



## Original article

# *Helicobacter pylori* cagA/vacAs1-m1 strain is associated with high risk of fibrosis in metabolic-dysfunction-associated steatotic liver disease

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## ABSTRACT

**Introduction and Objectives:** Recent studies have suggested an association between *H. pylori* and metabolic dysfunction associated steatotic liver disease (MASLD). We aim to evaluate the association of *H. pylori* virulence genes with non-invasive markers of liver injury and fibrosis in MASLD subjects.

**Patients and Methods:** A total of 362 dyspeptic patients who underwent gastroscopy were selected. Biochemical, clinical parameters, ultrasound, FIB-4 score, liver stiffness measurement (LSM) by vibration-controlled transient elastography (VCTE), gastric biopsies, and *H. pylori* virulence genes (cagA, vacA) were evaluated.

**Results:** A cohort comprised of 61 % women and 39 % men with a median age of 52 (40–60) years. MASLD was observed in 42 %, and *H. pylori*-positive in 45 %. No differences were observed regarding *H. pylori* status at co-morbid metabolic conditions. In MASLD cohort, *H. pylori*-positive was associated with higher AST, ALT, FIB-4 and LSM. Indeed, carriers of cagA/vacA-s1/m1-positive allelic combination were associated with higher AST, ALT, FIB-4 and LSM but not cagA/vacA-s1/m1-negative. The OR for high-risk of significant/advanced-fibrosis by VCTE ( $\geq 8$  kPa) with *H. pylori*-positive was 2.56 (95 % CI, 1.2–5.75) and for cagA/vacA-s1/m1-positive allelic carriers was 4.01 (95 % CI, 1.38–11.56), but non-significant association in cagA/vacA-s1/m1-negative. After adjusting for age, gender, diabetes, BMI and hypertension the OR for VCTE  $\geq 8$  kPa with *H. pylori*-positive was 2.43 (95 % CI, 1.88–12.44), and cagA/vacA-s1/m1-positive allelic carriers was 4.06 (95 % CI, 1.22–14.49).

**Conclusions:** In our cohort of functional dyspepsia (FD) patients with MASLD, *H. pylori* was associated with non-invasive markers of liver injury and fibrosis. Carriers of cagA/vacA-s1/m1-positive allelic combination showed an independent risk of significant/advanced fibrosis by VCTE.

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## 1. Introduction

Metabolic-dysfunction-associated steatotic liver disease (MASLD), previously known as Nonalcoholic Fatty Liver Disease

**Abbreviations:** ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; cagA, cytotoxin associated genes A; CI, confidence interval; DBT, Diabetes Mellitus; EPS, epigastric pain syndrome; FD, functional dyspepsia; GGT, gamma-glutamyl transferase; *H. pylori*, *Helicobacter pylori*; IQR, interquartile range; kPa, kilopascal; LSM, liver stiffness measurement; MASLD, metabolic dysfunction associated steatotic liver disease; MASH, metabolic dysfunction associated steatohepatitis; NSAID, non-steroidal anti-inflammatory drugs; OR, odds ratio; PCR, polymerase chain reaction; PDS, postprandial distress syndrome; PPI, proton pump inhibitors; SD, standard deviation; vacA, vacuolating cytotoxin A; VCTE, vibration controlled transient elastography

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(NAFLD) is currently the most common chronic liver disease [1,2]. The global prevalence of MASLD has increased from 25 % in 1990–2006 to 38 % in 2016–2019 [3]. Its prevalence is rising worldwide in parallel with increases in obesity and metabolic comorbid disease (insulin resistance, diabetes mellitus and obesity) [4,5]. MASLD encompasses a histological spectrum of disease characterized by macrovesicular hepatocyte steatosis that may be accompanied by mild inflammation, and metabolic dysfunction associated steatohepatitis (MASH), the more active form of MASLD characterized by the presence of hepatic steatosis, inflammation, and hepatocyte ballooning, is emerging as one of the leading causes of cirrhosis, cirrhotic complications, hepatocellular carcinoma (HCC), liver transplantation and liver-related death [1]. MASLD is a complex disorder that is influenced by multiple mechanisms, including genetic, environmental, and metabolic factors [6]. The pathogenesis, particularly

the drivers of progression in MASLD are incompletely understood [7].

*Helicobacter pylori* (*H. pylori*) is a gram-negative, microaerophilic bacteria that colonizes gastric mucosal epithelium [8]. Similarly to the high prevalence of MASLD, *H. pylori* has highly global prevalence, affects nearly 50 % the world's population with a higher prevalence in developing countries and is a key constituent of the human microbiome [8]. Clinical manifestations of *H. pylori* infection include peptic ulcer disease, gastric mucosa-associated lymphoid tissue lymphoma, and non-cardia gastric adenocarcinoma [9]. *H. pylori* is also the main infectious-related ethological cause of functional dyspepsia (FD) [10]. *H. pylori* infection not only affects gastric mucosa but is also linked to a number of extra-gastric diseases, indicating that *H. pylori* may cause disease far from the primary site of infection by a different pathogenic process [11]. Notably, recent attention has focused on whether *H. pylori* infection contributes to metabolic disorders including MASLD [12,13]. However, the existing literature on the association between *H. pylori* infection and MASLD is conflicting, with some studies reporting a positive association while others finding no significant relationship [14,15]. *H. pylori* is thought to contribute to the pathogenesis of MASLD by increasing insulin resistance, stimulating the release of proinflammatory cytokines, and increasing intestinal permeability [16,17]. The interaction between *H. pylori* virulence genes and human genetic polymorphisms in the pro- and anti-inflammatory cascade appear to play a role in the host's susceptibility to *H. pylori* gastritis and duodenitis [18,19]. Notably, *H. pylori* virulence genes, mainly cytotoxin-associated gene A (*cagA*) pathogenicity island (PAI) and vacuolating cytotoxin A (*vacA*), determine *H. pylori* pathogenicity and pro-inflammatory response [9,20]. Moreover, *H. pylori* internalization and injury in hepatocytes is also related on *cagA* and *vacA* status [21,22]. In this regard, previous data on chronic hepatitis C showed an association between *H. pylori cagA* status in the progression to cirrhosis and hepatocellular carcinoma [23]. It is noteworthy, that *H. pylori* infection per se is linked to gut dysbiosis including alterations in bacterial diversity and abundance that may influence metabolic derangements [17]. Viewing aforementioned data, we can consider that *H. pylori* virulence genes may contribute to MASLD severity. Thus, our aim was to evaluate the association of *H. pylori* virulence genes with non-invasive markers of severity in MASLD subjects. To address this subject, we examined the role *H. pylori* gastric infection, *cagA* and *vacA* virulence genes status in MASLD diagnosis, non-invasive markers of liver injury and fibrosis using a FD population who underwent upper endoscopy.

## 2. Patients and Methods

### 2.1. Patients

We consecutively recruited patients newly diagnosed with FD who were scheduled to undergo gastroscopy from January 2018 to December 2021 at IOT Medical Center, Simes Medical Institute (Posadas, Province of Misiones) and University Hospital San Juan Bautista (Santo Tomé, Province of Corrientes), Argentina.

