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# Short-term reduction of dietary gluten improves metabolic-dysfunction associated steatotic liver disease: A randomised, controlled proof-of-concept study

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## Summary

**Background:** The current management of metabolic dysfunction-associated steatotic liver disease (MASLD) relies on lifestyle intervention. Prior studies have shown that nutritional wheat amylase trypsin inhibitors (ATI) activate toll-like receptor 4 on intestinal myeloid cells to enhance intestinal and extra-intestinal inflammation, including the promotion of murine MASLD, insulin resistance and liver fibrosis.

**Aims:** We aimed to assess the impact of ATI (gluten)-free diet in liver as well as metabolic parameters of biopsy-proven MASLD patients.

**Methods:** We performed a 6-week, proof-of-concept 1:1 randomised controlled trial of an ATI-free diet. The controls followed a balanced diet recommended by the German Nutrition Society. We assessed changes in controlled attenuation parameter (CAP), body mass index (BMI) and homeostatic model assessment of insulin resistance (HOMA-IR). Patient-reported outcomes were assessed by the CLDQ-NASH questionnaire. Forty-five patients were consecutively enrolled (21 in the intervention arm and 24 in the control arm).

**Results:** Three patients from each arm discontinued the study. In the ATI-free diet group, a significant decrease in BMI ($p = 0.018$), CAP ($p = 0.018$) and HOMA-IR ($p = 0.042$) was observed at 6 weeks. The mean difference in CAP between the two arms at week 6 was 30.5 dB/m ($p = 0.039$), with a delta significantly higher in the ATI-free diet group ($p = 0.043$). Only an ATI-free diet could achieve a significant improvement in CLDQ-NASH domains ($p$ value for total scoring: 0.013).

**Conclusions:** A short-term ATI-free diet leads to significant improvements in liver and metabolic parameters, as well as patient-reported outcomes with good tolerability. A larger follow-up study is justified to corroborate these findings.

Clinical trial number: NCT04066400.

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[Correction added on 14 March 2024, after first online publication: The copyright line was changed.]

## 1 | INTRODUCTION

Metabolic-dysfunction associated fatty liver disease (MASLD), formerly known as non-alcoholic fatty liver disease (NAFLD), is currently the most frequent form of chronic liver disease, affecting about 30% of the adult population.[1] MASLD is tightly linked to the multiple features of metabolic syndrome, mainly obesity and type 2 diabetes (T2DM), with which it shares pathophysiological pathways and prognostic implications.[2,3] As a form of chronic liver injury, MASLD encompasses a wide spectrum of liver injury, ranging from simple intrahepatic fat accumulation to persistent chronic liver inflammation (metabolic-dysfunction associated steatohepatitis, MASH), which can eventually lead to cirrhosis and its complications, including portal hypertension and hepatocellular carcinoma.

So far, no pharmacological treatment is approved for MASLD, and current main management strategies are based on lifestyle intervention, aiming at weight loss and improvement in the metabolic profile (mainly insulin resistance), often combined with treatment of T2DM and cardiovascular risk factors.[4] Multiple dietary approaches have been investigated, with high heterogeneity across studies, with regard to populations, trial design and duration, and endpoints.[5]

Over the last years, the exploration of the gut-liver axis has provided increasing evidence on the tight relationship between gut permeability and gut-derived signals and the onset of liver and metabolic disease.[6] Alterations in the gut mucosal barrier or the onset of intestinal microbial dysbiosis (which is often a feature of the microbiota signature in obese individuals) can affect the liver by increased the flow of nutrient- and bacterial-derived pro-inflammatory antigen metabolites (e.g. lipopolysaccharide), combined with impaired enteric immune tolerance via continuous immune system activation.[7–9]

Amylase trypsin inhibitors (ATI), a family of related non-gluten proteins present in gluten-containing grains and products, have the ability to stimulate the enteric innate immune system via toll-like receptor 4 (TLR4) on monocytes, macrophages and dendritic cells. They are largely resistant to digestion and are partly taken up by the intestinal lamina propria, where the largest immune system of the body resides.[10–14]

Notably, ATI-derived immune activation and inflammation have been shown to promote intestinal and extra-intestinal inflammation in several murine models of metabolic and autoimmune diseases, including inflammatory bowel disease, celiac disease, intestinal and inhalative allergies, multiple sclerosis, Alzheimer's disease and MASLD.[10,11,15–21] Moreover, ATI directly impairs intestinal barrier function, inducing intestinal microbial dysbiosis.[10] Moreover, exploratory data from murine MASH models have suggested how a gluten containing, ATI-enriched diet has the ability to worsen MASH and associated metabolic features (weight gain, T2DM) independently of calorie intake.[17] These data have recently been confirmed in proof-of-concept studies of patients with familial Mediterranean fever, ulcerative colitis combined with primary sclerosing cholangitis and multiple sclerosis.[22–24]

In the present study, we designed a proof-of-concept, randomised study of MASLD patients undergoing a short-term ATI-free diet in order to test the hypothesis that a reduction in ATI-derived inflammation could lead to improvements in liver as well as metabolic features and additionally, in patient reported outcomes (PROs).

## 2 | MATERIALS AND METHODS

### 2.1 | Study population and trial design

This is a proof-of-concept, 6-week randomised, open label, controlled trial of an ATI-free diet in patients with MASLD (Figure 1). The randomisation ratio was 1:1. The control was represented by the recommendations of the German Nutrition Society (DGE—Deutsche Gesellschaft für Ernährung) for a balanced, gluten-containing diet.

An ATI-free diet is obtained by avoiding dietary sources of gluten, mainly wheat, to achieve a >90% reduction in daily regular ATI (gluten) intake, from a daily average of 0.7 g ATI (20 g gluten) to <0.05 g ATI per day. Patients were enrolled from March 2019 to March 2020 at the Outpatient Liver Clinic of the University Medical Center of Mainz. In the current analysis, patients with liver histology compatible with MASLD were included.

