Systematic Review and Meta-Analysis: Nonalcoholic fatty liver disease is associated with a history of osteoporotic fractures but not with low bone mineral density in middle-aged and elderly individuals

Alessandro Mantovani, MD[1], Marco Dauriz, MD, PhD[1], Davide Gatti, MD[2], Ombretta Viapiana, MD[2], Giacomo Zoppini, MD[1], Giuseppe Lippi, MD[3], Christopher D. Byrne, MB BCh, PhD[4,5], Fabrice Bonnet, MD[6], Enzo Bonora, MD[1], Giovanni Targher, MD[1]

[1]Section of Endocrinology, Diabetes and Metabolism, Department of Medicine, University and Azienda Ospedaliera Universitaria Integrata of Verona, Verona, Italy

[2]Section of Rheumatology, Department of Medicine, University and Azienda Ospedaliera Universitaria Integrata of Verona, Verona, Italy

[3]Section of Clinical Biochemistry, University and Azienda Ospedaliera Universitaria Integrata of Verona, Verona, Italy

[4]Nutrition and Metabolism, Faculty of Medicine, University of Southampton, UK

[5]Southampton National Institute for Health Research Biomedical Research Centre, University Hospital Southampton, Southampton General Hospital, Southampton SO16 6YD, UK

[6]Department of Endocrinology, INSERM UMR 991, University Hospital of Rennes, Rennes Cedex 9, France

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Address for correspondence:
Prof. Giovanni Targher, MD
Section of Endocrinology, Diabetes and Metabolism
Department of Medicine
University and Azienda Ospedaliera Universitaria Integrata
Piazzale Stefani, 1
37126 Verona, Italy
Phone: +39-045-8123110
E-mail: giovanni.targher@univr.it

ABSTRACT

Background: Several studies have explored the effect of nonalcoholic fatty liver disease (NAFLD) on bone mineral density (BMD) and risk of osteoporotic fractures in adults. However, the extent to which NAFLD adversely affects bone health remains uncertain.

Aim: To provide a quantitative estimation of the magnitude of the association of NAFLD with BMD or history of osteoporotic fractures in adults.

Methods: We searched PubMed, Web of Science and Scopus using predefined keywords to identify all observational studies, published until August 31, 2018, in which NAFLD was diagnosed by imaging or histology; BMD was measured by dual energy X-ray absorptiometry; and a self-reported history of osteoporotic fractures was collected with interviewer-assisted questionnaires. Data from selected studies were extracted, and meta-analysis was performed using random-effects modeling.

Results: Twelve cross-sectional or case-control studies with aggregate data on 30,041 adults of predominantly Asian ethnicity (30% with NAFLD) were included in the final analysis. No significant differences in BMD at different skeletal sites (whole body, lumbar spine or femur) were observed between individuals with and without NAFLD. Conversely, NAFLD was associated with increased odds of osteoporotic fractures, especially in older Chinese men (n=2 studies; random-effects odds ratio 2.10, 95%CI 1.36-3.25; I^2=0%). Sensitivity analyses did not alter these findings. Funnel plot and Egger test did not reveal significant publication bias.

Conclusions: This meta-analysis suggests that imaging-defined or biopsy-proven NAFLD is associated with a self-reported history of osteoporotic fractures (principally in Chinese men), but not with low BMD, in middle-aged and elderly individuals.

Keywords: NAFLD; fatty liver; bone mineral density; osteoporotic fractures; meta-analysis

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is the most common liver disease in both developed and developing countries, and is estimated to affect at least 25-30% of adults in the general population and up to 70-90% of persons with type 2 diabetes or obesity (1). NAFLD is associated not only with liver-related morbidity and mortality, but also with an increased risk of developing both cardiovascular disease and other extra-hepatic diseases (e.g., type 2 diabetes, chronic kidney disease and colorectal cancer) (2-6).

Osteoporosis is a skeletal disease characterized by low bone mineral density (BMD) and micro-architectural disruption, with a resulting increase in bone fragility and susceptibility to fractures (7). Osteoporosis is a public health problem due to the high cumulative rates of fractures with advancing age. For example, in the Global Longitudinal Study of Osteoporosis in Women, involving 60,393 non-institutionalized women aged ≥55 years, the proportion of incident osteoporotic hip fractures increased more than fivefold with age, from ~7% among women aged 55 to 59 years to ~35% among those aged ≥85 years (8). Notably, hip fractures are the most serious of all osteoporotic fractures, as they are strongly associated with an increased risk of morbidity and mortality, as well as with high healthcare costs (9). Identification of novel and potentially modifiable risk factors for osteoporosis is therefore a key issue in clinical practice.

In recent years, the existence of a possible association between NAFLD and osteoporosis has created considerable scientific interest (10). Several observational cross-sectional or case-control studies have examined the association between imaging-defined or biopsy-proven NAFLD and BMD measurement in middle-aged and elderly individuals (11-22). However, the findings from such observational studies have been inconsistent or conflicting so far, with some studies reporting a significant association between NAFLD and reduced BMD, especially in Chinese men (13,15,18), some studies reporting a significant association between NAFLD and increased BMD (12,16,19), and with other studies failing to find any significant association between NAFLD and BMD (11,14,17,20). Moreover, it remains uncertain whether an association also exists between low BMD and severity of NAFLD. Recently, some large cross-sectional studies of Chinese middle-aged and elderly individuals also showed that ultrasound-diagnosed NAFLD was significantly associated with a self-reported history of osteoporotic fractures in older men, but not in women

(21,22). Similarly to observations in patients with obesity or type 2 diabetes (23), these latter findings raise the possibility that BMD might underestimate the long-term risk of osteoporotic fractures in individuals with NAFLD (who are often obese or have diabetes).

