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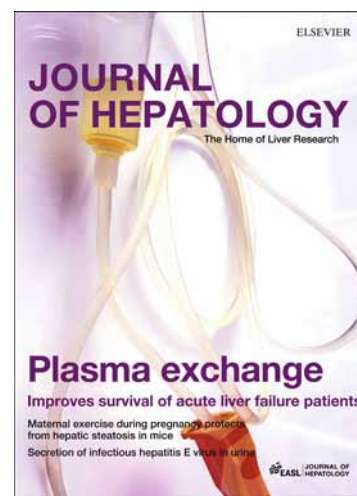
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**Nonalcoholic Fatty Liver Disease and Risk of Incident Cardiovascular Disease:
A Meta-Analysis of Observational Studies.**

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Abstract

Background & Aims: There have been many studies of the effects of nonalcoholic fatty liver disease (NAFLD) and the risk of cardiovascular disease (CVD), but these have produced conflicting results. We performed a meta-analysis of these studies to quantify the magnitude of the association between NAFLD (and NAFLD severity) and risk of CVD events.

Methods: We searched PubMed, Google scholar, and Web of Science databases using terms “NAFLD”, “cardiovascular events”, “cardiovascular mortality”, “prognosis” and their combinations to identify observational studies published through January 2016. We included only observational studies conducted in adults >18 years and in which NAFLD was diagnosed on imaging or histology. Data from selected studies were extracted and meta-analysis was then performed using random effects modelling.

Results: A total of 16 unique, observational prospective and retrospective studies with 34,043 adult individuals (36.3% with NAFLD) and approximately 2,600 CVD outcomes (>70% CVD deaths) over a median period of 6.9 years were included in the final analysis. Patients with NAFLD had a higher risk of fatal and/or non-fatal CVD events than those without NAFLD (random effect odds ratio [OR] 1.64, 95% CI 1.26-2.13). Patients with more ‘severe’ NAFLD were also more likely to develop fatal and non-fatal CVD events (OR 2.58; 1.78-3.75). Sensitivity analyses did not alter these findings. Funnel plot and Egger’s test did not reveal significant publication bias.

Conclusions: NAFLD is associated with an increased risk of fatal and non-fatal CVD events. However, the observational design of the studies included does not allow to draw definitive causal inferences.

Keywords: NAFLD; cardiovascular disease; CVD events; mortality; meta-analysis

Introduction

Non-alcoholic fatty liver disease (NAFLD) is a clinico-pathological syndrome that ranges from simple steatosis to nonalcoholic steatohepatitis (NASH) with varying amounts of fibrosis, and cirrhosis.¹ NAFLD is becoming the most common cause of chronic liver disease worldwide, affecting up to 30% of the adult population in the United States and Europe.¹⁻³ Over the past decade, it has become increasingly clear that NAFLD is not only associated with an increased risk of liver-related morbidity or mortality, but also it is a multisystem disease that affects a variety of extra-hepatic organ systems, including the cardiovascular system.³⁻⁷

A recent comprehensive meta-analysis involving 27 cross-sectional studies has shown that NAFLD was associated with various markers of subclinical atherosclerosis, such as increased carotid artery intimal-medial thickness, impaired flow-mediated vasodilation, increased arterial stiffness or increased coronary artery calcification.⁸ All these associations were independent of multiple cardio-metabolic risk factors across a wide range of patient populations.⁸

Several studies have also demonstrated that the prevalence of clinically manifest cardiovascular disease (CVD) was also significantly increased among patients with NAFLD (as reviewed elsewhere).^{5,6} Worryingly, NAFLD was also associated with a higher prevalence of high-risk and vulnerable coronary artery plaques, independently of traditional CVD risk factors and the extent and severity of coronary atherosclerosis.⁹

Although the cross-sectional association between NAFLD and increased CVD prevalence is strong and consistent, it remains uncertain whether the presence of NAFLD predicts incident CVD events or whether the more severe forms of NAFLD are associated with an even higher risk of future CVD events. Moreover, the mechanisms linking NAFLD to CVD are controversial and several putative mechanisms have been proposed which, however, are to be traced back to liver histologic changes, insulin resistance and oxidative stress.¹⁰

In this context, we have carried out a comprehensive systematic review and meta-analysis of published observational studies to gauge precisely the nature and magnitude of the association between NAFLD and the risk of incident CVD events. We have also investigated whether the severity of NAFLD is associated with a higher risk of CVD events. Clarification of

these issues may have important clinical implications for management of patients with NAFLD.

Methods

Registration of review protocol

The protocol for this review was registered in advance with PROSPERO (International Prospective Register of Systematic Reviews, #CRD42016033481).

Type of studies, inclusion and exclusion criteria and definition of severe NAFLD

Studies were included if they were observational, prospective or retrospective studies that reported fatal and/or non-fatal CVD events in adult patients (>18 years old) with NAFLD as compared with adult individuals without NAFLD. Study participants were of either sex with no restrictions in terms of comorbid conditions. We included only studies in which the diagnosis of NAFLD was based on either radiological imaging or histology in the absence of competing causes of hepatic steatosis. Exclusion criteria were as follows: 1) studies that used only serum liver enzyme levels to diagnose NAFLD; 2) studies conducted in paediatric population (<18 years old); 3) studies performed in patients with NAFLD who received liver transplants; and 4) studies that compared long-term adverse outcomes of fibrosing NASH and NASH-cirrhosis with patients with chronic liver diseases of other aetiology.

Based on data from the eligible studies, 'severe' NAFLD was defined either by presence of steatosis on radiological imaging *plus* either elevated serum gamma-glutamyltransferase (GGT) concentrations or high NAFLD fibrosis score or high hepatic ¹⁸F-fluoro-2-deoxyglucose (FDG) uptake on positron emission tomography or by increasing fibrosis stage on liver histology. All these histological and non-histological/imaging criteria can identify more 'severe' NAFLD, *e.g.*, NASH with varying amounts of fibrosis.^{1,2,11-14}

Included and excluded studies were collected following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow diagram. Additionally, because included studies were observational in design, we followed the Meta-analysis Of

Observational Studies in Epidemiology (MOOSE) guidelines for the meta-analysis of observational studies.

Search strategy and data extraction

Relevant studies were identified by systematically searching PubMed, Google scholar and Web of Science up to January 2016 using the terms “fatty liver” (OR “NAFLD” OR “NASH” OR “nonalcoholic fatty liver disease” OR “nonalcoholic steatohepatitis”) AND cardiovascular events, prognosis, cardiovascular mortality, mortality, CVD, myocardial infarction or stroke. No language restriction was applied. Reference lists of relevant papers and previous review articles were hand searched for other relevant studies. Two investigators independently examined all titles and abstracts, and obtained full texts of potentially relevant papers. Working independently and in duplicate, we read the papers and determined whether they met inclusion criteria. We resolved disagreement by consensus, and extracted data independently using an electronic spreadsheet. For all studies, we extracted information on study design, source of data, population characteristics, outcomes of interests, matching and confounding factors.

