Research Article

Effect of vitamin D on relapse-free survival in a subgroup of patients with p53 protein-positive digestive tract cancer: A post hoc analysis of the AMATERASU trial

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Disclosure of Potential Conflict of Interest

The authors declare no potential conflicts of interest.

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ABSTRACT

Background: The AMATERASU randomized trial of vitamin D3 supplementation (2000 IU/day) (UMIN000001977) showed the potential benefit of vitamin D in a subgroup of patients with digestive tract cancer. By conducting post hoc analyses of this trial, whether subgroups stratified by expression levels of p53, vitamin D receptor (VDR), and Ki-67 modify the effect of vitamin D supplementation was further explored.

Methods: The primary outcome was relapse-free survival (RFS). On immunohistochemistry using pathological specimens, the degree of p53 protein expression parallel with p53-missense mutations was classified as p53-positive (>10%) and p53-negative (≤10%). In addition, VDR and Ki-67 expression levels were divided into quartiles.

Results: The p53 status of 372 patients' pathological specimens was evaluated. In a subgroup of patients with p53-positive cancer (n=226), 5-year RFS was 79% in the vitamin D group, which was significantly higher than the 57% in the placebo group (hazard ratio, 0.52; 95% confidence interval, 0.31-0.88; P=0.02). In the subgroup of patients with p53-negative cancer, 5-year RFS in the vitamin D group vs. placebo group was 72% vs. 84% (not significantly different). Effect modification by p53-positivity was significant (P=0.02 for interaction). However, no significant effect modification by either VDR or Ki-67 was observed.

Conclusions: These results generate a hypothesis that vitamin D supplementation may improve RFS in patients with p53-positive digestive tract cancer.

Impact: The results are still preliminary, but potentially important, because p53 is

59 the most frequently mutated gene across cancers at all sites.

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61 **Registration**

62 Trial registry name: University Hospital Medical Information Network Clinical

63 Trials Registry (UMIN-CTR)

64 Registration identification number: UMIN000001977

65 Receipt No. R000002412

66 URL for the registry:

67 https://upload.umin.ac.jp/cgi-open-bin/ctr_e/ctr_view.cgi?recptno=R000002412

68 Date of disclosure of the study information: 2009/06/01

INTRODUCTION

Meta-analyses of randomized, clinical trials (RCTs) showed that vitamin D supplementation given pre-diagnostically reduced cancer mortality (1,2). On the other hand, recent RCTs, the SUNSHINE trial among patients with metastatic colorectal cancer (3), the AMATERASU trial involving patients with digestive tract cancers (4) and another trial involving patients with non-small cell lung cancer (5), did not show a significant difference in progression or relapse-free survival (RFS) between vitamin D and placebo in the primary results. However, these results of RCTs are not necessarily considered null. For example, the SUNSHINE trial (3) indeed showed a beneficial association with adjustment, and the AMATERASU (4) and the other trial (5) suggested that vitamin D was effective in certain subgroups. Thus, confirmatory RCTs are needed to evaluate these preliminary findings (6). Towards the next RCT, the target population showing a good response to vitamin D supplementation should be carefully examined.

Because beneficial effects of vitamin D have been reported in a variety of cancer sites, target molecules of vitamin D can be relatively common across cancers at all primary sites, e.g., p53 tumor suppressor, vitamin D receptor (VDR), and Ki-67. The p53 gene is the most frequently mutated gene, in about half of cancers, the majority of which are missense (7,8). The missense mutations of p53 are detectable by p53 nuclear accumulation on immunohistochemistry (IHC) with high sensitivity (9). The product of mutated p53 leads not only to loss of oncosuppressor function, but also to gain of oncogenic function (10), signaling of which may have cross-talk with vitamin D signaling (11,12). VDR was also widely

expressed in most cell types. Importantly, high VDR expression is significantly associated with improved survival of patients with prostate cancer (13) and non-small cell lung cancer (14). We thus hypothesized that vitamin D supplementation may further improve survival in patients with high VDR expression. Ki-67, a marker of cancer cell proliferation, is widely used in routine pathological investigations (15). Moreover, antiproliferative effects were suggested as a possible mechanism in the anticancer effects of vitamin D (16). Therefore, effect modification by pathological biomarkers, i.e., p53-positivity and quartiles of VDR or Ki67, was explored by conducting a post hoc analysis of the AMATERASU randomized trial of vitamin D3 supplementation (UMIN000001977).

PATIENTS AND METHODS

Trial design

This study was a post hoc analysis of the AMATERASU trial conducted in Japan, the details of which were previously reported (4). Briefly, 417 patients with digestive tract cancers from the esophagus to the rectum participated in a randomized, double-blind, clinical trial to compare the effects of vitamin D3 supplements (2,000 IU/day) and placebo on RFS as a primary outcome and overall survival (OS) as a secondary outcome at an allocation ratio of 3:2 at the International University of Health and Welfare Hospital in Japan between January 2010 and February 2018. The trial protocol was approved by the ethics committee of the International University of Health and Welfare Hospital, as well as the Jikei University School of Medicine. Written, informed consent was obtained from each

participating patient before surgery.

Participants

Details of the inclusion and exclusion criteria were described in the original report (4). Briefly, the trial included patients not taking vitamin D supplements, with stage I to III digestive tract cancers (48% colorectal, 42% gastric, and 10% esophageal) who underwent curative surgery with complete tumor resection.

Tissue microarrays

Tissue microarrays (TMAs) were constructed at the Department of Pathology, International University of Health and Welfare. First, hematoxylin and eosin (H&E)-stained slides were reviewed by a pathologist (S.O.) and an investigator (T.A.) to determine the evaluation site. This procedure was performed using the following criteria: the deepest tumor invasion site; the site where recurrence is expected, such as the resection margin or serosal exposure; and to avoid necrosis. Usually, one evaluation site was selected per patient, but two sites were selected if different histological subtypes were included in the same patient's H&E slides. A TMA set (Labro Co., Ltd., Seoul, Korea) was used for punching the core, enclosing it in a cassette in a block, and recreating a paraffin-embedded block. Five-millimeter tissue cores each from the tumor were contained in each TMA block that had a total of 20 cores.

