Influence of Vitamin D Status and Vitamin D_3 Supplementation on Genome Wide Expression of White Blood Cells: A Randomized Double-Blind Clinical Trial.

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Background

- Vitamin D is a group of fat-soluble secosteroids responsible for enhancing intestinal absorption of calcium and phosphate.

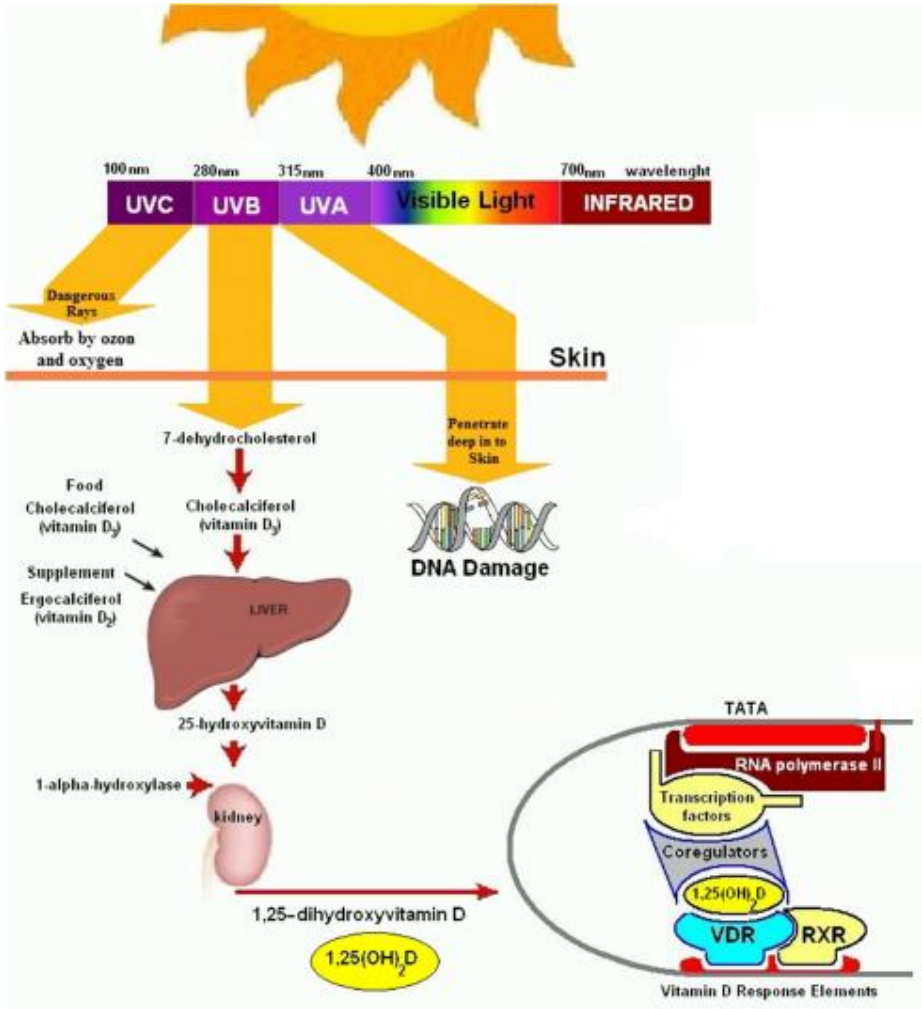
- Vitamin D_2 and D_3

- Can be ingested and synthesized by the skin from cholesterol under adequate sun exposure.

- Deficiency at [hydroxyvitamin D] < 20 ng/ml

- Insufficiency has been linked to cancers, autoimmune diseases, infectious diseases, type 2 diabetes and cardiovascular disease.

Background



Composition and Metabolism of Vitamin D_3

Objective

- To determine the effect of vitamin D status and subsequent supplementation on broad gene expression in healthy adults.