Assessment of risk of bias

Two authors assessed the risk of bias independently. Since all the included studies were nonrandomised and had a cohort design, the Newcastle–Ottawa Scale (NOS) was used to judge study quality, as recommended by the Cochrane Collaboration.¹⁵ This 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 four stars for selection, two stars for comparability, and three stars for outcome/exposure. Therefore, nine stars reflect the highest quality. Any discrepancies were addressed by a joint reevaluation of the original article with a third author. We recorded the review authors' judgments about the three NOS domains (selection, comparability and outcome) into the Risk of Bias tool of the Review Manager software of the Cochrane Collaboration. This tool allowed us to provide a graphical representation of quality ratings similar to that produced by Cochrane reviews for randomized studies, as suggested by Wells *et al.*¹⁶

Data synthesis

The outcome measure of this meta-analysis was the incidence of fatal and/or non-fatal CVD events in individuals with NAFLD in comparison with individuals without NAFLD. When possible, we pooled adjusted odds ratios or relative risks or hazard ratios, with their 95% confidence intervals, with the assumption that these are comparable measures of association given that CVD events are relatively rare.¹⁷ Visual inspection of graphs was used to investigate the possibility of statistical heterogeneity. This was supplemented using, primarily, the I-squared statistic. This provides an estimate of the percentage of variability due to heterogeneity rather than chance alone. According to Higgins *et al.*, a rough guide to interpretation is as follows: I-squared values of approximately 25% represent low heterogeneity; approximately 50% represent medium heterogeneity; and approximately 75% represent high heterogeneity.¹⁸

The results of studies were pooled and an overall estimate of odds ratio (OR) was obtained from a random effects model, as this methodology takes into account any differences between studies even if there is no statistically significant heterogeneity.¹⁹ Publication bias was evaluated using the funnel plot and Egger's regression test.²⁰

Primary and secondary analyses and meta-regression

The primary analysis included all clinical CVD events, stratified into CVD mortality, non-fatal CVD events (*i.e.*, myocardial infarction, angina, ischaemic stroke or coronary revascularization), or both. Sensitivity analyses were carried out to examine effect sizes when limiting the analysis to the following subgroups of studies: participants with 'severe' NAFLD only; studies with 8 or 9 stars at the NOS scale ('high-quality' studies); studies with full adjustment for covariates; excluding studies with the general population as reference group (*i.e.*, all those of the same age and sex living in the same country as each patient with NAFLD); excluding studies that enrolled exclusively participants with diabetes, hypertension or acute myocardial infarction. Additionally, we tested for possibly excessive influence of individual studies using a meta-analysis influence test that eliminated each of the included studies at a time.

Results

Characteristics of included studies

Based on the titles and abstracts of 4,569 citations, we identified 33 potentially relevant studies. Of these, we excluded 17 studies for the reasons reported in the MOOSE diagram (**Figure 1**). Thus, 16 unique observational studies, including 17 comparisons, were eligible for inclusion in the meta-analysis and were assessed for quality. As shown in **Table 1**, all the eligible studies had an observational retrospective or prospective design (either community-based or hospital-based or outpatient cohorts).²¹⁻³⁶ In the table we have also included the study by Kim *et al.*³⁷ who studied the same cohort of United States adults included in the study by Lazo *et al.*²⁶ and that we used only in the stratified analysis by NAFLD severity because the authors provided data on the NAFLD fibrosis score. The majority of the eligible studies recruited participants from approximately general populations in which NAFLD was mainly diagnosed by imaging (either ultrasonography or computed tomography) or by histology in a single study.³³ Four cohort studies selected participants on the basis of confirmed pre-existing medical conditions such as diabetes or coronary heart disease at baseline,^{22,25,31,34} whereas another cohort study was particularly enriched of patients with established hypertension.²⁹

Overall, in the 16 observational studies included in the meta-analysis there were 34,043 adult individuals (36.3% with NAFLD) with approximately 2,600 fatal and/or non-fatal CVD events (>70% CVD deaths). Studies were carried out in United States, Europe (Denmark, Finland, Germany, Italy, and Sweden), Asia (China, South Korea, Turkey, and Japan) and Africa (Egypt). Most of these studies included middle-aged subjects predominantly of male sex. The length of the follow-up period ranged from 3 to 26.4 years (except for the study by Emre *et al.*³⁴ who considered only in-hospital events as outcome); median follow-up was of 6.9 years (inter-quartile range: 4.5-10.6 years). Of the 17 comparisons, 7 employed fatal CVD events as outcome measure, 5 fatal and non-fatal CVD events (combined endpoint), and 5 non-fatal CVD events as outcome measure (**Table 1**). CVD events were validated by medical records, death certificates or endpoint committees using the International Classification of Diseases diagnosis codes.

Of the 16 included studies, 10 received eight or nine stars at the NOS, indicating low risk of bias (**supplementary Table 1**). Comparability of cohorts was judged at high risk of bias in two studies (**supplementary Figure 1** and **supplementary Table 2**).

NAFLD and risk of incident CVD events

The distribution of studies by estimate of the association between NAFLD and risk of CVD events is plotted in **Figure 2**. Sixteen studies (17 comparisons) provided data suitable for the pooled primary analysis. NAFLD was significantly associated with an increased risk of fatal and/or non-fatal CVD events (random effect OR 1.64, 95%CI 1.26-2.13, $I^2=86\%$). When this comparison was stratified by outcome (*i.e.*, analysing separately the published studies that had either nonfatal CVD events, or fatal CVD events, or both as primary outcomes), the presence of NAFLD was significantly associated with both an increased risk of fatal and non-fatal CVD events considered together (random effect OR 1.63, 95%CI 1.06-2.48, $I^2=83\%$) and an increased risk of non-fatal CVD events (random effect OR 2.52, 95%CI 1.52-4.18, $I^2=61\%$); however, the association between NAFLD and fatal CVD events (when the analysis was restricted only to studies with CVD mortality as the primary outcome) was not statistically significant (random effect OR 1.31, 95%CI 0.87-1.97, $I^2=90\%$) (**Figure 2**). The Egger's regression test did not show statistically significant asymmetry of the funnel plot, thus suggesting that publication bias was unlikely (**supplementary Figure 2**).

Severe NAFLD and risk of incident CVD events

Six studies (7 comparisons) reported data on patients with 'severe' NAFLD, defined either by presence of hepatic steatosis on imaging *plus* either elevated GGT levels or high NAFLD fibrosis score or high hepatic FDG uptake on positron emission tomography or by increasing fibrosis stage on liver histology. The distribution of studies by estimate of the association between severe NAFLD and risk of incident CVD events is plotted in **Figure 3**. Compared with the non-NAFLD group, the presence of more 'severe' NAFLD was significantly associated with an increased risk of CVD mortality (random effect OR 3.28, 95%CI 2.26-4.77, $I^2=0$) as well as with an increased risk of fatal and non-fatal CVD events considered together (random effect OR 1.94, 95%CI 1.17-3.21, $I^2=23\%$).