Vitamin D receptor, p53, and Ki-67 immunohistochemistry

IHC for p53, VDR, and Ki-67 was performed with the Histofine Histostainer 36A

(Nichirei Biosciences Inc., Tokyo, Japan). IHC with primary antibodies against p53 (DO-7) and Ki-67 (SP-6) (both from Nichirei Biosciences Inc.) was performed according to the manufacturer's protocols. For VDR IHC, antigen retrieval was performed by de-paraffin antigen retrieval solution pH 9.0 (Nichirei Bioscience Inc.) in a heat bath at 100 °C for 20 min. Tissue sections were incubated with 3% H_2O_2 (5 min) to block endogenous peroxidase. Primary antibody against VDR (D2K6W) Rabbit mAb #12550 (Cell Signaling Technology Japan, K.K, Tokyo, Japan) (dilution 1:200) was applied for 30 min at room temperature. The sections were then incubated with the Histo-fine Simple stain MAX-PO (Multi) (Nichirei Bioscience Inc.) for 30 min at room temperature according to the manufacturer's protocol. Sections were visualized by diaminobenzidine (10 min) and hematoxylin counterstain.

Detection of p53 protein nuclear accumulation by IHC

The degree of p53 expression based on immunoreactive nuclear cells in the cancer glandular component was evaluated by T.A., who was blinded to randomized group and outcomes. A cutoff point at 10% was applied because it has been shown to be 84-100% sensitive for the detection of p53 missense mutations and 86-97% specific for the absence of p53 missense mutations (9). Moreover, this 10% was found to be the most commonly chosen cutoff point in a meta-analysis of 36 studies on p53 IHC for p53 gene mutations (17). Thus, the study population was divided into two subgroups: >10% as "p53-positive" containing mainly missense mutations and ≤10% as "p53-negative" containing a mixture of mainly non-missense mutations and wild-type p53.

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166 **Scoring of vitamin D receptor expression by IHC**

167 For this study, the nuclear VDR expression level was measured in the glandular
168 cancer components. The level of nuclear VDR expression in the tumor tissue was
169 assessed using a semiquantitative immunoreactivity scoring (SIS) system (18).
170 The staining intensity was scored as 1 (no immunostaining), 2 (weak), 3
171 (moderate), to 4 (strong). When multiple intensity scores were mixed in the field
172 of view, the higher values were used (≥20%). The percentage of immunoreactive
173 nuclear cells was rated from 0% to 100%. The SIS scores were calculated by
174 multiplying the intensity scores with the percentage of positive cell nuclei,
175 resulting in score variation from 0 to 400. Two pathologists (S.O. and S.H.), who
176 were totally blinded to patients' information, independently assessed the SIS
177 scores. The correlation coefficient of the SIS scores was 0.83 (Spearman's
178 correlation coefficient test). The VDR score was the average of the two SIS
179 scores.

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181 **Scoring of Ki-67 expression by IHC**

182 The Ki-67 score ranged from 0-100 as a labeling index based on the percentage
183 of positive nuclear cells (19).

184

185 **Statistical analysis**

186 All patients who underwent randomization and for whom IHC evaluation was
187 available were included in this analysis. Relapse and death-related outcomes
188 were assessed according to the randomization group by whether or not

supplements were taken. The effects of vitamin D and placebo on risk of relapse or death and total death were estimated using Nelson-Aalen cumulative hazard curves for outcomes. A Cox proportional hazards model was used to determine hazard ratios (HRs) and 95% confidence intervals (CIs). To clarify whether vitamin D supplementation significantly affected these subgroups, the P for interaction was analyzed based on a Cox regression model including three variables: e.g., 1) vitamin D group; 2) the subgroup of patients with p53-positive cancer; and 3) both multiplied together as an interaction variable, by 2-way interaction tests comparing the p53-positive group and the p53-negative group. To evaluate the effects of vitamin D supplementation on relapse, cumulative incidence functions were applied by considering patient deaths due to causes other than relapse as a competing risk, and competing risk regression was performed using the subdistribution hazard ratio (SHR) and 95%CI (20). Values with two-sided $P<0.05$ were considered significant. All data were analyzed using Stata 14.0 (StataCorp LP, College Station, TX, USA).

RESULTS

Study population

Of the 417 patients with digestive tract cancers who were randomly assigned to receive vitamin D supplements (n=251: 60%) or placebo (n=166: 40%), IHC results for p53, VDR, and Ki-67 were available for 219 (87%) of the vitamin D group and 153 (92%) of the placebo group, for a total of 372 (89%), due to lack of tissue samples, no cancer tissue availability, a special tissue subtype such as

neuroendocrine tumor, or inappropriate samples during the tissue microarray process (Figure 1). Tissue samples were more frequently unobtainable in the vitamin D group than in the placebo group; this difference was considered to be caused by chance.

Expression of p53 protein

Images of typical p53 expression are shown in Figure 2. Positivity in cancerous regions ranged from 0 to almost 100%.

Patients' characteristics

A total of 372 patients were divided into the p53-positive group (n=226, 61%) and the p53-negative group (n=146, 39%). The participants' characteristics of these two subgroups are shown in Table 1. Expression levels of VDR and Ki-67 were high in patients in the p53-positive group. Patients with colorectal cancer were dominant in the p53-positive group, whereas patients with gastric cancer were dominant in the p53-negative group. Regarding pathological subtypes, well-differentiated adenocarcinoma was dominant in the p53-positive group, whereas moderately differentiated adenocarcinoma, poorly differentiated adenocarcinoma, and signet ring cell carcinoma were dominant in the p53-negative group. Other characteristics including intervention group, 25(OH)D levels, age, body mass index, and comorbid conditions were not different between the p53-positive and p53-negative groups.

Effect modification of vitamin D on RFS by p53-positive status

In the p53-positive group, relapse or death occurred in 26 (19%) vitamin D group patients and 30 (34%) placebo group patients; the 5-year RFS was significantly higher in the vitamin D group (79%) than in the placebo group (57%) (HR, 0.52; 95%CI, 0.31 to 0.88; P=0.02) (Figure 3A). In the p53-negative group, relapse or death occurred in 18 (23%) vitamin D and 10 (15%) placebo group patients; the 5-year RFS was 72% in the vitamin D group and 84% in the placebo group; the difference was not significant (Figure 3B). There was a significant 2-way interaction between vitamin D supplementation and the subgroup of patients with p53-positive cancers (P=0.02 for interaction), which remained significant even after adjusting with VDR expression levels, Ki-67 expression levels, the site of cancer, and pathological subtypes. Under methods for competing-risk analysis, the cumulative incidence of relapse was significantly lower in the vitamin D group than in the placebo group (SHR, 0.54; 95%CI, 0.30-0.98; P = 0.04) among those in the p53-positive group, but was not significant among those in the p53-negative group (SHR, 1.43; 95%CI, 0.63-3.27; P = 0.40).

