A significant risk of metabolic dysfunction-associated fatty liver disease plus diabetes on subclinical atherosclerosis

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Abstract

Background

This cross-sectional study aims to investigate the association between subclinical atherosclerosis and metabolic dysfunction-associated fatty liver disease (MAFLD) or non-alcoholic fatty liver disease (NAFLD), and a synergistic effect of diabetes mellitus (DM) and MAFLD on subclinical atherosclerosis.

Methods

Of 977 subjects who underwent health checkups with coronary artery calcification (CAC), carotid intima-media thickness, and brachial-ankle pulse wave velocity (ba-PWV), 890 were included in this study. They were classified as MAFLD, NAFLD, or Neither-FLD, and MAFLD was further categorized into three groups by three metabolic disorders (obesity, lean with metabolic dysregulation, DM), according to its new definition: Obesity-MAFLD, Lean-MAFLD and DM-MAFLD.

Results

In a multivariable analysis, MAFLD and NAFLD were significantly associated with subclinical atherosclerosis, except for an association between ba-PWV and NAFLD. MAFLD had higher odds for CAC than NAFLD (for CAC score > 100, odds ratio (OR) = 2.599, 95% confidence interval (CI) = 1.625–4.157; OR = 1.795, 95%CI = 1.145–2.814, respectively). In a sub-analysis, DM-MAFLD had higher odds for CAC (for CAC score > 100, OR = 5.833, 95% CI = 3.047–11.164) than the other groups of MAFLD, when compared to Neither FLD as a reference. Moreover, DM-MAFLD had a higher level of homeostasis model assessment of insulin resistance and high sensitive C-reactive protein, compared to the other groups of MAFLD.
Conclusions

MAFLD was significantly associated with subclinical atherosclerosis in the general population. Additionally, DM-MAFLD could be a significant risk factor for cardiovascular disease through insulin resistance and low-grade inflammation and requires careful follow-up or appropriate intervention.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is strongly associated not only with cirrhosis and carcinoma, but also with metabolic syndrome and its related components, leading to high morbidity and mortality from liver-related and extrahepatic diseases [1, 2]. The most common cause of death in patients with NAFLD is cardiovascular disease (CVD), independent of other metabolic comorbidities. Several studies have shown that NAFLD is a significant risk factor for atherosclerosis in the coronary and carotid arteries [3–6]. Furthermore, NAFLD has been suggested as an independent predictor of coronary artery calcification (CAC) using CAC score (CACS), a marker of a substitute for coronary arterial plaque burden evaluated by cardiac computed tomography (CT) [7–14]. In addition, NAFLD was reported to be strongly associated with subclinical atherosclerosis, including elevated brachial-ankle pulse wave velocity (ba-PWV) and carotid intima-media thickness (IMT) [15].

Very recently, an international expert group recommended “metabolic dysfunction-associated fatty liver disease (MAFLD)” as a more appropriate term to highlight the importance of the metabolic dysfunction risk for CVD [16]. The definition of MAFLD includes hepatic steatosis (> 5% liver fat) plus any one of the following three groups [16]: 1) overweight/obesity; 2) lean/normal weight with specific metabolic dysregulation; 3) DM. A subsequent study reported that MAFLD associated more strongly with CVD than NAFLD [17]. However, no studies have comprehensively investigated the correlation between asymptomatic atherosclerosis such as CAC, ba-PWV, or carotid IMT, and MAFLD or MAFLD with DM (DM-MAFLD). Thus, this cross-sectional study aims to investigate the correlation of MAFLD and subclinical atherosclerosis, and to further examine whether MAFLD and DM may have a synergistic effect on subclinical atherosclerosis.

Materials and methods

Study setting and population

This cross-sectional study was conducted as part of a comprehensive study of NAFLD, approved by the Institutional Review Board of Keio University Hospital (IRB No. 20170384). The need for informed consent was waived by the ethics committee, because this retrospective study is reporting medical records. We also have discussed whether all data were fully anonymized before we accessed them. The inclusion criteria for this study was a series of 977 individuals who evaluated subclinical atherosclerosis using cardiac CT scan, ba-PWV and carotid artery ultrasound, as part of a health checkup at the Center for Preventive Medicine, Keio University Hospital, between August 2012 and December 2018. The exclusion criteria were as follows: heart diseases such as chronic heart failure or arrhythmia (n = 85) and data missing (n = 2). Thus, 890 subjects were included in the final analysis of the study.

Competing interests: The authors have declared that no competing interests exist.
Collection of medical data

The following data were retrieved from their medical records: Demographics, medical history, obesity-related factors including body mass index (BMI) and visceral adipose tissue (VAT), and blood test data. BMI was calculated as weight (kg) divided by the square of height (m²). To measure VAT, umbilicus level Fat CT was performed and then calculated with AZE Virtual Place software (AZE Inc., Tokyo, Japan). The blood test included: total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), fasting blood sugar (FBS), hemoglobin A1c (HbA1c), homeostatic model assessment-insulin resistance (HOMA-R), estimated glomerular filtration rate (eGFR) and high sensitive C-reactive protein (hs-CRP). If the value of hs-CRP was greater than 0.14, it was defined as elevated.