Methods

- Trial design
  - Randomized, controlled, double-blind, investigator initiated, single center pilot trial.
  - Signed consent form
  - The study was conducted in winter months to minimize sun exposure as a confounding factor.
  - Group I: 400 IU/d for 8 weeks
    Group II: 2000 IU/d for 8 weeks

Methods

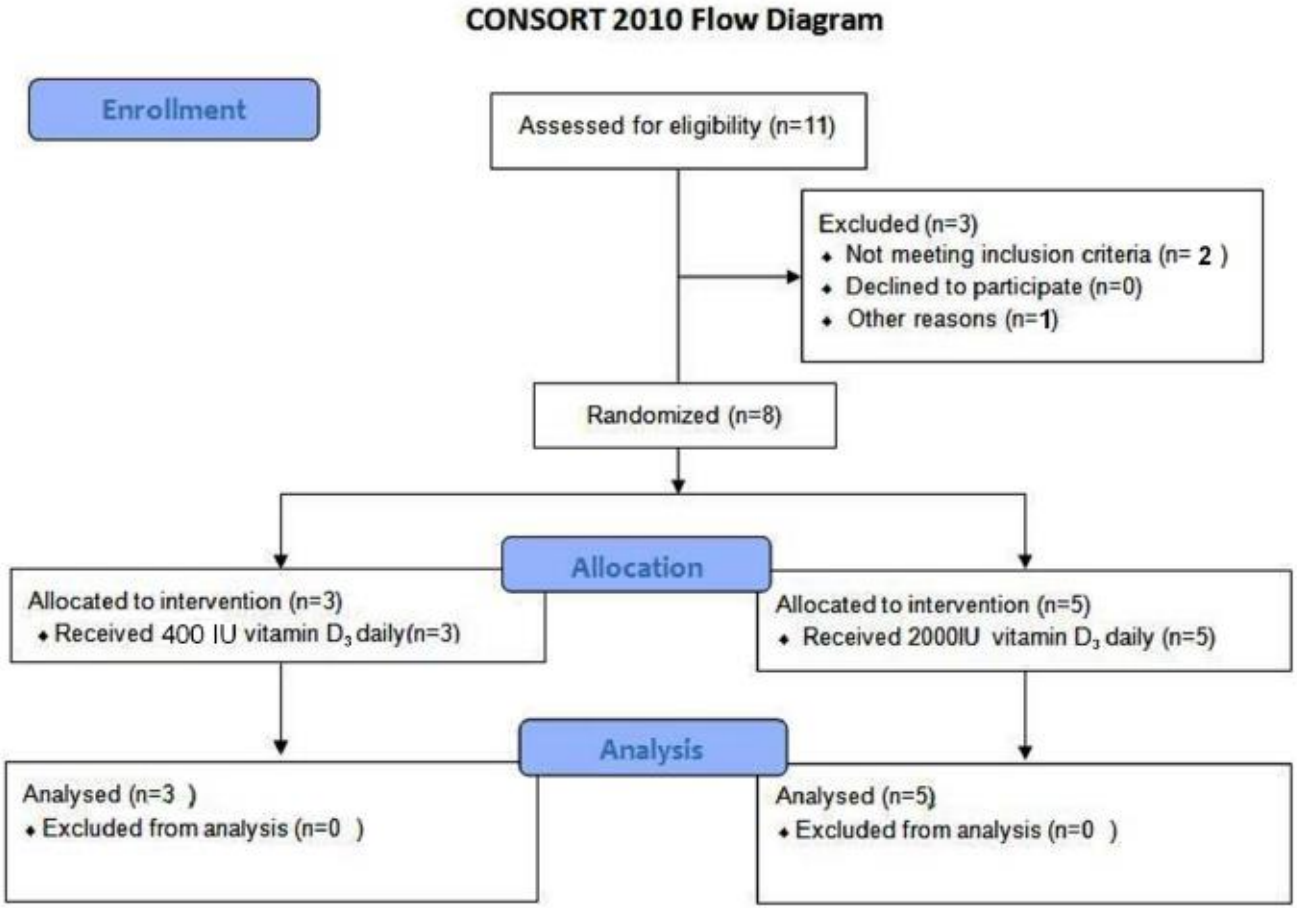


Figure 1. Flow Diagram of Study Subjects.
doi:10.1371/journal.pone.0058725.g001

Methods

- Study subjects:
  - Healthy, non-patient
  - English speaking
  - Adults
  - Males and females
  - Of all ethnic groups
- Exclusion criteria:
  - Pregnant/lactating women
  - Hepatic or renal disease
  - Vitamin D supplementation
  - Current antiseizure medications or glucocorticoids
  - Recent tanning
  - Intestinal malabsorption
  - Unwillingness to consent

