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Original Research

Colorectal cancer, Vitamin D and microbiota: A double-blind Phase II randomized trial (ColoViD) in colorectal cancer patients

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Abstract

Background

Several studies suggest a role of gut microbiota in colorectal cancer (CRC) initiation and progression. Vitamin D (vitD) blood levels are also inversely correlated with CRC risk and prognosis. However, these factors' interplay remains unknown.

Methods

74 CRC patients after standard treatment were randomized to 1-year 2000 IU/day vitD or placebo. Baseline and post-treatment fecal microbiota for shotgun metagenomics sequencing was collected. Coda-lasso and Principal Component Analysis were used to select and summarize treatment-associated taxa and pathways. Associations between vitD and taxa/pathways were investigated with logistic regression. Mediation analysis was performed to study if treatment-associated taxa mediated the effect of supplementation on 25(OH)D levels. Cox proportional-hazards model was used for disease-free survival (DFS).

Results

60 patients were analyzed. Change in alpha diversity (Shannon: $p = 0.77$; Simpson: $p = 0.63$) and post-treatment beta diversity ($p = 0.70$) were comparable between arms. Post-treatment abundances of 63 taxa and 32 pathways differed between arms. The 63 taxa also mediated the effect of supplementation on 25(OH)D ($p = 0.02$). There were sex differences in vitD levels, microbiota and

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pathways. Pathways of essential amino acids' biosynthesis were more abundant in supplemented women. *Fusobacterium nucleatum* presence at baseline was associated with worse DFS ($p = 0.02$). Those achieving vitD sufficiency (25(OH)D≥30 ng/ml) had lower post-treatment abundances ($p = 0.05$). Women were more likely to have *F. nucleatum* post-treatment ($p = 0.02$).

Conclusions

VitD supplementation may contribute shaping the gut microbiota and the microbiota may partially mediate the effect of supplementation on 25(OH)D. The observed sex-specific differences highlight the necessity of including sex/gender as a variable in microbiome studies.

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Keywords: Colorectal cancer, Microbiome, Microbiota, Vitamin D, Sex, Gender

Introduction

The microbiota contributes to regulating intestinal endocrine activities, neurologic signalling and the immunological response, protecting against pathogens, delivering vitamins and metabolites, and preserving the integrity of the gut mucosal barrier. The immune system appears to be the link between dysbiosis and several diseases, including cancer, diabetes, and cardiovascular or autoimmune diseases [1]. Recent studies have also revealed strong links between gut microbiota composition and colorectal cancer (CRC), with several proposed mechanisms that may promote CRC development [2–7].

Vitamin D also plays an essential role in human health. It is mainly produced by the synthesis in the skin in response to sun exposure and, to a lesser extent, absorbed through diet. Two distinct forms of vitamin D are available in nature: vitamin D_2 (ergocalciferol), which is mostly derived from vegetables and yeast, and vitamin D_3 (cholecalciferol), which is produced by sunlight on the skin and is present in animal derived-foods, particularly fatty fish. Vitamin D is hydroxylated in the liver into 25-hydroxyvitamin D (25(OH)D) and further hydroxylated to 1,25-dihydroxyvitamin D (1,25(OH)2D) in the kidney. 25(OH)D is the major circulating form of vitamin D and a fairly stable metabolite. For this reason, it is usually considered a reliable indicator of vitamin D status [8].

Several studies found an association between vitamin D and CRC risk. When comparing the highest versus the lowest levels of 25(OH)D, data revealed a significant reduction in risk with a strong dose-response effect [9,10]. In a meta-analysis summarizing 5,562 deaths out of 62,548 individuals, we showed a significant decrease in mortality risk in the general population as circulating 25(OH)D increased [11]. Consistently, in a meta-analysis of randomized clinical trials (RTCs), we found a statistically significant reduction of 7% in total mortality in healthy subjects supplemented with vitamin D [12].

Indirect evidence of a possible interaction between vitamin D and microbiota was provided by in-vitro findings, which identified a synergistic effect of butyrate and vitamin D in boosting phosphatase and tensin homolog (PTEN) expression and consequent cancer cell apoptosis [13,14]. Accordingly, the impact of vitamin D on gut bacteria composition could significantly affect the immune system function and, ultimately, human health [15]. To prove a causal relationship, however, it is important to investigate vitamin D extra-skeletal functions involving the immune system. One of these functions is mediated by gut microbiota, although little is still known about the direct effects of vitamin D on bacteria. Vitamin D inhibits the growth of specific mycobacterial species in vitro, [16] suggesting that its antimicrobial effect is compatible with its immune-regulating properties. Jahani et al. [17] showed that in mice exposed to high levels of vitamin D_3 during pregnancy and lactation, lower vitamin D levels were associated with reduced vitamin D receptors, increased expression of pro-inflammatory genes in the colon at 3 months and lower colonic Bacteroides/Prevotella at postnatal day 21. These results are confirmed by other rodent studies demonstrating that vitamin D deficiency through dietary restriction, lack of CYP27B1, or lack of vitamin D receptors (VDRs) promote increases in the Bacteriodetes [18–21] and Proteobacteria phyla [18,19,21]. Furthermore, VDR polymorphisms have been identified as important contributors to microbiome variance in a genome-wide association including a pooled cohort of 2029 individuals [22]. The human VDR polymorphisms influenced the Parabacterioides genus, and the subsequent evaluation of VDR−/− mice showed a corresponding increased abundance of Parabacteroides compared to wild-type mice [22].

