25-hydroxyvitamin D and MS activity during therapy with interferon beta-1b

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ABSTRACT

Background: Recently, various studies have been dissecting possible beneficial effects of 25-hydroxyvitamin D (25[OH]D) in reducing disease burden and MRI detectable disease activity in multiple sclerosis (MS).

Objectives: We studied the correlation between 25(OH)D levels under interferon beta-1b (IFNB-1b) treatment and global gene expression levels with respect to the activity of MS.

Methods: BENEFIT studied IFNB-1b in patients with a clinically isolated syndrome (CIS). Within the first 2 years of the study, contrast-enhanced cerebral MRI scans and 25(OH)D were obtained at the CIS and after 6, 12, and 24 months. In addition, gene expression profiles in whole blood were determined using Affymetrix HGU 133 plus 2 arrays at the CIS and after 2/3, 12, and 24 months. The association of ~19,000 genes with enhancing lesions, with 25(OH)D, and with IFNB-1b treatment was modeled with negative binomial and Gaussian generalized linear models. Gene set enrichment analysis (GSEA) was performed to test the association of previously described gene sets relevant for the function of IFNB-1b and 25(OH)D with the number of enhancing MRI lesions. For naïve, threshold-based gene-function classification, the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 was used.

Results: Higher 25(OH)D levels (p=0.0001) and IFNB-1b treatment (p<0.0001) were significantly and independently associated with a lower number of enhancing lesions. 63 genes were significantly associated (p<0.05) with 25(OH)D levels; all but one of them were also associated with IFNB-1b treatment, which was significantly associated with 770 genes. GSEA showed that 25(OH)D gene sets reflecting the impact of vitamin D receptor binding on respective target genes as well as some IFNB-1b response gene sets were highly significantly associated with enhancing lesions. IFNB-1b and 25(OH)D regulated similar genes and first-line immune regulatory processes as shown by DAVID-based gene-function classification.

Conclusions: The results support a beneficial role of 25(OH)D on MS activity. On a molecular level in whole blood, the most likely mechanistic explanation for this effect is a systemic gene regulation by 25(OH)D which is part of a larger systemic gene response to IFNB-1b therapy. Genes associated with either of the 2 are mainly steering immunological processes that impact on the inflammatory activity of MS.

Introduction

- Multiple sclerosis (MS) is a chronic demyelinating disorder in which 25-hydroxyvitamin D (25[OH]D) levels may play a role in disease activity and response to treatment[1]
  - However, studies reported in the available literature on the role of 25(OH)D in the disease are inconclusive and a mechanistic understanding of the potential effects of 25(OH)D is lacking
  - 25(OH)D has many roles within the body, and, importantly, it is known to be a potent immunomodulator[2,3]
- The BEtaferon®/BEtaseron® in Newly Emerging MS For Initial Treatment (BENEFIT) study examined the safety and efficacy of early interferon beta-1b treatment for patients who experienced their first neurological event suggestive of MS (a clinically isolated syndrome, or CIS, N=468)[4-6]
  - Earlier analyses from this trial showed significant improvements in clinical and radiological outcomes in patients who received early treatment relative to those who had delayed treatment with interferon beta-1b[4-6]
- Because of the size of BENEFIT and the length of follow-up (up to 8.7 years), data from the study could be used to examine both the effects of 25(OH)D levels on disease activity and the mechanistic effects of 25(OH)D in patients with MS

Objectives

- To examine the effects of 25(OH)D levels and treatment with interferon beta-1b on disease activity on a molecular level
- To assess changes in gene expression related to 25(OH)D levels and/or interferon beta-1b treatment

Methods

Design of the BENEFIT trial

- Patients with CIS in the BENEFIT study were initially randomized to treatment with interferon beta-1b 250 µg SC every other day (early treatment) or placebo (delayed treatment)
  - Patients remained on placebo for 2 years or until diagnosis of clinically definite MS (CDMS)
- After the placebo-controlled phase of the study, all patients were eligible for a follow-up study in which they were offered interferon beta-1b but could other or no medication
- Blood samples for RNA analyses were taken at baseline, 2 or 3 months, 12 months, and 24 months, or until the patients converted to CDMS in the placebo-controlled phase
  - Whole blood samples for analysis were taken at yearly intervals during the follow-up study
  - Overall, 955 gene expression profiles from 295 unique patients were analyzed in this study