The following self-administered questioners were routinely used to assess the lifestyle of subjects who underwent our health checkups: (Q1) Have you ever smoked? (Ever smoking, +/-). (Q2) Are you a non-drinker, or how much and how often do you drink alcohol? (Q3) Are you usually exercising over more than 30 minutes with a little sweat at least 2 days per week? (Exercise, +/-).

Assessment of subclinical atherosclerosis and fatty liver

CAC was detected with a VCT XTe-64 slice multidetector CT scanner (GE Healthcare, Tokyo, Japan) by using the standard scanning protocol [14]. The calcium content in each coronary artery was measured and summed, and the total CACS was determined using the method by Agatston [14, 18]. CACS were classified into one of two grades (0 versus > 0, or ≤ 100 versus > 100).

PWV between the brachial and ankle sites and IMT of common carotid artery were evaluated as previously reported [19]. PWV was measured by an automatic waveform analyzer (Colin Medical Technology Corporation; Komaki, Japan) and elevated arterial stiffness was defined as ba-PWV > 1400 cm/s. The maximum IMT was measured using a high-resolution Logiq S8 system (GE Healthcare; Tokyo, Japan) and was determined to be elevated if it was 1.1mm or greater.

Fatty liver was diagnosed by the following ultrasound (US) findings: liver brightness, echo contrast between the hepatic and renal parenchyma, vascular burring and deep attenuation [20]. Our experienced sonographers performed these US examinations, followed by confirmation by the radiologist.

NAFLD, and MAFLD and their grouping

NAFLD subjects were defined as those with (1) the presence of FL by US (2) daily alcohol consumption ≤ 30g for males and ≤ 20g for females (3) the absence of positive HBs antigen or HCV antibody [1]. MAFLD was diagnosed, according to the criteria [16], as those with FLD by US plus any one of the following three groups: 1) Obesity-MAFLD, overweight/obesity (≥ 23kg/m²); 2) Lean-MAFLD, lean/normal weight (< 23kg/m²) with specific metabolic dysregulation; 3) DM-MAFLD, type 2 DM. Metabolic dysregulation was defined as the presence of at least two metabolic conditions as follows: (1) waist circumference ≥ 90 cm in males and 80 cm in females, (2) blood pressure ≥ 130 mmHg for systolic, ≥ 85 mmHg for diastolic or specific drug treatment, (3) TG ≥ 150 mg/dl or specific drug treatment, (4) HDL-C < 40 mg/dl for males and < 50 mg/dl for females or specific drug treatment, (5) prediabetes (FBS 100–125 mg/dl or HbA1c 5.7–6.4%) (6) HOMA-R ≥ 2.5, and (7) CRP > 2 mg/L. Thus, MAFLD was subdivided into three groups: Obesity-MAFLD, Lean-MAFLD and DM-MAFLD.
**Evaluation of hepatic fibrosis by non-invasive hepatic fibrosis marker**

To assess the association between hepatic fibrosis and each group of MAFLD, the fibrosis-4 index (FIB-4) was calculated as follows: FIB-4 = [age (years) x AST (IU/L)] / [platelet count (x $10^9$/L) x ALT (IU/L)$^{1/2}$]. The cutoff points were chosen as 1.3 to divide low fibrosis and moderate-high fibrosis [21].

**Statistical analysis**

For continuous data, mean values were expressed with standard deviation (SD), and statistical differences between two groups were determined using the t-test or Mann-Whitney U in the univariate analyses. For categorical data, numbers were presented with percentage, and statistical differences were determined using the chi-square tests. Then, a binary regression analysis was used to analyze the correlation between the dichotomous outcome (CAC score = 0 versus > 0, or > 100 versus ≤ 100) and MAFLD or NAFLD. Two models were presented with progressive adjustments by covariates, which were chosen for clinical importance as well as statistical significance, including age, sex, hypertension, dyslipidemia, smoking status, physical activity and eGFR in this multivariable regression model. The presence of DM was not included in these models to avoid collinearity, since it was used to define DM-MAFLD. A sensitivity analysis was carried out to confirm the robustness of the associations by comparing using two types of controls. All statistical analyses were performed using SPSS software version 24 (SPSS, Inc., Chicago, Ill). All $p$-values less than 0.05 were considered statistically significant.

**Results**

**Clinical characteristics of study population**

The mean age of 890 subjects was 60.2 ± 12.3 years and 598 were males (67.2%). Fig 1 shows that MAFLD subjects were 384 (43.1%), which included 320 of Obesity-MAFLD, 63 of Lean-MAFLD and 84 of DM-MAFLD, whereas the prevalence of NAFLD subjects was 30.1%. Two hundred and fifty subjects (28.1%) belonged to both FLD. Table 1. demonstrates that MAFLD subjects were significantly associated with metabolic abnormalities and subclinical atherosclerosis, compared to those without (MAFLD-).

**The association of subclinical atherosclerosis with presence of MAFLD or NAFLD**

As shown in Table 2, MAFLD and NAFLD were significantly associated with CACS, respectively, compared to MAFLD- or NAFLD- as a reference: for CACS > 0, odds ratio (OR) = 1.821, 95% confidence interval (CI) = 1.331–2.492; OR = 1.825, 95% CI = 1.320–2.524; for CACS > 100, OR = 2.599, 95% CI = 1.625–4.157; OR = 1.795, 95% CI = 1.145–2.814, respectively. MAFLD was also significantly associated with elevated ba-PWV and carotid IMT (OR = 1.562, 95% CI = 1.128–2.161; OR = 1.823, 95% CI = 1.287–2.580), whereas NAFLD was correlated only with the latter (OR = 1.999, 95% CI = 1.407–2.840).

