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Indices of Hepatic Steatosis and Fibrosis in Prediabetes and Association with Diabetes Development in the Vitamin D and Type 2 Diabetes Study

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Abstract:	Aims: Non-alcoholic fatty liver disease (NAFLD) is a common comorbidity that leads to poor outcomes in people at high risk for development of type 2 diabetes (T2D). Vitamin D is a possible mediator. In the vitamin D and type 2 diabetes study (D2d), we investigated the relationship of baseline indices of NAFLD with incident T2D and whether vitamin D modified the association. Methods: Cross-sectional associations of indices of NAFLD with glycemia and vitamin D status were assessed in 3,972 individuals screened for the D2d study. In those with prediabetes randomized to vitamin D or placebo (n=2,423), we examined longitudinal associations of NAFLD indices with incident T2DM. We used validated non-invasive scores to assess steatosis [(hepatic steatosis index (HSI); NAFLD-liver fat score (NAFLD-LFS)] and advanced fibrosis [fibrosis-4 (FIB-4) index; AST to Platelet Ratio Index (APRI)]. Results: Eighty-five percent of participants had likely steatosis by HSI and 71% by NAFLD-LFS; 3% were likely to have advanced fibrosis by FIB-4 and 1.2% by APRI. FIB-4 indicated that 20.4% of individuals require further follow up of liver health. Steatosis and fibrosis scores were higher among participants with worse glycemia. The NAFLD-LFS and APRI predicted development of diabetes (hazard ratios [95%CI] 1.35 [1.07, 1.70]; p = 0.012) and 2.36 (1.23, 4.54; p = 0.010), respectively). The effect of vitamin D on diabetes risk was not modified by baseline NAFLD indices. Individuals with likely steatosis had a smaller increase in serum 25-hydroxyvitamin D level in response to vitamin D than those without steatosis. Conclusions: The predicted high prevalence of steatosis, the need for further fibrosis workup, and the relationship between liver health and incident T2D suggest that routine screening with clinically accessible scores may be an important strategy to reduce disease burden.
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Dear Reviewers,

We appreciate your valuable insights which have helped to improve the quality of our manuscript. Please find our responses to your comments below in blue font:

Reviewer #1:

GENERAL COMMENT

Observational studies have suggested that vitamin D plays a role in modulating diabetes risk (J Clin Endocrinol Metab. 2020 Dec 1;105(12):3721-33). However, more recent analysis found that studies remain inconclusive and controversial, in part, due to a lack of understanding of the threshold effects of vitamin D (World J Diabetes. 2021 Sep 15;12(9):1363-1385). As a potentially diabetogenic condition (Gut. 2021 May;70(5):962-969), NAFLD represents a disease model suitable for evaluating the effect of Vitamin D in preventing incident T2D if most of the variables under scrutiny are sufficiently characterized. For example, NAFLD is only one (although probably the most common in some areas of the world) among the various causes of hepatic steatosis. Indeed, this may occur owing to a variety of secondary causes (Dig Liver Dis. 2010 Apr;42(4):272-82) and differential diagnosis is important given that certain disease cofactors (Metab Target Organ Damage 2022;2:12. http://dx.doi.org/10.20517/mtod.2022.14) may themselves be diabetogenic.

A couple of years ago, a process in favor of renaming NAFLD to MAFLD has changed the diagnostic process aiming to move from a diagnosis of exclusion (nonalcoholic) to a positive criterion (metabolic) (Metabolism. 2020 Dec;113:154413. Lancet Gastroenterol Hepatol. 2022 May;7(5):388-390). Additionally, a consistent line of research strongly suggests personalized medicine approaches in the arena of NAFLD and metabolic disorders. The most basic initiative to promote such approaches is to process data by sex. Finally, a variety of diagnostic tools are available to identify hepatic steatosis noninvasively, spanning from imaging techniques to biomarkers. Collectively, these techniques hold promise to allow identification of presence and categorization of (fibrotic) severity although some caveats must be kept in mind (Aliment Pharmacol Ther. 2014 Nov;40(10):1209-22), with the population of those with T2D and those with advancing ages being particularly prone to complex interpretation of findings (Clin Gastroenterol Hepatol. 2022 Mar 11:S1542-3565(22)00248-8; Am J Gastroenterol. 2017 May;112(5):740-751.).

- We thank the reviewer for these important comments and agree that the relationships between diabetes, liver disease and metabolism are complex. The renaming and redefining of NAFLD is an important endeavor that is ongoing.

With this complex backset, submission JDC-D-01363 reports that, compared to placebo, vitamin D supplementation did not modulate the association between NAFLD-LFS and APRI with diabetes progression. Points of strength of the study include: topic is of clinical relevance; a large study population is evaluated. Points of weakness: further sex-based analysis of data is indicated; the manuscript should be re-written more consistently and concisely.

- Thank you. We address the comment about a sex-based analysis below. We have made significant edits to ensure the revised manuscript is clearer and more concise.

SPECIFIC COMMENT

MAJOR

1. Abstract - this section needs reworking given that it fails to specifically illustrate the rationale and aims of the study. Of concern, there seem to be no data regarding the number of individuals enrolled.

- We have clarified the rationale and aims of the study, and the “N” of each group in the abstract.

2. Introduction -

a) This section has long unreferenced statements, please add supporting bibliography where appropriate (e.g. lines 72-78).

- The journal has a reference limit of 50. To adhere to this, we included some comprehensive reviews as citations. The example you provide is cited with a comprehensive review that provides the definitions and pathophysiology of NAFLD, its relationship to complications, and comparison of complications in people with and without type 2 diabetes.

b) Lines 110-116: do these authors believe that points 1. And 2. Really deserve attention? It may be argued that these notions are universally accepted (Lancet Diabetes Endocrinol. 2022 Apr;10(4):284-296. J Diabetes. 2022 Sep;14(9):606-619. J Clin Invest. 2020 Jun 1;130(6):3305-3314. Ultrasound Q. 2019 Dec;35(4):330-338. Acta Diabetol. 2019 Apr;56(4):385-396) and listing too many aims may be misleading to the reader. All in all, it may be suggested focusing on points 3. And 4. Alone.