Inclusion criteria were: age >18 years and <75 years; MASLD on liver histology in the absence of other causes of liver injury; no changes in body weight >10% in the previous 6 months; for diabetic patients: HbA1c <8.6% and on stable therapy in the previous 6 months. Exclusion criteria were: other causes of liver disease, including viral (hepatitis B and C virus infection), autoimmune/cholestatic, drug-induced liver injury or therapy with steatogenic medications (including tamoxifen, steroids and methotrexate), genetic-driven liver disease, iron or copper storage disorders; alcohol intake >20 g/day in men and >10 g/day in women assessed by patient interview; pregnancy; indirect signs of advanced fibrosis as assessed by liver stiffness measurement (LSM) >12.5 kPa. The presence of celiac disease was excluded by negative serum transglutaminase IgA/IgG antibodies under a gluten-containing diet.

Nutritional counselling was done in groups of 2–4 on an ATI-free (Appendix S1) or DGE-based diet (Appendix S2). Both groups received consultation at baseline and a follow-up telephone-based consultation at week 3 of the intervention. Two on-site visits (baseline randomisation and end of treatment at week 6) were scheduled for each patient. Safety was assessed throughout and specifically at week 3 during telephone consultation, where patients were interrogated on adherence to the diet, willingness to maintain compliance to the study procedures, tolerability of the diet and potential doubts/concerns. Dietary compliance was assessed by self-reported weekly food protocols (Appendix S3). At baseline and end-of-study, clinical and anthropometrical parameters were collected, as well as fasting blood samples.

Fibrosis score-4 (FIB-4) was calculated as a surrogate test for liver fibrosis as described[25] according to the following formula: age (years) × AST (IU/L)/(platelet count ($\times 10^9$/L)) × radALT (IU/L).

Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) was calculated according to the following formula[26]:

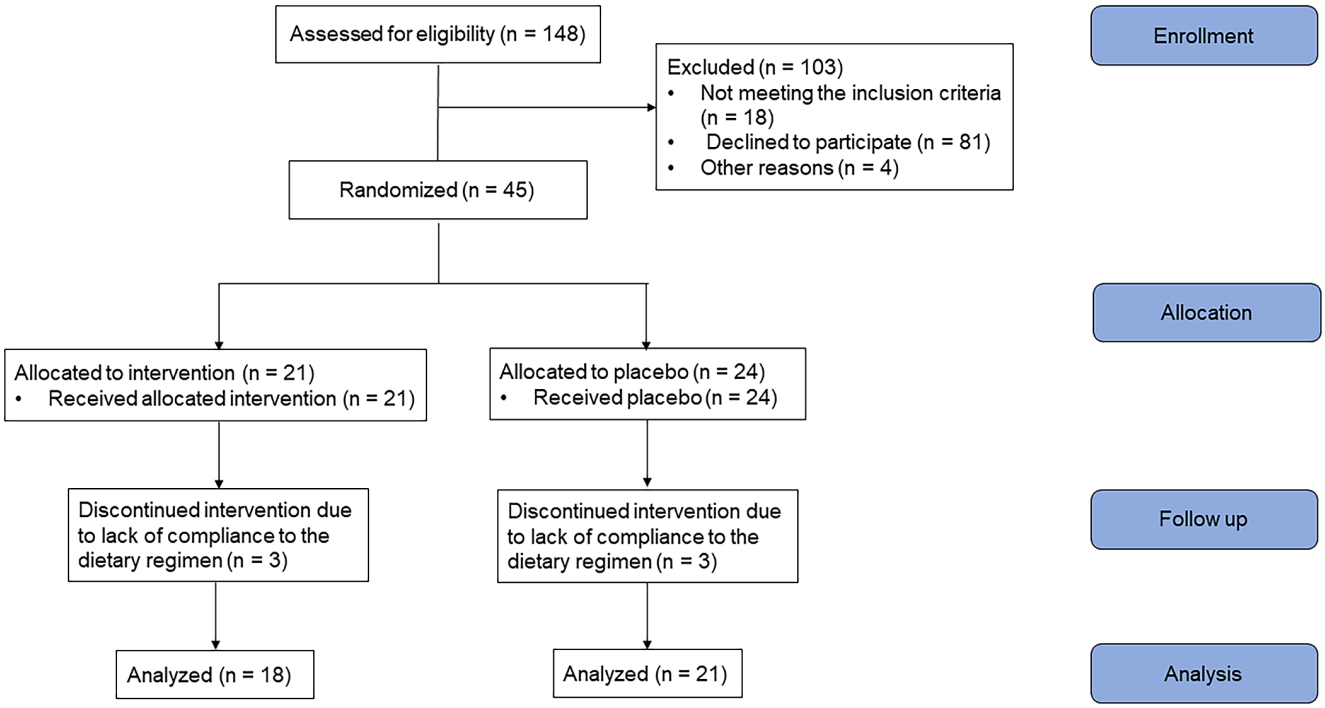


FIGURE 1 Flow chart of the study.

glucose (mg/dL) × insulin (mU/L)/405. Controlled attenuation parameter (CAP) and LSM were obtained for each patient using vibration-controlled transient elastography with a Fibroscan 530 device. The exam was performed fasted and by a single experienced operator (AM). Technical reliability was assessed by an IQR/median ratio <30% and a number of valid exams >10.

Enhanced liver fibrosis (ELF) measurements were performed at the MVZ Labordiagnostik Regensburg using the commercially available ELF™ assay.

The study was carried out according to the principles of the Declaration of Helsinki and was approved by the Ethics Committee of the State of Rhineland Palatinate (Landesärztekammer Rheinland-Pfalz 2018-13079). All patients provided written informed consent before study procedures.

## 2.2 | Primary and secondary endpoints

The predefined primary endpoint of the 'NASH (Non-Alcoholic Steatohepatitis)-ATI' study (NCT04066400) was ALT normalisation in patients with histologically defined NASH and elevated ALT at baseline. After 12 months of screening (January 2019 to February 2020), the study did not reach the prespecified sample size of 45 enrolled patients per arm with both histologically proven NASH and altered ALT for the predefined analysis of ALT normalisation. In particular, this was due to competing recruitment activities: patients with significant or advanced liver disease frequently declined to participate in this dietary trial and were enrolled in competing clinical trials.