**Sub-analysis of subclinical atherosclerosis in four groups with combination of MAFLD and/or DM**

Fig 2 shows the percentage of subclinical atherosclerosis in four groups, based on combination of MAFLD and/or DM by age (under 50, 50s, 60s, 70 and over): (1) no MAFLD and no DM (MAFLD-DM-, n = 464); (2) no MAFLD and DM (MAFLD-DM+, n = 42); (3) MAFLD
without DM (MAFLD+DM-, n = 300); (4) MAFLD with DM (DM-MAFLD, n = 84). The risk-positive percentage of DM-MAFLD was higher than that of MAFLD+DM- across all ages in all risk indicators of subclinical atherosclerosis, and the latter was higher than that of MAFLD-DM-. The multivariable analysis demonstrated that DM-MAFLD had higher odds for subclinical atherosclerosis than MAFLD+DM- (for CACS > 0, OR = 3.913, 95% CI = 2.249–6.810 vs OR = 1.546, 95% CI = 1.102–2.171; for CACS > 100, OR = 7.218, 95% CI = 3.784–13.767 vs OR = 1.990, 95% CI = 1.163–3.404; for c-IMT ≥ 1.1, OR = 2.231, 95% CI = 1.287–3.868 vs OR = 1.704, 95% CI = 1.164–2.495, respectively), except for ba-PWV, when MAFLD-DM- was a reference (Table 3).

Sub-analysis of subclinical atherosclerosis and hepatic fibrosis in the MAFLD groups

Lean-MAFLD and DM-MAFLD had a higher risk-positive percentage for subclinical atherosclerosis than Obesity-MAFLD, as shown in Table 4, and S1 Fig. When the Neither-FLD was a reference, the multivariable-adjusted odds [95% CI] for CACS > 0 and CACS > 100 were higher in DM-MAFLD (3.908 [2.258–6.764], and 5.833 [3.047–11.164]) than those in the other groups of MAFLD (Table 5). Regarding the probability of moderate to high hepatic fibrosis...
estimated by the FIB-4 index, there seemed to be no significant difference among the three groups of MAFLD (S2 Fig).

**Discussion**

We found that MAFLD is significantly associated with CAC, elevated ba-PWV and carotid IMT, even after adjusted by age, sex, hypertension, dyslipidemia, eGFR, smoking, and exercise, whereas NAFLD had a significant association with the above subclinical atherosclerosis, except for ba-PWV. Moreover, MAFLD had higher odds of CACS > 100, in comparison with NAFLD. Thus, MAFLD could identify subclinical atherosclerosis better than NAFLD in the general population.

In this cross-sectional study, the prevalence of MAFLD determined by the new definition was 43.1%, compared to the prevalence of 30.1% for NAFLD, which is almost the same as

| Table 1. Clinical characteristic by presence of MAFLD. |
|-----------------|-----------------|-----------------|-----------------|
| Characteristics | MAFLD+ | MAFLD- | P |
| Age (years)     | 384 (43.1) | 506 (56.9) | 0.341 |
| Male            | 308 (80.2) | 290 (57.3) | 0.000 |
| Body mass index (kg/m²) | 25.9 ± 3.7 | 21.8 ± 2.7 | 0.000 |
| VAT (cm²)       | 133.9 ± 52.0 | 73.2 ± 39.0 | 0.000 |
| Questioners     |          |          |     |
| Ever smoking    | 225 (58.6) | 228 (45.1) | 0.000 |
| Exercise        | 136 (35.4) | 199 (39.3) | 0.233 |
| Hypertension    | 175 (45.6) | 156 (30.7) | 0.000 |
| Diabetes mellitus | 84 (21.9) | 42 (8.3) | 0.000 |
| Dyslipidemia    | 138 (35.9) | 90 (17.7) | 0.000 |
| Blood test      |          |          |     |
| Total cholesterol (mg/dL) | 203.4 ± 36.1 | 209.2 ± 35.5 | 0.017 |
| LDL-C (mg/dL)   | 117.6 ± 30.3 | 115.5 ± 28.7 | 0.291 |
| HDL-C (mg/dL)   | 50.6 ± 11.8 | 63.4 ± 15.9 | 0.000 |
| Triglycerides (mg/dL) | 147.0 ± 97.2 | 89.5 ± 45.0 | 0.000 |
| Albumin (g/dl)  | 4.35 ± 0.29 | 4.27 ± 0.27 | 0.000 |
| Platelet (x10⁹/ul) | 22.3 ± 5.4 | 22.0 ± 5.4 | 0.294 |
| Fasting blood sugar (mg/dL) | 114.7 ± 23.3 | 102.9 ± 17.3 | 0.000 |
| Hemoglobin A1c (%) | 5.96 ± 0.68 | 5.66 ± 0.55 | 0.000 |
| HOMA-R          | 2.4 ± 2.4 | 1.1 ± 0.8 | 0.000 |
| Aspartate transaminase (U/L) | 26.9 ± 13.5 | 23.2 ± 17.5 | 0.000 |
| Alanine transaminase (U/L) | 29.4 ± 18.9 | 20.5 ± 40.4 | 0.000 |
| γ-GTP (U/L)     | 51.8 ± 68.4 | 33.9 ± 74.7 | 0.000 |
| Elevated hs-CRP | 73 (19.0) | 50 (9.9) | 0.000 |
| Subclinical atherosclerosis |          |          |     |
| CACS > 0        | 189 (49.2) | 161 (31.7) | 0.000 |
| CACS > 100      | 78 (20.3) | 44 (8.7) | 0.000 |
| ba-PWV > 1400 (cm/s) | 200 (52.1) | 227 (28.2) | 0.033 |
| c-IMT ≥ 1.1 (mm) | 122 (31.8) | 100 (19.7) | 0.000 |

