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Long-term Adverse Effect of Liver Stiffness on Glycaemic Control in Type 2 Diabetic Patients with Non-alcoholic Fatty Liver Disease: A Pilot Study

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Abstract: Currently, there is limited data regarding the long-term effect of liver stiffness on glycaemic control in patients with type 2 diabetes mellitus (T2DM) and non-alcoholic fatty liver disease (NAFLD). We prospectively followed an outpatient sample of 61 consecutive post-menopausal women with T2DM and NAFLD, who had baseline data on liver ultrasonography and Fibroscan®-assessed liver stiffness measurement (LSM) in 2017 and who underwent follow-up in 2022. Hemoglobin A1c (HbA1c) was measured both at baseline and follow-up. At baseline, 52 patients had NAFLD (hepatic steatosis) alone and 9 had NAFLD with coexisting clinically significant fibrosis (defined as LSM ≥7 kPa on Fibroscan®). At follow-up, 16 patients had a worsening of glycaemic control (arbitrarily defined as HbA1c increase ≥0.5% from baseline). The prevalence of NAFLD and coexisting significant fibrosis at baseline was at least three times greater among patients, who developed worse glycaemic control at follow-up, compared with those who did not (31.3% vs. 8.9%; p=0.030). In logistic regression analysis, the presence of NAFLD and clinically significant fibrosis was associated with an approximately 4.5-fold increased likelihood of developing worse glycaemic control at follow-up (odds ratio 4.66, 95% confidence interval 1.07-20.3; p=0.041), even after adjustment for baseline confounding factors, such as age, body mass index, hemoglobin A1c (or HOMA-estimated insulin resistance) and use of some glucose-lowering agents that may positively affect NAFLD and liver fibrosis. In conclusion, our results suggest that the presence of Fibroscan®-assessed significant fibrosis was associated with a higher risk of developing worse glycaemic control in post-menopausal women with T2DM and NAFLD.

Keywords: Nonalcoholic fatty liver disease; NAFLD; MAFLD; Metabolic associated fatty liver disease; Type 2 diabetes; T2DM; Fibrosis; Liver stiffness

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1. Introduction

Non-alcoholic fatty liver disease (NAFLD) has become the most common cause of chronic liver diseases worldwide, affecting up to ~30% of adults in the general population [1]. NAFLD also affects up to ~70% of patients with type 2 diabetes mellitus [T2DM], and almost all patients with severe obesity [2]. Worryingly, the global prevalence of NAFLD is expected to increase dramatically in the near future, in parallel with the increasing rates of obesity and T2DM globally [1]. T2DM and NAFLD

represent a "vicious circle", whereby the presence of one condition adversely affects the other and vice versa [3]. Compared with subjects without T2DM, patients with T2DM are more likely to have or develop the more advanced forms of NAFLD, such as non-alcoholic steatohepatitis (NASH), advanced fibrosis or cirrhosis [4,5]. In this context, a recent systematic review reported that the global prevalence of biopsy-confirmed NASH among patients with T2DM is nearly 40%, and that the global prevalence of advanced fibrosis in this patient population is around 15-20% [2]. On the other side of this "vicious circle", NAFLD may precede and/or promote the development of T2DM, possibly via worsening of systemic/hepatic insulin resistance and dysregulated production of several hepatokines and proinflammatory cytokines [4,6]. An updated meta-analysis of 33 observational cohort studies (including about 500,000 individuals) showed that NAFLD was significantly associated with a ~2.2-fold increased risk of new-onset T2DM over a median period of 5 years. This risk paralleled the underlying severity of NAFLD (especially higher stage of liver fibrosis) [7].

On this background of evidence, in 2022 the American Diabetes Association (ADA) guidelines recommended that individuals with T2DM and elevated serum liver enzyme levels or NAFLD on ultrasonography should be evaluated for the presence of liver fibrosis [8]. This presupposes also the need to better assess, on the one hand, how long-term glycaemic control may affect the development and progression of NAFLD and, on the other hand, how NAFLD and its more advanced forms may affect long-term glycaemic control in patients with T2DM. While convincing evidence indicates that suboptimal glycaemic control may predispose to development of advanced NAFLD forms [9-16], to date there is little information regarding the long-term effect of NAFLD with increasing levels of liver fibrosis on glycaemic control in patients with T2DM. Recognition of a possible adverse effect of NAFLD with coexisting liver fibrosis on glycaemic control might have important clinical implications, as it may further reinforce the need for a multidisciplinary, patient-centered approach to T2DM patients with advanced NAFLD, as well as the need for a tailored pharmacotherapy in this patient population (preferring the use of glucose-lowering agents with potential hepato-protective effects), in order to improve glycaemic control and prevent future NAFLD-related hepatic and extra-hepatic complications.

