(x) was calculated for percentage methylation (expressed as proportions), plasma 25(OH)D, calcium intake, vitamin D intake and plasma homocysteine, and log (x + 1) was calculated for alcohol (due to zero values). The remaining variables were approximately normally distributed. Standard least squares regression was used to assess the relationships between multiple parameters and variables of interest. All multivariable models were adjusted for age, sex, BMI, cigarette smoking history, alcohol intake, and cumulative irradiance. Adjusted R^2 values and p-values are reported for final models, and standardised parameter estimates (β values) and p-values reported for individual variables. Outcomes were considered to be statistically significant at $p \leq 0.05$. p-values for interaction of terms ($p_{interaction}$) were also calculated where appropriate.

3. Results

3.1. Percent methylation of CpG islands of vitamin D metabolism genes in PBCs is low, but highly variable

At all CpG sites tested percentage methylation ranged dramatically from almost fully unmethylated (0.01%) to almost fully methylated (99.80%). However, the distribution was heavily skewed to lower methylation values, and therefore the average methylation was low at all CpG sites (Fig. 2A) Percentage methylation values (expressed as proportions) were transformed (log10(x)) to approximately normalise the distribution for regression analyses (Fig. 2B).

The descriptive statistics for the remaining variables (blood measures, dietary and lifestyle) in this cohort are presented in Table 1.

3.2. Percentage methylation status is not correlated between the assessed CpG islands

The methylation statuses of the different sites were not related to each other (Fig. 3). Only *CYP2R1* and *CYP24A1* were significantly positively related ($r^2 = 0.17$, $p = 0.0001$).

3.3. Methylation of CpG islands in CYP2R1, CYP24A1 and VDR are related to plasma 25(OH)D

In bivariate analyses, plasma 25(OH)D levels (log(x)) demonstrated a weak negative correlation with *CYP2R1* ($r^2 = 0.05$, $p = 0.04$) and *CYP24A1* ($r^2 = 0.06$, $p = 0.02$), and a positive correlation with *VDR* ($r^2 = 0.12$, $p = 0.001$). *CYP27B1* methylation status was not significantly related to plasma 25(OH)D levels.

3.4. Methyl donor biochemistry does not influence the relationship between plasma 25(OH)D and methylation, but may be an independent predictor in some cases

As methylation potential may be influenced by methyl donor availability [27,39], multivariable regression analysis was conducted including serum folate, B12 and plasma homocysteine (Table 2), three biochemical variables important in the folate dependent one-carbon metabolism cycle. In the presence of these additional variables, *CYP2R1* and *CYP24A1* methylation remained negatively correlated to plasma 25(OH)D and *VDR* methylation remained positively correlated (Table 2). However, while these relationships were not altered the multi-variable modelling did identify a potential role for serum folate and B12 in methylation regulation at selected sites. Serum folate was positively related to *VDR* methylation status, and B12 was positively correlated with

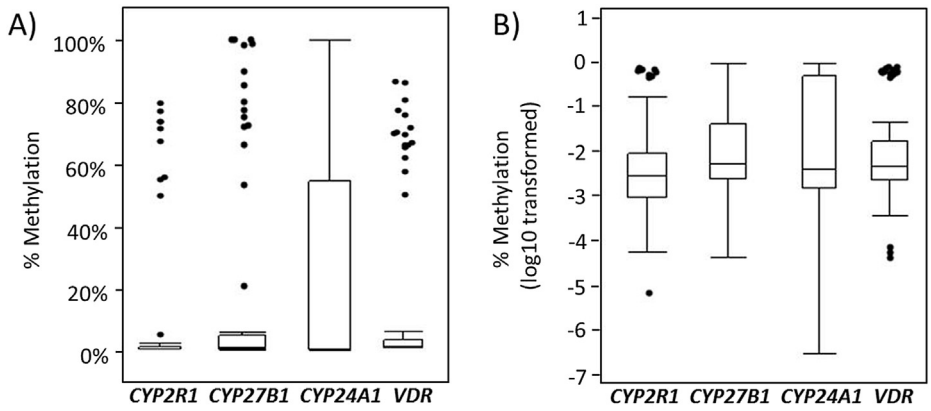

Fig. 2. Outlier box plots illustrating A) Percent methylation at each CpG island (raw data) and B) log10 transformed methylation status.

