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Peer Review Report for "Does Vitamin D Independently Lower the Severity of Nonalcoholic Fatty Liver Disease? A Case-Control Study in Khuzestan Province, Iran"

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Revision (0)

Here, you can see the **Reviewers**, **Associate Editors** and **EICs'** comments from the beginning to the end of the revision process.

Hossein Malekzadeh : Reviewer

18 Feb 2025

This manuscript presents a retrospective case-control study investigating the association between vitamin D levels and nonalcoholic fatty liver disease (NAFLD) in Khuzestan province, Iran. The study aims to determine whether vitamin D levels are independently associated with NAFLD, after controlling for potential confounding factors. Given the increasing prevalence of both hypovitaminosis D and NAFLD, this research is relevant and addresses an important public health issue. The focus on an Iranian population adds value due to potential differences in lifestyle, diet, and genetic factors compared to Western populations.

REFEREE : Reviewer

22 Feb 2025

Rrject

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Revision (1)

Reply to Reviewers

Ideally, the reviewing process can significantly improve the submitted manuscripts by allowing the authors to take into account the advice of reviewers. Author(s) must reply to all reviewers' comments in a separate Word file, point by point. A "**Reply to Reviewers**" document is submitted along with revised manuscript during submission of revised files, summarizing the changes that the authors made in response to the reviewers' comments. The responses to reviewers' comments specifies how the authors addressed each comment the reviewers made.

You can read the authors' responses to the reviewers' comments in the next page.

Reviewers' Comments

Reviewer 1:

The study successfully identifies a significant association between vitamin D deficiency and the severity of nonalcoholic fatty liver disease (NAFLD) in a cohort from Khuzestan province, Iran. By employing a robust case-control design and utilizing precise measurements of serum 25(OH)D levels, the research contributes valuable insights into the potential role of vitamin D in liver health. However, despite these noteworthy achievements, the manuscript requires major revisions to enhance clarity, consistency, and grammatical accuracy. Improvements in the methodology section, a more thorough discussion of findings, and refinements in language and structure will strengthen the overall impact of the work and ensure that the conclusions drawn are well-supported and clearly communicated.

1. Can you elaborate on how you determined the sample size of 400 participants? Was a power analysis conducted?

Action: I appreciate your insightful comment. The study conducted by Abbasi et al. (1), established that, with a 5% error margin and an 80% power, the required sample size for each group was calculated to be 190 individuals. Factoring in a 5% dropout rate, this resulted in an approximate total of 200 individuals per group. The calculation was based on the following formula:

$$n = \frac{\frac{Z^2 pq}{d^2}}{1 + \frac{1}{N} \left(\frac{Z^2 pq}{d^2} - 1 \right)}$$

1. Abbasi MMH ZM, Moghadam SD, Ghahestani FA, Robati FK. Vitamin D3 Deficiency in Non-Alcoholic Fatty Liver Disease. *Acta Medica Iranica*. 2019:709-13.

2. How did you ensure that the control group was adequately matched to the NAFLD group beyond BMI and age? Were other factors like lifestyle or dietary habits considered?

Action: I appreciate your thoughtful comment. In addition to age and body mass index, we considered comorbidities, including smoking and medication usage. Furthermore, considering the geographical context of the patient's residence and acknowledging that individuals who visit teaching hospitals typically share a similar socioeconomic status, it can be inferred that their lifestyles and dietary practices are likely to be analogous.

3. You mentioned controlling for confounding factors. Can you specify which factors were controlled for and how this was achieved?

Action: Thank you for your nice comment. Subjects with confounding factors were excluded, including those with diabetes, dyslipidemia, autoimmune hepatitis, chronic viral hepatitis, celiac disease, metabolic bone disorders, cholestatic liver disease, hemochromatosis, as well as smokers, and individuals taking vitamin D, insulin, or oral hypoglycemic drugs. The information presented above was derived from historical data, test results, and paraclinical findings.

4. How were serum 25(OH)D levels measured, and what steps were taken to ensure the accuracy and reliability of these measurements?

Action: Thank you for your valuable comment. The measurement of vitamin D levels can be conducted using two primary methods: chromatography, specifically mass spectrometry coupled with liquid chromatography (MS/LC-MS), and immunoassay techniques. At present, the absence or expensiveness of high-performance chromatography kits necessitates the use of the ELISA method, which exhibits an inaccuracy of up to 10 percent and an error margin of 15 percent. Therefore, we used this method to ensure the accuracy and reliability of the measurements.