The criteria for inclusion were: (1) age between 18 and 70 years, (2) symptoms meeting Rome-III criteria. The criteria for exclusion before gastroscopy were: (1) progressive, severe diseases requiring active medical management (e.g. advanced congestive heart failure, uncontrolled diabetes, decompensated cirrhosis, end-stage renal failure, neurological disease, advanced cancer, or psychiatric disorder), (2) those with known causes of chronic liver diseases and significant alcohol consumption (defined as  $\geq 140$  g/week for women and  $\geq 210$  g/week for men), (3) autoimmune medical conditions (inflammatory bowel disease, celiac disease, vasculitis, connective tissue disease), (4) atopic disease such as food allergies (milk, eggs, peanuts, tree nuts, fish, shellfish, fruit, and vegetables), asthma, allergic rhinitis or drug reaction, and eczema, (5) patients who had taken

steatogenic medications (corticosteroids, tamoxifen, amiodarone, methotrexate), (6) patients who had taken antibiotic within the past 3 weeks and (7) history of previous *H. pylori* infection and gastric or bariatric surgery. Patients receiving proton pump inhibitors (PPI), H2-blockers or non-steroidal anti-inflammatory drugs (NSAID) were advised to suspend them 14 days before endoscopy. The criteria for exclusion after upper endoscopy were: (1) evidence of active peptic ulcer disease or gastro-esophageal erosive esophagitis, (2) evidence of malignant gastric disease, (3) signs of celiac disease, and (4) not available gastric biopsies.

### 2.2. Clinical variables

Clinical co-morbid conditions and anthropometric variables were obtained by a pre-endoscopy interview, data regarding age, sex, body mass index (BMI), alcohol consumption, smoking habits, medical history, including presence of hypertension [24], pre-diabetes/type-2 diabetes [25], surgery, malignancy and chronic liver disease were collected. Medical history also involved the regular utilization of NSAIDs, PPI, H-2 blockers, anti-hypertensive medications and anti-diabetic medications. The following laboratory parameters were analyzed in this study: aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT), alkaline phosphatase (ALP), platelets and viral hepatitis serology (HBsAg, Anti-HBc and Anti-HCV). AST and ALT cut-off of upper limit of normal were defined as  $<40$  UI/mL [26]. The FIB-4 index was calculated using the formula:  $FIB-4 = \text{Age (years)} \times \text{AST (IU/L)} / [\text{PLT} (10^9/\text{L}) \times \sqrt{\text{ALT (IU/L)}}]$  [27]. Previously published FIB-4 cut-off were used to rule-out ( $<1.3$ ) advanced fibrosis (AF) [28]. Cut off of  $FIB-4 \geq 1.3$  is recommended to identify patients with MASLD as intermediate-high risk of AF requiring referral to the specialist liver clinic [28].

### 2.3. FD and MASLD definition

FD patients satisfied the Rome-III criteria for the past three months with symptom onset at least six months before diagnosis [29]. FD was divided into 2 subtypes depending on the symptoms: Epigastric pain syndrome (EPS) is associated with epigastric pain or epigastric soreness, and postprandial distress syndrome (PDS) is associated with early satiety or postprandial fullness. Those who meet both criteria were classified as EPS/PDS overlap syndrome. MASLD was defined according to the multi-society consensus statement on new fatty liver disease nomenclature [2], the diagnosis of MASLD required the following: (1) hepatic steatosis detected by ultrasonography; (2) no significant alcohol consumption (defined as  $<140$  g/week for women and  $<210$  g/d for men); (3) the presence of one cardiometabolic risk factor and (4) no other discernible cause of steatosis.

### 2.4. Vibration controlled transient elastography

Vibration controlled transient elastography (VCTE) was performed by FibroScan-402 (Echosens, Paris, France) medical device using the M or XL probe as appropriate. Liver stiffness measurement (LSM) was assessed after a diagnosis of MASLD by ultrasound, by one expert operator. Measurements were performed on the right lobe of the liver through intercostal spaces guided by ultrasonography with the patient lying in dorsal decubitus with the right arm in abduction. LSM was expressed in kilopascal (kPa) and calculated as the median value of ten successful acquisitions, defined by a success rate of at least 60 %, and by an interquartile range lower than 30 % [30]. Previously published LSM cut-offs were used to rule-out Fibrosis stage-2 or greater stage (significant fibrosis, SF)  $\geq 5.8$  kPa, and to rule-out Fibrosis stage-3 or greater stage (advanced fibrosis, AF)  $\geq 8$  kPa respectively [31,32,33].

## 2.5. Endoscopy

All recruited participants underwent gastroscopy performed by experienced endoscopists. Biopsy specimens were collected from the lesser curvature of the gastric body (two biopsies) and lesser curvature of the gastric antrum (two biopsies).

## 2.6. Histopathologic analysis

Biopsies were fixed in 10 % formalin and processed to paraffin embedding for hematoxylin and eosin (HE) staining by routine methods. The presence of *H. pylori* was assessed on gastric biopsies using Giemsa staining. Gastric pathology was recorded as per the Sydney system [34].

## 2.7. DNA extraction and polymerase chain reaction (PCR)

DNA extraction from gastric biopsies was performed according to the manufacturer's instructions (ADN PuriPrep-T kit, InbioHighway, Argentina). Samples were stored at  $-20^{\circ}\text{C}$  until used. PCR was performed by using specific primers. Target gene, amplicon size, primer names, and sequences are shown in Table 1. For PCR amplification, 50 ng of DNA samples was added to a PCR mixture containing 20  $\mu\text{mol}$  forward and reverse primers, 15  $\mu\text{L}$  of MINT Master Mix 2x (InbioHighway, Argentina) to the total volume of 25  $\mu\text{L}$ . PCR amplification was performed under the following conditions: initial denaturation at  $94^{\circ}\text{C}$  for 4 min followed by 35 cycles of denaturation at  $95^{\circ}\text{C}$  for 30 s, annealing for 30 s (Table 1), extension at  $72^{\circ}\text{C}$  for 30 s, and final extension at  $72^{\circ}\text{C}$  for 5 min (Labnet MultiGene MiniThermocycler). The PCR reaction products were electrophoresed on 2 % agarose gel (InbioHighway, Argentina) and the bands were visualized by ethidium bromide staining. The *cagA* [35] and *vacA* [36] statuses were determined from *H. pylori* positive samples by PCR using their respective primers (Table 1).

## 2.8. Statistical analysis

Sample size calculation was performed assuming a prevalence of non-alcoholic fatty liver disease in general population of 30 % [37] and 44.5 % in *H. pylori* infected subjects [38]. With 80 % of power and alpha level of 0.05, we calculated that at least 346 patients would be needed for the study.