Effect modification of vitamin D on OS by p53-positive status

In the p53-positive group, death occurred in 19 (14%) vitamin D and 17 (20%) placebo group patients; the 5-year OS was 82% in the vitamin D group and 72% in the placebo group, and the difference was not significant (Figure 3C). In the p53-negative group, death occurred in 14 (18%) vitamin D and 5 (8%) placebo group patients; the 5-year RFS was 77% in the vitamin D group and 86% in the placebo group, and the difference was not significant (Figure 3D). There was a significant 2-way interaction between vitamin D supplementation and the

subgroup of patients with p53-positive cancers (P=0.03 for interaction), which remained significant even after adjusting with VDR expression levels, Ki-67 expression levels, the site of cancer, and pathological subtypes.

Effect modification of p53-positive cancers stratified by cancer sites

The subgroup of patients with p53-positive cancers was further divided by cancer sites (Figure 4). Relapse or death occurred in 13 (17%) vitamin D group patients and 14 (28%) placebo group patients with colorectal cancer (Figure 4A), in 7 (15%) vitamin D group patients and 10 (36%) placebo group patients with gastric cancer (Figure 4B), and in 6 (43%) vitamin D group patients and 6 (67%) placebo group patients with esophageal cancer (Figure 4C). Thus, relapse and death were less in the vitamin D group than in the placebo group independent of cancer sites. However, only in patients with gastric cancer, the risk for relapse or death was significantly lower in the vitamin D group than in the placebo group: HR, 0.36; 95%CI, 0.14 to 0.95; P=0.04; it was not significant in patients with esophageal cancer and colorectal cancer.

Effect modification by VDR expression levels

VDR expression levels ranged between 15 and 400. These were divided into quartiles: quartile one (Q1) (≤100 mg/dL), quartile two (Q2) (101 to 180), quartile three (Q3) (181 to 272.5), and quartile four (Q4) (>272.5). Effect modification of vitamin D supplementation on RFS was explored in patients stratified by these quartiles of VDR expression levels (Figure 5). Vitamin D significantly reduced the risk for relapse or death in Q4, i.e., the highest level of VDR expression (HR, 0.30;

95%CI, 0.09 to 0.96; P=0.04). In contrast, significant effects of vitamin D supplementation on the risk of relapse or death were not observed in Q1, Q2, and Q3. There was no significant 2-way interaction between vitamin D supplementation and the Q4 subgroup (P=0.08 for interaction).

Effect modification by Ki-67 levels

Ki-67 expression levels ranged between 3 and 90. These were divided into quartiles: quartile one (Q1) (≤40), quartile two (Q2) (41 to 50), quartile three (Q3) (51 to 60), and quartile four (Q4) (>60). Effect modification by these quartiles of Ki-67 levels was explored (Supplementary Fig. S1). Vitamin D did not significantly reduce the risk for relapse or death in any of Q1 to Q4 of the Ki-67 levels.

DISCUSSION

In this post hoc analysis of the AMATERASU trial, daily supplementation with 2,000 IU of vitamin D significantly improved RFS in the subgroup of patients with p53-positive cancers. In the same subgroup, the 5-year OS was 10% higher in the vitamin D group than in the placebo group, although the difference was not significant. On the other hand, significant differences in both RFS and OS were not observed in the subgroup of patients with p53-negative cancers. There was significant effect modification by p53-positivity, both in RFS and OS. A negative feedback loop exists between p53 protein and mouse double minute 2 (MDM2): when cells are stressed, e.g., DNA damage, nuclear levels of p53 protein rise, stopping the cell cycle to repair damaged DNA or induce apoptosis to prevent

cancer development; alternatively p53 protein induces MDM2 protein to degrade p53 protein through the mechanism of posttranscriptional ubiquitination (7). The p53 is normally almost undetectable on IHC, but once the p53 gene has missense mutations within the DNA binding site, and the p53 protein has conformational changes, by which its ability to bind to the promotor region of the MDM2 gene is lost (21), it thus does not increase MDM2 protein, which allows the nuclear accumulation of p53 protein which may be effective as an oncogene (10). Vitamin D has been demonstrated to induce an 11-fold increase in MDM2 mRNA expression *in vitro* (22), reasoning that vitamin D supplementation may upregulate MDM2 protein independent of p53 missense mutation, decrease p53 levels accumulated in the nucleus, and improve patients' RFS. In contrast, vitamin D seemed to be harmful in the p53-negative group both in terms of RFS and OS, although the differences were not significant. Vitamin D is being extensively explored as a cancer-preventive and cancer-therapeutic agent (23), but a certain mutational status of p53 was suggested to convert vitamin D into an anti-apoptotic agent (24). Thus, for future RCTs, it may be better to exclude patients with p53-negative cancers and include patients with p53-positive cancers.

Vitamin D supplementation was associated with a reduced risk of relapse or death in patients with gastric cancer, but not in patients with either esophageal cancer or colorectal cancer. In patients with gastric cancer, p53-negative cases were more frequent than p53-positive cases. This positive effect of vitamin D may be due to chance or due to effect modification by another subgroup of patients, such as poorly differentiated adenocarcinoma and signet ring cell carcinoma,

rather than being related to p53-positive status. Indeed, activated vitamin D was demonstrated *in vitro* to inhibit gastric cancer cell growth through VDR and mutated p53 (24). However, P for interaction by cancer sites was not significant as observed in patients with p53-positive cancers, suggesting that vitamin D supplementation may be effective in p53-positive tumors across cancers at all sites rather than cancers at specific sites such as gastric cancer.

Vitamin D significantly reduced the risk for relapse or death in the subgroup of patients with the highest quartile of VDR expression without significant P for interaction. As shown in Table 1, VDR expression levels were higher in patients with p53-positive than p53-negative cancers. Mutations of p53 were demonstrated to increase the nuclear accumulation of VDR *in vitro* (23). Thus, this VDR result may be a reflection of the p53-positive subgroup. In patients with p53-positive cancers, well-differentiated adenocarcinoma was dominant, also shown in Table 1. VDR expression levels were shown to increase in well-differentiated adenocarcinoma of the colon (25), which may again be a reflection of the p53-positive subgroup. On the other hand, Ki-67 expression levels did not modify the effect of vitamin D supplementation, although Ki-67 expression levels were high in the p53-positive group.

There are several limitations to this study. First, this study performed an exploratory analysis that was not pre-specified in the original protocol of the AMATERASU trial. Thus, the findings must be considered as exploratory and interpreted with caution. Second, the sample size was not calculated for the subgroup analyses. Patients were divided into p53-positive and p53-negative groups. For VDR and Ki-67 expressions, patients were divided into quartiles.