There is also evidence that vitamin D supplementation affects the main gut microbial phyla - Firmicutes, Actinobacteria and Bacteroidetes - with either a decrease or an increase in relative abundance. Regarding alpha and beta diversity, a high dietary vitamin D intake seemed to shape bacterial composition in some studies and affect the species richness [23]. Through dysregulated colonic antimicrobial activity and decreased enteric bacterial homeostasis, vitamin D deficiency predisposes mice to colitis. This may be a key mechanism connecting vitamin D status with inflammatory bowel disease (IBD) in humans [24]. Furthermore, in response to a given dose of vitamin D, the effect on 25(OH)D concentration differs between individuals, so it is essential that the factors affecting this response are identified [25]. The microbiome may be a mediator of vitamin D, and the management of microbiota homeostasis may be an important facet of vitamin D function in the gut [26]. We conducted a case-control study [27] showing that several CRC-associated microbiome species, such as *Fusobacterium nucleatum*, were correlated with diet and serum inflammation biomarkers. Mediation analysis confirmed the significant role of the microbiome in mediating the effect of diet on CRC risk. Lastly, we found evidence that taxa associated with CRC could be indicators of early relapse.

Here, we present the results of a phase II clinical trial including CRC patients randomized to receive vitamin D 2000 International Units (IU) per day or placebo for one year. In this study, we investigated the change in the microbiome after vitamin D supplementation and we assessed whether the microbiome may be a mediator of 25(OH)D levels.

Materials and Methods

Study population

CRC patients after standard treatment (surgery with or without chemotherapy and/or radiotherapy when needed) were randomly assigned

to vitamin D_3 2000 IU a day or placebo and treated for 1 year. Stratification was made for chemotherapy (either adjuvant/neoadjuvant) versus no chemotherapy. The study received Istituto Europeo di Oncologia (IEO) institutional review board approval with the number IEO 223 and Eudract number 2015-000467-14.

Main inclusion criteria were: 35-75 years old patients with resected colorectal cancer stage I - III in the last 24 months. They signed informed consent according to the International Committee on Harmonization of Good Clinical Practice (ICH-GCP) guidelines. Main exclusion criteria were: baseline 25 (OH)D $\geq$ 30 ng/ml (external exams); history of cancer in the prior five years (other than cervical intra-epithelial neoplasia and non-melanoma skin cancer); clinical/radiological or laboratory/pathology evidence of neoplasia; current daily supplementation of vitamin D (e.g. calcium citrate with vitamin D); history of recurrent renal calculi; history of malabsorption syndrome (e.g., pancreatic insufficiency, celiac disease, Crohn disease, any chronic IBD); chronic liver disease and/or renal disease with altered biochemical functions, or renal dialysis; any medical condition that in the physician's opinion would potentially interfere with the subjects' health.

Study design

After signed informed consent was provided, the baseline visit included medical history, complete physical examination, concomitant medications, anthropometric measurements, smoking habit, food questionnaire, blood and stool samples collection. After eligibility was confirmed, the participant was stratified according to previous adjuvant/neoadjuvant treatment (yes versus no) and randomized to vitamin D_3 or placebo in a double-blind fashion. A computer-generated randomization list was prepared using permuted blocks of four to ensure that the 1:1 ratio was maintained. After randomization, a six-month drug supply was provided to the participant. Vitamin D_3 was provided in an oily solution and the placebo was made to be visually identical to the active formulation. The therapy boxes of vitamin D and matching placebo were prepared by the pharmacists at the European Institute of Oncology.

A three-month phone call was made to check safety and compliance. At six-month visit, safety, clinical examination and concomitant medications were assessed, and the new drug supply was provided. At 12-month final visit, safety, clinical examination, concomitant medications and all biological samples were collected.

In accordance with the study protocol, compliance was assessed using a self-reported diary collected at each visit and graded according to the level of adherence (1=83-100%, 2= 66-82%, 3=25-65%, 4=25%, 5=none). A patient was considered compliant if their level of adherence was of grade 1 or 2 at all times. Since we did not measure the returned leftover agent, 25(OH)D measurements were considered as an additional compliance control.

Sampling of biological specimens

Blood collection

Morning fasting samples of whole etheylenediaminetetraacetic acid (EDTA)-treated blood and serum were collected at baseline and after 12 months following storage at -80°C until biomarker measurement.

Stool collection

Freshly voided stool samples were collected at both timepoints. The stool sample was collected in a tube, stored at -20°C and then transported to the laboratory in a plastic bag containing an ice pack. Upon arrival to the laboratory, each sample was immediately frozen at -80°C.

Circulating biomarkers

Serum 25(OH)D concentrations were determined by a commercially available chemiluminescent immune assay (Immunodiagnostic Systems, Pantec S.r.l., Turin, Italy). This method recognizes both metabolites of vitamin D (D_2-D_3).

Microbiome Analyses

For metagenomic analysis, genomic bacterial DNA was isolated from feces of patients using G'NOME isolation kit (MP Biomedicals) following a published protocol [28]. Whole metagenome shotgun sequencing [29] was applied on the DNA samples. Metagenomic libraries were generated with a Nextera XT DNA Sample Prep Kit (Illumina, San Diego, CA, USA) and sequencing was carried out on the HiSeq2500 platform (Illumina) at a targeted depth of 5.0 Gb (100-bp paired end reads).

DNA sequences were aligned to a curated database containing all representative genomes in RefSeq [30] for bacteria with additional manually curated strains. Alignments were made at 97% identity against all reference genomes. Every input sequence was compared to every reference sequence in the CoreBiome Venti database using fully gapped alignment with BURST [31]. Ties were broken by minimizing the overall number of unique Operational Taxonomic Units (OTUs). For taxonomy assignment, each input sequence was assigned the lowest common ancestor that was consistent across at least 80% of all reference sequences tied for best hit. The number of counts for each OTU was normalized to the OTU's genome length. OTUs accounting for less than one millionth of all species-level markers and those with less than 0.01% of their unique genome regions covered (and < 1% of the whole genome) were discarded. Samples with fewer than 10,000 sequences were also discarded. Count data was converted to relative abundance for each sample. The normalized and filtered table was used for all downstream analyses.