Sensitivity analyses

Limiting the analysis to high-quality studies and limiting to studies with adjustment for multiple covariates provided overall estimates consistent with the primary analysis (**Table 2**). Additionally, excluding studies with the general population as reference group,^{17,29} and excluding studies that enrolled only participants with diabetes, hypertension or myocardial infarction^{22,25,29,31,34} had no effect on the comparison (**Table 2**). Finally, eliminating each of the included studies from the analysis had no effect on the overall risk of incident CVD events (data not shown).

Discussion

Several studies have assessed the association between NAFLD and the risk of CVD. The data on whether NAFLD by itself is associated with an increased risk of CVD events and death remains an issue of debate. The results from these studies have been conflicting partly due to variability in NAFLD definition and CVD ascertainment. A prior narrative review published in 2010 by Ghouri *et al.* concluded that a diagnosis of NAFLD was insufficient to consider patients as being at high risk for CVD, and that the evidence base for CVD risk screening based on the presence of NAFLD was weak.³⁸

The presented systematic review and results of the meta-analysis investigating the relationship between NAFLD and incident CVD events is the most comprehensive assessment of this relationship to date. The data provide robust evidence of the association between NAFLD (and NAFLD severity) and risk of incident CVD events. Indeed, the analysis involves a total of 16 unique observational studies with aggregate data on 34,043 adult participants (36.3% with NAFLD) and approximately 2,600 fatal and non-fatal CVD outcomes (>70% CVD deaths) followed-up over a median period of 6.9 years. We found that the presence of NAFLD was significantly associated with a 64% increased risk of a composite endpoint of CVD (*i.e.*, a combined outcome inclusive of CVD death and non-fatal CVD events such as myocardial infarction, angina, stroke or coronary revascularization). When this comparison was stratified by outcome, NAFLD was significantly associated with increased risks of non-fatal CVD events, and fatal and non-fatal CVD events considered together. Moreover, stratified analysis by NAFLD severity among the 6 cohort studies that reported

data on patients with more 'severe' NAFLD showed that patients with more 'severe' NAFLD had a higher risk of developing both CVD mortality (random effect OR 3.28, 95%CI 2.26-4.77, $I^2=0$) and fatal and non-fatal CVD events considered together (random effect OR 1.94, 95%CI 1.17-3.21, $I^2=23\%$) compared with subjects without NAFLD. Although further larger observational studies are needed, the imaging data also suggests that liver fat *per se* is (in our meta-analysis) associated with an increased risk of CVD events. Obviously, the results of this stratified analysis should be interpreted with caution, because we combined data from studies reporting histologic data (fully validated to be a surrogate for clinical outcomes) and studies that used imaging techniques with non-invasive scoring systems or serum biomarkers for defining NAFLD severity. In addition, the combination of studies that used different diagnostic modalities to establish NAFLD severity might introduce some bias and heterogeneity. Unfortunately, as discussed below, most of the published studies that used liver biopsy to diagnose NAFLD (*i.e.*, the 'gold standard' method to define NAFLD severity) lacked an adequate control group and cannot, therefore, be included in this meta-analysis. Finally, the key question of whether the prognostic value of NAFLD in CVD development is restricted to NASH or is also associated with simple steatosis remains unresolved. To date, there are only very few published studies with small sample sizes that have specifically compared the risk of incident CVD events between patients with more severe NAFLD and those with mild NAFLD,³³ and more research is definitely needed to address this issue. A prior meta-analysis did not find any association between the severity of NAFLD histology and risk of CVD events.³⁹

Collectively, our findings extend the results from two previous smaller meta-analyses.^{39,40} In the first study, Musso *et al.*³⁹ in 2011 reported that NAFLD (defined by either ultrasonography or histology; n=7 prospective studies included) was significantly associated with an increased risk of fatal and/or non-fatal CVD events (fixed effect OR 2.05, 95%CI 1.81-2.31). In the second meta-analysis, involving 6 studies (n=4 cross-sectional and n=2 prospective studies), Lu *et al.*⁴⁰ reported that NAFLD diagnosed on ultrasonography was associated with an increased risk of CVD (random effect OR 1.50, 95%CI 1.21-1.87). However, the meta-analysis by Lu *et al.* included also cross-sectional studies, and both of these meta-analyses (given the very small number of eligible studies) did not report analyses

stratified either by outcome (fatal vs. nonfatal CVD events) or by NAFLD severity.^{39,40} More recently, Younossi *et al.* have published a meta-analysis of the global prevalence, incidence, progression and outcomes of NAFLD.⁴¹ Although the authors did not show any specific forest plot of comparison between published studies, they stated that NAFLD was significantly associated only with liver-related mortality, but not with CVD mortality. In particular, in this meta-analysis of observational studies (n=6 prospective studies included for the analysis of CVD mortality) examining the association between NAFLD and CVD mortality, the authors stated that there was no significant association between NAFLD and risk of CVD mortality when they included studies that defined NAFLD by both ultrasonography and serum liver enzymes; in contrast, if NAFLD was diagnosed by ultrasonography, the incidence rate ratio for CVD mortality was increased at 1.37 (95%CI 1.23-1.54) in patients with ultrasound-diagnosed NAFLD as compared to the controls.⁴¹ However, it is important to note that in Dr. Younossi and colleagues' meta-analysis, the same population-based cohort of individuals, *i.e.*, the National Health Nutrition Examination Survey (NHANES)-III cohort, was included many times, and all studies that enrolled only patients with diabetes or obesity were excluded.⁴¹

As regards to this, we paid great attention in excluding from our meta-analysis all studies that lacked an adequate control group, or had inadequate data on outcomes of interest, or studies exhibiting any significant overlap of population. For example, we included only two published studies that used the same NHANES-III database (*i.e.*, the Lazo's study in the pooled analysis and the Kim's study that presented data on the NAFLD fibrosis score in the stratified analysis for examining the association between NAFLD severity and CVD mortality),^{26,37} but excluded other studies^{42,43} that have used the same NHANES-III database of two above-mentioned studies. For the same reason, we excluded the study published by Wong *et al.* in 2011,⁴⁴ given that it evaluated the same patient population as that included in a more recent study, with a longer follow-up period, that we included in our meta-analysis.³¹ Again, we did not consider the studies by Ekstedt *et al.*⁴⁵ and Söderberg *et al.*⁴⁶ given that both cohorts of patients with biopsy-confirmed NAFLD were included in the more recent study by Ekstedt *et al.* (included in our meta-analysis).³³ Finally, we excluded, for example, the retrospective studies by Matteoni *et al.*,⁴⁷ Rafiq *et al.*,⁴⁸ Adams *et al.*,⁴⁹ and Angulo *et*

al.,⁵⁰ who followed-up relatively small cohorts of patients with biopsy-proven NAFLD (recruited from tertiary gastroenterology centers), because of the lack of an adequate control group.