Patients were also divided into three cancer sites. Thus, these results may contain type II error due to the small sample size of each subgroup. Third, subgroup analyses of three biomarkers may increase the probability of type I error due to multiple comparisons. Fourth, the number analyzed was less than in the original study, because 11% of the pathological samples were not obtained, although the patients' characteristics of this post hoc study were not largely different from the original trial. Fifth, the cutoff value of p53 expression was set to 10% in this study, in reference to previous reports (9,17), but was not validated by sequencing using tumor DNA in this study population. Sixth, due to the nature of the TMA method, only a small part of each tumor sample was evaluated despite the heterogeneity of the cancer region. Moreover, each positive IHC evaluation sometimes differed by the depth or histological subtypes, even within the same patient. This may cause misclassification of p53 positivity and other biomarkers. Seventh, since the AMATERASU trial was conducted in Japan, the patients were Asian, most esophageal cancers were squamous cell carcinomas, the incidence of gastric cancer was still relatively high, and the bioavailable 25-hydroxyvitamin D could be different from that in other population groups (26). Thus, the results of this study are not necessarily generalizable to other populations.

In conclusion, these results generate the hypothesis that vitamin D supplementation may improve RFS in patients with p53-positive digestive tract cancers. However, the results are still preliminary, but potentially important, because p53 is the most frequently mutated gene across cancers at all sites.

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480 **Table 1. Participants' characteristics in subgroups stratified by p53-positive and p53-negative**

	No. (%) of Participants[a]	
Expression level of p53 protein, n (%)	p53-positive 226 (61)	p53-negative 146 (39)
Intervention		
Vitamin D, n (%)	139 (61)	80 (55)
Placebo, n (%)	87 (39)	66 (45)
VDR, median (IQR)	211 (125-290)	146 (80-213)
Ki-67, median (IQR)	60 (50-70)	50 (30-70)
Subgroup of 25(OH)D, n (%)		
Low: <20 ng/mL	92 (41)	60 (42)
Middle: ≥20 and ≤40 ng/mL	127 (57)	82 (57)
High: >40 ng/mL	3 (1.4)	2 (1.4)
25(OH)D, median (IQR), ng/mL	21 (16-27)	21 (15-27)
Sex		
Male, n (%)	152 (67)	96 (66)
Female, n (%)	74 (33)	50 (34)
Age quartile, n (%)		
Q1, 35–59 y	55 (24)	32 (22)
Q2, 60–65 y	48 (21)	35 (24)
Q3, 66–73 y	65 (29)	37 (25)
Q4, 74–90 y	58 (26)	42 (29)
BMI[b] quartile, n (%)		
Q1, 15.0–19.7 kg/m^2	64 (28)	27 (19)
Q2, 19.8–21.8 kg/m^2	53 (24)	38 (26)
Q3, 21.9–23.7 kg/m^2	52 (23)	43 (30)
Q4, 23.8–37.3 kg/m^2	56 (25)	37 (26)
History of other cancers, n (%)	8 (3.5)	5 (3.4)
Comorbid conditions, n (%)		
Hypertension	82 (36)	60 (41)
Diabetes mellitus	38 (17)	25 (17)
Endocrine disease	33 (15)	16 (11)
Cardiovascular disease	20 (8.9)	6 (4.1)
Chronic kidney disease	5 (2.2)	0 (0.0)
Asthma	2 (0.9)	1 (0.7)
Orthopedic disease	1 (0.4)	1 (0.7)
Site of cancer, n (%)		
Esophagus	23 (10)	11 (7.5)
Stomach	74 (33)	85 (58)
Small bowel[e]	1 (0.4)	1 (0.7)
Colorectal	128 (57)	49 (34)
Stage, n (%)		
I	89 (39)	71 (49)
II	68 (30)	33 (23)
III	69 (31)	42 (29)
Pathology[c]		
Adenocarcinoma		
Well-differentiated, n (%)	139 (62)	64 (44)
Moderately differentiated, n (%)	83 (37)	74 (51)
Poorly differentiated, n (%)	30 (13)	41 (28)
Signet ring cell, n (%)	15 (6.6)	26 (18)
Mucinous, n (%)	14 (6.2)	12 (8.2)
Squamous cell carcinoma, n (%)	20 (8.9)	7 (4.8)
Papillary carcinoma, n (%)	6 (2.7)	9 (6.2)
Others, n (%)	0 (0.0)	2 (1.4)
Adjuvant chemotherapy, n (%)	82 (36)	49 (34)

481 a. Percentages may not sum to 100% because of rounding.
482 b. Body mass index (weight [kg]/height squared [m^2])
483 c. As many patients had multiple histopathological components, histopathological subgroups were not mutually exclusive
484 of each other.
485

Figure legends

Figure 1. Flow diagram of patients

Figure 2. Typical p53 protein expression patterns on immunohistochemistry (x400).

A. Overexpressed p53: Nearly 100% of cellular nuclei in the cancerous region are showing strong p53 positivity (dark brown). Red arrows are pointing to typical strong nuclear accumulation of p53 protein. Slides counterstained in light purple by hematoxylin are negative for p53 protein. In this slide, normal cells in the interstitial area are negative for p53 protein.

B. Strongly expressed p53: Part (>10%) of the cellular nuclei in the cancerous region are showing strong p53 positivity (dark brown). Red arrows are pointing to typical strong nuclear accumulation of p53 protein.

C. Faintly expressed p53: Part of the cellular nuclei in the cancerous region are showing faint p53 positivity (light brown), and a few cells (≤10%) are showing strong p53 nuclear accumulation (dark brown). The red arrow is pointing to typical strong nuclear accumulation of p53 protein, but it is rare. Typical faint p53 staining is shown by the hollow red arrows.

D. Not expressed p53: Neither strong nor faint p53 cells are found in the cancerous region.

Figure 3. Nelson-Aalen cumulative hazard curves in the p53-positive and p53-negative groups

A. In the subgroup of patients with p53-positive cancers, hazard curves for relapse or death of patients taking vitamin D (black line) vs. those taking placebo (gray line) are compared.
B. In the subgroup of patients with p53-negative cancers, hazard curves for relapse or death of patients taking vitamin D (black line) vs. those taking placebo (gray line) are compared.
C. In the subgroup of patients with p53-positive cancers, hazard curves for death of patients taking vitamin D (black line) vs. those taking placebo (gray line) are compared.
D. In the subgroup of patients with p53-negative cancers, hazard curves for death of patients taking vitamin D (black line) vs. those taking placebo (gray line) are compared.

Figure 4. Nelson-Aalen cumulative hazard curves for relapse or death of patients with p53-positive cancers stratified by cancer sites
A. In the subgroup of patients with p53-positive colorectal cancer, hazard curves of patients taking vitamin D (black line) vs. those taking placebo (gray line) are compared.
B. In the subgroup of patients with p53-positive gastric cancer, hazard curves of patients taking vitamin D (black line) vs. those taking placebo (gray line) are compared.
C. In the subgroup of patients with p53-positive esophageal cancer, hazard curves of patients taking vitamin D (black line) vs. those taking placebo (gray line) are compared.

