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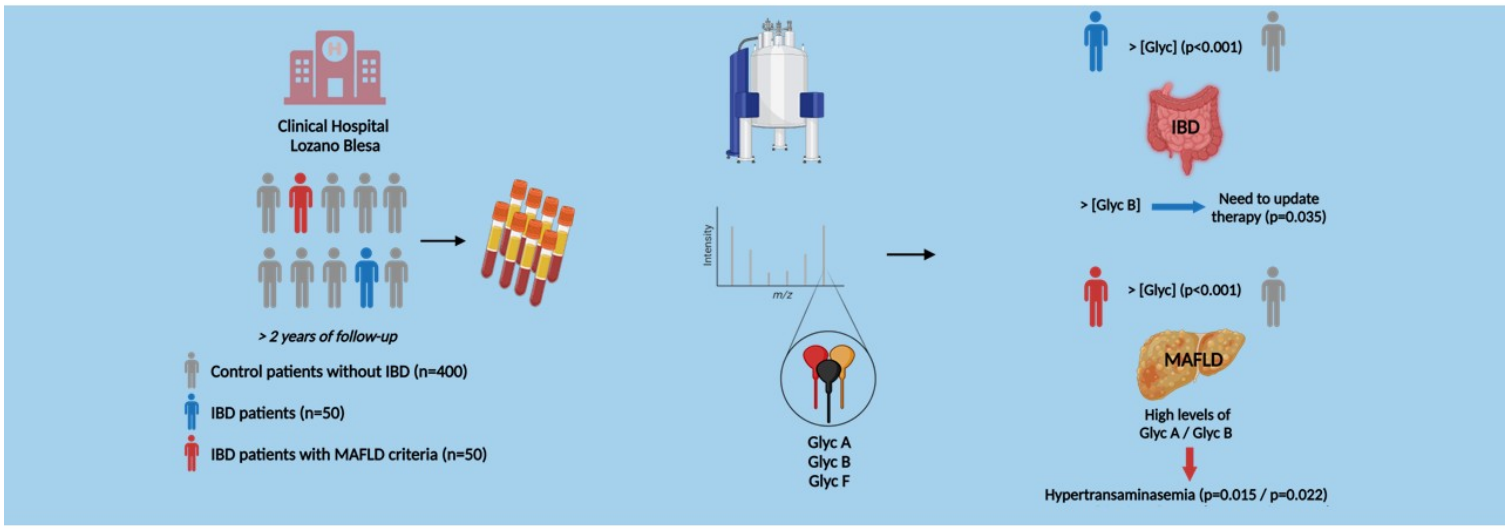
Unlocking the potential of plasma protein glycosylation: a promising biomarker for diagnosing and predicting Metabolic Dysfunction-Associated Steatotic Liver Disease in Inflammatory Bowel Disease.



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Plasma protein glycosylation: a biomarker for predicting metabolic dysfunction-associated steatotic liver disease in inflammatory bowel disease

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JM and PB: Help with the design of the protocol and corrections of the first draft of the paper. NA and NMM: Coordination, assess the transport and analysis of the samples and give important and intellectual content to the protocol. FG: Design the protocol, assess the data collection, correcting the last draft of the paper giving the final approval of the version to be submitted.

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Abbreviation list: Aspartate aminotransferase: AST**,** alanine aminotransferase: ALT**,** Body mass index: BMI**,** Controlled attenuation parameter (CAP ™)**,** Crohn's disease: CD**,** Cardiovascular: CV**,** C-reactive protein: CRP**,** DM: Diabetes Mellitus**,** Fatty liver

index: FLI, Gastrointestinal: GI, Inflammatory Bowel Disease: IBD, Metabolic Dysfunction-Associated Steatotic Liver Disease: MASLD, Metabolic dysfunction-associated steatohepatitis: MASH, Nuclear Magnetic Resonance: NMR, Steatotic Liver Disease: SLD, Ulcerative Colitis: UC.

ABSTRACT

Background: Plasma glycoproteins, influenced by systemic inflammatory conditions, hold promise as biomarkers for Metabolic Dysfunction-Associated Steatotic Liver Disease (MASLD) in patients with inflammatory bowel disease (IBD).