Therefore, we herein report the results of a comprehensive systematic review and meta-analysis of observational studies that have examined the association of NAFLD (as detected by imaging or histology) with either BMD measurements at different skeletal sites or a history of osteoporotic fractures in middle-aged and elderly individuals. Given the clinical and cost-effectiveness impact of osteoporosis, clarification of the magnitude of the potential adverse effect(s) of NAFLD on bone health might have relevant clinical implications for the prevention, diagnosis and treatment of this common skeletal disease.

MATERIALS AND METHODS

Registration of review protocol

The protocol for this systematic review and meta-analysis was registered in advance with PROSPERO (International Prospective Register of Systematic Reviews, no. CRD42018108317).

Data sources and searches

We conducted a systematic literature search in PubMed, Scopus and Web of Science databases for identifying all observational studies, published through August 31, 2018, which examined the association of NAFLD with either bone mineral density (BMD) or history of osteoporotic fractures. The search free text terms were “nonalcoholic fatty liver disease” (OR “fatty liver” OR “non-alcoholic fatty liver” OR “NAFLD” OR “nonalcoholic steatohepatitis”) AND "bone mineral density" OR "osteoporosis" OR "osteoporotic fractures" OR “fracture(s)”. We also searched for MeSH (Medical Subject Headings) terms. Searches were restricted to human studies. No language restrictions were applied. Additionally, we reviewed references from relevant original papers and review articles for identifying further eligible studies not covered by the original database searches. We performed a systematic review in accordance with the Preferred Reporting Items for Systematic

Reviews and Meta-Analyses (PRISMA) guidelines. Because the included studies were observational in design, we followed the Meta-analysis Of Observational Studies in Epidemiology (MOOSE) guidelines for the meta-analysis of these studies (24).

Study selection

Original studies were included if they met the following inclusion criteria: 1) observational cross-sectional or case-control studies that examined the association between NAFLD and BMD measurements or history of osteoporotic fractures; 2) all studies that reported data of either BMD (as measured by dual energy X-ray absorptiometry [DEXA] at various skeletal sites) that was expressed (or that could be calculated) as means ± standard deviation (SD), or odds ratios (OR) and 95% confidence intervals (CIs), for a history of osteoporotic fractures (collected with interviewer-assisted standardized questionnaires) in individuals with and without NAFLD; and 3) all studies in which the diagnosis of NAFLD was based on either biopsy or imaging techniques, in the absence of excessive alcohol consumption (>20 g/day for women and >30 g/day for men, respectively) and other competing causes of chronic liver disease. Study participants included in the meta-analysis were adult individuals (aged ≥18 years) of either sex without any restriction in terms of age, race or ethnicity. No prospective studies were available for the analyses.

Exclusion criteria were as follows: 1) congress abstracts, theses, case reports, reviews, practice guidelines, commentaries and editorials; 2) studies where NAFLD diagnosis was based exclusively on serum liver enzymes or other surrogate markers of NAFLD (e.g., the fatty liver index); 3) studies which did not exclude individuals with excessive alcohol consumption and other known causes of chronic liver disease; 4) studies in patients with end-stage renal disease, cancer, cirrhosis of any etiology or patients with end-stage liver disease awaiting liver transplantation; 5) studies in patients with rheumatoid arthritis, psoriatic arthritis or chronically treated with steroids; 6) studies which did not specifically report any BMD or OR values for the outcome measure(s) of interest; and 7) studies performed in pediatric population (aged <18 years).

Two investigators (AM and GT) independently examined all titles and abstracts, and obtained full texts of potentially relevant papers. Working independently and in duplicate, papers were read by

both investigators, and whether they met inclusion criteria were then assessed. Discrepancies were resolved by consensus, referring back to the original article, in consultation with a third author.

Data extraction and quality assessment

For all eligible studies, we extracted information on study design, study size, publication year, study country, participants characteristics (such as age, sex, menopausal status and body mass index [BMI]), methods used for diagnosing and staging NAFLD, outcomes of interest (i.e., whole body, lumbar or femoral BMD; and history of osteoporotic fractures), and list of covariates adjusted in multivariable regression analyses. In the case of multiple publications, the most up-to-date or comprehensive information was included.

Two investigators (AM and GT) assessed the risk of bias independently. Any discrepancies were addressed by a re-evaluation of original articles by a third author. Quality assessment was performed according to the Newcastle-Ottawa Quality Assessment Scale (NOS), which is a validated scale for non-randomized studies in meta-analyses (25). A NOS scale adapted for the cross-sectional studies was used (26). This NOS scale uses a star system to assess the quality of a study in three domains: selection, comparability, and outcome/exposure. The NOS assigns a maximum of five stars for selection (in the case of cross-sectional studies), two stars for comparability, and three stars for outcome/exposure. Studies achieving a score of at least eight stars were classified as being at low risk of bias (i.e., thus reflecting the highest quality).

Data synthesis and analysis

The primary outcome measures of the meta-analysis were either BMD measurements (i.e., whole body, lumbar, femoral neck or femoral hip), which were expressed as means±SD, or the odds of self-reported history of osteoporotic fractures between individuals with and without NAFLD (expressed as OR and 95% CI). When studies had several adjustment models, we extracted those values reflecting the maximum extent of adjustment for potentially confounding variables.

The effect sizes were expressed either as weighted mean difference (WMD) and 95% CI, for the eligible studies reporting BMD measurements between patients with and without NAFLD, or as

adjusted ORs and 95% CI for the studies reporting the history of osteoporotic fractures. The overall estimate of effect size was calculated using a random-effects model, as this methodology considers any differences between studies even in the absence of statistically significant heterogeneity (25).

Visual inspection of the forest plots was used to investigate the possibility of statistical heterogeneity. The statistical heterogeneity among studies was assessed by the I^2 statistic, which provides an estimate of percentage of variability across studies that is due to heterogeneity rather than chance alone. According to Higgins and Thompson (27), a rough guide to interpretation is as follows: I^2 values of approximately 25% represent low heterogeneity; approximately 50% represent medium heterogeneity; and approximately 75% represent high heterogeneity.