Table 1
Distribution of variables of interest in cohort.

Variable (units)	Mean	Lower Limit 95% CI	Upper Limit 95% CI	Range
Plasma vitamin D (ng/mL)	28.2	17.4	39.0	0.50–285.42
Serum folate (nmol/L)	27.2	24.9	29.4	8.8–55.3
Serum B12 (pmol/L)	240.8	216.7	265.0	83–604
Homocysteine (μM)	10.6	9.4	11.8	0.81–30.15
Vitamin D intake (μg/day)	5.5	4.0	7.0	0.23–28.6
Calcium intake (mg/day)	1020	917	1123	2.6–3.4
Age (years)	75.5	73.8	77.1	65–94
BMI (kg/m^2)	28.9	27.8	30.1	20.3–42.8
Alcohol intake (g/day)	9.9	6.4	13.4	0–87.8
Cumulative irradiance (mW/m^2/nm × 10^3)	1890	1675	2124	404–3362
Smoker status[a]	44%/56%	N/A	17.4	N/A

CI = confidence interval.
[a] percent ever smokers/never smokers.

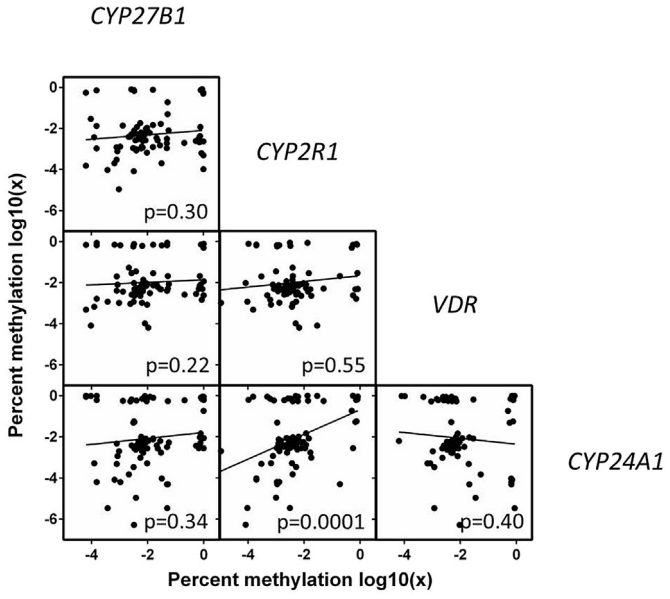

Fig. 3. Linear correlations between the methylation status of each CpG Island assessed.

CYP27B1 methylation status (Table 2). The overall predictive power of the adjusted model, however, was only significant for *VDR* methylation status (Table 2).

3.5. Methylation status of vitamin D metabolism enzyme genes may explain some of the variance in the relationship between vitamin D intake and plasma 25(OH)D levels

It has previously been reported that methylation status of *CYP2R1* and *CYP24A1* are negatively associated with plasma 25(OH)D levels in response to vitamin D supplementation [23]. Therefore, we investigated the role of methylation status in predicting plasma 25(OH)D levels, corrected for total reported vitamin D intake and other variables (calcium intake, age, sex, BMI, cigarette smoking history, alcohol intake, and cumulative irradiance). Modelling all methylation sites and adjustment variables together, resulted in a model ($R^2 = 0.54$, $p < 0.0001$) with better predictive value for plasma 25(OH)D than modelling without methylation status included ($R^2 = 0.46$, $p < 0.0001$) or with vitamin D intake alone ($r^2 = 0.41$, $p < 0.0001$). In the model including all variables, the methylation status at *CYP2R1* ($\beta = -0.20$, $p = 0.026$) was a significant independent negative predictor of plasma 25(OH)D, and *VDR* ($\beta = 0.26$, $p = 0.0046$) was a significant positive predictor of plasma 25(OH)D (Table 3). Modelling of the methylation status of the 4 genes alone significantly predicted plasma 25(OH)D ($R^2 = 0.18$, $p = 0.0008$).

As vitamin D intake, calcium intake, and cumulative irradiance may all individually contribute to plasma 25(OH)D levels, the interactions between methylation status at each gene site and these variables in predicting plasma 25(OH)D was assessed. $p_{interaction}$ values for each interaction are shown in Table 4. A significant interaction was found between the *CYP2R1* methylation status and vitamin D intake, calcium intake, and cumulative irradiance, in

Table 2
Multivariable regression analyses of the relationships between plasma 25(OH)D, serum folate, B12 and plasma homocysteine with methylation status of genes of key vitamin D metabolism enzymes.