5. The ROC curve indicates a sensitivity of 63.5% and specificity of 66.5%. How do you interpret these results in the context of clinical significance for diagnosing NAFLD?

Action: Thank you for your nice comment. A sensitivity of 63.5 percent and a specificity of 66.5 percent at this cutoff point provide evidence that persons with vitamin D levels below 26.4 are likely to have nonalcoholic fatty liver disease. This is proven by the fact that the data reveals that those with these levels are likely to have the desired condition.

6. You noted significant differences in vitamin D insufficiency between male and female patients. What might be the underlying biological or environmental factors contributing to this difference?

Action: Thank you for your valuable comment. In our study, the observed difference was exclusively present among individuals with health issues, while no significant variation was noted in healthy individuals or across different grades. This could be attributed to factors such as prolonged working hours in confined office settings for men, variations in dietary habits and lifestyle choices among men, and increased weight linked to obesity rather than muscle mass, as well as the types of clothing worn in professional environments and variations in skin color that the proof of it needs more comprehensive studies.

7. You mentioned the use of sonography rather than biopsy. How do you think this limitation affects the validity of your findings regarding the severity of NAFLD?

Action: Thank you for your insightful comment. You are right; the selection of that ultrasound technique to use for our research was impacted by factors such as accessibility and cost. The accuracy of this procedure is intrinsically limited in comparison to the findings of a biopsy since

it is greatly dependent on the quality of the equipment and the expertise of the sonographer. Despite this, a biopsy would be more expensive and would need an invasive process, both of which made it more difficult for us to get this statistical population.

8. How might the findings of this study be generalized to populations outside of Khuzestan province, considering regional dietary and lifestyle differences?

Action: Thank you for your nice comment. After taking into account the longer periods spent inside as a consequence of higher temperatures, it is not inconceivable that vitamin D levels might decrease. On the other hand, it seems that the present eating habits have not changed, even though they are impacted by both digital surroundings and economic concerns. This study must be carried out in a number of different places around the nation to come at a result that is reliable.

9. In scientific and statistical reporting, it is standard practice for the number of decimal places in the mean and the standard deviation (SD) to match. This consistency helps maintain clarity and precision in the presentation of data. For example, if the mean is reported as 89.85 ± 8.2 , it should be formatted as 89.85 ± 8.20 to ensure that both values have the same number of decimal places. Ensure that all means and SDs in Tables 1-3 follow this formatting rule. If the mean is presented with two decimal places, the SD should also be presented with two decimal places.

Action: Thank you for your helpful suggestion. We are sorry for making such a mistake. To ensure that all means and SDs in Tables 1-3 follow the same formatting rule, we have revised and highlighted the revised manuscript.

Here are several suggestions to improve the quality of the article:

a) Provide a more detailed overview of the global prevalence of NAFLD and vitamin D deficiency, including recent statistics and studies and summarize previous research findings more comprehensively to highlight gaps in the current literature that your study addresses.

Action: Thank you for your valuable suggestion. We provide a more detailed overview of the global prevalence of NAFLD and vitamin D deficiency, including recent statistics and studies, and summarize previous research findings more comprehensively and highlight it in the revised manuscript.

The most common cause of chronic liver disease globally is NAFLD. With greater rates seen in men (40%) than women (26%), a comprehensive analysis calculated the worldwide prevalence of NAFLD among adults to be around 32%. Especially, the frequency has changed with time; it started at 26% in research done in 2005 or before and rose to 38% in studies done from 2016 forward (2). According to reports from the United States, NAFLD prevalence there is 38%, a 50% rise over the previous three decades (3).