The possibility of publication bias was evaluated using the funnel plot and the Egger's regression asymmetry test (28).

To explore the possible sources of (expected) heterogeneity among the eligible studies and to test the robustness of associations, we conducted sensitivity analyses and meta-regression analyses. In particular, based on data from eligible studies, the effect of NAFLD on either BMD or history of osteoporotic fractures was assessed by stratifying the studies according to sex, menopausal status, study country, different skeletal sites of BMD measurement, methods used for diagnosing or staging NAFLD, or whether the studies had eight or nine stars on the NOS scale (i.e., the "high-quality" studies). We also performed univariable meta-regression analyses in order to examine the association of WMD in lumbar or femoral BMD with age, BMI and percentage of diabetes.

P values for chi-square tests are reported in all forest plots. A chi-square test p-value <0.10 was used to determine statistical significance considered for heterogeneity. The proportion of heterogeneity accounted for by between-study variability was also estimated using the I^2 index and adjudicated to be significant if I^2 was >50%. We used STATA® 14.2 (StataCorp, College Station, Texas) for all statistical analyses.

RESULTS

Literature search and study characteristics

Figure 1 summarizes the results of the literature search and study selection. After excluding duplicates, based on titles and abstracts of 148 citations (in accordance with the aforementioned exclusion criteria of the meta-analysis), we initially identified 17 potentially relevant studies (11-22,29-33) from PubMed, Web of Science and Scopus databases prior to August 31, 2018 (last date searched). After examining the full text of these 17 publications, we further excluded five studies (29-33), because of unsatisfactory inclusion criteria (i.e., use of fatty liver index for diagnosing NAFLD or inclusion of patients with end-stage liver disease awaiting liver transplantation) (29,31) or unavailability of extractable data for BMD (30,32,33), as specified in the PRISMA flow diagram. In **supplementary Table 1** are specified the syntax used and the records identified through database searching.

Overall, twelve studies were eligible for inclusion in the meta-analysis and were then assessed for quality (11-22). The main characteristics of the included studies are summarized in **Table 1**. All studies had a cross-sectional or case-control design, whereas no studies had a longitudinal design. The diagnosis of NAFLD was based on liver biopsy (n=1 study), ultrasonography (n=10 studies) or vibration-controlled transient elastography (Fibroscan) (n=2 study), in the absence of excessive alcohol consumption and other known causes of chronic liver disease. In ten of eligible studies the outcome measure was BMD (as measured by DEXA at different skeletal sites) between patients with and without NAFLD (11-20), whereas in the remaining two studies the outcome measure was the history of osteoporotic fractures in patients with NAFLD compared with those without the disease (21,22). Most eligible studies were carried out in Asia (*n*=10 studies mostly from China and South Korea) (11-18,21,22); one study was carried out in the United States (19), and another one was carried out in Italy (including only post-menopausal women with non-insulin treated type 2 diabetes) (20). Most of these studies included middle-aged and elderly individuals (age: 58.2 years, expressed as “mean of means” of all included studies), predominantly of female sex (63%) and Asian ethnicity (81%); their mean BMI was 25.5 kg/m^2.

The present meta-analysis involved a total of 30,041 individuals (19,353 women and 10,688 men), 30% (n=9,024) of whom had imaging-defined or biopsy-proven NAFLD (11-22). Finally, in the two

cross-sectional studies (conducted in China) examining the association between NAFLD and self-reported history of osteoporotic fractures, the total number of individuals included was 10,492, and the prevalence of osteoporotic fractures in those with NAFLD vs. those without NAFLD was 9% vs. 5.2% in men and 9.6% vs. 11% in women, respectively (21,22).

Of the twelve studies included in the meta-analysis (**supplementary Tables 2-4**), seven studies received at least eight stars on the NOS (indicating that those studies had a relatively low risk of bias), one study received seven stars, whilst the remaining four studies received four stars or less (i.e., being at high risk of bias).

Effect of NAFLD on BMD

Figure 2 shows the distribution of the ten cross-sectional or case-control studies (involving a total of 19,549 individuals) by effect size of the association between NAFLD and BMD measurements at various skeletal sites (11-20).

Overall, the meta-analysis did not show any significant difference in BMD for each skeletal site examined (i.e., whole body, lumbar spine or femur) between individuals with and without NAFLD. The pooled WMD were -0.04 (95% CI -0.16 to + 0.08; I^2=98.9%) for whole-body BMD, -0.01 (95% CI -0.03 to + 0.01; I^2=92.2%) for lumbar BMD, -0.01 (95% CI -0.02 to + 0.01; I^2=94.3%) for femoral-neck BMD, and +0.02 (95% CI -0.01 to +0.05; I^2=87.9%) for femoral-hip BMD, respectively. As specified in Table 1, for most of the eligible studies, the WMD was independent of common clinical risk factors for low BMD (e.g., age, sex, BMI, smoking, physical activity or diabetes status). The large majority of these eligible studies did not report data regarding either *T*-score or prevalence of osteoporosis. However, in those few studies in which this data was available (14,16,17,20), it was confirmed that individuals with NAFLD had either similar or slightly higher *T*-scores than those without NAFLD.

As shown in **supplementary Figure 1,** the Egger's regression test did not show any statistically significant asymmetry of the funnel plots (p=0.86), thus suggesting that publication bias was unlikely, although it should be noted that the numbers of included studies was small.

Subgroup analyses and meta-regression

We carried out some sensitivity analyses for exploring possible sources of heterogeneity across the eligible studies examining the association between NAFLD and BMD.

Stratifying either by sex or by menopausal status (**supplementary Figures 2** and **3**), we did not observe any significant differences in BMD either between women with and without NAFLD, *irrespective* of their menopausal status, or between men with and without NAFLD. These results were consistent for each skeletal site examined, except for a lower femoral-hip BMD in Chinese men with NAFLD than in those without (n=1 study; pooled WMD: +0.08 g/cm^2, 95% CI +0.05 to +0.11).