The current analysis, extrapolated from the consecutively enrolled biopsy-proven MASLD patients (intention to treat the population of the 'NASH-ATI' study), reports on changes in CAP and LSM (per protocol analysis on non-invasive tests of liver disease severity). Secondary analysis includes changes in body mass index (BMI), transaminases, lipid or glucose parameters (including HOMA-IR as an indirect marker of insulin resistance), FIB-4 and ELF in patients undergoing an ATI-free diet compared to the control group.

## 2.3 | Histology findings

All liver biopsies (average length 25 mm, with at least 11 portal tracts) were analysed by a local pathologist with experience in liver disease and blinded to clinical information. The histological features of MASLD included steatosis (>5% in the hepatocytes), ballooning, lobular inflammation and fibrosis, and were scored according to the Clinical Research Network (CRN) system.[27] The diagnosis of Metabolic dysfunction-Associated Steatohepatitis (MASH) was made according to the joint presence of steatosis, ballooning and lobular inflammation.

## 2.4 | Patient-reported outcomes

Patient-reported outcomes were evaluated through the administration of the CLDQ-NASH (Chronic Liver Disease Questionnaire) at baseline and after 6 weeks of treatment. CLDQ-NASH is a widely validated tool in the MASLD population,[28] including

36 items grouped into six domains: abdominal symptoms, activity/energy, emotional health, fatigue, systemic symptoms and worry (Appendix S4). A Likert scale of 1–7 is applied to all items: a score of 1 corresponds to a pervasive problem, whilst a score of 7 corresponds to the absence of the asked problem. The scores are calculated separately for each domain as an average of the domain's items.

### 2.5 | Statistical analysis

The primary hypothesis was based on an ATI-driven reduction in liver inflammation and a milder course of liver disease in animal models.[17] No similar studies have been so far published in the context of an ATI-free diet in MASLD; hence, the appropriate size effect could not be calculated. The current proof-of-concept study was based mechanistically on previously published data in animal models[17] and experience with lifestyle interventions,[29] as well as data from the literature on the effects of diets on liver fat content.[30] A total of 45 patients were included, based on a drop-out estimation of 15% and an expected size effect of a minimum 10% CAP reduction in a dietary intervention.[30] Based on $\alpha = 0.05$ and 80% power, a calculated sample size of 45 was required to observe an effect (22 patients in the interventional arm and 23 patients in the control arm in 1:1 randomisation).

Descriptive statistics were used to analyse differences in surrogate markers of liver and metabolic parameters in the two groups. Randomisation was made through Excel, using the '=RAND' function (block randomisation). Continuous variables are expressed as median [IQR] and categorical variables are expressed as frequency (%). Comparison between categorical variables was made by chi-squared test, while comparison between continuous variables was made through the Mann–Whitney test. The Wilcoxon rank sum test was used to assess the difference between baseline and follow-up of paired continuous variables (Hodges-Lehmann median difference). An analysis of variance (ANOVA) test was used to assess differences between the two arms in the changes from baseline to follow up of the variables of interest. An analysis of covariance (ANCOVA) test was used to compare differences in the variables of interest between the treatment arm and the control group adjusted for: age, sex, transaminases, type 2 diabetes and fibrosis stage. $p$-value <0.05 was considered statistically significant. All analyses were performed using MedCalc Software version 18.9.1 (Mariakerke, Belgium).

## 3 | RESULTS

### 3.1 | Baseline features of the study cohort (intention to treat population)

Out of 148 approached patients with suspected MASLD, a total of 45 patients (33%) were consecutively enrolled according to the inclusion criteria after informed consent, of which 21 were randomised to the ATI-free diet (ATI group) and 24 to the control diet according to the DGE diet (DGE group; Table 1). Overall, the median age was 45 [48.5–61.0] years, and 53.3% were male. BMI values did not differ between the two groups: median 29.3 [26.8–34.3] in the ATI group and median 33.7 [29.4–39.4] in the DGE group ($p = 0–054$). T2DM and arterial hypertension were present in 22.2% and 28.9% of the total cohort, respectively, without differences between the two diet groups. Similarly, median LSM values were 6.3 [5.3–10.1] kPa in the ATI group and median 5.3 [4.4–9.7] kPa in the DGE group ($p = 0.487$), while median CAP values were 320.0 [305–7–343-3] dB/m in the ATI group and 330.5 [302.5–351.0] in the DGE group ($p = 0.767$). The median HOMA-IR and FIB-4 scores were 3.2 [1–6–4.6] and 1.18 [0.9–1.3], respectively, without differences between the two groups. The median ELF value was 8.8 [8.3–9.4], with a similar distribution in the two groups ($p = 0.124$). Based on liver histology, metabolic dysfunction-associated steatohepatitis (MASH) was found in 68.9% of the total cohort, while significant fibrosis (fibrosis stage >1) was found overall in 55.5% of patients (Table 2).

### 3.2 | Evaluation after 6 weeks of intervention (per protocol analysis)

In the ATI group, a total of 18 patients (85.7%), and in the DGE group a total of 21 patients (87.5%) completed the study (Table 3). Discontinuation was based on a reported inability to adhere to dietary recommendations.

We explored changes in CAP and LSM values comparing baseline to end-of-treatment values in the study population. In the ATI group, a median of 6.1 kPa changed to 5.3 ($p = 0.118$), and in the DGE group, from 6.4 kPa to a median of .4 ($p = 0.474$). In parallel, no signal of relevant changes in fibrosis could be derived from the ELF score. ELF in the ATI group was 8.3 [8.1–8.8] at baseline and 8.5 [8.0–8.9] at end of treatment with no relevant differences between both arms.

CAP measurements improved significantly in the ATI-free group, decreasing from 317.5 [305–0–340.0] at baseline to 300.0 dB/m [278.0–334.0] after 6 weeks (median difference −27.5 dB/m, $p = 0.018$; Figure 2A). In the DGE group, no significant CAP changes were observed (median difference −6.2 dB/m, $p = 0.251$). The median difference in CAP between the two treatment arms at week 6 was 30.5 dB/m, which was statistically significant (ANOVA $p = 0.039$; Figure 3A).