Laboratory analysis

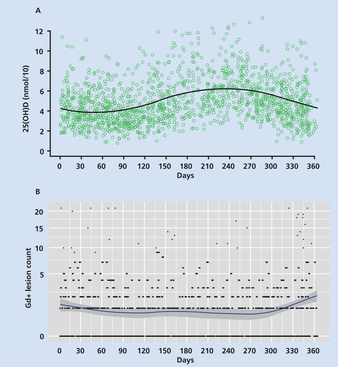
- Vitamin D
  - Levels of 25(OH)D were assessed using an enzyme immunoassay according to the manufacturer's protocol (Immunodiagnostic Systems Inc.; Fountain Hills, AZ)
- Gene Expression
  - Gene expression profiles were measured using Affymetrix HGU 133 plus 2.0 arrays according to the manufacturer's protocol
    - o Raw data were RMA background-corrected, quantile normalized, and transformed with a logarithm to base 2
- Statistical Analysis
  - To model gadolinium-enhancing (Gd+) lesions as a function of 25(OH)D and other covariates, generalized linear mixed models were applied
    - o Repeated measures were modeled by a compound symmetry correlation structure
    - o All modeling was done using proc glimmix in SAS version 9.2
  - Modeling of gene expression as a function of 25(OH)D and other covariates and of Gd+ lesion count as a function of gene expression used generalized linear models
    - o To correct for multiple testing, the Benjamini and Hochberg[7] or Bonferroni[8] methods were used
  - Gene set enrichment analysis (GSEA) was applied for gene set testing[9] using the R implementation available from the Broad Institute as a basic framework
    - o A permutation approach of gene labels was used to compute the probability of getting a specific enrichment score by chance
  - Database for Annotation, Visualization, and Integrated Discovery (DAVID)[10] was used to analyze gene sets for their biological functionality using the Affymetrix HGU 133 plus 2.0 platform as background

Results

25(OH)D levels, season, interferon beta-1b, and MS disease activity

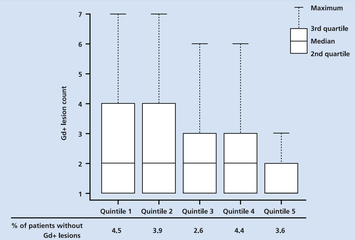
- As expected, 25(OH)D levels peaked between the end of July and the middle of August each year (Figure 1A)
  - The onset of Gd+ lesions also showed a seasonal variation, with a relatively constant minimum from April to late September followed by a rapid increase to a maximum in late December (Figure 1B)
- High levels of 25(OH)D correlated with low Gd+ lesion counts (Figure 2, Table 1)
  - Gd+ lesion incidence rate decreased by ~55% with an increase of 50 nmol/L in serum 25(OH)D
- Treatment with interferon beta-1b led to a 75% decrease in lesion rate

Figure 1. Seasonal distribution of (A) 25(OH)D levels[a] and (B) Gd+ lesions[b]



[a] All available 25(OH)D measurements were used.
[b] Locally weighted scatter plot smoothing line with 95% confidence intervals.

Figure 2. Gd+ lesions count as a function of 25(OH)D quintiles[a]



% of patients without Gd+ lesions	4.5	3.9	2.6	4.4	3.6

[a] Only Gd+ lesion measurements >0 were used. Upper cut offs for each quintile are quintile 1=30.91 nmol/L, quintile 2=40.25 nmol/L, quintile 3=50.49 nmol/L, quintile 4=66.20 nmol/L, and quintile 5=169.79 nmol/L.

Table 1. Incidence rate ratio (IRR) of 25(OH)D levels and Gd+ lesion counts

	IRR	Lower	Upper	p-value
	Unadjusted model			
25(OH)D	0.45	0.31	0.64	<0.0001
	Adjusted model[a]			
25(OH)D	0.43	0.28	0.66	0.0001
Interferon beta-1b	0.25	0.14	0.46	<0.0001

[a] Adjusted for age, sex, and interaction between 25(OH)D and interferon beta1-b.

Table 2. Number of genes that were significantly regulated by 25(OH)D and interferon beta-1b

	Nominal p-value≤0.05	BH corrected p-value≤0.1	Bonferroni corrected p-value≤0.05
25(OH)D	63	0	0
Interferon beta-1b	770	480	91
Gd+ lesions	5506	4337	351

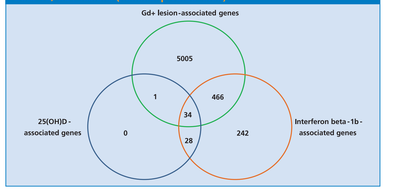
BH, Benjamini & Hochberg.

Results (cont)

Gene expression analyses

- In the uncorrected analysis (nominal p-value threshold 0.05), 63 genes were found to be regulated by 25(OH)D (Table 2) and 62 genes were regulated by both 25(OH)D and interferon beta-1b
- No genes were found to be regulated by 25(OH)D in the Benjamini and Hochberg-corrected analysis (corrected p-value threshold ≤0.1)
- The Bonferroni-corrected analysis identified 91 interferon beta-1b-associated genes and 351 Gd+ lesion-associated genes (corrected p-value threshold ≤0.05)
- Analysis of the overlap between the different gene sets (Figure 3) indicated that a large proportion of genes regulated by interferon beta-1b and/or 25(OH)D had a beneficial effect on Gd+ lesion count
  - Except for one 25(OH)D-associated gene, all of the effects of 25(OH)D on gene regulation were beneficial for reduction of Gd+ lesions
  - Genes regulated by interferon beta-1b were regulated in a way that would be associated with a reduction in Gd+ lesion count