Purpose: To explore the potential of these glycoproteins in identifying and predicting the course of MASLD in IBD patients.

Methods: An ambispective study was conducted involving patients with (cases) and without (controls) IBD. Of 947 IBD patients screened for inclusion in the study, 100 were selected (cases): 50 with IBD and MASLD and 50 without MASLD. Each IBD patient was matched with 4 controls without IBD. Additionally, each IBD patient was followed by a median of 25 (interquartile range IQR:22-28) months. Plasma samples were analyzed using Nuclear Magnetic resonance spectroscopy, examining the glycoproteins (Glyc-B, Glyc-F, and Glyc-A).

Main results: Glycoprotein concentrations were significantly higher in the IBD group ($p<0.001$), with IBD patients exhibiting MASLD showing the highest levels of glycoproteins compared to those without MASLD and the control group ($p<0.001$). Concerning MASLD outcomes, all Glyc-B ($p=0.032$), Glyc-F ($p=0.007$) and Glyc-A ($p=0.006$) showed significant associations with the persistence of hypertransaminasemia at the end of the monitoring, Glyc B ($p=0.033$) with the persistence of lower high density lipoprotein cholesterol, all glycoprotein groups ($p<0.001$) with hypertriglyceridemia and Glyc A ($p=0.015$) and Glyc B ($p=0.009$) with the development of impair fasting glucose during the follow-up.

Conclusions: Plasma glycoproteins emerge as a promising biomarker for the presence of MASLD in IBD patients.

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Keywords: Inflammatory bowel disease. Metabolic dysfunction-associated steatotic liver disease. Glycoproteins. Biomarkers. Diagnosis.

INTRODUCTION

Inflammatory bowel disease (IBD), which includes Ulcerative Colitis (UC) and Crohn's disease (CD), can be defined as chronic, systemic, and inflammatory illnesses that predominantly affect the gastrointestinal (GI) tract. However, it is known that they can present a wide range of comorbidities such as Metabolic Dysfunction-Associated Steatotic Liver Disease (MASLD) (1,2). The incidence of both IBD and MASLD has been growing alarmingly in recent years (3). Therefore, new non-invasive strategies are necessary to aid in diagnosing and monitoring patients with IBD and MASLD.

As is widely known, chronic inflammation is the main driver in the presence and disease progression of MASLD to Metabolic dysfunction-associated steatohepatitis (MASH) and liver fibrosis (2). Glycoproteins, could play an essential role as non-invasive biomarker for MASLD in IBD patients due to their potential advantages in clinical practice: less tendency to daily fluctuations and low intra-individual coefficient of variation when compared with inflammatory biomarkers such as C-reactive protein (CRP) (4), or proinflammatory cytokines which are involved on its pathogenesis (5) Moreover, although CPR has been shown a great correlation with biopsy proven fibrosis in those patients with obesity, but not with the disease progression (6).

Modifications in glycosylation have been found in autoimmune diseases (7,8) or type 2 diabetes (DM) (9). Although plasma glycoproteins were associated with the new development of MASLD in type 2 DM patients (10) their role as a potential biomarker for MASLD in IBD patients is unknown.

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Hence, our study endeavors to quantify protein glycosylation utilizing NMR spectroscopy in both, a cohort of IBD patients with and without MASLD and matched healthy controls with the aim of determining its association with the presence and prognosis of MASLD.

METHODS

Study design and study population

This observational and ambispective case-control study was performed from October 2020 to April 2021 in an IBD referral center in Zaragoza, Spain (Clinical Hospital Lozano Blesa).

The study population comprised all IBD outpatients except for those meeting the exclusion criteria: patients were under 18 years, a non-established diagnosis of UC or CD (11,12), time since IBD diagnosis < 1 year, patients with other common causes of chronic liver disease apart from MASLD (2), history of CV disease, fasting serum glucose ≥ 100 mg/dL or HbA1c ≥ 5.7% , DM or specific treatment, and patients being under treatment for dyslipidemia.