When comparing the delta CAP between the ATI group and the DGE group (Table 4), we observed a significant higher decrease in CAP in the ATI group ($p = 0.043$).

With regard to the secondary endpoint of this study (changes in BMI, HOMA-IR, FIB-4, lipid parameters and ELF), we found that overall 12 out of 18 patients (66.6%) in the ATI group showed a decrease in BMI, compared to only 9 out of 21 (42.8%) patients in the DGE group.

**TABLE 1** Characteristics of the study cohort.

| Characteristics | All patients ($n=45$) | ATI-free group ($n=21$) | DGE control group ($n=24$) | $p$ value |
|---|---|---|---|---|
| Age, years | 54 [48.5–61.0] | 53 [47.5–56.0] | 57.5 [48.0–67.0] | 0.058 |
| Male sex | 24 (53.3) | 11 (52.4) | 13 (54.2) | 0.905 |
| BMI (kg/m$^2$) | 31.02 [27.8–36.8] | 29.3 [26.8–34.3] | 33.7 [29.4–39.4] | 0.054 |
| Type 2 diabetes | 10 (22.2) | 3 (14.3) | 7 (29.2) | 0.236 |
| Hypertension | 22 (48.9) | 9 (42.9) | 13 (54.2) | 0.454 |
| Minor alcohol intake | 9 (20.0) | 4 (19.0) | 5 (20.8) | 0.882 |
| Statin therapy | 10 (22.2) | 3 (14.3) | 7 (29.2) | 0.236 |
| Metformin therapy | 5 (11.1) | 1 (4.8) | 4 (16.7) | 0.210 |
| GLP-1 receptor agonist or insulin therapy | 4 (8.9) | 1 (4.8) | 3 (12–5) | 0.368 |
| Liver stiffness (kPa) | 6.0 [4.6–10.1] | 6.3 [5.3–10.1] | 5.3 [4.4–9.7] | 0.487 |
| CAP (dB/m) | 326.0 [305.7–344.7] | 320.0 [305.7–343.3] | 330.5 [302.5–351.0] | 0.767 |
| HOMA-IR | 3.2 [1.6–4.6] | 2.7 [1.6–4.1] | 3.5 [2.1–5.5] | 0.236 |
| FIB-4 | 1.18 [0.9–1.3] | 1.12 [0.9–1.3] | 1.20 [1.0–1.51] | 0.300 |
| ELF | 8.8 [8.3–9.4] | 8.6 [8.2–9.2] | 9.0 [8.5–9.6] | 0.124 |

*Note*: Data are reported as frequency (%) for categorical variables and as median [interquartile range] for continuous variables.

Abbreviations: ATI, amylase trypsin inhibitor; BMI, body mass index; CAP, controlled attenuation parameter; DGE, Deutsche Gesellschaft für Ernährung; ELF, enhanced Liver Fibrosis test; FIB-4, fibrosis-4 score; GLP-1, glucagon-like peptide-1; HOMA-IR, homeostatic model assessment for insulin resistance.

In the ATI-free group, a small but significant decrease in BMI values was observed at the end of treatment with a median difference of −0.43 kg ($p=0.018$), from a median baseline of 28.9 [25.8–31.0] kg/m$^2$ to a median 28.5 [24.9–30.0] kg/m$^2$ after 6 weeks of diet (Figure 2B). Conversely, no changes were observed in the DGE group (median difference −0.11, $p=0.528$). A comparison of 6-week BMI between intervention and control arm showed that BMI values differed significantly between the two groups, with higher values in the DGE group (median difference 5.4 kg/m$^2$, ANOVA $p=0.004$; Figure 3B). Nonetheless, the delta in BMI values between the two groups was not statistically significant ($p=0.276$), despite a positive trend towards reduction (−0.38 in the ATI group versus 0.0 in the DGE group; Table 4).

A significant improvement in insulin resistance was observed. HOMA-IR values, changed from 2.7 [1.6–4.1] at baseline to 1.6 [1.3–3.7] after 6 weeks (median difference −0.7, $p=0.042$) in the ATI-arm (Figure 2C). Conversely, no changes in HOMA-IR were observed in the control group (median difference −0.1, $p=0.819$). HOMA-IR values after 6 weeks of treatment were significantly higher in the DGE group, when compared to the ATI group (median difference 1.17, ANOVA $p=0.032$; Figure 3C). No significant difference was found between the delta in HOMA-IR of the two groups, despite trends towards positive changes observed in the ATI group −0.41 versus −0.05, respectively (Table 4). No other explored metabolic parameter showed relevant changes after 6 weeks of the ATI-free diet.

## 3.3 | Post-hoc analysis

Next, we performed a post-hoc analysis comparing patients achieving any CAP reduction from the lifestyle intervention. A total of 27 out of 39 patients (69.2%) improved CAP (median reduction 12.5 [−25.0 to −1.5] dB/m). Of these, 14 (77.7%) occurred in the ATI group, while 13 (61.9%) occurred in the DGE group. Overall, patients achieving CAP reduction had significantly lower ELF values after 6 weeks of intervention, as compared to those who did not improve CAP values: median 8.6 [8.1–8.9] versus median 9.0 [8.9–9.6], respectively (median difference −0.6, $p=0.008$; Figure S1). Notably, baseline ELF values were similar in CAP responders versus non-responders (median difference 0.3, $p=0.124$). When comparing the delta in ELF values between CAP responders and non-responders, the difference was not statistically significant (median difference −0.1, $p=0.577$). No changes were observed with regard to the other metabolic parameters, including BMI and HOMA-IR (data not shown).

Finally, in order to assess the impact of potential confounders on the differences in BMI, CAP and HOMA-IR from baseline to the end of treatment between the two arms, we performed the ANCOVA test adjusted for age, sex, transaminases, type 2 diabetes and fibrosis stage. The adjusted models did not show any statistical differences between the two arms (Table S1; all $p>0.05$). Overall, ALT values were significantly associated with the delta CAP ($p=0.016$), while type 2 diabetes significantly impacted the HOMA-IR differences ($p=0.005$).