Thus, the main aim of our observational longitudinal pilot study was to examine whether T2DM patients with NAFLD and coexisting significant fibrosis (as non-invasively assessed by liver ultrasonography and vibration-controlled transient elastography [VCTE]) had a worsening of glycaemic control over time, compared with their counterparts with NAFLD alone.

2. Results

Of the 61 post-menopausal women with T2DM included in the study, 52 (85%) patients had NAFLD (hepatic steatosis) alone and 9 (15%) patients had NAFLD and coexisting clinically significant fibrosis [i.e., defined as liver stiffness measurement (LSM) ≥7 kPa on Fibroscan®] at baseline; seven of these 9 patients with NAFLD and coexisting significant fibrosis had a LSM ≥8.2 kPa (which is another more stringent cutoff used for defining the presence of clinically significant fibrosis).

Table 1 shows the main clinical and biochemical characteristics of the study participants at baseline (year 2017), who were stratified by worsening of glycaemic control at follow-up (year 2022). Baseline LSM values on Fibroscan® were significantly higher in patients who developed worse glycaemic control at follow-up compared with those who did not [median LSM: 6.6 (IQR 5.4-8.6) vs. 4.4 (3.6-5.6) kPa; p=0.005]. Similarly, the proportion of patients with NAFLD and significant fibrosis (i.e., LSM ≥7 kPa) at baseline was greater among those who developed worse glycaemic control at follow-up, compared with those who did not (31.3% vs. 8.9%; p=0.030). Again, the proportion of those with NAFLD and LSM ≥8.2 kPa at baseline was greater

among those who developed worse glycaemic control at follow-up compared with those who did not (25.0% vs. 6.7%; p=0.044). Conversely, the two patient groups did not significantly differ for other clinical and biochemical characteristics at baseline, such as age, BMI, smoking history, blood pressure, HbA1c, proportion of those with HbA1c from 7% to 8% or those with HbA1c >8%, plasma lipid profile, HOMA-IR score, serum liver enzymes, kidney function parameters, prevalence of ischaemic heart disease or stroke, and use of glucose-lowering, anti-hypertensive, lipid-lowering or anti-platelet agents.

Table 1. Main clinical and biochemical characteristics of post-menopausal women with type 2 diabetes at baseline, stratified by worsening of glycaemic control at follow-up.

	Patients with no worsening of glycaemic control at follow-up (n=45)	**Patients with worsening of glycaemic control at follow-up (n=16)**	***P* value**
Age (years)	70.9 ± 7.3	70.2 ± 9.0	0.738
BMI (kg/m^2)	29.7 ± 5.5	29.5 ± 3.9	0.897
Diabetes duration (years)	10 (6-15)	10 (7-16)	0.810
Current smokers (%)	13.3	6.2	0.357
Systolic blood pressure (mmHg)	135 ± 14	139 ± 17	0.391
Diastolic blood pressure (mmHg)	77 ± 7	75 ± 10	0.413
Fasting glucose (mmol/L)	7.1 ± 1.7	7.2 ± 1.4	0.835
Hemoglobin A1c (mmol/mol Hb)	52 ± 9	53 ± 10	0.711
Hemoglobin A1c (%)	6.9 ± 0.8	7.0 ± 0.9	0.711
Proportion of patients with hemoglobin A1c (%)			0.868
from 7% to 8% (53 to 64 mmol/mol Hb)	24.4	31.3	
>8% (>64 mmol/mol Hb)	6.7	6.3	
Total cholesterol (mg/dL)	159 ± 31	166 ± 41	0.457
LDL-cholesterol (mg/dL)	79 ± 29	85 ± 34	0.512
HDL-cholesterol (mg/dL)	59 ± 14	58 ± 13	0.849
Triglycerides (mg/dL)	112 (72-150)	119 (92-167)	0.279
HOMA-IR score	2.3 (1.5-4.0)	3.3 (1.1-6.4)	0.422
AST (IU/L)	23 ± 7	25 ± 9	0.387
ALT (IU/L)	12 (10-16)	13 (10-18)	0.503
GGT (IU/L)	16 (13-28)	26 (16-36)	0.139
Creatinine (umol/L)	64 ± 13	66 ± 15	0.699
eGFR$_{CKD-EPI}$ (ml/min/1.73 m^2)	82 ± 14	81 ± 16	0.716
Hypertension (%)	73.3	87.5	0.318
Ischaemic heart disease (%)	13.3	6.3	0.664
Ischaemic stroke (%)	2.2	6.3	0.459
Diabetic retinopathy, any degree (%)	6.3	4.4	0.606
Metformin (%)	80.0	87.5	0.711