Methods

- Upon visit to the investigation site:
  - Demographic data
  - Body weight
  - Height
  - BMI
  - Past vitamin D use
  - Diet
  - Medication usage
  - Urine pregnancy test
  - Vitamin D_3 capsules of either 400 IUs or 2000 IUs for the 8-week period.

Methods

- Blood sample collection
- LC-Tandem mass spectroscopy
- Microarray Data Acquisition and Preprocessing
- Real time PCR
- Sample size estimation
- Data analysis

Data analysis

- Arrays normalization with the RMA method.
- Principal Component Analysis
- 2-way ANOVA
- $P < 0.01$
- False Discovery Rate < 0.1
- Correction for multiple testing
- EASE software (version 2.0) with $P<0.05$

Results

Table 1. Subject demographics and total 25(OH)D levels before and after 400 IU/d or 2000 IU/d of vitamin D_3 supplementation for 8 weeks.

	400 IU/d (N = 3)	2000 IU/d (N = 5)
Sex (Women)	2	1
Age (years)	27.3 ± 2	26 ± 5.1
25(OH)D levels before supplementation (ng/ml)	18.3 ± 1.1	24 ± 10.7
25(OH)D levels after supplementation (ng/ml)	24 ± 5.2	33.8 ± 7.8

Demographic information including sex, average age and 25(OH)D levels are included (mean ± standard deviation).
doi:10.1371/journal.pone.0058725.t001

Results

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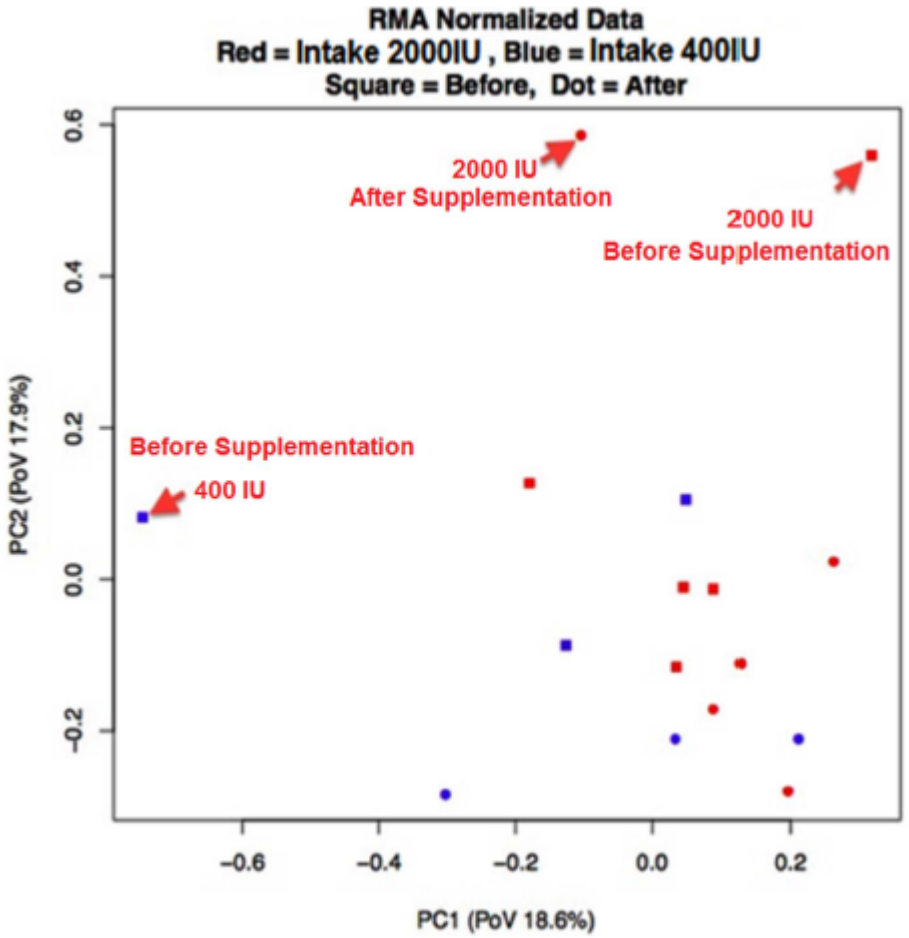