Data were presented as mean  $\pm$  SD, median (IQR, interquartile range), or number of subjects (% of total) as appropriate. Differences between groups were analyzed by Students' *t*-test or ANOVA for normal distribution, Wilcoxon rank sum test or Kruskal Wallis test for non-normal distribution. Categorical values were compared using Chi-square tests. The relationship with *H. pylori* and combined *cagA/vacAs1/vacAm1* allelic carriers with VCTE cut-off to rule-out intermediate-high risk of SF/AF were examined by logistic regression model (binary response variable). Univariate models and multiple predictor

variable models including age, gender, pre-DBT/type-II DBT, BMI and Hypertension as covariates were assessed. Data were analyzed using SPSS 22.0, and Jamovi 2.2.5. Two-tailed  $P < 0.05$  was considered statistically significant.

## 2.9. Ethical statement

Written informed consent was obtained from each participant enrolled in the study. The study protocol was approved by local ethical committee (Comité de Ética en Investigación Provincial, CEIP, N° 454/17). The study was performed in accordance with the ethical standards as laid down in the 1975 Declaration of Helsinki and its later amendments.

## 3. Results

### 3.1. Study population

Four hundred and one patients with FD who met Rome-III criteria were evaluated before upper endoscopy, 39 patients were excluded and 362 patients were included for analysis. A flowchart of the study and baseline characteristics of patients appear in Fig. 1 and Table 2. The cohort of patients comprised of 222 (61 %) women and 140 (39 %) men with a median age of 52 (IQR, 40–60) years. The FD syndromes were as follows: 176 (49 %) patients had EPS, 114 (31 %) PDS and 72 (20 %) were EPS/PDS overlap. *H. pylori* positive gastric biopsies were detected in 162 (45 %). About clinical comorbidities: 85 (23 %) patients had pre-DBT/type-II DBT, 111 (31 %) hypertension, median BMI was 26.5 (IQR, 24–29.8)  $\text{kg}/\text{m}^2$  and 89 (25 %) had obesity (BMI  $\geq 30 \text{ kg}/\text{m}^2$ ), regular smoking was present in 86 (24 %) patients, 193 (53 %) subjects referred moderate alcohol consumption daily or occasional. The prevalence of MASLD was 153/362 (42 %) (Table 2). Viral serologic status of the study population was negative. Then, we evaluated our cohort's association of *H. pylori* infection and clinical variables. No differences were observed regarding *H. pylori* status at age, BMI, obesity, pre-DBT/type-II DBT, hypertension, smoking, and alcohol consumption (Table 2). *H. pylori* infection was significantly associated with male gender and EPS. While PDS and EPS/PDS was associated with *H. pylori* negative FD. A non-significant trend to more proportion of MASLD subjects was observed under *H. pylori* positive (77/162, 48 %,  $p$  0.068) rather than *H. pylori* negative infection (76/200, 38 %).

### 3.2. *H. pylori* infection in MASLD subjects

Then, we evaluate the influence of *H. pylori* status in non-invasive markers of liver injury and fibrosis in our cohort of FD patients with MASLD ( $n$ : 153) (Table 3). *H. pylori* active infection status showed no difference at age, BMI, obesity, pre-DBT/type-II DBT, hypertension, platelets, ALP and GGT values in MASLD subjects. Serum AST and ALT values resulted in a significant increase in *H. pylori* positive subjects

**Table 1**  
Primer sets used for genotyping *H. pylori* virulence genes by PCR.

Target site	Amplicon size (bp)	Primer names and sequences	Annealing temperature	References
<i>cagA</i>	189	cagA-F (5-TTGACCAACAACCACAAACCGAAG-3)	62 $^{\circ}\text{C}$	[35]
		cagA-R (5-CTTCCCTTAATTGCGAGATTCC-3)		
<i>vacAs1</i>	259	VA1-F (5-ATGGAATACAACAACACAC-3)	56 $^{\circ}\text{C}$	[36]
		VA1-R (5-CTGCTTGAATGCCCAAAC-3)		
<i>vacAs2</i>	286	VA1-F (5-ATGGAATACAACAACACAC-3)	56 $^{\circ}\text{C}$	[36]
		VA1-R (5-CTGCTTGAATGCCCAAAC-3)		
<i>vacAm1</i>	290	VA3-F (5-GGTCAAATGCGGTATGG-3)	56 $^{\circ}\text{C}$	[36]
		VA3-R (5-CCATTGGTACCTGTAGAAC-3)		
<i>vacAm2</i>	352	VA4-F (5-GGAGCCCCAGGAAACATTG-3)	56 $^{\circ}\text{C}$	[36]
		VA4-R (5-ATAACTAGCCCTTGCAC-3)		

PCR primer sets, annealing temperature and size of the PCR products used for genotyping *H. pylori*. F – forward; R – reverse.