Figure 3. Overlap between genes associated with 25(OH)D, interferon beta-1b, Gd+ lesions (nominal p-value≤0.05)



DAVID-based gene set functional classification

- The 3 derived sets shown in Figure 3 were characterized using DAVID; all gene sets were highly enriched for processes/responses of the immune system (Table 3)
  - Enrichment scores for the top enrichment cluster related to immunological process were 3.89 (25[OH]D), 7.25 (interferon beta-1b), and 3.25 (Gd+ lesions)
- Genes associated with 25(OH)D were additionally enriched for serine-type peptidase activity (enrichment score 2.53) and interferon beta-1b-associated genes were additionally enriched for apoptosis regulation (enrichment score 2.95)

Table 3. Genes associated with 25(OH)D, interferon beta-1b, and Gd+ lesions

	p-value	Adjusted p-value
Term	25(OH)D-associated genes	
Defense response to bacterium	<0.001	0.024
Defense response	<0.001	0.026
Response to bacterium	<0.001	0.130
Serine-type endopeptidase activity	<0.001	0.022
Serine-type peptidase activity	<0.001	0.022
	Interferon beta -1b-associated genes	
Defense response	<0.001	<0.001
Response to wounding	<0.001	<0.001
Inflammatory response	<0.001	<0.001
Regulation of apoptosis	<0.001	0.004
Regulation of programmed cell death	<0.001	0.005
	Gd+ lesion-associated genes	
Defense response	<0.001	0.001
Inflammatory response	<0.001	0.095
MHC class I protein complex	<0.001	0.100
Antigen processing and presentation	<0.001	0.140
Regulation of f-kappaB kinase/NF-kappaB cascade	<0.001	0.140

Results (cont)

GSEA results

- 3 out of 10 analyzed 25(OH)D gene sets were highly enriched according to GSEA (Table 4), with an additional set close to the significance threshold, suggesting that 25(OH)D had a very specific mechanism of action
  - Genes regulated by 25(OH)D were associated with reduced lesion count in a systemic and concerted fashion
- 4 out of 6 interferon beta-1b-related gene sets showed a significant enrichment pattern, corresponding to a broad interferon beta-1b-induced gene response
  - Genes in this set were also associated with reductions in lesion count

Table 4. Enriched 25(OH)D gene sets according to GSEA

Gene set name	Description	p-value
KnightUp	Set derived from Ramagopalan et al.[11]: 25(OH)D-receptor ChIP-Seq and GEP in lymphoblastoid cell line after stimulation with calcitriol for 36h -> identification of genes that are bound and upregulated by the 25(OH)D-receptor	0.000
MS_vitD_genes0.05	Set derived from BENEFIT GEP and 25(OH)D data from the model *GeneExpression~25(OH)D *IFNB-covariates*. To enter the set, the particular gene had to have a nominal p-value of ≤0.05.	0.000
V$VDR_Q3	Genes with promoter regions [-2kb, 2kb] around transcription start site containing the motif GGGKNARNRRGGWSA which matches annotation for VDR: vitamin D (1,25- dihydroxyvitamin D3) receptor. From Broad Institute MSigDB v3.1	0.0055
V$VDR_Q6	Genes with promoter regions [-2kb, 2kb] around transcription start site containing the motif CNSNNTGAACCN which matches annotation for VDR: vitamin D (1,25- dihydroxyvitamin D3) receptor. From Broad Institute MSigDB v3.1	0.0604

Discussion

- In the BENEFIT study, genes associated with increased 25(OH)D levels and interferon beta-1b treatment were also associated with a decrease in Gd+ lesion counts
  - The beneficial effects of 25(OH)D may not only be concentration dependent, but also influenced by many other factors
- Approximately one quarter of the genes associated with Gd+ lesions (given a Bonferroni-corrected p-value threshold of ≤0.05) were also targeted by interferon beta-1b, with approximately 4% also targeted by 25(OH)D
  - These genes represent the starting point for inferring the mode of action of 25(OH)D and confirm the known mechanism of action of interferon beta-1b
  - GSEA results seem to suggest that 25(OH)D may reduce Gd+ lesion count, similar to the beneficial effect of interferon beta-1b
- Genes associated with Gd+ lesions, interferon beta-1b, or 25(OH)D were primarily associated with immune processes, suggesting that regulation of the immune system is key in treating MS
  - Interestingly, some processes appeared to be affected in an additive manner by 25(OH)D and interferon beta-1b, but further research is needed to understand the significance of this interaction

Conclusions

- The analyses presented here support the beneficial role of 25(OH)D in reducing disease activity in patients with MS as assessed by Gd+ lesions
- The findings provide some evidence on a molecular level for the mode of action of 25(OH)D in MS, which implies a causal role of 25(OH)D in reducing disease activity via regulating anti-inflammatory processes

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