Among the 410 patients with IBD who did not meet any exclusion criteria, those who met diagnostic criteria for MASLD were matched by sex and age in a 1:1 ratio with patients who did not meet diagnostic criteria for MASLD, and in a 1:4 ratio with non-IBD controls. This resulted in 50 patients with IBD and MASLD, as well as 50 patients with IBD and without MASLD and 400 non-IBD controls. Figure 1.

The diagnosis of IBD was based on clinical, endoscopic, and histological parameters according to ECCO guidelines following Lennard-Jones criteria (13).

MASLD was diagnosed in IBD patients according to the new Delphi consensus statement with ultrasound and controlled attenuation parameter (CAP ™) > 248 dB/min (2) fatty liver index (FLI) > 60 (14) was performed as a primary screening and non-invasive test in both patients with and without IBD.

Patients' demographic information, including age, sex, presence of comorbidities, smoking status, was collected when patients were included in the study.

Plasma samples were collected at the same time as clinical evaluations and were centrifuged at 1800 g during 15 minutes at room temperature and stored aliquoted at -80 ºC before their analysis through NMR spectroscopy (7). The samples must endure several phases before the glycoprotein quantification. First, it is necessary to apply pulsed sequences to suppress the water peak and acquire low molecular weight metabolites. The next step quantifies the region in which glycoprotein resonates. This signal is produced by the -COCH3 acetyl groups of N-acetylglucosamine and N-acetylgalactosamine.

Glycoprotein analysis was performed by Biosfer Teslab. The commercial provider was solely responsible for conducting the glycoprotein assays on patient samples, and had no access to patient clinical data or involvement in the statistical analysis of the results.

To evaluate the relationship of glycoproteins on MASLD course, a clinical follow-up period of a median of 25 (interquartile range IQR:22-28) months was conducted.

Taking into account the same exclusion criteria as for the IBD population, for each case, four non-IBD controls were included and matched by sex, age, body mass index (BMI), and FLI with IBD cases. The non-IBD patients were obtained from 4668 Spanish individuals participating in the Dia@bet.es trial (15).

Statistical analysis

All calculations were made by R bioconductor (version 4.1.1). Normality was assessed using Kolmogorov-Smirnov test, where $p \geq 0.05$ means normality. Initially, a descriptive

analysis of the sample was performed. Quantitative variables were expressed by median and range, whereas qualitative ones were shown in frequencies and percentages.

In order to compare different groups, Student's t test or Wilcoxon-U-Mann-Whitney was conducted.

The sample size was estimated considering the primary endpoint (diagnosis of MASLD in patients with IBD), and the expected number of outpatients with IBD seen in our unit during a year and based on the previous data published (16,17) assuming a desired power of 85% ($\beta = 0.020$) and a significance level of 5% ($\alpha = 0.005$). These calculations were performed using Epidat 4.2.

To assess the improvement in diagnostic performance by including glycoproteins, the area under the ROC curve (AUC) of a logistic regression model including only the FLI was compared to a model combining the FLI with glycoprotein levels.

To account for the issue of multiple comparisons, a Bonferroni correction was applied.

Associations between two quantitative and continuous variables were made using Pearson correlation tests. For all tests, a $p < 0.05$ was considered statistically significant.

AUC for every glycoprotein was calculated with SPSS (version 26), and differences between each AUC were studied by the DeLong method calculated by medCalc (18).

Ethical considerations

This study was carried out in agreement with the Helsinki Declaration and approved by the Ethics and Clinical Research Committee of Aragon (Spain) (CEICA) on February 24th, 2020 (CP - CI PI20/548). All participants signed an informed written consent before enrollment.

RESULTS

Characteristics of the study population

The median age of the sample is 51 years, with slightly over half of the patients being female. **Table 1**. When conducting a stratified analysis based on the presence or absence of MASLD, there were with no significant differences between groups. IBD characteristics were reflected in **table 2**.

Glycoprotein profile in IBD patients and non-IBD patients. Influence of MASLD on glycoprotein concentration

Glycoprotein levels exhibited a notable increase in IBD patients (n = 50), regardless of MASLD presence when compared to the non-IBD control group (n = 400) with $p < 0.001$ for the three groups. Remarkably, this rise encompassed all glycoprotein values, with the most elevated levels seen in patients with IBD and MASLD (n = 50), with significance after Bonferroni correction ($p = 0.005$) **Figure 2**.