Sulfonylureas (%)	24.4	18.8	0.742
Pioglitazone (%)	2.2	0	0.738
DPP-4 inhibitors (%)	22.2	31.3	0.510
GLP-1 analogues (%)	8.9	12.5	0.648
SGLT-2 inhibitors (%)	11.1	0	0.313
Anti-platelets drugs (%)	42.2	31.3	0.557
Beta-blockers (%)	28.9	37.5	0.543
ACE-inhibitors/ARBs (%)	53.3	75.0	0.152
Calcium-channel blockers (%)	20.0	18.8	0.914
Diuretics (%)	31.1	37.5	0.758
Statins (%)	75.6	75.0	0.965
Fibroscan®-assessed LSM (kPa)	4.4 (3.6-5.6)	6.6 (5.4-8.6)	**0.005**
Patients with NAFLD and significant fibrosis§ (%)	8.9	31.3	**0.030**
Patients with NAFLD and LSM ≥8.2% kPa (%)	6.7	25.0	**0.044**

Sample size, *n*=61. Data are expressed as means±SD, medians and IQRs (in parenthesis) or percentages. Differences between the two groups were tested by the Fisher's exact test for categorical variables, the unpaired Student's *t* test for normally distributed continuous variables and the Mann-Whitney test for non-normally distributed variables (*i.e.,* diabetes duration, plasma triglycerides, HOMA-IR score, ALT, GGT and LSM). For the sake of clarity, significant *p*-values have been highlighted in bold. §Clinically significant fibrosis was defined by LSM ≥7 kPa on Fibroscan®. Hypertension was defined as blood pressure ≥140/90 mmHg and/or specific drug treatment.

Abbreviations: ACE, angiotensin-converting-enzyme inhibitor; ARB, angiotensin II receptor blocker; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; DPP-IV, dipeptidyl peptidase-IV; $eGFR_{CKD\text{-}EPI}$, glomerular filtration rate estimated using the CKD-Epidemiology Collaboration equation; GGT, gamma-glutamyltransferase; GLP-1, glucagon-like peptide-1; HOMA-IR, homeostasis model assessment-insulin resistance; LSM, liver stiffness measurement; SGLT-2, sodium/glucose cotransporter-2.

Table 2 summarizes the main clinical and biochemical characteristics of the study participants at follow-up, who were stratified by severity of NAFLD at baseline. Compared with those with NAFLD alone, patients with NAFLD and clinically significant fibrosis had markedly higher levels of HbA1c at follow-up (HbA1c 8.4±2.1% vs. 6.9±0.9%; p<0.001). Moreover, the proportion of those with HbA1c >8% was significantly greater in patients with NAFLD and clinically significant fibrosis than in those with NAFLD alone (55.6% vs. 11.1%; p=0.003). All other clinical and biochemical characteristics recorded at follow-up were not significantly different between the two groups of patients, including also the use of glucose-lowering, antihypertensive, lipid-lowering or anti-platelet agents.

Table 2. Main clinical and biochemical characteristics of post-menopausal women with type 2 diabetes at follow-up, stratified by severity of NAFLD at baseline.

	Patients with NAFLD alone (n=52)	**Patients with NAFLD and coexisting significant fibrosis (n=9)**	***P* value**
Age (years)	75.7 ± 7.4	76.1 ± 8.9	0.874
Diabetes duration (years)	15 (11-21)	15 (11-18)	0.895
BMI (kg/m^2)	29.5 ± 3.9	29.6 ± 4.3	0.961
Fasting glucose (mmol/L)	7.4 ± 1.9	8.8 ± 3.8	0.131
Hemoglobin A1c (mmol/mol Hb)	52 ± 9	68 ± 22	**<0.001**
Hemoglobin A1c (%)	6.9 ± 0.9	8.4 ± 2.1	**<0.001**
Proportion of patients with hemoglobin A1c (%)			**0.003**
from 7% to 8% (53 to 64 mmol/mol Hb)	32.7	9.6	
>8% (>64 mmol/mol Hb)	11.1	55.6	
Total cholesterol (mg/dL)	153 ± 35	141 ± 32	0.415
HDL-cholesterol (mg/dL)	54 ± 9	51 ± 22	0.603
Triglycerides (mg/dL)	112 (88-148)	79 (59-215)	0.488
AST (IU/L)	23 ± 9	20 ± 5	0.474
ALT (IU/L)	22 (16-27)	21 (14-25)	0.859
GGT (IU/L)	18 (11-60)	17 (10-41)	0.761
Creatinine (umol/L)	69 ± 24	74 ± 34	0.117
$eGFR_{CKD-EPI}$ (ml/min/1.73 m^2)	77.0 ± 19.0	67.8 ± 28.7	0.238
Hypertension (%)	78.9	88.9	0.484
Ischaemic heart disease (%)	9.6	11.1	0.633
Ischaemic stroke (%)	1.9	11.1	0.159
Diabetic retinopathy, any degree (%)	9.6	11.1	0.889
Insulin (%)	15.4	0	0.207
Metformin (%)	78.9	100.0	0.127
Sulfonylureas (%)	15.4	33.3	0.196
Pioglitazone (%)	1.9	0	0.675
DPP-4 inhibitors (%)	30.8	22.2	0.604
GLP-1 analogues (%)	23.1	33.3	0.509
SGLT-2 inhibitors (%)	23.1	33.3	0.509
Anti-platelets drugs (%)	32.7	44.4	0.493
Beta-blockers (%)	40.4	33.3	0.689
ACE-inhibitors/ARBs (%)	67.3	66.7	0.970
Calcium-channel blockers (%)	23.1	11.1	0.418
Diuretics (%)	32.7	44.4	0.493

