# Resolution of, and risk of incident non-alcoholic fatty liver disease with changes in serum 25-hydroxy vitamin D status

**Short title:** Serum 25(OH)D and NAFLD

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**Abbreviation list**

25(OH)D, 25-hydroxyvitamin D; BMI, body mass index; BP, blood pressure; CI, confidence interval; CVD, cardiovascular disease; HEPA, health-enhancing physical activity; HOMA-IR, homeostasis model assessment of insulin resistance; HR, hazard ratio; hs-CRP, high-sensitivity C-reactive protein; NAFLD, non-alcoholic fatty liver disease; PY, person-years

**Keywords:** non-alcoholic fatty liver disease; cohort study; serum 25-hydroxy vitamin D; incidence; resolution

## ABSTRACT

**Context:** A protective or causative role of vitamin D status on the risk of non-alcoholic fatty liver disease (NAFLD) remains inconclusive.

**Objective**: To evaluate the association between changes in serum 25-hydroxyvitamin D [25(OH)D] status during follow-up and the risk of incident NAFLD and resolution of pre-existing NAFLD

**Design:** A retrospective cohort study

**Setting:** Kangbuk Samsung Health Study based on routine health screening examinations

**Participants:** Korean adults (mean age, 36.8 years; range, 18–96 years) who underwent comprehensive health examinations including assessment of serum 25(OH)D levels

**Main Outcome Measures:** The main outcomes were a) incidence and b) resolution of NAFLD assessed by liver ultrasound. Cox proportional hazard models were used to estimate hazard ratios (HRs) with 95% confidence intervals (CIs) for outcomes according to serum 25(OH)D levels.

**Results:** Among 139,599 participants without NAFLD at baseline, 27,531 developed NAFLD during follow-up. Serum 25(OH)D levels were significantly and inversely associated with NAFLD development. Among 48,702 participants with NAFLD at baseline, 13,449 showed NAFLD resolution. Multivariable-adjusted HR (95% CI) for NAFLD resolution comparing 25(OH)D 10–<20, 20–<30, and ≥30 ng/mL to <10 ng/mL were 1.09 (1.03–1.15), 1.13 (1.06–1.21), and 1.21 (1.09–1.35), respectively. Additionally, an increase in 25(OH)D levels between baseline and the subsequent visit (median, 1.8 years) was associated with decreased NAFLD incidence, while persistently adequate 25(OH)D levels over time was associated with decreased incidence and increased resolution of NAFLD.

**Conclusions:** Maintaining adequate serum 25(OH)D concentrations may be beneficial for both prevention as well as resolution of NAFLD.

## INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is considered a multisystem disease that is positively associated with cardiovascular disease (CVD) risk factors, CVD mortality, and all-cause mortality (1,2). Despite its burden and impact, the absence of approved pharmaceutical treatment indicates that management of NAFLD consists of lifestyle modifications, which are effective in ameliorating the early stages of liver disease and improving the associated cardiometabolic risk factors (3).

A growing body of evidence has suggested a link between vitamin D deficiency, characterized by low serum levels of 25-hydroxyvitamin D [25(OH)D], and increased risk of various cardiometabolic diseases, including metabolic syndrome (4), coronary artery disease (5), chronic liver disease (6-8), and mortality (9). The therapeutic potential of vitamin D supplementation in NAFLD has been investigated in clinical trials; however, the findings are conflicting and limited by small sample sizes and the short duration of follow-up. A meta-analysis of mainly cross-sectional and case-control studies has demonstrated an association between low vitamin D levels and the presence of NAFLD (10,11). However, we have not identified any previous cohort studies that have investigated the role of vitamin D levels in the development of incident NAFLD, or in the resolution of NAFLD.

The present study aimed to evaluate the association between repeated measurements of serum 25(OH)D levels and both the risk of incident NAFLD and the resolution of pre-existing NAFLD.

## METHODS

### *Study participants*

The present study was conducted as part of the Kangbuk Samsung Health Study which is a cohort study of Korean men and women aged ≥18 years who underwent

115 comprehensive annual or biennial examinations at Kangbuk Samsung Hospital Total
116 Healthcare Center in Seoul and Suwon, South Korea, as previously described. The present
117 cohort study included participants who underwent a comprehensive health examination
118 including serum vitamin D levels between January 2011 and December 2018 and had at least
119 one follow-up visit before 31 December 2019 (n = 251,687)

120 A total of 112,088 subjects were excluded based on the following criteria shown in
121 **Figure 1**. Exclusion criteria included: excessive alcohol consumption, liver steatogenic
122 medication, medication for hepatitis, history of hepatitis, serologic positivity for hepatitis B
123 virus and hepatitis C virus, liver cirrhosis based on ultrasound, history of cancer, and missing
124 information on alcohol consumption, fatty liver, or serum 25(OH)D levels. Some participants
125 satisfied more than one exclusion criterion, and a total of 139,599 participants with no
126 NAFLD were included in the NAFLD-free cohort, and 48,702 participants with NAFLD were
127 included in the NAFLD cohort. In the analyses regarding the association of 25(OH)D level
128 changes with the risk of incident NAFLD, and with the resolution of existing NAFLD, those
129 who had an initial baseline and subsequent visit and who did not have at least one follow-up
130 visit were further excluded (in addition to the aforementioned exclusion criteria). In addition,
131 subjects with missing data on 25(OH)D, as well as subjects who were diagnosed with
132 NAFLD (for assessing incident NAFLD) and who had NAFLD resolution (for assessing
133 NAFLD resolution) on the second visit were also excluded.

134 The study was approved by the institutional review board of Kangbuk Samsung
135 Hospital (IRB No. KBSMC 2021-08-045), which waived the requirement for informed
136 consent since de-identified retrospective data from routine health screening were used.