To study their potential role in assessing the development of MASLD in IBD patients sensitivity and specificity for each one based on the fixed cutoff were assessed recorded in **table 3.** Notably, in multivariate analysis, even after adjustment for BMI, FLI, sex, and age, both Glyc-B (OR per SD: 3.463, 95% CI (1.050-7.460), $p = 0.014$) and Glyc-F (OR per SD: 3.343, 95% CI (1.060-6.010), $p = 0.023$) sustained significant elevations.

If we study the AUC of the different glycoproteins with the aim of predicting the presence of MASLD in IBD patients, we obtain significant results for all of them with an AUC for Glyc-A, -B and -F of 0.711 (95% CI 0.607-0.815), 0.734 (95% CI 0.632-0.836) and 0.686 (95% CI 0.582-0.789) respectively. **Figure 3**.

To further investigate the diagnostic potential of glycoproteins, we constricted two logistic regression models. The FLI- based model alone showed an AUC of 0.800 (95% CI 0.709-0.891), while the model combining FLI with glycoprotein levels showed an AUC of 0.860 (95% CI 0.781-0.939). The difference in AUC ($\Delta AUC=0.006$) was not statistically significant ($p = 0.324$).

The role of glycoproteins in predicting the course of MASLD and its metabolic comorbidities

Of the 50 IBD patients with MASLD, 11 (22%) had hypertransaminasemia at the time of study enrollment. Of those patients, 9/50 (18%) continued to have hypertransaminasemia at the end of the follow-up period.

A statistically significant relationship was observed between higher initial values of glycoproteins and the persistence of hypertransaminasemia throughout the follow-up period. Related to that, Glyc-A showed median values of 868 (786-977) for MASLD patients without hypertransaminasemia and 1139 (876-1241) for patients with hypertransaminasemia (p=0.006) as the same way as Glyc-F [(310 (280-341) and 359 (320-466), p=0.007] and Glyc-B [(444 (407-468) and 485 (432-522), p = 0.032].

Focusing on the onset of metabolic complications among the patients during the follow-up, a significant relationship with high baseline values of glycoproteins was also detected **figure 4**. These results remained significant after applying a Bonferroni correction for multiple comparisons (p = 0.004).

In multivariate logistic regression analyses adjusted for sex, age, type of IBD and history of corticosteroid use in the previous 5 years, a significant association was found between Glyc B (OR per SD= 4.285, 95% CI 1.440-4.870) and Glyc A levels (OR per SD 6.172, 95% CI 1.199- 38.460) and the development of hypertriglyceridemia. However, no significant associations were found between glycoprotein levels and the development of impaired fasting glucose (Glyc B OR 1.005 (0.989-1.023), Glyc F OR 0.997 (0.982-1.013), Glyc A OR 1.003 (0.995-1.011)), the decrease of HDL (Glyc B OR 1.001 (0.986-1.017), Glyc F OR 0.997 (0.983-1.011), Glyc A OR 1.001 (0.994-1.008)) nor the increase in transaminases (Glyc B OR 0.997 (0.977-1.018), Glyc F OR 1.009 (0.991-1.028), Glyc A OR 1.001 (0.992-1.010)).

DISCUSSION

This pioneering ambispective study, which carefully examining glycoprotein profiles, presents a compelling potential as a non-invasive biomarker for detecting metabolic

complications such as MASLD among patients with IBD. Furthermore, it emerges as a valuable prognosis indicator. Importantly, our findings corroborate the discoveries underscored by Moreno-Vedia J et al. (10) about the association of glycoprotein profiles with the new onset of MASLD in patients with metabolic comorbidities.

Notably, glycoproteins have not only exhibited a significant correlation with MASLD when occurring as a comorbidity in IBD patients, as evidenced by the considerably elevated glycoprotein levels in those patients with both IBD and MASLD compared to IBD patients and controls as depicted in our study, but they also may be linked to the progression of metabolic comorbidities which compounds MASLD.