Pathway Analyses

To analyze the gut microbiome, we applied bioBakery tools [32] on whole shotgun metagenomic data of stool samples. To quantify the relative abundance of microbial species, we carried out Metagenomic Phylogenetic Analysis 3 (MetaPhlAn 3) pipeline [33] on raw reads. MetaPhlAn profiles the microbial community with 1.1 million microbial protein-coding gene markers (circa 50-400 marker genes for each bacterial species). The relative abundances of microbial pathways and functional potentials were computed utilizing the Human Microbiome Project Unified Metabolic Analysis Network 3 (HUMAnN 3[33]). HUMAnN provides the contribution of each species to the gene families and pathways.

Statistical methods

For a detailed description of the statistical methodology, see *Supplementary Statistical methods*.

Results

A total of 74 patients were enrolled, 36 in the placebo group and 38 in the vitamin D supplementation group (Fig. S1). Overall, 85% of the participants were compliant, and 77% took more than 83% of vitamin D/placebo. Clinical and demographic parameters by treatment arm are summarized in Table S1.

Levels of 25(OH)D significantly increased in the supplemented group, reaching a median concentration of 39.5 ng/ml (Interquartile Range (IQR): 33.7-44.6 ng/ml) at follow-up ($p < 0.001$). No significant change was observed in the placebo group ($p = 0.422$), although about 25% of the

Table 1

Distribution of 25(OH)D levels by timepoint and treatment arm in the overall population and in the analyzed sample.

	Baseline 25(OH)D	Post 25(OH)D	Change in 25(OH)D (Post-Baseline)	*p*-value*
All patients (N=74)				
Placebo, n=36				
Median [IQR]	24.0 [15.8, 26.3]	20.8 [14.5, 30.4]	0.750 [-2.85, 4.60]	0.422
Missing	2 (5.6%)	2 (5.6%)	2 (5.6%)	
Vitamin D supplementation, n=38				
Median [IQR]	20.5 [13.9, 26.3]	39.5 [33.7, 44.6]	19.4 [12.4, 25.8]	<0.001
Missing	6 (15.8%)	6 (15.8%)	6 (15.8%)	
Patients included in the analysis (N=60)				
Placebo, n=32				
Median [IQR]	24.2 [15.9, 26.5]	21.9 [14.6, 31.0]	0.750 [-2.98, 5.18]	0.432
Missing	0	0	0	
Vitamin D supplementation, n=28				
Median [IQR]	19.5 [13.8, 26.4]	40.4 [37.4, 46.6]	23.2 [18.2, 25.9]	<0.001
Missing	1 (3.6%)	1 (3.6%)	1 (3.6%)	

**p-values* derived from Wilcoxon signed-rank test for paired data that compared baseline 25(OH)D with post-treatment 25(OH)D within each group; 25(OH)D = 25-hydroxyvitamin D; IQR = Interquartile range. Interquartile range is reported as [First quartile (Q1) – Third quartile (Q3)]. 25(OH)D values were obtained from the post-enrollment evaluation on serum samples. For the enrolment, self-reported data were considered. Missing values regard post-enrollment evaluation.

patients reached vitamin D sufficiency by the end of the study (Third quartile (Q3): 30.4 ng/ml) (Table 1).

Vitamin D and microbiota

In *Supplementary Vitamin D and overall microbiome,* results on alpha diversity, beta diversity and overall microbiota composition by treatment arm are provided.

Because the primary endpoint of the study was to identify potential beneficial taxa that changed after one year of vitamin D supplementation, only the patients with available microbiota at both timepoints (n=65) were considered for analysis. Five drop-out patients in the supplementation group were further excluded. As a result, the final sample consisted of 60 individuals, 28 in the supplemented group and 32 in the placebo group. Except for two patients in the placebo group, all were compliant with the treatment.

As in the overall population, 25(OH)D levels significantly increased in patients who received vitamin D supplementation, reaching a median post-treatment concentration of 40.4 ng/ml (IQR: 37.4-46.6 ng/ml). No significant change was observed in the placebo group ($p = 0.432$), although about 25% reached vitamin D sufficiency by the end of the study (Q3: 31.0 ng/ml, Table 1).

Relative abundances of 980 taxa were available at both timepoints for each patient. They were clr-transformed after zero-value imputation. Out of the total 980 taxa, 75 were first selected because their normalized abundance at follow-up varied significantly between the two treatment groups. Twelve of these taxa were subsequently excluded because they were already significantly unbalanced between the groups at baseline, leaving 63 taxa for statistical analysis.

Principal component analysis (PCA) was performed on the clr-transformed abundances at follow-up of the 63 selected taxa. In Fig. S2a, the scaled PCA scores of the first two components, which together explained about 17% of the total variance, are plotted. As shown in the figure, the second component (PC2) significantly discriminated (Wilcoxon rank-sum test, $p < 0.001$) vitamin D-supplemented patients from those in the placebo group. Specifically, most of the supplemented patients fell within the component's negative axis (82% of the group), while the majority of patients in placebo (72%) had positive PC2 scores. These values, although not directly interpretable, identified two different microbiome-based clusters of PC2 that well discriminated between the two treatment groups. The biplot in Fig. S2b and the loadings barplot in Fig. S3 show the contribution of each of the 63 taxa on PC2: among the taxa that were correlated with the negative side of PC2, i.e., the one characterizing the supplemented patients, we found several from *Bacteroides* genus, *Faecalibacterium prausnitzii*, which is a well-known probiotic highly abundant in the gut microbiota of healthy adults, and *Holdemanella biformis*, which was found to have an anti-tumorigenic effect. In contrast, *Shigella boydii* and *Raoultella ornithinolytic*, as well as several species from *Streptococcus* and *Escherichia* genera, were the most correlated with the positive side of PC2, which mostly characterized the placebo group.