MAFLD, metabolic dysfunction-associated fatty liver disease; VAT, visceral adipose tissue; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; HOMA-R, homeostasis model assessment of insulin resistance; γ-GTP, gamma-glutamyl transferase; hs-CRP, high sensitive C-reactive protein; CACS, coronary artery calcification score; ba-PWV, brachial ankle pulse wave velocity; c-IMT, carotid intima media thickness.

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some previous epidemiological studies [17, 22, 23]. In the comparison of MAFLD but without NAFLD (MAFLD only) to NAFLD (S1 Table), the former was predominantly male, with a higher proportion of drinkers or smokers, and a higher frequency of metabolic disorders such as hypertension or diabetes. This difference is believed to be due to differences in the diagnostic criteria for MAFLD and NAFLD. The proportion of coexisting DM with MAFLD (i.e., DM-MAFLD) among MAFLD in this study was slightly higher than in previous reports [17].

Table 2. The association of subclinical atherosclerosis with presence of MAFLD or NAFLD.

| Subclinical atherosclerosis | Model1                          | Model2                          |
|-----------------------------|---------------------------------|---------------------------------|
|                             | CACS > 0                        | 1.980 (1.455–2.694) 0.000       | 1.821 (1.331–2.492) 0.000 |
|                             | MAFLD                           | 1.780 (1.293–2.452) 0.000       | 1.825 (1.320–2.524) 0.000 |
|                             | NAFLD                           | 3.201 (2.036–5.034) 0.000       | 2.599 (1.625–4.157) 0.000 |
|                             | CACS > 100                      | 1.974 (1.280–3.044) 0.002       | 1.795 (1.145–2.814) 0.011 |
|                             | MAFLD                           | 1.786 (1.302–2.449) 0.000       | 1.562 (1.128–2.161) 0.007 |
|                             | NAFLD                           | 1.786 (1.302–2.449) 0.000       | 1.562 (1.128–2.161) 0.007 |
|                             | CACS > 100                      | 1.976 (1.408–2.773) 0.000       | 1.823 (1.287–2.580) 0.001 |
|                             | MAFLD                           | 2.076 (1.472–2.927) 0.000       | 1.999 (1.407–2.840) 0.000 |
|                             | NAFLD                           | 1.671 0.091                    | 1.562 (1.128–2.161) 0.007 |

MAFLD, metabolic dysfunction-associated fatty liver disease; NAFLD, non-alcoholic fatty liver disease; CACS, coronary artery calcification score; ba-PWV, brachial ankle pulse wave velocity; c-IMT, carotid intima media thickness.

Model 1: adjusted for age, sex. Model 2: adjusted items in Model 1 plus for hypertension, dyslipidemia, estimated glomerular filtration rate, ever smoking, and exercise.

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Fig 2. (Upper) The percentage of CACS > 0 and > 100. (Lower) The percentage of ba-PWV > 1400 cm/s and carotid IMT ≥ 1.1 mm, based on the combination of MAFLD and/or DM by age (under 50, 50s, 60s, 70 and over). DM-MAFLD, MAFLD with DM; MAFLD+DM-, MAFLD without DM; MAFLD-DM+, no MAFLD and DM; MAFLD-DM-, no MAFLD and no DM. MAFLD, metabolic dysfunction-associated fatty liver disease; DM, diabetes mellitus; CACS, coronary artery calcification score; ba-PWV, brachial ankle pulse wave velocity; IMT, Intima media thickness.

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Table 3. The association of subclinical atherosclerosis with combination of MAFLD and/or DM.

| Subclinical atherosclerosis | Model1                  | P      | Model2                  | P      |
|-----------------------------|-------------------------|--------|-------------------------|--------|
| CACS > 0                    | MAFLD-DM-               | 1.00(ref) | MAFLD-DM-               | 1.00(ref) | 0.884 |
|                             | MAFLD+DM-               | 1.00(ref) | MAFLD+DM-               | 1.00(ref) | 0.884 |
|                             | DM-MAFLD                | 3.913(2.249–6.810) | 3.913(2.249–6.810) | 0.000 |
| CACS > 100                  | MAFLD-DM-               | 1.00(ref) | MAFLD-DM-               | 1.00(ref) | 0.175 |
|                             | MAFLD+DM-               | 2.464(1.468–4.135) | 2.464(1.468–4.135) | 0.001 |
|                             | DM-MAFLD                | 7.218(3.784–13.767) | 7.218(3.784–13.767) | 0.000 |
| ba-PWV > 1400               | MAFLD-DM-               | 1.00(ref) | MAFLD-DM-               | 1.00(ref) | 0.858 |
|                             | MAFLD+DM-               | 1.647(1.165–2.328) | 1.647(1.165–2.328) | 0.005 |
|                             | DM-MAFLD                | 2.459(1.470–4.422) | 2.459(1.470–4.422) | 0.001 |
| c-IMT ≥ 1.1                 | MAFLD-DM-               | 1.00(ref) | MAFLD-DM-               | 1.00(ref) | 0.652 |
|                             | MAFLD+DM-               | 1.870(1.289–2.711) | 1.870(1.289–2.711) | 0.004 |
|                             | DM-MAFLD                | 2.231(1.287–3.868) | 2.231(1.287–3.868) | 0.004 |