Although this updated meta-analysis of observational studies provides further support for existence of a significant association between NAFLD and increased risk of fatal and non-fatal CVD events, however, it is important to underline that the quality of published studies is not always high (only 10 studies received eight or nine stars at the Newcastle–Ottawa Scale; see supplementary Table 1 and supplementary Figure 1) and that causality remains to be proven in high-quality intervention studies. A clear understanding of the pathophysiological pathways linking NAFLD to CVD events remains elusive, because of the intricate interactions among NAFLD, abdominal obesity, oxidative stress and insulin resistance.^{3,51} However, there is now a growing body of evidence suggesting that NAFLD, especially its more severe forms (*i.e.*, NASH with varying amounts of fibrosis), exacerbates hepatic/peripheral insulin resistance, predisposes to atherogenic dyslipidemia and releases a variety of pro-inflammatory, vasoactive and thrombogenic factors that may promote the development of CVD.^{3-6,10} Accumulating evidence also suggests that NAFLD patients have early changes in cardiac substrate metabolism, producing myocardial functional, structural and arrhythmic consequences.^{6,52-54} Although all these mechanisms plausibly link NAFLD to CVD, no studies to date have proven a cause-and-effect relationship and further research is certainly needed to gain mechanistic insights into the pathophysiology linking NAFLD to CVD.

Collectively, our findings indicate that a diagnosis of NAFLD may identify a subset of the general population, which is exposed to an increased risk of CVD events. Additionally, our findings suggest that the more 'severe' forms of NAFLD are associated with an even greater risk of CVD events. Further to providing evidence for the need for selected case finding for NAFLD in certain high-risk groups,² our data also imply that patients with NAFLD should undergo careful cardiovascular surveillance. Moreover, those with the more severe forms of NAFLD need particular attention to ameliorate their high risk of CVD events.^{5,33,45,46,50,55} However, before this clinical practice strategy can be advocated, large and well-designed intervention trials are needed to test whether treating liver disease in NAFLD may decrease the risk of incident CVD events.

The main strengths and limitations of this study deserve mention. Our meta-analysis provides the most comprehensive assessment and robust evidence to date of the association between NAFLD (as diagnosed either by imaging or by histology) and incidence of major CVD events and death. It includes multiple cohort studies that had recruited participants from general populations, therefore reducing any effects of clinically evident pre-existing disease on NAFLD. In addition, as reported in Table 2, excluding studies that enrolled only patients with diabetes, hypertension or myocardial infarction provided overall estimates consistent with the primary analysis. Moreover, it is important to underline that we employed standardized risk estimates from all eligible studies to allow a consistent combination of estimates across studies. The large number of total CVD events provided high statistical power to quantitatively assess the association between NAFLD and CVD risk. Finally, selective reporting of studies was not a concern in our analyses, as our comprehensive search and contact with investigators made it unlikely that any published report was missed and visual inspection of plots and formal tests demonstrated no statistical evidence of publication bias.

As regards to the limitations of this meta-analysis, we were unable to fully examine the impact of adjustment for all known and potential CVD risk factors and also combine models in studies that adjusted for the same set of confounding factors, because of the varying degree of confounder adjustment across the individual studies (indeed, in a large part of the published studies the adjustment for established CVD risk factors and potential confounding factors is often incomplete – see Table 1). Despite using a composite endpoint of CVD to maximize comparability, we were unable to achieve this across all studies, as some studies reported cause-specific CVD outcomes, such as myocardial infarction, angina, stroke, or CVD death. However, we performed stratified analyses of specific CVD outcomes whenever possible. A plausible explanation for the differential relationship of NAFLD with CVD mortality in pooled vs. stratified analysis may be due, at least in part, to the duration of NAFLD, given that compared to NASH, simple steatosis takes a longer time to progress to advanced fibrosis or cirrhosis,⁵⁶ and the relatively short duration of follow-up of the population-based cohort studies that have evaluated mortality risk as an outcome. We consider that further follow-up studies in larger cohorts of patients with biopsy-confirmed

NAFLD (with an adequate group of control subjects) are needed in order to prove whether NAFLD severity can differentially affect risk of incident CVD events. Additionally, in this meta-analysis, it was not possible to correct the estimates for dynamic changes in NAFLD status over time. This may have led to underestimate the association between NAFLD and incident CVD events, because data involving repeat measurements of fatty liver were not performed in any of the eligible studies. Hence, it is plausible to hypothesize that the observed associations may be even stronger than those observed in the present study. Although we found significant heterogeneity between studies when investigating associations in all patients with NAFLD and CVD events, it is noteworthy that there was very low heterogeneity between studies, and stronger associations between NAFLD and CVD risk, when we restricted the analyses to studies with only the more 'severe' forms of NAFLD. Thus, it is likely that the high heterogeneity in the overall analysis reflects a mix of different people with NAFLD from subjects with simple steatosis who generally have not had the disease for long, to subjects with NASH and advanced fibrosis who have had the disease for many years. We systematically explored and identified possible sources of heterogeneity using stratified analyses, meta-regression and sensitivity analyses; more detailed analyses of the causes of heterogeneity will require collaborative pooling of individual participant data from prospective studies as these become available over time.

In conclusion, the findings of this updated and large meta-analysis of observational studies indicate that NAFLD is significantly associated with an increased risk of fatal and non-fatal CVD events, and that this risk is probably higher in presence of more severe liver disease. Some uncertainty, however, remains as to whether NAFLD is associated with an increased risk of fatal and non-fatal CVD events beyond the known cardiovascular risk factors.

Furthermore, it remains uncertain as to whether NASH is associated with greater CVD risk than simple steatosis. Additional well-controlled prospective studies of a more extensive panel of known CVD risk factors are needed to draw firm conclusions about any independent hepatic contribution to the increased CVD risk observed among patients with NAFLD. Further studies are also needed to establish whether adding NAFLD to the currently available risk scoring systems will improve CVD risk prediction. Finally, in order to assess causal

relationships between NAFLD and CVD events, randomized double-blind placebo controlled trials with CVD outcomes that focus on treatments for liver disease in NAFLD, are needed.

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FIGURE LEGENDS

Figure 1. Included and excluded studies: the MOOSE flow diagram.

Figure 2. Random-effects meta-analysis on the risk of incident CVD events (fatal, non-fatal or both) associated with NAFLD. Forest plot of comparison of patients with NAFLD *versus* those without NAFLD.

Figure 3. Random-effects meta-analysis on the risk of fatal and non-fatal CVD events associated with more 'severe' NAFLD (defined either by presence of fatty liver on imaging *plus* either elevated serum gamma-glutamyltransferase concentrations or high NAFLD fibrosis score or high FDG uptake on positron emission tomography, or by increasing fibrosis stage on liver biopsy).