Vitamin D is a common problem with a major risk factor for NAFLD. Between 2000 and 2022, a thorough meta-analysis projected 15.7% of worldwide serum 25-hydroxyvitamin D [25(OH)D] values below 30 nmol/L. Region, latitude, age, gender, and socioeconomic level all affect this shortfall (3). People who live farther from the equator have more vitamin D insufficiency. Serum 25(OH)D levels < 30 nmol/L, for example, were found in 14.9% of individuals residing at 40–60 degrees north latitude; this incidence rose to 57.4% among those living at 60–80 degrees north latitude (4). Lower-middle-income nations showed a greater prevalence of vitamin D insufficiency (26.7%) than upper-middle-income (10.2%) and high-income countries (15.1%); hence, socioeconomic elements also play a part. Age and gender also clearly show disparities; those under 18 years old had a greater frequency of blood 25(OH)D levels below 50 nmol/L (48.5%), and vitamin D insufficiency was more prevalent in women (17.8%) than in men (13.6%) (5). Furthermore, recent studies underlined that many people with type 2 diabetes lack

vital micronutrients like vitamin D. Type 2 diabetes increases the risk of developing severe liver diseases, including fibrosis, liver transplant, and death (6, 7).

b) Detailed Protocol: Include more specifics about the methodology used for participant selection, data collection, and analysis. This includes the exact procedures for measuring liver health and vitamin D levels.

Action: Thank you for your insightful suggestion. We included the Detailed Protocol, which includes the exact procedures for measuring liver health and vitamin D levels, in the Methods section and highlighted it in the revised manuscript.

Vitamin D measurement

The blood 25 (OH)D level was measured by the Immunology Research Lab at the Immunology Department of Ahvaz Jundishapur University of Medical Sciences Hospitals using a 25-HYDROXYVITAMIN D [25(OH)D] ELISA (Enzyme-linked immunosorbent assay) kit (CAN-VD-510) (8). Competitive ELISA compares the sample's 25(OH)D to a labeled form for binding to an antibody. The sample's target analyte concentration was negatively associated with its tagged concentration. Wells on a coated microtiter plate with anti-25(OH)D antibodies. Standard curves are created using 25(OH)D concentrations to assess performance; samples with known values are beneficial. The biotin-labeled 25(OH)D reagent battles the sample's 25(OH)D with an antibody. Streptavidin-based Horseradish Peroxidase (HRP) conjugates biotinylated 25(OH)D. Normal substrate solution tetramethylbenzidine (TMB) interacts with HRP to change color. Use sulfuric or phosphorous acid to inhibit enzymatic activity.

Detailed protocol of competitive ELISA

We diluted the concentrated solution to make wash buffer and diluted the biotinylated 25(OH)D reagent using the supplied diluent. Then, we pipetted 25 μ L of standards, controls, and samples into designated wells and added 100 μ L of diluted biotinylated 25(OH)D reagent to each well. We let the dish sit at room temperature with a cover for two hours and washed each with 300 μ L of wash buffer three times and removed the buffer by lightly tapping the plate with absorbent paper after the final wash. Place 100 μ L of streptavidin-HRP conjugate in each well. Washing repeated. After that we added 100 μ L of TMB substrate solution to each well and darkly

incubated at ambient temperature for 10–15 minutes. The color should develop—a blue tinge suggests. We added 50 μL of stop solution to each well. Yellow replaced blue. To measure absorbance at 450 nm 30 minutes after applying the stop solution, we used a microplate reader 5. We plotted the mean absorbance of each standard concentration on the y-axis against the corresponding 25(OH)D concentration on the x-axis to construct a standard curve. Interpolating absorbance values from the standard curve gives sample 25(OH)D concentrations. In this competitive test, greater sample absorbance indicated lower 25(OH)D concentrations (9).

Every additional laboratory test, including coagulation tests and assessments of kidney and liver function, was performed by standard protocols.

Detailed protocol of hepatic ultrasonography

Hepatic ultrasonography (UK) is a simple, affordable, radiation-free, and safe method of identifying liver fatty infiltration. A convex transducer operating at 2–5 MHz may be used for the examination. The liver parenchyma's homogenous echotexture and echogenicity are comparable to or somewhat higher than those of the spleen and renal cortex. Because of fatty infiltration, the liver exhibits stronger echogenicity than the spleen and renal cortex. Visual study of the echogenicity's intensity has led to the proposal of many (0–3) classes of steatosis, assuming that the gain setting is optimal. Grade I fatty infiltration occurs when the echogenicity is simply elevated; grade II occurs when the echogenic liver obscures the echogenic walls of portal vein branches; and grade III occurs when the echogenic liver obscures the diaphragmatic outline. However, there is inter-observer heterogeneity in these. USG has a sensitivity of 60 to 94% and a specificity of 84 to 95% in identifying hepatic steatosis. With a cut-off of 1.49, the hepatorenal sonographic index—the ratio of the liver's mean brightness level to that of the right kidney—has also been suggested as a gauge of hepatic steatosis. It has a very high sensitivity (100%) and specificity (91%) for the diagnosis of steatosis greater than 5% (10).

c) Use graphs or charts to visually represent key findings, such as the differences in vitamin D levels between NAFLD patients and controls.