Although the inclusion of glycoproteins in the model appeared to offer a trend towards improved diagnostic performance for MASLD in IBD patients, the difference compared to the FLI-based model alone did not reach statistical significance in our cohort. This suggests that while glycoproteins may have the potential to enhance MASLD detection, further studies with larger sample sizes and diverse populations are needed to confirm these findings and to fully elucidate the clinical utility of incorporating glycoprotein measurements into diagnostic algorithms.

Protein glycosylation emerges as a candidate for a readily accessible screening tool of disease progression. The linkage between elevated protein glycosylation and an increased probability of new development of impaired fasting glucose, or persistence or development of hypertriglyceridemia or low HDL cholesterol, is intrinsically tied to its strong association with systemic inflammation in the context of metabolic disorders, which may contribute to developing other metabolic comorbidities (19). The relationship between glycoproteins with inflammatory status in IBD (16) and the components of metabolic syndrome such as DM, impaired fasting glucose has been extensively studied in cross-sectional analyses (20–22). Our work adds to this knowledge by highlighting the prognostic value that these glycoproteins may offer in the follow-up of these conditions.

The detection of the onset or maintenance of these metabolic conditions (2) may play an essential role to anticipate and alerting clinicians about whose patients who are potentially at risk of developing early states of liver fibrosis or CV disease.

Although no standardized non-invasive diagnostic method has been approved for MASH apart from the biopsy (23), it is essential to highlight that elevated transaminase levels, in the absence of other causes, are generally associated with MASH in patients with SLD(24). Our study found a strong correlation between higher glycoprotein levels in the initial assessment and the persistence of hypertransaminasemia at the end of the follow-up period. This association suggests a close link between glycoprotein variations and the probable susceptibility to developing medium term MASH, and indirectly liver fibrosis or hepatocellular carcinoma. Our results match with previous published data about non-invasive biomarkers which was associated with the development of hypertransaminasemia in patients with metabolic comorbidities (25,26). Our findings suggest a potential link between Glyc-B and Glyc-A levels and the development of hypertriglyceridemia in IBD patients, even after adjusting for potential confounders. However, the lack of significant associations between glycoproteins and other metabolic outcomes, such as impaired fasting glucose, decreased HDL, and elevated transaminases, highlights the complexity of the relationship between glycosylation, inflammation, and metabolic dysfunction in this population. Further research and validation in more extensive cohorts are needed to elucidate the specific mechanism by which Glyc-B and Glyc-A may influence triglyceride metabolism in IBD, and to determine whether these associations are consistent across different IBD subtypes and patient populations. A key limitation of our study stems from the sample size, although withing the expected size, curtailed due to rigorous exclusion criteria thereby preventing the enlargement of the case group. Moreover, all the patients enrolled were assessed by FLI with no differences between groups, while CAP measurements were obtained in the IBD group, it was not performed in the control group. The control group was selected to have a similar distribution of FLI to avoid differences in metabolic comorbidity at baseline(27,28). Related to the outcomes during the follow-up, the small number of patients with metabolic outcomes as well as the potential for residual confounding despite multivariate adjustment, limits the statistical power of our analyses. Increase in glycoprotein levels could not be explicitly linked to any of the aforementioned comorbidities. Instead, its clinical relevance appears to primarily involve non-active IBD patients who are susceptible to CV and

metabolic complications. Moreover, the differences found between glycoprotein profiles in the control group displayed limited strength, thus limiting its use as a non-invasive diagnostic test in these patients. While our findings suggest that glycoproteins have potential as biomarkers for MASLD in IBD patients, the moderate AUC values and the strict exclusion criteria, indicate that further research is needed to improve their diagnostic accuracy and generalizability of the findings to a wider IBD population.

Conversely, a notable strength of this study lies in its pioneering proposition. For the first time, our findings underscore the potential of protein glycosylation as a tool for physicians to discern IBD patients at risk of not only developing metabolic syndrome but also progressing toward MASLD – a condition strongly related to MASH, liver fibrosis and CV diseases, as previous outlined. Moreover, the prospective nature of our data lends a heightened degree of reliability to the results compared to conclusions derived from retrospective studies.

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TABLES

Table 1. General characteristics of IBD patients (cases) and non-IBD patients (controls).