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LAY SUMMARY

- The data on whether NAFLD by itself is associated with increased cardiovascular events and death remains an issue of debate.
- The findings of this updated and large meta-analysis of observational studies indicate that NAFLD is significantly associated with an increased risk of fatal and non-fatal cardiovascular events.
- However, the observational design of the studies included does not allow us to prove that NAFLD causes cardiovascular disease.
- Clinicians who manage patients with NAFLD should not focus only on liver disease but should also consider the increased risk of cardiovascular disease and undertake early, aggressive risk factor modification.

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Figure 1

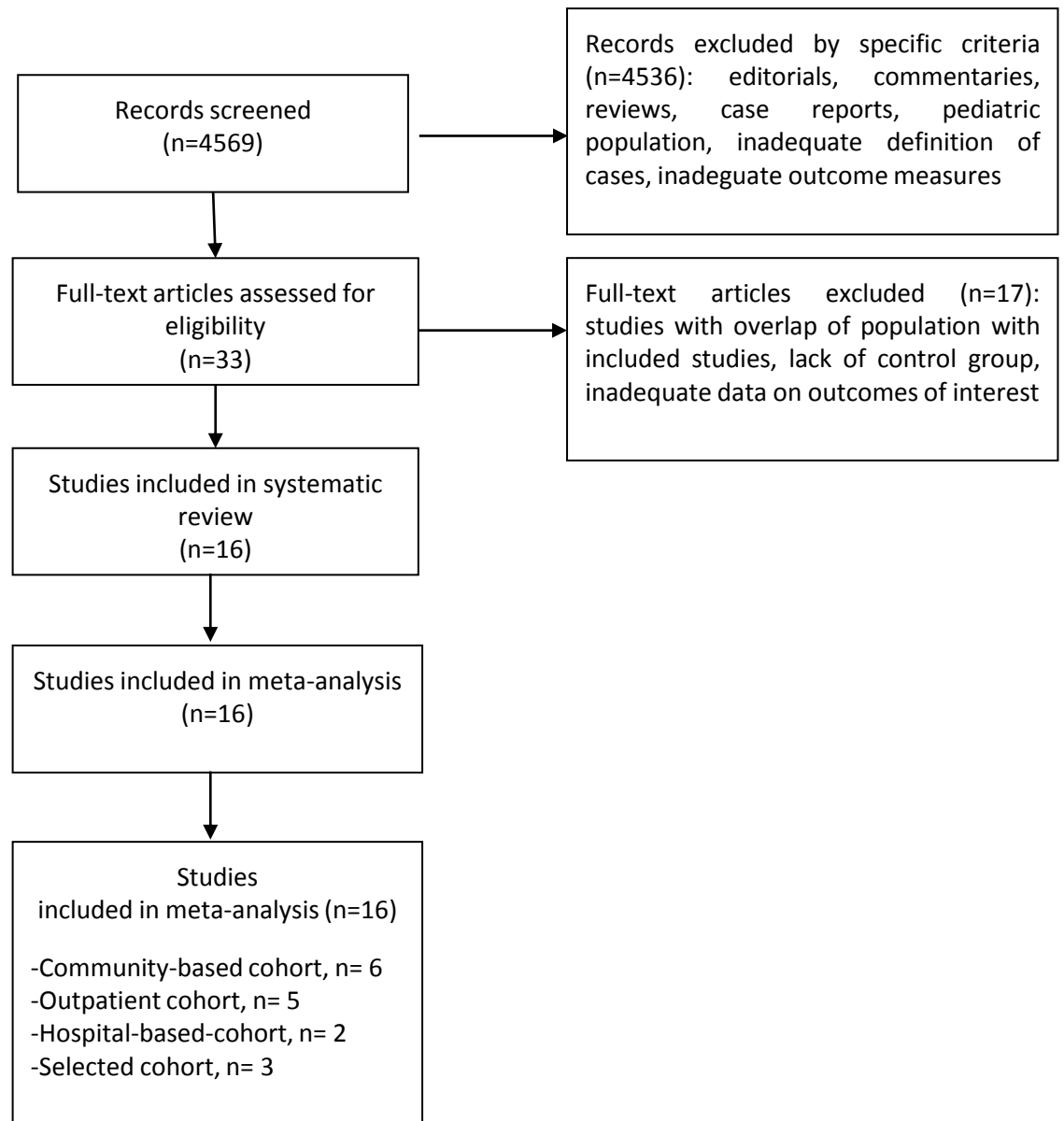


Figure 2

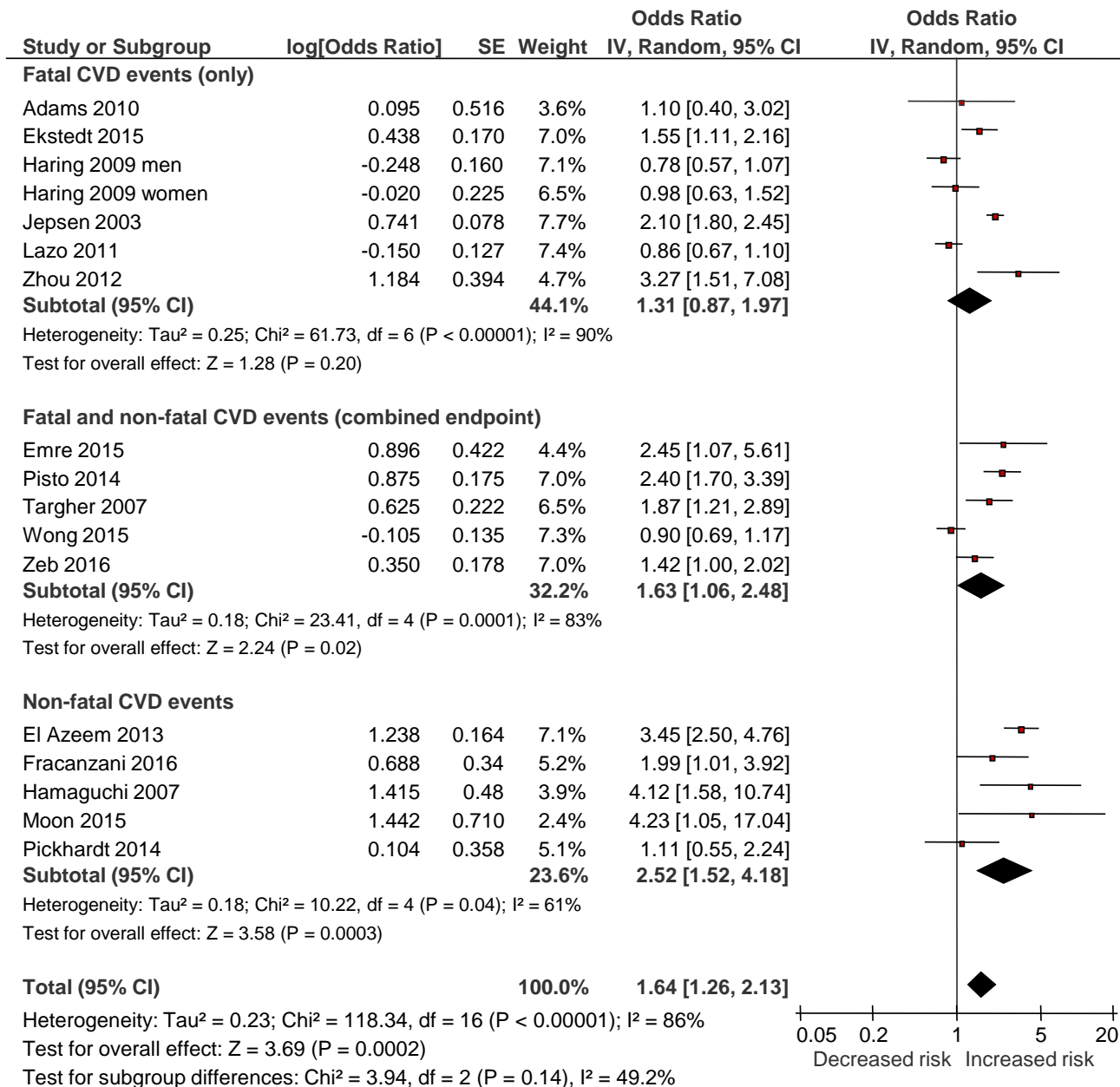


Figure 3

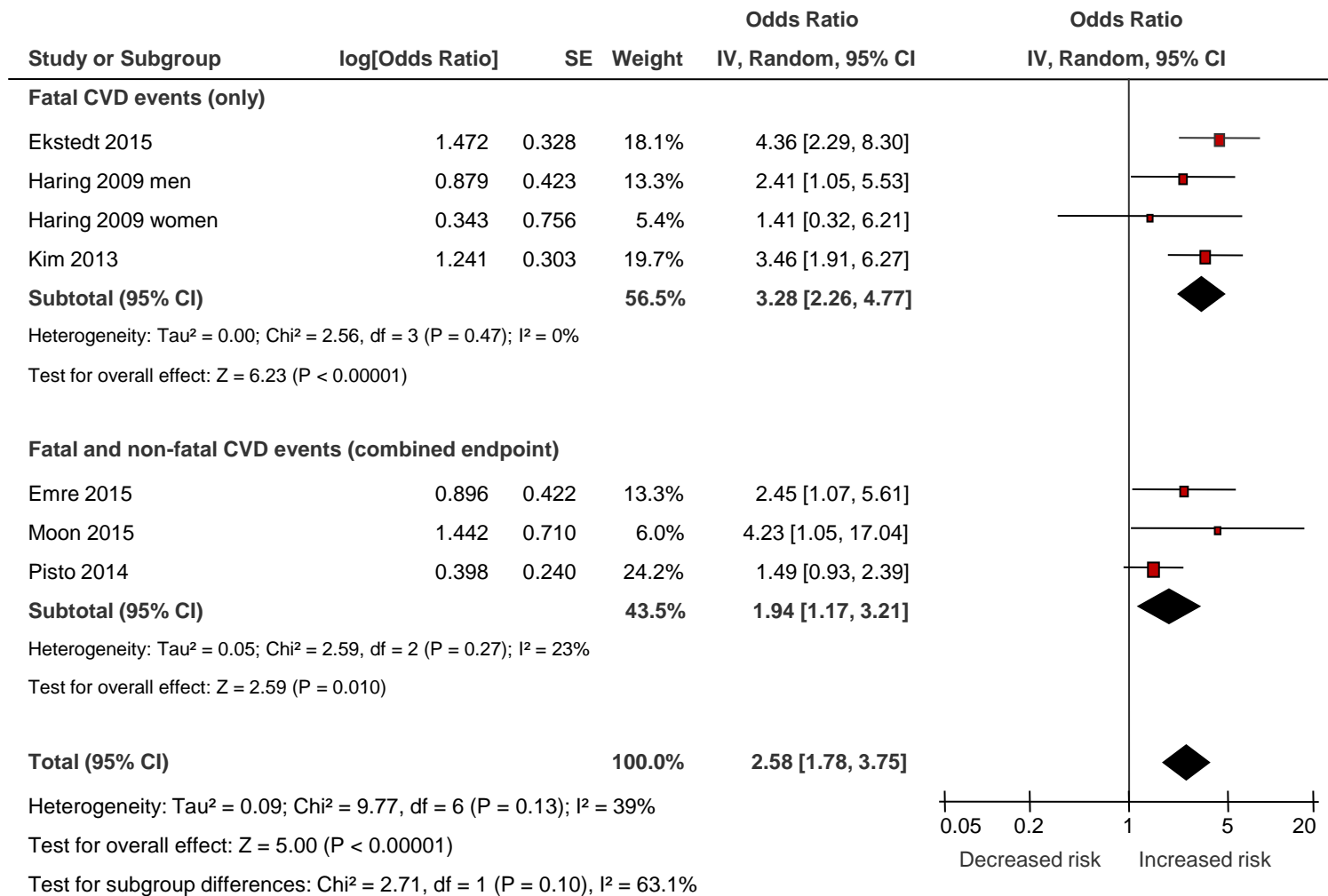


Table 1. Characteristics of observational cohort studies assessing the risk of fatal and/or non-fatal CVD events associated with NAFLD (as diagnosed by imaging or histology).