**TABLE 2** Histological findings of the study cohort.

| Feature | All patients ($n=45$) | ATI-free group ($n=21$) | DGE control group ($n=24$) |
|---|---|---|---|
| Steatosis | | | |
| Grade 1 | 14 (31.1) | 7 (33.3) | 7 (29.2) |
| Grade 2 | 24 (53.3) | 10 (47.6) | 14 (58.3) |
| Grade 3 | 7 (15.6) | 4 (19.0) | 3 (12.5) |
| Ballooning | | | |
| Grade 0 | 10 (22.2) | 8 (38.1) | 2 (8.3) |
| Grade 1 | 32 (71.1) | 11 (52.4) | 21 (87.5) |
| Grade 2 | 3 (6.7) | 2 (9.5) | 1 (4.2) |
| Lobular inflammation | | | |
| Grade 0 | 11 (24.4) | 8 (38.1) | 3 (12.5) |
| Grade 1 | 30 (66.7) | 13 (61.9) | 17 (70.8) |
| Grade 2 | 4 (8.9) | 0 (0.0) | 4 (16.7) |
| Fibrosis stage | | | |
| Stage 0 | 2 (4.4) | 1 (4.8) | 1 (4.2) |
| Stage 1 | 18 (40.0) | 10 (47.6) | 8 (33.3) |
| Stage 2 | 18 (40.0) | 8 (38.1) | 10 (41.7) |
| Stage 3 | 6 (13.3) | 2 (9.5) | 4 (16.7) |
| Stage 4 | 1 (2.2) | 0 (0.0) | 1 (4.2) |
| MASH | 31 (68.9) | 11 (52.4) | 20 (83.3) |

*Note*: Data are reported as frequency (%).

Abbreviation: MASH, Metabolic dysfunction-associated steatohepatitis.

### 3.4 | Patient reported outcomes

Both diets led to numerical improvements in the CLDQ-NASH domains (Table S2). However, only an ATI-free diet could achieve a significant improvement in the final scoring scheme, from a median baseline of 5.5 [5.0–6.1] to 5.9 [5.3–6.4] after 6 weeks ($p=0.013$). In particular, significant changes were observed in the Abdominal Symptoms domain, from median baseline 5.6 [4.6–6.6] to 6.5 [6.0–6.6] after 6 weeks ($p=0.003$), in the Emotional Health domain (from median 5.2 [4.5–6.2] to 6.0 [5.6–6.4], $p=0.001$), and in the Systemic Symptoms domain (from median 5.8 [4.6–6.6] to 6.2 [5.2–6.8], $p=0.012$). In the control group, no significant changes were observed in any domain.

## 4 | DISCUSSION

Easy, implementable dietary interventions to treat MASLD are urgently needed. A number of studies have explored different concepts.[5] One aspect of nutrition-induced metabolic inflammation and obesity[31] assumes that certain nutritional compounds promote immune activation and insulin resistance, leading to liver inflammation. Pre-clinical evidence highlights the potential role of wheat-containing anti-trypsin inhibitors (ATIs), which act as activators of intestinal TLR4 and, as such, the metabolic syndrome and MASLD.[17] The comparison of mice fed a high fat diet inducing mild MASLD with 25% of all dietary protein from gluten versus gluten-free mice

**TABLE 3** Comparison between baseline and 6-week (end of treatment) parameters of interest in the intervention (ATI-free) and control (DGE) arm.

| | ATI-free group | | | DGE control group | | |
|---|---|---|---|---|---|---|
| Parameter | Baseline ($n=21$) | Follow-up ($n=18$) | $p$ value | Baseline ($n=24$) | Follow-up ($n=21$) | $p$ value |
| BMI (kg/m$^2$) | 28.9 [25.8–31.0] | 28.5 [24.9–30.0] | 0.018 | 34.7 [30.2–39.4] | 33.2 [30.0–38.7] | 0.528 |
| Waist circumference (cm) | 99.5 [92.0–108.0] | 99.0 [94.0–105.0] | 1.000 | 114.0 [104.7–124.0] | 113.0 [107.0–124.5] | 0.283 |
| ALT (U/L) | 71.0 [48.0–102.0] | 71.0 [48.0–112.0] | 0.181 | 58 [46.0–97.5] | 49.0 [41.7–83.0] | 0.175 |
| AST (U/L) | 44.5 [32.0–66.0] | 36.5 [31.0–62.0] | 0.143 | 43 [30.7–60] | 38.0 [29.7–60.0] | 0.294 |
| Triglycerides (g/dL) | 147.0 [117.0–176.0] | 140.0 [109.0–177.0] | 1.000 | 130.0 [107.7–179.2] | 112.0 [96.7–164.2] | 0.198 |
| Total cholesterol (g/dL) | 231.5 [172.0–254.0] | 214.5 [169.0–238.0] | 0.089 | 214.0 [173.5–259.5] | 211.0 [188.0–237.7] | 0.164 |
| HDL cholesterol (g/dL) | 50.0 [43.0–55.0] | 47.0 [39.0–56.0] | 0.391 | 49.0 [45.7–56.5] | 49.0 [44.5–58.0] | 0.368 |
| Ferritin (µg/mL) | 194.5 [99.0–310.0] | 216.5 [93.0–275.0] | 0.579 | 123 [93.7–233.0] | 125.0 [103.0–195.2] | 0.388 |
| HbA1c (%) | 5.6 [5.2–6.0] | 5.5 [5.3–6.2] | 0.302 | 6.0 [5.5–6.6] | 5.9 [5.4–6.2] | 0.579 |
| Liver stiffness (kPa) | 6.1 [5.4–6.8] | 5.3 [4.1–7.2] | 0.118 | 6.4 [4.5–10.6] | 7.4 [6.1–9.9] | 0.474 |
| CAP (dB/m) | 317.5 [305.0–340.0] | 300.0 [278.0–334.0] | 0.018 | 331.0 [320.2–356.7] | 330.0 [305.2–364.2] | 0.251 |
| HOMA-IR | 2.7 [1.6–4.1] | 1.6 [1.3–3.7] | 0.042 | 3.5 [2.1–5.5] | 2.9 [1.7–5.3] | 0.819 |
| FIB-4 | 1.10 [0.9–1.3] | 1.04 [0.6–1.4] | 0.284 | 1.21 [1.05–1.64] | 1.23 [1.04–1.46] | 0.862 |
| ELF | 8.3 [8.1–8.8] | 8.5 [8.0–8.9] | 0.403 | 9.1 [8.5–9.4] | 9.0 [8.6–9.3] | 0.953 |

*Note*: Data are reported as median [interquartile range].