Action: Thank you for your kind suggestion. We also input graphs to visually represent key findings, such as the differences in vitamin D levels between NAFLD patients and controls with different ages and genders in Figures 2 and 3.

d) Provide a more thorough interpretation of the results in the context of existing literature. Discuss potential mechanisms linking vitamin D deficiency to NAFLD.

Action: Thank you for your insightful suggestion. We included and discussed potential mechanisms linking vitamin D deficiency to NAFLD and highlighted it in the revised manuscript.

Considered the most common cause of chronic liver disease globally is NAFLD. With greater rates seen in men (40%) than women (26%), a comprehensive analysis calculated the worldwide prevalence of NAFLD among adults to be around 32%. Especially, the frequency has changed with time; it started at 26% in research done in 2005 or before and rose to 38% in studies done from 2016 forward (2). According to reports from the United States, NAFLD prevalence there is 38%, a 50% rise over the previous three decades (3).

Deficiency of vitamin D is a common problem with a major risk factor for NAFLD. Between 2000 and 2022, a thorough meta-analysis projected 15.7% of worldwide serum 25-hydroxyvitamin D [25(OH)D] values below 30 nmol/L. Region, latitude, age, gender, and socioeconomic level all affect this shortfall (3). People who live farther from the equator have more vitamin D insufficiency. Serum 25(OH)D levels < 30 nmol/L, for example, were found in 14.9% of individuals residing at 40–60 degrees north latitude; this incidence rose to 57.4% among those living at 60–80 degrees north latitude (4). Lower-middle-income nations showed a greater prevalence of vitamin D insufficiency (26.7%) than upper-middle-income (10.2%) and high-income countries (15.1%); hence, socioeconomic elements also play a part. Age and gender also clearly show disparities; those under 18 years old had a greater frequency of blood 25(OH)D

levels below 50 nmol/L (48.5%), and vitamin D insufficiency was more prevalent in women (17.8%) than in men (13.6%) (5). Furthermore, recent studies underlined that many people with type 2 diabetes lack vital micronutrients like vitamin D. Type 2 diabetes increases the risk of developing severe liver diseases, including fibrosis, liver transplant, and death (6, 7).

Here's an evaluation of the English writing and grammar of the article, along with specific examples and suggestions for improvement:

1. Clarity and Conciseness

- Example: "Hypovitaminosis D is becoming a global epidemic."
- Suggestion: Consider rephrasing for clarity. For instance, "Vitamin D deficiency is increasingly recognized as a global health issue."

Action: Thank you for your valuable suggestion. We checked the Clarity and Conciseness and highlighted the sentences in the revised manuscript.

2. Sentence Structure

- Example: "Therefore, its absence may enhance hepatocyte fat formation."
- Suggestion: This sentence could be more concise. You could say, "Thus, a deficiency may promote fat accumulation in hepatocytes."

Action: Thank you for your valuable suggestion. We checked the Sentence Structure and highlighted the sentences in the revised manuscript.

3. Word Choice

- Example: "The results showed that, in contrast to age, vitamin D insufficiency, gender, and a higher BMI were substantially linked to an increased risk of NAFLD."
- Suggestion: Replace "substantially linked" with "significantly associated" for a more precise scientific tone: "The results indicated that, unlike age, vitamin D insufficiency, gender, and higher BMI were significantly associated with an increased risk of NAFLD."

Action: Thank you for your valuable suggestion. We checked the Word Choice and replaced and highlighted them in the revised manuscript.

4. Consistency in Terminology

- Example: "Vitamin D insufficiency is defined by serum 25(OH)D below 20 ng/ml."
- Suggestion: Ensure consistency in units throughout the article. If you use "ng/mL" in one instance, maintain that format throughout. Change to "Vitamin D insufficiency is defined as serum 25(OH)D levels below 20 ng/mL."