- We agree that points 1 and 2 are reasonably well established; however, they are important to document in the D2d cohort which is a modern cohort of people with prediabetes and to set the stage for the remaining aims. We have combined and streamlined these aims. We provide more clarity in our presentation of the aims and provide results and discussion clearly within each aim.

c) This paragraph is devoted to NAFLD. However, title focuses on "Indices of Hepatic Steatosis and Fibrosis" (not NAFLD) and (line 158) these authors clearly state that "Secondary causes of liver steatosis (i.e., alcohol use, medications etc.) were not assessed". To avoid these elements of ambiguity and inconsistency, it may be suggested to work on the relatively MAFLD definition (as opposed to NAFLD).

- We appreciate the opportunity to clarify. We avoided the use of NAFLD in the title because the scores are not diagnostic and did not want to give the wrong impression to the readers. The indices are indicators of potential steatosis or fibrosis. The scores were developed prior to the current debate about the appropriate nomenclature change, which is still ongoing, and the change is not widely accepted yet. Hence, we are opting to retain NAFLD.

3. Method -

a) a robust line of research has identified sex differences as a major modifier both in chronic liver disease/NAFLD and in deranged glucose metabolism (Lancet. 2020 Aug 22;396(10250):565-582). On these grounds, it is recommendable conducting analyses separately by sex rather than using sex as a covariate. This personalized approach has also been envisaged as a definite priority in NAFLD/MAFLD arena research.

- We have elected not to show results by sex because (1) the HSI index has sex in the calculation (2) We examined whether there is an interaction by sex for NAFLD-LFS, FIB-4 and APRI and there was no interaction (p for interaction 0.68, 0.81, and 0.96, respectively) (3) sample sizes, especially for the high fibrosis group, would be small and results might be misleading. In the revision, we have included the reviewer's point, the results above and enhanced the Discussion.

b) among the various surrogate indices of steatosis Fatty Liver Index first described by Bedogni & Bellentani (Metab Target Organ Damage 2021;1:10. http://dx.doi.org/10.20517/mtod.2021.08) should also be tested (PLoS One. 2021 Apr 6;16(4):e0249221.BMJ Open. 2021 Aug 25;11(8):e045498. PLoS One. 2018 Jun 1;13(6):e0198327. J Clin Endocrinol Metab. 2016 Nov;101(11):4030-4038.).

- The FLI is an excellent surrogate index. We are unable to evaluate the FLI because we do not have all the variables within our dataset. We added as a potential limitation in the discussion.

c) it would be important to address indices of cardiovascular risk and renal function as related to liver health (Lancet Gastroenterol Hepatol. 2021 Nov;6(11):903-913.Clin Mol Hepatol. 2022 Jul;28(3):565-574. Int J Mol Sci. 2022 Nov 1;23(21):13320).

- We agree with the reviewer that there is a relationship between liver health and cardiovascular risk and renal function. We have elected not to show such data in this paper to maintain focus. We added a comment in the discussion. We will consider examining the relationship between liver health and cardiovascular risk and renal function in a future paper.

MINOR

1. "The D2d design and methods have been described" it would be reader-friendly adding "in short…."
   - We provide some additional details of the D2d study in the Methods section.
2. It would be important to evaluate the role, if any, played by GGT in identifying specific patient populations (Metab Target Organ Damage 2022;2:17. http://dx.doi.org/10.20517/mtod.2022.20).
   - We agree. Unfortunately, GGT is not available in our dataset.
3. Conclusions fail to comment on the most important (negative) findings.
   - We dramatically simplified the discussion for clarity and added this specific point to the conclusion.

Reviewer #2:

The present study investigated an association between potential steatosis and advanced fibrosis at baseline and or vitamin D status, whether the estimated presence of steatosis and advanced fibrosis at baseline predicts development of diabetes, and whether this association is modulated by vitamin D supplementation.

The authors concluded that populations at high risk for T2D are also at high risk for hepatic steatosis and, to a lesser extent, fibrosis.

Minor Comments

1. Did you measure serum insulin levels at baseline? If you were doing, please add HOMA-IR to Table 1.
   - We have added HOMA2-%S in table 1. We opted to add HOMA2%S, which is the inverse of HOMA-IR, to be consistent with another paper in the D2d cohort that has reported such data (https://doi.org/10.1210/clinem/dgab649).
2. Please add platelet counts to Table 1.
   - We have added platelets in Table 1.

Highlights

- In people with prediabetes, predicted hepatic steatosis is high.
- ~1 in 5 people require further investigation for advanced fibrosis.
- Glycemic control was related to predicted prevalence of steatosis/fibrosis
- Predicted presence of hepatic steatosis/fibrosis associated with increased incident T2D
- Vit D did not modulate association of predicted steatosis/fibrosis and incident T2D

Indices of Hepatic Steatosis and Fibrosis in Prediabetes and Association with Diabetes Development in the Vitamin D and Type 2 Diabetes Study

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Short Title: Steatosis and Fibrosis in Prediabetes

Key Words: Vitamin D, NAFLD, FIB-4, APRI, NAFLD-LFS, HSI

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Trial Registration: NCT01942694

Abbreviations: 25-hydroxyvitamin D- 25(OH)D; AST to platelet ratio index- APRI; fibrosis-4 index- FIB-4; hepatic steatosis index- HSI; impaired fasting glucose- IFG; impaired glucose tolerance- IGT; non-alcoholic fatty liver disease- NAFLD; non-alcoholic fatty liver disease liver

fat score- NAFLD-LFS; non-alcoholic steatohepatitis- NASH; proton magnetic resonance spectroscopy- 1H-MRS; type 2 diabetes- T2D; Vitamin D and Type 2 Diabetes Study- D2d

Abstract

Aims: Non-alcoholic fatty liver disease (NAFLD) is a common comorbidity that leads to poor outcomes in people at high risk for development of type 2 diabetes (T2D). Vitamin D is a possible mediator. In the vitamin D and type 2 diabetes study (D2d), we investigated the relationship of baseline indices of NAFLD with incident T2D and whether vitamin D modified the association.

Methods: Cross-sectional associations of indices of NAFLD with glycemia and vitamin D status were assessed in 3,972 individuals screened for the D2d study. In those with prediabetes randomized to vitamin D or placebo (n=2,423), we examined longitudinal associations of NAFLD indices with incident T2DM. We used validated non-invasive scores to assess steatosis [(hepatic steatosis index (HSI); NAFLD-liver fat score (NAFLD-LFS)] and advanced fibrosis [fibrosis-4 (FIB-4) index; AST to Platelet Ratio Index (APRI)].