Authors, Year [Ref.]	Study Design, Sample Size, and Population	Years of Follow-up	Diagnosis of NAFLD, and Number of NAFLD patients	Study Outcomes, and Number of Clinical CVD Events	Adjustments considered	Main Findings, and Adjusted ORs or HRs (\pm 95% CI) for CVD Events	Degree of Adjustment *
Jepsen et al. 2003 ²¹	Retrospective hospital-based cohort, n=1804 patients discharged with a diagnosis of NAFLD from a Danish hospital between 1977 and 1993; patients with cirrhosis were excluded from analysis; 53% men.	6.4 (mean)	Ultrasonography (N=1804 with NAFLD)	All-cause and CVD mortality N=561 total deaths (197 CVD deaths)	The general population comprised all those of the same age and sex living in the same county as each patient with NAFLD at baseline	Increased standard mortality rates of all-cause, liver-related and CVD-related mortality (SMR 2.1, 95%CI 1.8-2.5) in NAFLD compared with the general population	+
Targher et al. 2007 ²²	Prospective outpatient cohort, n=2103 Italian type 2 diabetic patients without known liver diseases or established CVD (Valpolicella Heart Diabetes Study); mean 60 years, 62% men	6.5	Ultrasonography (N=1417 with NAFLD)	Fatal and non-fatal CVD events (myocardial infarction, ischemic stroke, coronary revascularizations or CVD death) N=384 CVD events (121 CVD deaths)	Age, sex, smoking, duration of diabetes, hemoglobin A1c, LDL cholesterol, medication use (i.e., hypoglycemic, anti-hypertensive or lipid-lowering agents), and metabolic syndrome	NAFLD was independently associated with fatal and non-fatal CVD events (adjusted HR 1.87, 95%CI 1.21-2.64)	+++
Hamaguchi et al. 2007 ²³	Population-based cohort, n=1637 Japanese apparently healthy individuals (health check-up program); 1221 participants available for outcome analyses; mean 48 years, 59% men	5	Ultrasonography (N=312 with NAFLD)	Non-fatal CVD events (CHD, ischemic stroke and cerebral hemorrhages) N=22 CVD events	Age, sex, systolic blood pressure, smoking, LDL cholesterol, and metabolic syndrome	NAFLD was independently associated with non-fatal CVD events (adjusted OR 4.12, 95%CI 1.58-10.75 for the entire cohort; adjusted OR 3.56, 95%CI 1.16-10.95 for men, and adjusted OR 7.32, 95%CI 1.22-43.8 for women, respectively)	+++
Haring et al. 2009 ²⁴	Population-based cohort, n=4160 German individuals after excluding those with known liver diseases (Study of Health in Pomerania); mean 49 years, 49% men	7.3 (median)	Ultrasonography & liver enzymes (serum GGT) (N=1249 with NAFLD)	All-cause and CVD mortality N=307 total deaths	Age, waist circumference, alcohol consumption, physical activity, educational level, civil status, equalized income, and functional comorbidity index	NAFLD was not independently associated with CVD mortality (adjusted HR 0.78 95%CI 0.57-1.04 for in men, and adjusted HR 0.98, 95%CI 0.63-1.53 for women). However, presence of fatty liver and elevated serum GGT levels were associated with increased risk of CVD mortality in men (adjusted HR 2.41, 95% CI 1.05-5.55), but not in women (HR 1.41; 95% CI 0.32-6.22)	++
Adams et al. 2010 ²⁵	Retrospective outpatient cohort, n=337 United States patients with type 2 diabetes (from the Olmsted county) after excluding those with known liver diseases; mean 58 years, 49% men	10.9 years (mean)	Ultrasonography, Computed tomography or Histology (N=116 with NAFLD)	All-cause and CVD mortality N=99 total deaths (36 CVD deaths)	Sex, age, duration of diabetes, and obesity	NAFLD was independently associated with increased all-cause mortality, but not with CVD mortality (adjusted HR 1.10, 95% 0.4-3.1)	++
Lazo et al. 2011 ²⁶	Population-based cohort, n=11371 United States adults (NHANES 1988-94); mean 43 years, 48% men	14.5 (median)	Ultrasonography (N=2515 with NAFLD; those with mild steatosis were considered as not	All-cause and cause-specific mortality N=1836 total deaths (716 CVD deaths)	Sex, race, education, smoking, alcohol intake, physical activity, body mass index, hypertension, dyslipidemia,	NAFLD was not associated with increased all-cause and cause-specific (CVD, cancer and liver) mortality. Adjusted HR 0.86, 95%CI 0.67-1.12 for CVD mortality	+++

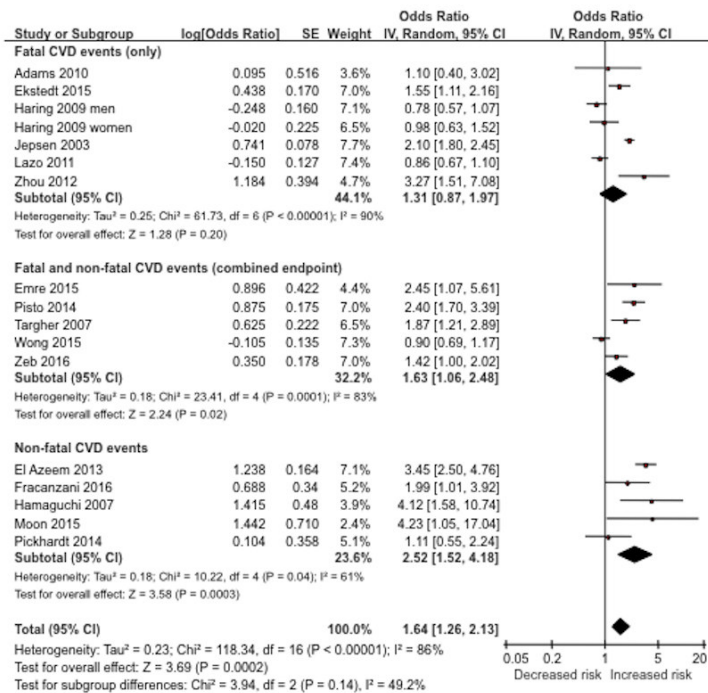
			having NAFLD)		and diabetes		
Zhou et al. 2012 ²⁷	Population-based cohort, n=3324 Chinese individuals without known liver diseases	4 (median)	Ultrasonography (N=467 with NAFLD)	All-cause and CVD mortality N=32 total deaths (29 CVD deaths)	None	NAFLD was significantly associated with CVD mortality (unadjusted HR 3.27, 95%CI 1.51-7.07)	0
El Azeem et al. 2013 ²⁸	Prospective observational cohort, n=1150 Egyptian subjects with normal liver function and without history of CVD (747 subjects completed the follow-up); mean 51 years, 49% men	3 years	Ultrasonography (N=268 with NAFLD)	Non-fatal CVD events (CHD, ischemic stroke and cerebral hemorrhage) N=246 CVD events	None	NAFLD was significantly associated with an increased risk of non-fatal CVD events (unadjusted OR 3.46, 95%CI 2.51-4.76)	0
Pisto et al. 2014 ²⁹	Prospective observational cohort, n=988 middle-aged Finnish participants (OPERA study), enriched of patients with established hypertension (50%); mean 51 years, 49% men	17.7 (median)	Ultrasonography (N=268 with NAFLD)	Fatal and non-fatal CVD events N=169 CVD events (54 CVD deaths)	Age, sex, study group, smoking, alcohol intake, LDL cholesterol, body mass index, systolic blood pressure, and insulin resistance (by QUICKI index)	NAFLD was significantly associated with fatal and non-fatal CVD events (unadjusted HR 2.40, 95%CI 1.70-3.39). However, moderate-severe NAFLD was not independently associated with fatal and non-fatal CVD events (adjusted HR 1.49, 95%CI 0.93-2.18)	++
Pickhardt et al. 2014 ³⁰	Retrospective cohort study of United States adults undergoing abdominal computed tomography selected among 4412 consecutive adults scanned with computed tomography for clinical reasons over a 12-month period: 282 NAFLD patients and 768 non-steatotic controls after exclusion of those with known liver diseases or <1 year of follow-up; mean 51 years, 46% men	7.5 (mean)	Unenhanced computed tomography (N=503 with NAFLD)	Non-fatal CVD events (myocardial infarction, stroke, transient ischemic attacks or coronary bypass or stent) N=73 CVD events	Diabetes, obesity and elevated serum transaminases	NAFLD was not independently associated with non-fatal CVD events (adjusted OR 1.11, 95%CI 0.55-2.23)	+
Wong et al. 2015 ³¹	Prospective outpatient cohort, n=612 consecutive Chinese patients undergoing coronary angiograms without known liver diseases; mean 63 years, 71% men	6 (mean)	Ultrasonography (N=356 with NAFLD)	Fatal and non-fatal CVD events, heart failure or secondary coronary interventions N=225 CVD events (106 CVD deaths)	Age and sex	NAFLD was associated with significant CHD needing percutaneous coronary interventions at baseline, but NAFLD was not significantly associated with fatal and non-fatal CVD events (age- and sex-adjusted HR 0.90, 95%CI 0.69-1.18). NAFLD was associated with lower CVD mortality (age- and sex-adjusted 0.33, 95% CI 0.15-0.73)	+
Moon et al. 2015 ³²	Retrospective observational cohort, n=815 consecutive South Korean asymptomatic participants who underwent a general health screening program (to screen for possible malignancies) that included liver ultrasonography, positron emission tomography and carotid intima-media thickness measurements after excluding those with known liver diseases and a plasma glucose level >200 mg/dl; mean 52 years, 94% men	4.2 (mean)	Ultrasonography & positron emission tomography with F-18 fluoro-2-deoxyglucose (FDG) (N=394 with NAFLD)	Non-fatal CVD events (myocardial infarction, angina, coronary revascularization) N=9 CVD events	Age, sex, and serum triglycerides	NAFLD with high hepatic FDG uptake was independently associated with non-fatal CVD events (adjusted HR 4.23; 95% CI 1.05-17.04)	+