Figure 2. Principal Component Analysis across 16 microarray samples. There is no grouping of samples along the first or second principal components (representing 18.6% and 17.9% of the variance in gene expression, respectively) based on the expression of these genes. Sample types of each group before or after vitamin D_3 supplementation are color-coded for the dose of vitamin D_3 supplementation. Red = 2000 IUs and blue = 400 IUs (PoV = Possibility of Variance.)
doi:10.1371/journal.pone.0058725.g002

Results

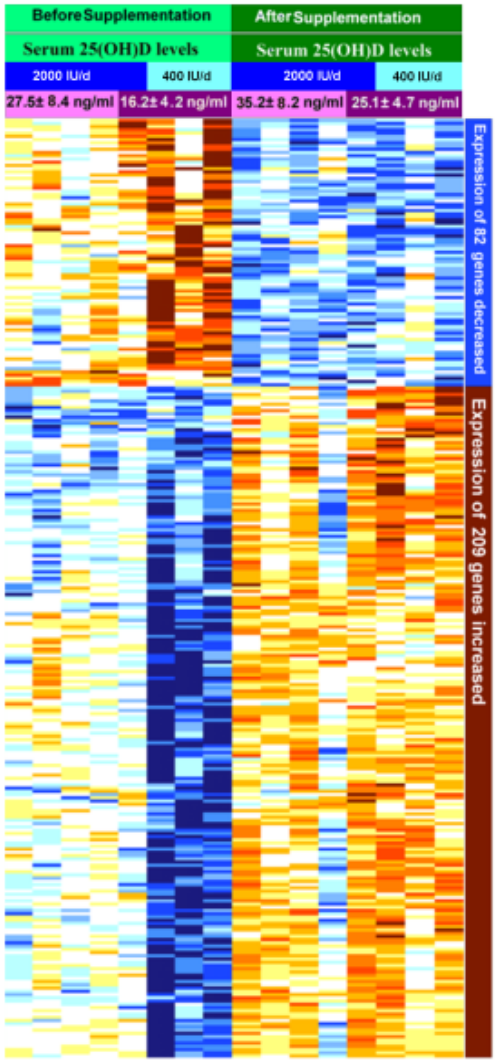


Figure 3. Heatmaps of vitamin D responsive genes whose expression levels change after 2 months vitamin D_3 supplementation. Before supplementation (light green) four subjects were vitamin D deficient with 25(OH)D of 16.2±4.2 ng/ml (dark purple) and the other four subjects were insufficient or sufficient with a 25(OH)D of 27.5±8.4 ng/ml(light purple). After supplementation (dark green) serum levels of 25(OH)D in vitamin D insufficient/sufficient subjects increased to 35.2±8.2 ng/ml (light purple) and in the vitamin deficient subjects increased to 25.1± 4.7 ng/ml(dark purple). Two groups of gene-expression changes are seen based on stimulation (brown) or inhibition (blue) of gene expression post vitamin D_3 supplementation. (Colors ranged from blue to brown; High expression = brown, average expression = white, low expression = blue). Clustering of the 291 genes affected by vitamin D_3 supplementation was based on stimulation (brown) or inhibition (blue) of gene expression. The list of the 291 genes is shown in Table S1.
doi:10.1371/journal.pone.0058725.g003