	CYP2R1[a]		*CYB27B1*[a]		*CYP24A1*[a]		*VDR*[a]	
	β	p	β	P	β	p	β	P
Plasma 25(OH)D[a]	**−0.27**	**0.03**	0.03	0.77	**−0.33**	**0.04**	**0.25**	**0.03**
Serum folate (nmol/L)	−0.07	0.56	−0.01	0.91	−0.07	0.61	**0.30**	**0.01**
Serum B12 (pmol/L)	0.04	0.67	**0.36**	**0.004**	−0.04	0.80	−0.10	0.59
Homocysteine (μM)[a]	−0.11	0.37	−0.02	0.84	0.04	0.98	0.04	0.86
Model R^2 (p-values)	0.003 (0.47)		0.03 (0.30)		0.04 (0.21)		**0.21 (0.006)**	

Boldface highlights those variables that are statistically significant.
[a] log(x) of raw values used in regression analysis. Analyses corrected for age, sex, BMI, cigarette smoking history, alcohol intake, and cumulative irradiance.

Table 3
Individual predictive value of DNA methylation status for plasma 25(OH)D levels in a model including all assessed methylation sites.

Methylation site	Parameter estimate (β)	p-value
CYP2R1	**−0.20**	**0.03**
CYP27B1	−0.01	0.92
VDR	**0.26**	**0.005**
CYP24A1	0.02	0.79

Boldface highlights those variables that are statistically significant; Analysis adjusted for vitamin D and calcium intake, age, sex, BMI, cigarette smoking history, alcohol intake, and cumulative irradiance.

addition to the direct association with plasma 25(OH)D (Table 3). *VDR* methylation status showed an interaction with calcium intake, but no interaction with vitamin D intake or cumulative irradiance (Table 4). An interaction was found between *CYP24A1* methylation status and vitamin D intake (Table 4), despite there being no direct association between *CYP24A1* status and plasma 25(OH)D levels (Table 3).

4. Discussion

This research considers the relationship of plasma 25(OH)D status, alone and in the context of vitamin D intake, with the methylation status of vitamin D metabolism enzyme genes (*CYP2R1, CYP27B1* and *CYP24A1*) and *VDR* in a single cohort. Previous studies [19,23] on the relationship between DNA methylation and vitamin D metabolism have not included *VDR*. Consideration of *VDR* in addition to those enzymes directly involved in the metabolism pathway is important, as complex feedback loops exist involving the activated receptor.

Importantly, we have shown that when the methylation status of all 4 genes is modelled together, methylation status alone predicts plasma vitamin D, explaining about 18% of variance, and improves the predictive relationship of vitamin D intake on plasma 25(OH)D levels. In this model the methylation status of *CYP2R1* and *VDR* each remain independent significantly predictive variables. Additionally, this is the first study to incorporate an analysis of the influence of biochemical variables relevant to folate dependent one-carbon metabolism, the origin of approximately half of the methyl groups required for biochemical reactions.

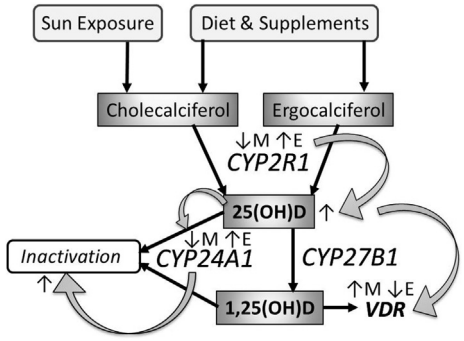

Fig. 4. Potential points of methylation mediated regulation in the vitamin D metabolism pathway. M = methylation status, E = hypothesised expression level.

A weak but significant negative association was found between *CYP2R1* methylation status and plasma 25(OH)D status when considered alone and in the context of vitamin D intake, supporting the findings of the two previous studies [19,23]. These data are further supported by the significant interactions found between *CYP2R1* methylation status and the major determinants of plasma 25(OH)D levels, vitamin D intake, calcium intake and cumulative irradiance. This may suggest that *CYP2R1* methylation status is modulating the contribution of these factors to the rise in plasma 25(OH)D levels (Fig. 4).