MAFLD, metabolic dysfunction-associated fatty liver disease; DM, diabetes; CACS, coronary artery calcification score; ba-PWV, brachial ankle pulse wave velocity; c-IMT, carotid intima media thickness.

Model 1: adjusted for age, sex. Model 2: adjusted items in Model 1 plus for hypertension, dyslipidemia, estimated glomerular filtration rate, ever smoking, and exercise.

Table 4. Clinical characteristic of each group of MAFLD.

| Characteristics    | Obesity-MAFLD | Lean-MAFLD | DM-MAFLD | P       |
|--------------------|---------------|------------|----------|---------|
| Age (years)        | 59.8 ± 11.0   | 64.8 ± 9.6 | 60.9 ± 9.4 | 0.000   |
| Male               | 264 (82.5)    | 43 (68.3)  | 77 (91.7) | 0.010   |
| Ever smoking       | 189 (59.0)    | 35 (55.6)  | 65 (77.4) | 0.606   |
| Exercise           | 113 (35.3)    | 22 (34.9)  | 29 (34.5) | 0.953   |
| Non-drinker        | 80 (25.0)     | 19 (30.2)  | 13 (15.5) | 0.393   |
| Hypertension       | 150 (46.9)    | 25 (39.7)  | 54 (64.3) | 0.285   |
| Diabetes mellitus  | 106 (33.1)    | 18 (28.6)  | 84 (100.0)| 0.146   |
| Dyslipidemia       | 138 (35.9)    | 32 (50.8)  | 40 (47.6) | 0.008   |
| AST                | 27.7 ± 14.5   | 23.0 ± 5.6 | 30.6 ± 21.6| 0.000   |
| ALT                | 30.9 ± 20.0   | 24.5 ± 34.3| 33.5 ± 28.3| 0.000   |
| γGTP               | 53.6 ± 72.2   | 43.0 ± 43.8| 78.0 ± 128.7 | 0.261  |
| HOMA-R             | 2.47 ± 1.98   | 2.13 ± 3.84| 3.40 ± 4.13 | 0.305  |
| Elevated hs-CRP    | 67 (20.9)     | 6 (9.5)    | 18 (21.4) | 0.014   |
| FIB-4 moderate-high| 163 (50.9)    | 38 (60.3)  | 49 (58.3) | 0.173   |
| CACS > 0           | 153 (47.8)    | 36 (57.1)  | 58 (69.0) | 0.176   |
| CACS > 100         | 62 (19.4)     | 17 (27.0)  | 32 (38.1) | 0.172   |
| ba-PWV > 1400      | 166 (51.9)    | 41 (65.1)  | 53 (63.1) | 0.058   |
| c-IMT ≥ 1.1        | 90 (28.1)     | 32 (50.8)  | 32 (38.1) | 0.000   |

MAFLD, metabolic dysfunction-associated fatty liver disease; AST, Aspartate transaminase; ALT, Alanine transaminase; γGTP, gamma-glutamyl transferase; HOMA-R, homeostasis model assessment of insulin resistance; hs-CRP, high sensitive C-reactive protein; CACS, coronary artery calcification score; ba-PWV, brachial ankle pulse wave velocity; c-IMT, carotid intima media thickness.

*P, P-value for Obesity-MAFLD vs Lean-MAFLD
24] (21.8% vs 15.8–20.6%), whereas the number of non-MAFLD subjects with DM (i.e., MAFLD-DM+) was very small (n = 42).

The most common cause of death in patients with NAFLD is CVD [5, 6] and CACS has been shown to be a strong predictor of atherosclerotic CVD events; Compared to CACS of 0, CACS of 1–100 has four times higher risk of the event, and a CACS cutoff above 100 increases the risk of the event seven times [11, 13]. To date, three meta-analyses regarding CACS have reported that subjects with NAFLD had a significant association with subclinical coronary atherosclerosis compared to those without [9, 10, 25]. Specifically, a recent meta-analysis on diabetic population showed 2.2-fold increased risk for CVD in the NAFLD group, compared with the non-NAFLD group, suggesting that NAFLD and DM might have a synergistic effect on the risk of CVD [26]. However, as far as we know, no studies have comprehensively reported the relationship between subclinical atherosclerosis and MAFLD or its components, especially, DM-MAFLD. Therefore, in the sub-analysis, we compared DM-MAFLD to MAFLD-DM- or Neither FLD as a reference to examine the impact of DM on subclinical atherosclerosis. Importantly, DM-MAFLD had significantly higher odds for subclinical atherosclerosis compared to MAFLD-DM-, although MAFLD-DM+ did not significantly increase the risk of subclinical atherosclerosis. The small sample size belonging to the MAFLD-DM+ group may have affected this unexpected result. Also, subsequent sensitivity analysis demonstrated similar results: DM-MAFLD had higher odds for subclinical coronary atherosclerosis than the other groups of MAFLD, when compared to Neither FLD as a reference. Thus, the present study suggested that MAFLD and DM as comorbidities have a synergistic effect on the risk of subclinical atherosclerosis. On the other hand, Lean-MAFLD is composed of a population that is older and has more complications of hyperlipidemia compared to Obesity-MAFLD (Table 4). Lean-MAFLD had higher odds ratio of subclinical atherosclerosis than Obesity-MAFLD except ba-PWV (Table 5). Therefore, the location and extent of ectopic fat accumulation, such as