Results

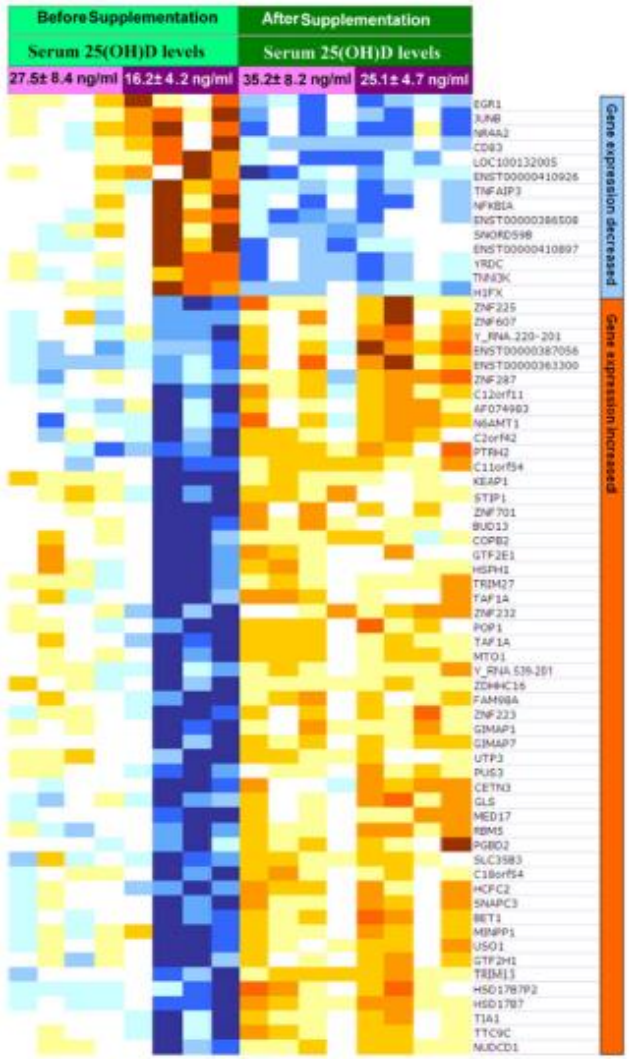


Figure 5. Heatmaps of vitamin D responsive genes affected by vitamin D status. Before supplementation (light green) four subjects were vitamin D deficient with 25(OH)D of 16.2±4.2 ng/ml (dark purple) and the other four subjects were insufficient or sufficient with a 25(OH)D of 27.5±8.4 ng/ml(light purple). After supplementation (dark green) serum levels of 25(OH)D in vitamin D insufficient/sufficient subjects increased to 35.2±8.2 ng/ml (light purple) and in the vitamin deficient subjects increased to 25(OH)D of 25.1±4.7 ng/ml(dark purple). Two groups of gene-expression changes are seen based on stimulation (brown) or inhibition (blue) of gene expression post vitamin D_3 supplementation. (Colors ranged from blue to brown; High expression = brown, average expression = white, low expression = blue).Expression of 66 genes before supplementation was significantly different in the vitamin D deficient group (dark purple) compared to the vitamin D insufficient/sufficient group (light purple). Clustering of the 66 genes affected by vitamin D status and vitamin D_3 supplementation was based on stimulation (brown) or inhibition (blue) of gene expression.
doi:10.1371/journal.pone.0058725.g005