Results: Eighty-five percent of participants had likely steatosis by HSI and 71% by NAFLD-LFS; 3% were likely to have advanced fibrosis by FIB-4 and 1.2% by APRI. FIB-4 indicated that 20.4% of individuals require further follow up of liver health. Steatosis and fibrosis scores were higher among participants with worse glycemia. The NAFLD-LFS and APRI predicted development of diabetes (hazard ratios [95%CI] 1.35 [1.07, 1.70]; p = 0.012) and 2.36 (1.23, 4.54; p = 0.010), respectively). The effect of vitamin D on diabetes risk was not modified by baseline NAFLD indices. Individuals with likely steatosis had a smaller increase in serum 25-hydroxyvitamin D level in response to vitamin D than those without steatosis.

Conclusions: The predicted high prevalence of steatosis, the need for further fibrosis workup, and the relationship between liver health and incident T2D suggest that routine screening with clinically accessible scores may be an important strategy to reduce disease burden.

Key Words: Vitamin D, NAFLD, FIB-4, APRI, NAFLD-LFS, HSI

1.0 Introduction

Non-alcoholic fatty liver disease (NAFLD) is a common chronic liver disorder worldwide without approved pharmacologic therapies. It encompasses a range of pathologies including uncomplicated hepatic steatosis, an inflammatory phenotype (non-alcoholic steatohepatitis-NASH), and more advanced stages including fibrosis and cirrhosis. People with advanced NAFLD, fibrosis in particular, have a higher risk of liver-related and all-cause mortality, including cardiovascular mortality. NAFLD is a common comorbidity in people with type 2 diabetes (T2D) and is associated with complications such as cardiovascular disease. Thus, early detection of NAFLD is important in populations at high-risk for advanced stages including people with metabolic diseases such as obesity, cardiovascular disease, metabolic syndrome, prediabetes, and T2D.[1]

Steatotic and fibrotic features of NAFLD are highly prevalent in people with prediabetes and T2D.[2, 3] NAFLD independently predicts prediabetes and T2D[4, 5] and improvement in NAFLD is associated with reduced T2D incidence.[6] Often the diagnosis of NAFLD is made at advanced stages, and early identification of NAFLD and intervention may be important for high-risk populations with prediabetes and early T2D. Although it is well established that T2D is a risk factor for NAFLD[1], the link between NAFLD and risk of T2D in people with prediabetes is not fully understood. It is important to establish how common NAFLD is in people with prediabetes using clinical tools that are readily available in clinical practice and determine whether identifying NAFLD adds predictive value to risk of progressing from prediabetes to T2D.

A complementary approach to early diagnosis of NAFLD is to advance understanding of modifiable risk factors and mechanisms of disease. Vitamin D deficiency could be a unifying mechanism between NAFLD and progression from prediabetes to T2D. Vitamin D deficiency is common in patients with chronic liver disease[7] and is also implicated in dysglycemia.[8] Preclinical

data suggest therapeutic efficacy of vitamin D supplementation in NAFLD.[9] Results from human studies have been mixed with respect to mechanisms linking vitamin D deficiency or supplementation and NAFLD/NASH.[10] In addition, among patients with NAFLD and low vitamin D levels, response to vitamin D supplementation is attenuated in people with advanced liver disease,[11] which could be due to an impairment in hepatic vitamin D hydroxylation.[12]

The Vitamin D and type 2 diabetes study (D2d) is the largest clinical trial examining the effect of vitamin D, as compared to placebo, for diabetes prevention in a modern population at high risk for T2D.[13] We performed a post-hoc analysis of the D2d study with the following aims: (1) report the prevalence of steatosis and advanced fibrosis in prediabetes and across the glycemic spectrum from normal glucose tolerance (NGT) to diabetes using validated, non-invasive indices; (2) examine the cross-sectional association between steatosis and advanced fibrosis across the spectrum of vitamin D status; (3) examine whether baseline steatosis and advanced fibrosis modify the response of vitamin D on serum 25-hydroxyvitamin D (25[OH]D level; and (4) test whether baseline steatosis and advanced fibrosis predict incident T2D and (5) whether scores modify the effect of vitamin D on development of T2D.

2.0 Subjects

In this post-hoc analysis, we studied two populations: 1) participants fully screened for participation in D2d (n=3972) and 2) those randomized to vitamin D or placebo (n=2423). The Screened D2d population included all participants with complete baseline data, irrespective of whether they were subsequently randomized.[14] At baseline, these participants had either normal glucose tolerance, pre-diabetes diagnosed with one, two or three pre-diabetes glycemic criteria, or newly recognized diabetes (met at least one diabetes glycemic criterion). This population was suitable for evaluating cross-sectional associations between NAFLD scores and clinical characteristics, including glycemia and vitamin D status (assessed by 25[OH]D) (aims 1-2). The

Randomized D2d population included participants that met at least two of three glycemic criteria for prediabetes (fasting plasma glucose level, 100 to 125 mg/dL; plasma glucose level 2 hours after a 75-g oral glucose load of 140 to 199 mg/dL; and glycated hemoglobin level of 5.7 to 6.4%) and met no diagnostic criteria for diabetes. They were randomized to vitamin D (N=1211) or placebo (N=1212) and followed for a median of 2.5 years. The Randomized D2d population was used to evaluate whether NAFLD scores modified the response to vitamin D on serum 25(OH)D (aim 3), predicted incident diabetes (aim 4), and whether the presence of NAFLD modified the effect of vitamin D on incident T2D (aim 5). People taking diabetes or weight loss medications and those with liver transaminases >3 times the upper limit of normal were excluded from the parent study.

3.0 Materials and Methods

3.1 Overview of D2d Study

The design and primary outcome of the D2d parent study from which the data were derived for the post-hoc analyses presented herein have been published.[13, 14] Briefly, the D2d study is a US-based multicenter, randomized, primary prevention trial that compared oral vitamin D_3 at 4000 IU/day versus placebo in participants at high risk for developing diabetes who were followed for incident diabetes. Participants were recruited and followed at 22 academic medical centers (d2dstudy.org/sites). The institutional review board at each clinical site approved the protocol, and all the participants provided written informed consent.