137

138 ***Measurement***

139 Standardized, self-administered questionnaires, diet, physical measurements,

abdominal ultrasonography, and serum biochemical measurements were collected at each visit as part of the basic health check-up program (12,13). The current average alcohol consumption per day was assessed using the frequency of alcohol consumption per week and the amount of alcohol consumed per drinking day. Physical activity levels were assessed using the validated Korean version of the International Physical Activity Questionnaire Short Form (14). Physical activity levels were classified into three categories: inactive, minimally active, and health-enhancing physical activity (HEPA). HEPA was defined as follows: (1) vigorous activity ≥3 days/week with ≥1,500 accumulated metabolic equivalent (MET)-min/week, or (2) a combination of walking, moderate, or vigorous-intensity activities for seven days and accumulating ≥3,000 MET-min/week (14).

Sitting blood pressure (BP), height, weight, and waist circumference were measured by trained nurses. Obesity was defined as a body mass index (BMI) ≥ 25 kg/m$^2$, the proposed cut-off for the diagnosis of obesity in Asians (15). Hypertension was defined as a systolic BP ≥140 mmHg, diastolic BP ≥90 mmHg, or current use of antihypertensive medications.

Blood and urine specimens were collected after at least 10 h of fasting. Fasting blood sample measurements included: total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, triglycerides, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT), glucose, hs-CRP, albumin, and platelet count. The serum total cholesterol and triglyceride concentrations were determined using an enzymatic colorimetric assay. High-density lipoprotein and low-density lipoprotein cholesterol levels were directly measured using a homogenous enzymatic colorimetric assay. AST, ALT, and GGT were measured using the modified IFCC method, and serum fasting glucose levels were measured using the hexokinase method on Modular DPP systems (Roche Diagnostics, Tokyo, Japan) until 2015, and the Cobas 8000 c702 (Roche Diagnostics) thereafter. Hemoglobin A1c levels were determined using a turbidimetric inhibition

immunoassay on the Cobas Integra 800 (Roche Diagnostics) until January 2018 and the Cobas 8000 c513 (Roche Diagnostics) thereafter (RRID: AB_2909460 and AB_2909459). Serum insulin levels were measured using an electrochemiluminescence immunoassay with the sandwich principle on the Modular E170 (Roche Diagnostics, Tokyo, Japan) until April 2015, and the Cobas 8000 e801 (Roche Diagnostics) thereafter (RRID: AB_2756877 and AB_2909455). The homeostatic model assessment of insulin resistance (HOMA-IR) index was calculated as follows: fasting blood insulin (mU/mL) × fasting blood glucose (mmol/L)/22.5.

Total 25(OH)D measurement using the Elecsys Vitamin D Total assay demonstrated acceptable performance compared to using liquid chromatography-tandem mass spectrometry, the reference standard for 25(OH)D measurement (16-19). When the analytical performance for precision was evaluated according to CLSI-EP15-A2 guidelines (20), the inter-assay coefficients of variation for quality control specimens of lower and higher levels of total 25(OH)D were 2.01-5.94% and 2.69%-5.03%, respectively, during the study period. The detection limit was determined according to the CLSI EP17-A2 guidelines (21) and was reported to be <3 ng/mL.

To assess serum 25(OH)D status, total 25(OH)D levels, including $25(OH)D_2$ and $25(OH)D_3$, were measured with a competitive immunoassay using an Elecsys Vitamin D Total assay on the Modular E170 (Roche Diagnostics, Tokyo, Japan) until April 2015 and Cobas e801 (Roche Diagnostics) thereafter (18). (RRID: AB_2909604 and AB_2909456)

Serum 25(OH)D levels were categorized as <10, 10-<20, 20-<30, and ≥30 ng/mL (For conversion to SI units: ng/mL×2.5=nmol/L; e.g., <25, 25-<50, 50-<75, and ≥75 nmol/L) (22). Vitamin D insufficiency is defined as serum 25(OH)D level <20 ng/mL; serum 25(OH)D levels ≥20 ng/mL were considered vitamin D sufficient, according to the recommendation for the healthy general population (23-27). The change in 25(OH)D status

from baseline to the second visit was analyzed in the following four groups based on the presence/absence of insufficient serum 25(OH)D (defined as serum 25(OH)D level <20 ng/mL [50 nmol/l]): a) insufficient 25(OH)D level at baseline and follow-up (persistently low); b) insufficient 25(OH)D level at baseline but no insufficiency at follow-up (increased); c) no insufficiency at baseline but insufficiency at follow-up (decreased); and d) no 25(OH)D insufficiency at baseline and also follow-up (persistently adequate).

***Diagnosis of hepatic steatosis***

Diagnosis of fatty liver was made based on an abdominal ultrasound performed by an experienced radiologist using standard criteria, including a diffuse increase in fine echoes in the liver parenchyma in comparison with the kidney or spleen, deep beam attenuation, and bright vessel walls (28). NAFLD was defined as the presence of mild to severe fatty liver in the absence of excessive alcohol use (<20 and <30 g/day for women and men, respectively) or any other identifiable cause (29). The inter-observer and intra-observer reliability values for hepatic steatosis diagnosis were substantial (kappa statistic of 0.74) and excellent (kappa statistic of 0.94), respectively (13). The severity of hepatic steatosis was also recorded as mild, moderate, or severe steatosis on sonography.

***Statistical analyses***

Descriptive statistics were used to summarise the participants' characteristics according to 25(OH)D levels as follows: <10, 10–19, 20–29 and ≥30 ng/mL (<25, 25–50, 50–75, and ≥75 nmol/L) based on categories of 25(OH)D levels with adequate levels defined as ≥20 ng/mL (≥50 nmol/L) (22,30). To describe potential linear trends in NAFLD incidence, the four categories were treated as a continuous variable in regression models.