Results

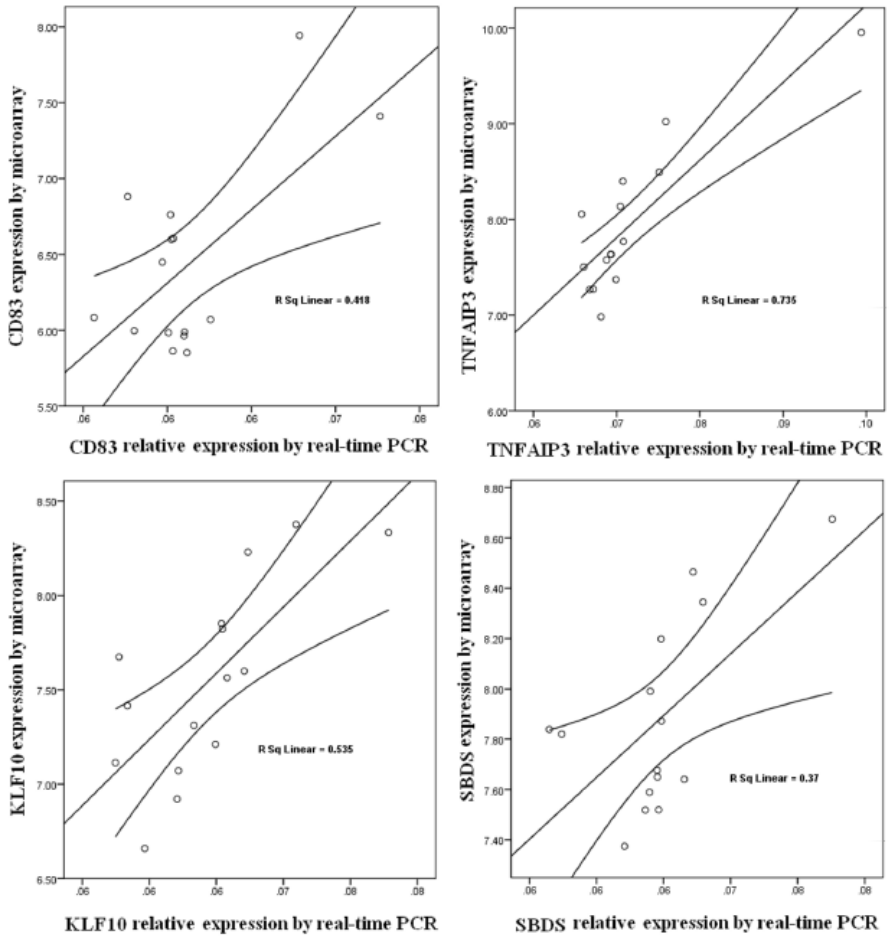


Figure 4. Verification of microarray gene expression by Real-time PCR. For verification of gene expression real-time PCR was performed for four genes including CD83, TNFAIP3, KLF10 and SBDS. Relationship between two sets of data from microarray and real-time PCR is shown by linear regression with 95% mean prediction interval. The results showed the relative expression of these genes was consistent with the expression observed from the broad gene expression by microarray.
doi:10.1371/journal.pone.0058725.g004