Similar to Zhou et al., but contradictory to Zhu et al., a negative association was found between plasma 25(OH)D and *CYP24A1* methylation. However, this relationship was lost when plasma 25(OH)D prediction was corrected for vitamin D intake. Assuming the normal paradigm of reduced methylation increasing gene expression, this may indicate that reduced methylation of *CYP24A1* is a response to increased availability of vitamin D as a homeostatic measure. The fact that the relationship between *CYP24A1* methylation was lost when corrected for intake suggests that impaired methylation is not the direct cause of plasma deficiency as proposed by Zhu et al., but occurs in response to increased availability

Table 4
Statistical significance of the interaction between methylation status and vitamin D intake, calcium intake and cumulative irradiance in the prediction of plasma 25(OH)D levels.

Methylation site	$p_{interaction}$ vitamin D intake × methylation	$p_{interaction}$ calcium intake × methylation	$p_{interaction}$ cumulative irradiance × methylation
CYP27B1	0.71	0.74	0.94
CYP2R1	**0.0009**	**0.003**	**0.009**
VDR	0.47	**0.03**	0.86
CYP24A1	**0.04**	0.62	0.41

Boldface highlights those variables that are statistically significant; log(x) of raw values used in regression analysis for all variables.

(Fig. 4), as hypothesised by Zhou et al. [23].

Both previous studies found no relevant associations for *CYP27B1* methylation [19,23]. Our data support these findings, with no direct correlation found between *CYP27B1* methylation and plasma 25(OH)D, and no interaction found between plasma 25(OH)D predicting variables and *CYP27B1* methylation. However, B12 was identified as being potentially positively associated with *CYP27B1* status, although with a small effect size. Additional studies are required to determine if this association is functionally relevant and identify other variables explaining the range of methylation found at this CpG site. Potentially, *CYP27B1* methylation and expression may be modulated by 1,25(OH)D status, as this is the metabolite produced by the enzyme.

This is the first study, to our knowledge, to consider the relationship between *VDR* methylation status and plasma 25(OH)D status. *VDR* methylation was positively associated with plasma 25(OH)D independently, and when considered in the context of vitamin D intake, and when corrected for methyl donor biochemistry. Increased *VDR* methylation in response to increased plasma 25(OH)D may reflect a potential mechanism for a negative feedback loop, where increased ligand availability decreases receptor expression to maintain homeostasis of vitamin D signalling. Aberrant methylation in this pathway could therefore have consequences for modulating risk of vitamin D sensitive diseases, and the maintenance of calcium homeostasis.

This is the first study to consider the role of methyl donor biochemistry in the modulation of methylation in the genes of the vitamin D metabolism pathway. Whilst the relationships noted between plasma 25(OH)D and methylation status were maintained when serum folate, B12 and plasma homocysteine levels were included in the model, serum B12 was identified as positively associated with *CYP27B1* methylation status, and serum folate was linked to *VDR* methylation. Although the effect size is small, given their role in folate-dependent one-carbon metabolism, it is logical that increased folate and B12 could result in increased methylation via increased availability of methyl donors [31]. It is not clear how or why this relationship would be specific to particular genes, thus additional larger studies are required to confirm or discount this result. However, the lack of correlation between methyl-donor biochemistry and *CYP2R1* and *CYP24A1*, and the lack of correlation between the percentage methylation of each assayed gene site suggest that the results are not due to modulated global methylation, as is commonly suggested.

In this study, homocysteine was not related to the methylation status of any gene assessed, however, given its position in the one-carbon metabolism methylation cycle, changes in homocysteine could be both a cause and a consequence of altered *de novo* methylation, which may be confounding the analysis. The size of the present study may mean this analysis is insensitive to these effects in the context of the complex nature of the interactions between the methyl-donor nutrients.

The hypothesised alterations in gene expression presented in Fig. 4 are based on the standard paradigm of increased methylation leading to decreased gene expression. It is important to note that DNA methylation is only one part of the bigger gene regulation picture, which includes other mechanisms such as histone methylation, 5-hydroxymethylcytosines, and post-transcriptional regulation. Additional studies are required to determine the impact of this altered methylation on gene expression, and how this regulatory mechanism interacts with others factors, including genetic variance in the genes of interest, and variance in the genes involved in maintenance of epigenetic marks.