We examined the association between serum 25(OH)D levels and the development

and resolution of NAFLD. The primary endpoints were a) the development of NAFLD and b) NAFLD resolution. The follow-up duration for each participant extended from the baseline examination until the development of the endpoint or the last health examination conducted prior to 31 December 2019; whichever came first. Incidence rates were calculated as the number of incident cases divided by person-years of follow-up. Cox proportional hazard models were used to estimate the hazard ratios (HRs) with 95% confidence intervals (CIs) for the development of incident NAFLD, or resolution of existing NAFLD. We initially adjusted for age and sex. Model 1 was further adjusted for the study center (Seoul, Suwon), year of screening examination, alcohol consumption, smoking, physical activity, total energy intake, education level, and BMI. Model 2 was further adjusted for medications for hyperlipidemia, medications for diabetes, multi-vitamin supplements, 25(OH)D supplements, and calcium supplements. To evaluate the effects of changes in serum 25(OH)D levels and other covariates during the follow-up period, we performed additional analyses by introducing serum 25(OH)D levels, season, BMI, and other factors as time-varying covariates in the models. For each analysis, we further adjusted for HOMA-IR, glucose, and waist circumference, in addition to the variables considered in model 2, and tested the effects of serum 25(OH)D and its change on incident NAFLD or resolution of existing NAFLD. The proportional hazards assumption was assessed via estimated log (-log) survival curves, and no violation of the assumption was found.

To assess the relationship between the serum 25(OH)D as a continuous variable and NAFLD risk, we modelled the serum 25(OH)D as restricted cubic splines with knots at the $5^{th}$, $35^{th}$, $65^{th}$, and $95^{th}$ percentiles of the sample distribution to provide a flexible estimate of the concentration-response relationship between the serum 25(OH)D concentration and incident NAFLD. Models were adjusted for age, sex, center, year of screening exam, alcohol consumption, smoking, physical activity, total energy intake, education level, medication for

hyperlipidemia, glucose-lowering medication, multi-vitamin supplements, vitamin D supplements, calcium supplements, season, and BMI.

Statistical analyses were performed using STATA version 16.0 (StataCorp LP, College Station, TX, USA). Statistical significance was set at $P < 0.05$.

## RESULTS

The baseline characteristics of the study participants are presented according to 25(OH)D levels at baseline and subsequent visits (**Table 1, eTables 1–3)** (31). The mean (SD) age of the subjects was 36.8 (7.3) years. The 25(OH)D levels were positively associated with age, alcohol intake, HEPA, education level, medication use for hyperlipidemia, use of multi-vitamin supplements, vitamin D supplements, calcium supplements, BP, total cholesterol, and ALT (**Table 1**). The baseline characteristics of participants according to NAFLD status are described in **eTables 4** and **5** (31). Compared to those who did not develop NAFLD, those who developed NAFLD were older, more likely to be male, alcohol drinkers, current smokers, obese, have a history of diabetes, hypertension, or CVD, receive glucose-lowering medication or hyperlipidemia medication, and take multi-vitamin supplements; these individuals also had higher BP, total cholesterol, glucose, GGT, ALT, HOMA-IR, and total energy intake (**eTable 4)** (31).

Compared to those with no NAFLD resolution, individuals with NAFLD resolution were more likely to be: older, female, alcohol drinkers, regular exercisers, have higher education levels, take multi-vitamin supplements, and have a higher total energy intake (**eTable 5**) (31).

**Table 2** shows the risk of NAFLD development according to 25(OH)D levels among the cohort of people without NAFLD at baseline (n=139,599). Within 581,021 person-years of follow-up (median, 4.1 years; interquartile range, 2.1–6.0 years), 48,702 subjects

developed NAFLD (incidence rate, 45.9 per 1,000 person-years). The median (interquartile range) follow-up frequencies for the NAFLD-free and NAFLD cohorts were 4 visits (3-5) and 4 visits (3-6), respectively. Overall, baseline 25(OH)D levels were inversely associated with the risk of incident NAFLD. After adjusting for age, sex, center, year of screening exam, alcohol consumption, smoking, physical activity, total energy intake, education level, and BMI (Model 1), the HRs (95% CI) for incident NAFLD at baseline 25(OH)D levels of 10–<20, 20–<30, and ≥30 ng/mL compared to <10 ng/mL (reference group) were 0.91 (0.88–0.94), 0.85 (0.81–0.88) and 0.75 (0.70–0.81), respectively. The associations remained significant when the model was further adjusted for medications for hyperlipidemia, medications for diabetes, multi-vitamin supplements, vitamin D supplements, and calcium supplements (Model 2), as well as when 25(OH)D levels, BMI and other potential confounders were treated as time-varying covariates. After further adjustment for glucose and HOMA-IR (**eTable 6**) (31) and waist circumference (**eTable 7**) (31), all associations remained statistically significant. In spline regression models, the NAFLD risk decreased across the range of the 25(OH)D levels (**Figure 2**).

**Table 3** presents the association between 25(OH)D levels and resolution of NAFLD. Among 48,702 participants with NAFLD at baseline, 13,449 had resolution of NAFLD. The multivariable-adjusted HRs (95% CI) in subjects with 25(OH)D levels of 10–<20, 20–<30, and ≥30 ng/mL for NAFLD resolution were 1.09 (1.03–1.15), 1.13 (1.06–1.21), and 1.21 (1.09–1.35), respectively, as compared with the reference group (Model 2). All associations were similar after further adjustment for glucose and HOMA-IR (**eTable 6**) (31) and waist circumference (**eTable 7**) (31) as well as when 25(OH)D levels, BMI and other potential confounders were treated as time-varying covariates. When participants were stratified by vitamin D supplement use status, we found significant associations between higher serum 25(OH)D and NAFLD resolution only in vitamin D supplement non-users, whereas the

associations were non-significant among vitamin D supplement users (**eTable 8**) (31).