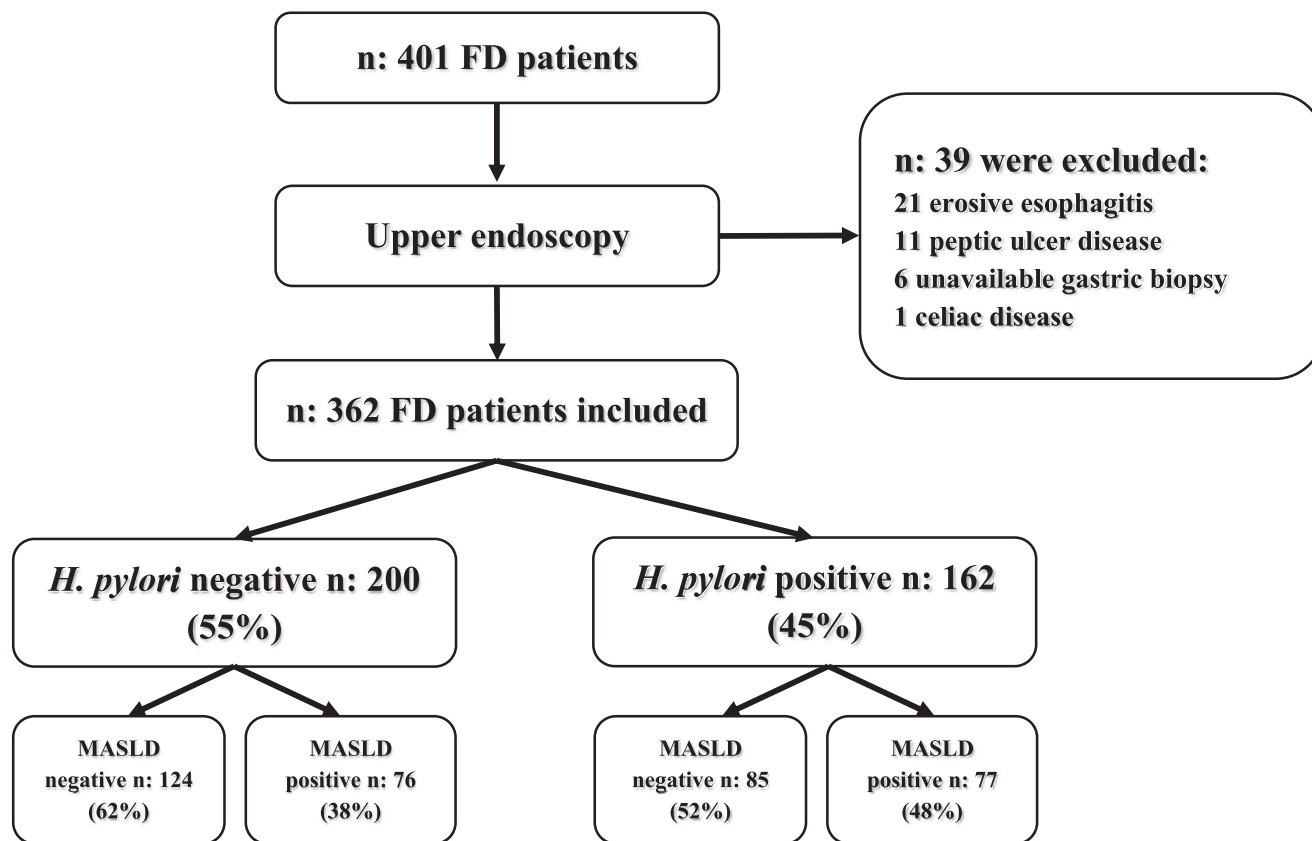


Fig. 1. Study flow-chart. Functional dyspepsia (FD), *H. pylori* negative, *H. pylori* positive, Non-alcoholic fatty liver disease (MASLD).

Table 2  
*H. pylori* infection and clinical variables in FD subjects.

Variable	Total (n: 362)	<i>H. pylori</i> negative (n: 200)	<i>H. pylori</i> positive (n: 153)	p
Age (IQR)	52 (40–60)	52 (41–61)*	51 (39–59)	ns
Gender (Fem%)	222 (61 %)	133 (66 %)*	89 (55 %)	<0.05
BMI (IQR)	26.5 (24–29.8)	26.1 (23.7–29.6)	26.8 (24.2–30.3)	ns
Obesity (%)	89 (25 %)	46 (23 %)	43 (27 %)	ns
PDS (%)	114 (31 %)	78 (39 %)*	36 (22 %)	<0.05
EPS (%)	176 (49 %)	64 (32 %)*	112 (69 %)*	<0.05
EPS/PDS (%)	72 (20 %)	58 (29 %)*	14 (9 %)	<0.05
Pre-DBT/Type-II DBT (%)	85 (23 %)	45 (22 %)	40 (25 %)	ns
Hypertension	111 (31 %)	64 (32 %)	47 (29 %)	ns
Smoking	86 (24 %)	44 (22 %)	42 (26 %)	ns
Moderate Alcohol consumption	193 (53 %)	101 (50 %)	92 (57 %)	ns
MASLD (%)	153 (42 %)	76 (38 %)	77 (48 %)	ns

Data are expressed as median (IQR, interquartile range), or percentage (%) of the total. For continuous variables, Wilcoxon rank sum test was used. For categorical variables, Chi-square test was used.

\*  $p < 0.05$ .

(Table 3), though median values fall into de normal range. Next, to evaluate the clinical impact of AST and ALT in our cohort of MASLD, we used a cut-off of 40 IU/mL to categorize patients with upper limit of normal values (ULN). Indeed, more proportion of patients with ALT>ULN was attained in *H. pylori* positive but no difference at AST>ULN was observed (Table 3). To further evaluate liver injury, non-invasive markers of fibrosis — FIB-4 score and liver stiffness measurement by vibration control transient elastography (VCTE) — was determined. Consistent with AST and ALT levels data, FIB-4 and VCTE were significantly increased in *H. pylori* positive versus *H. pylori* negative subjects (Table 3). Furthermore, using cut-off values to rule-out of significant/advanced fibrosis (FIB-4  $\geq 1.3$ , VCTE  $\geq 5.8$  kPa and VCTE  $\geq 8$  kPa), we observed a significant trend of more proportion of *H. pylori* positive with FIB-4  $\geq 1.3$  (38/77, 49 %,  $p 0.003$ ), VCTE  $\geq 5.8$  kPa (47/77, 61 %,  $p 0.002$ ) and VCTE  $\geq 8$  kPa (25/77, 32 %,  $p$

0.016) compared to *H. pylori* negative patients (FIB-4  $\geq 1.3$  20/76, 26 %; VCTE  $\geq 5.8$  kPa 27/76, 36 %, VCTE  $\geq 8$  kPa 12/76, 16 %) (Table 3). These data may indicate that MASLD subjects with *H. pylori* active infection have elevated markers of liver injury and fibrosis.

### 3.3. *H. pylori* virulence genes in MASLD subjects

Next, we ascertained the influence of *H. pylori* virulence genes *cagA* and *vacA* from gastric biopsies on non-invasive markers of liver injury and fibrosis in our MASLD cohort. We first explored *cagA*, where 32 out of 77 (41 %) *H. pylori* positive gastric biopsies were carriers of *cagA* strain. Serum AST and ALT levels were significantly increased in *cagA* positive carriers, and no difference was observed at ALP nor GGT levels (Table 4). Likewise, using ULN cut-off analysis a significant more proportion of patients with ALT>ULN was attained

**Table 3**  
**H. pylori infection in MASLD subjects.**

Variable	H. pylori negative (n: 76)	H. pylori positive (n: 77)	p
Age (IQR)	53 (47–61)	54 (47–59)	ns
BMI (IQR)	29.9 (27.1–32.5)	29.5 (26.5–31.7)	ns
Obesity (%)	38 (50 %)	34 (43 %)	ns
Hypertension (%)	37 (49 %)	34 (44 %)	ns
Pre-DBT/Type-II DBT (%)	35 (46 %)	31 (40 %)	ns
AST, UI/L	22 (17–31)	30 (22–43)*	<0.001
AST>ULN (%)	13 (17 %)	21 (27 %)	ns
ALT, UI/L	25 (18–35)	32 (22–43)*	0.004
ALT>ULN (%)	11 (14 %)	29 (38 %)*	0.001
ALP, UI/L	90 (72–145)	103 (75–141)	ns
GGT, UI/L	32 (21–52)	36 (22–60)	ns
Platelets, 10 <sup>9</sup> /L	250 (212–287)	210 (178–250)	ns
FIB-4	0.97 (0.73–1.34)	1.29 (0.9–1.9)*	<0.001
FIB-4 ≥ 1.3 (%)	20 (26 %)	38 (49 %)*	0.003
VCTE, kPa	4.8 (4.2–7.4)	6.4 (4.8–8.1)*	0.011
VCTE ≥ 5.8 kPa (%)	27 (36 %)	47 (61 %)*	0.002
VCTE ≥ 8 kPa (%)	12 (16 %)	25 (32 %)*	0.016

Data are expressed as median (IQR, interquartile range) or percentage (%) of the total. For continuous variables, the Wilcoxon rank sum test was used. For categorical variables, the Chi-square test was used. H. pylori, Helicobacter pylori; MASLD, metabolic associated steatotic liver disease; IQR, interquartile range; %, percentage; p, probability value.