Stratifying by study country (**supplementary Figure 4**), significant differences (but with *opposite* results) were found in BMD at different skeletal sites between individuals with and without NAFLD for studies performed in China (n=2 studies), which showed lower lumbar and femoral BMD in patients with NAFLD, compared to studies performed in the United States (n=1 study), India (n=1 study) or Turkey (n=1 study) that showed higher whole-body or lumbar BMD in patients with NAFLD. In contrast to this finding, no significant differences were observed in lumbar and femoral BMD between individuals with and without NAFLD in studies performed in South Korea or Italy.

Again, the meta-analysis did not show any significant difference in BMD between individuals with and without NAFLD when the comparison was stratified by the methods used for diagnosing or staging the severity of NAFLD, or whether the studies had eight or nine stars on the NOS scale (i.e., the "high-quality" studies) (**supplementary Figures 5-7**). In particular, these results were consistent for each skeletal site examined, except for a higher lumbar BMD in patients with biopsy-proven NAFLD (n=1 study published by Kaya *et al*.).

Finally, in **supplementary Figure 8** we report the results of univariable meta-regression analyses of the eligible studies, showing no significant associations of WMD in BMD measurement at the level of lumbar spine or femoral neck with age, BMI or percentage of diabetes.

Effect of NAFLD on risk of osteoporotic fractures

Figure 3 shows the distribution of the only two available retrospective cross-sectional studies (involving a total of 10,492 middle-aged Chinese individuals) by effect size of the association between NAFLD and self-reported history of osteoporotic fractures (21,22).

A significant association between ultrasound-defined NAFLD and increased odds of osteoporotic fractures was observed (random-effects OR 1.43, 95% CI 1.0-2.06; I^2=55.1%). This risk was particularly increased among older Chinese men (random-effects OR 2.10, 95% CI 1.36-3.25; I^2=0%). Since we used the fully adjusted OR estimates for each eligible study (as specified in Table 1), this random-effects OR was independent of common clinical risk factors (e.g., age, sex, BMI, lipids, smoking history, physical activity or diabetes).

DISCUSSION

The novel findings of this meta-analysis are as follows: (*i*) there were no significant differences in BMD for each skeletal site examined by DEXA (i.e., whole body, lumbar spine or femur) between individuals with and without NAFLD; (*ii*) these results were consistent in subgroup analyses (and meta-regression analyses) by age, sex, different skeletal sites of BMD measurement, menopausal status, pre-existing diabetes, and methods used for diagnosing or staging NAFLD; (*iii*) when the data were stratified by study country, significant differences (but with *opposite* results) were observed for BMD measurements between individuals with and without NAFLD, especially for studies performed in China (i.e., lower lumbar and femoral BMD in patients with NAFLD) and in the United States (i.e., higher lumbar BMD in patients with NAFLD); and (*iv*) a significant association was observed between NAFLD and self-reported history of osteoporotic fractures, especially among older Chinese men (n=2 studies; random-effects OR 2.10, 95% CI 1.36-3.25; I^2=0%).

The present systematic review and meta-analysis aimed to quantify the magnitude of the association between NAFLD and BMD or history of osteoporotic fractures, and this review and

analysis is the most comprehensive assessment to date. Indeed, our study has involved a total of 12 observational, cross-sectional or case-control studies with aggregate data on ~30,000 middle-aged and elderly individuals of predominantly Asian ethnicity, and 9,024 (30% of total) patients with imaging-defined or biopsy-proven NAFLD (11-22).

Overall, the present findings corroborate and further extend by a ~25-fold increased sample size the results of the only meta-analysis currently available in the literature (34). In this previous meta-analysis, the authors incorporated five cross-sectional studies published up to 2014 (involving a total of nearly 1,300 Asian adult individuals), and failed to find any significant difference in BMD between patients with and without imaging-defined NAFLD (34). However, compared to this smaller meta-analysis, we have now included large observational studies published between 2015 and 2018, which examined the association between NAFLD and either BMD or history of osteoporotic fractures, and conducted a more thorough statistical analysis. As a result our meta-analysis provides evidence that there are no significant differences in whole-body, lumbar or femoral BMD measurements between individuals with and without NAFLD, even after stratifying by age, sex, menopausal status, pre-existing diabetes or methods used for diagnosing or staging NAFLD.

However, in contrast, after stratifying the studies by country, a significant difference in BMD (although with opposite results) was observed between individuals with and without NAFLD principally for studies performed in the United States and China. Notably, our meta-analysis is also the first to show a significant and independent association between the presence of ultrasound-diagnosed NAFLD and a self-reported history of osteoporotic fractures in middle-aged and elderly individuals; this risk was particularly increased among older Chinese men with NAFLD, who reported a greater likelihood of osteoporotic fractures at the level of ankle or lumbar spine (21,22).

It should also be noted that after the study selection process and the final extraction of data for this meta-analysis were concluded (as of the August 31, 2018), a further retrospective cohort study was published examining the association between NAFLD and risk of developing osteoporosis (35). Using the National Health Insurance Research Database in Taiwan (including two separate cohorts: 4,318 patients with NAFLD and 17,272 patients without NAFLD, who were

followed for a median period of ~10 years), Chen *et al*. reported that the risk of newly-diagnosed osteoporosis (based on the diagnostic codes of the International Classification of Diseases [ICD, 9th revision]) was 1.35 times higher in patients with ICD-defined NAFLD, especially among those with coexisting chronic pulmonary diseases and diabetes, than in their counterparts without NAFLD (35). However, further long-term and well-designed prospective studies are needed to address the association between NAFLD and risk of incident osteoporosis.