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; ATI, amylase trypsin inhibitor; BMI, body mass index; CAP, controlled attenuation parameter; DGE, Deutsche Gesellschaft für Ernährung; ELF, enhanced Liver Fibrosis test; FIB-4, Fibrosis-4 score; GLP-1, Glucagon-like peptide-1; HDL, high density lipoprotein; HOMA-IR, homeostatic model assessment for insulin resistance.

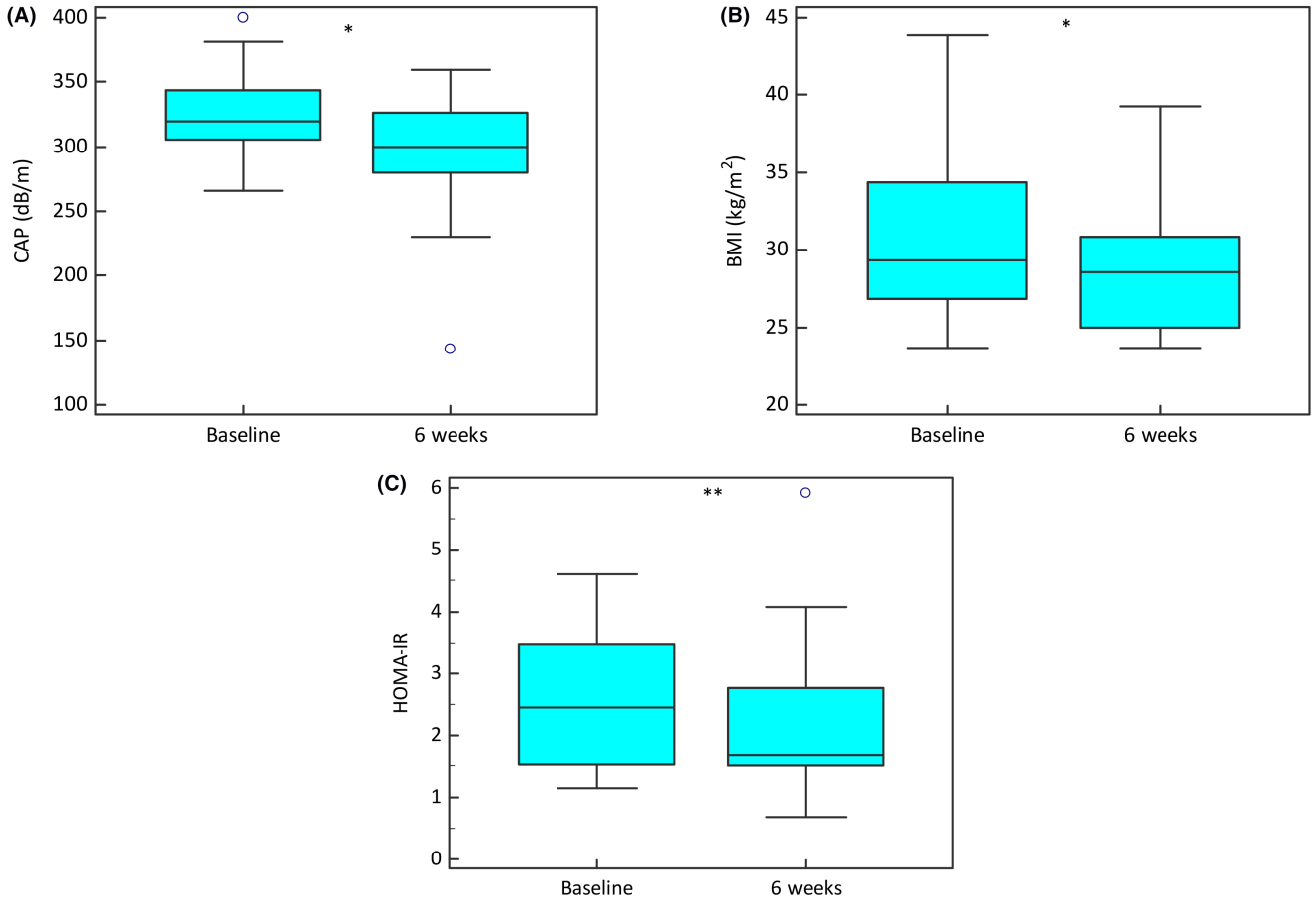


FIGURE 2 Changes in controlled attenuation parameter (CAP) values (A), body mass index (BMI) (B) and Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) (C) in the intervention arm (amylase trypsin inhibitor [ATI]-free diet) from randomization to end of treatment. *$p = 0.018$; **$p = 0.042$.

showed significant differences in hepatic inflammation and liver fat.[17] Notably, mice fed purified ATI or a whole wheat equivalent to the average human daily intake developed significantly more steatosis, higher serum transaminases, and higher parameters of activated liver fibrogenesis. This was accompanied by marked hepatic and adipose tissue inflammation, including neutrophils, macrophages and T cells, as well as an elevation of proinflammatory cytokines and chemokines including tumour necrosis factor (TNF)-α, macrophage chemotactic protein-1 (MCP-1, CCL2) and interleukin-8.[17] This model suggests close communication between the gut and the liver in the onset of metabolic inflammation and its promotion by ATIs.[32]

Based on this pre-clinical evidence, we performed a short, 6-week proof-of-concept, randomised, open label trial of an ATI (gluten)-reduced diet, aiming for at least a 90% reduction in patients with histological MASLD. The comparator was nutritional counselling according to the recommendations of the German Nutrition Society (DGE diet). Even in this short intervention and compared to an evidence-based dietary recommendation of healthy eating in the control arm, the reduction of ATI (gluten-containing food) led to a significant reduction in BMI, CAP levels and HOMA-IR index. The gluten/ATI reduction was well tolerated, and no serious adverse events occurred. In the intervention arm, 3 patients dropped out for non-adherence to the gluten-reduced diet. Notably, the BMI, CAP and HOMA-IR values of the ATI-reduced diet arm displayed a significant difference at the end of treatment, as compared to the DGE diet controls, confirming the strong impact of this specific diet on these 3 parameters.