\* p < 0.05.

in *cagA* positive carriers, but no difference was observed at AST>ULN (Table 4). Non-invasive markers of liver fibrosis, FIB-4 and VCTE, were increased in *H. pylori cagA* positive patients (Table 4). Furthermore, *cagA* carriers were associated with more proportion of patients with VCTE ≥ 5.8 kPa but not at VCTE ≥ 8 kPa (Table 4). More proportion of subjects with FIB-4 ≥ 1.3 was associated in *H. pylori* positive status irrespective of *cagA* carriage (Table 4).

Then, we explored *vacA-s1/s2* and *-m1/-m2*, 47/77 (61 %) and 30/77 (39 %) of *H. pylori* positive gastric biopsies were carriers of *vacA-s1* and *-s2* strain, 34/77 (44 %) and 43/77 (56 %) were carriers of *vacA-m1* and *-m2* strain respectively. AST and ALT serum levels were significantly increased and more proportion of patients with ALT>ULN were associated with *vacA-s1* carriers, no difference was observed at ALP, GGT levels and AST>ULN between groups (Table 4). In addition, *vacA-m1* carriers showed a strong association with increased AST and ALT with more proportion of patients with ALT>ULN (62 %) and AST>ULN (38 %) (Table 4). Also, FIB-4 was increased in *vacA-m1* carriers, but was similarly increased in *vacA-s1* and *-s2* groups. Higher liver stiffness by VCTE was associated with *H. pylori* infection regardless *vacA-s1/s2* and *-m1/-m2* carrier status. Remarkably, carriers of *vacA-s1* (36 %) and *vacA-m1* (38 %) were associated with more proportion of patients with VCTE ≥ 8 kPa.

**3.4. H. pylori *cagA/vacA-s1/m1*-positive allelic carriers in MASLD subjects**

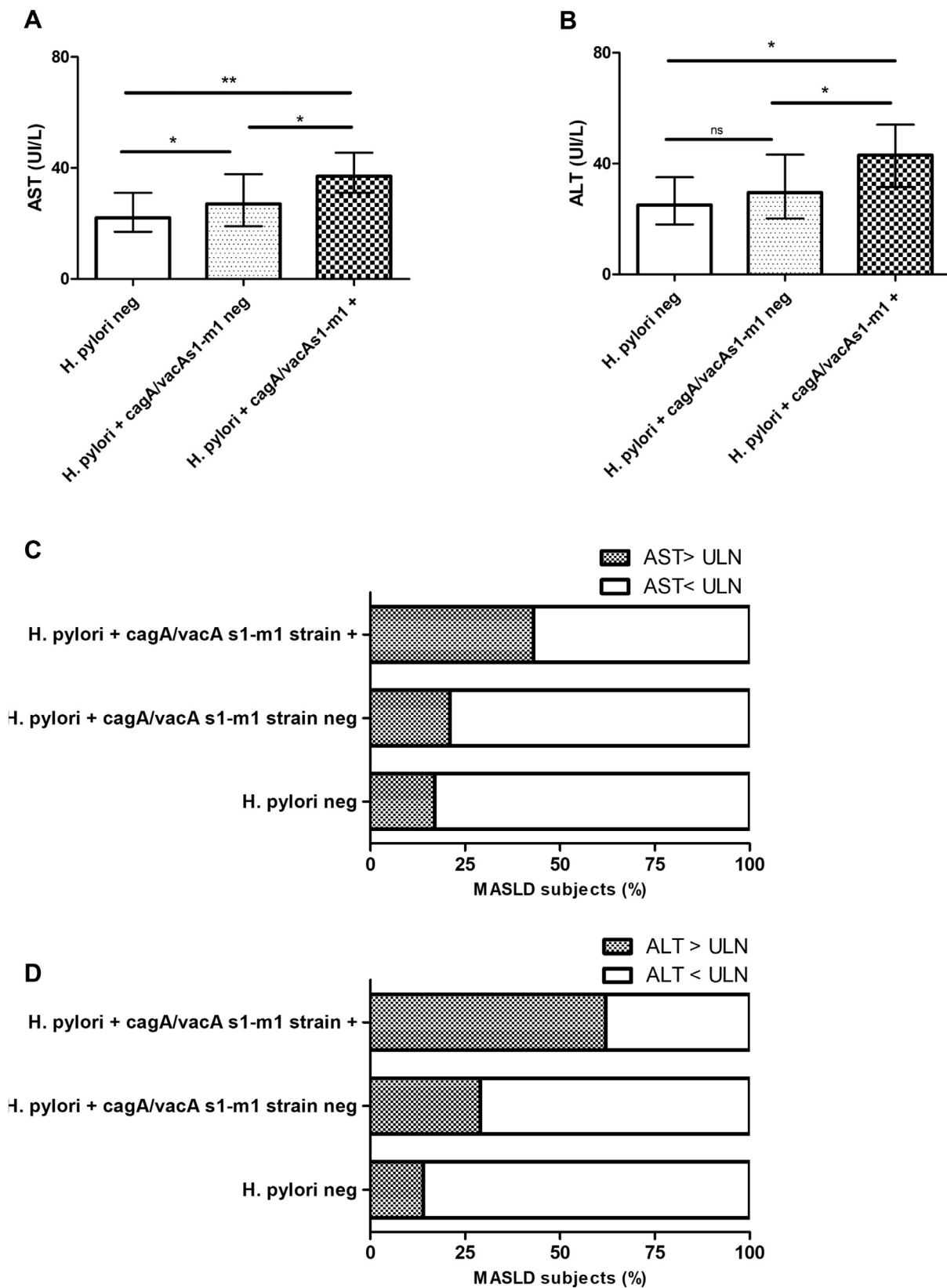
Next, we evaluated the allelic combination analysis with *cagA/vacA-s1/m1*. A significant association with higher AST, ALT, AST>ULN (43 %, p 0.042) and ALT>ULN (62 %, p < 0.001) was attained in combined *cagA/vacA-s1/m1*-positive allelic carriers (Fig. 2A, B, C and D). Likewise, FIB-4 and LSM by VCTE was increased and a clear association with more proportion of patients with FIB-4 ≥ 1.3 (57 %, p 0.009), VCTE ≥ 5.8 kPa (67 %, p 0.006) and VCTE ≥ 8 kPa (43 %, p 0.023) was observed in combined *cagA/vacA-s1/m1*-positive allelic carriers (Fig. 3A, B, C and D).