Figure 5. Nelson-Aalen cumulative hazard curves for relapse or death stratified by quartiles of vitamin D receptor (VDR) protein expression

A. In the subgroup of patients in the lowest quartile of VDR (Q1), hazard curves of patients taking vitamin D (black line) vs. those taking placebo (gray line) are compared.

B. In the subgroup of patients in the low quartile of VDR (Q2), hazard curves of patients taking vitamin D (black line) vs. those taking placebo (gray line) are compared.

C. In the subgroup of patients in the high quartile of VDR (Q3), hazard curves of patients taking vitamin D (black line) vs. those taking placebo (gray line) are compared.

D. In the subgroup of patients in the highest quartile of VDR (Q4), hazard curves of patients taking vitamin D (black line) vs. those taking placebo (gray line) are compared.

Figure 1

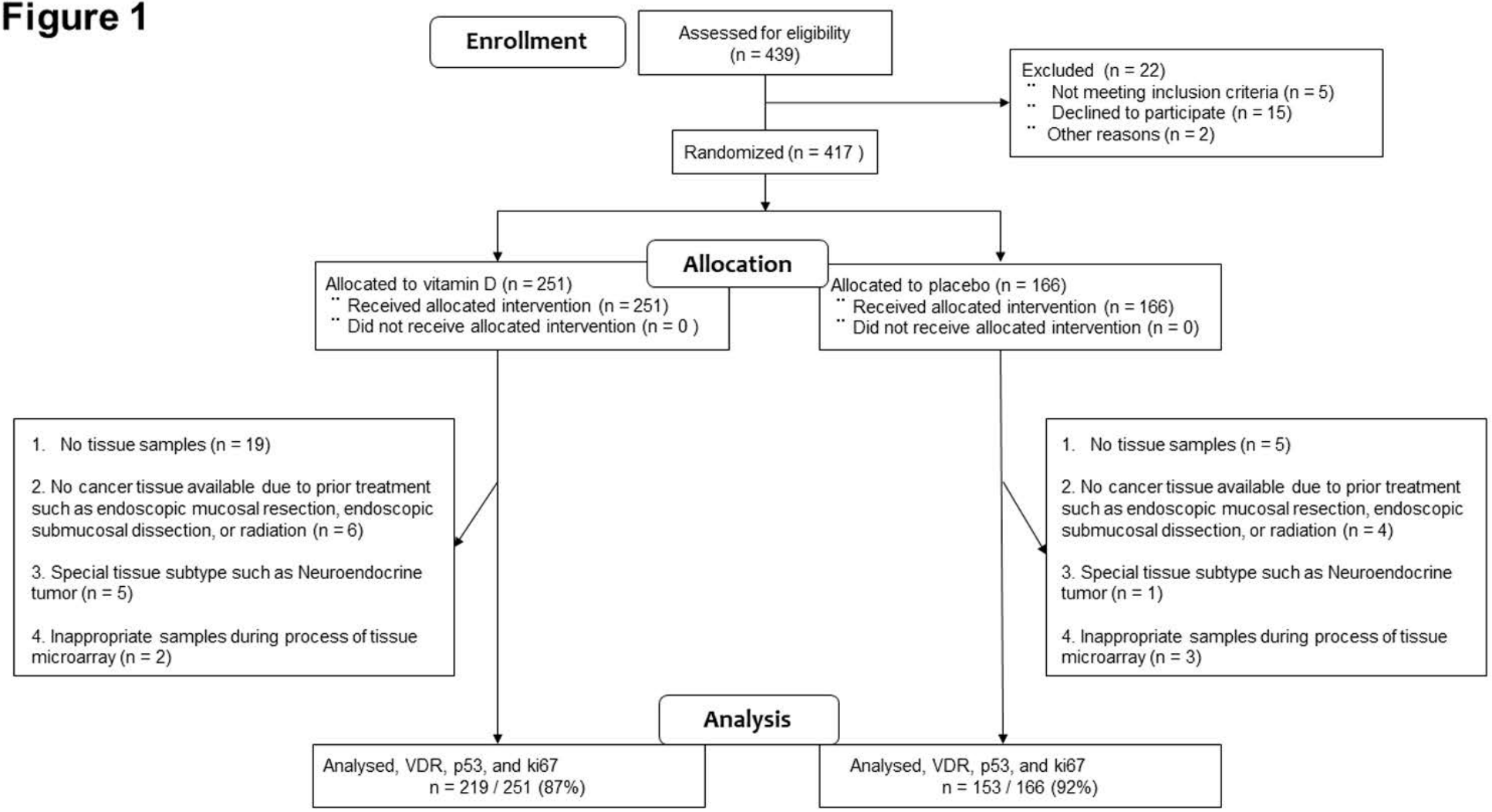

Enrollment
Assessed for eligibility (n = 439)
Excluded (n = 22)
Not meeting inclusion criteria (n = 5)
Declined to participate (n = 15)
Other reasons (n = 2)
Randomized (n = 417)
Allocation
Allocated to vitamin D (n = 251)
Received allocated intervention (n = 251)
Did not receive allocated intervention (n = 0)
Allocated to placebo (n = 166)
Received allocated intervention (n = 166)
Did not receive allocated intervention (n = 0)
1. No tissue samples (n = 19)
2. No cancer tissue available due to prior treatment such as endoscopic mucosal resection, endoscopic submucosal dissection, or radiation (n = 6)
3. Special tissue subtype such as Neuroendocrine tumor (n = 5)
4. Inappropriate samples during process of tissue microarray (n = 2)
1. No tissue samples (n = 5)
2. No cancer tissue available due to prior treatment such as endoscopic mucosal resection, endoscopic submucosal dissection, or radiation (n = 4)
3. Special tissue subtype such as Neuroendocrine tumor (n = 1)
4. Inappropriate samples during process of tissue microarray (n = 3)
Analysis
Analysed, VDR, p53, and ki67
n = 219 / 251 (87%)
Analysed, VDR, p53, and ki67
n = 153 / 166 (92%)