Results

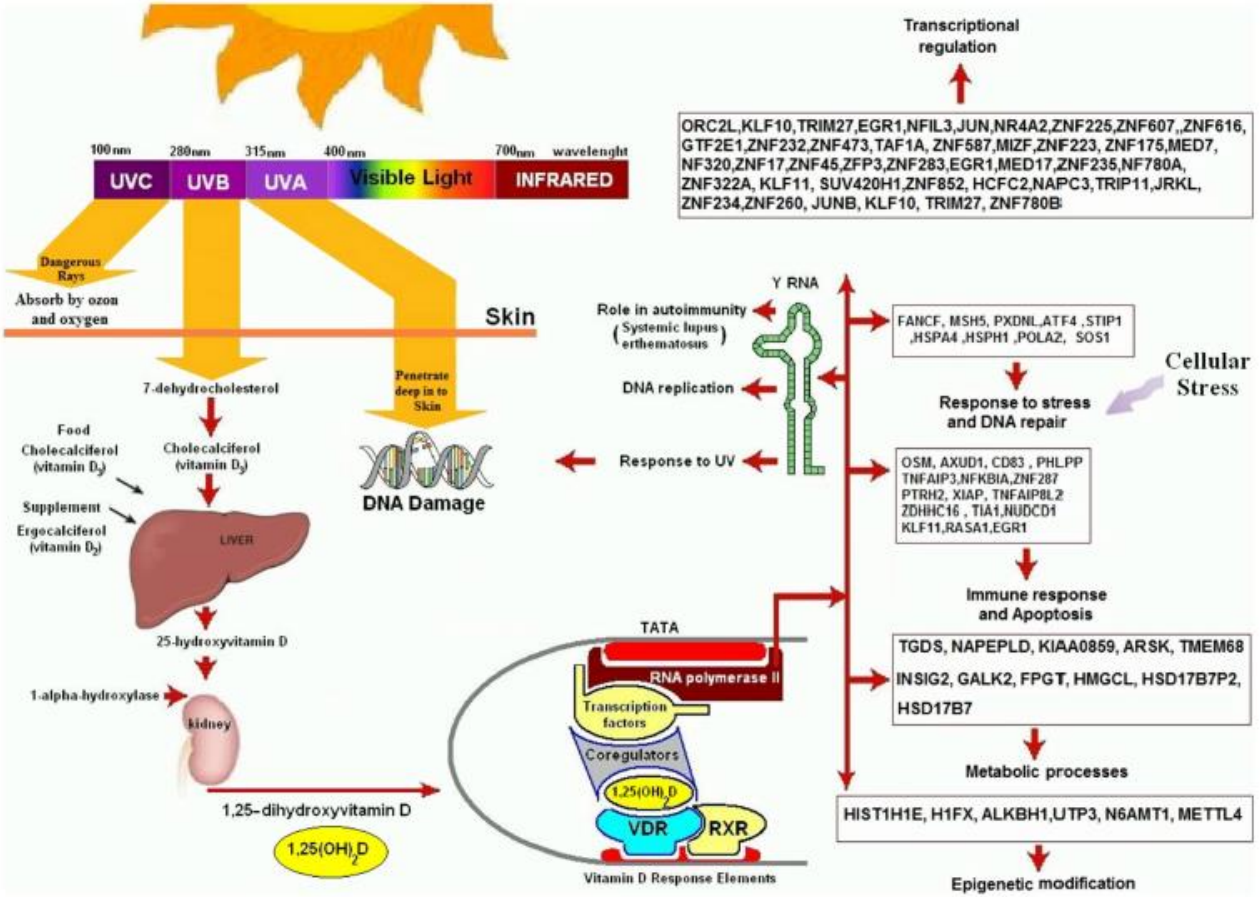


Figure 7. Biological functions for genes whose expression levels were altered after 2 months of vitamin D_3 supplementation. After receiving vitamin D_3 supplementation we identified 291 genes whose expression was significantly decreased or increased. Some of these genes influence several pathways that are involved in response to stress and DNA repair, DNA replication, immune regulation, epigenetic modification, transcriptional regulation and other biological functions. In addition vitamin D_3 supplementation influenced the expression of Y RNA and CETN3 that are involved in DNA repair in response to UVR exposure.

doi:10.1371/journal.pone.0058725.g007

Critical appraisal

Study design:

- Randomized
- Controlled
- Double-blind
- Single center
- Investigator-initiated
- Pilot trial
- Two groups – parallel trials

→ Gold standard in evaluating healthcare interventions

Critical appraisal

Research question:

- How does vitamin D status and vitamin D3 supplementation affects broad gene expression in humans (healthy adults)?