Since, fibrosis in MASLD is the most important prognostic factor and LSM by VCTE is one of the most validated non-invasive diagnostic tool for liver fibrosis evaluation [1,28]. We then, assessed by logistic regression model the impact of *H. pylori* status and combined *cagA/vacAs1/vacAm1* allelic carriers on the VCTE ≥ 8 kPa cut-off to rule-out intermediate-high risk of SF/AF (Table 5). The OR for VCTE ≥ 8 kPa

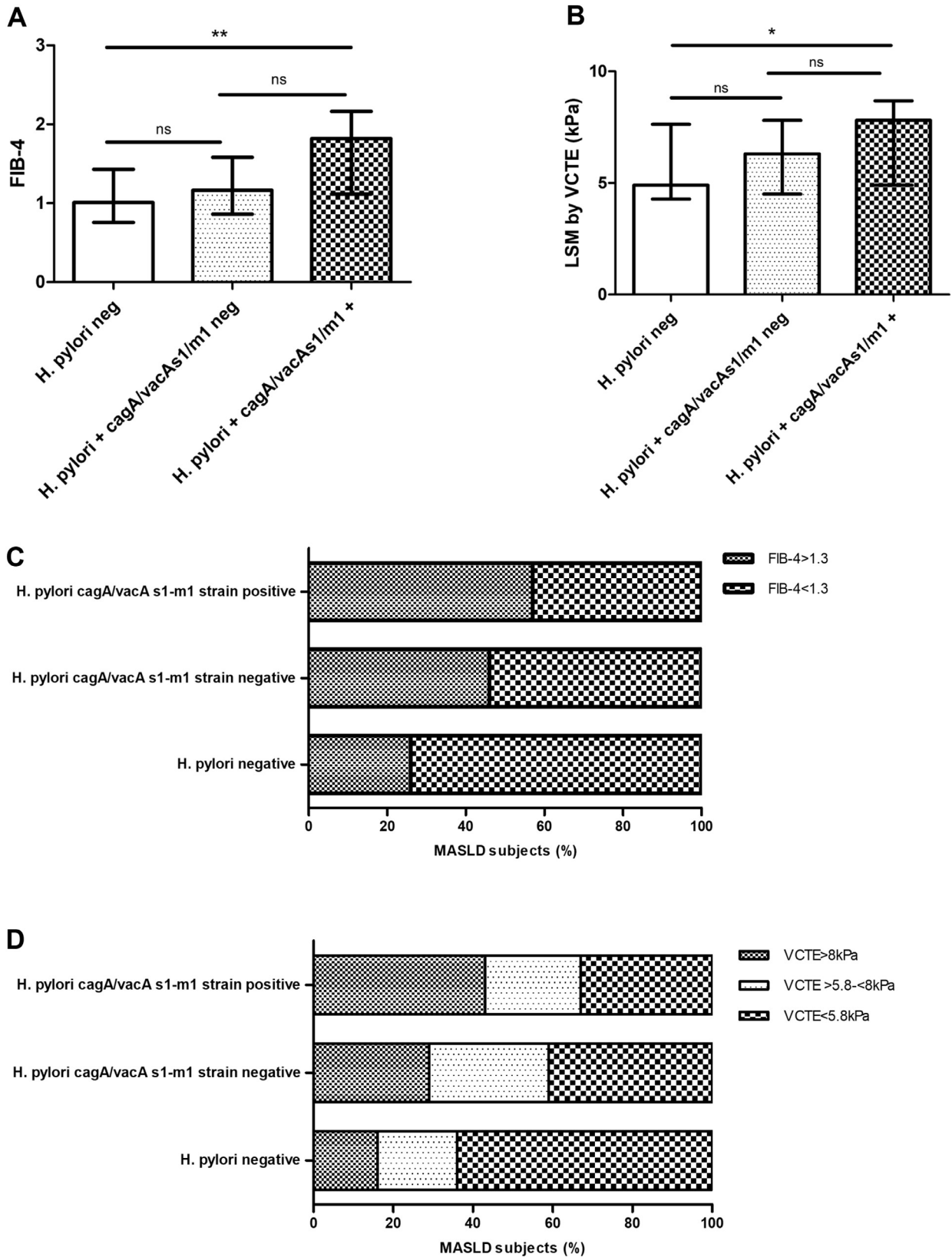
**Table 4**  
**H. pylori virulence genes *cagA*, *vacA s1/s2* and *m1/m2* in MASLD subjects.**

Variable	H. pylori positive			H. pylori positive			H. pylori positive			H. pylori positive		
	H. pylori negative (n: 76)	<i>cagA</i> neg (n: 45)	<i>cagA</i> pos (n: 32)	H. pylori negative (n: 76)	<i>vacA s2</i> (n: 30)	<i>vacA s1</i> (n: 47)	H. pylori negative (n: 76)	<i>vacA m2</i> (n: 43)	<i>vacA m1</i> (n: 34)	H. pylori negative (n: 76)	<i>vacA m2</i> (n: 43)	<i>vacA m1</i> (n: 34)
AST, UI/L (IQR)	22 (17–31)	27 (19–38)*a	34 (28–44)*a	22 (17–31)	28 (23–38)	34 (22–44)*b	22 (17–31)	27 (18–36)	34 (23–46)*c	22 (17–31)	27 (18–36)	34 (23–46)*c
AST>ULN (%)	13 (17 %)	11 (24 %)	10 (31 %)	13 (17 %)	7 (23 %)	14 (30 %)	13 (17 %)	8 (19 %)	13 (38 %)*c	13 (17 %)	8 (19 %)	13 (38 %)*c
ALT, UI/L (IQR)	25 (18–35)	30 (21–45)	40 (26–49)*a	25 (18–35)	28 (20–38)	34 (24–48)*b	25 (18–35)	28 (20–34)	44 (27–52)*c	25 (18–35)	28 (20–34)	44 (27–52)*c
ALT>ULN (%)	11 (14 %)	13 (29 %)	16 (50 %)	11 (14 %)	7 (23 %)	22 (47 %)*b	11 (14 %)	8 (19 %)	21 (62 %)*c	11 (14 %)	8 (19 %)	21 (62 %)*c
ALP, UI/L (IQR)	90 (72–145)	98 (75–119)	111 (79–144)	90 (72–145)	102 (78–143)	103 (74–138)	90 (72–145)	98 (74–118)	110 (77–152)	90 (72–145)	103 (75–141)	110 (77–152)
GGT, UI/L (IQR)	32 (21–52)	34 (22–53)	40 (24–64)	32 (21–52)	32 (22–70)	40 (22–56)	32 (21–52)	34 (22–52)	40 (22–65)	32 (21–52)	34 (22–52)	40 (22–65)
FIB-4 (IQR)	0.97 (0.73–1.34)	1.25 (0.84–1.65)	1.30 (1–1.92)*a	0.97 (0.73–1.34)	1.29 (0.85–1.61)	1.29 (0.92–1.92)*b	0.97 (0.73–1.34)	1.18 (0.86–1.64)	1.34 (1.11–1.92)*c	0.97 (0.73–1.34)	1.18 (0.86–1.64)	1.34 (1.11–1.92)*c
FIB-4 ≥ 1.3 (%)	20 (26 %)	22 (49 %)	16 (50 %)	20 (26 %)	15 (50 %)	23 (49 %)	20 (26 %)	20 (47 %)	18 (53 %)*c	20 (26 %)	20 (47 %)	18 (53 %)*c
FIB-4 > 2.67 (%)	5 (6.6 %)	4 (8.9 %)	0 (0 %)	5 (6.6 %)	2 (6.7 %)	2 (4.3 %)	5 (6.6 %)	4 (9.3 %)	0 (0 %)	5 (6.6 %)	4 (9.3 %)	0 (0 %)
VCTE, kPa (IQR)	4.8 (4.2–7.4)	6.3 (4.4–8.1)	6.5 (4.9–8.0)*a	4.8 (4.2–7.4)	6.2 (4.8–8)	6.6 (4.6–8.3)	4.8 (4.2–7.4)	6.4 (4.6–8.1)	6.7 (5.0–8.3)	4.8 (4.2–7.4)	6.4 (4.6–8.1)	6.7 (5.0–8.3)
VCTE > 5.8 kPa (%)	27 (36 %)	26 (58 %)	21 (66 %)*a	27 (36 %)	19 (63 %)*b	28 (60 %)*b	27 (36 %)	26 (60 %)*c	21 (62 %)*c	27 (36 %)	26 (60 %)*c	21 (62 %)*c
VCTE > 8 kPa (%)	12 (16 %)	15 (33 %)	10 (31 %)	12 (16 %)	8 (27 %)	17 (36 %)*b	12 (16 %)	12 (28 %)	13 (38 %)*c	12 (16 %)	12 (28 %)	13 (38 %)*c