Figure 2

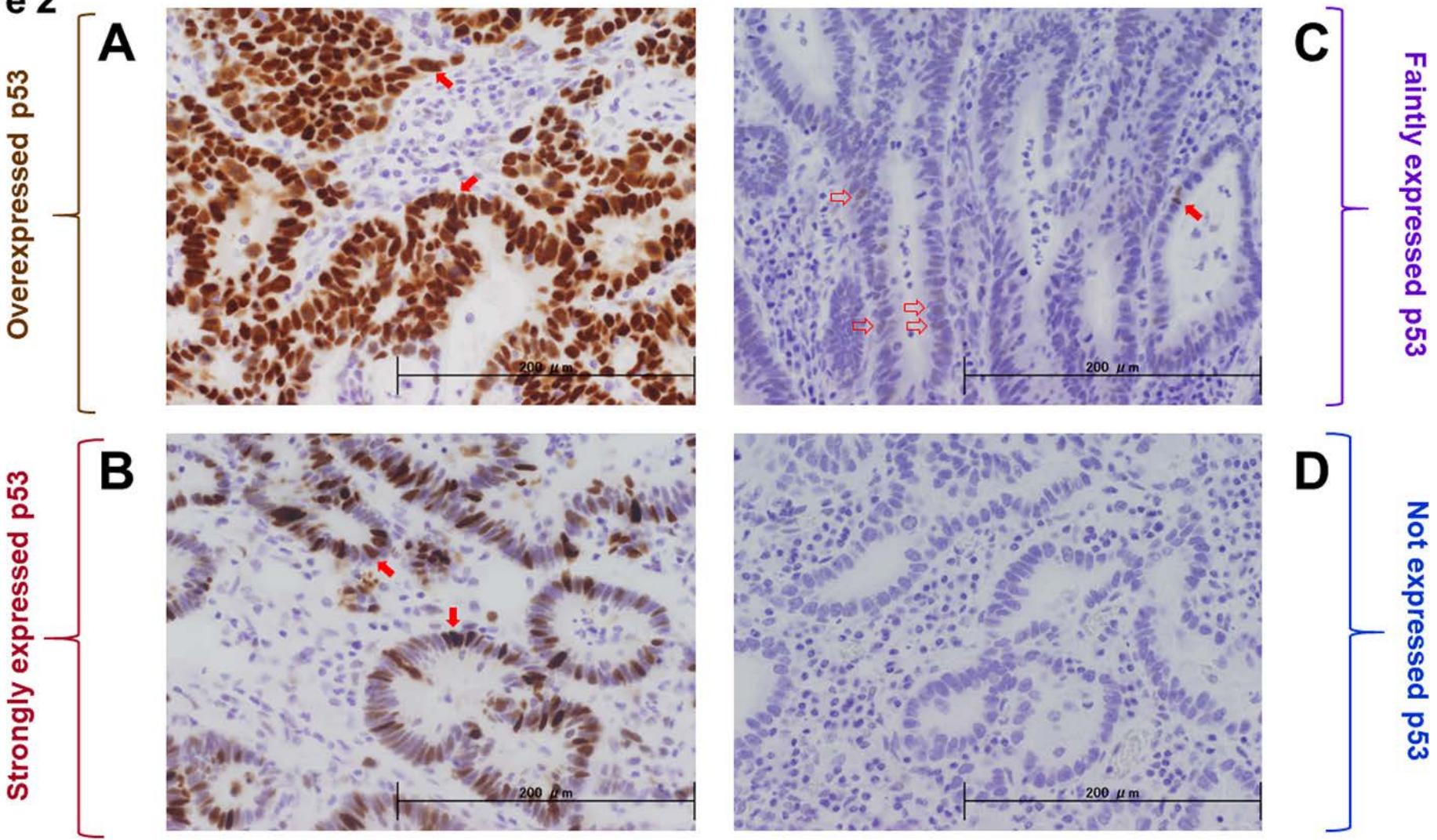
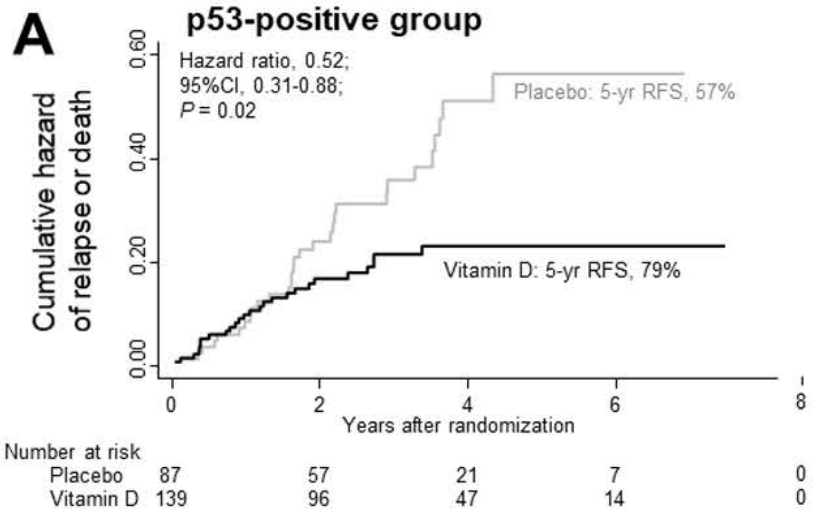
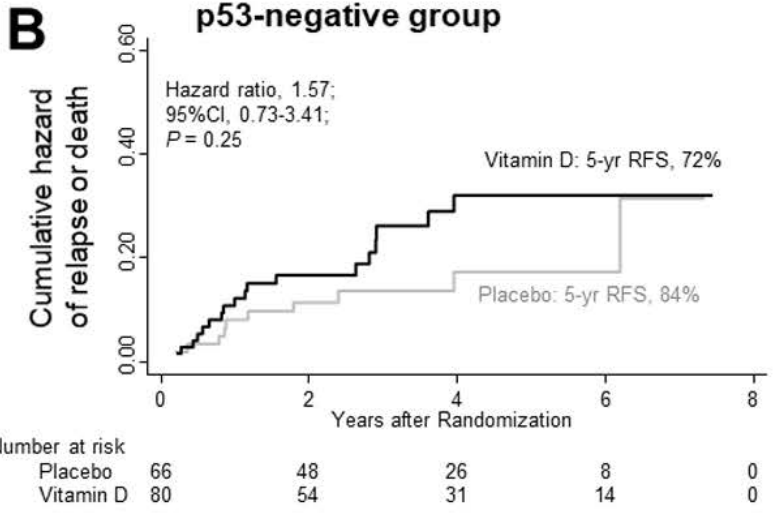
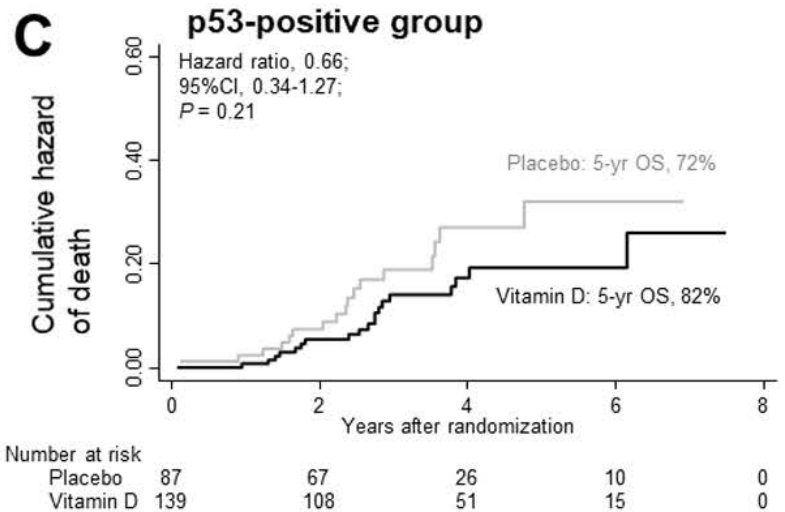