There is now emerging evidence of biological plausibility that NAFLD may increase risk of osteoporotic fractures (10,36,37). Indeed, NAFLD (especially nonalcoholic steatohepatitis [NASH] with varying levels of hepatic fibrosis) may exacerbate insulin resistance and causes the release of multiple pro-inflammatory cytokines and bone-influencing molecules that may promote bone demineralization and osteoporosis (10,36,37). According to the findings of this meta-analytic study, it is possible to speculate that there may also be a sex-related differential effect of NAFLD on fracture risk. However, it is currently uncertain whether these findings can also be extrapolated to other ethnic populations, given that an increased odds of self-reported osteoporotic fractures among patients with NAFLD has been observed only in (two) retrospective, cross-sectional studies performed in China. Conceptually, it could also appear somewhat paradoxical that individuals for whom it cannot be documented low BMD measurements should incur in more osteoporotic fractures over time. However, it is plausible to speculate that the presence of excessive body weight, i.e., a common feature of patients with NAFLD (1), may prevent bone loss, possibly through increased mechanical loading and enhanced cortical bone formation. Similarly to observations in patients with obesity or type 2 diabetes (23,38), BMD could underestimate the long-term fracture risk in patients with NAFLD (who are frequently obese or diabetic), thus making risk assessment challenging. In recent years, serum levels of 25-hydroxyvitamin D_3 and/or bone turnover biomarkers (e.g., osteocalcin, osteoprotegerin, sclerostin, dickkopf-related protein-1, C-terminal telopeptide of type collagen or procollagen type 1 N-terminal propeptide) have been measured in individuals with NAFLD (10,18,20,31,39-42), but none of these bone biomarkers (except for low vitamin D_3 levels) has been shown to correlate with BMD in patients with NAFLD or other chronic liver diseases (43-46). It should also be noted that some studies have suggested that increased serum levels of bone turnover biomarkers are predictive of greater long-term risk of osteoporotic fractures, but not of lower BMD (10,43,47). However, we believe that more

prospective investigations, particularly in non-Asian populations, and mechanistic studies are needed to better elucidate this important topic.

Although our meta-analysis did not show any significant and consistent difference in BMD at various skeletal sites according to NAFLD severity, it is known that osteoporosis is a frequent complication in patients with cirrhosis, especially in those with alcoholic cirrhosis or advanced cholestatic liver disease (37,48). In this clinical setting, the main risk factors are disease severity, older age and duration of cholestasis. Abnormalities in endocrine system and homeostasis, such as hypogonadism and vitamin D_3 deficiency, as well as malnutrition and drug-induced effects, may also be involved in osteoporosis development among cirrhotic patients (37). Less is currently known about the presence of bone and mineral disorders in NAFLD. However, since the findings of this meta-analysis were based only on three small cross-sectional studies that used either liver biopsy or vibration-controlled transient elastography (Fibroscan) for staging NAFLD (14,17,20), and where patients with established cirrhosis were mostly excluded, we believe that the question of whether the severity of NAFLD (especially non-cirrhotic NASH with varying levels of fibrosis) adversely affects fracture risk remains largely unsolved. More prospective studies with larger cohorts of individuals with biopsy-confirmed NAFLD are needed to investigate this issue.

Our systematic review and meta-analysis has some important limitations, which are inherent to the design of the included studies. First, no prospective studies were available in the literature (prior to August 31, 2018) for this meta-analysis, and the cross-sectional design of the eligible studies limits our ability to establish causality and temporality of the observed associations. Second, most studies originate from Asian countries (mostly China and South Korea), where large populations undergo regular health check-up programs, including liver ultrasonography and DEXA measurements. As Asian and non-Asian populations have different adipose tissue distributions, dietary habits and genetic/cultural backgrounds that might adversely impact on the risk of both osteoporosis and osteoporotic fractures, further studies should be conducted in non-Asian populations. The aforementioned differences in adipose tissue distributions, dietary habits or genetic/cultural backgrounds might also partly explain why Chinese patients with NAFLD appear to be at higher risk of osteoporosis in comparison to the American population. However, it is also plausible to assume that the screening programs in the US/Europe might not be easily compared with the health check-up programs in Asian countries, and thus any definitive conclusion could be

misleading at this time. Third, the overall quality of the included studies is not consistently high, and some of these studies have reported incomplete adjustments for established risk factors and potential confounding variables for osteoporosis (e.g., waist circumference, physical activity or serum 25-hydroxyvitamin D_3 levels). Fourth, although we used a random-effects model, the interpretation of some results of this meta-analysis requires some caution, given the high heterogeneity observed, principally in the pooled primary analysis of cross-sectional studies examining the association between NAFLD and BMD; in contrast, there was a relatively low heterogeneity for the two cross-sectional studies examining the association between NAFLD and history of osteoporotic fractures. From the results of our sensitivity analyses, it is plausible to assume that this high heterogeneity most likely reflects differences in demographic and ethno-racial characteristics of study populations. However, we believe that more detailed analyses of the causes of heterogeneity will require collaborative pooling of individual participant data from large prospective studies, when these will become available in the future. Finally, another potential limitation of this meta-analysis is that most studies used liver ultrasonography, which is the recommended first-line imaging method for diagnosing NAFLD in clinical practice, whereas only one study used biopsy, which is the reference method for diagnosing and staging NAFLD (49-52).

Despite these limitations, our meta-analytic study has also important strengths. We believe that the topic of our meta-analysis is clinically relevant, given the conflicting literature on the risk of osteoporosis in NAFLD and the emerging data regarding underlying biological mechanisms linking NAFLD with both osteoporosis and long-term fracture risk. As discussed previously, our systematic review and meta-analysis provides the most comprehensive and updated assessment on the association of NAFLD with either BMD or self-reported history of osteoporotic fractures. Moreover, we have used standardized risk estimates from all eligible studies to allow consistent combination of estimates across studies. Finally, selective reporting bias of studies was not a major concern in our analyses, as our comprehensive search have made it unlikely that any published report was missed, whilst visual inspection of the funnel plot and the Egger's regression test did not show any significant publication bias.