Statins (%)	76.9	77.8	0.955

Sample size, *n*=61. Data are expressed as means±SD, medians and IQRs (in parenthesis) or percentages. Differences between the two groups were tested by the Fisher's exact test for categorical variables, the unpaired Student's *t* test for normally distributed continuous variables and the Mann-Whitney test for non-normally distributed variables. For the sake of clarity, significant *p*-values have been highlighted in bold.

Abbreviations: ACE, angiotensin-converting-enzyme; ARB, angiotensin II receptor blocker; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; DPP-IV, dipeptidyl peptidase-IV; $eGFR_{CKD\text{-}EPI}$, glomerular filtration rate estimated using the CKD-Epidemiology Collaboration equation; GGT, gamma-glutamyltransferase; GLP-1, glucagon-like peptide-1; SGLT-2, sodium/glucose cotransporter-2.

Table 3 shows the association between the severity of NAFLD at baseline and worsening of glycaemic control at follow-up (arbitrarily defined as HbA1c increase ≥0.5% from baseline). The presence of NAFLD and significant fibrosis was significantly associated with an approximately 4.5-fold increased risk of worsening of glycaemic control at follow-up (unadjusted-OR 4.66, 95% CI 1.07-20.3; p=0.041). The adjustment for age and BMI (model 1) or for the baseline use of some specific glucose-lowering agents (such as glucagon-like peptide 1 (GLP-1) receptor agonists [model 2], sodium-glucose cotransporter-2 (SGLT2) inhibitors [model 3], or pioglitazone [model 4]) that might favorably affect NAFLD and liver fibrosis did not weaken the strength of this association. Almost identical results were found even when we used LSM ≥8.2 kPa instead of ≥7 kPa (by excluding two patients from the analysis) for defining the presence of clinically significant fibrosis (data not shown). Further adjustment for baseline HbA1c levels did not change the strength of the association between the severity of NAFLD at baseline and risk of developing worsening of glycaemic control at follow-up (**Supplementary Table 1**). Almost identical results were also observed when we included HOMA-IR score (instead of HbA1c) as covariate in these multivariable logistic regression models (data not shown).

Table 3. Association between the severity of NAFLD at baseline and risk of developing worsening of glycaemic control at follow-up in post-menopausal women with type 2 diabetes.

Logistic regression models	**Odds Ratios (95% CI)**	***P* value**
Unadjusted model		
NAFLD and clinically significant fibrosis§	4.66 (1.07-20.3)	**0.041**
Adjusted model 1		
NAFLD and clinically significant fibrosis	4.72 (1.07-20.7)	**0.040**
Age (years)	0.98 (0.91-1.06)	0.678
BMI (kg/m^2)	0.99 (0.88-1.12)	0.866
Adjusted model 2		
NAFLD and clinically significant fibrosis	4.70 (1.07-20.8)	**0.041**
Age (years)	0.99 (0.91-1.08)	0.724
BMI (kg/m^2)	0.98 (0.87-1.11)	0.827
GLP-1 receptor agonist use	1.42 (0.21-10.1)	0.724
Adjusted model 3		
NAFLD and clinically significant fibrosis	4.99 (1.14-21.9)	**0.033**
Age (years)	0.96 (0.88-1.05)	0.376

BMI (kg/m²)	0.99 (0.87-1.12)	0.942
SGLT-2 inhibitor use	0.12 (0.10-3.07)	0.198
Adjusted model 4		
NAFLD and clinically significant fibrosis	4.11 (1.03-16.4)	**0.045**
Age (years)	0.99 (0.92-1.06)	0.744
BMI (kg/m²)	1.00 (0.89-1.11)	0.958
Pioglitazone use	1.21 (0.45-33.2)	0.907

Sample size, *n*=61. Data are expressed as odds ratio and 95% confidence intervals (CI) as tested by logistic regression analyses. The presence of worsening of glycaemic control at follow-up (arbitrarily defined as HbA1c increase ≥0.5%) was the dependent variable in these logistic regression models. Covariates included in these regression models were recorded at baseline. §Clinically significant fibrosis was defined by LSM ≥7 kPa on Fibroscan®. For the sake of clarity, significant *p*-values have been highlighted in bold.