Data are expressed as median (IQR, interquartile range), or percentage (%) of total. For continuous variables, Kruskal-Wallis test was used. For categorical variables, Chi-square test was used. For pairwise comparisons between groups Dwass-Steel-Critchlow-Fligner non-parametric test was used. (a), *cagA* positive or negative carriers versus *H. pylori* negative. (b), *vacA-s1* or *-s2* carriers versus *H. pylori* negative. (c), *vacA-m1* or *-m2* carriers versus *H. pylori* negative. \* p < 0.05.



**Fig. 2.** AST and ALT in patients with MASLD stratified according to *H. pylori* negative status, *H. pylori* positive *cagA/vacA s1/m1*-negative carrier and *H. pylori cagA/vacA s1/m1*-positive carrier. A) AST serum levels. B) ALT serum levels. C) AST > or < ULN. D) ALT > or < ULN. Data are expressed as median and interquartile range (IQR), and (%) percentage of MASLD subjects. For continuous variables pairwise comparisons between groups Dwass-Steel-Critchlow-Fligner pairwise non-parametric test was used. For categorical variables, Chi-square test was used. \*  $p < 0.05$ , \*\*  $p < 0.001$ .



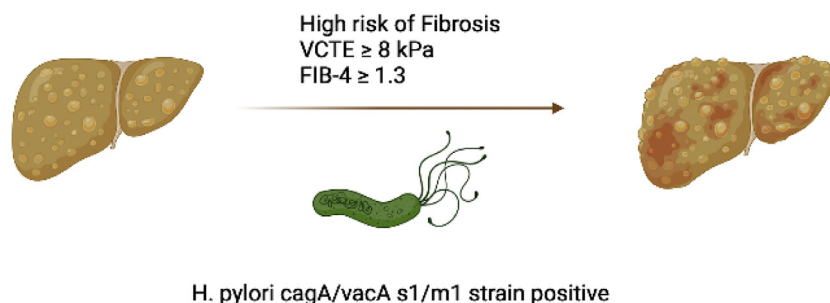
**Fig. 3. Non-invasive markers of high risk of significant/advanced fibrosis in patients with MASLD stratified according to *H. pylori* negative status, *H. pylori* positive *cagA/vacA s1/m1*-negative carrier and *H. pylori cagA/vacA s1/m1*-positive carrier. A) FIB-4 values. B) Liver stiffness measurement by vibration controlled transient elastography (LSM by VCTE). C) FIB-4 score  $\geq 1.3$  or  $< 1.3$ . D) LSM by VCTE distribution by LSM cut-offs to rule-out significant fibrosis  $< 5.8$  kPa, intermediate risk  $\geq 5.8$  kPa- $< 8$  kPa, and to rule-out advanced fibrosis  $\geq 8$  kPa [31,32,33]. Data are expressed as median and interquartile range (IQR), and (%) percentage of MASLD subjects. For continuous variables pairwise comparisons between groups Dwass-Steel-Critchlow-Fligner pairwise non-parametric test was used. For categorical variables, Chi-square test was used. \*  $p < 0.05$ , \*\*  $p < 0.001$ .**

**Table 5**  
***H. pylori*, combined *cagA/vacA s1/m1* carrier and VCTE  $\geq$  8 kPa.**

		Univariate Results		Adjusting for Age, Gender, Pre-DBT/Type-II DBT, BMI and Hypertension	
		OR (95 % CI)	<i>p</i>	OR (95 % CI)	<i>p</i>
VCTE $\geq$ 8 KPa	<i>H. pylori</i> negative				
	<i>H. pylori</i> positive	2.56 (1.2–5.75)	0.018	2.43 (1.88–12.44)	0.001
VCTE $\geq$ 8 KPa	<i>H. pylori</i> negative				
	<i>H. pylori</i> positive <i>cagA/vacA s1/m1</i> negative	1.87 (0.66–5.31)	ns		
	<i>H. pylori cagA/vacA s1/m1</i> positive	4.01 (1.38–11.56)	0.01	4.06 (1.22–14.49)	0.002

OR: odds ratio, CI: confidence interval, ns: non-significant.

\* *p* < 0.05.



**Fig. 4.** *H. pylori cagA/vacA s1/m1*-positive carrier association with non-invasive markers of high-risk of fibrosis in non-alcoholic fatty liver disease. Figure created with Bio-Render.com.

with *H. pylori* positive gastric biopsies was 2.56 (95 % CI, 1.2–5.75; *p* = 0.018) and for *cagA/vacA-s1/m1*-positive allelic carriers was 4.01 (95 % CI, 1.38–11.56, *p* = 0.01), but non-significant association was observed by *cagA/vacA-s1/m1*-negative allelic carriers. After adjusting for age, gender, pre-DBT/type-II DBT, BMI and Hypertension the OR for VCTE  $\geq$  8 kPa with *H. pylori* positive gastric biopsies was 2.43 (95 % CI, 1.88–12.444; *p* = 0.009) and for *cagA/vacA-s1/m1*-positive allelic carriers was 4.06 (95 % CI, 1.22–14.49, *p* = 0.004). Collectively, these data suggest that, carriers of allelic combination *cagA/vacA-s1/m1* are associated with markers of liver injury and may increase the risk of significant/advanced fibrosis in MASLD subjects (Fig. 4).