In conclusion, the results of this comprehensive meta-analysis of observational cross-sectional or case-control studies (involving middle-aged and elderly individuals of predominantly Asian ethnicity) suggest that NAFLD as detected by imaging or histology is significantly associated with a

self-reported history of osteoporotic fractures (especially in elderly Chinese men), but not with low BMD. However, the cross-sectional design of the eligible studies does not allow establishing temporal and causal relationships between NAFLD and risk of osteoporotic fractures. Future large prospective studies, particularly in American and European cohorts, and mechanistic studies are required to better understand the link between NAFLD, bone demineralization and long-term fracture risk.

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FIGURE LEGENDS

Figure 1. The PRISMA flow diagram for search and selection processes of the meta-analysis.

Figure 2. Forest plot of comparison of bone mineral density (BMD) values measured by dual energy X-ray absorptiometry at different skeletal sites (i.e., whole body, lumbar spine and femur) between NAFLD and control groups. The effect size was expressed as weighted mean difference (WMD) and 95% confidence interval for all eligible studies (n=10).

Figure 3. Forest plot and pooled estimates of the effect of NAFLD on the rate of self-reported history of osteoporotic fractures in two eligible cross-sectional studies, stratified by sex.

Supplementary Figure S1. Funnel plot of standard errors by weighted mean difference in BMD. P-value=0.86 by the Egger's regression test. In this analysis were included only the studies with available lumbar BMD measurements (n=9 studies in total, three of whom had also extractable data either for both men and women or for menopausal status).

Supplementary Figure S2. Forest plot of comparison of BMD values measured by dual energy X-ray absorptiometry at different skeletal sites (i.e., lumbar spine and femur) between women with and without NAFLD, stratified by menopausal status. The effect size was expressed as weighted mean difference (WMD) and 95% confidence interval for all studies.

Supplementary Figure S3. Forest plot of comparison of BMD values measured by dual energy X-ray absorptiometry at different skeletal sites (i.e., lumbar spine and femur) between men with and without NAFLD. The effect size was expressed as weighted mean difference (WMD) and 95% confidence interval for all studies.

Supplementary Figure S4. Forest plot of comparison of BMD values measured by dual energy X-ray absorptiometry at different skeletal sites (i.e., whole body, lumbar spine and femur) between NAFLD and control groups, stratified by study country. The effect size was expressed as weighted mean difference (WMD) and 95% confidence interval for all studies.

Supplementary Figure S5. Forest plot of comparison of BMD values measured by dual energy X-ray absorptiometry at different skeletal sites (i.e., whole body, lumbar spine and femur) between NAFLD and control groups, stratified by methods used for diagnosis of NAFLD (liver ultrasonography or Fibroscan vs. biopsy). The effect size was expressed as weighted mean difference (WMD) and 95% confidence interval for all studies.

Supplementary Figure S6. Forest plot of comparison of BMD values measured by dual energy X-ray absorptiometry at different skeletal sites (i.e., lumbar spine and femur) between NAFLD and control groups, stratified by severity of NAFLD (liver biopsy vs. Fibroscan). The effect size was expressed as weighted mean difference (WMD) and 95% confidence interval for all studies.

Supplementary Figure S7. Forest plot of comparison of BMD values measured by dual energy X-ray absorptiometry at different skeletal sites (i.e., whole body, lumbar spine and femur) between NAFLD and control groups, stratified by Newcastle-Ottawa quality assessment Scale (NOS). The effect size was expressed as weighted mean difference (WMD) and 95% confidence interval for all studies.

Supplementary Figure S8. Univariable meta-regression analysis. A meta-analysis of the association of age, body mass index, and percentage of patients with type 2 diabetes (for studies in which this latter data was available; n=6) with weighted mean difference (WMD) in BMD values (measured at the level of lumbar spine or femoral neck) between NAFLD and control groups.

Table 1. Principal observational, cross-sectional and case-control studies examining the association between NAFLD (detected by imaging or histology) and either bone mineral density (BMD) measurements or self-reported history of osteoporotic fractures in middle-aged and elderly individuals.

Author, Year (Ref.)	Study Design, Sample Size, Population Characteristics	Diagnosis of NAFLD, Prevalence of NAFLD	Skeletal Sites examined on DEXA	Outcome Measures; BMD (Mean ± SD); Self-reported history of osteoporotic fractures in no-NAFLD vs. NAFLD group	Covariate Adjustment(s)	Main Findings
			BMD measurements (n = 10)			
Moon SS *et al*. 2012 (11)	Cross-sectional retrospective study of 481 South Korean adult women (216 premenopausal and 265 postmenopausal; mean age 52±13 years, mean BMI 23±8.8 kg/m^2; 14.8% type 2 diabetics)	Ultrasonography; 217 (54 premenopausal and 163 post-menopausal; 45.1% of total) subjects had NAFLD	Lumbar spine BMD	Lumbar BMD: 1.01±0.2 vs. 0.98±0.1 g/cm2, p=0.046 in post-menopausal women Lumbar BMD: 1.15±0.1 vs. 1.18±0.2 g/cm2, p=0.13 in premenopausal women	Age, BMI, smoking, alcohol intake, serum ALT	Lumbar BMD was significantly lower in postmenopausal women with NAFLD than those without NAFLD
Bhatt SP *et al*. 2013 (12)	Case-control study of 162 Indian overweight/obese adult patients with NAFLD and 173 controls matched for age and sex (238 men and 97 women; mean age 37.5±7 years, mean BMI 27.5±3 kg/m^2)	Ultrasonography, 162 subjects had NAFLD	Whole body, lumbar spine BMD (*plus* arm, ribs, trunk and pelvis)	Whole body BMD: 1.10±0.1 vs. 1.20±0.1 g/cm2, p=0.02 Lumbar BMD: 1.05±0.09 vs. 1.08±0.1 g/cm2, p=0.02 Leg BMD: 1.31±0.14 vs. 1.33±0.2 g/cm2, p=0.10	Age, sex, BMI	NAFLD patients had significantly higher BMD at the lumbar spine and whole body sites