| Subclinical atherosclerosis | Model1          | P       | Model2          | P       |
|-----------------------------|-----------------|---------|-----------------|---------|
| CACS > 0                    | *Neither FLD    | 1.00(ref) | 1.00(ref)       |         |
| Obesity-MAFLD              | 1.888(1.361–2.621) | 0.000   | 1.720(1.231–2.404) | 0.001   |
| Lean-MAFLD                 | 2.261(1.268–4.032) | 0.006   | 2.261(1.268–4.032) | 0.006   |
| DM-MAFLD                   | 3.908(2.258–6.764) | 0.000   | 3.908(2.258–6.764) | 0.000   |
| CACS > 100                 | Neither FLD     | 1.00(ref) | 1.00(ref)       |         |
| Obesity-MAFLD              | 3.011(1.863–4.868) | 0.000   | 2.510(1.528–4.123) | 0.000   |
| Lean-MAFLD                 | 3.478(1.741–6.947) | 0.000   | 2.682(1.300–5.532) | 0.008   |
| DM-MAFLD                   | 6.886(3.663–12.946) | 0.000   | 5.833(3.047–11.164) | 0.000   |
| ba-PWV > 1400              | Neither FLD     | 1.00(ref) | 1.00(ref)       |         |
| Obesity-MAFLD              | 1.856(1.323–2.603) | 0.000   | 1.582(1.116–2.244) | 0.010   |
| Lean-MAFLD                 | 0.075           |         | 0.084           |         |
| DM-MAFLD                   | 2.603(1.501–4.512) | 0.001   |                 | 0.104   |
| c-IMT ≥ 1.1                | Neither FLD     | 1.00(ref) | 1.00(ref)       |         |
| Obesity-MAFLD              | 1.714(1.187–2.475) | 0.004   | 1.565(1.075–2.278) | 0.019   |
| Lean-MAFLD                 | 3.769(2.116–6.713) | 0.000   | 3.769(2.116–6.713) | 0.000   |
| DM-MAFLD                   | 2.307(1.341–3.970) | 0.003   | 2.307(1.341–3.970) | 0.003   |

MAFLD, metabolic dysfunction-associated fatty liver disease; DM, diabetes CACS, coronary artery calcification score; ba-PWV, brachial ankle pulse wave velocity; c-IMT, carotid intima media thickness.

Model 1: adjusted for age, sex. Model 2: adjusted items in Model 1 plus for hypertension, dyslipidemia, estimated glomerular filtration rate, ever smoking, and exercise.

*Neither FLD was defined as neither MAFLD nor non-alcoholic fatty liver disease.
as fatty liver and epicardial adipose tissue, may contribute to the pathogenesis of subclinical atherosclerosis rather than obesity as defined by BMI.

Our study also suggests that there seemed to be no significant difference in the probability of moderate to high hepatic fibrosis among the three groups of MAFLD, although DM-MAFLD might have a slightly high probability of moderate to high fibrosis in subjects under the age of 60, compared to the other subgroups of MAFLD. Since most of DM-MAFLD was included in Obesity-MAFLD, the two groups were not independent. Therefore, it was difficult to statistically compare difference in the variables in FIB-4.

The underlying pathophysiological candidates linking the association between NAFLD and coronary atherosclerosis in an earlier stage include insulin resistance and low-grade hepatic and systematic inflammation [27, 28]. The main focus of the definition of MAFLD is metabolic dysfunction as a core element along with the accumulation of hepatic steatosis [16]. Serum level of AST, ALT and HOMA-R, and the proportion of elevated hs-CRP were significantly higher in subjects with MAFLD than those without. In addition, their level and the proportion were higher in the DM-MAFLD group than the other groups of MAFLD. Therefore, unlike the direct effects on the liver by fatty liver-induced inflammation and insulin resistance, their effects on coronary atherosclerosis might be amplified, especially due to inflammation and insulin resistance induced by metabolic dysfunction such as DM.

Our study has some limitations. First, this cross-sectional study cannot conclude a causal link between subclinical atherosclerosis and MAFLD. Second, the diagnosis of FLD was determined by US, not by more sensitive and specific modalities such as magnetic resonance imaging or US elastography. Third, the number of subjects in the MAFLD-DM+ group is very small, especially only five under the age of 60. Lastly, since our results are derived from Japanese health checkup data, the findings may not be generalized to other races. Thus, more longitudinal studies are needed to determine if there is a synergistic effect on subclinical atherosclerosis between MAFLD and DM.

**Conclusion**

MAFLD, especially DM-MAFLD, was significantly associated with subclinical atherosclerosis in an asymptomatic general population. This study suggests that DM-MAFLD could be a significant risk factor for CVD through insulin resistance and low-grade inflammation, and requires careful follow-up or appropriate intervention.