Vitamin D-supplemented patients had significantly higher abundances of *Leuconostoc pseudomesenteroides, Bacteroides gallinarum, Christensenella timonensis* and *Ruminococcus YE78* (Fig. 1a).

Comparing the patients with vitamin D sufficiency (25(OH)D >= 30 ng/ml, n=36) versus those deficient (n=23) at follow-up, we found that *Leuconostoc pseudomesenteroides* and *Ruminococcus YE78* were also significantly more abundant in vitamin D-sufficient patients, regardless of treatment arm, together with *Faecalibacterium prausnitzii* and *Bacteroides clarus* (Fig. 1b). Conversely, *Eubacterium brachy* and *Bacteroides coprocola* were significantly more prevalent in placebo-treated patients and in those not reaching vitamin D sufficiency at the end of the study (Fig. 1a-b).

Taxa-mediated effect of vitamin D supplementation on 25(OH)D levels

Since PCA analysis suggested differences in the 63 taxa between supplemented and non-supplemented patients, we performed a mediation analysis to see if these taxa also mediated the effect of the supplementation on post-treatment 25(OH)D levels. To do this, we employed the counterfactual approach to mediation analysis, assuming an interaction between vitamin D supplementation (exposure) and the selected taxa (mediator) on 25(OH)D levels at follow-up (outcome). Both Natural Direct Effect (NDE) and Natural Indirect Effect (NIE) of vitamin D supplementation on post-treatment 25(OH)D levels were hypothesized and graphically represented using a directed acyclic graph (DAG), with baseline 25(OH)D levels and sex as confounders (Fig. 2). Because PC2 was the component that best discriminated the supplemented from the non-supplemented, we used it as a proxy for the 63 taxa abundances. After performing the analysis, we found that vitamin D supplementation significantly and directly affected the final

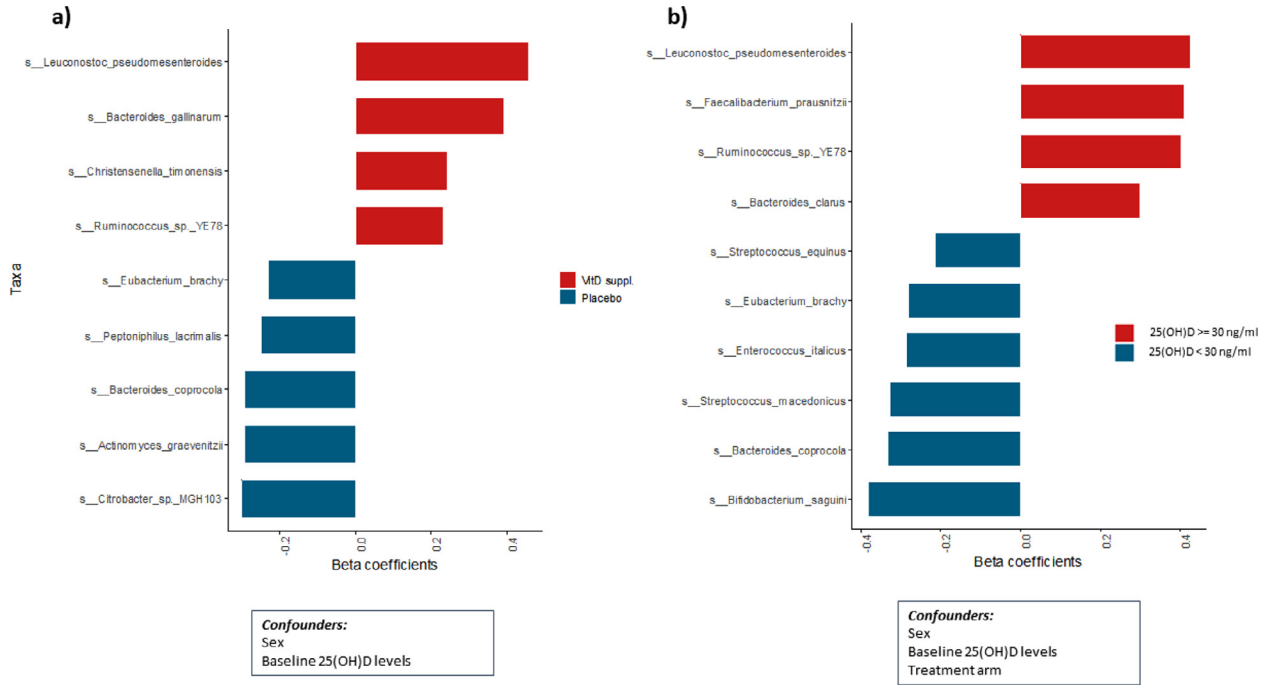

Fig. 1. Taxa significantly associated with **a.** treatment arm **b.** post-treatment vitamin D sufficiency (25(OH)D ng/ml). For each taxon, results are obtained from a multivariable logistic model including the post-treatment clr-transformed abundance of the taxon as covariate and adjusted for confounders. The bar length indicates the significant beta-coefficient of the taxon ($p < 0.05$). If positive, the taxon was significantly more abundant in patients **a.** supplemented with vitamin D **b.** reaching vitamin D sufficiency at the end of the treatment. If negative, the taxon was significantly more abundant in patients **a.** in the placebo group **b.** not reaching vitamin D sufficiency at the end of the treatment. 25(OH)D = 25-hydroxy vitamin D.