Patient's characteristics	**Non-IBD controls (n=400)**	**IBD patients (n=100)**		**p-value***
Age, m (IQR)	51.0 (41.0-59.0)	51.0 (41.0-59.0)		0.982
Female, n (%)	206 (51.5)	51 (51.0)		1.000
BMI, m (IQR)	27.0 (24.4-30.14)	26.4 (23.1-29.9)		0.165
FLI, m (IQR)	41.8 (18.9 -67.3)	42.1 (13.8-66.9)		0.637
		No MASLD	**With MASLD**	<0.001
		67.2 (46.8-81)	14.0 (7.0-29.7)	

m: mean, IQR: Interquartile range, IBD: inflammatory bowel disease, BMI: body mass index, FLI: fatty liver index. MASLD: metabolic dysfunction-associated steatosis liver disease.

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*Univariate analysis with Student's t test or Mann-Whitney U test, as appropriate.

Table 2: Baseline characteristics of IBD patients (cases) and its association with MASLD.

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Patient's characteristics	**Cases without MASLD n=50**	**Cases with MASLD n=50**	***p-value**
Smoking habit			
Smokers, n (%)	8 (16)	3 (6)	0.110
Former-smoker, n (%)	24 (48)	27 (54)	0.548
Hypertension, n (%)	5 (10)	12 (24)	0.062
Disease location, n (%)			
Crohn's disease (CD)			
Colonic CD	5 (10)	3 (6)	
Ileocolonic CD	18 (36)	25 (50)	
Ulcerative colitis (UC)			0.156
Left side colitis	8 (16)	12 (24)	
Extensive colitis	19 (38)	10 (20)	
CD behaviour, n (%)			
Stricturing	7 (14)	9 (18)	0.688
Inflammatory	12 (24)	16 (32)	
Penetrating	4 (8)	3 (6)	
Extraintestinal disease, n (%)	14 (28)	10 (20)	0.349
Need of surgery, n (%)	10 (20)	11 (22)	0.806
Therapy, n (%)			
Mesalazine	17 (34)	18 (36)	
Anti-TNF	11 (22)	12 (24)	
Other biologics*	7 (14)	8 (16)	0.935
Tiopurines	6 (12)	4 (8)	
Tiopurines + Anti-TNF	4 (8)	2 (4)	
Without treatment	5 (10)	6 (12)	
Steroid-dependent course, n (%)	18 (36)	14 (28)	0.391
Calprotectin (µg/g), m (IQR)	86.6 (24-446.8)	125.3 (39.8-326.4)	0.632

Ustekinumab, vedolizumab or tofacitinib. MASLD: metabolic dysfunction-associated steatotic liver disease. MD: Mediterranean diet. CRP: C-reactive protein. UC: ulcerative colitis. CD: Crohn's disease. M: mean. IQR: interquartile range. **Univariate analysis with Student's t test or Mann-Whitney U test, as appropriate.

Table 3. AUC, specificity and sensitivity for glycoprotein cutoff values and the detection of MASLD in IBD patients

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MASLD presence	AUC	95% CI	Cutoff (umol/L)	Sensitivity (%)	Specificity (%)
Glyc-A	0.711	(0.607-0.815)	802	80	68
Glyc -B	0.734	(0.632-0.836)	413	76	68
Glyc-F	0.686	(0.582-0.789)	307	62	70

MASLD: metabolic dysfunction-associated steatotic liver disease, AUC: Area under the curve, Glyc: glycoprotein. CI: confidence interval.