Cui R *et al*. 2013 (13)	Cross-sectional retrospective study of 224 Chinese adult individuals (99 men and 125 post-menopausal women; mean age 59.4±5.5 years, mean BMI 25.4±3.5 kg/m^2)	Ultrasonography; 122 (46 men and 76 women; 54.4% of total) subjects had NAFLD	Lumbar spine, right hip and femoral neck BMD	Lumbar BMD: 1.043±0.18 vs. 0.987±0.16 g/cm2, p=0.11 in men, and 0.844±0.15 vs. 0.812±0.14 g/cm2, p=0.22 in women Hip BMD: 0.930±0.12 vs. 0.852±0.12 g/cm2, p=0.007 in men, and 0.805±0.15 vs. 0.725±0.14 g/cm2, p=0.005 in women Femoral neck BMD: 0.812±0.13 vs. 0.736±0.12 g/cm2, p=0.01 in men, and 0.695±0.13 vs. 0.651±0.13 g/cm2, p=0.067 in women	In women: body weight, BMI, waist circumference, HDL-cholesterol and ALT In men: body weight, BMI, waist circumference, plasma lipids	BMD was significantly lower in elderly male and female individuals with NAFLD
Kaya M *et al*. 2013 (14)	Case-control study of 38 Turkish adult patients with biopsy-proven NASH and 42 healthy controls matched for age and sex (49 men and 31 women, mean age 42±11 years, mean BMI 28±4.5 kg/m^2)	Biopsy, 38 subjects had NAFLD	Lumbar spine and femoral neck BMD	Lumbar BMD: 0.941±0.13 vs. 1.057±0.12 g/cm2, p=0.001 Femoral neck BMD: 0.972±0.13 vs. 1.004±0.12 g/cm2, p=0.30	Age, sex, BMI	Lumbar BMD was significantly higher in patients with NASH than in controls. In the NASH group, there was no significant association between BMD and fibrosis stage on histology
Xia MF *et al*. 2016 (15)	Cross-sectional retrospective study of 1,659 Chinese elderly individuals	Ultrasonography; 528 (31.8%) subjects had NAFLD	Whole body, lumbar spine and hip BMD	Whole body BMD: 1.071±0.11 vs. 1.051±0.11 g/cm2, p=0.001	Age, sex, body weight, smoking, alcohol intake,	Patients with NAFLD had significantly lower BMD at the lumbar

	(755 men and 1,028 postmenopausal women; mean age 62±5 years, mean BMI 24.3±3.3 kg/m^2)			Lumbar BMD: 1.062±0.17 vs. 1.036±0.17 g/cm2, p<0.005 Total hip BMD: 0.817±0.12 vs. 0.804±0.11 g/cm2, p=0.07 in 1st vs. 4th quartile of liver fat content on ultrasound	lipid profile, fasting glucose, uric acid and trunk to appendicular fat ratio	spine and whole body sites. When both NAFLD and elevation of serum ALT were present, there was a significant synergistic worsening of the BMDs at all bone sites. After adjustment for potential confounding factors, NAFLD was associated with lower BMD only in men
Lee SH *et al*. 2016 (16)	Cross-sectional retrospective study of 6,634 South Korean adult individuals (3,306 men and 3,328 post-menopausal women; mean age 59±8 years, mean BMI 24.5±2.7 kg/m^2; 10.3% type 2 diabetics)	Ultrasonography; 2,505 (1,288 men and 1,217 women; 37.8% of total) subjects had NAFLD	Lumbar spine, femoral neck BMD	Lumbar BMD: 1.183±0.19 vs. 1.234±0.17 g/cm2, p<0.001 in men, and 0.993±0.15 vs. 1.031±0.15 g/cm2, p<0.001 in women Femoral neck BMD: 0.935±0.14 vs. 0.960±0.13 g/cm2, p<0.001 in men, and 0.794±0.11 vs. 0.811±0.11 g/cm2, p<0.001 in women Prevalence of osteoporosis: 3.3% vs. 0.9% in men and 10.4%	Age, BMI, waist circumference, blood pressure, ALT, smoking status, alcohol intake, physical activity, fasting glucose, eGFR, plasma lipids	In both sexes, the NAFLD group had significantly higher BMD at the lumbar spine and femur. Multivariate linear regression analysis showed a significant negative association between femoral neck BMD and NAFLD in men. However, there was a positive association between lumbar

				vs. 4.6%		BMD and NAFLD in postmenopausal women
Kim G *et al*. 2017 (17)	Cross-sectional retrospective study of 231 South Korean elderly individuals (71 men and 160 women; mean age 61.6±8.4 years, mean BMI 24.5±3.3 kg/m^2; 18.6% type 2 diabetics)	Transient elastography (Fibroscan) with CAP measurement; 129 (55.8%) subjects had NAFLD	Lumbar spine, total hip and femoral neck BMD	Lumbar BMD: 0.896±0.17 vs. 0.921±0.18 g/cm2, p=0.28 Total hip BMD: 0.827±0.14 vs. 0.847±0.15 g/cm2, p=0.32 Femoral neck BMD: 0.685±0.12 vs. 0.701±0.13 g/cm2, p=0.37	Age, sex	BMD measurements at all sites were comparable between those with and those without NAFLD. However, those patients (n=28) with NAFLD and significant fibrosis (i.e. LSM >7 kPa) had significantly lower BMD at lumbar spine (0.858±0.15 g/cm2), total hip (0.786±0.14 g/cm2) and femoral neck (0.655±0.11 g/cm2) compared to those with hepatic steatosis alone
Lee DY *et al*. 2018 (18)	Cross-sectional retrospective study of 3,739 South Korean post-menopausal women (mean age 54.6±2.5 years, mean BMI 23.5±2.7 kg/m^2; 3.6%	Ultrasonography; 605 (16.2%) subjects had NAFLD	Lumbar spine, femoral neck and hip BMD	Lumbar BMD: 1.063±0.14 vs. 1.050±0.13 g/cm2, p=0.017) Femoral neck BMD: 0.858±0.11 vs. 0.835±0.1 g/cm2, p<0.001	Age, age at menarche, years since menopause, BMI, ALT, hemoglobin A1c, HOMA-IR score,	Postmenopausal women with NAFLD had significantly lower BMD at the lumbar spine and femur