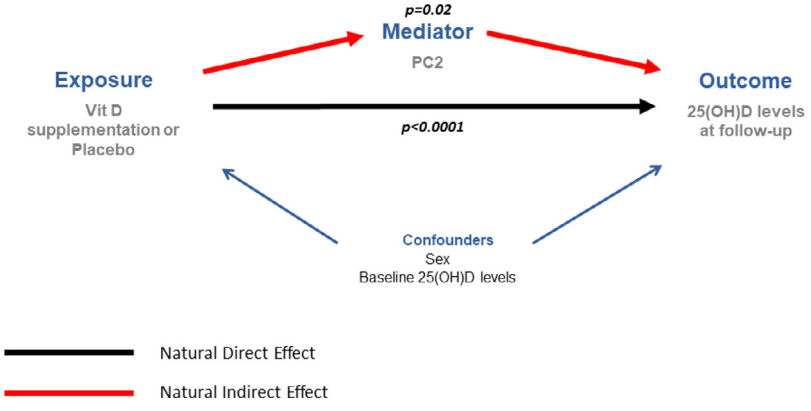

Fig. 2. Direct acyclic graph (DAG) of mediation model analyses. The 63 selected taxa (summarized with PC2) as mediator of the effect of vitamin D supplementation (exposure) on post-treatment 25(OH)D levels (outcome). In black, natural direct effect (NDE); in red, natural indirect effect (NDE); in blue, the effect of confounders on the exposure–outcome relationship. *p*-value obtained from mediation analysis. Significant direct effect of vitamin D supplementation on post-treatment 25(OH)D ($p < 0.0001$). The 63 taxa significantly mediate the effect of supplementation on post-treatment 25(OH)D ($p = 0.02$). 25(OH)D = 25-hydroxy vitamin D.

25(OH)D levels (NDE: $p < 0.0001$), but part of its overall effect was also significantly mediated by the 63 taxa (NIE: $p = 0.02$) (Fig. 2).

Fusobacterium nucleatum, vitamin D and disease progression

Fusobacterium nucleatum is a common bacterium in the oral cavity known to be significantly associated with CRC and oral diseases. Data on the bacterium prevalence at both timepoints is provided in Fig. S5, according to treatment arm and clinical event (median follow-up$p = 3.7$ years). Due to the short follow-up period, we considered a clinical event not only death and cancer relapse but also colorectal adenoma.

In univariate analysis, the Disease-Free Survival (DFS) of patients with *F. nucleatum* only at baseline was significantly worse ($p = 0.047$) (Fig. S6). However, after adjusting for baseline 25(OH)D and post vitamin D sufficiency (which, in this instance, was a proxy for the treatment effect, having also included those not treated or drop-outs), the association between *Fusobacterium nucleatum* at baseline and an increased risk of event was significant, regardless of the presence of the bacterium post-treatment (Hazard Ratio (HR) yes versus no: 3.19; 95% Confidence Interval (CI) 1.21-8.35; $p = 0.019$). Body Mass Index (BMI) at baseline was also significantly and inversely correlated with risk of recurrence (HR: 0.88; 95%CI: 0.78-0.99; $p = 0.033$) (Table 2). However, no significant association was found

Table 2

Cox multivariate results for disease-free survival.

Characteristic	HR[1]	95% CI[1]	*p*-value
F. nucleatum at baseline (yes vs no)	3.18	1.21, 8.35	0.019
Baseline BMI	0.88	0.78, 0.99	0.033
Vitamin D sufficiency at f.u. (yes vs no)	1.16	0.49, 2.76	0.70
Baseline 25(OH)D ng/ml	0.99	0.94, 1.05	0.80

[1] HR = Hazard Ratio; CI = Confidence Interval; *F. nucleatum* = *Fusobacterium nucleatum*; f.u. = follow-up: BMI = Body Mass Index; 25(OH)D = 25-hydroxy vitamin D.

between the post-treatment presence of the bacterium and tumor progression (data not shown). Overall, women were more likely to have *Fusobacterium nucleatum* at the end of the treatment (Odds Ratio (OR) female versus male: 5.55; 95%CI: 1.37-29.9; $p = 0.025$), regardless of whether they had it at baseline (Table S2). Post-treatment abundances in those with the bacterium were significantly and inversely correlated with age (beta: -0.14; 95%CI: -0.21; -0.08; $p = 0.001$), significantly higher in those carrying it from baseline (beta: 2.8; 95%CI: 1.3-4.2; $p = 0.003$) and borderline significantly lower in those reaching vitamin D sufficiency at the end of the treatment (beta: -1.3; 95%CI: -2.7-0.02; $p = 0.05$) (Table S3; Fig. S7a). In addition, an inverse correlation between *F. nucleatum* and post-treatment 25(OH)D levels was observed, with abundances decreasing as vitamin D levels increased (Fig. S7b).

Functional pathways and vitamin D

We considered community-level pathway abundances for microbial function. At both timepoints, the abundances of 1465 pathways were computed for each patient and normalized using the counts per million (CPM) technique. Normalized abundances were clr-transformed after zero-imputing. Only the pathways present in at least 10% of the analyzed patients (n=60) at the end of the treatment were considered (n=237 pathways). We initially selected 40 pathways with post-treatment CPM-abundances significantly associated with the treatment arm, although 8 were already significantly unbalanced between arms at baseline. Consequently, 32 pathways were eventually selected for investigation. One male patient in placebo was excluded from the statistical analysis because he had zero abundances for all the selected pathways.