Ekstedt et al. 2015 ³³	Retrospective outpatient cohort, n=229 Sweden patients with NAFLD and elevated serum liver enzyme levels (49% NASH); mean 49 years, 66% men	26.4 (mean)	Histology (N=229 with NAFLD)	All-cause and CVD mortality N=96 total deaths (41 CVD deaths)	The reference population comprised all those of the same age and sex living in the same county as each patient with NAFLD at baseline	Increased rates of all-cause, liver-related and CVD mortality (adjusted HR 1.55, 95% CI 1.11-2.15) in patients with NAFLD compared with the general control population. Fibrosis stage on histology significantly predicted the risk of all-cause, liver-related and CVD mortality (adjusted HR 4.36, 95% CI 2.29-8.29)	+
Emre et al. 2015 ³⁴	Retrospective hospital-based cohort, n=186 consecutive Turkish non-diabetic patients undergoing primary percutaneous coronary interventions for ST-segment elevation myocardial infarction after excluding those with known liver diseases or established diabetes; mean 58 years, 76% men	In-hospital cardiac events	Ultrasonography (N=75 with NAFLD)	In-hospital CVD events (acute myocardial infarction, acute heart failure or death) N=32 CVD events (8 CVD deaths)	Age, body mass index, total cholesterol, HDL cholesterol, triglycerides, presence of anterior wall infarction, and multi-vessel coronary disease	Moderate-severe NAFLD was independently associated with increased in-hospital CVD events (adjusted OR 2.45, 95% CI 1.07-4.87). Moderate-severe NAFLD was not independently associated with CVD death (adjusted OR 2.24, 95% CI 0.97-5.16)	+++
Zeb et al. 2016 ³⁵	Prospective cohort study, n=4119 United States participants aged 45 to 84 years who were free of CVD and known liver diseases at baseline (The Multi-Ethnic Study of Atherosclerosis); mean 62 years, 45% men	7.6 years (median)	Non-enhanced computed tomography (N=728 with NAFLD)	All-cause mortality and non-fatal CVD events (myocardial infarction, resuscitated cardiac arrest, angina or coronary revascularization procedures) N=253 deaths and 209 nonfatal CVD events	Age, sex, ethnicity, diabetes, hypertension, body mass index, lipids, smoking, family history of CHD, statin use, C-reactive protein, and coronary artery calcium score on cardiac CT scans	NAFLD was independently associated with a composite endpoint inclusive of all-cause death and nonfatal CVD events (adjusted HR 1.42, 95% CI 1.00-2.03)	+++
Fracanzani et al. 2016 ³⁶	Prospective cohort study, n=125 Italian patients with NAFLD and 250 age- and sex-matched control individuals without known liver diseases; mean 52 years, 87% men	10 years	Ultrasonography or histology (N=125 with NAFLD)	Non-fatal CVD events (acute coronary syndrome, coronary revascularization procedures, ischemic stroke or transitory ischemic attacks) N=35 CVD events	Sex, smoking history, diabetes, hypertension, and carotid atherosclerotic plaques on ultrasound	NAFLD was independently associated with non-fatal CVD events (adjusted HR 1.99, 95% CI 1.01-3.91)	+++
Kim et al. 2013 ³⁷	Population-based cohort, n=11154 United States adults (NHANES 1988-94); mean 43 years, 48% men	14.5 (median)	Ultrasonography (N=4083 with NAFLD those with mild steatosis were considered as having NAFLD)	All-cause and CVD mortality N=1795 total deaths (673 CVD deaths)	Age, sex, race, education, income, diabetes status, hypertension, pre-existing CVD, lipid-lowering medications, smoking, waist circumference, alcohol intake, caffeine intake, total cholesterol, HDL cholesterol, transferrin saturation, C-reactive protein	NAFLD was not associated with increased all-cause and CVD mortality (adjusted HR 0.75 95%CI 0.56-1.01) in the whole cohort. However, NAFLD with advanced hepatic fibrosis (defined by the NAFLD fibrosis score) was independently associated with increased all-cause and CVD mortality (adjusted HR 3.46, 95%CI 1.91-6.25)	+++

* **Degree of adjustment:** 0 unadjusted; + adjusted for age and/or sex; ++, further adjustment for traditional CVD risk factors; +++, further adjustment for non-traditional CVD risk factors and/or metabolic syndrome.

Table 2. Risk of fatal and/or non-fatal CVD events associated with NAFLD (as diagnosed either by imaging or by histology): sensitivity analyses.

Analysis	Number of comparisons	Overall ORs or HRs (with 95% confidence intervals)	P values	I ² values
Including only high-quality studies at the Newcastle-Ottawa scale	11	1.54 (1.13-2.11)	<0.001	86%
Including only studies with full adjustment for covariates	6	1.69 (1.11-2.58)	<0.001	78%
Excluding studies with the general population as the reference group	15	1.63 (1.19-2.22)	<0.001	86%
Excluding studies with cohorts of participants with diabetes, hypertension or acute myocardial infarction	13	1.57 (1.15-2.14)	<0.001	89%



Graphical abstract