	type 2 diabetics)			Hip BMD: 0.921±0.11 vs. 0.921±0.1 g/cm2, p=0.92	potassium, eGFR, uric acid, estradiol, osteocalcin, and C-telopeptide	
Umehara T 2018 (19)	Cross-sectional analysis of data from NHANES 1988-1994, including 6,089 United States adults aged 40–75 years, selected after excluding those with virus hepatitis, excessive alcohol consumption, decreased renal function, steroid use, or pregnant women (2835 men; mean age 54.5±9 years; mean BMI 27.5±3 kg/m^2; 6% type 2 diabetics)	Ultrasonography; 1,690 (27.7%) subjects had NAFLD	Femoral neck BMD	Femoral neck BMD (weighted mean): 0.77 vs. 0.80 g/cm2, p<0.01; Femoral neck BMD in men: 0.813±0.1 vs. 0.839±0.1 g/cm2, p<0.01 In postmenopausal women: 0.701±0.1 vs. 0.741±0.1 g/cm2, p<0.01 Overall prevalence of osteoporosis: 5.6% vs. 3.6%, p=0.01	Age, sex, post-menopausal status, BMI and race/ethnicity	Patients with NAFLD had significantly higher lumbar BMD. After adjusting for age, sex, BMI and menopausal status, race/ethnicity, presence of NAFLD was not associated with BMD. However, NAFLD with higher levels of ALT was associated with lower BMD
Mantovani A *et al*. 2018 (20)	Cross-sectional study of 77 consecutive Italian post-menopausal women with not-insulin treated type 2 diabetes without significant alcohol intake and known causes of liver diseases and without self-reported history	Ultrasonography and transient elastography (Fibroscan) for staging hepatic fibrosis; 62 (80.5%) patients had NAFLD, 10 of whom had clinically significant fibrosis (i.e. LSM >7 kPa)	Lumbar spine, hip and femoral neck BMD	Lumbar BMD: 1.05±0.15 vs. 1.06±0.17 vs. 1.15±0.18 g/cm2, p=0.59 Hip BMD: 0.85±0.14 vs. 0.93±0.14 vs. 0.97±0.18 g/cm2, p=0.81 Femoral neck BMD: 0.78±0.13 vs. 0.84±0.13 vs. 0.87±0.14 g/cm2, p=0.81 Prevalence of	Age, waist circumference, HOMA-IR score, hemoglobin A1c, serum 25(OH)D levels	No significant differences were found in BMD or prevalence of osteoporosis at all sites between the three groups of patients

	of osteoporosis, osteoporotic fractures or treatment with anti-osteoporotic agents (all women; mean age 72±8 years; mean BMI 29±5 kg/m^2; 100% type 2 diabetics)			osteoporosis at column: 13% vs. 14% vs. 10%, and at femur: 24.7% vs. 16% vs. 20% in patients without NAFLD (n=15), patients with hepatic steatosis alone (n=52) and those with NAFLD and significant fibrosis (n=10), respectively		
			Self-reported history of osteoporotic fractures (n = 2)			
Li *et al*. 2012 (21)	Cross-sectional retrospective study of 7,797 Chinese adult individuals (2,441 men and 5,356 women; mean age 58.4±9.5 years, mean BMI 25.5±2.8 kg/m^2)	Ultrasonography; 2,352 (30.2%) subjects had NAFLD	Self-reported recent history of osteoporotic fractures (i.e. fractures that occurred due to low-trauma in 2 years prior to the study)	Prevalence of osteoporotic fractures was higher in those with NAFLD (3.6 vs. 1.7%, p<0.003); however, no difference was found in women (3.4 vs. 2.6%, p=0.14). The presence of NAFLD was associated with increased fracture risk in men (adjusted-odds ratio 2.53; 95% CI 1.26–5.07) but not in women (adjusted-odds ratio 1.19; 95% CI 0.78–1.83)	Age, BMI, waist circumference, smoking, menopausal status, alcohol intake, physical activity, eGFR, plasma lipids, HOMA-IR, diabetes status, hormone replacement treatment or use of oral steroids and osteoporosis drugs	NAFLD was significantly associated with a recent history of osteoporotic fractures in middle-aged and elderly men. The skeletal sites of the most common fractures were ankle in men (20% of all fractures) and wrist in women (25% of all fractures)
Wang *et al*. 2018 (22)	Cross-sectional retrospective study of 2,695 Chinese elderly individuals (950 men and 1,709 women; mean age 73±6 years, mean	Ultrasonography; 614 (22.8%) subjects had NAFLD	Self-reported history of osteoporotic fractures (i.e. fractures that occurred due to low-trauma at age >45 years)	Presence of NAFLD was independently associated with increased fracture risk in men (adjusted-odds ratio 1.86; 95% CI 1.06–3.27) but not in women	Age, BMI, waist circumference, smoking, alcohol intake, physical activity, lipids, diabetes, hypertension,	NAFLD was significantly associated with a history of osteoporotic fractures (especially lumbar

	BMI 24.5±3.1 kg/m^2; 15.9% type 2 diabetics)		(adjusted-odds ratio 1.05; 95% CI 0.74–1.48)	cardiovascular disease, family history of fracture, BMD	fractures) only in men

Abbreviations: ALT, alanine aminotransferase; BMD, bone mineral density; BMI, body mas index; CAP, controlled attenuation parameter; eGFR, estimated glomerular filtration rate; HOMA-IR homeostasis model assessment of insulin resistance; LSM, liver stiffness measurement; NASH, nonalcoholic steatohepatitis; 25(OH)D, 25-hydroxyvitamin D.