FIGURE LEGENDS

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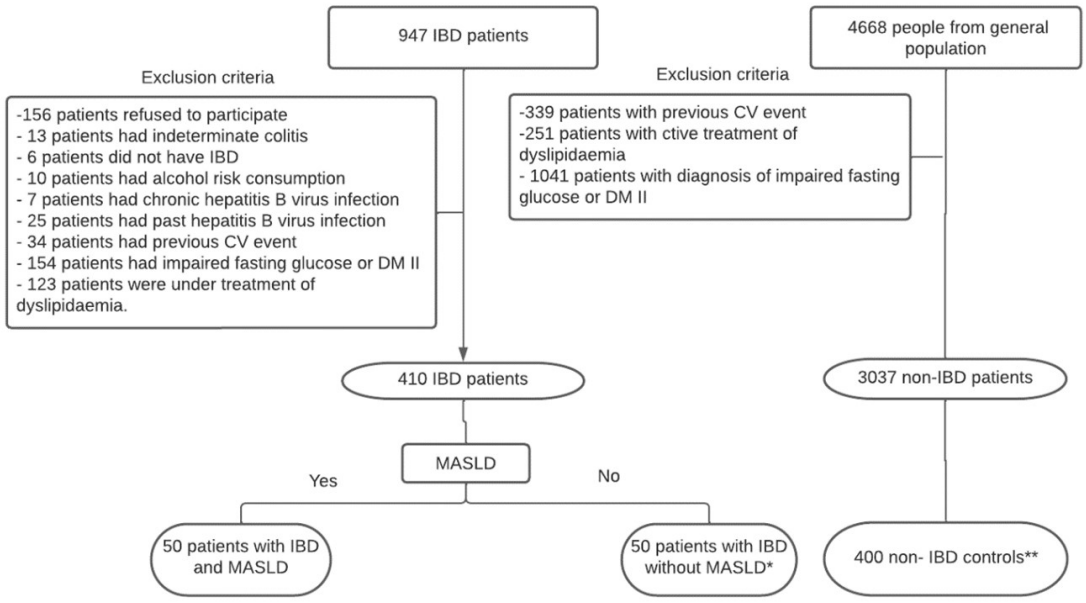


IBD: Inflammatory bowel disease, CV: Cardiovascular event, MASLD: metabolic dysfunction-associated steatotic liver disease, DM II: type II diabetes
*Matched by sex and age with IBD patients with MASLD **Matched by sex, age, body mass index and fatty liver index with IBD patients

Figure 1. Flow-chart of the study. *IBD patients with MASLD were matched by sex and age with IBD patients without MASLD. ** IBD patients and non-IBD controls were matched by sex, age, BMI and FLI. IBD: Inflammatory bowel disease, BMI: body mass index CV: Cardiovascular event, FLI: fatty liver index, MASLD: Metabolic dysfunction-associated steatotic liver disease, DM II: type 2 DM.

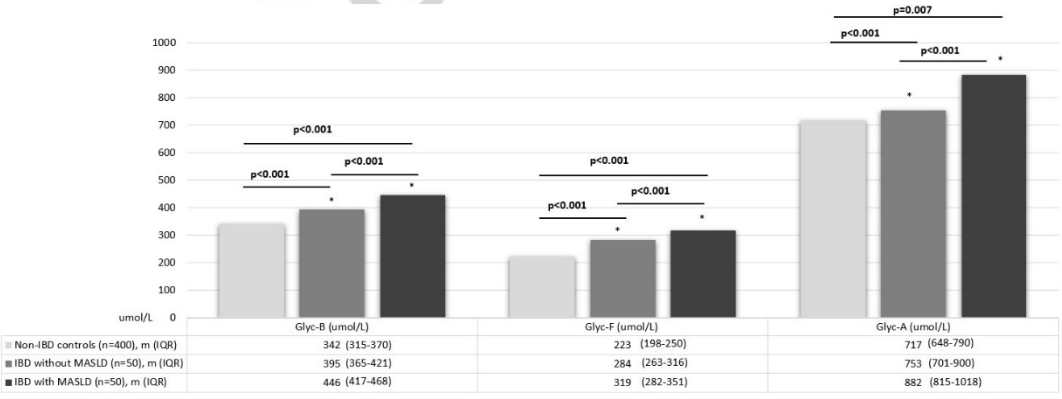


Figure 2. Glycoprotein profile of IBD patients with and without MASLD and non-IBD controls. A progressive increase in glycoprotein values of all glycoprotein subclasses is noted. *Statistical significance with Mann Whitney-U test. Glyc: glycoprotein, IBD:

Inflammatory bowel disease, IQR: Interquartile range, MASLD: Metabolic dysfunction-associated steatotic liver disease, m: median.