A
B
C
D
Overexpressed p53
Strongly expressed p53
Faintly expressed p53
Not expressed p53
200 μm
200 μm
200 μm
200 μm

Figure 3


A
p53-positive group
Hazard ratio, 0.52;
95%CI, 0.31-0.88;
P = 0.02
Placebo: 5-yr RFS, 57%
Vitamin D: 5-yr RFS, 79%
Cumulative hazard of relapse or death
0.00
0.20
0.40
0.60
0
2
4
6
8
Years after randomization
Number at risk
Placebo 87 57 21 7 0
Vitamin D 139 96 47 14 0


B
p53-negative group
Hazard ratio, 1.57;
95%CI, 0.73-3.41;
P = 0.25
Vitamin D: 5-yr RFS, 72%
Placebo: 5-yr RFS, 84%
Cumulative hazard of relapse or death
0.00
0.20
0.40
0.60
0
2
4
6
8
Years after Randomization
Number at risk
Placebo 66 48 26 8 0
Vitamin D 80 54 31 14 0


C
p53-positive group
Hazard ratio, 0.66;
95%CI, 0.34-1.27;
P = 0.21
Placebo: 5-yr OS, 72%
Vitamin D: 5-yr OS, 82%
Cumulative hazard of death
0.00
0.20
0.40
0.60
0
2
4
6
8
Years after randomization
Number at risk
Placebo 87 67 26 10 0
Vitamin D 139 108 51 15 0

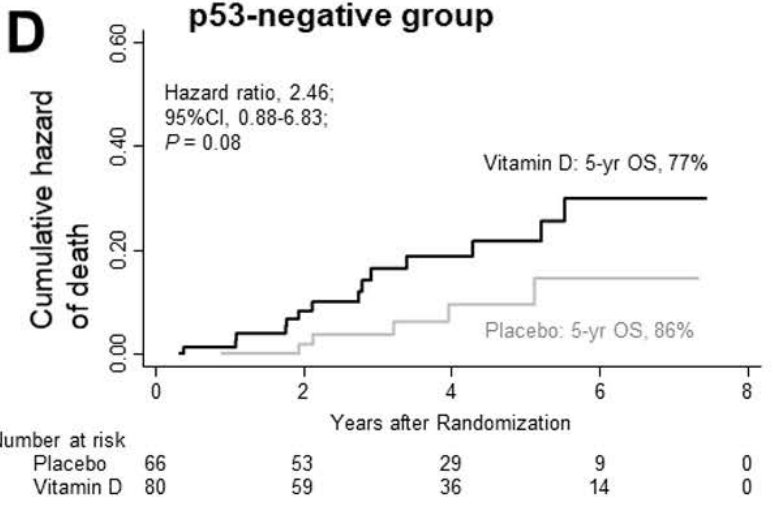

D
p53-negative group
Hazard ratio, 2.46;
95%CI, 0.88-6.83;
P = 0.08
Vitamin D: 5-yr OS, 77%
Placebo: 5-yr OS, 86%
Cumulative hazard of death
0.00
0.20
0.40
0.60
0
2
4
6
8
Years after Randomization
Number at risk
Placebo 66 53 29 9 0
Vitamin D 80 59 36 14 0