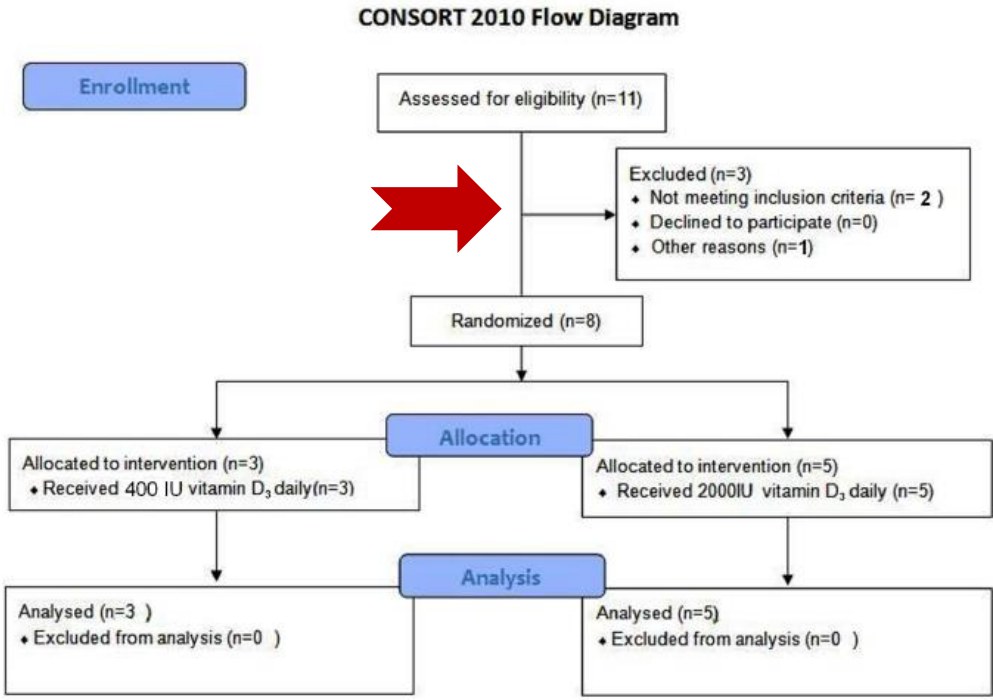
Critical appraisal

- P: healthy, non-patient, english-speaking, adults, males and females, of all ethnic groups, white, age 18 and older, (n=8) → HOW RECRUITED?
- I: vitamin D3 supplementation daily for 8 weeks in two groups: - 400IUs (n=3)
  - 2000IUs (n=5)
- C: no control group? (suppl. in both groups)
- O: vitamin D status affects gene expression (nonskeletal health benefits of vitamin D)

Critical appraisal

Randomization:

- 11 subjects recruited
- 2 subjects dropped out
- Computer-generated simple randomization scheme (double-blind)
  → MORE INFORMATION?
- group I: 400IU/d (n=4), group II: 2000IU/d (n=5)
- 1 subject of group I dropped out (→ n=3)



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Critical appraisal

- Double-blind study
  - Subjects received a bottle containing vitamin D3 capsules (400IUs or 2000IUs)
- Two groups
  - Comparable
  - Except mean of vitamin D status (one group deficient, one group insufficient/sufficient in mean at baseline)

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Critical appraisal

- Two groups
  - Same treatment (except: 400 or 2000IUs)
- Subjects
  - All included in analysis (16 microarrays – 2 of every subject (baseline and after 2 months suppl.))
  - Analysis in group I and group II (ITT/PP)
  - AND in other two groups (subgroup analysis):
    - Group A: vitamin D deficient at baseline (n=4)
    - Group B: vitamin D sufficient or insufficient at baseline (n=4)

  → HOW MANY DEFICIENT/(IN)SUFFICIENT SUBJECTS IN GROUP I OR II ?

Critical appraisal

Endpoints:

- Gene expression affected by vitamin D3 supplementation
- Vitamin D status after vitamin D3 supplementation
- Comparison between group I/II, subgroup A/B, baseline/follow-up

Critical appraisal

Clinical relevance:

- Additional 47 genes influenced by vitamin D3 status
- Effect on immune function, transcriptional regulation, cell cycle activity, DNA replication, stress, …
- Vitamin D influences up to 5% of the human genome!

→ Vitamin D as prevention for diseases!

Critical appraisal

Efficacy:

- Statistically significant difference in expression from baseline to follow-up (291 genes) and between subgroups (66 genes)
- NO significant difference between group I/II (400 or 2000lUs)

→ don‘t know which dose is better (trend for a larger change in group II (2000lUs))

→ reasons could be:

- small number of subjects
- any improvement in serum 25(OH)D can lead to significant changes

(→ Maximize vitamin D‘s effect → even higher doses)

Critical appraisal

Major limitation of the study:

- Small number of subjects
- Only a few of 291 genes verified by real time PCR
- Didn't identify actual VDR binding sites with a biologic function

→ But it does support vitamin D responsive genes from in vitro studies

→ 17 novel candidate VDREs in vitamin D regulated genes

→ Need to confirm with experimental studies!

Critical appraisal

Strengths of the study:

- Measuring serum 25(OH)D concentrations by the gold standard
  → liquid chromatography tandem mass spectroscopy assay
- Comparing gene expression in the same individual
  → baseline and follow-up
- In winter (no influence of sun tanning)
- Real-time PCR analysis of 4 genes
  → CD83, TNFAIP3, KLF10, SBDS

Critical appraisal

Summary:

- Good explained and described (analysis)
- Sometimes confused which groups are compared
- Sometimes redundant
- Small number of subjects
- More information about each subject
- No table to subgroup analysis

Thank you for your attention!