The associations of the changes in 25(OH)D levels from baseline to the second visit with the risk of incident NAFLD are presented in **Table 4**. The mean interval between the 1st and 2nd visits was 1.8 years (interquartile range, 1.1–2.1 years). The multivariable-adjusted HRs (95% CI) for "decreased", "increased", and "persistently adequate" groups versus "persistently low" group for NAFLD development were 0.92 (0.87–0.98), 0.87 (0.82–0.91), and 0.76 (0.76–0.85), respectively (Model 2). For the association between 25(OH)D level change with NAFLD resolution in subjects with NAFLD at baseline (**Table 5**), the multivariable-adjusted HRs (95% CI) for "decreased", "increased", and "persistently adequate" 25(OH)D groups versus the "persistently low" group for NAFLD development were 0.97 (0.88–1.07), 1.02 (0.94–1.11), and 1.10 (1.01–1.19), respectively. All associations remained materially unchanged after further adjustment for glucose and HOMA-IR (**eTable 9**) (31) and waist circumference (**eTable 10**) (31). In the analysis of the effect of the changes in 25(OH)D levels on NAFLD resolution by vitamin D supplement use status, persistently adequate serum 25(OH)D levels were significantly associated with NAFLD resolution only in the supplement non-users (**eTable 11**) (31).

We also performed subgroup analyses stratified by glucose-lowering medication status (no medication usage vs. medication usage) and assessed the association between serum 25(OH)D levels and both NAFLD development and resolution. In this analysis, we found a similar inverse association between 25(OH)D levels and incident NAFLD, and a significant positive association with NAFLD resolution in the "no medication use" group; the trends of these associations remained similar in the "medication use" group, and no significant differences were found between the two groups ($P$ = 0.674 for incident NAFLD; $P$ = 0.152 for NAFLD resolution) (**eTable 12**) (31). Similarly, the trends of associations observed in the original analyses were mostly retained for both groups when the effects of

25(OH)D changes were assessed, with no significant differences between the groups ($P$ = 0.944 for incident NAFLD; $P$ = 0.490 for NAFLD resolution) (**eTable 13**) (31).

In analyses after further exclusion of individuals with abnormally high ALT levels (ALT > 35 IU/L (32,33), as the upper limit of normal is usually regarded as 35 IU/L), the main results were similar, showing a significant decrease in the risk of incident NAFLD and an increase in NAFLD resolution with increasing 25(OH)D levels (**eTable 14**) (31). Consistent trends were observed when we assessed the changes in 25(OH)D levels (**eTable 15**) (31). In addition, when the outcome was defined as high ALT level instead of an ultrasonographic diagnosis of NAFLD, the trends were similar, showing decreased risks of high ALT and increased resolution of high ALT with increasing categories of 25(OH)D levels (**eTable 16**) (31). Regarding the changes in 25(OH)D levels, persistently adequate serum 25(OH)D levels were associated with a significantly lower risk of elevated ALT; no significant associations were found between the changes in serum 25(OH)D levels and high ALT (**eTable 17**) (31).

## DISCUSSION

In this large cohort study of 139,599 individuals without NAFLD at baseline, serum 25(OH)D levels were inversely associated with the development of NAFLD, while in those with NAFLD at baseline, serum 25(OH)D levels were positively associated with the resolution of NAFLD in a dose-response manner. Importantly, an increase in 25(OH)D from an insufficient level (<20 ng/mL) at baseline to adequate levels (≥20 ng/mL) at subsequent visits; as well as persistently adequate levels of 25(OH)D at both visits, were both associated with a decreased risk of incident NAFLD. In addition, persistently adequate levels of ≥20 ng/mL were also associated with the resolution of NAFLD in subjects with pre-existing NAFLD at baseline. Our study results suggest that maintaining sufficient serum 25(OH)D

levels may be an effective approach to both primary and secondary prevention of NAFLD; a strategy that could be easily achieved by the use of supplements (or potentially also by increased sun exposure).

The protective or causative role of vitamin D in the risk of NAFLD has been highly controversial with conflicting results. It is relatively well established that vitamin D deficiency is prevalent among patients with NAFLD (6,10,34). A recent meta-analysis suggested that low serum vitamin D levels might play a role in NAFLD pathogenesis (11). Although data on the potential effect of vitamin D to prevent NAFLD are scarce, in a recent cross-sectional study, higher levels of serum vitamin D were associated with a decreased prevalence of controlled attenuation parameter-defined NAFLD, compared to low levels of serum vitamin D (35). However, not only are existing studies limited by small sample sizes and a cross-sectional study design, but there is also considerable heterogeneity between existing studies with respect to the prevalence of comorbidities. Our study, the largest cohort study to date, supports the idea that higher serum 25(OH)D levels are prospectively associated with a reduced risk of incident NAFLD in relatively healthy young adults whose mean age was 36.8 years (interquartile range, 31.3-40.5 years), with a much lower prevalence of comorbid conditions than in previous studies. Although outdoor activities and sunlight exposure were not specifically measured in our study, our population predominantly consisted of urban office workers who were likely to spend most of the daytime indoors and whose sun exposure was likely to be particularly insufficient even in the summer (36-38). Moreover, the association of serum 25(OH)D with the risk of NAFLD was not significantly altered and remained significant when we accounted for other, possibly more relevant, factors, such as BMI, physical activity, vitamin D, or calcium supplementation, season during the venesection, and the changing status of 25(OH)D. However, owing to the observational nature of our study, we were not able to establish causality, though our study findings align

with a recent Mendelian randomization study of three populations of European descent that reported a significant inverse correlation between genetically predicted serum 25(OH)D levels and NAFLD (39).