3.2 Intervention

The Randomized D2d population was assigned to take a single, once-daily soft-gel pill containing either 4000 IU of vitamin D_3 or matching placebo.[14]

3.3 Scoring Models of Liver Disease

In the absence of histology, imaging or liver-specific biomarkers, we used several liver disease scoring models that can be calculated with commonly available clinical data. Although all scores have limitations, we selected a suite of scores based on availability of data in the D2d study and published performance characteristics.[15, 16] Secondary causes of liver steatosis (i.e., alcohol use, medications etc.) were not assessed. Each score is briefly described below.

3.3.1 *Hepatic Steatosis Index (HSI):* This score correlates with liver fat (measured with proton magnetic resonance spectroscopy; 1H-MRS) and insulin resistance.[17] The formula is: HSI= 8 x ALT/AST + BMI (+ 2 if type 2 diabetes yes, + 2 if female). Scores can be interpreted as follows: <30 steatosis can be ruled out; 30-<36 is indeterminate; ≥36 steatosis is highly likely.[18]

3.3.2 *NAFLD/Liver Fat Score (NAFLD-LFS):* This score has been confirmed to predict liver fat (AUROC 0.786).[19] The formula is: NAFLD/LFS = - 2.89 + 1.18 x Metabolic Syndrome (Yes: 1, No: 0) + 0.45 x Type 2 Diabetes (Yes: 2, No: 0) + 0.15 x Insulin in mU/L + 0.04 x AST in U/L – 0.94 x AST/ALT. Scores can be interpreted as follows: ≤ -0.640 steatosis can be ruled out > -0.640 steatosis is highly likely.[20]

3.3.3 *Fibrosis-4 (FIB-4) Index:* The FIB-4 index has been validated in NAFLD, including comparison to histologically defined fibrosis.[21] The performance characteristics of this score make it particularly valuable for risk stratification and determination of need for further diagnostics. This score is not recommended for people aged ≤ 35. The formula is: FIB-4 = (Age x AST) / (Platelets in 1000/uL x √(ALT)). Scores can be interpreted as follows: for age 36-64, <1.3 advanced fibrosis excluded; 1.3 to 2.67 further investigation needed; > 2.67 advanced fibrosis likely; for age ≥ 65, <2.0 advanced fibrosis excluded; 2.0 to 2.67 further investigation needed; > 2.67 advanced fibrosis likely.[22]

3.3.4 *AST to Platelet Ratio Index (APRI):* The APRI score has been validated in NAFLD, including comparison to histologically defined fibrosis.[23] The formula is: (AST (IU/L)/upper limit of normal)/platelets (X 10^9/L)) X 100. The scores can be interpreted as follows: <0.7 fibrosis excluded; 0.7 to <1.0 significant fibrosis; ≥ 1.0 severe fibrosis/cirrhosis.[24]

3.4 Statistical Methods

We examined the distribution of steatosis and fibrosis scores measured at the baseline visit (aim 1). Descriptive statistics included means ± standard deviation (SD), medians, the range between the 25th and 75th percentiles, and the minimum and maximum values. For score cutoffs above or below a given threshold, percentages are given. Cross-sectional comparisons of steatosis and fibrosis scores across the spectrum of glycemia used Wilcoxon rank-sum tests for continuous scores and chi-square tests for dichotomous cutoffs (aim 1). The cross-sectional correlation between the scores and continuous serum 25(OH)D levels used R-square statistics from ordinary least squares regression (aim 2). Cross-sectional comparisons across vitamin D status groups defined by specific serum 25(OH)D thresholds used Spearman's rank correlation.

To examine whether steatosis and fibrosis scores influence change in serum 25(OH)D level in response to vitamin D, we tested for an interaction between dichotomous score cutoffs and vitamin D on serum 25(OH)D levels in the Randomized D2d population (aim 3). The average percent change in serum 25(OH)D level from baseline and 95% confidence interval (95% CI) between the dichotomous score categories was compared using linear mixed effects regression models to account for repeated measurements within participants over time.

Time-to-event Cox proportional hazards regression models were used to describe the relationship between the steatosis and fibrosis scores measured at baseline and development of T2D overall and in response to vitamin D (aims 4 and 5). We report hazard ratios and 95% CI and model p-values with the dichotomous score as the only independent variable in the model.

Statistical analyses were performed using SAS version 9.4 (SAS Institute Inc). Two-tailed tests with alpha-level of 0.05 were used for p-values to determine statistical significance.

4.0 Results

4.1 Prevalence of Steatosis and Advanced Fibrosis in Prediabetes and by Glycemic Category (aim 1)

The Screened and Randomized D2d populations were similar in age, sex, race, ethnicity, and body mass index. Glycemic parameters aligned with a population of people at risk for diabetes. (**Table 1**). In the Screened population, the HSI showed that steatosis was likely in about 85% of participants while the NAFLD-LFS showed that >70% were likely to have steatosis. The FIB-4 Score showed that >3% of the population was likely to have advanced fibrosis and ~22% needed further investigation. The APRI showed that ~1.0% were likely to have advanced fibrosis (**Table 1**). The distribution of the liver indices was similar in the Randomized D2d population and scores did not differ between the vitamin D and placebo groups (not shown). There is known sexual dimorphism in diabetes and chronic liver diseases. We did not stratify our analyses by sex for various reasons. First, it is not appropriate to do this for the HSI because it has sex in the calculation. Second, we examined whether there was an interaction by sex in the relationship between NAFLD-LFS, FIB-4 and APRI (dichotomous cutoffs) and development of T2D overall and there was no interaction between biological sex and the scores dichotomized by disease

threshold (p for interaction 0.68, 0.81 and 0.96, respectively). Lastly, sample sizes, especially for the high fibrosis group, would be small and results might be misleading.

We compared the estimated prevalence of steatosis and advanced fibrosis in groups defined by normal glucose tolerance (NGT, all three criteria in the normal range), prediabetes (any criterion for prediabetes and none in the diabetes range) and diabetes (any criterion in the diabetes range). All four scores increased as the degree of glycemia worsened (**Table 2**). These differences were statistically significant for most of the pairwise comparisons (Prediabetes vs. NGT; T2D vs. Prediabetes; T2D vs. NGT). The only exceptions were that FIB-4 was not significant when comparing prediabetes vs. T2D, and APRI was only significant when comparing T2D vs. NGT. For all the scores, the percentage of individuals above the disease cutoff increased as glycemia worsened with significant differences for HSI (NGT vs. T2D and prediabetes vs. T2D), NAFLD-LFS (all comparisons) and APRI (NGT vs. T2D and prediabetes vs. T2D) (**Table 2**).