**Supporting information**

**S1 Data.**
(XLSX)

**S1 Fig. The risk-positive percentage of subclinical atherosclerosis in Neither FLD and the MAFLD groups.** MAFLD, metabolic dysfunction-associated fatty liver disease; DM, diabetes mellitus; CACS, coronary artery calcification score; ba-PWV, brachial ankle pulse wave velocity; IMT, Intima media thickness.
(TIF)

**S2 Fig. The percentage of moderate-high hepatic fibrosis evaluated by the FIB-4 index in the MAFLD groups by age (under 50, 50s, 60s, 70 and over).** MAFLD, metabolic dysfunction-associated fatty liver disease; DM, diabetes mellitus; FIB-4, fibrosis-4.
(TIF)

**S1 Table. Clinical characteristic of MAFLD only and NAFLD.** MAFLD, metabolic dysfunction-associated fatty liver disease; VAT, visceral adipose tissue; LDL-C, low-density lipoprotein
cholesterol; HDL-C, high-density lipoprotein cholesterol; HOMA-R, homeostasis model assessment of insulin resistance; γGTP, gamma-glutamyl transferase; hs-CRP; high sensitive C-reactive protein; CACS, coronary artery calcification score; ba-PWV, brachial ankle pulse wave velocity; IMT, Intima media thickness.

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References
1. Chalasani N, Younossi Z, Lavine JE, Charlton M, Cusi K, Rinella M, et al. The diagnosis and management of nonalcoholic fatty liver disease: Practice guidance from the American Association for the Study of Liver Diseases. Hepatology. 2018; 67: 328–357. https://doi.org/10.1002/hep.29367 PMID: 28714183
2. EASL-EASD-EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease. J Hepatol. 2016; 64: 1388–1402. https://doi.org/10.1016/j.jhep.2015.11.004 PMID: 27062661
3. Targher G, Day CP, Bonora E. Risk of cardiovascular disease in patients with nonalcoholic fatty liver disease. N Engl J Med. 2010; 363: 1341–1350. https://doi.org/10.1056/NEJMra0912063 PMID: 20879883
4. Targher G, Byrne CD, Lonardo A, Zoppini G, Barbui C. Non-alcoholic fatty liver disease and risk of incident cardiovascular disease: A meta-analysis. J Hepatol. 2016; 65: 589–600. https://doi.org/10.1016/j.jhep.2016.05.013 PMID: 27212244
5. Dunn W, Xu R, Wingard DL, Rogers C, Angulo P, Younossi ZM, et al. Suspected nonalcoholic fatty liver disease and mortality risk in a population-based cohort study. Am J Gastroenterol/ 2008; 103: 2263–2271.
6. Kim D, Kim WR, Kim HJ, Therneau TM. Association between noninvasive fibrosis markers and mortality among adults with nonalcoholic fatty liver disease in the United States. H epatology. 2013; 57: 1357–1365. https://doi.org/10.1002/hep.26156 PMID: 23175136
7. Kim D, Choi SY, Park EH, Lee W, Kang JH, Kim W, et al. Nonalcoholic fatty liver disease is associated with coronary artery calcification. Hepatology. 2012; 56: 605–613. https://doi.org/10.1002/hep.25593 PMID: 22271511
8. Chhabra R, O’keefe JH, Patil H, O’keefe E, Thompson RC, Ansari S, et al. Association of coronary artery calcification with hepatic steatosis in asymptomatic individuals. Mayo Clin Proc. 2013; 88: 1259–1265. https://doi.org/10.1016/j.mayocp.2013.06.025 PMID: 24138963
9. Jaruvongvanich V, Wirunawanyka V, Sangwankeo A, Upala S. Nonalcoholic fatty liver disease is associated with coronary artery calcification: A systematic review and meta-analysis. Dig Liver Dis. 2016; 48: 1410–1417. https://doi.org/10.1016/j.dld.2016.09.002 PMID: 27697419

10. Kapuria D, Takyar VK, Etzion O, Surana P, O’keefe JH, Koh C. Association of hepatic steatosis with subclinical atherosclerosis: systematic review and meta-analysis. Hepatol Commun. 2018; 2: 873–883. https://doi.org/10.1002/hep4.1199 PMID: 30094399

11. Greenland P, LaBree L, Azen SP, Doherty TM, Detrano RC. Coronary artery calcium score combined with Framingham score for risk prediction in asymptomatic individuals. JAMA. 2004; 291: 210–215. https://doi.org/10.1001/jama.291.2.210 PMID: 14722147

12. Pletcher MJ, Tice JA, Pignone M, Browner WS. Using the coronary artery calcium score to predict coronary heart disease events: a systematic review and meta-analysis. Arch Intern Med. 2004; 164: 1285–1292. https://doi.org/10.1001/archinte.164.12.1285 PMID: 15226161

13. Budoff MJ, Achenbach S, Blumenthal RS, Carr JJ, Goldin JG, Greenland P. Assessment of coronary artery disease by cardiac computed tomography: a scientific statement from the American Heart Association Committee on Cardiovascular Imaging and Intervention, Council on Cardiovascular Radiology and Intervention, and Committee on Cardiac Imaging, Council on Clinical Cardiology. Circulation. 2006; 114: 1761–1791. https://doi.org/10.1161/CIRCULATIONAHA.106.178458 PMID: 17015792