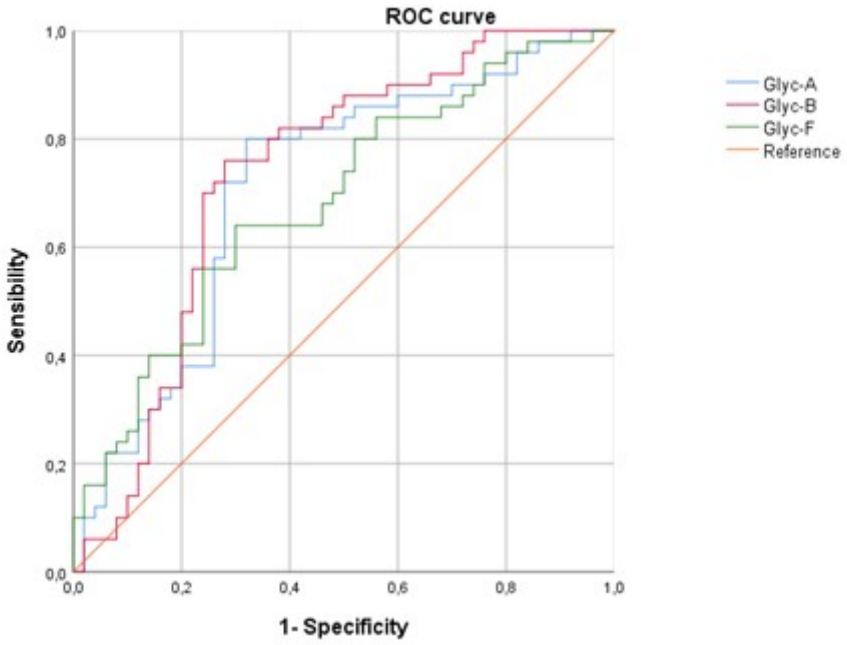
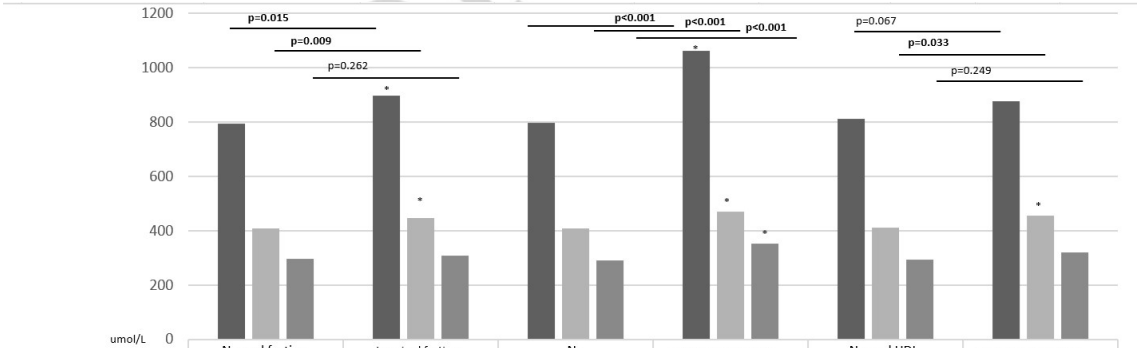


Figure 3. ROC curve for glycoproteins as possible predictors of MASLD in IBD patients.



	Normal fasting glucose	Impaired fasting glucose n= 28/100	No hypertriglyceridemia	Hypertriglyceridemia n= 18/100	Normal HDL cholesterol	Low HDL cholesterol n=13/100
Glyc A (umol/L), m (IQR)	793 (712-916)	896 (802-1066)	797 (708-914)	1061 (869-1230)	812 (721-952)	875 (825-1150)
Glyc B (umol/L), m (IQR)	409 (378-450)	447 (409-475)	407 (370-449)	471 (439-488)	411 (378-457)	455 (422-476)
Glyc F (umol/L), m (IQR)	295 (271-329)	309 (274-350)	290 (268-326)	352 (311-427)	293 (272-328)	321 (269-355)

Figure 4. Glycoprotein levels and their relationship to the persistence or development of metabolic comorbidities during the follow-up. Glyc: glycoproteins. M: median, IQR: Interquartile range. * Mann-Whitney- U test.

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