#### 4. Discussion

The main findings of this observational cross-sectional study, pertain to the potential association of *H. pylori* virulence genes and non-invasive markers of severity in MASLD. Our results suggest that *H. pylori* infection in MASLD subjects is significantly associated with, (i) higher values of non-invasive markers of liver injury and fibrosis, (ii) carriers of *cagA*, *vacA-s1* and *vacA-m1* showed increased values of AST, ALT, FIB-4 and LSM by VCTE, and (iii) carriers of *H. pylori* with *cagA/vacA-s1/m1*-positive allelic combination showed a strong association with elevated AST, ALT and independent risk of significant/advanced fibrosis by VCTE.

Etiopathogenic association between *H. pylori* infection and MASLD is a matter of debate that was intended to be answered by various researcher worldwide from the bench, and clinical stand point [39,40]. First, is important to note that murine models of *H. pylori* infection do not have a fatty liver phenotype [41], but under a high fat diet *H. pylori*-positive mice had more hepatic steatosis and release of inflammatory mediators [41]. Noteworthy, in a cell cultures approach, *H. pylori* mediated hepatocyte injury is dependent upon *cagA* and *vacA* internalization [21,22]. Furthermore, in a liver fibrosis mice model using carbon tetrachloride, *H. pylori*-infected mice had increased fibrosis scores, and ALT and AST levels compared to uninfected mice [42]. For instance, local inflammation in the gastric and duodenal mucosa may lead to release of proinflammatory cytokines that increase gut permeability and access to portal circulation [17]. In

this regard, *H. pylori* and other bacteria and toxins as well as cytokines may directly affect the hepatic parenchyma leading to and inflammatory milieu that may activate stellate cells to generate fibrosis [17]. A recent study, pointed toward an association between active *H. pylori* gastric infection and MASLD severity in morbidly obese patients subjected to bariatric surgery, in this study histological diagnostic “gold standard” for both main variables of interest (*H. pylori* gastric infection and MASLD) was used. Particularly, the rates of MASH by NAS score, as well as hepatic steatosis, inflammation, and fibrosis, were higher in *H. pylori*-positive than *H. pylori*-negative patients, however virulence genes status was not evaluated. Also, in study from China observed that *H. pylori* infection was not associated with steatosis but may be a risk factor for increased liver stiffness [38]. Our study showed a correlation with *H. pylori*-infection and elevated non-invasive markers of liver injury, and fibrosis by FIB-4 score and VCTE, with a strong association in carriers of *cagA/vacA-s1/m1*-positive allelic combination. Fibrosis is the most relevant prognostic factor in patients with MASLD [43]. We found a prevalence of high liver stiffness suggestive of significant/advanced fibrosis in 43 % of MASLD patients with *H. pylori cagA/vacA-s1/m1*-positive carriers. Our observation was further independent of indicators of disease severity or progression such as age, pre-DBT/type-II DBT, BMI and Hypertension [44]. Perhaps, the interaction between *H. pylori* and host genetic polymorphism delineates low-grade gastric and duodenal inflammation with eventually leading to hepatic pro-inflammatory milieu, and our results revealed that *cagA/vacA-s1/m1*-positive allelic combination strain significantly impact this observation. Since, the pathophysiology of MASLD is only partially revealed, it is considered as a multifactorial disorder, attributed to multiple or parallel “hits”, both genetic and environmental [6]. The precise mechanisms underlying the connection between *H. pylori* gastric infection and MASLD remain unclear. While the correlation between increased non-invasive markers of liver injury and fibrosis, *H. pylori* gastric infection and *cagA/vacA-s1/m1*-positive allelic combination strain does not prove causality. Confirmation from mechanistic studies is needed to clarify the clinical role of *H. pylori* virulence genes and fibrogenesis in MASLD. In this regard, the interaction between host genetic polymorphism background, *H. pylori* virulence genes and low-grade duodenal



inflammation could explain high-risk of fibrosis in MASLD, and will be evaluated in our future research.

Second, multiple observational, prospective and meta-analysis studies led to opposed conclusion on whether *H. pylori* infection contributes to MASLD [12,13] to no association between *H. pylori* infection and diagnosis of MASLD [14,15]. Our study failed to show an association between *H. pylori* infection and MASLD diagnosis by ultrasound. Perhaps, the prevalence of MASLD and *H. pylori* infection may not be the same in different regions, races, social customs, genetical background and eating habits, which makes the results of their correlation inconsistent. Therefore, the comprehensive analysis of data from multiple regions is needed. It is beyond the scope of this study to evaluate the impact of *H. pylori* eradication on MASLD non-invasive markers of liver injury and fibrosis. However, information is available from four clinical controlled studies [45,46,47,48]. While this four studies had disparate design, follow-up and end points making it difficult to compare, it seems that eradication therapy as adjunct to lifestyle intervention may improve non-invasive markers of steatosis, fibrosis and inflammation. Therefore, whether *H. pylori* eradication influences the occurrence or progression of MASLD is still controversial and needs to be confirmed by multicentric studies in the future.

The strengths of this study are that it is a prospective evaluation from a well-defined MASLD cohort. Endoscopists and Pathologists were blinded to medical history before evaluation. Non-invasive marker of liver fibrosis, LSM by VCTE was performed in all subjects. *H. pylori* virulence genes were genotyped in all *H. pylori* positive gastric biopsies. We choose FD patients for the current study because gastric biopsy sample is mostly available, which is the gold standard for *H. pylori* infection and virulence genes evaluation [49]. Also, FD etiopathogenesis is not related to MASLD and may not increase selection bias of the cohort [7,10].

The limitations of the study include: (1) population studied: Our cohort comprised of South-American population of functional dyspepsia, though we tried to investigate a cohort whose characteristics could resembled those of the general population, the modality of cohort recruitment did not allow us to affirm that our cohort was fully representative of the general population. However, our cohort was recruited independently from the hypothesis concerning high risk of MASLD severity. (2) Based on the non-invasive nature of the study design in FD-cohort, hepatic steatosis was detected by ultrasonography and fibrosis by VCTE but not liver histology, so the absence of histologic data prevents us from reporting the exact prevalence of steatohepatitis and advanced fibrosis according to the gold standard technique. Noteworthy, Noteworthy, VCTE is the most validated non-invasive method to accurately screen high risk of advanced fibrosis in MASLD [28,50]. Furthermore, LSM by VCTE can predict the occurrence of liver-related events [50].

## 5. Conclusions

In conclusion, the results of the current study suggest that in MASLD subjects with active *H. Pylori* infection is associated with elevated markers of liver injury and fibrosis, and carriers of *cagA/vacA-s1/m1*-positive allelic combination showed a higher risk of significant/advanced fibrosis by VCTE.

## Author contributions

FJB: study concept and design, statistical analysis and drafting of the manuscript. Guarantor of the article

FJB and AS: obtained funding.

FM, MN, AS and GV: acquisition of data, analysis and interpretation of data.

MVC, KE: acquisition of histological data, analysis and interpretation of data.

PDZ: analysis and interpretation of data, critical revision of the manuscript.

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## Conflicts of interest

The authors declare that they have no conflict of interest. All authors have approved the final version of the manuscript.

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