We also observed that higher serum 25(OH)D concentration was associated with resolution of NAFLD in people with pre-existing NAFLD at baseline; and to date, there have been no comparable studies. Several clinical trials have examined the benefits of vitamin D supplementation in individuals with NAFLD, with conflicting results (40-45). These studies are mostly small clinical trials with a short follow-up duration ranging from 4 to 48 weeks. Also, due to the limitations such as heterogeneity of NAFLD at diagnosis, the presence of comorbidities, and the use of biochemical parameters to define NAFLD, such as liver enzyme levels, with sub-optimal specificity and sensitivity, it is difficult to draw a conclusion from these findings (46,47). Large statistical power and longer duration of follow-up in our study allowed us to account for potential confounders and observe the incidence and resolution of hepatic steatosis, and these data suggest potential temporality of the association between 25(OH)D status and NAFLD. Although there remains a possibility of residual confounding due to the observational nature of our study, based on our findings, we suggest that vitamin D may confer therapeutic benefits for those with established NAFLD. Thus, we suggest that our study results highlight that further longer-term studies are still needed to confirm (or refute) whether there are causal associations between serum 25(OH)D levels and NAFLD.

In the present study, both groups of participants whose serum 25(OH)D levels increased from low to adequate levels and those with persistently adequate 25(OH)D levels over time showed a reduced risk of NAFLD development compared to the group with persistently low levels. As many of the previous clinical trials examining the effects of vitamin D did not assess vitamin D levels by repeat measurements (48), the effect of the changes in vitamin D status on NAFLD risk has been uncertain. A strength of our study is

that we incorporated the changes in serum measurements of vitamin D status based on repeated measurements at each follow-up visit. Another important consideration is that a substantial proportion (approximately 77%) of our study cohort had sub-optimal 25(OH)D levels (<20 ng/mL) at baseline. This proportion is markedly higher than the prevalence range of 24%–40% in the US and European countries (48). That said, the prevalence of sub-optimal 25(OH)D levels in our cohort is similar to the nationwide prevalence of suboptimal 25(OH)D levels, reported from the Korea National Health and Nutrition Examination Survey (38,49). The reason for the disparity might be a substantially higher number of indoor office workers or differences in nutrition or patterns of supplement use with respect to other populations. This is likely to be clinically relevant since it underlines the benefit of improving 25(OH)D status in the prevention of NAFLD in a population with a high prevalence of 25(OH)D deficiency. We also observed that people with persistently adequate serum 25(OH)D levels were more likely to experience resolution of NAFLD than those with persistently low levels, whereas those in the "increased group" who had insufficient 25(OH)D levels at baseline but no insufficiency at follow-up, did not. The implication of this observation is not clear, but a previous meta-analysis of randomized-controlled trials has suggested that sufficient duration of vitamin D supplementation is essential in achieving vitamin D levels that can have therapeutic effects on NAFLD (50). Based on our study results, it may be speculated that having an adequate level of serum 25(OH)D, even within a low-normal range, at any point in time may be beneficial in protecting against incident NAFLD, but maintaining the levels for a prolonged duration of time would be needed in order to reverse the disease course in patients with pre-existing NAFLD. Further large-scale observational and interventional studies are warranted to confirm our findings.

The mechanisms by which serum 25(OH)D exerts benefits in NAFLD are not fully understood. Numerous studies have suggested that a pivotal pathway whereby vitamin D

improves liver parameters, involves the resolution of insulin resistance and reduction in blood glucose levels (45,51). However, in our study, the associations between 25(OH)D levels and NAFLD were not fully attenuated by adjustment for glucose or insulin resistance. An alternative explanation may involve the anti-inflammatory and immunomodulatory properties of 25(OH)D. In pre-clinical studies in rats, low 25(OH)D levels led to the exacerbation of hepatic steatosis and lobular inflammation (52). Low serum 25(OH)D levels are implicated in upregulating the hepatic inflammatory response via the effects of adipocytokines, such as adiponectin, leptin, and resistin; which can directly affect the pathogenesis of NAFLD (53). Low serum 25(OH)D levels may also activate the Toll-like receptor signaling pathway as well as downstream inflammatory signaling molecules, subsequently leading to the accumulation of hepatic fat (52). Importantly, it has been reported that activation of vitamin D receptors in liver macrophages by vitamin D ligands ameliorated liver inflammation as well as hepatic steatosis in a mouse model, which may partly explain the resolution of NAFLD (54). Further studies are required to elucidate the mechanisms involved in the benefits of vitamin D in NAFLD pathophysiology.

Several limitations of our study must be considered. First, ultrasonography was used instead of liver biopsy and histology, which is still the reference standard for the diagnosis of NAFLD. However, the use of liver ultrasound to diagnose liver fat is an accepted proxy in large epidemiological studies, and the use of liver biopsy was not feasible or ethically acceptable in the routine health screening setting involving repeat measurements in our cohort study. Second, although we had information on the use of vitamin D or multivitamin supplements, we did not have detailed information on dose, type, frequency of supplementation, outdoor activities, or sunlight exposure, and therefore, the potential for residual confounding remains. Nevertheless, we directly measured serum 25(OH)D levels, which are considered to reflect the overall vitamin D status in the body and the cumulative

effect of sunlight exposure and dietary intake of vitamin D. Third, our study participants mainly represent a relatively young and healthy Korean working population. Although this could be perceived as a limitation, it also represents a strength of our study, since relatively few study participants had existing comorbidities that are known to be associated with low levels of serum 25(OH)D. Fourth, detailed information on glucose-lowering medications, such as specific types of medication and their treatment duration, was not collected. Finally, the generalizability of our findings to other populations with different sociodemographic characteristics and other ethnic groups is limited. The body's capacity to synthesize vitamin D, vitamin D status, and even optimal vitamin D levels may differ across different ethnicities. In addition, genetic polymorphisms, dietary and sociocultural factors, and geographic locations can also influence vitamin D status (55-57). Future investigations should focus on extending our findings to large populations comprising different ethnicities in diverse geographical locations.