In a secondary analysis, we explored the improvement of liver fat content, measured by CAP. In patients who decreased CAP values in both groups (median reduction 12.5 dB/m), a significant reduction of the ELF test, a direct marker algorithm for liver fibrosis and fibrogenesis, was observed. This is remarkable, as short, dietary interventions, even when affecting liver fat content, are unlikely to profoundly change histological fibrosis stages.[17] However, the improvement of parameters that reflect the dynamics of liver fibrogenesis, as represented by ELF, may represent a valuable tool to assess response to treatment, despite the short duration of the study limiting the robustness of this finding. On the other hand, long-term reduction of dietary ATI may also beneficially affect liver fibrosis in patients with MASLD, as already demonstrated in murine MASLD.[17]

Mechanistically, ATI reduction can lead to reduced activation of the enteric innate immune system, including myeloid antigen-presenting cells.[10,12–14] ATI proteins represent about 3% of the total grain proteins, the second most abundant after gluten (80–90%). Modern

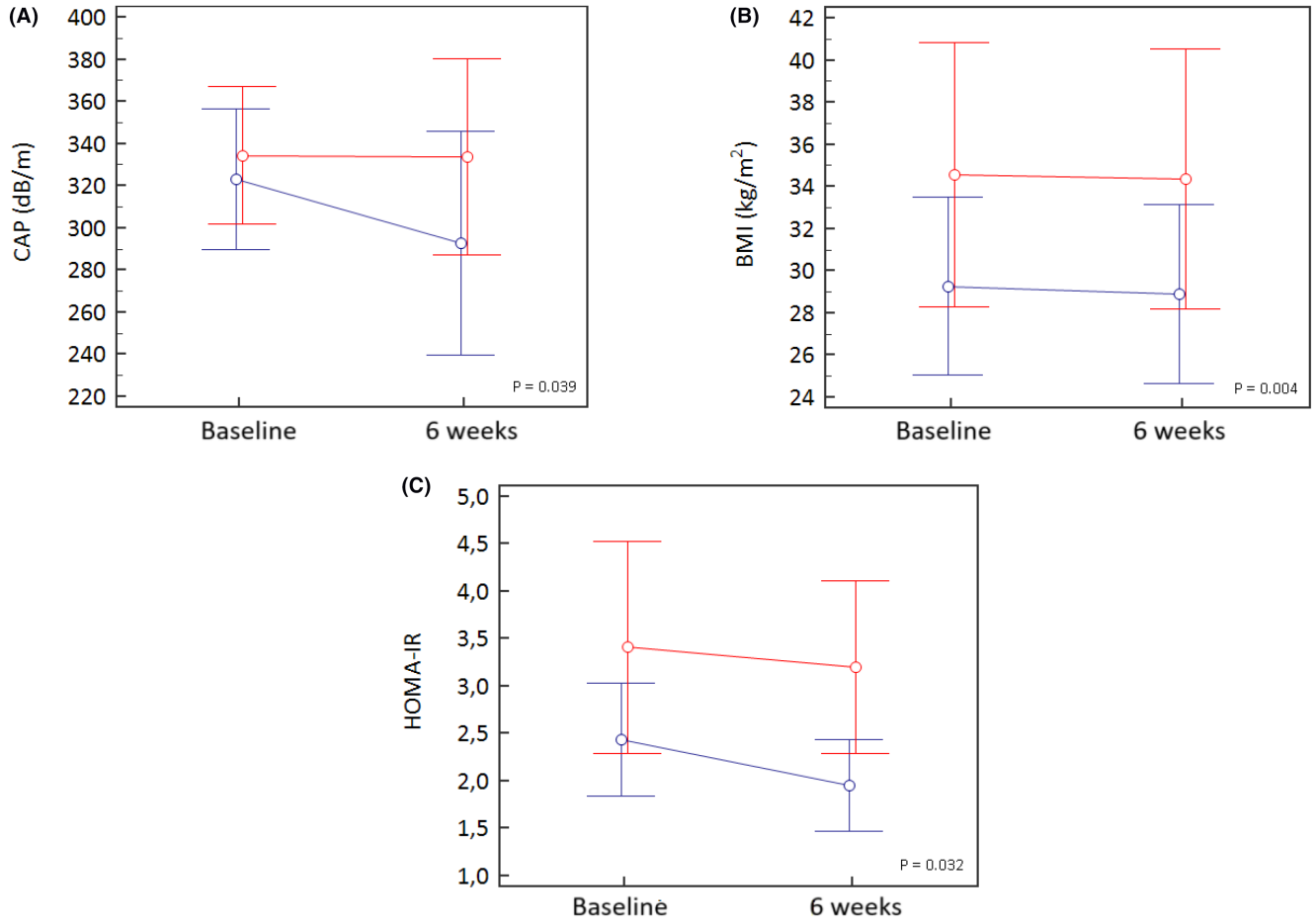


**FIGURE 3** Comparison between Amylase Trypsin Inhibitor (ATI)-free diet arm (blue draw) and control diet arm (DGE—Deutsche Gesellschaft für Ernährung) (red draw) with regard to changes in Controlled Attenuation Parameter (CAP) values (A), Body Mass Index (B) and Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) (C). *p* values referred to ANOVA 2×2 test.