A limitation of this study is the use of PBCs as the source of DNA for methylation analysis. This may reduce the relevance of the results for *CYP2R1*, *CYP27B1* and *CYP24A1* as most of the actions of these enzymes occur in the liver and kidneys, and we cannot directly relate the results presented here to these organs. This might explain why no significant relationship to plasma 25(OH)D was found for *CYP27B1*. However, extra-renal and extra-hepatic expression of these genes, including the expression of *CYP2R1* [40], *CYP27B1* [40,41] and *CYP24A1* [40–42] has been demonstrated in immune cells [40]. Additionally, increased expression of *CYP24A1* has been demonstrated in PBCs following stimulation with 1,25(OH)D [41,42], and 25(OH)D production from precursors in PBCs has also been demonstrated [41], indicating a potential functional relevance of these genes in this tissue. VDR is ubiquitously expressed, and has relevance to immune signalling pathways, however, it must be acknowledged that this result is not necessarily transferable to other VDR expressing organs. Despite the lack of generalisability, regulation of these enzymes in PBCs may contribute to the overall regulation of this pathway, or may be reflective of its status in other organs.

Additionally, while we have attempted to correct for sun-exposure, due to its potential influence on both vitamin D synthesis and methylation, the precision of the cumulative irradiance measurements used only takes into account location exposure for the time of year of collection, and does not factor in each individuals time spent outdoors, clothing habits, use of sunscreen and other potential modulating factors. Unfortunately, due to the nature of the original study from which this cohort was drawn, this information was not available. It is also important to note this study was conducted in an elderly cohort. Whilst this result is therefore not automatically generalizable to all age groups, synthesis of vitamin D in the skin is reduced in the elderly (due to lifestyle changes and reduced precursor availability) and intake from dietary and supplementary sources is therefore a more important contributor.

Whilst the sample size is also a potential limitation, it is significantly larger than those used by Zhu et al. [19], and in the Zhou et al. [23] pilot study, and similarly sized relative to the validation study conducted by Zhou et al. [23]. Numerous methods are available for the assessment of methylation at CpG islands. The EpiTect II assays are more sensitive than methylation arrays, and more accurate than methylation specific PCR (MSP) [28]. The CpG Islands assessed in the current study span the same regions as the MSP conducted by Zhou et al. [23], and the methylation array conducted by Zhu et al. [19], for CYP2R1 and CYP24A1. For CYP27B1 the CpG Island assessed by the Epitect II assay is located further away from the transcription start site from that assayed using MSP by Zhou et al. [23]. However, advantage of the present study is that the Epitect II assays represent density of methylation across the assayed CpG Island, on a continuous scale. Whereas, by comparison, MSP only compares fully methylated versus fully unmethylated DNA in the primer regions [23].

While bisulfite conversion and sequencing used in the validation study by Zhou et al. [23] is far more detailed, the lack of understanding of the role of methylation at individual CpG sites, as opposed to the island in general, means this level of detail may be excessive at this stage. Sequencing is also far more expensive than enzyme based methods, as such, the sequencing for CYP2R1 and CYP24A1 conducted in the confirmation study by Zhou et al., covered only 14–16 CpG sites. Therefore although the enzyme based method presented here does not achieve single base resolution, assessment of percentage methylation (methylation density) allows assessment across a larger range.

In summary, this study has confirmed the inverse association between *CYP2R1* methylation status and plasma 25(OH)D, and confirmed the lack of association for *CYP27B1*. As the association between plasma 25(OH)D and *CYP24A1* is only apparent in the absence of correction for vitamin D intake, this supports the

hypothesis that altered *CYP24A1* methylation occurs in response to plasma 25(OH)D levels, rather than methylation status being an explanatory factor for deficiency as previously hypothesised.

We have extended the understanding of the role of methylation in this pathway by demonstrating, for the first time, a positive association between *VDR* methylation and plasma 25(OH)D. This may contribute to the broader understanding of the maintenance of vitamin D and calcium homeostasis, and the role that aberration of this pathway could play in disease. Additionally, we have identified a potential limited role for methyl-donor biochemistry as an independent predictor of *CYP27B1* and *VDR* methylation, without modification of the plasma 25(OH)D effect. The gene specificity of this relationship and the consequences of excess, deficiency or supplementation of these factors should be further investigated.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jnim.2016.04.010.

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