14. Alluri K, Joshi PH, Henry TS, Henry T, Blumenthal RS, Nasir K, et al. Scoring of coronary artery calcium scans: history, assumptions, current limitations, and future directions. Atherosclerosis. 2015; 239: 109–117. https://doi.org/10.1016/j.atherosclerosis.2014.12.040 PMID: 25585030

15. Huang Y, Bi Y, Xu M, Ma Z, Xu Y, Wang T, et al. Nonalcoholic fatty liver disease is associated with atherosclerosis in middle-aged and elderly Chinese. Atherosclerosis. 2012; 32: 2321–2316. https://doi.org/10.1161/ATVBAHA.111.252957 PMID: 22814750

16. Eslam M, Sanyal AJ, George J. International consensus P. MAFLD, a consensus-driven proposed nomenclature for metabolic associated fatty liver disease. Gastroenterology. 2020; 158: 1999–2014. e1. https://doi.org/10.1053/j.gastro.2019.11.312 PMID: 32044314

17. Lee H, Lee YH, Kim SU, Kim HC. Metabolic dysfunction-associated fatty liver disease and incident cardiovascular disease risk: A National Cohort Study. Clin Gastroenterol Hepatol. 2021; 19: 2138–2147. https://doi.org/10.1016/j.cgh.2020.12.022 PMID: 33348045

18. Agatston AS, Janowitz WR, Hildner FJ, Zusmer NR, Viamonte M Jr, Detrano R. Quantification of coronary artery calcium using ultrafast computed tomography. J Am Coll Cardiol. 1990; 15: 827–832. https://doi.org/10.1016/0735-1097(90)90282-t PMID: 2407762

19. Kashiwagi K, Yamaguchi A, Shiba S, Taniki N, Inoue N, Takeishi H, et al. Moderate alcohol consumption is not associated with subclinical cardiovascular damage but with hepatic fibrosis in non-alcoholic fatty liver disease. Alcohol. 2021; 89: 1–7.

20. Hamaguchi M, Kojima T, Itoh Y, Harano Y, Fuji K, Nakajima T, et al. The severity of ultrasonographic findings in nonalcoholic fatty liver disease reflects the metabolic syndrome and visceral fat accumulation. Am J Gastroenterol. 2007; 102: 2708–2715. https://doi.org/10.1111/j.1572-0241.2007.01526.x PMID: 17894848

21. Shah AG, Lydecker A, Murray K, Tetr BN, Contos MJ, Sanyal AJ; Nash Clinical Research Network. Comparison of noninvasive markers of fibrosis in patients with nonalcoholic fatty liver disease. Clin Gastroenterol Hepatol. 2009; 7: 1104–1112.

22. Ciardullo S, Perseghin G. Prevalence of NAFLD, MAFLD and associated advanced fibrosis in the contemporary United States population. Liver Int. 2021; 41: 1290–1293. https://doi.org/10.1111/liv.14828 PMID: 33590934

23. Younossi ZM, Koenig AB, Abdelatif D, Fazel Y, Henry L, Wymer M. Global epidemiology of nonalcoholic fatty liver disease—metaanalytic assessment of prevalence, incidence, and outcomes. Hepatology. 2016; 64: 73–84. https://doi.org/10.1002/hep.28431 PMID: 26707365

24. Yoneda Y, Yamamoto T, Honda Y, Imaho K, Ogawa Y, Kessoku T, et al. Risk of cardiovascular disease in patients with fatty liver disease as defined from the metabolic dysfunction associated fatty liver disease or nonalcoholic fatty liver disease point of view: a retrospective nationwide claims database study in Japan. J Gastroenterol. 2021; 56: 1022–1032. https://doi.org/10.1007/s00535-021-01828-6 PMID: 34601620

25. Zhou YY, Zhou XD, Wu SJ, Fan DH, Poucke SV, Chen YP, et al. Nonalcoholic fatty liver disease contributes to subclinical atherosclerosis: A systematic review and meta-analysis. Hepatol Commun. 2018; 2: 376–392. https://doi.org/10.1002/hep4.1155 PMID: 29619417

26. Zhou YY, Zhou XD, Wu SJ, Hu XQ, Tang B, van Poucke S, et al. Synergistic increase in cardiovascular risk in diabetes mellitus with nonalcoholic fatty liver disease: a meta-analysis. Eur J Gastroenterol Hepatol. 2018; 30: 631–636. https://doi.org/10.1097/MEG.0000000000001075 PMID: 29351115

A significant risk of MAFLD plus DM on subclinical atherosclerosis
27. Francque SM, van der Graaff D, Kwanten WJ. Non-alcoholic fatty liver disease and cardiovascular risk: Pathophysiological mechanism and implications. J Hepatol. 2016; 65: 425–443. https://doi.org/10.1016/j.jhep.2016.04.005 PMID: 27091791

28. Stahl EP, Dhindsa DS, Lee SK, Sandesara PB, Chalasani NP, Sperling LS. Nonalcoholic fatty liver disease and the heart: JACC state-of-the-art review. J Am Coll Cardiol. 2019; 73: 948–963. https://doi.org/10.1016/j.jacc.2018.11.050 PMID: 30819364