Action: Thank you for your valuable suggestion. We checked the Consistency in Terminology and corrected and highlighted them in the revised manuscript.

5. Punctuation and Formatting

- Example: "Vitamin D insufficiency was found in 70% of NAFLD patients and 20% of controls."
- Suggestion: Add a comma before "and" for clarity: "Vitamin D insufficiency was found in 70% of NAFLD patients, and 20% of controls."

Action: Thank you for your valuable suggestion. We checked the Punctuation and Formatting and corrected and highlighted them in the revised manuscript.

6. Use of Passive Voice

- Example: "Receiver operating characteristic (ROC) was employed in this analysis."
- Suggestion: Active voice can make sentences stronger. Consider: "We employed receiver operating characteristic (ROC) analysis in this study."

Action: Thank you for your valuable suggestion. We checked the Use of Passive Voice in the text and corrected and highlighted them in the revised manuscript.

7. Ambiguity

- Example: "The study included 200 NAFLD patients and 200 healthy controls."
- Suggestion: Clarify how these groups were selected. For example: "The study comprised 200 NAFLD patients, diagnosed based on specific criteria, and 200 age- and gender-matched healthy controls."

Action: Thank you for your valuable suggestion. We checked the Ambiguity in the text and corrected and highlighted them in the revised manuscript.

8. Redundancy

- Example: "Research on NAFLD detected by sonography indicates that the illness is becoming more common in Iran."
- Suggestion: Avoid redundancy in phrasing. You could simplify it to: "Sonographic studies show that NAFLD is increasingly prevalent in Iran."

Action: Thank you for your valuable suggestion. We checked the Redundancy in the text and corrected and highlighted them in the revised manuscript.

9. Grammar and Tense Consistency

- Example: "Additionally, the population-based research found that NAFLD is more prevalent in males, and vitamin D insufficiency is more common in women."
- Suggestion: Maintain consistent tense. Change to: "Additionally, population-based research has found that NAFLD is more prevalent in males, while vitamin D insufficiency is more common in females."

Action: Thank you for your valuable suggestion. We checked the Grammar and Tense Consistency and corrected and highlighted them in the revised manuscript.

10. Conclusion Strengthening

- Example: "Low levels of 25(OH) vitamin D are independently associated with NAFLD, regardless of any confounding factors."
- Suggestion: Strengthen the conclusion by summarizing implications: "Our findings suggest that low levels of 25(OH) vitamin D are independently associated with NAFLD, highlighting the need for further research on vitamin D supplementation as a potential therapeutic approach."

Action: Thank you for your valuable suggestion. We checked the Conclusion Strengthening and corrected and highlighted it in the revised manuscript.

Reviewer 2:

Reject

Reviewer 3:

This manuscript presents a retrospective case-control study investigating the association between vitamin D levels and nonalcoholic fatty liver disease (NAFLD) in Khuzestan province, Iran. The study aims to determine whether vitamin D levels are independently associated with NAFLD, after controlling for potential confounding factors. Given the increasing prevalence of both hypovitaminosis D and NAFLD, this research is relevant and addresses an important public health issue. The focus on an Iranian population adds value due to potential differences in lifestyle, diet, and genetic factors compared to Western populations.

Action: Thank you for your kind comment.

Associate Editor's Comments:

Associate Editor 1:

Comments to the Authors

1. In abstract section, mention the specific cutoff value for vitamin D that was found to be associated with increased NAFLD risk in the abstract (26.4 ng/ml).

Action: Thank you for your valuable suggestion. We corrected and highlighted them in the revised manuscript.

Conclusions: Regardless of any confounding variables, low levels of 25(OH) vitamin D are independently linked to NAFLD, and 26.4 ng/ml was the precise cutoff value for vitamin D that was associated with an elevated risk of NAFLD.

2. In the introduction section, provide more specific data on the prevalence of NAFLD in Iran, with references to relevant epidemiological studies.

Action: Thank you for your valuable suggestion. We corrected and highlighted them in the revised manuscript.

According to recent thorough research, 33% of the 41,971 Iranian participants had NAFLD overall. For both men and women, the frequency was 35% and 37%, respectively. The study's findings indicate that NAFLD is very prevalent in Iran (11).

3. In introduction section, expand on the potential mechanisms by which vitamin D may influence liver health and NAFLD development. Discuss the role of vitamin D in regulating lipid metabolism, inflammation, and insulin sensitivity.

Action: Thank you for your valuable suggestion. We corrected and highlighted them in the revised manuscript.

Vitamin D helps maintain liver health by regulating lipid metabolism, suppressing pro-inflammatory cytokines (e.g., TNF- α and IL-6), enhancing anti-inflammatory cytokines (e.g., IL-

10), and improving insulin sensitivity. This may impact the onset and progression of nonalcoholic fatty liver disease (NAFLD). Increases insulin receptor expression and glucose absorption in the liver and peripheral organs. Vitamin D may regulate lipid metabolism, reduce inflammation, and improve insulin sensitivity to prevent or slow NAFLD and its development into more severe liver illnesses, including NASH and fibrosis (12, 13).

4. In introduction section, clearly state the rationale for conducting this study in Khuzestan province, Iran. Are there any unique characteristics of this population that make this research particularly important?

Action: Thank you for your valuable suggestion. We corrected and highlighted them in the revised manuscript.

In light of the high incidence of vitamin D deficiency and the widespread occurrence of NAFLD in Iran, coupled with the scarcity of relevant studies, we resolved to investigate the correlation between vitamin D3 levels and the severity of NAFLD among patients. This study specifically excluded confounding variables, such as individuals with diabetes, dyslipidemia, and smokers, from the beginning, while also considering the unique climatic conditions, clothing styles, and dietary habits of the Khuzestan region. We examined the vitamin D levels of those with NAFLD and those of healthy people as an independent factor.

5. Provide more details on the criteria used to diagnose NAFLD based on abdominal ultrasonography. What specific features were assessed?

Action: Thank you for your valuable suggestion. We corrected and highlighted them in the revised manuscript.

Detailed protocol of hepatic ultrasonography

Hepatic ultrasonography (UK) is a simple, affordable, radiation-free, and safe method of identifying liver fatty infiltration. A convex transducer operating at 2–5 MHz may be used for the examination. The liver parenchyma's homogenous echotexture and echogenicity are comparable to or somewhat higher than those of the spleen and renal cortex. Because of fatty infiltration, the liver exhibits stronger echogenicity than the spleen and renal cortex. Visual study of the echogenicity's intensity has led to the proposal of many (0–3) classes of steatosis, assuming that the gain setting is optimal. Grade I fatty infiltration occurs when the echogenicity is simply elevated; grade II occurs when the echogenic liver obscures the echogenic walls of portal vein branches; and grade III occurs when the echogenic liver obscures the diaphragmatic outline. However, there is inter-observer heterogeneity in these. USG has a sensitivity of 60% to 94% and a specificity of 84% to 95% in identifying hepatic steatosis. With a cut-off of 1.49, the hepatorenal sonographic index—the ratio of the liver's mean brightness level to that of the right kidney—has also been suggested as a gauge of hepatic steatosis. It has a very high sensitivity (100%) and specificity (91%) for the diagnosis of steatosis greater than 5% (10).

6. Clarify the matching criteria used to select healthy controls. Were controls matched individually to cases based on specific characteristics, or was frequency matching used?

Action: Thank you for your valuable suggestion. We corrected and highlighted them in the revised manuscript.

The control group consisted of patients aged 18 to 65, all of whom had ultrasound results classified as usual (grade 0). Similar to the patient group, we controlled for confounding factors, ensuring that they experienced the same conditions as the patient group, with the sole exception of the ultrasound. The control group consisted of patients aged 18 to 65, all of whom had ultrasound results classified as usual (grade 0). Similar to the patient group, we controlled for confounding factors, ensuring that they experienced the same conditions as the patient group, with the sole exception of the ultrasound. We ensured that they were matched based on pre-existing comorbidities, smoking status, alcohol usage, height, weight, age, gender, alanine

transferase (ALT), aspartate transferase (AST), alkaline phosphatase (ALP), and body mass index (BMI).

7. While the study collected data on the grade of NAFLD (Grades 0-III), there is no mention of how this was incorporated into the analysis. Was the association between vitamin D and NAFLD analyzed separately for different grades of NAFLD? This could provide valuable insights into the relationship between vitamin D and NAFLD severity.