Post-treatment abundances of the 32 pathways were investigated in relation to both vitamin D supplementation and vitamin D sufficiency. In multivariate analysis, we observed significantly increased *D-fructuronate degradation, superpathway of glycerol degradation to 1,3-propanediol, acetyl-CoA fermentation to butanoate II, superpathway of thiamin diphosphate biosynthesis II, guanosine nucleotides degradation II* in patients that were vitamin D supplemented, with *superpathway of glycerol degradation to 1,3-propanediol, superpathway of thiamin diphosphate biosynthesis II* and *guanosine nucleotides degradation II* significantly more abundant also in those with post-treatment 25(OH)D levels≥30 ng/ml. Conversely, *L-histidine biosynthesis* and *pyrimidine deoxyribonucleosides salvage* pathways were significantly more abundant in placebo patients, while the pathway of *L-ornithine de novo biosynthesis* was more prevalent in those with vitamin D deficiency at follow-up (Fig. 3a-b).

Vitamin D, microbiome and sex

Looking at the distribution of 25(OH)D levels at both timepoints, we found that women in the supplementation group had lower vitamin D levels at baseline than men (p_{base}=0.04). However, the supplementation restored this gap, and by the end of the study both post-treatment levels and the change in 25(OH)D levels from baseline were comparable between men and women (p_{post}=0.70; p_{change}=0.95) (Fig. S8d-f). Conversely, no significant difference in 25(OH)D levels at baseline was found between women and men in the placebo group (p_{base}=0.15), although vitamin D levels increased significantly more in men throughout the year of treatment (p_{change}=0.03) (Fig. S8a-c). Because these findings suggested an interaction between sex and vitamin D, all the multivariate models in this study were also adjusted for sex.

We also looked at a potential interaction between sex and the treatment-associated taxa on post 25(OH)D levels, using PC2 scores as a proxy for taxa abundances. The predicted regression lines stratified by sex are shown in Fig. S9. We found a statistically significant interaction between sex and PC2 on 25(OH)D levels ($p < 0.001$), suggesting that men and women had a different taxa composition at follow-up and that this difference also affected the final 25(OH)D levels (Fig. S9).

As for taxonomic data, PCA was computed on the selected 32 clr-tranformed pathways at follow-up. The first two components (Fig. S10a-b), which explained about 30% of the total variance, did not differ between treatment groups. However, a difference between men and women could be detected in the first component (PC1), where the majority of men were distributed alongside the negative axis of PC1 (59% of men), whereas most of the women (74%) were in the positive side. This difference was further investigated in multivariate analysis, where an interaction between vitamin D supplementation and sex was introduced. Results from the model showed that, while the abundances of the selected pathways summarized by PC1 was comparable between men and women in the placebo group, a significant difference was present between men and women after the supplementation ($p = 0.006$, Fig. S11).

The contribution of each pathway on PC1 was summarized in Fig. S12 with their corresponding loading. Because women had mostly positive PC1 scores, a pathway with a positive loading was expected to be more abundant in women. Conversely, a pathway with a negative loading was expected to be more prevalent in men. *Superpathway of L-lysine, L-threonine and L-methionine biosynthesis II* and *L-histidine biosynthesis* were the pathways with the two largest positive loadings. Both pathways involve the biosynthesis of essential amino acids. Multivariate regression analysis confirmed that *Superpathway of L-lysine, L-threonine and L-methionine biosynthesis II* was significantly more abundant in supplemented women compared to supplemented men ($p = 0.002$, Fig. S13) while *L-histidine biosynthesis* was significantly less abundant in supplemented men than supplemented women ($p = 0.002$, Fig. S14). However, both pathways looked comparable among non-supplemented men and women.

Looking at the opposite side of the loadings barplot, *superpathway of thiamin diphosphate biosynthesis II* and *6-hydroxymethyl-dihydropterin diphosphate biosynthesis I* were the pathways with the largest negative loadings, so with the highest inverse contribution on PC1. In multivariate analysis, *6-hydroxymethyl-dihydropterin diphosphate biosynthesis I* was borderline significantly associated to treatment ($p = 0.051$), with an indication of decreasing levels in the supplementation group, but no significant association with sex ($p = 0.14$) or interaction between vitamin D supplementation and sex was observed ($p = 0.09$). *Superpathway of thiamin diphosphate biosynthesis II*, on the other hand, was also not significantly different by