Figure 4

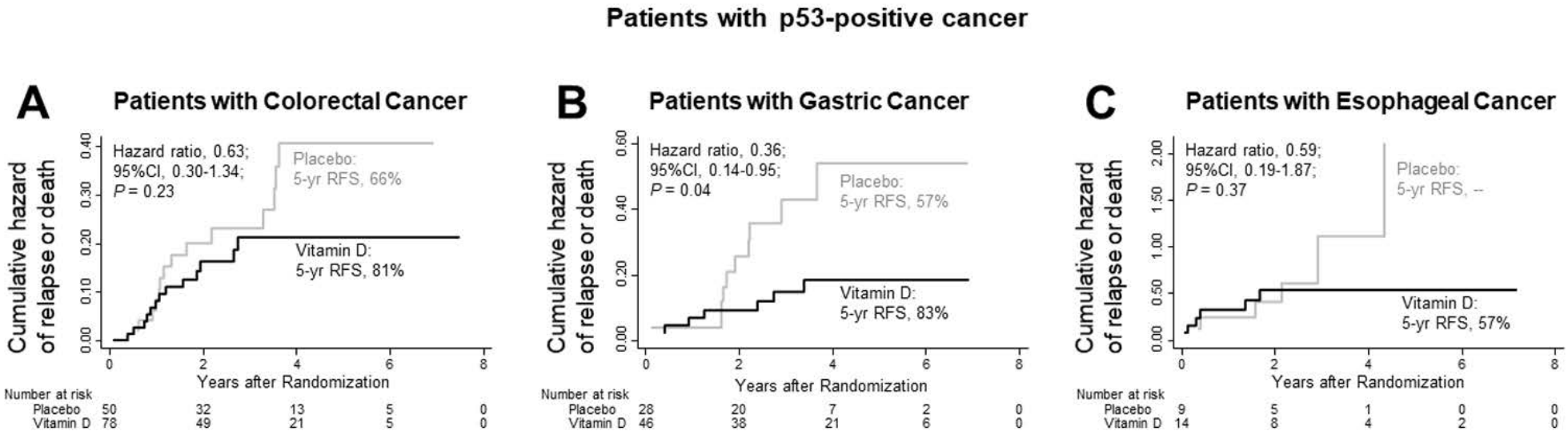
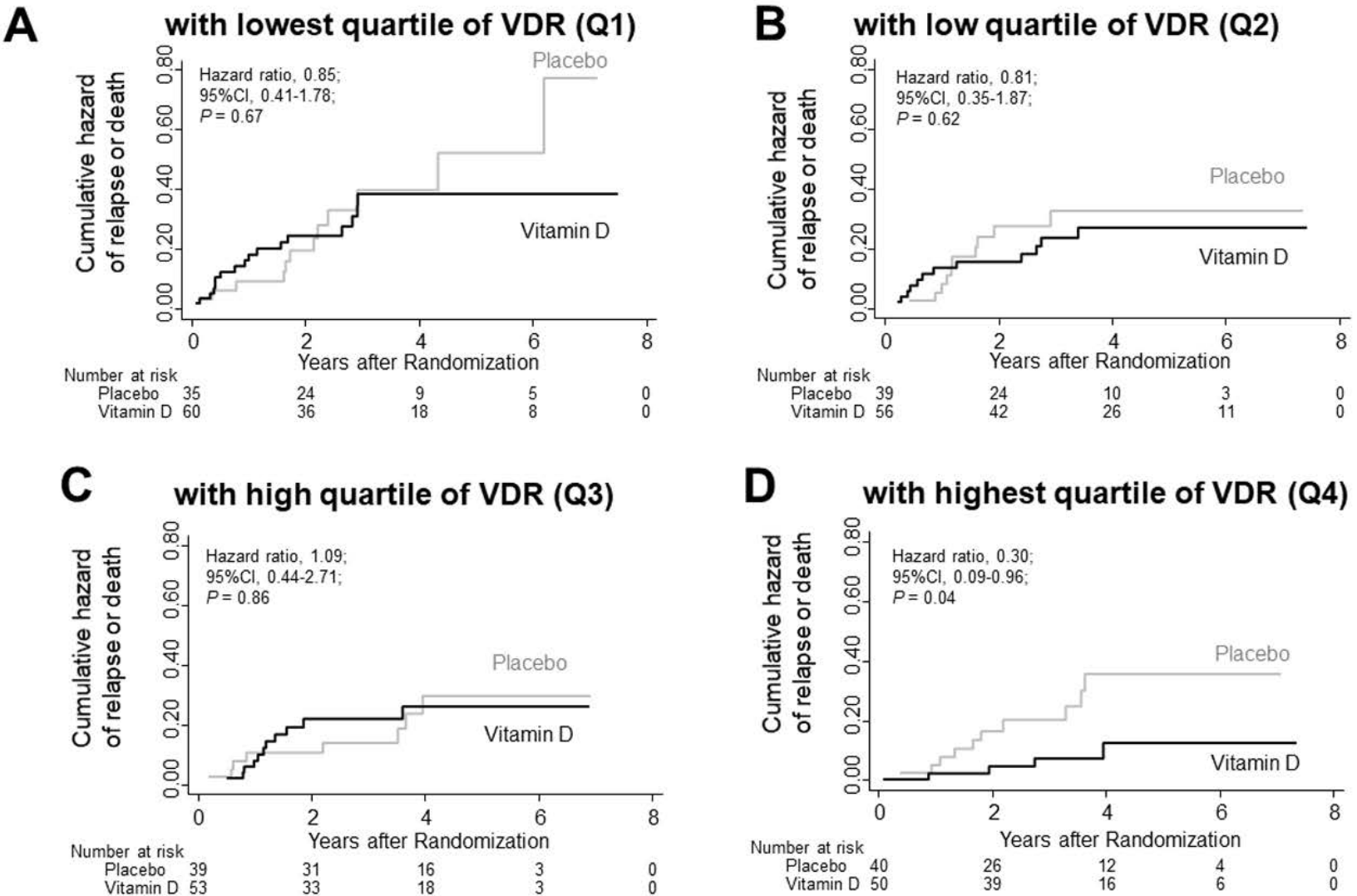

Patients with p53-positive cancer
A
Patients with Colorectal Cancer
Cumulative hazard of relapse or death
Hazard ratio, 0.63;
95%CI, 0.30-1.34;
P = 0.23
Placebo:
5-yr RFS, 66%
Vitamin D:
5-yr RFS, 81%
0.00
0.10
0.20
0.30
0.40
0
2
4
6
8
Years after Randomization
Number at risk
Placebo 50 32 13 5 0
Vitamin D 78 49 21 5 0
B
Patients with Gastric Cancer
Cumulative hazard of relapse or death
Hazard ratio, 0.36;
95%CI, 0.14-0.95;
P = 0.04
Placebo:
5-yr RFS, 57%
Vitamin D:
5-yr RFS, 83%
0.00
0.20
0.40
0.60
0
2
4
6
8
Years after Randomization
Number at risk
Placebo 28 20 7 2 0
Vitamin D 46 38 21 6 0
C
Patients with Esophageal Cancer
Cumulative hazard of relapse or death
Hazard ratio, 0.59;
95%CI, 0.19-1.87;
P = 0.37
Placebo:
5-yr RFS, --
Vitamin D:
5-yr RFS, 57%
0.00
0.50
1.00
1.50
2.00
0
2
4
6
8
Years after Randomization
Number at risk
Placebo 9 5 1 0 0
Vitamin D 14 8 4 2 0

Figure 5


A
with lowest quartile of VDR (Q1)
Hazard ratio, 0.85;
95%CI, 0.41-1.78;
P = 0.67
Placebo
Vitamin D
Cumulative hazard of relapse or death
0.00
0.20
0.40
0.60
0.80
0
2
4
6
8
Years after Randomization
Number at risk
Placebo 35 24 9 5 0
Vitamin D 60 36 18 8 0
B
with low quartile of VDR (Q2)
Hazard ratio, 0.81;
95%CI, 0.35-1.87;
P = 0.62
Placebo
Vitamin D
Cumulative hazard of relapse or death
0.00
0.20
0.40
0.60
0.80
0
2
4
6
8
Years after Randomization
Number at risk
Placebo 39 24 10 3 0
Vitamin D 56 42 26 11 0
C
with high quartile of VDR (Q3)
Hazard ratio, 1.09;
95%CI, 0.44-2.71;
P = 0.86
Placebo
Vitamin D
Cumulative hazard of relapse or death
0.00
0.20
0.40
0.60
0.80
0
2
4
6
8
Years after Randomization
Number at risk
Placebo 39 31 16 3 0
Vitamin D 53 33 18 3 0
D
with highest quartile of VDR (Q4)
Hazard ratio, 0.30;
95%CI, 0.09-0.96;
P = 0.04
Placebo
Vitamin D
Cumulative hazard of relapse or death
0.00
0.20
0.40
0.60
0.80
0
2
4
6
8
Years after Randomization
Number at risk
Placebo 40 26 12 4 0
Vitamin D 50 39 16 6 0