Action: Thank you for your nice comment. The investigation was conducted, and the results are detailed in Table 3. The Vitamin D levels recorded for grade one patients were 27.3 ± 10.20 , for grade two they were 22.68 ± 6.98 , and for grade three, the levels were 19.93 ± 7.60 .

8. Include p-values for all comparisons between cases and controls in Table 1.

Action: Thank you for your nice comment. In the sections where the P-value is not present, the low incidence of cases related to the underlying diseases within the total sample size of 400 individuals provided it insignificant, resulting in an overall calculation of 0.68, which is shown in Table 1.

9. Provide more details on Spearman's rank correlation coefficients. Which variables were significantly correlated with 25(OH)D levels?

Action: Thank you for your nice comment. The data presented in Table 4 indicates a notable correlation between BMI and various stages of fatty liver. The analysis revealed no significant differences in gender between patients with varying grades of fatty liver and healthy subjects. Notable variations in vitamin D levels were identified among patients classified in grades 1, 2, and 3 compared to healthy individuals. Table 5 indicates a significant correlation between the gender of NAFLD patients and varying vitamin D levels when compared to healthy subjects.

This study demonstrates a significant relationship between the age of healthy subjects and varying vitamin D levels in contrast to NAFLD patients. All of them have been methodically identified in the results section of the manuscript.

Table 4 illustrates a significant relationship between BMI and the various stages of fatty liver. The analysis revealed no significant differences in gender between patients with varying degrees of fatty liver and healthy individuals. Notable variations in vitamin D levels were observed among patients classified in grades 1, 2, and 3 compared to healthy individuals.

Results demonstrate a significant correlation between the gender of NAFLD patients and varying levels of vitamin D when compared to healthy individuals. The study shows a significant relationship between the age of healthy individuals and varying levels of vitamin D in contrast to patients with NAFLD (Table 5, Figure 2, 3).

10- Provide more details on the ROC curve analysis, including the area under the curve (AUC) and the optimal cutoff value for vitamin D.

Action: Thank you for your nice comment. The analysis of the ROC curve led us to determine a cut-off value of 26.4. A serum 25-hydroxyvitamin D level below 26.4 is associated with an increased risk of NAFLD, exhibiting a sensitivity of 63.5% and a specificity of 66.5%. The area under the curve (AUC) is measured at 0.66, reflecting the diagnostic efficacy of the test, characterized by a true positive rate and a suboptimal overall performance.

An ROC curve was generated to evaluate the balance between specificity and sensitivity concerning the association between vitamin D levels in NAFLD patients and healthy individuals. The threshold was established at 26.4 (AUC = 0.66, $P < 0.001$), demonstrating a sensitivity of 63.5% and a specificity of 66.5% (Figure 4).

11. Discuss the clinical implications of the findings for the prevention and management of NAFLD. Should clinicians routinely screen for vitamin D deficiency in patients with NAFLD?

Action: Thank you for your valuable suggestion. In this study, the association between vitamin D deficiency and the severity of NAFLD is evident, with fatty liver patients exhibiting significantly lower vitamin D levels compared to healthy individuals. Estimating vitamin D levels may contribute to a reduction in the risk of NAFLD. Vitamin D supplementation may play a role in decelerating the progression of NAFLD. Nonetheless, the small sample size in our study restricts the applicability of the findings to the broader population. In cross-sectional studies, the complete elimination of selection bias may not always be achievable. Therefore, we suggested further multicenter studies with larger sample sizes be done to validate these findings.

12. Proofread the manuscript carefully to correct any typos or grammatical errors.

Action: Thank you for your valuable suggestion. We checked the carefully to correct any typos or grammatical errors and corrected and highlighted them in the revised manuscript.

13. Ensure consistency in the units used throughout the manuscript.

Action: Thank you for your valuable suggestion. We checked the carefully to ensure consistency in the units used throughout the manuscript and corrected and highlighted them in the revised manuscript.

Best regards

1. Abbasi MMH ZM, Moghadam SD, Ghahestani FA, Robati FK. Vitamin D3 Deficiency in Non-Alcoholic Fatty Liver Disease. *Acta Medica Iranica*. 2019;709-13.
2. Teng ML, Ng CH, Huang DQ, Chan KE, Tan DJ, Lim WH, et al. Global incidence and prevalence of nonalcoholic fatty liver disease. *Clinical and molecular hepatology*. 2022;29(Suppl):S32.
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