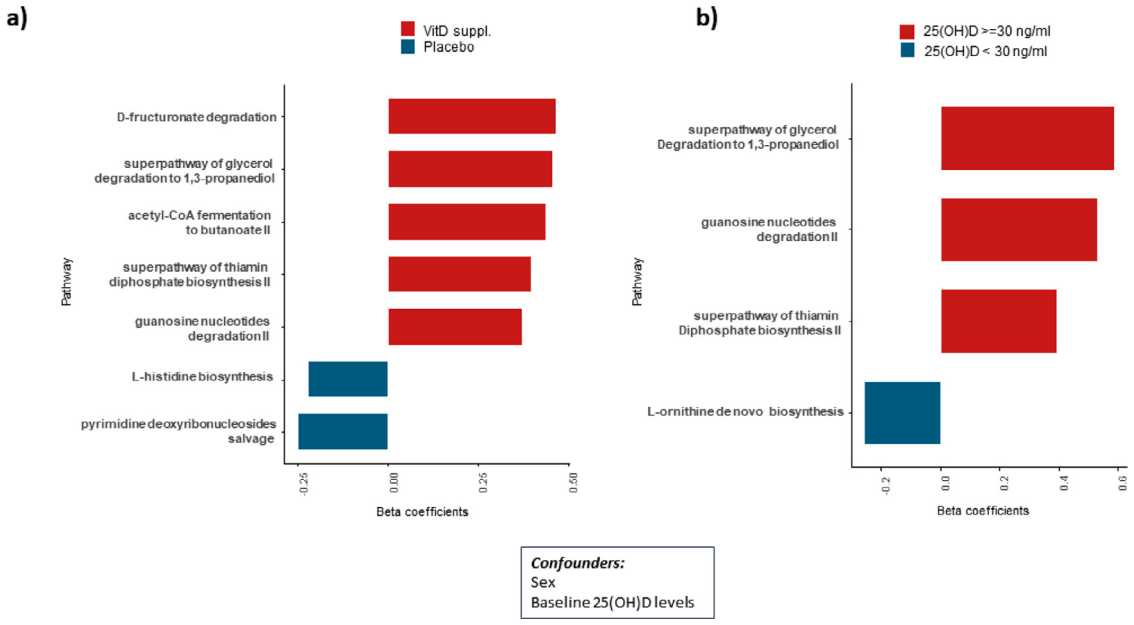

Fig. 3. Pathways significantly associated with **a.** treatment arm **b.** post-treatment vitamin D sufficiency (25(OH)D ng/ml). For each pathway, results are obtained from a multivariable logistic model including the post-treatment clr-transformed abundance of the pathway as covariate and adjusted for confounders. The bar length indicates the significant beta-coefficient of the pathway ($p < 0.05$). If positive, the pathway was significantly more abundant in patients **a.** supplemented with vitamin D **b.** reaching vitamin D sufficiency at the end of the treatment. If negative, the pathway was significantly more abundant in patients **a.** in the placebo group **b.** not reaching vitamin D sufficiency at the end of the treatment. 25(OH)D = 25-hydroxy vitamin D.

sex, although, overall, it was significantly more abundant in supplemented patients ($p = 0.001$, Fig. S15).

Discussion

In recent years, several studies have reported an association between gut microbiota and both vitamin D supplementation and 25(OH)D, suggesting that vitamin D and its VDR may shape the microbiota [34]. Vitamin D and gut microbiota are also known to be associated with CRC risk, [3,5,10] so the investigation of their interplay has become more and more appealing. However, it is often difficult to identify reproducible results in microbiome studies due to their methodological limitations, which typically include a small sample size and an observational study design that, unlike RCTs, tends to provide estimates affected by confounding bias and problems of reverse causality.

We designed a RCT involving vitamin D supplementation for CRC survivors to investigate if and how the supplementation modulates the species known to be beneficial for human health. For vitamin D supplementation, a daily dose regimen was adopted. Unlike large-bolus dosing, daily dosing was found to be significantly associated with reduced total cancer mortality and with reduced cancer incidence in normal-weight individuals in a recent meta-analysis of RTCs [35]. Overall, we found that several species at the end of the treatment were different between supplemented and non-supplemented patients. Among the taxa that most contributed to defining the microbiota-based cluster of supplemented patients, we found several from *Bacteroides* genus, like *Bacteroides clarus* and *Bacteroides gallinarum. Bacteroides* seem to play an important role in modulating the human immune system by metabolizing polysaccharides and oligosaccharides [36]. Moreover, these results are consistent with those collected in a recent review, which identified *Bacteroidetes* as one of the most frequently increasing phyla following vitamin D supplementation [23]. Another taxon positively correlated with the cluster was *Holdemanella biformis*, which was shown to have an anti-tumorigenic effect by producing fatty acids that control tumor cell proliferation [6]. *Faecalibacterium prausnitzii* also positively correlated with the microbiota of the supplemented and was significantly more abundant in vitamin D-sufficient patients. *F. prausnitzii* is highly abundant in the human gut and one of the major gut's butyrate producer, with well-known anti-inflammatory properties, [37] especially in IBD, Crohn's disease, and ulcerative colitis [38]. However, recent findings also suggest a potential protective effect of the bacterium on CRC initiation and progression [39].

Results from our mediation analysis suggested that vitamin D supplementation modulated a subgroup of taxa, and that this modulation significantly affected the final 25(OH)D levels. These findings are consistent with those from our previous case-control study, where we observed that a high consumption of fatty fish – so a vitamin D-rich diet – significantly increased the levels of *Bifidobacteria/Escheria* ratio (an indicator of "good" intestinal health), thus reducing the risk of CRC [27].

Differences related to vitamin D were also assessed in functional analysis, with *superpathway of glycerol degradation to 1,3-propanediol, superpathway of thiamin diphosphate biosynthesis II* (with thiamin diphosphate also known as vitamin B1) and pathway of *guanosine nucleotides degradation II* significantly more abundant both in supplemented patients and in those reaching vitamin D sufficiency by the end of the treatment. The expected taxonomic ranges [40] of *superpathway of glycerol degradation to 1,3-propanediol* and *acetyl-CoA fermentation to butanoate II*, both significantly increased in the supplementation group, were *Firmicutes* and *Proteobacteria*, and *Firmicutes*, respectively. Both phyla were frequently reported as increasing following vitamin D supplementation [23].