Our study has several notable strengths. The longitudinal, prospective design enabled us to examine temporal associations of serum 25(OH)D status and changes in 25(OH)D levels with the risk of incident NAFLD and NAFLD resolution. In addition, we were able to control for the effects of changes with different covariates during follow-up in our time-dependent model in which 25(OH)D levels and other covariates were treated as time-dependent variables. Also, the large sample size, the use of carefully standardized clinical, imaging, and laboratory procedures, the inclusion of lifestyle factors, and repeated measurements allowed us to account for possible confounders such as supplementation use in investigating the associations between change in vitamin D status and both incident NAFLD and resolution of NAFLD.

In conclusion, in this large cohort study, we demonstrated that serum 25(OH)D levels were inversely associated with the risk of incident NAFLD and were positively associated

with NAFLD resolution. In addition, maintaining sufficient serum levels of 25(OH)D, even at a low-normal range, had favorable effects not only in prevention but also in the resolution of NAFLD. With the recent prevalence of vitamin D deficiency occurring in parallel with the rising incidence of NAFLD, our findings highlight that improved serum 25(OH)D levels may be beneficial in NAFLD prevention and treatment. These data also emphasize that better-designed, longer term, vitamin D intervention trials are required, in order to prove unequivocally whether inexpensive vitamin D supplements are beneficial in the primary and secondary prevention of NAFLD, in patients with low levels of vitamin D.

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**Data availability statement:** The data will not be made available to other researchers for purposes of reproducing the results. However, analytical methods are available from corresponding author on reasonable request.

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**Figure 1. Flow chart for the selection of the study participants**

**Figure 2. Multivariable-adjusted hazard ratios for NAFLD**

Curves represent adjusted hazard ratios for NAFLD based on restricted cubic splines with knots at the $5^{th}$, $35^{th}$, $65^{th}$, and $95^{th}$ percentiles of serum 25(OH)D distribution.

**Table 1.** Estimated[a] mean values (95% confidence intervals) and adjusted[a] proportion (95% confidence intervals) of baseline characteristics by baseline 25(OH)D levels among participants without NAFLD at baseline (n = 139 599)

| Characteristics | Vitamin D levels (ng/mL) | | | | *p*–trend |
|---|---|---|---|---|---|
| | <10 | 10-19 | 20-29 | ≥30 | |
| Number of participants | 25 975 | 81 269 | 26 348 | 6 007 | |
| Age (years) | 35.2 (35.2–35.3) | 35.9 (35.8–35.9) | 37.1 (37.0–37.2) | 39.2 (39.0–39.3) | <0.001 |
| Male (%) | 23.0 (22.5–23.5) | 42.3 (42.0–42.7) | 53.8 (53.2–54.4) | 48.0 (46.7–49.3) | <0.001 |
| Alcohol intake (%) [b] | 22.6 (22.1–23.2) | 24.8 (24.5–25.1) | 27.3 (26.8–27.7) | 27.5 (26.4–28.5) | <0.001 |
| Current smoker (%) | 11.7 (11.2–12.2) | 12.6 (12.4–12.9) | 14.2 (13.9–14.6) | 13.8 (13.1–14.6) | <0.001 |
| HEPA (%) | 12.3 (11.9–12.7) | 14.5 (14.2–14.7) | 17.3 (16.9–17.8) | 19.1 (18.1–20.1) | <0.001 |
| Education level (%) [c] | 80.6 (80.1–81.1) | 84.5 (84.3–84.8) | 86.2 (85.7–86.6) | 86.5 (85.6–87.3) | <0.001 |
| History of diabetes (%) | 0.6 (0.5–0.7) | 0.7 (0.6–0.7) | 0.7 (0.6–0.8) | 0.7 (0.6–0.9) | <0.001 |
| History of hypertension (%) | 3.7 (3.4–3.9) | 3.4 (3.3–3.5) | 3.6 (3.4–3.8) | 3.3 (2.9–3.6) | <0.001 |
| History of CVD (%) | 0.6 (0.5–0.7) | 0.5 (0.5–0.6) | 0.6 (0.5–0.7) | 0.6 (0.5–0.8) | <0.001 |
| Glucose-lowering medication (%) | 0.4 (0.3–0.5) | 0.4 (0.4–0.5) | 0.4 (0.3–0.5) | 0.4 (0.3–0.5) | 0.635 |
| Anti-lipid medication use (%) | 0.9 (0.8–1.1) | 1.0 (0.9–1.0) | 1.0 (0.9–1.1) | 1.4 (1.2–1.6) | <0.001 |
| Multi-vitamin supplement (%) | 6.7 (6.4–7.0) | 11.5 (11.3–11.7) | 17.9 (17.5–18.4) | 22.7 (21.7–23.8) | <0.001 |
| Vitamin D supplement (%) | 0.4 (0.3–0.5) | 0.9 (0.8–1.0) | 2.4 (2.2–2.6) | 6.3 (5.7–6.9) | <0.001 |
| Calcium supplement (%) | 0.3 (0.2–0.4) | 0.7 (0.6–0.8) | 1.5 (1.4–1.7) | 2.9 (2.5–3.4) | <0.001 |
| Season | | | | | |
| Spring | 38.3 (37.7–38.9) | 26.9 (26.6–27.2) | 16.4 (15.9–16.8) | 17.5 (16.5–18.4) | <0.001 |
| Summer | 22.1 (21.6–22.6) | 31.2 (30.9–31.5) | 39.6 (39.0–40.2) | 38.4 (37.2–39.7) | <0.001 |
| Fall | 20.9 (20.4–21.4) | 30.2 (29.9–30.5) | 36.5 (36.0–37.1) | 34.1 (32.9–35.3) | <0.001 |
| Winter | 19.1 (18.6–19.6) | 11.7 (11.5–12.0) | 7.7 (7.4–8.1) | 10.2 (9.3–10.8) | <0.001 |
| Obesity (%) [d] | 12.2 (11.7–12.6) | 12.8 (12.6–13.0) | 13.6 (13.2–14.0) | 12.0 (11.2–12.7) | <0.001 |
| BMI ($kg/m^2$) | 21.8 (21.8–21.9) | 22.0 (21.9–22.0) | 22.0 (22.0–22.0) | 21.8 (21.7–21.8) | <0.001 |
| SBP (mmHg) | 104.3 (104.1–104.4) | 104.6 (104.5–104.7) | 104.8 (104.7–104.9) | 104.9 (104.7–105.2) | <0.001 |
| DBP (mmHg) | 66.6 (66.5–66.7) | 66.8 (66.8–66.9) | 66.9 (66.9–67.0) | 66.9 (66.7–67.1) | <0.001 |
| Glucose (mg/dl) | 91.4 (91.3–91.5) | 91.5 (91.4–91.6) | 91.4 (91.3–91.5) | 91.1 (90.9–91.3) | 0.075 |
| Total cholesterol (mg/dl) | 183.4 (183.1–183.8) | 186.8 (186.6–187.1) | 188.0 (187.6–188.3) | 188.3 (187.5–189.0) | <0.001 |
| GGT (U/L) | 19.5 (19.2–19.7) | 20.6 (20.4–20.7) | 21.7 (21.5–21.9) | 21.2 (20.8–21.7) | <0.001 |
| ALT (U/L) | 16.6 (16.4–16.7) | 17.2 (17.2–17.3) | 18.0 (17.8–18.1) | 18.5 (18.2–18.8) | <0.001 |
| HOMA-IR | 1.21 (1.20–1.22) | 1.20 (1.19–1.20) | 1.19 (1.18–1.20) | 1.18 (1.16–1.20) | <0.001 |