4.2 Steatosis and Advanced Fibrosis Scores across the Spectrum of Vitamin D Status (aim 2)

We evaluated the correlation between serum 25(OH)D level and steatosis and advanced fibrosis scores. There was a statistically significant but weak inverse correlation between serum 25(OH)D and HSI and NAFLD-LFS steatosis scores (**Table 3**). There was also a statistically significant but weak correlation between 25(OH)D levels and FIB-4. There was no significant association between 25(OH)D and APRI. When evaluated within categories of serum 25(OH)D level (**Table 3**), the associations were similar: in categories of higher 25(OH)D level, the HSI and NAFLD-LFS scores were lower. In categories of higher 25(OH)D level, FIB-4 score was higher. There was no meaningful difference in mean APRI scores across categories by 25(OHD) level despite a significant

p-value for the correlation. The mean scores for the HSI were above the threshold for steatosis in all 25(OH)D categories. For the NAFLD-LFS, the score was below the disease cutoff in people with serum 25(OH)D ≥ 30 ng/ml. The FIB-4 score and the APRI had mean values that were below the disease threshold for all 25(OH)D categories.

4.3 Change in Serum 25(OH)D in Response to Vitamin D according to Baseline Steatosis and Advanced Fibrosis Scores (aim 3)

We next evaluated whether change in serum 25(OH)D in response to vitamin D was modified by baseline steatosis or fibrosis scores. In the Randomized D2d population, the group treated with vitamin D had significant increases from baseline in serum 25(OH)D level at 12, 24, 36 and 48 months, and the percent change was higher in participants with liver scores below the disease threshold for HSI and NAFLD-LFS (**Table 4**). There was no interaction between vitamin D and advanced fibrosis scores (FIB-4, APRI) on change in serum 25(OH)D level.

4.4 The Relationship Between Steatosis and Advanced Fibrosis Scores and Incident Diabetes (aims 4 & 5)

In the parent study, 293 study participants in the vitamin D group and 323 in the placebo group developed diabetes.[13] In the entire cohort, we evaluated whether baseline steatosis or advanced fibrosis scores predicted development of diabetes (**Figure 1**). The hazard ratios (95%CI) for development of diabetes were 1.33 (0.94, 1.87), 1.35 (1.07, 1.70), 1.17 (0.75, 1.83), and 2.36 (1.23, 4.54) for HSI, NAFLD-LFS, FIB-4 and APRI, respectively, when comparing the scores above vs. below the disease thresholds. We did not find an interaction between baseline steatosis or fibrosis scores and vitamin D on the development of T2D (aim 5; **data not shown**).

5.0 DISCUSSION

Our results in a modern at-risk for diabetes cohort showed that steatosis, assessed by non-invasive scores, is likely common in people with prediabetes with the prevalence being higher among those with worse glycemia. We also found that in this cohort, NAFLD-LFS and APRI scores were associated with incident diabetes and those with likely steatosis at baseline had a lower rise in blood 25OHD following supplementation with vitamin D. Compared to placebo, vitamin D supplementation did not modulate the association between NAFLD-LFS and APRI with diabetes progression.

5.1 Steatosis Evaluated by Non-Invasive Scores is Common in People with Prediabetes (aim 1)

This study shows that individuals with prediabetes have a high prevalence of steatosis, assessed by non-invasive scores and, therefore, are at risk for progression to fibrosis. The predicted prevalence of advanced fibrosis was low (~1-3% depending on score used). Given that the D2d study excluded people with liver enzymes >3 times the upper limit of normal, these low proportions represent a group at risk for having advanced fibrosis that could have gone undetected on clinical screening. Indeed, it is known that people with established T2D have a high prevalence of NAFLD and NASH despite having normal liver enzymes,[25] and our data suggest that this may also be the case in people with prediabetes. In addition, our findings highlight that a large proportion of people (~22%) with prediabetes fall in the FIB-4 category where “further investigation” by imaging and/or liver biopsy are indicated. This group may benefit from early intervention to reduce the burden of liver disease.[25]

We found that people with prediabetes had an intermediate score-predicted prevalence of steatosis and advanced fibrosis, suggesting the possibility of lower liver disease burden before overt diabetes occurs. These findings suggests that risk for liver disease begins before

overt diabetes. Indeed, recent data from the Diabetes Prevention Program Outcome Study show that in a population with prediabetes followed longitudinally for 14 years, hepatic steatosis occurred almost twice as often in people who developed T2D vs. those who did not.[26] Our results suggest that in addition to NAFLD surveillance in populations with T2D, there is a need to begin surveillance at the prediabetes stage so that cardiometabolic risk factors for both development of diabetes and NAFLD can be appropriately managed with weight loss or pharmacological interventions

5.2 NAFLD Indices and Vitamin D Status (aim 2)

Several mechanisms have been postulated to mediate the relationship between vitamin D metabolism and hepatic lipid balance, primarily in non-human model systems.[10] We observed a relationship between low vitamin D status, assessed by serum 25(OHD) level, and higher steatosis and fibrosis scores, but the correlations were very weak. These weak correlations may be because vitamin D-mediated NAFLD is a pathophysiological mechanism in only a subset of individuals such as those with vitamin D receptor impairments or marked vitamin D deficiency.[27, 28] Some studies have reported associations between low vitamin D status and presence of NAFLD/NASH[29, 30] while some studies – including ours - did not show this association.[31, 32] Future studies with more robust liver phenotyping methods that are designed to specifically test these relationships are needed.

5.3 Interaction Between Steatosis Scores and Response to Vitamin D Supplementation (aim 3)

For vitamin D to have a biological effect, it needs to be converted to 25(OH)D by CYP2R1 and recent studies have shown that obesity represses vitamin D bioactivation by CYP2R1 leading to reduced production of 25(OH)D.[33] Through similar mechanisms, we hypothesized that people with steatosis and advanced fibrosis would have impaired CYP2R1 activity

leading to lower increase in serum 25(OH)D level in response to supplementation over time. We found this to be true based on the steatosis scores, but the effect was modest. There was no significant interaction between advanced fibrosis scores and vitamin D supplementation, likely because our study was underpowered to detect a mediating effect of fibrosis on response to vitamin D supplementation.