In **Table 4** are reported the associations between the severity of NAFLD at baseline and four increasing categories of worsening of glycaemic control at follow-up (arbitrarily defined as HbA1c increases ≤0.19%, from 0.20% to 0.49%, from 0.50% to 0.99%, and ≥1%, respectively)**.** The results of these ordered logistic regression models were superimposable to those reported in Table 3, showing that the presence of NAFLD and clinically significant fibrosis at baseline was strongly associated with an increased risk of worsening of glycaemic control at follow-up, even after adjustment for potential confounding factors. This association remained significant even when we further adjusted the data for baseline HbA1c levels (**Supplementary Table 2**). Almost identical results were also observed when we included HOMA-IR score (instead of HbA1c) as covariate in these ordered logistic regression models (data not shown).

Table 4. Association between the severity of NAFLD at baseline and increasing levels of worsening of glycaemic control at follow-up in post-menopausal women with type 2 diabetes.

Ordered logistic regression models	Odds Ratios (95% CI)	P value
Unadjusted model		
NAFLD and clinically significant fibrosis§	6.16 (1.48-25.7)	**0.013**
Adjusted model 1		
NAFLD and clinically significant fibrosis	6.15 (1.47-25.9)	**0.013**
Age (years)	1.01 (0.95-1.08)	0.733
BMI (kg/m²)	0.97 (0.87-1.06)	0.462
Adjusted model 2		
NAFLD and clinically significant fibrosis	6.11 (1.46-26.6)	**0.014**
Age (years)	1.01 (0.95-1.09)	0.696
BMI (kg/m²)	0.96 (0.86-1.06)	0.431
GLP-1 receptor agonist use	1.53 (0.28-8.21)	0.621
Adjusted model 3		
NAFLD and clinically significant fibrosis	6.97 (1.62-30.0)	**0.009**
Age (years)	0.99 (0.92-1.06)	0.801
BMI (kg/m²)	0.96 (0.86-1.06)	0.385
SGLT-2 inhibitor use	0.14 (0.01-1.69)	0.123

Adjusted model 4		
NAFLD and clinically significant fibrosis	5.96 (1.42-25.1)	**0.015**
Age (years)	1.01 (0.95-1.08)	0.665
BMI (kg/m^2)	0.96 (0.87-1.07)	0.481
Pioglitazone use	1.25 (0.40-31.0)	0.982

Sample size, *n*=61. Data are expressed as odds ratio and 95% confidence intervals (CI) as tested by ordered logistic regression analyses. The presence of four increasing categories of worsening of glycaemic control at follow-up (i.e., arbitrarily defined as HbA1c increases ≤0.19%, from 0.20% to 0.49%, from 0.50% to 0.99%, and ≥1%, respectively) was the ordinal dependent variable in all these models. All covariates included in these regression models were recorded at baseline. §Clinically significant fibrosis was defined by LSM ≥7 kPa on Fibroscan®. For the sake of clarity, significant *p*-values have been highlighted in bold.

3. Discussion

The main findings of our longitudinal pilot study involving post-menopausal women with T2DM and NAFLD are as follows: (a) compared with NAFLD alone, the presence of NAFLD and clinically significant fibrosis at baseline (as non-invasively assessed by liver ultrasound and VCTE examinations) was significantly associated with an approximately 4.5-fold increased risk of glycaemic worsening at follow-up (5 years later); and (b) this significant association persisted even after adjusting for baseline confounding factors such as age, BMI, HbA1c (or HOMA-IR score) and use of some glucose-lowering agents (GLP-1 receptor agonists, SGLT-2 inhibitors, or pioglitazone) that may positively affect hepatic steatosis and fibrosis. In this study, we used an increase in HbA1c of at least 0.5% from baseline to define glycaemic worsening. This HbA1c increase has been used as a marker of worsening of glycaemic control in patients with T2DM in other published studies [17,18].

In 2022, the ADA scientific guidelines recommended that patients with T2DM and elevated serum liver enzymes or NAFLD on ultrasonography should be evaluated for presence of liver fibrosis [8], thereby supporting the need for a better understanding of how long-term glycaemic control may impact on the risk of NAFLD and, on the other hand, how NAFLD may impact on long-term glycaemic control in T2DM.

To date, there is evidence showing that poor glycaemic control is associated with a higher likelihood of having NASH or advanced fibrosis [4,10]. For instance, in a cross-sectional study of 713 patients with biopsy-confirmed NAFLD (~50% of whom had established T2DM), Angelopoulos *et al.* reported that patients with poor glycaemic control were more likely to have NASH and advanced fibrosis compared with those with good glycaemic control [19]. In a small study, involving 39 patients with biopsy-proven NAFLD, who were followed for a median period of 2.4 years, Hamaguchi *et al.* showed that insulin use and lower HbA1c levels were associated with a significant improvement in liver fibrosis, independent of age, sex and BMI [20]. In a cross-sectional study of nearly 1,900 individuals with ultrasound-detected NAFLD, Tanaka *et al.* reported that a HbA1c level ≥6.5% (≥48 mmol/mol) was associated with greater levels of liver fibrosis, as assessed non-invasively by fibrosis (FIB)-4 index [21].

Little information is available to date about the long-term effect of NAFLD and its more advanced forms on long-term glycaemic control in people with T2DM. In this context, in a small cross-sectional study of 230 individuals, who underwent Fibroscan®, Patel *et al.* showed that patients with NAFLD were more likely to have HbA1c levels ≥7% (≥53 mmol/mol) and to be treated with insulin [13]. Preliminary

cross-sectional evidence has also shown that liver fat content (as assessed by proton spectroscopy) may be the principal factor explaining the daily amount of insulin required to achieve good glycaemic control in patients with insulin-treated T2DM [11].

Collectively, therefore, the results of our study corroborate and expand the aforementioned findings, showing that the baseline presence of NAFLD and coexisting significant fibrosis (as assessed by ultrasonography and VCTE) was strongly associated with a worsening of glycaemic control at follow-up, irrespective of age, BMI, HbA1c, HOMA-IR score and baseline use of certain glucose-lowering agents.

The most obvious explanation for our findings is that the association between advanced NAFLD and worsening of glycaemic control at follow-up might arise from shared metabolic risk factors. However, it is important to note that in our study the association of NAFLD and clinically significant fibrosis with worsening of glycaemic control remained statistically significant even after adjusting for some important confounding factors at baseline, including also the use of GLP-1 receptor agonists, SGLT-2 inhibitors or pioglitazone that may positively affect NAFLD (steatosis) and liver fibrosis. Notably, as reported in Tables 1 and 2, the use of these and other glucose-lowering agents, both at baseline and at follow-up, did not significantly differ between patients with NAFLD alone and those who had NAFLD and coexisting significant fibrosis at baseline. Hence, on the basis of the results of our study, it is also possible to hypothesize that the presence of NAFLD and coexisting clinically significant fibrosis might (partly) contribute to glycaemic worsening, possibly through exacerbation of systemic/hepatic insulin resistance, and increased production of multiple hepatokines (such as, for example, fetuin A, fetuin B or fibroblast growth factor-21) and proinflammatory cytokines (such as tumor necrosis factor-α or interleukin-6) [4,6,9].

Although further research is certainly needed, our findings may have important clinical implications, as they further support the need for a multidisciplinary and holistic approach to patients with T2DM and advanced NAFLD, as well as the need for a tailored treatment of NAFLD in this specific patient population [22-24]. In particular, although there are no licensed treatments for NAFLD, three different classes of glucose-lowering drugs (peroxisome proliferator-activated receptor agonists, GLP-1 receptor agonists and SGLT-2 inhibitors) showed promise in the treatment of this common liver disease. Specifically, pioglitazone and GLP-1 receptor agonists (mostly subcutaneous liraglutide and semaglutide) improved individual histological features of NASH or achieved histological resolution of NASH without worsening of fibrosis. SGLT-2 inhibitors (mostly dapagliflozin and empagliflozin) improved plasma aminotransferase levels and liver fat content, as assessed by magnetic resonance-based techniques [25-28]. A recent consensus report by the ADA and the European Association for the Study of Diabetes on management of T2DM also suggested for the first time that individuals with T2DM at intermediate to high risk of liver fibrosis should be considered for treatment with pioglitazone and/or a GLP-1 receptor agonist with evidence of benefit [29].

Our study has some important limitations that should be mentioned. First, the observational design of the study precludes to making any causal inferences. Second, the number of participants was small and the study included only Caucasian postmenopausal women with T2DM and NAFLD. Hence, our results cannot be necessarily generalizable to other patient groups, including, for example, men with T2DM (the investigation of possible sex-related differences is now becoming a priority in NAFLD research [30]). Third, we used only two HbA1c measurements for each study participant, one performed in 2017 (at baseline) and one performed in 2022 (at follow-up). Hence, the lack of repeat measurements of HbA1c between 2017 and 2022 does not allow us to have detailed information about the temporal trends of HbA1c levels. Fourth, although the further adjustment for HOMA-score did not attenuate the sig-

nificant association we observed between the presence of NAFLD and clinically significant fibrosis at baseline and the risk of worsening of glycaemic control at follow-up, larger prospective studies are needed to better elucidate the long-term effect of insulin resistance on glycaemic control in patients with T2DM and advanced NAFLD. In addition, due to the small sample size of the study, it is important to note that time-varying covariates, such as changes in glucose-lowering agents over the follow-up, cannot be included in multivariable logistic regression models. Fifth, we did not perform a liver biopsy or magnetic resonance elastography for staging liver fibrosis at baseline, nor a VCTE examination at follow-up. Hence, the possible differential effects of hepatic steatosis, inflammation, ballooning and fibrosis on glycaemic worsening over time cannot be accurately assessed in our study. However, both ultrasonography and VCTE (Fibroscan®) are two non-invasive methods that are widely used for diagnosing and staging NAFLD in clinical practice [31], although ultrasonography is characterized by inter-observer and intra-observer variability [32,33] and Fibroscan®-assessed LSMs may be affected not only by hepatic fibrosis but also by severe steatosis and inflammation [34]. Finally, we cannot definitely exclude the possibility that other unmeasured factors might partly explain the observed associations.

Despite these limitations, our study has some important strengths, including the consecutive enrolment of the study population and the completeness of our database. Additionally, both liver ultrasound and VCTE examinations were performed by a single expert physician, who was blinded to participants' clinical and biochemical details, thereby eliminating possible assessment bias and inter-observer variability. However, we cannot exclude a certain degree of intra-observer variability in the diagnosis of hepatic steatosis on ultrasonography [32,33]. Finally, we excluded T2DM patients with important comorbidities (e.g., cirrhosis, cancer and advanced kidney disease), as we believe that the inclusion of patients with such comorbidities might have confounded the interpretation of data.

In conclusion, the results of this longitudinal pilot study suggest that the presence of NAFLD and clinically significant fibrosis at baseline was associated with a markedly higher risk of worsening of glycaemic control at follow-up (5 years later) in post-menopausal women with both T2DM and NAFLD. The strength of this association was not weakened by adjustment for important baseline confounding factors such as age, BMI, HbA1c, HOMA-IR score and use of some specific glucose-lowering agents (such as pioglitazone, GLP-1 receptor agonists or SGLT-2 inhibitors) that might favorably affect NAFLD and liver fibrosis. Further studies are certainly needed to confirm these data in other patient cohorts and to better understand whether the prescription of certain glucose-lowering drugs with potential hepatoprotective effects may increase the probability of achieving good glycaemic control in patients with T2DM and advanced NAFLD.

4. Methods

4.1. Patients

We studied 61 Caucasian post-menopausal women with T2DM and NAFLD consecutively attending our diabetes outpatient service, who had data on liver ultrasonography and VCTE that were performed in the year 2017 (baseline), and who subsequently underwent a diabetic visit in the first 6 months of 2022 (follow-up). The exclusion criteria of the study were as follows: (a) history of significant alcohol consumption (defined as >20 grams of alcohol per day) and other competing causes of hepatic steatosis (e.g., virus, drugs, autoimmunity, or hemochromatosis); (b) cirrhosis, cancer and end-stage kidney disease; and (c) chronic use of potentially hepatotoxic drugs. Considering the technical limitations of VCTE methodology, patients with congestive heart failure or free abdominal fluid were also excluded from the

study. Most patients enrolled in this study have also been included in our previous studies [5,35].

The local Ethics Committee approved the study protocol. All patients gave their written informed consent for participation in this research.

4.2. Clinical and laboratory data

Body mass index (BMI) was measured as kilograms divided by the square of height in meters. Blood pressure was measured with a standard sphygmomanometer after the patient had been seated quietly for at least 5 minutes. Patients were considered to have hypertension if their blood pressure was ≥140/90 mmHg or if they were taking any anti-hypertensive drugs.

Venous blood samples were collected in the morning after an overnight fast. Complete blood count, glucose, lipids, creatinine, liver enzymes and other biochemical blood parameters were measured using standard laboratory procedures (Roche Cobas 8000; Roche Diagnostics, Basel, Switzerland) at the central Laboratory of our hospital. Hemoglobin A1c (HbA1c) was measured using the high-performance liquid chromatography analyzer Tosoh-G7 (Tosoh Bioscience Inc., Tokyo, Japan). Homeostasis model assessment-insulin resistance (HOMA-IR) score was used for estimating insulin resistance. Glomerular filtration rate (GFR) was estimated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) study equation [36].

Presence of ischaemic heart disease was defined as a documented history of myocardial infarction, angina or coronary revascularizations. Presence of ischaemic stroke was based on medical history and examination, and was confirmed by reviewing hospital medical records of patients, including radiology imaging results. Presence of diabetic retinopathy, diagnosed with fundoscopy after pupillary dilation, was also recorded in all patients. All these data were collected both at baseline and at follow-up, except for HOMA-IR score that was available only at baseline.

4.3. Liver ultrasonography and VCTE

A single expert physician, who was blinded to participants' clinical and biochemical details, performed both liver ultrasonography and VCTE examinations at baseline. Hepatic steatosis was diagnosed by using ultrasonography (using an Esaote MyLab 70 ultrasound with a 4 MHz probe, Esaote Group, Genova, Italy), according to specific ultrasonographic characteristics, such as diffuse hyperechogenicity of the liver relative to kidneys, ultrasound beam attenuation, and poor visualization of intra-hepatic vessel borders and the diaphragm [5,35]. Semi-quantitative ultrasonographic indices of hepatic steatosis were not available in this study

Liver stiffness measurement (LSM) was performed by VCTE using Fibroscan® (Echosens, Paris, France) and an M probe [5,35]. We did not have the Fibroscan® XL probe for patients with severe obesity. The accuracy of the Fibroscan® M probe to identify significant liver fibrosis is excellent in those with overweight or grade 1 obesity (BMI ≤35 kg/m^2). In our study, only four patients had a BMI >35 kg/m^2. Our Fibroscan® system was not equipped with the controlled attenuation parameter (CAP) technology for measuring hepatic steatosis [5,35]. LSMs were performed in each patient after at least eight hours of fasting and in the same day of the liver ultrasound examination [5,35]. Further details of the technical background and examination procedures have been described elsewhere [31]. Briefly, each patient's LSM was considered adequate if it included at least 10 valid measurements, with a success rate >60% and measurement variability <30% of the median [5,35]. The presence of clinically significant hepatic fibrosis was defined by the presence of LSM ≥7 kPa (that corresponds to Kleiner's stage F≥2 fibrosis on liver histology) [5,37].

4.4. Statistical analysis

Given the exploratory design of the study, we did not perform an *a priori* sample size calculation. Continuous variables were expressed as means±SD or medians and inter-quartile ranges (IQR) when indicated, while categorical variables were expressed as proportions. The Fischer's exact test for categorical variables, the unpaired Student's *t* test for normally distributed continuous variables and the Mann-Whitney test for non-normally distributed continuous variables (i.e., diabetes duration, plasma triglycerides, HOMA-IR score and Fibroscan®-assessed LSM) were used to examine the inter-group differences in main clinical and biochemical characteristics of the study participants, who were stratified either by severity of NAFLD at baseline (NAFLD alone *vs*. NAFLD and coexisting significant fibrosis) or by an overall worsening of glycaemic control at follow-up (arbitrarily defined as HbA1c increase ≥0.5% from baseline).

We tested the independent association between the severity of NAFLD at baseline and worsening of glycaemic control at follow-up (i.e., HbA1c increase ≥0.5%) by using logistic regression analyses. We performed four adjusted logistic regression models. Model 1 was adjusted for age and BMI at baseline; model 2 was adjusted for age, BMI and baseline use of GLP-1 receptor agonists; model 3 was adjusted for age, BMI and baseline use of SGLT2 inhibitors; and, finally, model 4 was adjusted for age, BMI and baseline use of pioglitazone. We also repeated the same multivariable logistic regression models after further adjustment for HbA1c or HOMA-IR score at baseline. Additionally, we performed an ordered logistic regression analysis (also called the ordered logit model, which is a subtype of logistic regression where the Y-category is categorical and ordered) using four increasing categories of worsening of glycaemic control (arbitrarily defined as HbA1c increases at follow-up ≤0.19%, from 0.20% to 0.49%, from 0.50% to 0.99%, and ≥1%, respectively) that was included as the ordinal dependent variable in all ordered logistic regression models. The ordered logistic regression models were adjusted for the same list of covariates that were included in the four aforementioned logistic regression models. Covariates included in logistic regression models were selected as potential confounding factors based on their significance in univariable analyses or based on their biological plausibility.

All statistical tests were two-sided, and a *p*-value of <0.05 (two-tailed) was considered to be statistically significant. Statistical analyses were performed using STATA software, version 16.1 (STATA, College Station, Texas, USA).

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, **Table S1**: Association between the severity of NAFLD at baseline and risk of developing worsening of glycaemic control at follow-up in post-menopausal women with type 2 diabetes; **Table S2**: Ordered logistic regression analyses - Association between the severity of NAFLD at baseline and increasing levels of worsening of glycaemic control at follow-up in post-menopausal women with type 2 diabetes.

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Informed Consent Statement: Written informed consent was obtained from all subjects involved in the study.

Data Availability Statement: All data of the study are available in the manuscript.

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