**TABLE 4** Delta of Body Mass Index (BMI), Controlled Attenuation Parameter (CAP) and Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) between baseline and week 6 for the two groups.

| Parameter | Median delta [IQR] ATI-free group | Median delta [IQR] DGE group | *p* value |
|---|---|---|---|
| BMI (kg/m$^2$) | −0.38 [−0.83 to 0.0] | 0.0 [−0.36 to 0.32] | 0.276 |
| CAP (dB/m) | −27.5 [−63.0 to −9.0] | −7.5 [−19.0 to 7.5] | 0.043 |
| HOMA-IR | −0.41 [−1.32 to 0.41] | −0.05 [−1.07 to 1.02] | 1.000 |

Abbreviations: ATI, amylase trypsin inhibitor; DGE, Deutsche Gesellschaft für Ernährung; IQR, interquartile range.

grain contains up to 17 different types of ATI, which have a similar, highly conserved, compact and therefore intestinal protease-resistant secondary structure that is stabilised by 5 intramolecular disulfide bonds.[33,34] In general, all our pre-clinical in vitro and in vivo studies have shown that ATI, but not gluten, specifically activates TLR4 on myeloid cells, which are also important antigen presenting cells (mainly dendritic cells, but also macrophages) of the intestinal mucosa, with the subsequent release of pro-inflammatory cytokines and chemokines, including interleukin (IL)-6, IL-8, TNF-α and CCL2.[10,11,18] These activated cells also appear to leave the gut via the mesenteric lymph nodes.[16] Notably, the immunogenic activity of ATI is present only in gluten-containing grains, and even a small (calorically irrelevant) consumption of these ATI dose dependently promotes either intestinal or extra-intestinal immune-mediated diseases, often through a secondary T-cell-specific response. In particular, ATI intake has proven to boost gluten-driven immune responses in celiac disease and in multiple autoimmune and other chronic diseases in mice,[10,11,15–21] which is increasingly confirmed in human studies.[21–23] Moreover, ATI has the ability to shape the gut microbiota, by directly inhibiting anti-inflammatory and favouring pro-inflammatory bacterial strains.[18] These aspects may have a relevant impact in the clinical field, in particular when considering the PROs, which are expressions of systemic inflammation or disease burden. In our study, only an ATI-free diet could achieve a significant improvement in PROs evaluated by the CLDQ-NASH questionnaire. Interestingly, significant changes were observed with regard to abdominal symptoms, which may be a direct consequence of gluten removal from the diet but also in emotional health and, overall, in the burden of systemic symptoms. These aspects need to be considered in the context of a dietary trial, since improvements in PROs are tightly connected to compliance reinforcement and willingness to adhere.

This pilot study is the first exploring the efficacy of a short-term ATI-free (reduced) diet on liver and metabolic features in patients with biopsied-proven MASLD. Notably, CAP changes have provided good evidence on the overall improvement of liver disease in clinical MASLD studies using pharmacological treatments[18] and can therefore be used for short-term, non-invasive monitoring of treatment response. In addition, the positive changes in HOMA-IR values underline the beneficial effects of the wheat- and ATI- free diet on insulin resistance, again as observed in our murine MASLD models,[17] which is a key driver of MASH onset and progression. The mild but significant reduction in BMI that has been observed in the present study may be partly responsible for the improvement in insulin sensitivity and the other metabolic improvements.

There are a number of limitations to our study. The recruitment of a sufficiently large number of patients agreeing to undergo liver biopsy was not possible. We explored a limited set of novel biomarkers, but not all are fully validated.[35–39] Nonetheless, the concordance of CAP and ELF reductions, potentially linking fibrogenesis and hepatic steatosis/steatohepatitis in response to a lifestyle diet is reassuring. This concordance of ELF changes and improvement in liver histology with regard to disease activity has been shown in a post-hoc analysis of the PIVENS trial,[40] as well as ELF could predict changes in liver fibrosis in patients with HCV infection undergoing antiviral treatment with pegylated interferon.[41] Additionally, the study was influenced by an inherent selection bias towards early disease stages, considering the parallel conduct of clinical studies exploring histological disease stages (fibrosis F2-F3) at the University Medical Center of Mainz at that time.

Independent of the potential efficacy, the willingness to undergo and adhere to a gluten-reduced diet was a barrier to patients. The compliance with the ATI-free diet is likely to decrease over time and the means to monitor adherence need to be improved. Additional limitations of the current study with regard to this aspect are linked to the self-reporting methods that were used to assess compliance, which are subjected to recall bias. This would also lead to inaccurate evaluation of total daily calorie intake as well as the potential impact of physical exercise in the outcomes.

Currently available measurements to monitor gluten intake include immunogenic peptides in the stool or, more practicably, in the urine, may serve as a surrogate of significant wheat and thus ATI consumption,[42] as we used recently in 2 clinical trials in patients with primary sclerosing cholangitis/ulcerative colitis and multiple sclerosis.[22,23] However, these tests are unreliable tools to monitor compliance or to quantify the amount of gluten (as a surrogate for ATI) in the diet.[43,44] Importantly, unlike in celiac disease, the proinflammatory effect of ATI in MASLD is dose-dependent, and even consumption of 10% of the normal daily intake of gluten containing foods, for example 20 g of wheat flower containing about 2 g of gluten and 80 mg of ATI, will significantly reduce the stimulation of intestinal innate immunity and produce a major treatment benefit.[15–20]

In conclusion, this small, proof-of-concept study suggests that even a short course of an ATI (wheat or gluten)-reduced diet leads to improvements in body weight, liver fat content and insulin resistance. The short-term tolerability is acceptable, and we did not observe adverse events. A larger sample size and study duration, including emerging additional biomarkers of diet compliance and inflammation and fibrosis, are warranted to corroborate these findings.

## AUTHOR CONTRIBUTIONS

**Angelo Armandi:** Data curation; formal analysis; software; writing – original draft. **Helena Bespaljko:** Investigation. **Alexander Mang:** Investigation. **Yvonne Huber:** Investigation. **Maurice Michel:** Investigation. **Christian Labenz:** Investigation. **Peter R. Galle:** Resources. **Manjusha Neerukonda:** Investigation. **Elisabetta Bugianesi:** Supervision; visualization. **Detlef Schuppan:** Conceptualization; supervision; visualization; writing – review and editing. **Jörn M. Schattenberg:** Conceptualization; investigation; supervision; visualization; writing – review and editing.

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## SUPPORTING INFORMATION

Additional supporting information will be found online in the Supporting Information section.

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