5.4 Steatosis and Advanced Fibrosis Scores are Related to Incident T2D with No Impact of Vitamin D Supplementation (aims 4 and 5)

It is plausible that having both prediabetes and NAFLD represents a high-risk phenotype for progression to T2D. We found that there was a relationship between scores for steatosis and advanced fibrosis and development of diabetes irrespective of vitamin D supplementation with two of the scores tested: HSI and APRI. Larger studies with deeper phenotyping, including change in liver disease over time and including participants with overt vitamin D deficiency, are needed to better understand these relationships.

5.5 Strengths and Limitations

Our study has several strengths. The modern cohort of people with prediabetes, the cohort's wide range of glycemia, the long-term intervention with vitamin D and longitudinal follow-up at multiple timepoints are key strengths. In addition, our findings support the use of liver indices for screening purposes of individuals with prediabetes. This is of public health significance because those with higher scores warrant further diagnostic evaluation and may be at increased risk for progression to diabetes and the development of cardiovascular disease.

There are also limitations. First, the predictive values of non-invasive scores have known inherent limitations. For HSI and NAFLD-LFS, although areas under the curve have been

reported to be 0.81 and 0.80 (respectively) in comparison to liver biopsy for detecting any level of steatosis, they are not able to distinguish between different levels of steatosis.[34] Their ability to detect change in response to an intervention has also come into question.[35] The inclusion of glycemic parameters in the scores may confound our results. As far as fibrosis prediction, APRI and FIB-4 perform best for excluding advanced fibrosis (fibrosis stage ≥ 3), but are unable to discern lower levels of fibrosis that are clinically meaningful and more likely to respond to interventions.[36] We did not calculate other scores, such as the fatty liver index[37], due to the lack of all necessary data for the calculation.

There were several important domains that were not fully addressed by our analyses. We did not find that sex modified the observed results; however, future studies should address this since multiple biological and behavioral/societal constructs related to biological sex are known to impact outcomes in diabetes and NAFLD.[37] Complications such as renal and cardiovascular disease are more prevalent in people with T2D and NAFLD and impact outcomes.[38, 39] Future studies should address how these pathophysiological conditions interact.[38] The lack of longitudinal evaluation of steatosis and fibrosis scores is also a limiting factor, as repeated measures can add insight related to whether changes in the scores are linked to vitamin D supplementation and transition from prediabetes to T2D. This population was not selected based on vitamin D status and excluded people with high liver enzymes, which may have limited the study's power to detect differences. In addition, people with BMI > 42 were excluded, and studies show higher levels of liver fat in extreme obesity.[40] Lastly, several variables within the scores are also risk factors for prediabetes and T2D, and they may be driving some of the associations observed in our analyses.

5.6 Conclusions

Populations at high risk for T2D are also at high risk for hepatic steatosis and, to a lesser extent, fibrosis. We uncovered a relationship between baseline steatosis and fibrosis scores and the progression from prediabetes and diabetes that warrants further study. Participants with likely steatosis had a smaller increase in serum 25(OH)D level in response to vitamin D than those without steatosis; however, the effect of vitamin D on diabetes risk was not modified by baseline NAFLD indices. Our study shows that in a population at high risk for developing T2D, evaluation of NAFLD using non-invasive, clinically available scores can further delineate risk. Given that NAFLD is associated with poor health outcomes, close monitoring and appropriate management with weight loss interventions and risk-factor modification are essential. An open question in the fields of both endocrinology and hepatology is whether vitamin D metabolism is a causal pathway in disease in a subset of individuals. The link between liver health, vitamin D and T2D is complex and warrants further study.

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Research Data for this Article

The data underlying this article and the associated data dictionary are not publicly available. Requests for datasets analyzed in the current study can be made by bona fide researchers by submitting a research proposal to the D2d Publications and Presentation Subcommittee for review. Individual participant data will be shared in a deidentified/anonymized format using a specialized SAS data platform. Protocol synopsis, contact details, publications, and the process for collaboration and data requests can be found on the website (d2dstudy.org).

Data D2d Research Group collaborators.

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Figure Legend

Figure 1: The Relationship Between Baseline Steatosis and Fibrosis Scores on Incident Diabetes in the Randomized D2d Population. The hazard ratio for incident diabetes was derived from a time-to-event Cox proportional hazards regression model.

Tables

Table 1: Baseline Characteristics

	Screened (n=3,972)	Randomized (n=2,423)
Demographic		
Age, years	59.4 ± 10.2	60.0 ± 9.9
Female, no. (%)	1817 (45.7)	1086 (44.8)
Race, no. (%)		
Asian	193 (4.9)	130 (5.4)
Black	1016 (25.6)	616 (25.4)
White	2658 (66.9)	1616 (66.7)
Other	105 (2.6)	61 (2.5)
Hispanic or Latino ethnicity, - no (%)	387 (9.7)	225 (9.3)
Anthropometric		
Body Mass Index, kg/m^2	31.9 ± 4.5	32.1 ± 4.5
Laboratory Assessments		
Fasting plasma glucose, mg/dL	106.7 ± 10.7	107.9 ± 7.4
2hour post-load plasma glucose, mg/dL	137.9 ± 44.9	137.2 ± 34.3
Glycated hemoglobin, %	5.9 ± 0.3	5.9 ± 0.2
Serum 25-hydroxyvitamin D		
Mean ± SD, ng/ml	28.1 ± 10.2	28.0 ± 10.2
Distribution, n (%)		
<12 ng/ml	156 (3.9)	103 (4.3)
12-19 ng/ml	682 (17.2)	422 (17.4)
20-29 ng/ml	1420 (35.9)	876 (36.2)
≥ 30 ng/ml	1699 (42.9)	1021 (42.2)
AST, U/L	26.3 ± 10.8	26.3 ± 10.5
ALT, IU/L	29.8 ± 15.7	30.0 ± 15.6
Platelets, $10^9/L$	244.8 ± 57.8	243.8 ± 57.6
HOMA2%S, insulin	67.4 ± 45.8	71.5 ± 50.0
Steatosis Scores		
Hepatic Steatosis Index		
Mean ± SD	42.2 ± 5.9	42.2 ± 5.8
Distribution, n (%)		
<30: steatosis ruled out	17 (0.4)	10 (0.4)
30 to <36: indeterminate	568 (14.4)	332 (13.7)
≥36: steatosis likely	3360 (85.2)	2078 (85.9)
NAFLD Liver Fat Score		
Mean ± SD	0.56 ± 2.14	0.48 ± 1.89
Distribution, n (%)		
≤ -0.64: steatosis ruled out	1003 (29.2)	555 (25.7)
> -0.64: steatosis likely	2429 (70.8)	1608 (74.3)
Advanced Fibrosis Scores		
Fibrosis-4 Score		
Mean ± SD	1.29 ± 0.64	1.31 ± 0.63
Distribution, n (%)		
<1.3 (age 36-64) or <2.0 (age ≥ 65): advanced fibrosis excluded	2902 (74.3)	1782 (74.4)
1.3 (age 36-64) or <2.0 (age ≥ 65): further investigation needed	873 (22.4)	527 (22.0)
>2.67 (age ≥ 36): advanced fibrosis likely	129 (3.3)	87 (3.6)
AST to Platelet Ratio Index		
Mean ± SD	0.27 ± 0.14	0.27 ± 0.13
Distribution, n (%)		
<0.7: advanced fibrosis excluded	3915 (98.8)	2393 (98.8)
0.7 to <1.0: significant fibrosis	38 (1.0)	25 (1.0)
≥1.0: severe fibrosis/cirrhosis	9 (0.2)	3 (0.1)