In the era of precision medicine, sex and gender must be taken into account. Regarding vitamin D metabolism, it has been shown that women have lower vitamin D absorption compared to men. Moreover, a sex-specific fatty acid metabolism was observed after vitamin D supplementation [41]. Gender may also affect the microbiome, specifically through sex hormones exposure. A higher *Firmicutes/Bacteroidetes* ratio was found in pre-menopausal women compared to post-menopausal women, with men similar to post-menopausal women [42]. Also, our results showed gender differences with an inverse relationship between men and women in both vitamin D levels and microbiome. We also found significant differences in pathway abundances

between men and women. In recent years, emerging evidence has shown that both biological sex and gender significantly affect gut microbiota for reasons that seem related not only to sex hormones but also to host metabolism, gut-brain communication, diet and environmental factors [43–47]. In our study, we found a significant interaction between gut microbiota and gender on post-treatment 25(OH)D. Moreover, the abundances of pathways related to the biosynthesis of essential amino acids were significantly different between men and women, but only if supplemented. Sex-specific associations in short-chain acylcarnitines and branched-chain amino acid metabolites were also observed in a metabolomics cohort study of critically-ill patients supplemented with high doses of oral vitamin D_3 [41]. In addition, a mouse study investigating the relationship between dietary vitamin B6 supplementation and colon luminal environment identified significant differences by sex on colonic free amino acids such as *threonine, ornithine, aspargine/aspartate ratio and glutamine/glutamate* ratio [48].

Fusobacterium nucleatum is a proinflammatory [49] bacterium of the oral cavity that is highly abundant in CRC patients [50]. However, it is still unclear whether this relationship is just an association or implies a causal involvement of the bacterium in CRC prognosis and progression. In our study, *Fusobacterium nucleatum* was present in 14 patients at baseline and in 12 patients post-treatment. Women were more likely to have the bacterium at the end of the treatment. Looking at preliminary data on clinical events, we found that only patients with *Fusobacterium nucleatum* at baseline had worse DFS, whereas no association between the bacterium after the treatment and events was observed. This result could indicate that the bacterium is only an indicator of the patient's health status rather than a promoter of tumor carcinogenesis. Moreover, post-treatment abundances of *Fusobacterium nucleatum* were lower in those reaching vitamin D sufficiency, probably confirming the anti-inflammatory effect of vitamin D on tumorigenesis [51]. However, due to the still short follow-up and the resulting small number of advanced tumour recurrences observed to date, we carried out the DFS analysis considering as a clinical event not only cancer recurrence or death, but also colorectal adenomas, which are mostly benign types of tumours. Therefore, a longer follow-up with more events of cancer relapses is necessary to establish a causal link between *Fusobacterium nucleatum* and advanced tumours.

The main limitation of our study is the relatively small sample size compared to the high number of variables. To address this problem, we employed a step of variable selection before running the analysis. While this approach considerably reduced data dimensionality and noisy information, it also made it easier to detect significant results, as only the microbiota-related variables significantly associated with the treatment arm were included in the analysis. Moreover, without a validation set, we could not assess the reproducibility of the results. However, the randomization procedure and the multivariate statistical approaches allowing for confounders guaranteed a certain degree of estimates reliability. Another limitation is the formulation of the agent, as, at least in Italy, at the beginning of the trial pure vitamin D formulation was available only in an oily solution. Unlike tablets, drops at each intake are not easily counted, and this resulted in few patients finishing the agent supply before the last visit. Nevertheless, we still considered these participants to be compliant because of the depot effect of vitamin D accumulating in adipose tissue. Moreover, the essay we used to quantify 25(OH)D levels could not distinguish between vitamin D_2 and vitamin D_3. Because of this, we could not further investigate the factors influencing the increase in 25(OH)D levels in the quarter of placebo-treated patients who reached vitamin D sufficiency by the end of the study. We assume that this is partly attributable to modifications in diet and, to a bigger extent, to lifestyle changes that resulted in increased sunlight exposure (such as the resumption of physical and work activities).

Overall, these results suggest that vitamin D supplementation affects several taxa in gut microbiota and that these taxa also significantly mediate the effect of the supplementation on 25(OH)D levels. We also showed differences between men and women in response to vitamin D supplementation that affected both microbiota and pathways, especially those involved in the biosynthesis of essential amino acids. We also confirmed the association between *Fusobacterium nucleatum* and CRC, although we found no evidence of causality. In the future, we plan to integrate these data with information on diet, serum biomarkers related to inflammation and adipose tissue (like adiponectin, leptin, C-reactive protein, etc.), and gene expression data related to the immune system and evaluated in the tumour tissue, to investigate how the interplay between all these risk factors affects both microbiota and tumour progression.

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Declaration of Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Federica Bellerba: Investigation, Methodology, Visualization, Formal analysis, Data curation, Writing – original draft. **Davide Serrano:** Conceptualization, Investigation, Funding acquisition, Supervision, Writing – original draft. **Johansson Harriet:** Data curation. **Chiara Pozzi:** Data curation, Formal analysis, Investigation, Writing – review & editing. **Nicola Segata:** Methodology, Supervision, Writing – review & editing. **Amir NabiNejad:** Data curation, Software. **Elisa Piperni:** Data curation, Software. **Patrizia Gnagnarella:** Data curation. **Debora Macis:** Data curation. **Valentina Aristarco:** Data curation. **Chiara A. Accornero:** Data curation. **Paolo Manghi:** Data curation, Software. **Aliana Guerrieri Gonzaga:** Data curation. **Roberto Biffi:** Data curation. **Luca Bottiglieri:** Data curation. **Cristina Trovato:** Data curation. **Maria Giulia Zampino:** Data curation. **Federica Corso:** Data curation. **Rino Bellocco:** Methodology, Writing – review & editing. **Sara Raimondi:** Investigation, Visualization, Data curation, Writing – review & editing. **Maria Rescigno:** Data curation, Formal analysis, Investigation. **Sara Gandini:** Conceptualization, Investigation, Funding acquisition, Methodology, Data curation, Visualization, Supervision, Writing – original draft.

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Federica Bellerba, Amir Nabinejad and Elisa Piperni are PhD students at the European School of Molecular Medicine (SEMM), Milan, Italy.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.neo.2022.100842.

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