| | | | | | |
|---|---|---|---|---|---|
| Total energy intake (kcal/d)[f] | 1502 (1494–1511) | 1505 (1500–1510) | 1471 (1463–1480) | 1415 (1396–1433) | <0.001 |

[a]Adjusted for age and sex; [b] ≥10 g/day; [c] ≥ college graduate; [d] BMI ≥ 25 kg/m$^2$; [e] waist circumference ≥90 cm for men and ≥85 cm for women; [f] among 103,514 participants with plausible estimated energy intake levels (within three standard deviations from the log-transformed mean energy intake).
Abbreviations: ALT, alanine aminotransferase; BMI, body mass index; CVD, cardiovascular disease; DBP, diastolic blood pressure; GGT, gamma-glutamyl transferase; HEPA, health-enhancing physical activity; hs-CRP, high-sensitivity C-reactive protein; HOMA-IR, homeostasis model assessment of insulin resistance; NAFLD, non-alcoholic fatty liver disease; SBP, systolic blood pressure.

**Table 2.** Development of non-alcoholic fatty liver disease (NAFLD) by 25(OH)D levels among participants without NAFLD at baseline (n = 139 599)

| 25(OH)D levels (**ng/mL**) | Person-years (PY) | Incident cases | Incidence density (/ $10^3$ PY) | Age sex-adjusted HR (95% CI) | Multivariable-adjusted HR[a] (95% CI) | | HR (95% CI)[b] in a model with time-dependent variables |
|---|---|---|---|---|---|---|---|
| | | | | | Model 1 | Model 2 | |
| <10 | 114 688.6 | 4310 | 37.6 | 1.00 (reference) | 1.00 (reference) | 1.00 (reference) | 1.00 (reference) |
| 10-19 | 343 136.6 | 16 487 | 48.0 | 0.95 (0.92–0.99) | 0.91 (0.88–0.94) | 0.89 (0.86–0.92) | 0.86 (0.83–0.89) |
| 20-29 | 102 627.3 | 5740 | 55.9 | 0.91 (0.88–0.95) | 0.85 (0.81–0.88) | 0.81 (0.78–0.85) | 0.74 (0.71–0.77) |
| ≥30 | 20 569.0 | 994 | 48.3 | 0.76 (0.71–0.82) | 0.75 (0.70–0.81) | 0.72 (0.67–0.77) | 0.60 (0.56–0.64) |
| *p*–trend | | | | <0.001 | <0.001 | <0.001 | <0.001 |

[a] Estimated from Cox proportional hazards models. Multivariable model 1 was adjusted for age, sex, center, year of screening examination, alcohol consumption, smoking, physical activity, total energy intake, education level, and BMI; model 2: model 1 plus adjustment for medication for hyperlipidemia, glucose-lowering medication, multi-vitamin supplements, vitamin D supplements, and calcium supplements.

[b] Estimated from Cox proportional hazard models with quintiles of vitamin D levels, smoking, alcohol consumption, physical activity, total energy intake, BMI, medication for hyperlipidemia, medication for hyperlipidemia, glucose-lowering medication, multi-vitamin supplements, season, vitamin D supplements, and calcium supplements as time-dependent variables, baseline age, center, year of screening exam, and education level as time-fixed variables.

Abbreviations: BMI, body mass index; CI, confidence interval; HR, hazard ratio

**Table 3.** Resolution of non-alcoholic fatty liver disease (NAFLD) by 25(OH)D levels among subjects with NAFLD at baseline (n = 48 702)

| 25(OH)D levels (**ng/mL**) | Person-years (PY) | Resolution cases | Resolution rate (/ $10^3$ PY) | Age sex-adjusted HR (95% CI) | Multivariable-adjusted HR[a] (95% CI) | | HR (95% CI)[b] in a model with time-dependent variable |
|---|---|---|---|---|---|---|---|
| | | | | | Model 1 | Model 2 | |
| <10 | 25 318.4 | 1819 | 71.8 | 1.00 (reference) | 1.00 (reference) | 1.00 (reference) | 1.00 (reference) |
| 10-19 | 118 651.4 | 8202 | 69.1 | 1.12 (1.06–1.18) | 1.09 (1.03–1.14) | 1.09 (1.03–1.15) | 1.07 (1.01–1.12) |
| 20-29 | 41 262.9 | 2929 | 71.0 | 1.17 (1.10–1.24) | 1.13 (1.06–1.20) | 1.13 (1.06–1.21) | 1.07 (1.01–1.14) |
| ≥30 | 6140.4 | 499 | 81.3 | 1.23 (1.12–1.36) | 1.20 (1.08–1.33) | 1.21 (1.09–1.35) | 1.03 (0.93–1.13) |
| *p*–trend | | | | <0.001 | <0.001 | <0.001 | 0.265 |

[a] Estimated from Cox proportional hazards models. Multivariable model 1 was adjusted for age, sex, center, year of screening examination, alcohol consumption, smoking, physical activity, total energy intake, education level, and BMI; model 2: model 1 plus adjustment for medication for hyperlipidemia, glucose-lowering medication, multi-vitamin supplements, vitamin D supplements, and calcium supplements.
[b] Estimated from Cox proportional hazard models with quintiles of vitamin D levels, smoking, alcohol consumption, physical activity, total energy intake, BMI, medication for hyperlipidemia, medication for hyperlipidemia, glucose-lowering medication, multi-vitamin supplements, season, vitamin D supplements, and calcium supplements as time-dependent variables, baseline age, center, year of screening exam, and education level as time-fixed variables.
Abbreviations: BMI, body mass index; CI, confidence interval; HR, hazard ratio

**Table 4.** Development of non-alcoholic fatty liver disease (NAFLD) by binary categories of 25(OH)D concentration at two examinations, in subjects without NAFLD at baseline (n = 92 792)

| 25(OH)D concentrations (**ng/mL**) | | Person-years (PY) | Incident cases | Incidence density (/ $10^3$ PY) | Age sex-adjusted HR (95% CI) | Multivariable-adjusted HR[a] (95% CI) | |
|---|---|---|---|---|---|---|---|
| Visit 1 | Visit 2 | | | | | Model 1 | Model 2 |
| <20 | <20 | 215 164.6 | 8419 | 39.1 | 1.00 (reference) | 1.00 (reference) | 1.00 (reference) |
| ≥20 | <20 | 30 123.8 | 1390 | 46.1 | 0.96 (0.90–1.01) | 0.94 (0.89–1.00) | 0.92 (0.87–0.98) |
| <20 | ≥20 | 45 558.3 | 1678 | 36.8 | 0.89 (0.84–0.94) | 0.87 (0.82–0.91) | 0.87 (0.82–0.91) |
| ≥20 | ≥20 | 35 618.6 | 1656 | 46.5 | 0.87 (0.82–0.92) | 0.82 (0.78–0.87) | 0.80 (0.76–0.85) |

[a] Estimated from Cox proportional hazards models. Multivariable model 1 was adjusted for age, sex, center, year of screening examination, alcohol consumption, smoking, physical activity, total energy intake, education level, and BMI; model 2: model 1 plus adjustment for medication for hyperlipidemia, glucose-lowering medication, multi-vitamin supplements, vitamin D supplements, and calcium supplements.
Abbreviations: BMI, body mass index; CI, confidence interval; HR, hazard ratio

**Table 5.** Resolution of non-alcoholic fatty liver disease (NAFLD) by binary categories of 25(OH)D levels at two visits, among subjects with NAFLD at baseline (n = 30 848)

| 25(OH)D concentrations (ng/mL) | | Person-years (PY) | Resolution cases | Resolution rate (/ $10^3$ PY) | Age sex-adjusted HR (95% CI) | Multivariable-adjusted HR[a] (95% CI) | |
|---|---|---|---|---|---|---|---|
| Visit 1 | Visit 2 | | | | | Model 1 | Model 2 |
| <20 | <20 | 68 831.8 | 3352 | 48.7 | 1.00 (reference) | 1.00 (reference) | 1.00 (reference) |
| ≥20 | <20 | 11 386.1 | 521 | 45.8 | 0.98 (0.90–1.08) | 0.98 (0.89–1.07) | 0.97 (0.88–1.07) |
| <20 | ≥20 | 12 970.5 | 620 | 47.8 | 1.02 (0.93–1.11) | 1.02 (0.94–1.11) | 1.02 (0.94–1.11) |
| ≥20 | ≥20 | 14 786.6 | 772 | 52.2 | 1.11 (1.02–1.20) | 1.10 (1.02–1.20) | 1.10 (1.01–1.19) |

[a] Estimated from Cox proportional hazards models. Multivariable model 1 was adjusted for age, sex, center, year of screening examination, alcohol consumption, smoking, physical activity, total energy intake, education level, and BMI; model 2: model 1 plus adjustment for medication for hyperlipidemia, glucose-lowering medication, multi-vitamin supplements, vitamin D supplements, and calcium supplements.
Abbreviations: BMI, body mass index; CI, confidence interval; HR, hazard ratio