Plus-minus values are mean ± SD.
The Screened D2d population comprised people in the entire glycemic spectrum (normal glucose tolerance, prediabetes, diabetes) and was used in cross-sectional analyses. The Randomized D2d population comprised people with 2 or 3 criteria for prediabetes and none in the diabetes range and was used in the longitudinal analyses. Randomized is a subset of Screened.

Table 2: Steatosis and Fibrosis Scores across the Glycemia Spectrum in the Screened D2d population

Steatosis and Fibrosis Scores	NGT	Prediabetes	Type 2 Diabetes	P-value Prediabetes vs. NGT	P-value Prediabetes vs. T2D	P-value Type 2 Diabetes vs. NGT
Hepatic Steatosis Index						
N	152	3324	469	**0.004**	**<0.001**	**<0.001**
mean ± sd	40.48 ± 5.31	41.95 ± 5.78	44.67 ± 5.91			
median (IQR)	39.58 (36.74 to 43.99)	41.53 (37.65 to 46.02)	44.61 (40.1 to 48.18)			
range	28.75 to 54.77	28.43 to 81.4	32.07 to 74.3			
n (%) above cutoff	120 (78.9%)	2797 (84.1%)	443 (94.5%)	0.088	**<0.001**	**<0.001**
NAFLD Liver Fat Score						
N	130	2902	400	**<0.001**	**<0.001**	**<0.001**
mean ± sd	-0.72 ± 1.45	0.35 ± 1.91	2.53 ± 2.67			
median (IQR)	-1.11 (-1.81 to -0.11)	0.05 (-0.87 to 1.17)	2.31 (0.76 to 3.59)			
range	-2.66 to 4	-6.05 to 20.15	-1.95 to 21.59			
n (%) above cutoff	48 (36.9%)	2007 (69.2%)	374 (93.5%)	**<0.001**	**<0.001**	**<0.001**
Fibrosis-4 Score						
N	152	3321	467	**0.004**	0.095	**<0.001**
mean ± sd	1.13 ± 0.52	1.29 ± 0.62	1.38 ± 0.78			
median (IQR)	1.06 (0.76 to 1.36)	1.15 (0.87 to 1.56)	1.22 (0.9 to 1.66)			
range	0.3 to 3.94	0.25 to 7.26	0.41 to 9.69			
n (%) above cutoff	2 (1.4%)	107 (3.3%)	20 (4.3%)	0.217	0.249	0.107
AST to Platelet Ratio Index						
N	152	3321	467	0.181	0.084	**0.024**
mean ± sd	0.24 ± 0.1	0.26 ± 0.13	0.29 ± 0.19			
median (IQR)	0.24 (0.17 to 0.28)	0.24 (0.18 to 0.31)	0.24 (0.18 to 0.35)			
range	0.09 to 0.59	0.06 to 1.9	0.08 to 2.57			
n (%) above cutoff	0 (0)	35 (1.1%)	12 (2.6%)	0.203	**0.006**	**0.046**

SD, standard deviation; IQR, interquartile range.
Comparison across glycemic groups for continuous measures is based on Wilcoxon rank-sum tests and for dichotomous cutoffs is based on chi-square tests.

Table 3: Steatosis and Fibrosis Scores Across the Spectrum of Vitamin D Status in the Screened D2d population

	Serum 25(OH)D: continuous variable		Serum 25(OH)D				
Steatosis and Fibrosis Scores	**R^2**	**P-value**	**<12ng/ml**	**12-19 ng/ml**	**20-29 ng/ml**	**≥ 30 ng/ml**	**Spearman correlation P-value**
Hepatic Steatosis Index	0.023	**<0.001**					-0.15 (**<0.001**)
N			155	682	1412	1690	
mean ± sd			43.93 ± 5.77	43.36 ± 6.24	42.56 ± 5.74	41.3 ± 5.65	
median (IQR)			44.27 (40.36 to 47.2)	42.76 (38.89 to 47.77)	42.39 (38.39 to 46.55)	40.67 (37.23 to 45.2)	
range			29.85 to 58.78	29.97 to 74.3	28.45 to 81.4	28.43 to 66.6	
NAFLD Liver Fat Score	0.010	**<0.001**					-0.09 (**<0.001**)
N			128	576	1225	1501	
mean ± sd			0.79 ± 2.09	0.82 ± 2.27	0.7 ± 2.25	0.33 ± 1.97	
median (IQR)			0.25 (-0.55 to 1.58)	0.44 (-0.72 to 1.86)	0.32 (-0.73 to 1.6)	0.02 (-0.91 to 1.18)	
range			-2.69 to 10.41	-3.36 to 12.37	-3.95 to 21.59	-6.05 to 20.15	
Fibrosis-4 Score	0.026	**<0.001**					0.19 (**<0.001**)
N			156	679	1419	1693	
mean ± sd			1.07 ± 0.57	1.16 ± 0.6	1.24 ± 0.61	1.4 ± 0.66	
median (IQR)			0.96 (0.72 to 1.31)	1.05 (0.77 to 1.4)	1.12 (0.84 to 1.49)	1.27 (0.95 to 1.7)	
range			0.32 to 5.45	0.3 to 6.09	0.25 to 7.26	0.27 to 9.69	0.05
AST to Platelet Ratio Index	0.001	0.097					(**0.001**)
N			156	679	1419	1693	
mean ± sd			0.27 ± 0.2	0.26 ± 0.14	0.26 ± 0.12	0.27 ± 0.14	
median (IQR)			0.24 (0.17 to 0.31)	0.23 (0.18 to 0.31)	0.23 (0.18 to 0.31)	0.24 (0.19 to 0.32)	
range			0.07 to 1.88	0.07 to 1.75	0.06 to 0.98	0.08 to 2.57	

SD, standard deviation; IQR, interquartile range.
For continuous serum 25(OH)D R-square and p-value are from unadjusted linear regression model. Comparison across groups is based on Spearman's rank correlation.

630 **Table 4: Interaction between liver health indices and vitamin D supplementation on serum**

631 **25(OH)D among D2d participants randomized to vitamin D.**

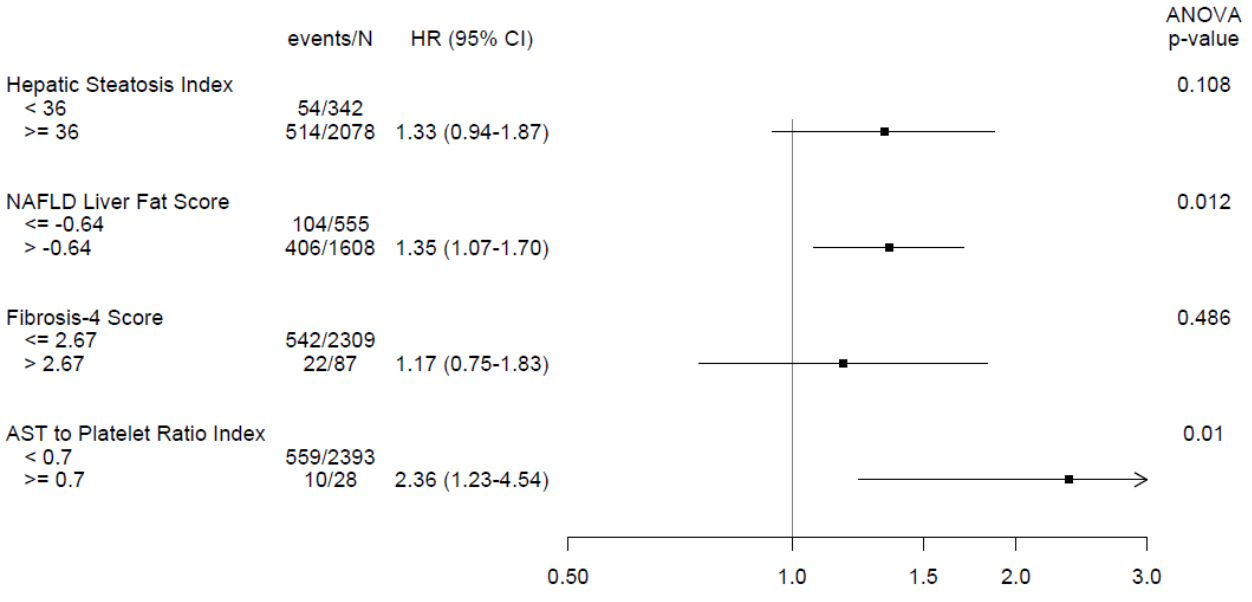
632

Score Category	25-hydroxyvitamin D Level (ng/ml)					Average % change compared to baseline (95% CI)	Interaction P-value
	Baseline	Month 12	Month 24	Month 36	Month 48		
Hepatic Steatosis Index							
≥ 36	27.4 ± 10.1 n = 1035	51.4 ± 14.6 n = 958	53.4 ± 15.2 n = 844	56.4 ± 16.1 n = 524	60.7 ±17.7 n = 204	118 (115, 121)	**0.005**
<36	29.5 ± 10.6 n = 174	56.6 ± 15.8 n = 161	57.7 ± 15.9 n = 140	59.4 ± 16.7 n = 82	60.8 ±20.3 n = 33	130 (122, 138)	
NAFLD-Liver Fat Score							
> -0.640	27.5 ± 10.1 n = 805	51.4 ± 14.6 n = 745	53.0 ± 14.5 n = 656	55.8 ± 16.3 n = 390	60.2 ± 18.0 n = 138	115 (111, 119)	**<0.001**
≤ -0.640	29.1 ± 10.6 n = 277	55.3 ± 14.9 n = 264	57.4 ± 16.2 n = 227	59.2 ± 15.8 n = 146	62.7 ± 16.7 n = 67	129 (124, 135)	
Fibrosis-4 Score							
> 2.67	27.2 ± 9.3 n = 41	55.4 ± 12.9 n = 40	57.4 ± 15.9 n = 36	59.1 ± 19.6 n = 22	64.2 ± 22.0 n = 10	127 (112, 143)	0.310
≤ 2.67	27.9 ± 10.2 n = 1156	52.3 ±14.8 n = 1070	54.0 ±15.2 n = 943	56.9 ±16.0 n = 580	60.7 ±17.8 n = 226	119 (116, 122)	
AST to Platelet Ratio Index							
≥ 0.7	22.9 ± 10.2 n = 13	49.5 ± 17.9 n = 12	54.4 ± 14.6 n = 10	57.3 ± 15.1 n = 8	59.0 ± 29.7 n = 2	135 (107, 163)	0.286
< 0.7	27.8 ± 10.2 n = 1198	52.2 ± 14.8 n = 1109	54.0 ± 15.4 n = 976	56.9 ± 16.2 n = 600	60.7 ± 18.1 n = 237	119 (116, 122)	

633 Average percent change compared to baseline within group is based on linear mixed effects model to account for repeated
634 measures of longitudinal clustering within individual participant.

Figures

Figure 1: Incident Diabetes according to Baseline Steatosis and Fibrosis Scores on in the Randomized D2d Population



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Declaration of interests

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

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:
