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Women in science - Gastroenterology 2021

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Women in science - Gastroenterology 2021

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Editorial: Women in science—Gastroenterology 2022

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KEYWORDS

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Editorial on the Research Topic
Women in science—Gastroenterology 2022

This Editorial encompasses highlights from a Research Topic of articles published in *Women in science—Gastroenterology 2022*. The main aim of this Research Topic of Frontiers in Medicine is to promote high quality research by women in the field of gastroenterology. Articles were selected based on the relevance of the Research Topic to the current practices in gastroenterology and female authorship. Over the years, females have been instrumental in advancements in the field of gastroenterology including the improvement in endoscopic procedures, the investigative approach of patients with underlying gastrointestinal pathology and drugs used in the management of such patients.

Females who choose to pursue a career in gastroenterology are faced with challenges, at times struggling to publish and to progress further in their career. Here we celebrate the inspiration of these authors, their resilience and dedication, qualities that have allowed them to be successful and to meet the competitive demands of their workplaces. We hope that their example will serve as a role model for younger male and female gastroenterologists alike in their everyday clinical practice.

Role of galectins in the liver diseases: A systematic review and meta-analysis (An et al.)—Galectins are responsible for the regulation of pre-messenger RNA (mRNA) splicing, cell cycle, cell growth, and cell apoptosis and the development and/or progression of cancer. Carbohydrate recognition domains in galectins play a regulatory role in liver diseases by binding to glycoconjugates expressed in hepatocytes. This meta-analysis explores the role of galectins in liver related disorders, namely in predicting prognosis in hepatocellular carcinoma that has a poor 5 years survival. Identifying these biomarkers provides a stepping stone for potential therapeutic targets to manage chronic liver disorders and hepatocellular carcinoma.

Genetic polymorphisms and clinical features in diabetic patients with fatty liver: Results from a single-center experience in Southern Italy (Villani et al.)—Non-alcoholic fatty liver disease (NAFLD) has a high prevalence in patients with diabetes and the metabolic syndrome. It is a major burden in the medical field in view of the ongoing monitoring that this condition entails and the potential requirement for liver transplantation. This single study highlights the role of genetic studies in the early identification of patients at

a higher risk of cirrhosis and a lower estimated glomerular filtration rate who will require closer surveillance.

Exposure to plasma from non-alcoholic fatty liver disease patients affects hepatocyte viability, generates mitochondrial dysfunction, and modulates pathways involved in fat accumulation and inflammation (Grossini et al.)—In this study, the authors determine whether factors in the plasma of NAFLD patients may induce a similar phenotype in hepatocytes by activation of intracellular inflammatory pathways. Although further studies are required, this mechanism can influence cell viability and results in activation of pathways causing inflammation and tissue damage.

Risk factors of invasive fungal infection in recipients after liver transplantation: A systematic review and meta-analysis (Liu et al.)—An improvement in surgical technique has resulted in an improved outcome following liver transplantation. However, fungal infection remains a major cause of morbidity and mortality in this cohort of patients. Results from existent studies are limited by geographical limitations and sample sizes. In this meta-analysis, the authors analyzed existent studies to identify risk factors for fungal infection following liver transplantation, thus providing a basis for clinical prevention.

Combined estrogen alpha and beta receptor expression has a prognostic significance for colorectal cancer patients (Topi et al.)—This study investigated the prognostic significance of the combined expression of estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) in female patients with colorectal cancer. The authors investigated survival, local recurrence and liver metastasis in relation to the estrogen receptor expression. This study provides a basis for future research on the early detection of colorectal cancer, targeted therapies and prognostic markers to predict the recurrence risk more accurately.

An assessment of physicians' recommendations for colorectal cancer screening and international guidelines awareness and adherence: results from a Thai national survey (Pausawasdi et al.)—Screening for colorectal cancer (CRC) is a proven strategy to improve prognosis and survival as lesions can be detected in a precancerous phase and there is the ability to cure precancerous lesions mostly by endoscopically. The uptake of CRC screening is generally low in the Asia Pacific region. The aim of this study was to understand the recommendations by physicians in Thailand for CRC screening and the awareness and adherence to international guidelines by means of a questionnaire. The authors tried to understand how recommendations vary across different specialities and if low uptake of CRC screening could potentially be improved by understanding trends in recommendations by physicians.

Circulating fibroblast activation protein as potential biomarker in patients with inflammatory bowel disease (Corsi et al.)—This study explores the role of circulating Fibroblast activation protein (FAP), a serum biomarker that correlates inversely with disease activity in inflammatory bowel disease (IBD). It can serve an important role as a non-invasive test in those with suspected IBD and in predicting recurrence following surgery. Unlike other biomarkers such as faecal calprotectin, it is significantly specific to IBD, distinguishing IBD related inflammation from other causes of colitis. Serum biomarkers are increasingly sought after in current medical practice as they minimize the requirement for invasive procedures and their associated complications, and improve cost effectiveness.

Are steroids still useful in immunosuppressed patients with inflammatory bowel disease? A retrospective, population-based study (Sicilia et al.)—Patients with IBD who are on immunosuppressants may require systemic steroids to manage moderate to severe flare ups. The aim of this study was to assess the effectiveness of steroids in this scenario and to determine whether the use of steroids can impact on the requirement for further escalation of therapy (the requirement for biologics). The authors question whether such treatment that has been used for a long time in the management of patients with IBD still plays an important role in managing flare ups and minimizes the need for escalation of therapy.

Helicobacter pylori eradication therapy affect the gut microbiota and ghrelin levels (Martín-Núñez et al.)—In this prospective study, the authors analyzed the effect of *Helicobacter pylori* eradication eherapy on ghrelin levels that correlate with the changes in diversity and abundance of gut microbiota. Ghrelin can affect stimulation of food intake, growth hormone secretion, adiposity, gastric motility, acid secretion and insulin secretion inhibition. Studying the association between ghrelin levels and gut microbiota can shed light on the pathogenesis of obesity, varying gut microbiota and ghrelin levels.

Author contributions

The author confirms being the sole contributor of this work and has approved it for publication.

Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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ORIGINAL RESEARCH
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Are Steroids Still Useful in Immunosuppressed Patients With Inflammatory Bowel Disease? A Retrospective, Population-Based Study

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Background: Effectiveness of corticosteroids in immunosuppressed patients with inflammatory bowel disease (IBD) has not been completely elucidated.

Aims: To assess the effectiveness and examine the long-term follow-up of systemic or low-bioavailability oral steroid treatment for moderate flare-ups in patients treated with immunosuppressive drugs.

Methods: Immunosuppressed patients with inflammatory bowel disease (IBD) from our population-data registry were analyzed. For statistical analysis, the chi-square test, Mann-Whitney U test, and Kaplan-Meier survival analysis were used as appropriate.

Results: A total of 392 patients with IBD and a median of 82 (range, 6–271) months of immunosuppressive (IMM) treatment were identified. The mean follow-up was 87 months (range, 6–239 months). A total of 89 patients (23%) needed at least one steroid course during their follow-up. Average time from IMM to steroid treatment was 26 (range, 6–207) months. In patients with CD, fibrostenotic (B2) and fistulizing (B3) behaviors [$p = 0.005$; odds ratio (OR): 2.284] were risk factors for using steroids after IMM treatment. In patients with UC, no statistically significant variables were identified. Of the 89 patients who received one first steroid course, 49 (55%) stepped up to biological treatment or surgery after a median of 13 months (range, 0–178), 19 (21%) were treated with repeated steroid courses, and 31 (35%) required no further treatment. Patients with CD had a higher risk ($p = 0.007$; OR: 3.529) of receiving biological treatment or surgery than patients with UC. The longer the patients with UC (more months) spent using steroids, the greater the risk of requiring treatment with biological drugs or surgery ($p = 0.009$).

Conclusion: A total of 23% of the immunosuppressed patients with IBD received at least one course of steroid treatment. In patients under immunosuppression treated with at least a course of steroids, CD patients were more likely stepped up to biologics and/or surgery than UC patients. In patients with CD, B2/B3 behavior pattern were significant risk factors. After one course of steroids only 35% of immunosuppressed IBD patients remained in remission without needing treatment scalation.

Keywords: immunosuppression, corticosteroids, Crohn's disease, ulcerative colitis, inflammatory bowel diseases

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INTRODUCTION

In 1955, Truelove and Witts (1) were the first to demonstrate the efficacy of corticosteroids to induce remission in patients with inflammatory bowel disease (IBD): moderate to severe ulcerative colitis (UC) patients treated with hydrocortisone (100 mg/day) did have a clearly better response than those receiving placebo, with statistically significant differences in hard endpoints as mortality. Until now corticosteroids continue to be the most widely used drugs for the treatment of moderate and severe flare-ups in both Crohn's disease (CD) and UC (2, 3).

The main effects of systemic corticosteroids (prednisone or equivalent) are to induce remission of moderate and severe outbreaks in patients with CD [evidence level (EL) 1] (4) and UC (EL 1) (5), with high quality of evidence (QL) and strong recommendation (6). High doses of prednisone (40 mg/day−1 mg/kg) are usually prescribed. Different systemic drugs require specific doses, and parenteral route is sometimes preferred in severe cases. For low-bioavailability systemic corticosteroids, 9 mg of oral budesonide is the treatment of choice to induce remission in patients with ileal CD, both in mild (EL 2) and moderate (EL 1) flares (4). In patients with UC and mild-to-moderate flare-ups without a response to mesalamine, beclomethasone at a minimum dose of 5 mg/day (EL 2) (5) (low QL, weakly in favor) (6) or multimatrix budesonide at a dose of 9 mg/day (EL 2) can be used (**Table 1**). Steroid-resistance (primary failure) and steroid-dependence are common and defined in ECCO guidelines (4).

Thiopurine immunosuppressant drugs (azathioprine or mercaptopurine) are effective for patients with corticosteroid-dependence and have helped patients achieve a withdrawal rate from corticosteroids of almost 60% (7). Corticosteroids are frequently used in patients already under immunosuppressive (IMM) treatment for controlling a new flare-up. However, scant data are available on the efficacy of corticosteroid use in this scenario. In fact, main published studies have shown that 40–50% of patients receive corticosteroid treatment and that 20–30% receive immunomodulatory treatment (8–11). A recent Canadian study of 3,312 patients with IBD in clinical practice between 1994 and 2014 (12) showed that corticosteroids were prescribed in the first year of IMM treatment for 20% of patients with CD and up to 40% of patients with UC. Steroids have significant toxicity, especially in the long-term. Data on their effectiveness in daily clinic could help for designing new protocols of use.

We do present our clinical experience with the use of steroids in a retrospective study of all the patients from our IBD Unit.

AIMS

Our first aim was to evaluate the effectiveness of systemic oral (prednisone) and low-bioavailability (beclomethasone/budesonide) corticosteroids to induce remission of moderate IBD flare-ups in patients with at least 6 months of IMM treatment (azathioprine, mercaptopurine, or methotrexate). Our second aim was to assess the long-term effectiveness of this treatment in avoiding the need for biological drugs and/or surgery and analyzing the predictors of response in this specific scenario.

METHODOLOGY

We used a population database to identify patients diagnosed with IBD (both UC and CD) between 1966 and 2016 from a reference area of 176,208 inhabitants [Spanish Statistical Office (INE) 2016]; we included only patients who had at least 6 months of IMM treatment in our study.

We performed a descriptive analysis and collected the following variables:

- Type of IBD (UC/CD)
- Year of diagnosis
- Location of IBD according to the Montreal classification for UC and CD:
  - Extensive UC (E3)/left-sided (E2)/ulcerative proctitis (E1)
  - Ileal CD (L1)/colonic (L2)/ileocolonic (L3) with/without isolated upper disease (L4)
  - Presence of perianal disease
- Behavior pattern according to the Montreal classification for CD:
  - Inflammatory pattern (B1)/fibrostenotic (B2)/penetrating (B3)
- Appendectomy
- Smoking habit at diagnosis:
  - Smoker: Smoker at the start of IMM and current treatment
  - Former smoker: At least 6 months without smoking
  - Non-smoker: Never smoked or >10 years without smoking
- Previous surgery
- Use of corticosteroids at diagnosis
- IMM treatment
  - Type of IMM treatment: azatioprine, mercaptopurine, methotrexate
  - IMM treatment time until the use of corticosteroids and until the end of follow-up
- Treatment with biological drugs prior to immunosuppression

During the follow-up, we recorded the following:

- Treatment with systemic oral or low-bioavailability corticosteroids, dosage, and time
- The efficacy of treatment with corticosteroids, which was defined as not requiring further cycles of corticosteroids, escalation of treatment, or surgery
- The amount of time in which no rescue therapy was necessary after the use of corticosteroids
- Any need for biologic drugs, surgery, or further corticosteroid cycles.

Abbreviations: CD, Crohn's disease; CI, confidence interval; EL, evidence level; IBD, inflammatory bowel disease; IMM, immunosuppressive; OR, odds ratio; UC, Ulcerative colitis.

TABLE 1 | Level of evidence and indications for use of corticosteroids in patients with IBD.

	Flare-up	Corticosteroid	ECCO	GRADE
UC	Mild-moderate	Beclomethasone 5 mg/d	EL 2	QL low RG Weak for
	Mild-moderate	Budesonide MMX 9 mg/d	EL 2	–
	Mild-Moderate	Prednisone 1 mg/kg	EL 1	QL moderate RG Strong for
	Severe	Prednisone 1 mg/kg	EL 1	QL high RG Strong for
CD	Mild ileocecal	Budesonide 9 mg/d	EL 2	–
	Moderate ileocecal	Budesonide 9 mg/d	EL 1	–
	Moderate-severe	Prednisone 1 mg/kg	EL 1	–
	Severe	Prednisone 1 mg/kg	EL 1	–

QL, Quality of evidence; EL, Evidence level; RG, Recommendation Grade.

TABLE 2 | Baseline patient demographics and disease characteristics.

IMM Treatment >6 Months	CD 260 (66%)	UC 132 (34%)	Total 392 (100%)
Gender (male)	136 (52%)	71 (54%)	207 (53%)
Location	46% L1/18% L2/36% L3/ 17% L4	52% E3/45% E2/3% E1	
Behavior	57% B1/43% B2-3 25% perianal disease		
Appendectomy	91 (77%)	6 (5%)	97 (25%)
Extra-intestinal manifestations	49 (19%)	23 (17%)	72 (19%)
Smoke habit	Smoker 84 (32%)	Smoker 17 (13%)	Smoker 101 (26%)
	Former smoker 50 (19%)	Former smoker 12 (9%)	Former smoker 62 (16%)
	Non-smoker 126 (48%)	Non-smoker 103 (78%)	Non-smoker 229 (58%)
Steroids at Dg	200 (77%)	104 (79%)	304 (78%)
Biological before IMM treatment	4 (2%)	0 (0%)	4 (2%)
Surgery before IMM treatment	62 (24%)	2 (16%)	64 (16%)
Steroids post IMM treatment	Classical 38 (63%)	Classical 18 (62%)	Classical 56 (65%)
	Low-bioavailability 22 (37%)	Low-bioavailability 11 (38%)	Low-bioavailability 33 (37%)
Surgery follow-up	13 (5%)	1 (0.8%)	14 (3.5%)
Biologic follow-up	27 (10%)	9 (7%)	35 (9%)

Activity of the disease was defined clinically first with physician-based subjective evaluation and then Harvey-Bradshaw index in CD (<4 points defined as clinical remission) and Truelove-Wits index in UC (remission if <3 bowel movements/day and no rectal bleeding). The goal of treatment was clinical remission, and steroids prescribed to obtain remission if clinical activity present. Protocol for steroids in our hospital followed textbook and GETECCU (www.geteccu.org) recommendations in the first years (1966–2006) and ECCO guidelines from 2006 to 2016. In brief, moderate to severe CD or UC were treated with prednisone, with 1 mg/kg/day starting dose, and tapering from week 4 (usually reducing 10 mg/day every week). Mild to moderate ileitis was treated with budesonide (9 mg/day, tapering from week 4). Mild to moderate cases of UC were treated with oral beclomethasone (5–10 mg/day; 1–2 months course).

Patients were treated at the discretion of the responsible physician. In most cases IBD patients were under the care of the same expert gastroenterologist from 1966 to 2010. After 2010 the team responsible for IBD patients has remained constant, following ECCO guidelines as gold-standard. Biologics were available from several months (<12) after EMA approval. Azathioprine was given at 2.5–3 mg/kg/day in one dose, and mercaptopurine at 1.5 mg/kg/day. In both cases doses were adjusted if needed by frequent (every 3–6 months) blood tests follow-up, but no metabolites determination was available.

For statistical analysis, all data were processed using the IBM SPSS 19 statistical software with a confidence interval of 95%. The data had been previously collected and processed using Microsoft Office Excel 2010. Descriptive analysis of the sample was performed and showed the means (standard deviation), medians (interquartile range), and frequency (percentage) according to the characteristics and distributions of variables. Differences between variables were evaluated using the chi-square (Fisher) test for qualitative variables and Student's *t*-test, provided it verified the conditions of use; otherwise, the corresponding

TABLE 3 | Variables associated with the need for treatment with corticosteroids in patients who have received at least 6 months of immunosuppressant treatment.

Variables		Post-IMM treatment corticosteroids		
		No ($n = 303$)	**Yes ($n = 89$)**	***p***
Sex	Male	160 (53%)	47 (53%)	1.000
	Female	143 (47%)	42 (47%)	
Age		47.6	49.66	0.242
Corticosteroids at diagnosis	No	72 (24%)	14 (16%)	0.102
	Yes	229 (76%)	75 (84%)	
Current diagnosis	UC	103 (34%)	29 (33%)	0.805
	CD	200 (66%)	60 (67%)	
UC location	E3	49 (48%)	20 (69%)	0.097
	E2	50 (49%)	9 (31%)	
	E1	4 (4%)	0 (0%)	
CD location	L3	41 (21%)	6 (10%)	0.105
	L2	67 (34%)	27 (45%)	
	L1	92 (46%)	27 (45%)	
L4	No	170 (85%)	46 (77%)	0.131
	Yes	30 (15%)	14 (23%)	
Pattern	**B1**	**76 (38%)**	**35 (58%)**	**0.005 OR: 2.284**
	B2/B3	**124 (62%)**	**25 (42%)**	
Perianal disease	No	258 (85%)	69 (78%)	0.089
	Yes	45 (15%)	20 (22%)	
Extra-intestinal manifestations	No	244 (81%)	72 (82%)	0.918
	Yes	56 (15%)	16 (18%)	
Biological drugs prior to IMM treatment	**No**	**302 %)**	**86 (97%)**	**0.038**
	Yes	**1 (0%)**	**3 (3%)**	
Surgery prior to IMM treatment	No	257 (85%)	71 (80%)	0.258
	Yes	46 (15%)	18 (20%)	
Smoker	No	169 (56%)	60 (67%)	0.147
	Yes	83 (27%)	18 (20%)	
	Former smoker	51 (17%)	11 (12%)	
Appendectomy	No	225 (76%)	59 (69%)	0.151
	Yes	70 (24%)	27 (31%)	

The bold values highlight the variables which are statistically significative.

non-parametric tests were used, Mann-Whitney U test or Kruskal-Wallis, if one of the variables was quantitative.

Finally, survival analysis was performed using the Kaplan-Meier curve and comparing different survival curves according to the diagnosis of UC or CD using the log-rank hypothesis test, which tests the null hypothesis that the two groups compared have the same survival curves.

RESULTS

We identified 904 patients with IBD in our population database from 1966 to 2016 and selected 392 (43.3%) who had at least 6 months of IMM treatment, with a mean duration of 82 months (6–271). Of these 392 patients, 260 were diagnosed with CD (66%) and 132 were diagnosed with UC (34%). We describe the demographic and clinical characteristics of the patients in **Table 2**.

A total of 89 patients (23%) were treated with at least one course of oral corticosteroids during their follow-up, with an average duration of 4 months (1–168 months). Of these 89 patients, 63% received treatment with systemic corticosteroids and 37% with low-bioavailability oral corticosteroids; a total of 29 patients (33%) had UC, and 60 patients (67%) had CD ($p = 0.805$).

A comparison of the variables associated with the need for treatment with corticosteroids (**Table 3**) showed no differences with regard to sex, age, location of the disease, perianal disease, appendectomy, extra-intestinal manifestations, smoking habits, previous use of corticosteroids, and previous surgery. Fibrostenotic (B2) and fistulizing (B3) patterns of CD ($p = 0.005$) behaved as a statistically significant variable. A patient with a fibrostenotic or fistulizing pattern of CD (B2-B3) was twice as likely to take corticosteroids while on IMM treatment than a patient with an inflammatory pattern of CD (B-1) [odds ratio (OR): 2.284] (**Figure 1**). A total of 4 patients (1%) with CD required treatment with a biological drug prior to starting their IMM treatment (a top-down strategy), and this was also associated with the need for taking corticosteroids during evolution ($p = 0.038$).

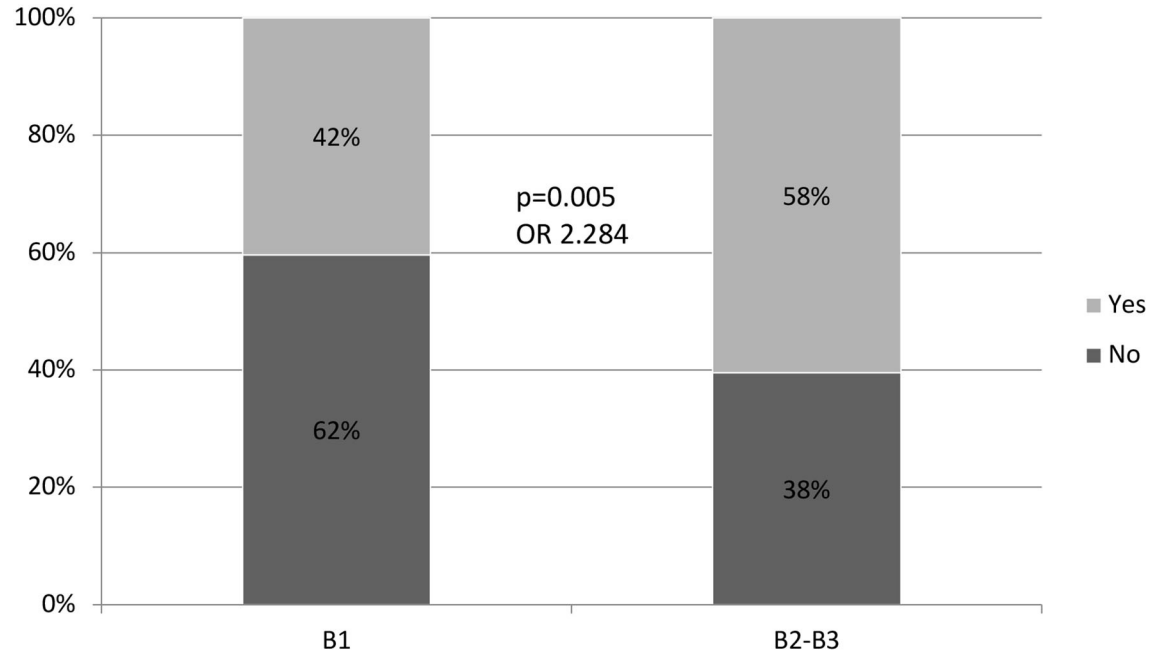

FIGURE 1 | Relation between the pattern of behavior and the need for treatment with corticosteroids.

A total of 49 patients (55%), who were on IMM treatment and treated with corticosteroids for a moderate flare-up of their disease, required biological treatment or surgery during their average follow-up period of 40 months (1–178 months); a total of 19 patients (21%) required more than 1 cycle of corticosteroids. The mean length of time from taking the immunosuppressant drug to the use of corticosteroids was 26 months (6–207). There is no predictive variable of corticosteroid efficacy in patients with CD, but there is a directly proportional association between the time of corticosteroid use and the need for treatment with biological drugs or surgery in the follow-up of patients with UC (**Tables 4**, **5**).

A single cycle of corticosteroids was effective in 31 patients (35%), and no other type of treatment was needed in these patients during their follow-up period, such as further cycles of corticosteroids, biologics, or surgery.

A comparison of the variables associated with the need for biological drugs or surgery showed a higher risk of treatment with a biological drug or surgery after initial corticosteroid treatment in patients with CD than in patients with UC ($p = 0.007$; OR: 3.529) (**Figure 2**). Of the 89 patients who required treatment with corticosteroids, 10 with UC (20%) and 39 with CD (80%) needed rescue therapy throughout their follow-up. This difference was maintained when analyzing the probability of not requiring any type of treatment, including repeat cycles of corticosteroids ($p = 0.005$) (**Figure 3**).

In the survival analysis (**Figure 4**), we observed a 50% chance of not requiring biological drugs or surgery at 130 months. When comparing survival between patients with CD and those with UC (**Figure 5**), we observed a clear separation between both curves, but they crossed at certain periods ($p = 0.078$); however, survival without salvage therapy was higher in patients with UC. The 50% chance of not receiving any type of treatment after receiving corticosteroids lasted 83 months longer (range 97–180) in patients with UC compared to those with CD (**Table 6**). We observed a 75% probability of not needing any additional treatment for 62 months in patients with UC and for 36 months in patients with CD (**Table 7**).

DISCUSSION

The use of corticosteroids in our patients who had responded to IMM treatment reached 55%, with no differences between patients with UC and patients with CD, and 21% required more than one course of corticosteroids. We believe this percentage is likely higher than in current clinical practice, because our review was retrospective and included years when biological drugs were not yet in use. The pivotal published studies (8–11) report corticosteroid treatment in 40–50% of patients enrolled in clinical trials, and ∼30% did not respond to IMM treatment. The only randomized clinical trial that reported this associated data was the GEMINI 1 (9), where 17–21% of the patients included in the different treatment arms were receiving IMM treatment and taking corticosteroids concurrently.

Reinforcing the results of other published cohorts (13), the results of our study show that the fibrostenotic and fistulizing patterns in CD are statistically significant factors associated with the need for corticosteroid treatment in immunosuppressed patients; likely because this reflects a greater severity in the clinical evolution of this behavior pattern and has been reflected in follow-up, epidemiological-incident cohort studies (14). However, compared to the inflammatory pattern, this pattern

TABLE 4 | Requirement for rescue therapy with biological drugs/surgery in immunosuppressed patients with UC who have needed treatment with corticosteroids.

Corticosteroids after IMM treatment (*n* = 29)		**Biological drugs/surgery UC**				
		No (*n* = 19)		**Yes (*n* = 10)**		
		Median/n	**Range/%**	**Median/n**	**Range/%**	***p***
Age (mean/deviation)		53.63	±11.63	44.2	±12.67	0.054
Sex	Male	11	58%	5	50%	0.714
	Female	8	42%	5	50%	
Corticosteroids at diagnosis	No	1	5%	3	30%	0.105
	Yes	18	95%	7	70%	
Location	E3	13	68%	7	70%	1.000
	E2	6	32%	3	30%	
	E1	0	0%	0	0%	
Extra-intestinal manifestations	No	17	89%	8	80%	0.592
	Yes	2	11%	2	20%	
Smoker	No	18	95%	7	70%	–
	Yes	0	0	1	10%	
	Former smoker	1	5%	2	20%	
Appendectomy	No	19	100%	8	80%	0.111
	Yes	0	0%	2	20%	
Corticosteroids	Low-bioavailability	9	47%	2	20%	0.234
	Standard	10	53%	8	80%	
Months on corticosteroids		**2**	**(2–7)**	**5**	**(3–44)**	**0.004**

The bold values highlight the variables which are statistically significative.

TABLE 5 | Requirement for rescue therapy with biological drugs/surgery in immunosuppressed patients with CD who have needed treatment with corticosteroids.

Corticosteroids after IMM treatment (*n* = 60)		**Biological drugs/surgery CD**				
		No (*n* = 21)		**Yes (*n* = 39)**		***p***
		Median/n	**Range/%**	**Median/n**	**Range/%**	
Age (mean/deviation)		48.71	±15.60	49.64	±13.90	0.814
Sex	Male	10	48%	21	54%	0.645
	Female	11	52%	18	46%	
Corticosteroids at diagnosis	No	5	24%	5	13%	0.298
	Yes	16	76%	34	87%	
Location	Colonic	4	19%	2	5%	–
	Ileal	9	43%	18	46%	
	Ileocolonic	8	38%	19	49%	
L4	No	18	86%	28	72%	0.340
	Yes	3	14%	11	28%	
Pattern	B2/B3	12	57%	23	59%	0.891
	B1	9	43%	16	41%	
Extra-intestinal manifestations	No	15	75%	32	82%	0.524
	Yes	5	25%	7	18%	
Perianal disease	No	14	67%	26	67%	1.000
	Yes	7	33%	13	33%	
Smoker	No	12	57%	23	59%	
	Yes	5	24%	12	31%	
	Former smoker	4	19%	4	10%	
Appendectomy	No	11	58%	21	55%	0.850
	Yes	8	42%	17	45%	
Corticosteroids	Low-bioavailability	10	48%	12	31%	0.196
	Standard	11	52%	27	69%	
Months on corticosteroids		4	(2–30)	4	(1–168)	0.511

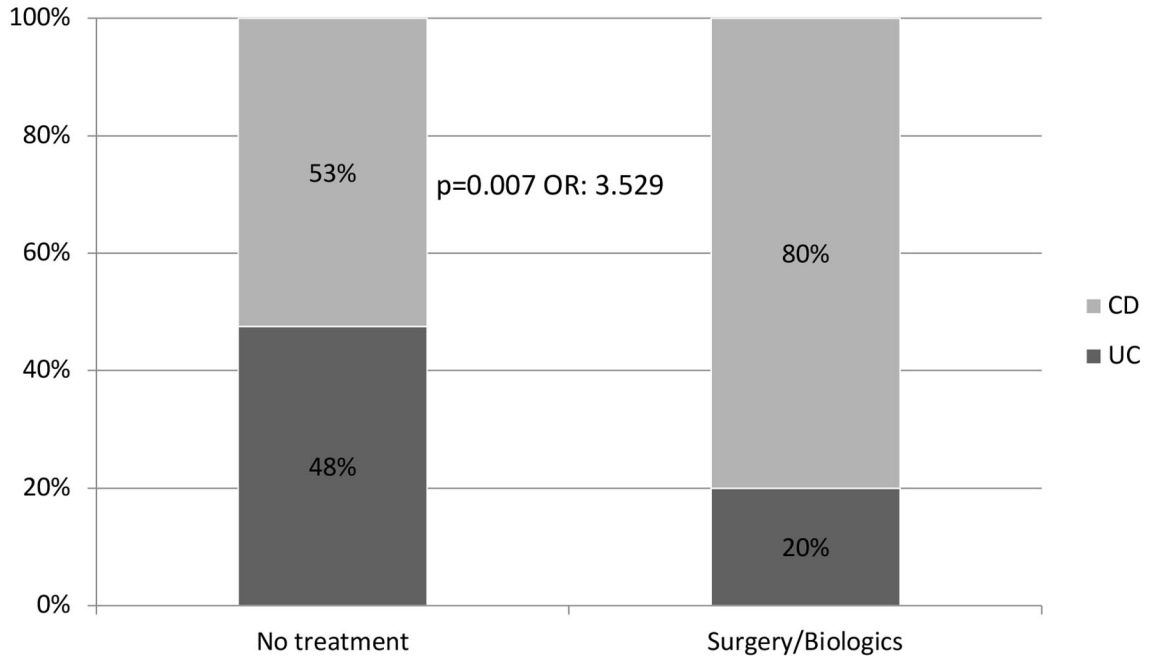

FIGURE 2 | Probability of receiving biological treatment and/or surgery during follow-up after receiving steroids (89 patients).

does not predict the efficacy of this strategy, which is defined as the subsequent requirement of treatment with biological drugs or surgery.

Perianal disease, young age at diagnosis, and the need for corticosteroids appear to be risk factors in patients with CD; therefore, we attempted to identify the criteria and clinical factors associated with a more aggressive course to coincide with the emergence of more effective therapies (15, 16). In our cohort, this variable was not associated with a subsequent need for corticosteroids once the patient is already on immunosuppressive therapy. In addition, the use of corticosteroids at diagnosis did not predict the need for subsequent rescue therapies once these corticosteroids were used in our patients. However, once the patient is receiving IMM treatment, as is the case with our cohort, the use of corticosteroids should be carefully evaluated. At no instance should more than 1 cycle of corticosteroid (preferably of low-bioavailability) be used if other therapeutic options that are more effective in the long-term have not been optimized, such as the use of biological drugs and surgery.

The overall efficacy of corticosteroid use in already-immunosuppressed patients with UC or CD, which was defined in our study as having no need for any other type of treatment throughout the follow-up, was only 35%; therefore, 65% of patients receiving corticosteroids will require a new cycle of treatment with corticosteroids, biological drugs, or surgery.

The efficacy of this course of corticosteroids for the long-term in our cohort was significantly different between patients with UC and CD. A patient with CD who needs corticosteroids is 3.5 times more likely to need rescue therapy with biological drugs or surgery than a patient with UC ($p = 0.007$, OR: 3.529). In addition, patients with CD also have a significantly higher risk of requiring more than one cycle of corticosteroids compared to patients with UC.

The role of low-bioavailability corticosteroids in immunosuppressed patients has not been studied. In our cohort, treatment with low-bioavailability corticosteroids in patients with either UC or CD did not predict the use of subsequent salvage therapy when compared to treatment with systemic corticosteroids. Thus, the severity of the flare-up is inadequate to identify the subgroup of patients in which a corticosteroid cycle will be effective. Once we used corticosteroids, we found no differences in the different variables when trying to predict which patients would benefit from this strategy.

Our results indicate that the patients who will need to use corticosteroids the most in the immunosuppressed situation (EC B2-B3) will also be the ones less responsive in the long-term and require early biological treatment or rescue surgery. Therefore, the strategy of using corticosteroids in patients already on IMM treatment may be used most effectively in patients with UC whose flare-up is controlled with low-bioavailability corticosteroids (fewer side-effects). Moreover, the durability of the effect of corticosteroids is greater in these patients with UC than patients with CD.

There are no many studies in the literature that analyze the efficacy of corticosteroids in patients with IMM treatment with which to compare our results; therefore, we believe that data from other larger population cohorts or prospective scenarios are needed to confirm our results. Our work is the first in the literature to show efficacy data and risk factors that are predictive of favorable clinical progress in this specific scenario. We acknowledge the that there are limitations in our study. In fact, there have been many changes in practice

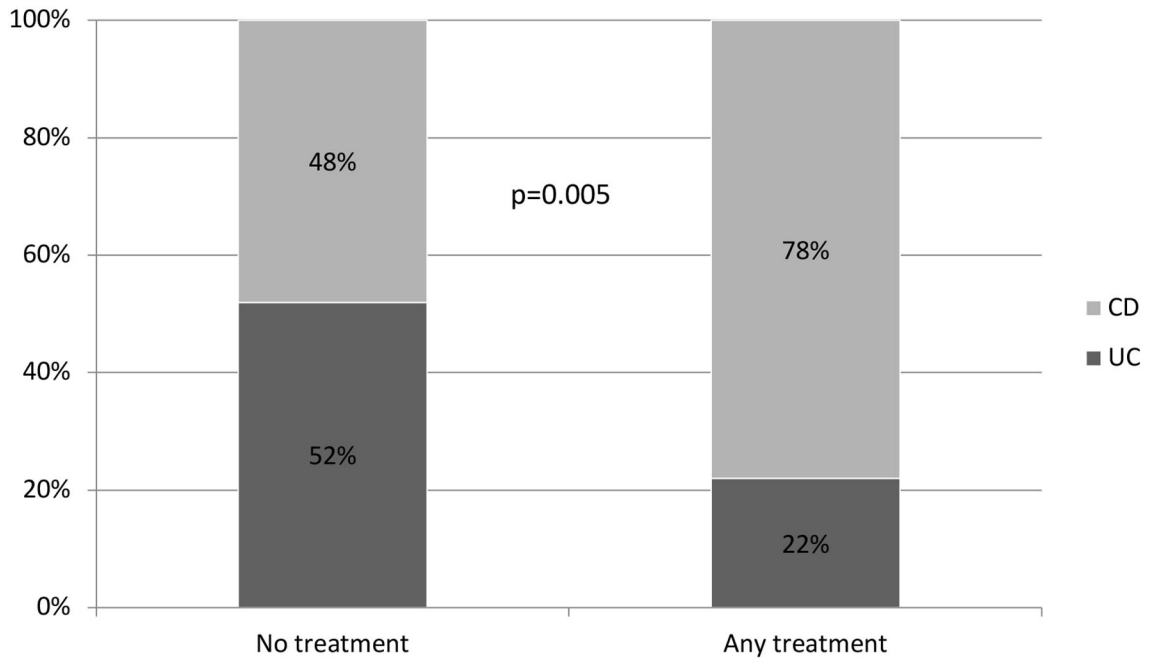

FIGURE 3 | Probability of NO treatment at all (No biological drug, surgery, or corticosteroids).

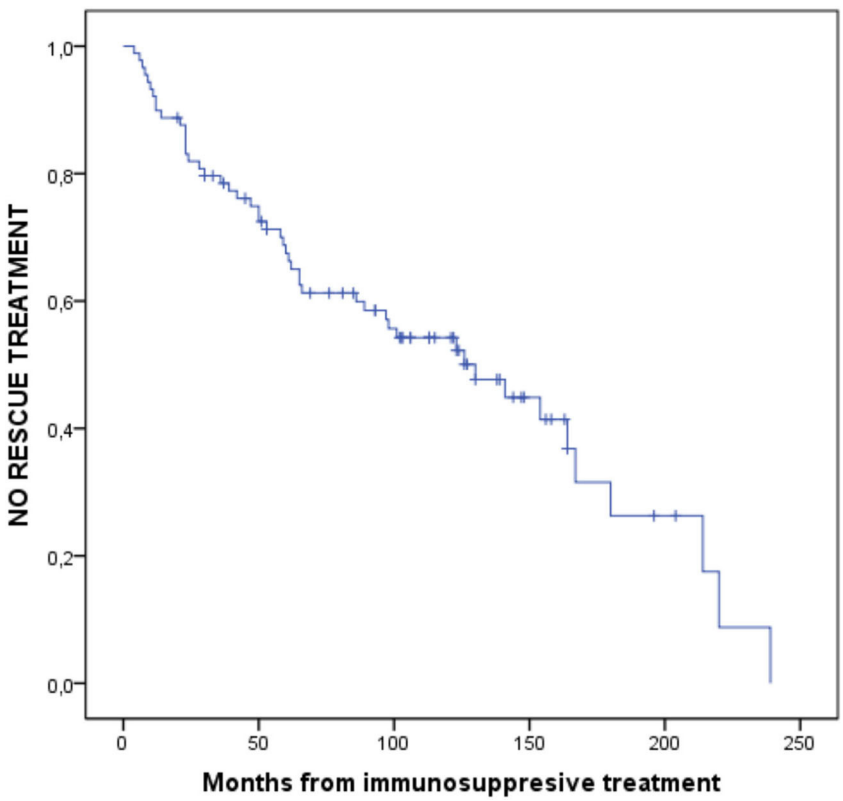

FIGURE 4 | Probability of NO rescue therapy from IMM treatment to the end of follow-up.

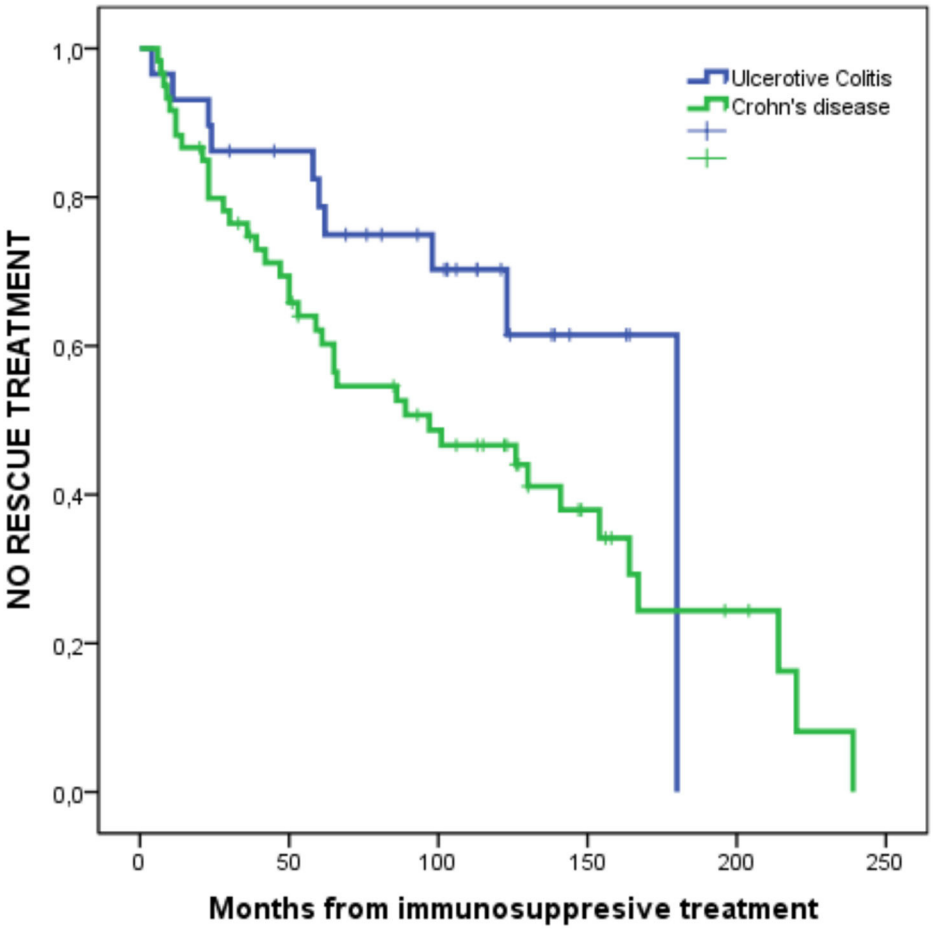

FIGURE 5 | Probability of NO rescue therapy from IMM treatment to the end of follow-up in UC and CD. $P = 0.078$.

TABLE 6 | Means and medians of survival times.

Current diagnosis	Mean[a] (months)			Median (months)		
	Estimate	95% confidence interval		Estimate	95% confidence interval	
		Lower limit	Upper limit	Lower limit	Lower limit	Upper limit
Ulcerative colitis	134,96	109,21	160,73	180,00	–	–
Crohn's disease	111,10	88,47	133,75	97,00	32,12	161,87
Total	122,27	102,87	141,68	130,00	82,79	177,21

[a] *The estimate is restricted to the longest survival time if this was recorded.*

during the 50 years observation period, and the retrospective design limits the analysis. However, we think these data add information to our knowledge of natural history of IBD under immunosuppressive treatment.

CONCLUSIONS

More than half of our patients who were in established IMM treatment required treatment with systemic or low-bioavailability corticosteroids throughout their subsequent follow-up. The patients' B2-B3 CD pattern and previous use of biological drugs were the only associated risk factors.

This drug strategy was clearly effective only in 35% (1/3) of the patients treated with corticosteroids; the remaining patients needed further courses of corticosteroids, biological drugs, or surgery. It seems that most flare-ups in IBD patients under immunosuppressants lead finally to biological and/or surgical therapies, and as steroids have significant toxicity, a different strategy could be more adequate, such as directly

TABLE 7 | Percentils.

Current diagnosis	25.0%	50.0%	75.0%
	Estimate (months)	Estimate (months)	Estimate (months)
Ulcerative colitis	180	180	62
Crohn's disease	167	97	36
Total	214	130	47

switching to biologics or considering surgery, depending on individual factors.

Patients with CD who require corticosteroids in this specific scenario are 3.5 times more likely to need rescue therapy with biological drugs or surgery than patients with UC; this is reflected in survival curves during follow-up, where the 50% probability of not receiving any rescue therapy is 83 (180–97) months longer in patients with UC than patients with CD.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee Hospital Universitario de Burgos. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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frontiers in Medicine

BRIEF RESEARCH REPORT
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Exposure to Plasma From Non-alcoholic Fatty Liver Disease Patients Affects Hepatocyte Viability, Generates Mitochondrial Dysfunction, and Modulates Pathways Involved in Fat Accumulation and Inflammation

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Changes of lipidic storage, oxidative stress and mitochondrial dysfunction may be involved in the pathogenesis of non-alcoholic fatty liver disease (NAFLD). Although the knowledge of intracellular pathways has vastly expanded in recent years, the role and mechanisms of circulating triggering factor(s) are debated. Thus, we tested the hypothesis that factors circulating in the blood of NAFLD patients may influence processes underlying the disease. Huh7.5 cells/primary human hepatocytes were exposed to plasma from 12 NAFLD patients and 12 healthy subjects and specific assays were performed to examine viability, H_2O_2 and mitochondrial reactive oxygen species (ROS) release, mitochondrial membrane potential and triglycerides content. The involvement of NLRP3 inflammasome and of signaling related to peroxisome-proliferator-activating-ligand-receptor-γ (PPARγ), sterol-regulatory-element-binding-protein-1c (SREBP-1c), nuclear-factor-kappa-light-chain-enhancer of activated B cells (NF-kB), and NADPH oxidase 2 (NOX2) was evaluated by repeating the experiments in the presence of NLRP3 inflammasome blocker, MCC950, and through Western blot. The results obtained shown that plasma of NAFLD patients was able to reduce cell viability and mitochondrial membrane potential by about 48 and 24% ($p < 0.05$), and to increase H_2O_2, mitochondrial ROS, and triglycerides content by about 42,

19, and 16% ($p < 0.05$), respectively. An increased expression of SREBP-1c, PPARγ, NF-kB and NOX2 of about 51, 121, 63, and 46%, respectively, was observed ($p < 0.05$), as well. Those effects were reduced by the use of MCC950. Thus, in hepatocytes, exposure to plasma from NAFLD patients induces a NAFLD-like phenotype by interference with NLRP3-inflammasome pathways and the activation of intracellular signaling related to SREBP-1c, PPARγ, NF-kB and NOX2.

Keywords: biomarker, inflammasome, mitochondria, NAFLD, oxidative stress

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is currently the most common liver disease in the world and the second most common cause of liver transplantation in the United States (1, 2).

Our understanding of NAFLD pathogenesis and natural history has greatly expanded in the last decade, however, many issues remain unsolved. The liver plays a central role in regulating lipid homeostasis through processes that are strictly regulated by complex interactions between hormones, nuclear receptors, and transcription factors-related pathways. Their alteration may cause the retention of fat within the liver and the subsequent development of NAFLD (3, 4).

Another important player in the onset of NAFLD and its progression to non-alcoholic steatohepatitis (NASH) is mitochondrial dysfunction (5) that can alter the balance between prooxidants/antioxidants, leading to an increase of non-metabolized free fatty acids (FFAs) in the cytosol and the consequent induction of reactive oxygen species (ROS) production. A "primary" mitochondrial dysfunction has been proposed as one of the mechanisms of FFAs accumulation in the hepatocytes of NAFLD patients (6), however, an overload of FFAs into mitochondria may be able by itself to lead to dissipation of the membrane potential, loss of ATP synthesis capacity, and enhanced ROS generation (7). In addition, the accumulation of lipotoxic intermediates may promote inflammation and alter insulin signaling, facilitating the establishment of an insulin resistance state (5, 8).

Further topics of major interest regard the interference with pathways associated to recognition receptors including Toll-like receptors (TLRs) and NOD-like receptors (NLRs) (9), and downstream signaling involving peroxisome proliferator activating ligand receptors (PPAR), sterol regulatory element binding protein 1c (SREBP-1c), nuclear factor kappa-light-chain enhancer of activated B cells (NF-kB), and NADPH oxidase 2 (NOX2) (1, 10, 11).

In an "hormonocentric" view of NAFLD pathogenesis, all these processes may be triggered by circulating factors (12), that may represent diagnostic or prognostic biomarkers.

Based on these premises, we aimed to evaluate whether factors circulating in the blood of NAFLD patients may modulate one or more of the above-mentioned processes. Hence, in the present study we examined the effects of plasma of NAFLD patients on the viability and function of hepatocytes.

MATERIALS AND METHODS

Patients and Controls

Experiments were conducted between October 2018 and March 2019 using plasma from 12 NAFLD patients and 12 healthy subjects. The use of excess plasma remaining from blood samples taken for the routinary clinical monitoring of NAFLD patients has been approved by the local Ethics Committee (Ethical Committee of the "Azienda Ospedaliera Maggiore della Carità" University Hospital in Novara). Patients and controls have given written informed consent for experimental use of pseudonymized clinical data and blood specimens. The work was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki).

The clinical monitoring and plasma sampling of patients and controls were performed at Liver Clinic Unit, "Azienda Ospedaliera Maggiore della Carità" University Hospital in Novara.

Culture of Huh7.5 Cells

The human hepatocellular carcinoma cell line Huh7.5 (Apath L.L.C New York, USA) (13) was maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma, Milan, Italy) supplemented with 10% fetal bovine plasma (FBS; Euroclone, Pero, Milan, Italy), 2 mM L-glutamine (Euroclone), 1% penicillin-streptomycin (P/S; Euroclone), at 37°C with 5% CO_2 in incubator.

Isolation of Hepatocytes From Human Liver Biopsy Specimens

From October 2018 until November 2019, 10 liver samples (i.e., 50–100 g each) were obtained from patients undergoing surgery at General Surgery Unit, Azienda Ospedaliera Maggiore della Carità University Hospital, in Novara, due to primary or metastatic liver resectable tumor.

The specimens were acquired from the non-neoplastic portion of the liver parenchyma resected during the surgery. All tissue donors gave written informed consent for experimental use of pseudonymized clinical data and liver specimen prior to surgery. Then fresh samples were transferred on ice cold physiologic saline solution to Physiology laboratory, and hepatocytes were immediately isolated, as previously performed (14, 15). Briefly, liver specimens were minced with a scalpel and undergone a two steps collagenase procedure; the cell pellet was, then, centrifugated at 300 g for 5 min and the cell pellet was filtered through a nylon mesh. Thereafter, hepatocytes were pooled

and seeded into plates coated with collagen-I (Sigma), and maintained in DMEM/HAM'S F-12 (Euroclone) supplemented with 10% FBS (Euroclone), 100 U/ml penicillin (Sigma), 0.1 mg/ml streptomycin (Sigma), and 2 mM L-glutamine (Sigma). Finally, they were transferred into 75-cm^2 culture flasks (Euroclone) in incubator under standard conditions. The culturing was performed until passage 15 (16).

Experimental Protocol

To evaluate the effects of plasma samples taken from NAFLD patients and healthy subjects, on cell viability (MTT Assay), mitochondrial membrane potential (JC-1 Assay), H_2O_2 release (ROS-Glo H_2O_2,) mitochondrial ROS (mitoROS) release, triglycerides content (Triglyceride assay) and protein expression (Western Blot) in Huh7.5 cells/primary human hepatocytes, co-culture experiments were performed, by using specific Transwell inserts (**Supplementary Figure 1A**).

For the experiments, plasma samples from 12 NAFLD patients and 12 healthy subjects were plated in the apical compartment of the insert and left to act for 3 h, while, Huh7.5 cells/primary human hepatocytes were plated in the basal compartment. The 3 h time limit for stimulation was chosen based on preliminary time-course experiments during which Huh7.5 cells were exposed to plasma for 3, 12, and 24 h, showing that cell viability was drastically reduced already at 12 h. In addition, preliminary experiments were performed on both Huh7.5 cells and primary human hepatocytes with 5, 10, and 20% plasma calculated in relation to the total volume of each insert. In those experiments the effects of plasma from NAFLD patients and healthy subjects on cell viability and H_2O_2 release were measured (**Supplementary Figure 1B**). The results obtained allowed us to select the proper plasma concentration to be used for all next experiments performed on Huh7.5 cells. Furthermore, in some experiments, 1 nM inflammasome NLRP3 inhibitor (MCC950) was administrated to Huh7.5 cells for 30 min alone or before 200 pM TNFα (for 3 h) in co-stimulation with plasma. Some samples of Huh7.5 cells and primary human hepatocytes were not treated with plasma and were used as "control." At the end of stimulations, various assays were performed (**Supplementary Figure 1A**). All experiments were conducted in triplicate and repeated at least five times.

1) Cell Viability

Cell viability was examined in Huh7.5 cells and primary human hepatocytes by using the 1% 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT; Life Technologies Italia, Monza, Italy; catalog number CT02) dye, as previously performed (17–21) and described in **Supplementary Material**. Cell viability was determined by measuring the absorbance through a spectrometer (VICTOR™ X Multilabel Plate Reader; PerkinElmer) with a wavelength of 570 nm and cell viability was calculated by setting control cells as 100%.

Mitochondrial Membrane Potential Measurement

Mitochondrial membrane potential measurement in Huh7.5 cells was performed with JC-1 assay (17, 19–22). The detailed description of methods is reported in **Supplementary Material**. The mitochondrial membrane potential was determined by measuring the red (excitation 550 nm/emission 600 nm) and green (excitation 485 nm/emission 535 nm) fluorescence through a spectrometer (VICTOR™ X Multilabel Plate Reader; PerkinElmer). The data were normalized vs. control cells.

2) Mitochondrial ROS Quantification

MitoROS production was determined through the Cayman's Mitochondrial ROS Detection Assay Kit (Cayman Chemical; catalog number 701600), as previously performed (23) and described in **Supplementary Material**. The MitoROS production was measured with an excitation and emission wavelength of 480 and 560 nm, respectively, by using a spectrophotometer (VICTOR™ X Multilabel Plate Reader; PerkinElmer). The data were normalized vs. control cells.

3) ROS-Glo H_2O_2 Quantification

H_2O_2 production was determined by the ROS-Glo H_2O_2 Assay, following the manufacturer's instructions (Promega Corporation; Padova, Italy; catalog number G8820) (24, 25). The detailed description of methods is reported in **Supplementary Material**. The H_2O_2 production was quantified as relative luminescence by using a spectrophotometer (VICTOR™ X Multilabel Plate Reader; PerkinElmer). The data were normalized vs. control cells.

4) Triglycerides Quantification

Triglycerides measurement was performed with a specific kit (Cayman Chemical; catalog number 10010303) and as described in **Supplementary Material**. The triglycerides content was detected following the manufacturer's instructions through a spectrometer (VICTOR™ X Multilabel Plate Reader; PerkinElmer) at excitation/emission wavelengths of 530–550 nm (22). The value of each sample was quantified in respect to triglycerides standard curve and expressed as triglycerides content (mg/dl).

5) Cell Lysates

For protein expression/activation, Huh7.5 cells were stimulated as described for various assays. For the experiments, 400000 Huh7.5 cells/insert in 6-Transwell plate were plated. At the end of stimulation, Huh7.5 cells were lysed in iced Ripa buffer supplemented with sodium orthovanadate (2 mM; Sigma) and protease inhibitors cocktail (1 mM; Thermo Fisher Scientific) and phenylmethanesulfonyl fluoride (1 mM; Sigma). The extracted proteins were quantified through bicinchoninic acid protein (BCA, Pierce) and used for electrophoresis and immunoblotting studies (16–22). Due to shortage of plasma specimens, experiments were conducted with plasma taken from three NAFLD patients and three healthy subjects and were repeated at least three times.

Western Blot Analysis

Cell lysates (30 μg protein each sample) were dissolved in 5× Laemmli buffer, boiled for 5 min and resolved in 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis gels (Bio-Rad Laboratories). After electrophoresis they were transferred to polyvinylidene fluoride membranes (Bio-Rad

Laboratories) and incubated overnight at 4°C with specific primary antibodies: anti Nf-kB (p-50; 1:1,000; Santa Cruz Biotechnology, catalog number sc-8414), anti gp91-phox (NOX2; 1:1,000; Santa Cruz Biotechnology, catalog number sc-130543), anti PPAR-γ (1:1,000; Santa Cruz Biotechnology, catalog number sc-271392), anti SREBP-1c (1:1,000; Santa Cruz Biotechnology, catalog number sc-13551). The membranes were washed and then incubated with horseradish peroxidase-coupled goat anti-rabbit IgG (Sigma), peroxidase-coupled rabbit anti-goat IgG and horseradish peroxidase-coupled goat anti-mouse IgG (Sigma) for 45 min and were developed through a non-radioactive method using Western Lightning Chemiluminescence (PerkinElmer Life and Analytical Sciences). Phosphorylated protein expression was calculated as a ratio toward β-actin (1:5,000; Santa Cruz Biotechnology; catalog number sc-47778) detection.

Statistical Analysis

Statistical analysis was performed using STATVIEW version 5.0.1 for Microsoft Windows (SAS Institute Inc., Cary NC, USA). Data were checked for normality before statistical analysis. All data are presented as means ± standard deviation (SD) of five independent experiments for each experimental protocol. Differences between groups were analyzed by one-way ANOVA and Bonferroni *post hoc* tests. The threshold for statistical significance was 0.05 (two-tails).

RESULTS

NAFLD patients were seven males and five females, aged 51 ± 14 years, had a body mass index (BMI) of 31.1 ± 4.0 kg/m^2, plasma alanine aminotransferase (ALT) concentration of 43 ± 29 U/L, plasma total cholesterol of 182 ± 30.4 mg/dl, liver stiffness and a controlled attenuation parameter measured by FibroScan® of 7.1 ± 1.9 kPa and 297 ± 45 dB/m, respectively. Healthy subjects were seven males and five females, aged 24.2 ± 0.4 year, had a BMI 22.9 ± 2.9 kg/m^2, plasma ALT concentration of 23 ± 4 U/L, plasma total cholesterol of 167 ± 19 mg/dl, liver stiffness and a controlled attenuation parameter measured by FibroScan® of 5.4 ± 1 kPa and 186 ± 27 dB/m, respectively. Significant differences were observed in age, BMI, ALT, controlled attenuation parameter and liver stiffness measured between NAFLD and non-NAFLD patients ($p < 0.05$).

A dose response study was executed in order to choose the proper plasma concentration for the *in vitro* experiments. As shown in **Supplementary Figure 2**, plasma from NAFLD patients was able to reduce cell viability and increase H_2O_2 release in both Huh7.5 cells and primary human hepatocytes. In addition, a grading response was seen by using 5–20% plasma. Indeed, as regarding primary human hepatocytes viability, it amounted to 25, 34, and 43% about, in cells treated with 20, 10, and 5% NAFLD plasma, respectively. At the same time ROS release increased nearly by 100, 76, and by 56%. Moving on Huh7.5 cells, cell viability amounted to 34, 43, and 53% about, in cells treated with 20, 10, and 5% NAFLD plasma, respectively, whereas ROS release increased nearly by 80, 60, and 40%. Although the highest effects were observed after the treatment with the 20% plasma concentration, we decided to choose the 5% plasma concentration for all subsequent experiments in order to save plasma. As described in **Figure 1**, in Huh7.5 cells, plasma of NAFLD patients was able not only to reduce the cell viability but also mitochondrial membrane potential, by 47.25 ± 13.2% and 23.5 ± 0.8%, respectively. The finding of a reduction of mitochondrial membrane polarization would confirm the harmful effects elicited by NAFLD plasma. It is to note that those effects were accompanied by an increase not only of H_2O_2 release amounting to 45 ± 1.3%, but also of mitoROS release of 20.2 ± 0.7%. In addition, an increase of triglycerides content of 27.8 ± 2.1% was observed, as well.

In the presence of MCC950, the NLRP3 inflammasome inhibitor, the effects of NAFLD plasma on Huh7.5 cells were counteracted. Hence, cell viability and mitochondrial membrane potential were increased in comparison with findings obtained in Huh7.5 cells treated with NAFLD plasma alone (**Figures 1A,B**). Moreover, ROS and mitoROS release were reduced, as well as, the triglycerides content (**Figures 1C–E**).

It is to note that all the effects of NAFLD plasma on Huh7.5 cells were higher than those caused by plasma from healthy subjects (**Figure 2**). Also, TNFα was able to reduce cell viability, mitochondrial membrane potential, to increase triglycerides content and promote hydrogen peroxide and mitoROS release in a stronger way in the presence of NAFLD plasma than healthy subjects' plasma. The NLRP3 inflammasome inhibitor, MCC950, was so effective in preventing the deleterious effects of NAFLD plasma that no differences could be observed between NAFLD plasma and healthy subjects' plasma, as regarding mitochondrial membrane potential, triglycerides content and mitoROS release.

Western blot analysis performed on Huh7.5 cells treated with NAFLD plasma at 5% concentration showed the involvement inflammatory pathways and of an intracellular signaling related to lipid accumulation in liver during NAFLD. Indeed, in Huh7.5 cells treated with plasma of NAFLD patients, an increase in the expression of NF-kB, NOX2, PPARγ and SREBP-1c of 28.7 ±14.8%, 31,8 ± 16,1%, 55 ± 13%, 34,7 ± 13%, respectively, was observed (**Figures 3**, **4**).

In our experiments we used TNFα as positive "inflammatory" control to stimulate Huh7.5 cells treated with plasma of both NAFLD patients and healthy subjects. As observed in previous experiments, also in Western blot analysis all effects of plasma of NAFLD patients were potentiated by TNFα and reduced by MCC950 (**Figures 1**–**4**).

In addition, all the above effects observed in Huh7.5 cells treated with plasma of NAFLD patients were accompanied by an increase in the expression of NF-kB, NOX2, PPARγ and SREBP-1c of 28.7 ±14.8%, 31,8 ± 16,1%, 55 ± 13%, 34,7 ± 13%, respectively (**Figures 3**, **4**). All effects of plasma of NAFLD patients were potentiated by TNFα and reduced by MCC950 (**Figures 1**–**4**).

DISCUSSION

The present paper documents that exposure of hepatocytes to plasma from NAFLD patients was able to affect cell viability

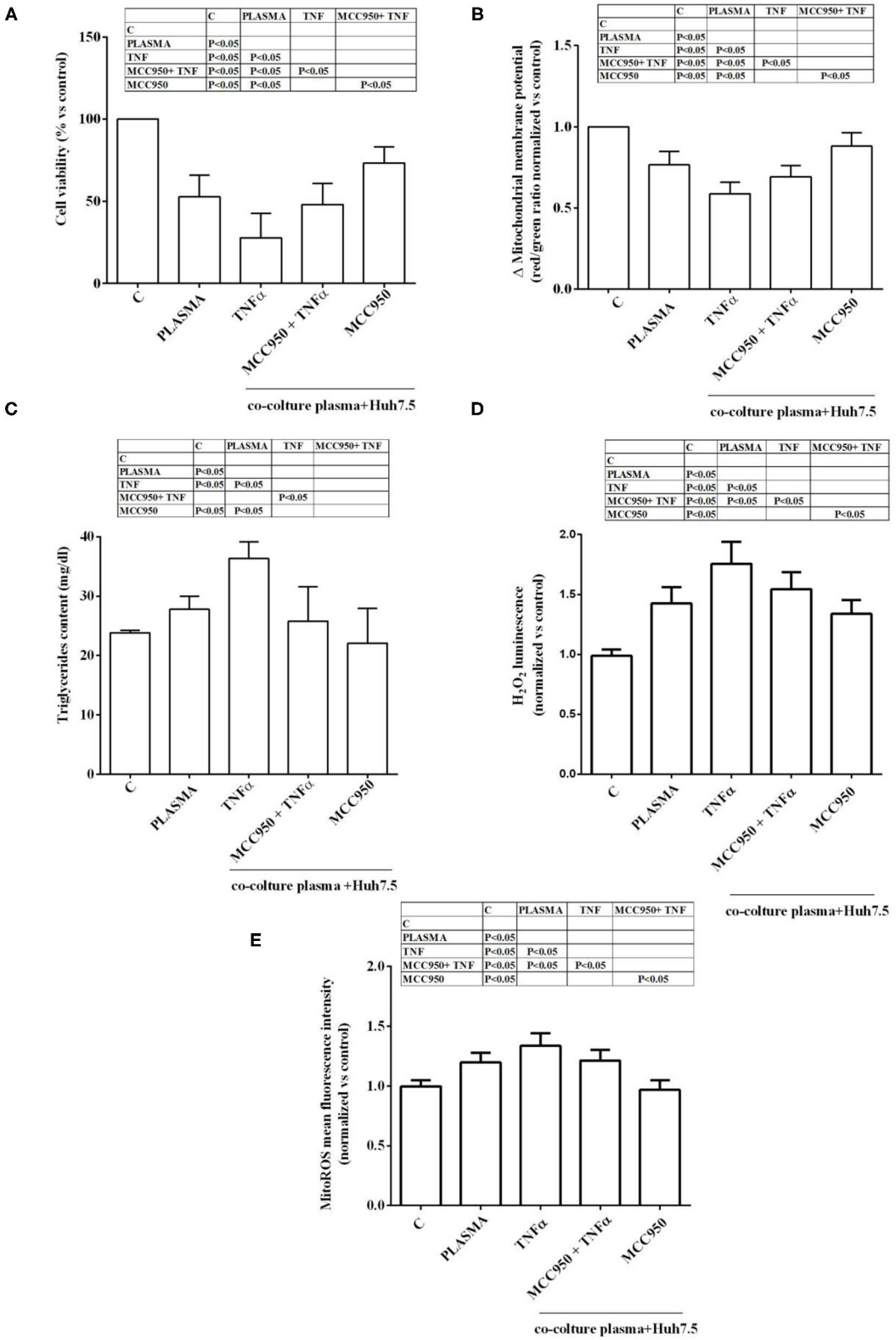


FIGURE 1 | Effects of NAFLD plasma on Huh7.5 cell viability **(A)**, mitochondrial membrane potential **(B)**, triglycerides content **(C)**, H_2O_2 release **(D)** and mitochondrial ROS (mitoROS, **E**). C=non-treated cells. MCC950 (NLRP3 inflammasome inhibitor, 1 nM for 30 min); TNFα (200 pM for 3 h). Reported data are means ± SD of five independent experiments for each experimental protocol.

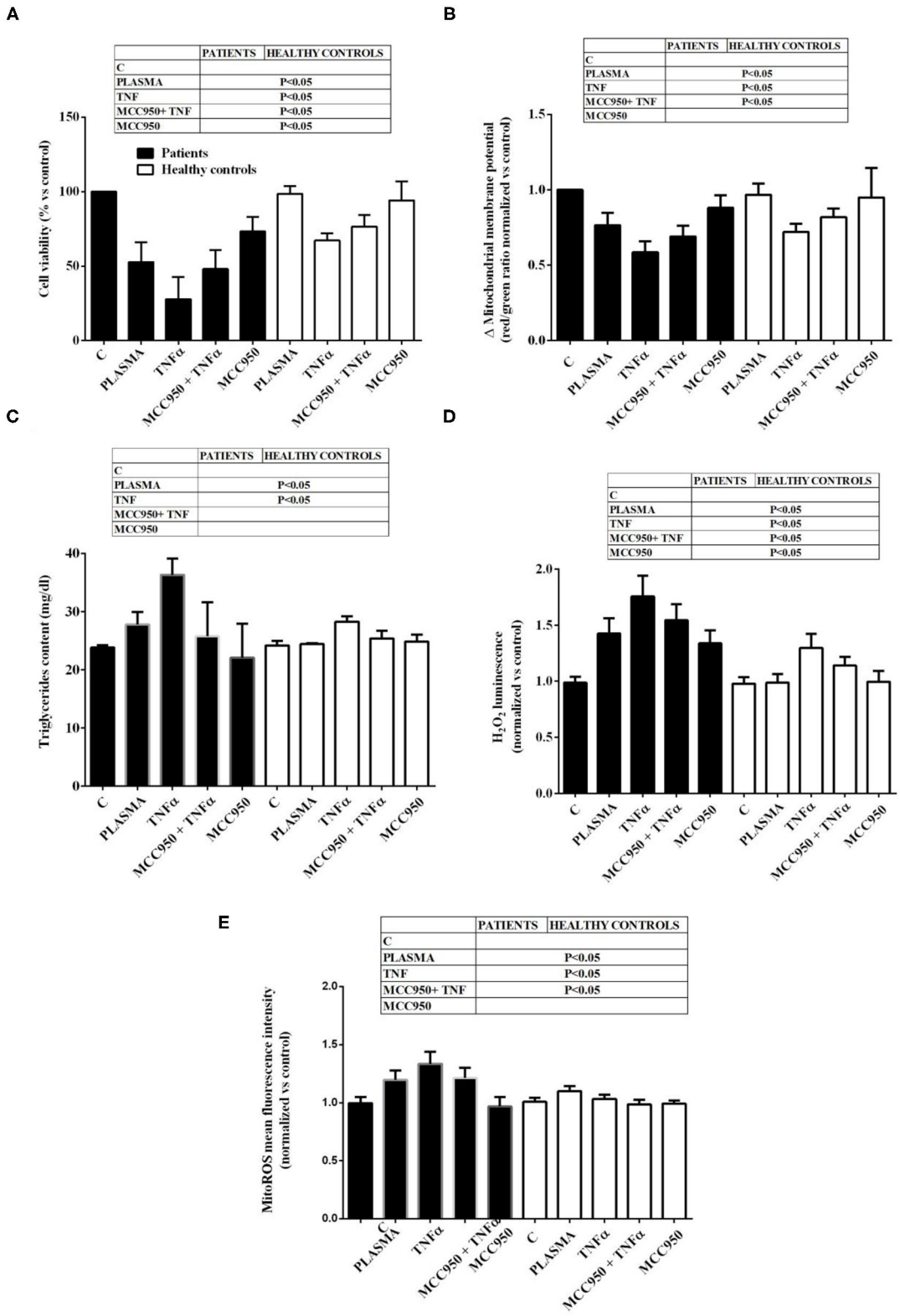
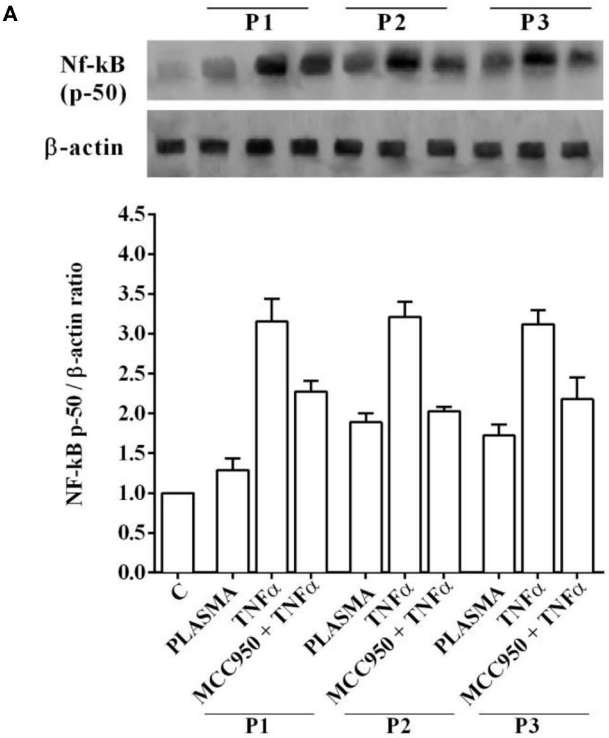
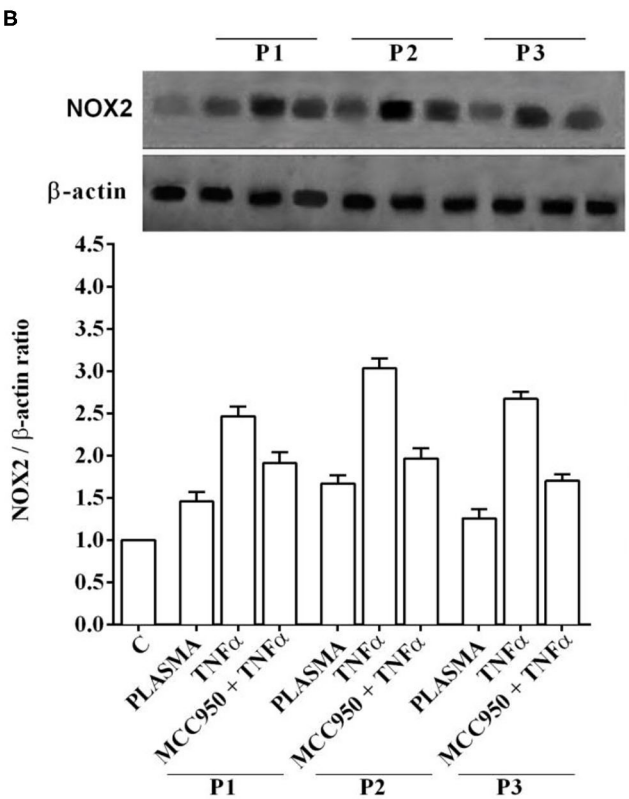


FIGURE 2 | Comparison between the effects of NAFLD plasma and of plasma from healthy subjects on Huh7.5 cell viability **(A)**, mitochondrial membrane potential **(B)**, triglycerides content **(C)**, H_2O_2 release **(D)** and mitochondrial ROS (mitoROS, **E**). Abbreviations are as described in **Figure 1**. Reported data are means ± SD of five independent experiments for each experimental protocol.

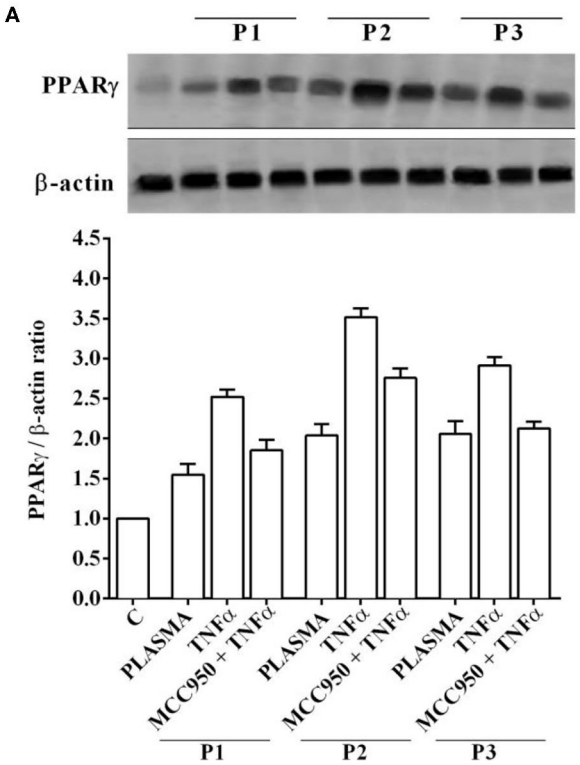


		C	PLASMA	TNF	MCC950+TNF
P1	PLASMA	P<0.05			P<0.05
	TNF	P<0.05	P<0.05		
	MCC950+TNF	P<0.05	P<0.05	P<0.05	
P2	PLASMA	P<0.05			
	TNF	P<0.05	P<0.05		P<0.05
	MCC950+TNF	P<0.05			
P3	PLASMA	P<0.05			P<0.05
	TNF	P<0.05	P<0.05		
	MCC950+TNF	P<0.05	P<0.05	P<0.05	

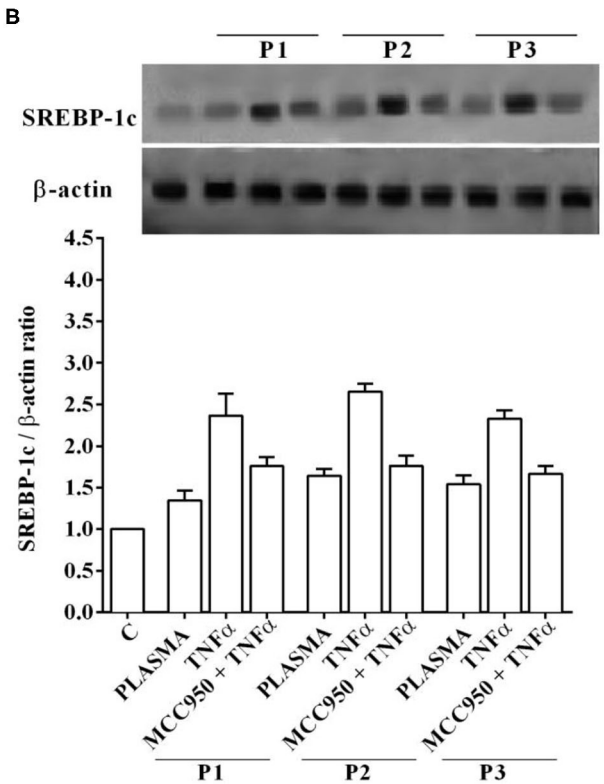


		C	PLASMA	TNF	MCC950+TNF
P1	PLASMA	P<0.05			P<0.05
	TNF	P<0.05	P<0.05		
	MCC950+TNF	P<0.05	P<0.05	P<0.05	
P2	PLASMA	P<0.05			
	TNF	P<0.05	P<0.05		P<0.05
	MCC950+TNF	P<0.05	P<0.05	P<0.05	
P3	PLASMA	P<0.05			p<0.05
	TNF	P<0.05	P<0.05		
	MCC950+TNF	P<0.05	P<0.05	P<0.05	

FIGURE 3 | Effects of NAFLD plasma on NF-kB **(A)** and NOX2 **(B)** expression in Huh7.5 cells. P, patient; M, MCC950 (1 nM, for 30 min). Other abbreviations are as described in previous Figures. In the densitometric analysis, all values are normalized vs. control which is normalized, as well, and considered as 1. They are shown as fold changes vs. control. Reported data are means ± SD of five independent experiments for each experimental protocol.



		C	PLASMA	TNF	MCC950+TNF
P1	PLASMA	P<0.05			P<0.05
	TNF	P<0.05	P<0.05		
	MCC950+TNF	P<0.05	P<0.05	P<0.05	
P2	PLASMA	P<0.05			
	TNF	P<0.05	P<0.05		P<0.05
	MCC950+TNF	P<0.05	P<0.05	P<0.05	
P3	PLASMA	P<0.05			P<0.05
	TNF	P<0.05	P<0.05		
	MCC950+TNF	P<0.05		P<0.05	



		C	PLASMA	TNF	MCC950+TNF
P1	PLASMA	P<0.05			P<0.05
	TNF	P<0.05	P<0.05		
	MCC950+TNF	P<0.05	P<0.05	P<0.05	
P2	PLASMA	P<0.05			
	TNF	P<0.05	P<0.05		P<0.05
	MCC950+TNF	P<0.05			
P3	PLASMA	P<0.05			P<0.05
	TNF	P<0.05	P<0.05		
	MCC950+TNF	P<0.05	P<0.05	P<0.05	

FIGURE 4 | Effects of NAFLD plasma on PPARγ **(A)** and SREBP-1c **(B)** expression in Huh7.5 cells. P, patient; M, MCC950 (1 nM, for 30 min). Other abbreviations are as described in previous Figures. In the densitometric analysis, all values are normalized vs. control which is normalized, as well, and considered as 1. They are shown as fold changes vs. control. Reported data are means ± SD of five independent experiments for each experimental protocol.

and to modulate intracellular pathways *in vitro* in the direction predicted by current hypotheses on NAFLD pathogenesis.

In recent years, several *in vitro* model of NAFLD of increasing complexity have been proposed, from culture of a single cell type to 3D cocultures and body-on-a-chip systems (26). Human hepatoma cell lines are easy to maintain and may be apt for multiparametric evaluation of steatosis (27), though their enzymatic profile may differ significantly from that of normal hepatocytes. On these premises, we decided to set a system in which human hepatoma cells could be maintained at ease varying conditions in which they were grown by using specific stimulant and inhibitor molecules. It should be of particular interest and represent a touch of originality the use of plasma for treating hepatocytes, Hence, almost all previous studies have been performed by simulating an *in vitro* condition of NAFLD milieu, using mixed FFAs solutions. Here, we aimed to examining the role of any circulating factors playing a pathophysiological role in the onset of NAFLD. The Huh7.5 cell line that we employed in our experiments has gained popularity for being highly permissive for hepatitis C virus replication *in vitro* (28). Moreover, some experiments have been performed in human primary hepatocytes, as well, to set up the optimal experimental conditions and to confirm the results obtained in Huh7.5 cells.

It is to note that the method employed for hepatocytes isolation is the one that was reported to result in hepatocytes populations of high purity with retained physiological activity *in vitro* (14).

In both Huh7.5 cells and human primary hepatocytes we tested 5, 10, and 20% NAFLD and non-NAFLD plasma samples on cell viability and H_2O_2 release, so that we could select the proper plasma concentration to be used in all other experiments performed on Huh7.5 cells only. To culture medium of Huh7.5 cells we also added TNFα, to reproduce *in vitro* conditions mimicking the steatohepatitis milieu (29), where TNFα plays a major role (30).

Furthermore, experiments were conducted in the presence or absence of the potent and selective inhibitor MCC950, which targets directly the NLRP3 ATP-hydrolysis motif for inflammasome inhibition (31).

Regarding the loss of cell viability that we observed after exposure to NAFLD plasma, it was presumably due to the differences in triglycerides content and oxidants release observed in Huh7.5 cells. It is to note that H_2O_2 and mitoROS have widely been considered as main contributors to liver injury and disease progression in NAFLD (32–35).

These effects were amplified in the presence of TNFα and reduced by the pretreatment with MCC950. It is noteworthy that in similar *in vitro* models, TNFα appears to mediate an increase of mitochondrial Ca^{2+} leading to production of ATP and ROS, with the shedding of TNF receptor 1 (which acts as a decoy) limiting the propagation of the inflammatory response (36). Our results would indicate the potentiation by TNFα of the harmful effects elicited by not yet clarified "inflammatory" factors circulating in the plasma of NAFLD patients, a mechanism that was counteracted by the NLRP3 inflammasome inhibition.

Following the "multiple-hit" hypothesis, hepatic lipid overload would induce the overproduction of oxidants that could be detrimental for DNA, lipids and proteins and lead to the accumulation of the so called "damage-associated molecular pattern" (DAMPS), which would induce liver injury. In addition, the impairment of the electrons transfer chain and the fall of mitochondrial membrane potential could be followed by cell death (35).

Mitochondrial membrane potential is essential for cells, enabling them to store energy: a long-lasting drop of mitochondrial potential is deleterious to cell viability, possibly more because of interference by products of ATP hydrolysis than by ATP in itself. About this issue it is to note that the increased mitoROS release we have observed was accompanied by the fall of mitochondrial membrane potential in Huh7.5 cells.

Interestingly, we also found an increase of SREBP-1c and PPARγ expression, which are involved in the intracellular triglycerides accumulation (1) and an increase of NF-kB and NOX2. As concerning SREBP-1c, it has been found to be involved in mitochondrial oxidants release through AQP8 upregulation (36). NF-kB is responsible for the transcription of pro-inflammatory molecules; among other stimuli like SREBP-1c (37). Also, NOX enzymes, especially NOX1, NOX2, and NOX4, have been associated with liver injuries in NAFLD both *in vivo and in vitro*, through the increased ROS release and the reciprocal interaction between different NOX enzymes and mitochondria (38).

The data we have obtained with MCC950 highlight the involvement of NLRP3 inflammasome in eliciting the harmful effects of NAFLD plasma in Huh7.5 cells. Our findings are in agreement with previous observations about the role of NOD-like receptors in NAFLD pathogenesis. In fact, the release by dying hepatocytes of DAMPS can be detected by members of NOD-like receptors, such as the NLRP3 inflammasome. Its activation is followed by cleavage of pro-caspase-1 into active caspase-1, which, in turn, cleaves pro-IL-1β into mature IL-1β (39, 40). Overall, the activation of NLRP3 inflammasome may potentiate a proinflammatory condition, resulting into stellate cells activation and transformation in a myofibroblastic phenotype. The fact however, that the only some effects of NAFLD plasma were abolished by MCC950, whereas others were just reduced, would suggest, on the one side, the important role played by NLRP3 inflammasome, and on the other side, the existence of other mechanisms which could trigger the damage. As also previously reported and confirmed by our results, intracellular pathways related to SREBP-1c and PPARγ could be involved. In addition, the modulation of inflammatory/anti-inflammatory cytokines, like interleukin 6 or adiponectin, could play a role and could be object of future studies.

Interestingly, the present data suggest the existence of an association between the activation of the NLRP3 inflammasome and that of intracellular pathways associated with SREBP-1c, PPARγ, NOX2 and NF-kB. In the presence of the inhibitor MCC950, the expression of all the above proteins was reduced in Huh7.5 cells exposed to NAFLD plasma. Data about SREBP-1c are also in agreement with previous findings in HepRG cells (41). Thus, it could be hypothesized a role for the NLRP3 inflammasome in the regulation of triglycerides accumulation via involvement of SREBP-1c and PPARγ, and in the onset

of oxidative stress. Concomitantly or alternatively, the NLRP3 inflammasome may be responsible for oxidants release through signaling involving NOX2 and NF-kB. Future studies could be organized aimed at the analysis of other intracellular pathways related to all the above pathways and their relationships. In this context, it would be of interest to examine the role of AMP-activated protein kinase (AMPK), which is crucial for the regulation of fat mebabolism in liver.

One limitation of the present study is that NAFLD patients and healthy subjects were different as regarding BMI and age. This bias is somewhat inevitable, since NAFLD and NAFLD progression are strongly related to BMI and age. Moreover, sample size could be increased. The hepatoma cell line we used is not fully comparable to those of normal hepatocytes, though preliminary experiments performed on primary human hepatocytes (data not shown) confirmed the results obtained regarding cell viability and oxidant release. One may also argue that the model does not allow the identification of any specific pathogenic factor(s) and/or of putative biomarker(s); on the other hand, it has the advantage of being free of bias on the kind of substances involved. As said, our aim was to examine the role of any circulating factor possibly involved in the onset of NAFLD without venturing in the attempt of identifying all possibleculprits. Indeed, this is the first study performed by using NAFLD plasma on Huh7.5 cells and aimed at the analysis of cell viability, mitochondrial function and oxidants release. In addition, the role of main pathways involved in NAFLD onset has been investigated by performing experiments in the presence of an inhibitor and through Western Blot. The findings of harmful effects elicited by NAFLD plasma on hepatocytes would represent the starting point for the execution of future studies aimed at deepening the aforementioned issues. In particular, the application of proteomic and metabolomic profiling technologies to NAFLD plasma might contribute to identify any circulating factor(s) (microRNA, extracellular vesicles), whereas, *in vitro* experiments could be organized to increase the knowledge about the intracellular mechanisms (SREBP-1c, PPARγ, inflammasome molecules, AMPK).

CONCLUSIONS

In summary, we have established an *in vitro* model in which exposure to NAFLD plasma, in agreement with what predicted by "hormonocentric" theories on NAFLD pathogenesis (12, 42) resulted in loss of cell viability and activation of pathways known to be involved in inflammation and tissue damage. Substances present in NAFLD plasma could have affected lipid metabolism of Huh7.5 cells: triglycerides accumulation activated as downstream signaling involving SREBP-1c and PPARγ may have acted as a starting point. Hence, accumulation of triglycerides may have hampered mitochondrial function either directly or through increased oxidants release, NOX2 and NLRP3 inflammasome-related pathways.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethical Committee of the Azienda Ospedaliera Maggiore della Carità University Hospital in Novara. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

EG and MP: concenptualization and funding acquisition. DG, RM, RR, CR, CS, and MB: methodology and investigation. DS, MB, and MP: resources. DG, GC, CR, CS, and MB: formal analysis. EG, DG, GC, RR, CR, RM, CS, DS, MB, and MP: supervision, validation, visualization, writing/review, and editing. EG, GC, and MP: writing/original draft preparation. All authors involved in editing the paper and had final approval of the submitted and published versions.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.693997/full#supplementary-material

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Helicobacter pylori Eradication Therapy Affect the Gut Microbiota and Ghrelin Levels

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Background: Antibiotic therapy used to eradicate *Helicobacter pylori* has been associated with changes in plasma ghrelin and alterations in the gut microbiota. On the other hand, changes in ghrelin levels have been related to changes in gut microbiota composition. Our aim was to evaluate the relationship between changes in the gut microbiota and ghrelin levels in *H. pylori* infected patients who received antibiotic treatment for its eradication.

Methods: A prospective case-control study that included forty *H. pylori*-positive patients who received eradication therapy (omeprazole, clarithromycin, and amoxicillin) and twenty healthy *H. pylori* antigen-negative participants. Patients were evaluated, including clinical, anthropometric and dietary variables, before and 2 months after treatment. Gut microbiota composition was analyzed through 16S rRNA amplicon sequencing (IlluminaMiSeq).

Results: Changes in gut microbiota profiles and decrease in ghrelin levels were identified after *H. pylori* eradication treatment. Gut bacteria such as *Bifidobacterium longum*, *Bacteroides, Prevotella*, *Parabacteroides distasonis*, and *RS045* have been linked to ghrelin levels fasting and/or post meals. Changes in the abundance of *Lachnospiraceae*, its genus *Blautia*, as well as *Prevotella stercorea*, and *Megasphaera* have been inversely associated with changes in ghrelin after eradication treatment.

Conclusions: Eradication treatment for *H. pylori* produces changes in the composition of the intestinal microbiota and ghrelin levels. The imbalance between lactate producers such as *Blautia*, and lactate consumers such as *Megasphaera*, *Lachnospiraceae*, or *Prevotella*, could trigger changes related to ghrelin levels under the alteration of the eradication therapy used for *H. pylori*. In addition, acetate producing bacteria such as *B. longum*, *Bacteroides*, and *P. distasonis* could also play an important role in ghrelin regulation.

Keywords: *Helicobacter pylori*, gut microbiota, ghrelin, eradication treatment, antibiotic

INTRODUCTION

Ghrelin, known as the hunger hormone, is predominantly produced in the stomach (1), but it is also produced in other organs such as the pituitary, hypothalamus, adrenal gland, placenta, and pancreas (2, 3) as well as the small and large intestines (4). *Via* binding to the growth hormone secretagogue receptor 1a (GHSR1a), ghrelin performs multiple physiological functions, including the stimulation of food intake, growth hormone secretion (GH) (5), adiposity (6), gastric motility, acid secretion (7), and insulin secretion inhibition (8). Serum ghrelin concentrations increase during fasting (when the stomach is empty), and decrease after eating (9). Moreover, ghrelin levels have been related to body mass index (BMI) in a complicated manner, even showing a certain level of ghrelin-resistance (6, 10).

The gastrointestinal microbiota have the potential to modulate the energy metabolism by altering hormone levels affecting their secretion capacities (11) or through the intestine-brain axis (12). The interaction between the microbiome and ghrelin was corroborated by findings showing that germ-free mice have different ghrelin concentrations than conventionalized mice (13). Experimental studies revealed changes in circulating ghrelin levels linked to changes in gut microbiota composition, suggesting that the ghrelinergic system may be under regulation of the gut commensal microbes (14).

Helicobacter pylori is known to infect the gastric mucosa, induce inflammation, and alter both gastric and intestinal microbiota resulting in a broad spectrum of alterations, including metabolic syndrome-related disorders (15). At the same time, the therapeutic strategies used to eradicate *H. pylori* have also been associated with alterations in the gut microbiota (16). However, there are currently only several studies relating alterations in the gut microbiota by *H. pylori* and its eradication with changes in metabolic parameters. Our group have previously published alterations in the microbiota due to *H. pylori* infection and eradication therapy, and more importantly, these alterations have been related to glucose metabolism (17), GLP-1 levels (18), and blood lipid levels (19).

H. pylori eradication with antimicrobials has been associated with changes in plasma ghrelin (20–22), and weight gain (23). Moreover, *H. pylori* may alter ghrelin levels through the induction of damage to endocrine hormone-producing cells from the gastric mucosa (24), but also through the alteration of the gut microbiota composition (25). However, the relationship between gut microbiota and ghrelin levels after *H. pylori* therapy has been little explored (26). Accordingly, our objective was to study the relationship between the gut microbiota and ghrelin levels in *H. pylori* infected patients who received antibiotic treatment for its eradication.

MATERIALS AND METHODS

Study Subjects and Design

Forty patients with positive *H. pylori* antigen in stool determined by immunochromatography and 20 healthy adults (control group) were recruited. The inclusion criteria were: (1) age 18–65 years, and (2) first *H. pylori* infection (for positive *H. pylori*). Exclusion criteria were (1) type 1 or 2 diabetes diagnosis; (2) prior documented treatment of *H. pylori*; (3) antibiotic use within 3 months previous to enrollment; (4) informed consent could not be obtained; (5) eradication therapy failure. Sample size was assessed considering a reduction in richness of 16% because of the antibiotic therapy based on previous microbiota studies (27, 28) and a pilot study (non-published).

The study included two visits, before and 2 months after treatment (omeprazole 20 mg, clarithromycin 500 mg, amoxicilin 1,000 mg twice daily for 10 days) for patients and only one visit for the control group. All visits included a physical examination, a fasting blood sample, and a 75 g oral glucose tolerance test (OGTT) at 30, 60, and 120 min.

Also, stool samples were collected during each visit and frozen at −80°C until DNA extraction. The study protocol was approved by the Medical Ethics Committee at Virgen de la Victoria University Hospital and conducted in accordance with the Declaration of Helsinki. Written informed consent was provided by all participants, who also were verbally informed of the characteristics on the study.

Anthropometric, Biochemical, and Dietetics Measurements

Body weight, height, and waist circumferences were measured according to standardized procedures (29). Total cholesterol (mg/dl), high-density lipoprotein (HDL) cholesterol (mg/dl), triglycerides (mmol/L), and serum glucose were measured using a standard enzymatic method (Randox Laboratories Ltd.). Low-density lipoprotein (LDL) cholesterol (mg/dl) was calculated by using the Friedewald formula. Plasma insulin (pmol/L) was assessed by electrochemiluminescence (E170 module, Roche Diagnostics). Plasma glucagon-like peptide-1 (GLP-1) (ng/mL) and total ghrelin levels (pg/mL) were measured manually using commercial kits (human GLP-1 EIA Kit, GENTAUR Belgium, Kampenhout, Belgium, and Human Ghrelin Fluorescent EIA Kit, Phoenix Pharmaceuticals, Burlingame, CA, USA, respectively) and expressed in ng/mL and pg/mL, respectively. The area under the ghrelin curve (ghrelin AUC) (pg*min/mL) was calculated from the serum ghrelin concentrations at time points 0, 30, 60, and 120 min after the oral glucose tolerance test, using the trapezoidal rule. Food intake was evaluated by using seven 24-h dietary recalls for case and control groups. Total energy (kcal/day), macronutrients [proteins, fats, total carbohydrates, dietary fiber, and sugars (g/day)] and micronutrients [total polyphenols (mg/day)] for each participant were obtained using DIAL© nutrition program and the professional Diet Balancer software (Cardinal Health Systems Inc.).

Gut Microbiota Analysis

The determination of the microbiota has been described in detail in a previous study (17). Briefly, the fecal bacterial microbiota composition was determined using tag-encoded 16S rRNA gene Miseq-based (Illumina, CA, USA) high throughput sequencing. The 16S rRNA V3-V4 amplicon (amplicon size ∼460 bp) was amplified by polymerase chain reaction (PCR) using the universal primers reported by Klindworth et al. (30). Dual indices and Illumina sequencing adapters were attached to sequence the

amplicons, using the Nextera XT Index Kit (Illumina, CA, USA). Paired-end sequencing of amplicons was conducted on the Illumina MiSeq platform using the v3 kit generating 2 × 301 nucleotide reads (Illumina, San Diego, USA).

The merged paired-end reads were analyzed using the Quantitative Insights Into Microbial Ecology (Qiime) tool (version 1.9.1, open source software). The operational taxonomic units (OTUs) were generated by clustering sequences with 97% similarity and the representative sequences, selected as the most abundant in each cluster, underwent taxonomic alignment by UCLUST consensus (http://drive5.com/usearch/manual/uclust_algo.html) to obtain the taxonomic assignment and relative abundance of each OTU using the Greengenes 16S rRNA gene database (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi). Raw data can be found in the SRA database public repository from NCBI within the BioProject accession number PRJNA517270.

Statistical Analysis

The statistical analysis was performed with SPSS 22.0 (SPSS Inc., Chicago, IL, USA) and QIIME (version 1.9.1; open source software). The data were expressed as mean ± standard deviation. Statistical comparisons between the means for independent samples and paired samples (pre- and post-eradication treatment) were performed using the Student's *t*-test. Non-parametric variables were evaluated by Mann–Whitney and Wilcoxon signed-rank tests. The correlation between quantitative variables including analytical, clinical, and microbial populations was analyzed using the Spearman bivariate correlations test. Linear regression models (both univariate as multivariate adjusted by age, sex, and BMI) were applied to identify bacterial changes as independent predictors of the selected variables (ghrelin levels and AUC grhelin). Statistical significance was established at $p < 0.05$. *P*-values were corrected for multiple comparisons using the Benjamini–Hochberg method when appropriate.

RESULTS

General Characteristics

The anthropometric and clinical characteristics of the subjects included in this study have been previously described elsewhere (17). In summary, no significant differences were found in BMI, waist circumference, plasma glucose, plasma insulin, and triglycerides between study groups. However, HDL cholesterol and GLP-1 levels increased significantly (55.36 ± 16.36 vs. 52.97 ± 12.9, $p = 0.021$ and 4.2 ± 0.4 vs. 3.6 ± 0.3; $p < 0.001$, respectively) in patients after *H. pylori* eradication with antibiotic therapy, while LDL cholesterol levels increased in *H. pylori*-infected subjects compared to controls (121.45 ± 35.8 vs. 102.05 ± 34, $p = 0.036$) (17). There were no statistically significant differences in dietary intake of energy and macro or micronutrients, as well as in dietary fiber ($p > 0.05$) between the study groups (data not shown).

Ghrelin Levels

Focusing on ghrelin levels, after *H. pylori* eradication treatment, we have observed a statistically significant decrease of the fasting plasma ghrelin levels respect to patients pre-treatment (12.97 ± 9.51 vs. 17.82 ± 13.38, $p = 0.017$) and controls (12.97 ± 9.51 vs. 23.13 ± 19.27, $p = 0.04$; **Figure 1**). After the functional glucose test, the ghrelin AUC decreased significantly after the *H. pylori* eradication therapy (1,065.85 ± 550.87 vs. 1,273.73 ± 717.67, $p = 0.03$). However, no statistically significant differences were detected in the ghrelin AUC between patients (pre- and post-treatment) and controls (**Figure 1**). Interestingly, after 120 min of the ingestion of the glucose bolus, ghrelin levels remained significantly lower in post-treatment patients compared to controls (10.82 ± 8.67 vs. 18.99 ± 18.92, $p = 0.039$), but no statistically significant changes were detected between *H. pylori* positive patients (pre-eradication treatment) and controls.

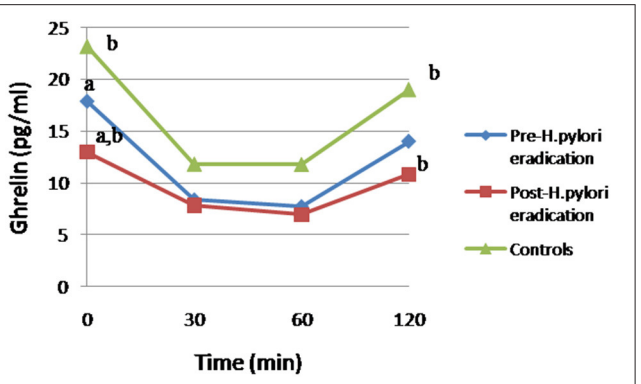

FIGURE 1 | Changes in ghrelin levels after 75g-OGTT in patients and controls. Equal letter means differences between groups. (a) Differences between *H. pylori*-infected individuals before and after antibiotic treatment, $p < 0.05$. (b) Differences in patients undergoing antibiotic therapy compared to the control group, $p < 0.05$.

To delve into these results, through a simple linear correlation analysis we have related BMI and ghrelin levels, indicating that the ghrelin AUC and fasting ghrelin levels were negatively associated with BMI ($r = -0.289$, $p = 0.004$ and $r = -0.242$, $p = 0.017$, respectively). Reinforcing these results, a univariate regression analysis, associated ghrelin AUC ($R^2 = 0.056$, $\beta = -0.238$, and $p = 0.019$) and fasting ghrelin levels ($R^2 = 0.057$, $\beta = -0.239$, $p = 0.018$) with BMI (as an independent variable).

Fasting Ghrelin Levels and Gut Microbiota

In a previous study, we already reported alterations in richness, diversity (Chao 1 and Shannon indices, respectively) and specific bacteria related to *H. pylori* infection and eradication treatment in these subjects (17). Our study model in subjects without metabolic abnormalities or other diseases allows us to evaluate changes in specific bacteria and ghrelin levels, as well as the relationship between both variables, avoiding possible confounding factors associated with these variables.

A simple linear correlation analysis, which included the whole sample population (*H. pylori* positive, post-treatment and control), associated fasting ghrelin levels with specific intestinal bacteria (**Table 1**). In addition, these relationships between the different bacteria and ghrelin levels were explained by regression model. Thus, the abundance of *RS045* ($R^2 = 0.062$, $\beta = 0.249$,

TABLE 1 | Linear correlation between bacteria and fasting ghrelin levels.

Phyla	Families/Genera/Species	Ghrelin	
		Spearman's Rho	*p*-value
Actinobacteria	*Bifidobacterium longum*	0.219	0.033
Firmicutes	*Megasphaera*	−0.266	0.009
	Roseburia faecis	0.235	0.022
Bacteroidetes	*Bacteroides*	0.207	0.044
	Bacteroides ovatus	0.221	0.031
	Bacteroides plebeius	−0.216	0.035
	Prevotellaceae	−0.321	0.001
	Prevotella	−0.295	0.004
	Prevotella stercorea	−0.215	0.037
	Rikenellaceae	0.209	0.040
	Parabacteroides distasoni	0.240	0.019
Saccharibacteria (formed TM7)	*RS045*	0.274	0.007
Proteobacteria		0.228	0.026

Spearman correlation test used to compare bacterial abundance and fasting ghrelin levels, including the 3 groups studied. Statistically significant data is shown ($p \leq 0.05$).

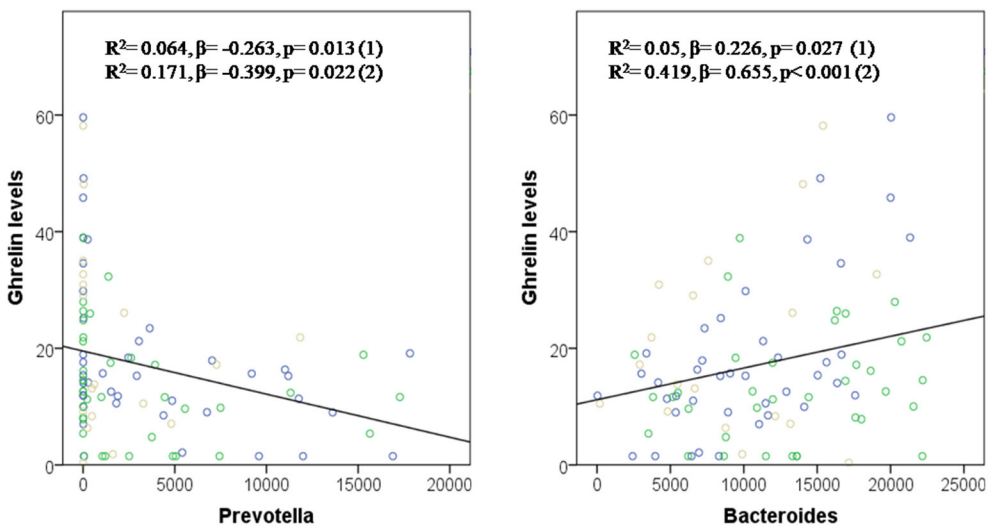

FIGURE 2 | *Prevotella* and *Bacteroides* in the prediction of fasting ghrelin levels. Dependent variable: ghrelin levels. Model (1): Linear Univariate regression model. Model (2): Linear multivariate regression model adjusted by age, sex, and BMI. The brown circles belong to the control group; blue circles to patients before treatment; and green circles to patients after treatment.

and $p = 0.015$), *Bacteroides* ($R^2 = 0.05$, $\beta = 0.226$, $p = 0.027$ and $R^2 = 0.419$, $\beta = 0.655$, $p \leq 0.001$ adjusted), *Bifidobacterium longum* ($R^2 = 0.070$, $\beta = 0.264$, $p = 0.010$), and *Parabacteroides distasonis* ($R^2 = 0.043$, $\beta = 0.207$, $p = 0.044$, and $R^2 = 0.192$, $\beta = 0.416$, $p = 0.014$ adjusted) predicted positively fasting ghrelin levels, while the abundance of *Prevotellaceae* ($R^2 = 0.068$, $\beta = -0.260$, $p = 0.011$ and $R^2 = 0.171$, $\beta = -0.399$, $p = 0.022$ adjusted), its genus *Prevotella* ($R^2 = 0.064$, $\beta = -0.263$, $p = 0.013$ and $R^2 = 0.171$, $\beta = -0.399$, $p = 0.022$ adjusted), and its species *Prevotella stercorea* ($R^2 = 0.055$, $\beta = -0.235$, $p = 0.022$) predicted negatively fasting ghrelin levels. **Figure 2** graphically represents the association found between fasting ghrelin and genera.

Post-meal Ghrelin Levels and Gut Microbiota

A significant univariate correlation between the abundance of specific bacteria and the post-meal ghrelin levels (referred to 120 min after the ingestion of the glucose bolus) were found. The analysis showed a significant positive correlation between *Streptococcus* ($r = -0.217$, $p = 0.35$), *Bacteroides ovatus* ($r = 0.217$, $p = 0.36$), *RS045* ($r = 0.294$, $p = 0.004$) and post-meal ghrelin levels, while *Bacteroides coprophilus* ($r = -0.302$, $p = 0.003$), *Megasphaera* ($r = -0.234$, $p = 0.023$) showed a significant negative correlation with post-meal ghrelin levels. In the univariate regression analysis, the genus *RS045*

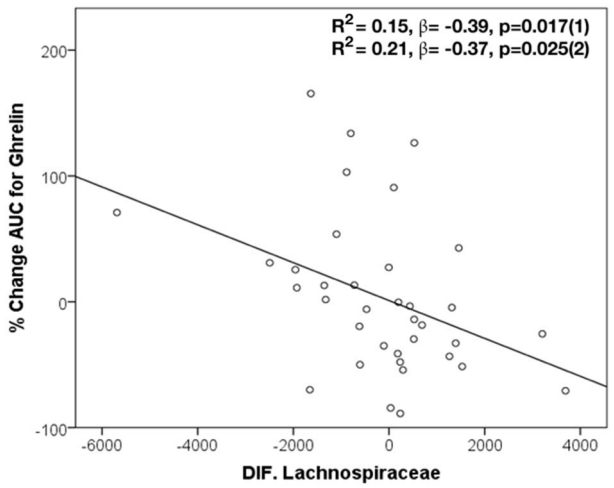

FIGURE 3 | Changes in *Lachnospiraceae* in the prediction of modifications in AUC for ghrelin after *H. pylori* eradication treatment. Dependent variable: ghrelin AUC. Model (1): Linear Univariate regression model. Model (2): Linear multivariate regression model adjusted by age, sex, and BMI.

($R^2 = 0.079$, $\beta = 0.282$, and $p = 0.006$) predicted post-meal ghrelin levels.

Bacterial Changes Associated With Changes in Post-treatment Ghrelin Levels

In this study, changes in the abundance of specific bacteria after *H. pylori* eradication treatment have been correlated with changes in the ghrelin AUC. Specifically, an inverse correlation between ghrelin AUC and changes in the abundance of *Lachnospiraceae* ($r = -0.43$, $p = 0.008$), its genus *Blautia* ($r = -0.52$, $p = 0.001$), and *P. stercorea* ($r = -0.34$, $p = 0.038$) after the triple therapy have been identified. In addition, through linear regression analysis, changes in *Lachnospiraceae* ($R^2 = 0.15$, $\beta = -0.39$, $p = 0.017$ and $R^2 = 0.21$, $\beta = -0.37$, $p = 0.025$ adjusted) predicted the proportion of changes in the ghrelin AUC, in patients after eradication treatment (**Figure 3**). On the other hand, changes in the abundance of *Megasphaera* were associated with changes in post-eradication fasting ghrelin levels ($r = -0.374$, $p = 0.02$).

DISCUSSION

Although it is known that *H. pylori* infection and its eradication therapy cause perturbations in the gut microbiome, in spite of the existence of several studies that point to metabolic changes in these patients, including alterations in BMI, how these disturbances are able to influence host metabolism is a little studied field. In this study, changes in the gut microbiota have been related to ghrelin levels in otherwise healthy *H. pylori*-infected subjects receiving the standard triple therapy.

Ghrelin levels are altered by the *H. pylori* eradication therapy. In fact, our study, in accordance to other studies (26, 31), have shown a decrease in plasma ghrelin levels after eradication therapy. In addition, other authors have shown a decrease in plasma desacyl-ghrelin levels (32) but an increase in plasma acyl-ghrelin levels (21, 32). The decrease in ghrelin levels could be attributed to the damages caused to ghrelin-producing endocrine cells by *H. pylori* (33, 34). However, different aspects point out that microbiota, a direct target of antimicrobials, could be partly involved in alterations observed in the ghrelin levels. First of all, the use of antimicrobials is known to alter the profile of the microbiota (35) and changes in the composition of the gut microbiota have been linked to ghrelin levels (14). Moreover, a mouse-study has demonstrated that *H. pylori* and gut microbiota-associated modulation of metabolic gut hormones was independent and preceded *H. pylori*-induced histopathological changes in the gut of infected mice (25). Finally, patients included in this study eradicated *H. pylori* post-therapy, and it has been reported that the successful *H. pylori* eradication treatment in subjects without severe gastric atrophy or intestinal metaplasia leads to improvements in gastric mucosal patterns in the short term (36). However, a plasma ghrelin reduction have also been observed in patients who failed *H. pylori* eradication therapy (26).

As mentioned in our previous study, *H. pylori* infection and its eradication with triple therapy decreased diversity and richness, as well as changed the abundance of specific bacteria, with persisting effects after 2 months of the *H. pylori* eradication treatment (17). In the current study, specific bacterial changes have been associated with both fasting and postprandial ghrelin levels. In line with our results, previous studies have positively associated ghrelin levels with *Bacteroides* (37, 38), *Bifidobacterium* (39), and *Parabacteroides* (40), and negatively with *Prevotellaceae* (41), *Blautia* (42), and *Streptococcus* (42). But data have also been reported in the other direction, with negative associations between ghrelin and *Bifidobacterium* (37, 43), *Bacteroides* (42) and positive with *Prevotella* (37, 44). However, most of these relationships have been found as the result of interventions with non-antibiotic therapies or in animals (14). To date, only one previous study has linked changes in ghrelin and gut bacteria (Bacteroidetes/Firmicutes) after *H. pylori* eradication therapy in humans (26). Therefore, our results could contribute to broadening the knowledge on the eradication therapy-gut microbiota-ghrelin relationship. Furthermore, many of the ghrelin-related taxa identified in our study are novel.

How gut microbiota is able to mediate its influence to the host is a matter of continuous study. Evidence suggests that microbial metabolites could be the mediators between the gut microbiota and host homeostasis. Among these metabolites, short-chain fatty acids (SCFAs), mainly acetate, propionate, butyrate, are known to exert multiorgan effects on the host energy metabolism (45). Therefore, microbial imbalance by *H. pylori* eradication treatment could affect the production of these bacterial metabolites, and consequently affect host metabolism, including ghrelin regulation. In fact, although more studies are necessary, changes in the microbiome and its metabolites have been related to alterations in ghrelin expression, secretion, activation, and signaling and appetite regulation through ghrelin (14).

Colonic SCFAs production can negatively affect serum ghrelin concentration (46) probably *via* SCFA receptors on enteroendocrine cells, as already shown for example for GLP-1 (47). Some studies have observed an increase in fecal butyrate and a decrease in plasma ghrelin (40, 46). Butyric acid is one of the most abundant and important SCFAs in the gut due to its multiple effects, like its participation in energy homeostasis by regulating appetite and energy intake (48). In addition, butyrate has been involved in ghrelin signaling (49). In our study, changes in the abundance of butyrate-producing bacteria such as *Lachnospiraceae* and *Megasphaera* have been inversely related with changes both in ghrelin AUC and fasting ghrelin levels after eradication therapy. Interestingly, changes in *Lachnospiraceae* after triple therapy predicted changes in ghrelin AUC, regardless of age, gender and BMI. *Lachnospiraceae* and *Megasphaera* are considered health promoting bacteria (50).

Lactate is another bacterial metabolite that can be produced by many intestinal bacteria, but its accumulation in the colon is often an indicator of microbiota perturbation or dysbiosis (51). Lactate is able to suppress ghrelin through the inhibition of the secretory function of ghrelin producing gastric cells, in addition to its possible involvement in modulation signaling through the ghrelin receptor (52). In our study, lactate-producing bacteria such as *Streptococcus* and *Blautia* were negatively associated with ghrelin. Interestingly, increase in *Blautia* after triple therapy was associated with decrease in ghrelin AUC. *Blautia* has been associated with metabolic disorders, including obesity (53), but it has also been inversely associated with visceral fat accumulation (54). The imbalance between lactate-producing and utilizing bacteria or a reduction in lactate-utilizing bacteria leads to dysbiosis. In fact, communities with low numbers of lactate-utilizing bacteria are inherently less stable and more prone to lactate-induced perturbations (51). *Lachnospiraceae* and *Megasphaera,* in addition to their ability to degrade complex polysaccharides to butyrate, are able to use lactate to generate propionate through the acrylate pathway (55), therefore, these bacteria could be categorized as lactate-utilizing bacteria. Other bacteria inversely related to ghrelin in our study, such as *Prevotella*, can also use lactate to produce succinate, a precursor to propionate (56), which could contribute to the reduction of colonic lactate levels, and therefore, increase ghrelin levels. However, propionate has been related to satiety by decreasing in ghrelin levels (57). On the other hand, propionate has been related to lipid metabolism (58), and in our previous study, propionate precursor bacteria such as members of *Lachnospiraceae* have been positively related to HDL cholesterol levels (19). Since, *Lachnospiraceae* was negatively associated with ghrelin in this study, and this hormone is involved in the regulation of lipid metabolism, promoting fat storage, in addition to exerting direct peripheral effects on lipid metabolism (59), we speculate that members of *Lachnospiraceae* could be related to lipid metabolism through propionate levels and variations in ghrelin levels.

In contrast, the lactate and acetate producer *Bifidobacterium longum*, belonging to the Actinobacteria phylum, predicted positively fasting ghrelin levels, in our study. *B. longum* has been previously linked to the ghrelinergic signaling, which is an important signaling pathway modulating central appetite regulation and metabolism (49). Specifically, *B. longum* may decrease the GHSR-1a internalization and has been correlated with its higher acetate content (49), but also with decrease body weight gain, fat depot size, glucose tolerance, and leptin levels in a preclinical mouse model of HFD-induced obesity (60). In the current study, we have observed a marked decrease in *Bifidobacterium*, including *B. Longum* at post-eradication therapy compared to controls and/or *H. pylori* positive (17) which could be contributing to the decline in nocturnal plasma ghrelin observed. Declining ghrelin levels contributes to this reduction in food intake and lean body mass (61). However, *H. pylori* eradication has been related to an increase in BMI (23). Although, we did not observe significant changes in BMI after eradication, possibly due to the short follow-up period, we observed a negative association between fasting ghrelin levels and BMI. These discrepancies could be related to the gut microbiota and its ghrelinergic effects previously shown (49). We speculate that the higher levels of ghrelin in controls and *H. pylori* positive together with the presence of *B. Longum* would have an attenuated response on its receptor and therefore on food intake and energy metabolism. Although Actinobacteria represent only a small percentage of the gut microbiota, this phylum is pivotal in the maintenance of gut homeostasis, and an unbalanced abundance has been evidenced in several pathological conditions (62).

Other acetate-producing bacteria, such as *Bacteroides*, *P. distasonis*, positively predicted fasting ghrelin levels regardless of age, gender, and BMI, and these bacteria have been associated with obesity (53). It has been suggested that acetate could be mediating changes in ghrelin signaling, which could indicate an interaction between the microbiota and ghrelin (13). In fact, increased acetate production by an altered gut microbiota leads to activation of the parasympathetic nervous system which in turn promotes increased glucose-stimulated insulin secretion (GSIS), increased ghrelin secretion, hyperphagia, obesity, and its related sequelae (13). It has also been suggested that the acetate reduces GHSR-1a internalization and acetate, propionate and lactate inhibit ghrelin-mediated receptor internalization (49).

Finally, we have observed that the abundance of *RS045*, belonging to the former TM7 phylum and currently known as *Saccharibacteria*, predicted fasting and post-meal ghrelin levels. TM7 has an ultra-small size and lives on the surface of its host bacterium, and lacks the ability to synthesize any of its own amino acids, vitamins, or cell wall precursors, thereby parasitizing other bacteria. TM7 has been strongly associated with all adiposity markers (63).

In the present study, there are several limitations that must be taken into consideration. *H. pylori* could intervene in ghrelin secretion and therefore be a confusing factor. However, the strength of the current study is the fact that it was performed in otherwise healthy patients with no other confounding variables. Although, the inclusion of a group of subjects without *H. pylori* infection exposed to eradication treatment could have provided more detailed information on the role of antibiotic treatment in the association found, it was not possible for ethical reasons. In

this manner, it would be also of interest to study the alterations produced by the different drugs used in the *H. pylori* eradication therapy separately, but since it is a procedure not validated in clinical practice, this approach only could be carried out in the form of a particular clinical trial for ethical reasons. On the other hand, the sample size could be increased, although previous calculations of the sample size were performed to ensure a realistic approach. Lastly, the 16s ribosomal RNA gene sequencing used has limitations in identifying genetically specific species and strains.

In summary, eradication treatment for *H. pylori* could decrease ghrelin levels, and this alteration could be mediated through the changes produced in the gut microbiota composition by the antibiotic therapy. These results could indicate that the imbalance between lactate producers and its utilizers could trigger changes related to ghrelin levels under alteration of *H. pylori* eradication therapy. In addition, acetate, and butyrate producing bacteria identified in the study could also play an important role in ghrelin regulation. We suggest that ghrelin secretion and signaling may be under regulation by intestinal commensal microbes and affected by antibiotics. More studies are required to clarify the effect of the antibiotic on ghrelin.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/ (PRJNA517270).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Medical Ethics Committee at Virgen de la Victoria University Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

GMM-N performed the metagenomic analysis, statistical analysis and interpretation of data, drafting, and reviewing of the manuscript. IC-P performed the recruitment follow-up of the patients, and revision of the manuscript. MC-P performed laboratory analysis and was involved in the revision of the manuscript. FJT contributed to the study concept and design, interpretation of data, reviewed, and critically revised the article for important intellectual content. IM-I contributed to the study concept, interpretation of data, bioinformatic analysis, and critical revision of the manuscript. All authors contributed to the article and approved the submitted version.

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frontiers in Medicine

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Circulating Fibroblast Activation Protein as Potential Biomarker in Patients With Inflammatory Bowel Disease

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A major concern in the management of Inflammatory Bowel Disease (IBD) is the absence of accurate and specific biomarkers to drive diagnosis and monitor disease status timely and non-invasively. Fibroblast activation protein (FAP) represents a hallmark of IBD bowel strictures, being overexpressed in stenotic intestinal myofibroblasts. The present study aimed at evaluating the potential of circulating FAP (cFAP) as an accessible blood biomarker of IBD. Quantitative determination of cFAP was performed by enzyme-linked immunosorbent assay on plasma samples prospectively collected from patients with IBD and control subjects. A discrimination model was established on a training set of 50% patients and validated on independent samples. Results showed that cFAP concentration was reduced in patients with IBD when compared to controls ($p < 0.0001$). Age, sex, smoking, disease location and behavior, disease duration and therapy were not associated with cFAP. The sensitivity and specificity of cFAP in discriminating IBD from controls were 70 and 84%, respectively, based on the optimal cutoff (57.6 ng mL^{-1}, AUC = 0.78). Predictions on the test set had 57% sensitivity, 65% specificity, and 61% accuracy. There was no strong correlation between cFAP and routine inflammatory markers in the patients' population. A subgroup analysis was performed on patients with Crohn's disease undergoing surgery and revealed that cFAP correlates with endoscopic mucosal healing. In conclusion, cFAP deserves attention as a promising blood biomarker to triage patients with suspected IBD. Moreover, it might function as a biomarker of post-operative remission in patients with Crohn's disease.

Keywords: inflammatory bowel disease, fibroblast activation protein, blood biomarkers, diagnosis, mucosal healing, chronic patient

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INTRODUCTION

Currently, no accurate serum biomarker of inflammatory bowel disease (IBD) is available to aid clinicians in establish a diagnosis properly (1–3). As a consequence, several patients complaining about gastrointestinal symptoms undergo invasive and costly diagnostic procedures, and only a small subset of them receives a diagnosis of IBD (4, 5). Definitive diagnosis relies on the histological assessment of bowel biopsies from endoscopy, which remains highly uncomfortable for patients and requires expert gastroenterologists and pathologists. In addition, a diagnostic delay frequently triggers a delay in the establishment of appropriate therapies, with an impact on disease progression and increased risk for complications.

A few serum biomarkers have been described in the literature, although controversial results about their utility exist (2, 6, 7). Among them, the anti-*Saccharomyces cerevisiae* antibody (ASCA) is the most well-known. However, its sensitivity is not optimal (∼39–44%). Perinuclear antineutrophil cytoplasmic antibodies (pANCAs) are frequently found in serum samples from patients with ulcerative colitis (UC), but they are less frequent in patients with Crohn's disease (CD) (8, 9). Therefore, ASCA and pANCAs are used in combination to better define the IBD type affecting the patient, rather than to diagnose IBD itself. Patients with ASCA+/pANCA- are more likely to have CD, but the sensitivity achieved by the combined use of these two markers is 55%. Indeed, a generally low sensitivity limits the overall utility of the identified serological markers for IBD diagnosis.

Fecal markers, such as fecal calprotectin (FC), appear more specific for intestinal inflammation. FC dosage may be helpful in the evaluation of disease exacerbations and monitoring of therapy responsiveness. However, FC cannot distinguish IBD from other causes of intestinal inflammation, and it is strongly associated with colonic inflammation, though much less with ileal inflammation (6, 10, 11). Furthermore, considerable intra-individual variability of FC levels is observed, thus adding critical issues for the correct interpretation of the results (12, 13).

Repeated endoscopy is required not only for IBD diagnosis or surveillance, but it also allows to follow up medically or surgically treated IBD patients, especially patients with CD undergoing surgery. Recently, mucosal healing (MH) has been suggested as the real therapeutic goal in these patients, as it is associated with less frequent relapses, reduced hospitalization and lower risk of further surgery (14). However, the only way to assess MH is currently ileocolonoscopy, and both clinicians and patients would highly desire a less invasive biomarker.

In the last years, fibroblast activation protein (FAP) has been identified as a hallmark of intestinal fibrosis in CD (15–17). FAP is an inducible cell surface glycoprotein belonging to the postprolyl dipeptidyl aminopeptidase enzyme family, and it is a well-recognized marker of reactive fibroblasts in different contexts (18). In CD, FAP expression is specifically up-regulated on intestinal strictured myofibroblasts (15, 16, 19). FAP also exists as a soluble enzymatically active form, which can be detected in human blood. Some studies have associated altered circulating FAP (cFAP) levels with certain disorders, such as cancer and liver fibrosis (20, 21). However, the significance of cFAP in IBD has never been explored.

The present study aimed to investigate the potential of cFAP as a reliable serological biomarker of IBD, assessing its plasma levels in a cohort of patients with IBD vs. control subjects. Moreover, a subgroup analysis was performed on a subset of patients with CD undergoing surgery to correlate cFAP with post-operative endoscopic disease activity.

MATERIALS AND METHODS

Patient Population

From April 2018 to February 2020, all consecutive patients affected by IBD and referred to the ASST Fatebenefratelli Sacco, "Luigi Sacco" University Hospital (Milano, Italy) were eligible to participate in the study. Inclusion criteria were: proven histopathological diagnosis of CD or UC, any disease pattern and localization, 18–85 years old. Patients were excluded from the study if they had an unclear IBD diagnosis (indeterminate colitis), displayed rheumatologic disease, chronic liver diseases, chronic heart failure or other concurrent gastrointestinal and autoimmune diseases. Two cohorts of patients were considered: (1) patients with controlled IBD undergoing routine outpatient evaluation; (2) patients with active IBD undergoing surgery for complicated disease. Indication for surgery was established during a formal multidisciplinary meeting involving gastroenterologists, surgeons, pathologists, and radiologists. Patients were excluded if they were referred in emergency, if they displayed severe sepsis, and in case they were under steroids in the last month or under immunosuppressants or anti-TNF antibodies in the last 3 months. A control group was formed of healthy volunteers without any gastrointestinal or autoimmune disorder. In addition, a cohort of patients with diverticulitis was enrolled as part of the study to make a comparison between IBD and another intestinal disease (baselines features of patients with diverticulitis are reported in **Supplementary Table 1**).

Blood Samples Collection and cFAP Detection

From each subject, 10 mL blood sample was collected in EDTA-coated tubes at the time of outpatient visit or as part of the pre-operative assessment in case patients underwent surgery. Plasma was isolated by centrifuge at 1,000 × g for 10 min, transferred in sterile vials and stored at −80°C until usage. FAP concentration was determined by double-antibody sandwich enzyme-linked immunosorbent assay, according to the manufacturer's instructions (Human FAP DuoSet ELISA, R&D systems). Each plasma sample was diluted in Reagent Diluent (1:200) and run in a 96-well microplate as duplicates. A calibration curve was performed using seven-point dilutions of recombinant human FAP as standard. Absorbance was read using a testing wavelength of 450 nm and a correction wavelength of 570 nm. The intra-assay coefficient of variability (CV) was 2.8% (±0.6, $n = 14$); the inter-assay CV was 5.4% (±2.4, $n = 10$).

Clinical Assessment

For all the patients, demographic and clinical data were collected at baseline in a prospective database. Age at diagnosis, disease location and clinical phenotypes were evaluated with the Montreal classification (22). Laboratory data on blood analyses were exported as electronic medical record from the hospital management system (clinical electronic repositories). For a subgroup of patients with CD undergoing ileocolonic resection, endoscopic procedures were performed at 12 months after surgical intervention, in the setting of routine clinical practice. The endoscopic reports were reviewed by an IBD physician to grade endoscopic activity through Rutgeerts score. Scores of i0 and i1 were regarded as post-surgery remission; scores of i2, i3, and i4 were considered post-surgery recurrence. At the time of endoscopy, a second blood sample was withdrawn from the patient and analyzed for cFAP as previously described. Rutgeerts score and paired cFAP dosage were analyzed by Spearman's rank correlation coefficient.

Statistical Methods

Variables were reported as means ± standard deviations (SD) or as absolute numbers and percentages. Categorical variables were compared using χ^2-test or exact Fisher's, while continuous variables were compared using Student's *T*-test or non-parametric Wilcoxon test in case of non-normal distribution of the variable. If it was necessary to apply regression models on non-normal variables, an appropriate transformation was applied to make them follow a Gaussian distribution.

To define a diagnostic model, the original dataset was divided into two independent samples with the same size. To this aim, temporal criterion was used as previously described: (23) the first half of enrolled patients formed the training set; the second half of enrolled patients generated the test set. The first sample was used to develop the diagnostic model. In order to estimate the diagnostic accuracy, the area under curve (AUC) of the receiver operating characteristic (ROC) curve was designed. An internal validation for AUC was performed with bootstrap method. Briefly, the original patient population was re-sampled 500 times and the optimism index (the mean of differences between AUC on bootstrap sample and AUC on original sample) was calculated. Optimism is the amount by which the AUC (or "the apparent prediction accuracy") overestimates the true prediction accuracy of the model. Then, the corrected AUC after bootstrap was reported. The second sample was used to externally validate the developed diagnostic model in order to evaluate its performance (accuracy, sensitivity, specificity, positive/negative predictive value) in other independent dataset and to determine generalizability of the derived diagnostic rule to new patients.

Data analysis was performed using SAS software (v. 9.4, SAS Institute Inc., Cary, USA) and R software (v. 3.5.1, © The R Foundation).

Ethics Approval

The study was authorized by the Ethical Committee of ASST Fatebenefratelli Sacco (Milano, Area 1) as protocols n. 545/2016 and n. 24916/2019. The study protocol was conducted in accordance with the International Conference on Harmonization (ICH) Good Clinical Practice (GCP) guidelines. Informed consent was obtained from each subject included in the study.

TABLE 1 | Baseline variables of the two cohorts of patients with IBD and of the healthy controls (HC) included in the study.

Variable	HC ($n = 160$)	IBD no surgery ($n = 152$)	IBD surgery ($n = 120$)	*p*-value
Age (mean ± SD, years)	44.2 ± 16.0	46.8 ± 14.7	46.2 ± 16.1	0.22
Gender, *n* (%)				
Female	100 (62.5)	60 (39.5.)	47 (39.2)	<0.0001
Male	60 (37.5)	92 (60.5)	73 (60.8)	
Smoke, *n* (%)				
Yes	62 (38.8)	64 (42.1)	38 (31.7)	0.21
No	98 (61.2)	88 (57.9)	82 (68.3)	

Age of patients is expressed as continuous variable (mean ± SD); gender and smoke habit are expressed as total number of subjects and frequency distribution.

RESULTS

Baseline Characteristics of Study Population

A total of 432 subjects were included in the study: 272 patients had IBD, and 160 were healthy controls (HC). Patients with IBD attending routine outpatient consultation ($n = 152$) and patients with IBD undergoing surgery ($n = 120$) were analyzed separately not to introduce any bias due to the non-homogeneity of disease status among the two groups. In the first group, 86 patients (56.6%) had CD and 66 patients (43.4%) had UC. Their mean age was 46.8 (±14.7) years, 60 patients (39.5%) were female, 92 (60.5%) were male, 64 patients (42.1%) were smokers. The mean disease duration was 11.6 (±8.0) years. In the second group, 96 patients (80.0%) had confirmed diagnosis of CD and 24 (20.0%) of UC. Their mean age was 46.2 (±16.1) years; 47 patients (39.2%) were female, and 73 (60.8%) were male. Thirty-six patients (30%) declared to be smokers. The mean disease duration was 12.0 (±10.1) years. The HC group had an average age of 44.2 (±16.0) years and displayed a slight prevalence of females (62.5%) than males (37.5%). In the group, 54 people (33.8%) were smokers. HC and IBD groups were similar in terms of age ($p = 0.22$) and smoking habitude ($p = 0.21$). There was a male predominance in the IBD groups as compared with HC ($p < 0.0001$), while no different gender distribution was observed in the two IBD groups ($p = 0.96$). The distribution of baseline variables in the study population is shown in **Table 1**.

Plasma FAP in Patients With IBD

Mean cFAP concentration was significantly lower in patients with IBD, both in the group attending outpatient visit (55.7 ± 26.8 ng mL^{-1}) and in the surgery group (42.4 ± 26.7 ng mL^{-1}) than in HC (76.5 ± 32.5 ng mL^{-1}, $p < 0.0001$) (**Figure 1**). Levels of cFAP in patients with IBD undergoing surgery were reduced as compared to patients with stable disease that did not require

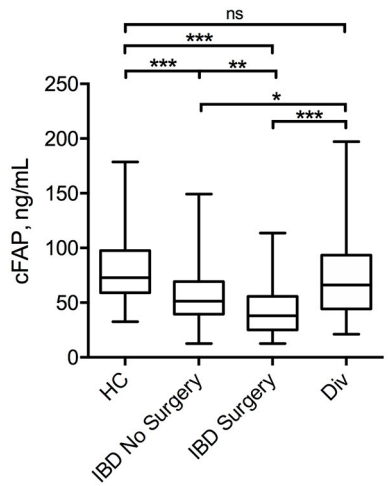

FIGURE 1 | Box plot displaying the concentration of cFAP in patients with IBD attending routine outpatient consultation ($n = 152$) or undergoing surgery ($n = 120$) as compared with healthy controls (HC, $n = 160$) and patients with diverticulitis (Div, $n = 20$). Statistical analysis was performed by Student *t*-test, $^{*}p = 0.04$; $^{**}p < 0.001$; $^{***}p < 0.0001$.

surgery ($p < 0.001$). There was no correlation between cFAP levels and age across the groups ($\rho = 0.02$, $p = 0.78$ in HC; $\rho = 0.11$, $p = 0.18$ in patients with IBD attending outpatient visit; $\rho = 0.08$, $p = 0.39$ in patients with IBD undergoing surgery). Levels of cFAP were not significantly associated with sex ($p = 0.09$, $p = 0.19$, and $p = 0.58$ in HC and IBD groups, respectively). Moreover, age at diagnosis, location and behavior of the disease, disease duration or biological therapy were not related to cFAP, thus not ascribing cFAP reduction to some specific clinical features but rather to the presence of IBD (**Table 2**). Patients with CD and patients with UC were both characterized by reduced levels of cFAP as compared to controls ($p < 0.0001$). No significant cFAP difference was observed between CD and UC subtypes in the group of IBD attending outpatient visit (52.9 ± 22.2 ng mL^{-1} in CD, 59.3 ± 31.7 ng mL^{-1} in UC, $p = 0.14$) and in IBD undergoing surgery (42.2 ± 24.7 in CD, 43.4 ± 33.8 in UC, $p = 0.83$, **Supplementary Figure 1**). Disease features from patients with CD and UC were analyzed separately and showed no dependence on cFAP levels (see **Supplementary Table 2**).

In order to investigate whether cFAP is specific for IBD or a marker common with other intestinal diseases, we measured cFAP levels also in a cohort of patients with diverticulitis ($n = 20$). The results, reported in **Figure 1**, showed that cFAP concentration in diverticulitis is not significantly different from that measured in HC ($p = 0.27$). By contrast, it is significantly higher than cFAP levels observed in IBD ($p = 0.04$ vs. IBD with controlled disease; $p < 0.0001$ vs. IBD undergoing surgery).

Diagnostic Value of cFAP

To investigate whether cFAP could help to discriminate patients with IBD from HC, a diagnostic model was set up. In order to avoid any bias due to complicated disease, patients with IBD undergoing surgery were excluded from this analysis. A ROC curve was computed using data from HC and patients with IBD assigned to the training set ($n = 156$). The analysis showed that cFAP is able to distinguish patients with IBD from HC with an AUC of 0.78 (CI 0.69–0.84). The sensitivity of cFAP was as high as 70%, and the specificity reached 84% based on the optimal cut-off (57.6). cFAP was able to identify true IBD cases with a positive predictive value (PPV) of 80.3% and a negative predictive value (NPV) of 74.4%. The optimism index was equal to 0.01; the calculated AUC after bootstrap was 0.77 (**Figure 2**). The as-designed diagnostic model was applied to an independent set of patients ($n = 156$), used for validation. The discrimination matrix revealed a capability to differentiate IBD and HC with 57% sensitivity, 65% specificity, and 61% accuracy (**Supplementary Figure 2**).

Association Between cFAP and Other Inflammatory Markers

Association studies were performed between cFAP levels and the main routine markers of inflammation from blood analysis in the patients' population. The analysis showed a weak inverse correlation between cFAP and erythrocyte sedimentation rate {ESR, $r = -0.31$ [C.I. (-0.47, -0.13), $p = 0.0008$, $n = 116$]}, and between cFAP and C-reactive protein {CRP, $r = -0.39$ [C.I. (-0.51, -0.27), $p < 0.0001$, $n = 211$]}. No other significant correlations were observed with total white blood cells, the relative amount of neutrophils, nor markers derived from protein electrophoresis in peripheral blood (see **Supplementary Table 3**). Beyond blood markers, the dosage of FC was evaluated as an additional measure of intestinal inflammation activity. FC did not show any correlation with cFAP values in the study population {$r = -0.14$ [C.I. (-0.39, 0.13), $p = 0.29$, $n = 59$]}.

cFAP as Biomarker of Recurrence in CD

In order to further analyze the potential of cFAP as a biomarker of IBD, a subgroup analysis was performed in 21 patients with CD who concluded a regular follow up of at least 12 months post-surgery. Baseline characteristics of this subgroup of patients with CD is shown in **Supplementary Table 4**. Mean cFAP concentration was significantly increased at 12 months post-surgery as compared to preoperative values ($p = 0.02$, **Figure 3**). Interestingly, there was a significant inverse correlation between cFAP dosage and disease activity at 12 months post-surgery, as graded by Rutgeerts score upon endoscopic examination {$r = -0.52$ [C.I. (-0.78, -0.09), $p = 0.017$]}. Higher cFAP values were associated with lower scores, thus suggesting that increased cFAP could be a biomarker of post-operative remission. We thus compared cFAP concentrations in those patients who attained endoscopic remission (Rutgeerts i0, i1) vs. those who experienced recurrence at 12 months post-surgery (Rutgeerts i2, i3, i4). Data showed that cFAP was significantly increased in patients with endoscopically-assessed remission ($p = 0.03$, **Figure 4**).

DISCUSSION

Early and definite diagnosis of IBD is a major point of concern for clinical management of these disorders (4, 24–26). Currently,

TABLE 2 | Baseline characteristics and levels of cFAP in HC and IBD groups.

Variable	HC ($n = 160$)			IBD no surgery ($n = 152$)			IBD surgery ($n = 120$)		
	n (%)	cFAP (ng mL^{-1})	*p*-value	*n* (%)	cFAP (ng mL^{-1})	*p*-value	*n* (%)	cFAP (ng mL^{-1})	*p*-value
Gender									
Female	100 (62.5)	72.6 ± 30.1	0.09	60 (39.5)	52.4 ± 24.6	0.23	47 (39.2)	39.6 ± 17.7	0.58
Male	60 (37.5)	83.1 ± 32.2		92 (60.5)	57.8 ± 28.1		73 (60.8)	43.7 ± 23.8	
Smoking									
Yes	62 (38.8)	74.9 ± 37.1	0.28	64 (42.1)	55.5 ± 26.8	0.96	38 (31.7)	38.7 ± 23.7	0.07
No	98 (61.2)	77.6 ± 29.4		88 (57.9)	55.8 ± 27.0		82 (68.3)	43.6 ± 20.6	
Family history of IBD									
Yes				17 (12.2)	56.9 ± 21.9	0.94	16 (13.8)	35.9 ± 16.5	0.25
No				122 (87.8)	56.4 ± 27.1		100 (86.2)	42.8 ± 21.3	
Disease duration									
≤10 years				73 (51.8)	56.5 ± 29.2	0.42	56 (50.5)	42.0 ± 21.4	0.96
>10 years				68 (48.2)	53.0 ± 22.2		55 (49.5)	41.2 ± 18.7	
Age at diagnosis									
A1, <16 years				7 (4.9)	51.0 ± 18.5	0.64	14 (12.1)	41.9 ± 20.1	0.44
A2, 17–40 years				93 (64.6)	53.8 ± 26.3		66 (56.9)	40.3 ± 21.4	
A3, >40 years				44 (30.5)	57.8 ± 26.1		36 (31)	44.6 ± 20.3	
Montreal location*									
L1, terminal ileum				23 (27.4)	51.6 ± 22.2	0.23	62 (66)	41.0 ± 17.9	0.44
L2, colon				10 (11.9)	66.9 ± 31.6		9 (9.6)	51.3 ± 29.5	
L3, ielocolon				43 (51.2)	51.9 ± 20.6		21 (22.3)	40.3 ± 22.1	
L4, upper gastrointestinal tract				8 (9.5)	50.0 ± 10.3		2 (2.1)	55.6 ± 10.4	
Montreal behavior*									
B1, non-stricturing non-penetrating				36 (43.4)	52.5 ± 23.5	0.71	5 (5.3)	58.4 ± 37.4	0.67
B2, stricturing				35 (42.2)	56.0 ± 20.9		43 (45.7)	41.4 ± 17.8	
B3, penetrating				12 (14.4)	50.8 ± 21.5		46 (49)	41.1 ± 19.6	
Perianal disease*									
Yes				8 (10.3)	34.2 ± 23.5	0.006	21 (20)	36.1 ± 15.2	0.14
No				70 (89.7)	61.8 ± 26.8		68 (80)	43.6 ± 19.9	
Montreal extent#									
E1, proctitis				7 (12.5)	39.4 ± 16.4	0.16	2 (9.5)	45.5 ± 7.9	0.22
E2, left-sided colitis				19 (29.7)	64.5 ± 37.1		1 (4.8)	70.8 (± 0.0)	
E3, extensive colitis				37 (57.8)	59.5 ± 30.2		18 (85.7)	34.8 ± 19.0	
Medications									
Biological therapy				119 (88.8)	55.6 ± 24.8	0.71	32 (27.1)	39.3 ± 20.5	0.01
Other therapy				9 (6.7)	51.1 ± 27.7		50 (42.4)	37.7 ± 20.1	
None				6 (4.5)	62.1 ± 35.7		36 (30.5)	49.3 ± 19.9	

cFap is expressed as mean ± SD.
**Applicable to patients with CD only.*
#Applicable to patients with UC only.

IBD diagnosis is based on the complex interpretation of history, clinical signs, endoscopic and histopathologic data from biopsies, whose reliability often depends on extremely few expert operators on the territory. Therefore, easy-accessible blood biomarkers would be a tool of paramount importance for clinicians to timely triage patients suspected to have IBD, before prescribing more invasive and costly imaging procedures in a reference center (1–3).

In this study, the plasma levels of FAP were analyzed in patients with IBD and found to be significantly reduced compared to HC (**Figure 1**). Reduced cFAP levels were confirmed in both patients with CD and UC. Moreover, no correlations between cFAP and any recorded disease characteristic were found, thus supporting the hypothesis that cFAP is a general marker of IBD rather than an indicator of a particular IBD subtype or pattern. In the present study, we have included patients with controlled IBD under treatment and patients with active IBD undergoing surgery. Surgery in IBD is indicated when an aggressive disease presents with symptoms, or in case of uncontrolled disease after therapy failure, as often happens

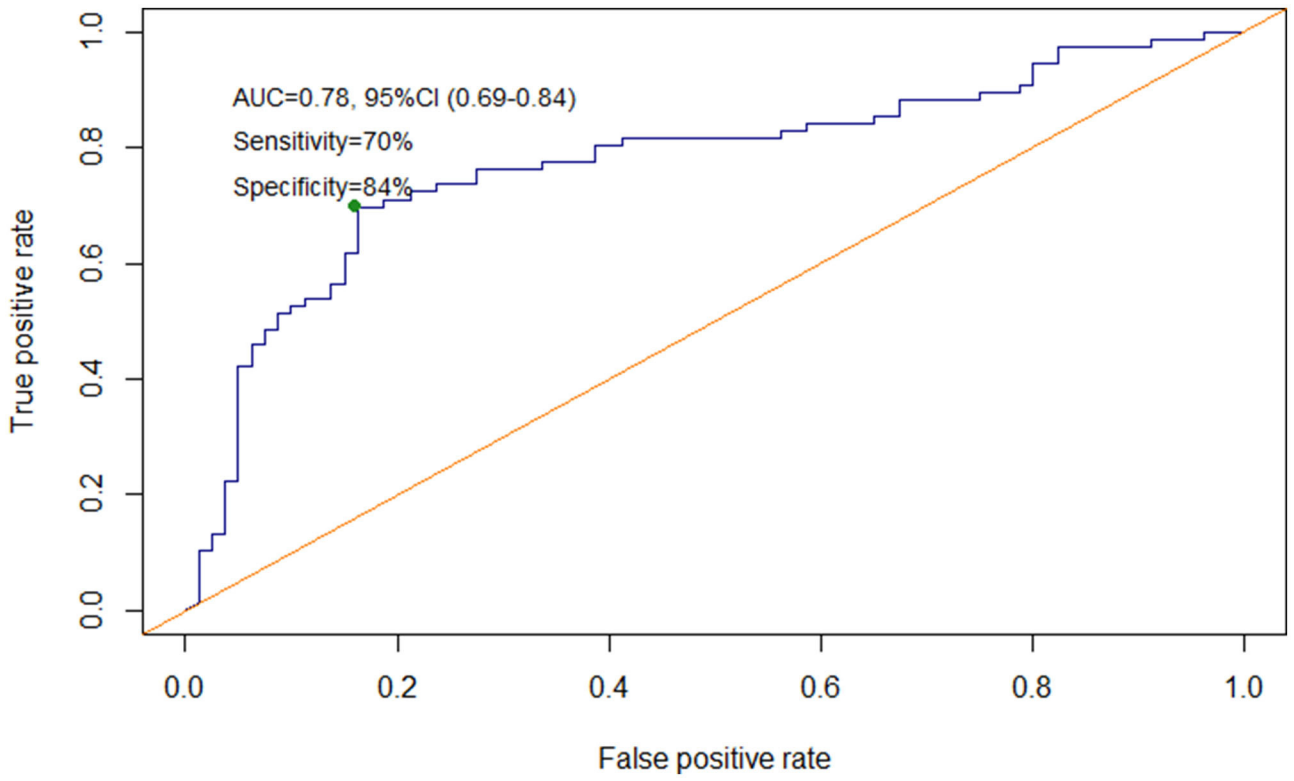

FIGURE 2 | Receiver operating characteristic (ROC) curve for cFAP level's accuracy to distinguish patients with IBD from HC.

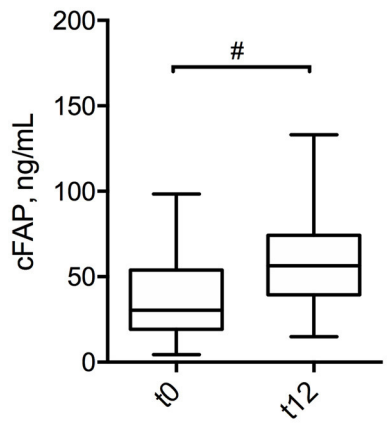

FIGURE 3 | Box plot displaying the concentration of cFAP in patients with CD at pre-operative stage (t0) and at 12 months post-surgery (t12). Statistical analysis was performed by paired Wilcoxon test, $^{\#}p = 0.02$.

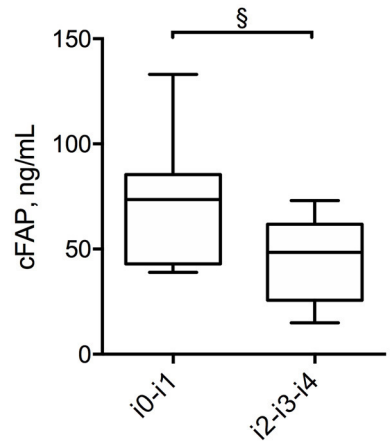

FIGURE 4 | Box plot displaying the concentration of cFAP in patients with CD who have attained endoscopic remission (Rutgeerts score i0, i1) and in those with post-surgery recurrence (Rutgeerts score i2, i3, i4). Statistical analysis was performed by Mann Whitney test, $^{\S}p = 0.03$.

with UC or stricturing CD. Our findings revealed that cFAP is lower in both IBD cohorts as compared to HC. Furthermore, cFAP was significantly lower in patients undergoing surgery than patients with controlled disease ($p < 0.0001$), thus suggesting a role for cFAP as a biomarker of IBD activity and remission: the more cFAP is reduced, the more disease is active. By contrast, patients with diverticulitis had similar cFAP levels than HC. Despite preliminary, this observation indicates that reduced cFAP may be a specific marker of IBD over other inflammatory gastrointestinal disorders.

Other observations in the literature already reported reduced levels of cFAP in pathological contexts characterized by stroma

reactivity, such as some cancers and myocardial infarction (27). To the best of our knowledge, this is the first investigation of cFAP in a cohort of patients with IBD. The cause for reduced cFAP level still remains poorly elucidated, but it could be that a systemic reaction to the disease occurs. Indeed, the origin of cFAP is not completely clarified (28, 29). FAP extracellular domain can be shed from cells as soluble form, but cause and actors involved in this proteolytic cleavage have not been demonstrated yet. Activated fibroblasts, myofibroblasts, and the hepatobiliary system appear as the primary physiological sources of FAP, though it is likely that multiple organs may contribute to the circulating levels of FAP (27, 30). There is literature showing that proteins of the dipeptidyl peptidase family, such as the FAP paralog DPP4, are expressed in gut epithelial cells and that their expression increases in cells that display an enterocytic differentiation (31–33). Since enterocytes are damaged and partly destroyed in IBD, it could be hypothesized that one of the cellular sources of these proteins fails to produce and secrete the proteins to the same extent as it happens in the healthy physiological state. However, studies on FAP secretion by enterocytes in IBD are lacking and this hypothesis cannot yet be confirmed. Moreover, results from our study revealed that patients with stricturing disease had similar cFAP levels compared to penetrating disease and non-stricturing non-penetrating disease, both in IBD patients controlled with therapy ($p = 0.71$) and in patients treated by surgery ($p = 0.67$). This finding is particularly relevant, since it further suggests that cFAP could be not only related with the fibroblasts' local presence in bowel lesions, but also with other unknown pathways related to FAP independent from strictures. Further preclinical studies are required to uncover the mechanisms responsible for cFAP production and to explain why it is reduced in IBD.

In the present study, a predictive diagnostic model based on ROC curve assessed the capability of cFAP to discriminate IBD and healthy groups with 78.0% accuracy (**Figure 2**). In our tested population, cFAP was able to identify real IBD cases with a PPV of 80.3% and a NPV of 74.4%. Despite preliminary, these results suggest that cFAP could be a valuable, non-invasive solution to triage patients suspected to have IBD in primary care diagnostic. The future clinical potential of cFAP may be intended to accelerate clinical diagnosis for patients ending up with reduced cFAP, who will promptly undergo more invasive and costly imaging procedures. The absence of any strong correlation between cFAP and traditional though a specific inflammatory markers, such as ESR, CRP, FC, indicates that cFAP may be a more specific IBD biomarker than other aspecific inflammatory indexes. It also means that information derived from cFAP is different and non-redundant with currently available inflammatory markers.

It has to be noted that discriminative performances deriving from predictions on the independent test set achieved 57% sensitivity, 65% specificity, and 61% accuracy. These parameters certainly highlighted some limitations of cFAP as a stand-alone biomarker for IBD diagnosis. However, a performance's decrease is often expected in external validation and accuracy remains significantly higher than the null model (AUC = 61% with CI 0.54–0.72). Moreover, we need to consider that no other single serological test is currently available to guide IBD diagnosis in primary care. The Prometheus IBD Sgi diagnostic® combines serologic, genetic and inflammatory markers to aid decision-making in IBD diagnosis. Despite being welcomed as a "holy Graal," this multi-marker panel presents several concerns. First, only three markers appeared as really predictive of IBD: pANCA, ASCA IgA, and ASCA IgG (34). Secondly, the accuracy of its serologic markers was assessed in cohorts with a high prevalence of IBD (up to 62%), thus its value in a real-world setting with a low-prevalence of IBD remains controversial (35, 36). Recently, the Prometheus test was applied in a series of patients with IBD seen at a tertiary referral center. The sensitivity for CD was 52%, with an accuracy of 61.5%. A better performance was observed for UC (sensitivity 67%, accuracy 80%), but the overall conclusion was that the test is not robust enough for initial diagnostics of IBD (37). In this context, cFAP could be extremely promising, mainly because it represents a much simpler dosage of a single plasma protein, which has high relevance for IBD pathophysiology. After the present study, further trials, including other centers and community hospitals, should be conducted to validate FAP as a blood biomarker of IBD.

Interestingly, in the present study, increased cFAP was demonstrated to be associated to MH in patients with CD treated by surgery and undergoing follow-up ileocolonoscopy ($p = 0.03$, **Figure 4**). Patients with UC requiring surgery were excluded from this analysis because, once operated of proctocolectomy, these patients should be considered cured, so any recurrence is not expected. MH is currently considered the therapeutic goal for patients with CD, and today it is the endpoint of several trials to estimate the success rate of novel therapies (38, 39). However, the definition of MH is quite ambiguous, depending on precise endoscopic evaluation and reporting. Recently, a combined blood test called the endoscopic healing index has been developed to assess endoscopic remission, but it requires the quantitative determination of 13 different proteins (40). Preliminary results from our study suggest that cFAP might deserve attention as an ease-to-get, stand-alone blood biomarker of MH after surgery in CD, since a concordance rate with endoscopic findings was found. A limitation for this observation consists in the small number of patients for which endoscopic data were available at 12 months post-surgery. A larger study with a longer longitudinal follow up is now required to confirm the observed correlation and validate cFAP as a biomarker of post-operative MH.

In conclusion, the present study provides evidence that cFAP is reduced in patients with IBD as compared to controls. Since no accurate serum biomarker of IBD is currently available, cFAP deserves attention as a potential non-invasive solution to triage patients with suspected IBD. Moreover, this study provides a preliminary indication that cFAP increases in patients with CD experiencing endoscopic remission, thus

suggesting exploration of this protein as a novel biomarker of MH.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethical Committee of ASST Fatebenefratelli Sacco (Milano, Area 1) as protocols n. 545/2016 and n. 24916/2019. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

FCor and MT planned the study. FCol, SAr, and GS recruited patients. MC, AM, CM, SM, FP, and MT collected data. FCor, LS, SAl, FCol, and MT analyzed data. FCor, SAl, and MT drafted the manuscript. All the authors have approved the final draft submitted.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.725726/full#supplementary-material

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SYSTEMATIC REVIEW
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Risk Factors of Invasive Fungal Infection in Recipients After Liver Transplantation: A Systematic Review and Meta-Analysis

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Objectives: Invasive fungal infection (IFI) remains an important cause of mortality in liver transplantation (LT). The objective of this meta-analysis was to identify the risk factors for IFI after LT.

Methods: We searched for relevant studies published up to June 2020 from PubMed, Web of Science, Embase, and the Cochrane Library. Odds ratios (ORs) and their corresponding 95% CIs were used to identify significant differences in the risk factors. Heterogeneity between studies was evaluated by the I^2 test, and potential publication bias was assessed with Egger's test. The quality of included studies was evaluated with the Newcastle-Ottawa Scale (NOS).

Results: A total of 14 studies enrolling 4,284 recipients were included in the meta-analysis. Reoperation (OR = 2.18, 95% CI: 1.61–2.94), posttransplantation dialysis (OR = 2.03, 95% CI: 1.52–2.72), bacterial infection (OR = 1.81, 95% CI: 1.33–2.46), live donor (OR = 1.78, 95% CI: 1.20–2.63), retransplantation (OR = 2.45, 95% CI: 1.54–3.89), and fungal colonization (OR = 2.60, 95% CI: 1.99–3.42) were associated with the risk factors of IFI after LT.

Conclusions: Despite some risk factors that have been identified as significant factors for IFI post-LT, which may inform prevention recommendations, rigorous and well-designed studies with adequate sample sizes should be conducted to solve the limitations of this study.

Keywords: invasive fungal infection, liver transplantation, risk factors, meta-analysis, end-stage liver diseases

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INTRODUCTION

Since the first liver transplantation (LT) by Thomas Starzl in 1963, the prognosis was significantly improved. LT can be considered the most effective therapy for nearly all causes of end-stage liver diseases (ESLDs) (1). The incidence of invasive fungal infections (IFIs) in liver transplant recipients is surpassed only by small bowel and lung transplant recipients (2). IFIs occur in 7–42% of patients with liver transplant (3). Despite the advance in surgical techniques and antifungal prophylactic strategies, IFI is still a major cause of postoperative morbidity and mortality for patients undergoing LT (4). The reported mortality associated with IFI range from 25 to 69% (5).

Several clinical trials have shown that the causative organisms of IFIs are variable. *Candida* species are the most common causes of IFI post-LT, followed by *Aspergillus* species (4). Recipients with active or latent infection in the recipient or donor at the time of transplantation, or early graft dysfunction or rejection, are at particularly high risk of developing opportunistic infections (6). Therefore, the incidence of IFI is strongly associated with the hospital microbiological environment, level of immunosuppression, the clinical condition of the patient, surgical factors, complicated transplant operation, and use of high-dose antibiotics (6, 7).

Many studies have reported the risk factors for IFI post-LT, including bacterial infections in the first month and absence of antifungal prophylaxis (7), reoperation, or retransplantation (5). Model for End-Stage Liver Disease (MELD) Score, cytomegalovirus reactivation (8), Roux-en-y anastomosis, and hemodialysis (9). However, because of geographical limitations and sample size, the outcomes of studies of risk factors for IFI post-LT are controversial. For instance, three studies reported that the MELD score was a risk factor for IFI posttransplant (7, 10, 11), but another study reported that the MELD score was not related to IFI post-LT (12). To address the inconsistency in the results, the present meta-analysis measured the quantitative combined effect of all the related studies and increased the power of statistical analysis by merging multiple single studies about the risk factors of IFI in recipients after LT. Finally, this meta-analysis involving 14 studies and provided more reliable evidence for the risk factors of IFI post-LT.

METHODS

Data Source Collection

A literature search was performed in PubMed, Web of Science, Embase, the Cochrane Library to identify studies related to risk factors of IFI after LT that were published up to June 2020. The terms searched were "liver transplantation or hepatic transplantation or liver grafting" and "invasive fungal infection or IFI" and "risk factors".

Inclusion Criteria

Studies were selected in accordance with the "Preferred reporting items for systematic reviews and meta-analyses" (PRISMA) statement (13). The inclusion criteria were as follows: (1) the study was related to the risk factors of IFI in recipients post-LT; (2) IFI was defined as proven or probable according to the European Organization for Research and Treatment of Cancer/Mycoses Study Group (EORTC/MSG) Criteria (14); (3) the study was a case-control or cohort study in design; (4) availability of the published data; (5) the study was written in English.

Exclusion Criteria

The following were the exclusion criteria: (1) duplicate articles; (2) reviews, meeting abstracts, letters, or case reports; (3) no diagnostic or no defined criteria for IFIs; (4) studies were related to the risk factors of IFIs after the organ transplantation but did not report the relevant data on the LT subgroup; (5) studies included data of risk factors for LT infection but did not show the information for IFI.

Data Extraction

Relevant information was extracted independently by the two reviewers (ML and LYS). A final check was confirmed by another researcher (ZJZ). The extracted data included the first author, publication year, country of origin, study time and design, number of patients and controls, and risk factors for IFIs with odds ratio (OR) and 95% CI from the multivariate analyses.

Quality Assessment

The quality of each included study was independently evaluated by the three reviewers (ML, LYS, and ZJZ) based on the Newcastle–Ottawa Scale (NOS) (15). The NOS consists of three domains and a total of nine points: four for selection of study groups, three for outcome and exposure, and two for comparability of the cases. Only studies with scores> 6 were considered as high quality and finally included (16).

Statistical Analysis

Statistical analysis was performed using Review Manager 5.3 and Stata 12.0. Heterogeneity was considered significant with $I^2 > 50\%$ or $P < 0.1$ (17). The fixed-effects model was used to calculate the 95% CI and its pooled ORs for the homogeneous data ($I^2 < 50\%$ or $P > 0.05$). Otherwise, the random-effects model was used. Sensitivity analysis was conducted by excluding one study at a time. Publication bias was assessed with Egger's test. $P < 0.05$ suggested that there was a publication bias in this study. The population attributable risk proportion (PARP) was calculated as follows: $\text{PARP} = P_e\,(\text{OR}-1)/[P_e\,(\text{OR}-1)+1]$. P_e was defined as the pooled exposure rate of controls that represented the overall population exposure rate.

RESULTS

Study Selection and Characteristics

The literature search yielded 2,405 results; most studies were excluded because they were duplicate studies or because they were not relevant to our meta-analysis. Then, 84 studies were excluded after the full-text articles were reviewed because they did not match the criteria. Finally, 14 studies involving 4,284 recipients (533 cases and 3,751 controls) were included in this meta-analysis (7, 10–12, 18–27). The study selection process is shown in **Figure 1**.

The specific characteristics of the studies included in the meta-analysis are presented in **Table 1**. These 14 studies were published from 2003 to 2020. They were conducted in the USA (10, 22, 25, 27), Australia (26), China (20), Japan (11, 19, 21), Korea (12), Italy (23, 24), Spain (18), and France (7). A total of seven studies were cohort studies (10, 12, 18, 19, 23, 25, 26) and seven were case–control studies (7, 11, 20–22, 24, 27). All the included studies were evaluated as high quality after being assessed by the NOS.

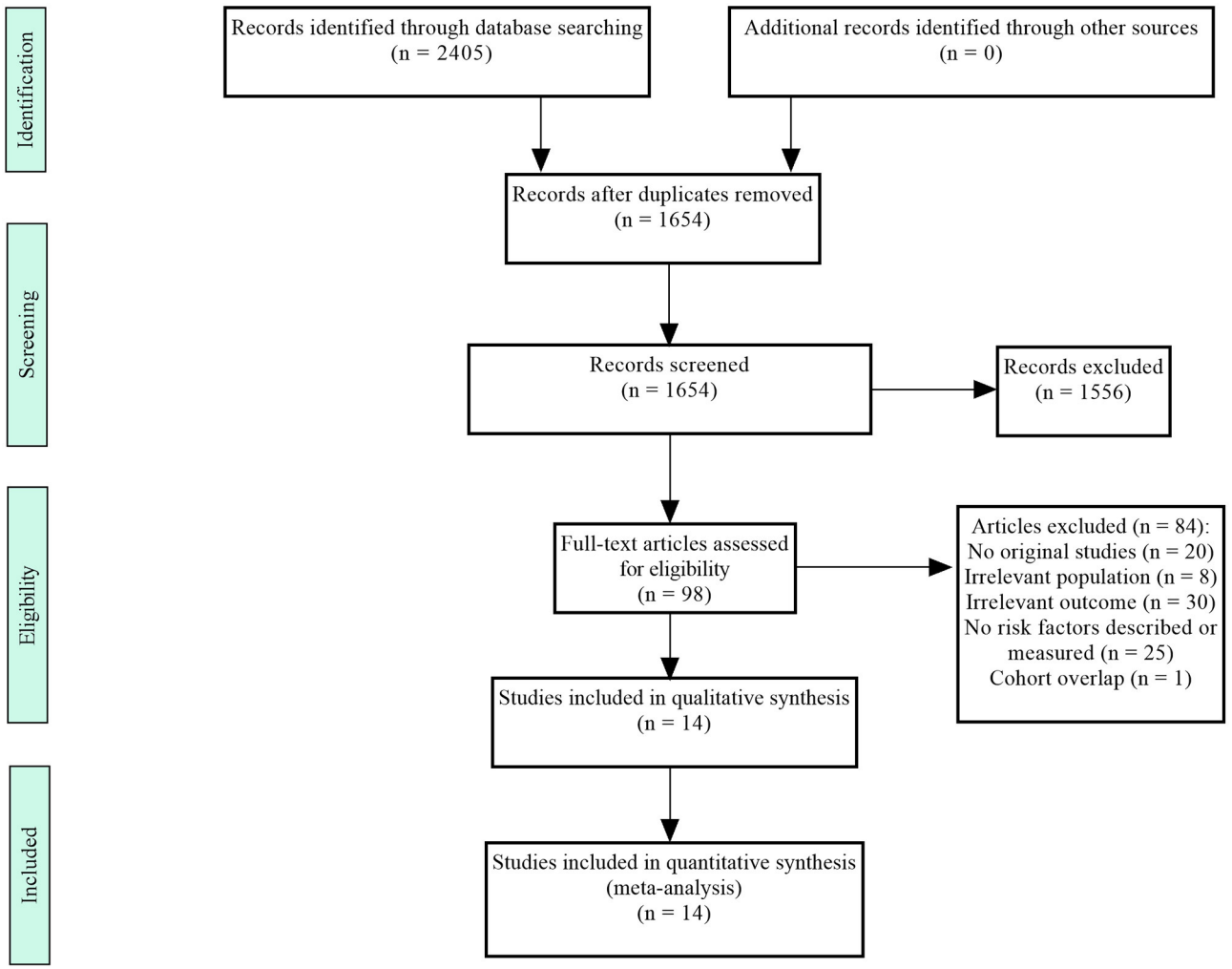

FIGURE 1 | Flowchart of the selection process.

Risk Factors of IFI

The risk factors for IFI post-LT are shown in **Table 2**. Several risk factors were identified, including reoperation, post-transplant dialysis, bacterial infection, live donor, the MELD score, retransplantation, fungal colonization, Roux-en-Y anastomosis. The risk factors with significant differences in IFIs after LT were as follows: reoperation (OR = 2.18, 95% CI: 1.61–2.94), posttransplantation dialysis (OR = 2.03, 95% CI: 1.52–2.72), bacterial infection (OR = 1.81, 95% CI: 1.33–2.46), live donor (OR = 1.78, 95% CI: 1.20–2.63), retransplantation (OR = 2.45, 95% CI: 1.54–3.89), and fungal colonization (OR = 2.60, 95% CI: 1.99–3.42). A forest plot describing the association between risk factors for IFIs after LT is presented in **Figure 2**.

PARP of Risk Factors

Population attributable risk proportion was used to represent the proportion of cases in a population that was attributable to the exposed factor. The PARP of risk factors such as reoperation, posttransplant dialysis, bacterial infection, live donor, fungal colonization, and retransplantation is shown in **Table 3**. The PARP ranged from 6.3 to 37.5%. Reoperation had the highest PARP, whereas live donor had the lowest. Bacterial infection, reoperation, and fungal colonization were strong risk factors for IFIs after LT.

Sensitivity Analysis

Sensitivity analysis was used to evaluate the potential effect of heterogeneity conducted by eliminating one study in each turn. Sensitivity analyses manifested no significant changing of heterogeneity when one study was eliminated at a time.

Publication Bias

Egger's test was conducted for statistical investigation to evaluate potential publication bias (**Table 2**). The publication bias was considered to exist for $P < 0.05$. Egger's test showed that most risk factors did not have a publication bias ($P > 0.05$).

TABLE 1 | Characteristics of studies included in the meta-analysis.

Study	Year	Type of study	Country	Study period	N	Age (years)	Gender (% men)	Cases/controls	Quality assessment[a]
Ohkubo et al. (21)	2012	Case-control	Japan	over a 6-year period	156	24.5	46	19/137	6 points
Eschenauer et al. (22)	2015	Case-control	USA	November 2008 to December 2012	382	55.7±10.7	65	20/362	6 points
Kawagishi et al. (19)	2006	Cohort	Japan	July 1991 to November 2005	96	18.67	41	8/88	7 points
Utsumi et al. (11)	2019	Case-control	Japan	January 2005 and April 2012	153	55	54	15/128	8 points
Lavezzo et al. (24)	2017	Case-control	Italy	January 2011 to December 2015	268	NA	NA	16/252	6 points
Fortún et al. (18)	2003	Cohort	Spain	January 1994 to December 2001	131	NA	70	22/109	8 points
Raghuram et al. (27)	2012	Case-control	USA	January 2003 to December 2007	502	56	65	58/444	6 points
Lum et al. (26)	2020	Cohort	Australia	January 2005 to October 2015	554	NA	NA	28/56	7 points
Alexander et al. (10)	2006	Cohort	USA	January 1997 to December 1999	153	51	61	28/125	7 points
Kim et al. (12)	2019	Cohort	Korea	January 2009 and February 2012	482	53	76.8	196/286	7 points
Giannella et al. (23)	2016	Cohort	Italy	June 2010 to December 2014	303	53	68.6	19/284	7 points
Jorgenson et al. (25)	2019	Cohort	USA	July 2009 to June 2017	189	54.4±9.9	60.4	50/139	6 points
Zhou et al. (20)	2011	Case-control	China	April 2008 to March 2010	248	50.14±9.68	77.4	44/204	7 points
Saliba et al. (7)	2013	Case-control	France	January 1999 to December 2005	667	46.8±13.4	65.6	171/496	7 points

NA, not available. [a] High-quality research, 6–9 points.

TABLE 2 | Meta-analysis of risk factors of invasive fungal infection in recipients after liver transplantation.

Risk factors	Combination studies	Cases/controls	OR (95%CI)	Z	*P*	Heterogeneity of study design			Analysis model	Egger's test
						Chi^2	*p*	I^2		
Reoperation	4	85/554	2.18[1.61,2.94]	5.08	<0.05[a]	0.42	0.94	0%	Fixed	0.508
Post-transplant dialysis	3	54/673	2.03[1.52,2.72]	4.75	<0.05[a]	1.08	0.58	0%	Fixed	0.747
Bacterial infection	2	190/633	1.81[1.33,2.46]	3.79	0.0002[a]	0.00	0.99	0%	Fixed	NA
Live donor	2	70/501	1.78[1.20,2.63]	2.86	0.004[a]	0.83	0.36	0%	Fixed	NA
MELD score	3	214/749	1.02[0.99,1.05]	1.12	0.26	0.18	0.91	0%	Fixed	0.782
Retransplantation	2	218/395	2.45[1.54,3.89]	3.78	0.0002[a]	0.63	0.43	0%	Fixed	NA
Fungal colonization	2	254/730	2.60[1.99,3.42]	6.92	<0.05[a]	0.73	0.39	0%	Fixed	NA
Roux-en-Y anastomosis	2	199/552	1.83[0.78,4.28]	1.40	0.16	2.64	0.1	62%	Random	NA

[a] P < 0.05 stands for significant. NA, not available.

DISCUSSION

Invasive fungal infection is associated with poor outcomes in recipients with posttransplant (28). Therefore, identifying risk factors is essential for preventing IFI post-LT. Accordingly, targeted prophylaxis should be performed only in high-risk recipients (22). Several studies have reported potential risk factors for IFI post-LT. However, there has been inconsistency about the risk factors, perhaps because of the different studies using different designs or inclusion criteria. The

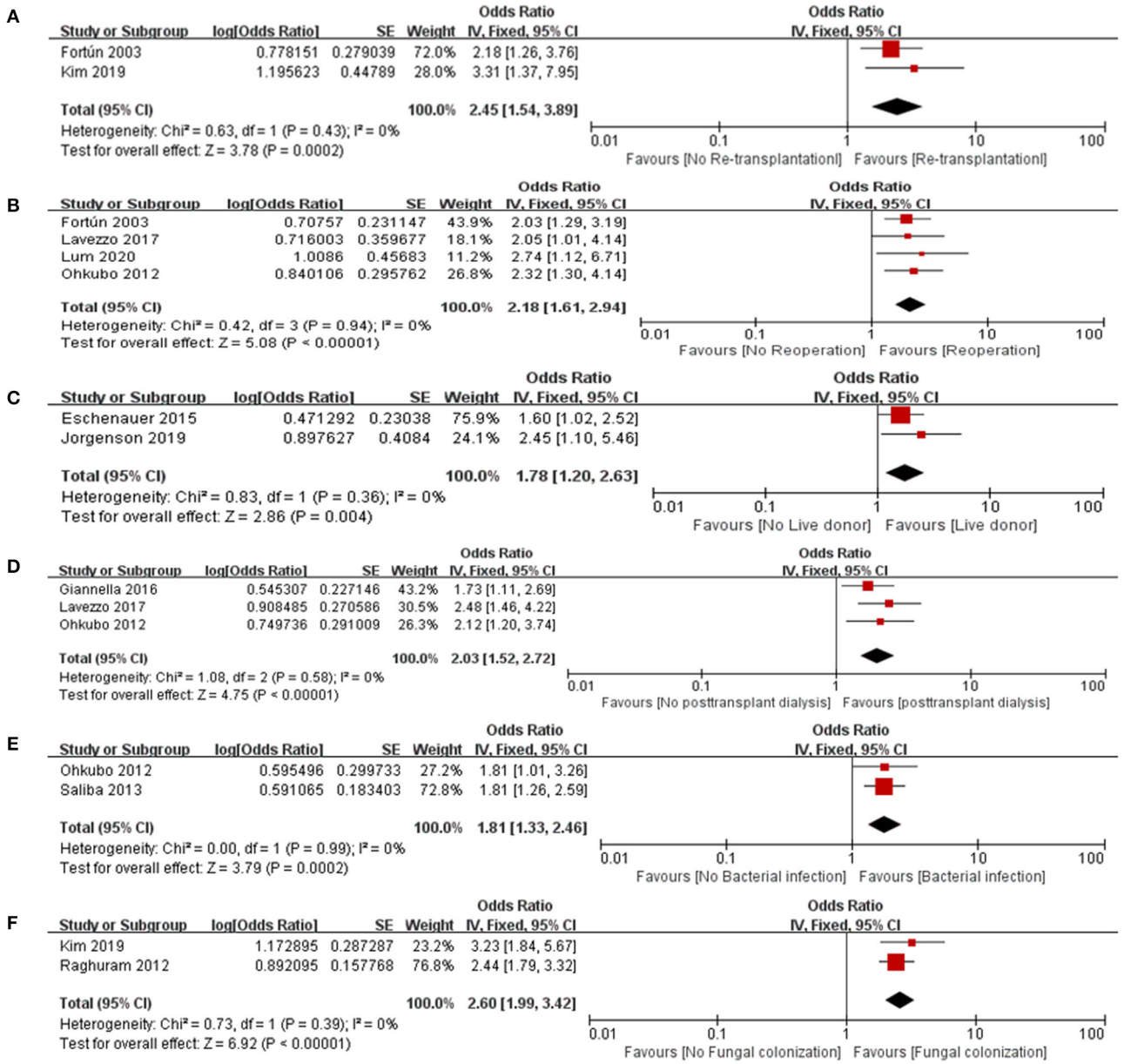

FIGURE 2 | Forest plot for the association between IFI after LT and **(A)** Retransplantation, **(B)** Reoperation, **(C)** Live donor, **(D)** Posttransplant dialysis, **(E)** Bacterial infection and **(F)** Fungal colonization.

current meta-analysis was conducted to identify the risk factors for IFI post-LT and provide the best evidence for the clinical applications.

According to the inclusion and exclusion criteria, we identified 14 studies enrolling 4,284 patients. In our meta-analysis, risk factors for IFI after LT included reoperation, posttransplant dialysis, bacterial infection, retransplantation, live donor, and fungal colonization. As summarized in **Figure 3**, 14 studies identified 506 pathogens that caused IFI post-LT. *Candida* species were the most common causative organism of IFI among recipients of the LT, and *Aspergillus* species was the second most common.

Reoperation and retransplantation, which indicated a more complicated intraoperative and postoperative procedure, were risk factors for IFI. Meta-analysis showed that the risk of IFI in recipients with reoperation was 2.18 times higher than the recipients without reoperation, which was consistent with the results of previous studies (29, 30). However, among the studies included in this meta-analysis, Eschenauer et al. (22) and Utsumi et al. (11) concluded that IFI was not associated with reoperation, but the researchers did not give an explanation for this result. Some included studies reported a higher risk of IFI in recipients who underwent retransplantation (12, 18). Our result was consistent with these studies. Several studies

TABLE 3 | Population-attributable risk proportion of risk factors of invasive fungal infections in recipients after liver transplantation.

Risk factors	OR (95% CI)	P_e (%)	PARP (%)
Reoperation	2.18[1.61,2.94]	36.1	29.9
Posttransplant dialysis	2.03[1.52,2.72]	8.5	8.1
Bacterial infection	1.81[1.33,2.46]	74.1	37.5
Live donor	1.78[1.20,2.63]	8.6	6.3
Retransplantation	2.45[1.54,3.89]	10.6	13.3
Fungal colonization	2.60[1.99,3.42]	14.4	18.7

OR, odds ratio; CI, confidence interval; P_e, pool exposure rate; PARP, population-attributable risk proportion.

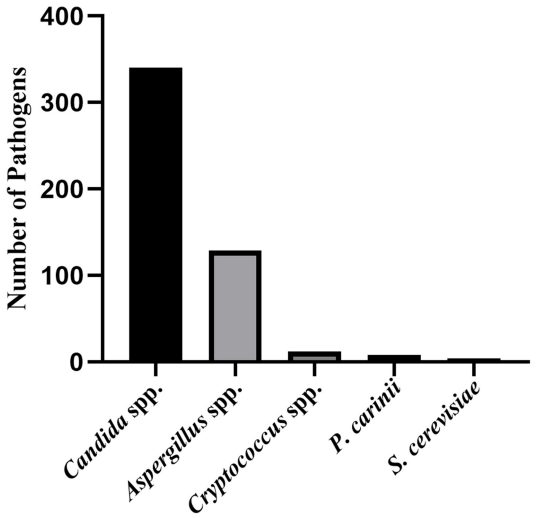

FIGURE 3 | The pathogen composition of IFI after LT. spp, species; *P*. carinit, Pneumocystis cariniee; S. Cerevisiae, Saccharomyces cerevisiae.

reported that retransplantation was not a significant risk factor (22–24), which was likely due to the small number of recipients who underwent retransplantation. The results showed that Roux-en-y anastomosis was not a risk factor for IFI after LT, possibly because of the small number of patients who underwent retransplantation, which was consistent with the results of a previous study (26).

Recipients of the live donor were at a higher risk of IFI after LT, which was consistent with the previous studies (22, 31). In the case of the living donor liver transplant (LDLT), the graft is smaller than that recovered from a deceased donor, and it involves complex surgery and carries a risk of bile leakage. Therefore, antifungal prophylaxis should be given to patients with LDLT to counter the risks of IFI.

Our study also found that the risk of IFI increased with the posttransplant dialysis that is consistent with the studies of Ohkubo et al. (21), Lavezzo et al. (24), and Giannella et al. (23), but several studies (10, 12, 18, 22) found that the posttransplant dialysis was not a risk factor for IFI post-LT; however, the authors did not give any explanation for this negative outcome.

We used PARP to estimate the percentage of IFI in recipients of LT attributed to one kind of risk factor. We found that the PARP of the bacterial infection and reoperation was high. Thus, we infer that these risk factors were important for IFI post-LT.

Fungal colonization was defined as the presence of fungus before LT without clinical symptoms or evidence of infection. We found that the patients with fungal colonization were at a higher risk of IFI. Several studies have shown that antifungal prophylaxis dramatically reduces fungal colonization, mortality caused by a fungal infection, and the overall incidence of fungal infection (5, 32). Further investigation of pretransplant screening to identify fungal colonization is warranted. Therefore, we recommend that post-LT, the recipients should have targeted antifungal prophylaxis to reduce antibiotic exposure.

There were several limitations to this meta-analysis. First, we only included English language literature from four databases, and there may have been incomplete retrieval. Second, there may have been mistakes in the data conversion because some study data required to be recalculated. Third, because of the limitations of the included date, we did not conduct subgroup analyses and funnel plots. At last, analyses were limited by the sample size included in this meta-analysis, so the combined effect size may have been exaggerated to draw the opposite conclusion.

In conclusion, this meta-analysis identified some risk factors for IFI post-LT and might provide a basis for clinical prevention. However, a well-designed prospective cohort study should be conducted to validate our findings.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

ML and LYS designed the study and wrote the first draft of the manuscript. ML and ZJZ verified data extraction, data analysis, and reviewed the manuscript. LYS and ZJZ supervised the data acquisition, data analysis, and interpretation. All the authors read and approved the final manuscript.

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Genetic Polymorphisms and Clinical Features in Diabetic Patients With Fatty Liver: Results From a Single-Center Experience in Southern Italy

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Genetic background may be involved in the promotion and progression of non-alcoholic fatty liver disease (NAFLD). Previous studies have suggested that the single nucleotide polymorphisms (SNPs) may be associated with the specific clinical features in the patients with hepatic steatosis; however, data on the patients with diabetes from Southern Italy are lacking. We enrolled 454 patients and 260 of them had type 2 diabetes. We studied the *PNPLA3 rs738409*, *LPIN1 rs13412852*, *KLF6 rs3750861*, *SOD2 rs4880*, *TM6SF2 rs58542926*, and *ZNF624 rs12603226* SNPs and their distribution in the study population. Lipid profile, liver stiffness, and kidney function were also studied to understand the potential role of the SNPs in the development of clinical phenotypes. No differences were observed in the distribution of polymorphisms between the diabetic and non-diabetic subjects. Carriers of risk allele G for *PNPLA3 rs738409* SNP showed a lower mean value of serum triglycerides and a higher liver stiffness. Risk allele for *KLF6 rs3750861* and *SOD2 rs4880* polymorphism had a lower estimated glomerular filtration rate (eGFR) value, whereas no differences in the glucose and glycated hemoglobin level were observed in the subgroups by the different genotypes. Genetic polymorphisms are useful to identify the patients at higher risk of development of liver fibrosis and lower eGFR values in the patients with diabetes and NAFLD. Their use in clinical practice may help the clinicians to identify the patients who require a more strict follow-up program.

Keywords: diabetes, NAFLD, SNPs, fatty liver, polymorphisms

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a major cause of chronic liver disease and the second leading cause of liver transplantation (1). NAFLD has been predominantly associated with diabetes and obesity, and its worldwide prevalence has been estimated to be up to 33% in the general population and 75% in diabetic patients (2). From a clinical point of view, NAFLD encompasses a spectrum of diseases ranging from simple steatosis without liver inflammation to steatohepatitis and, finally, liver cirrhosis (3).

There is a significant heterogeneity in the clinical phenotype and natural history of NAFLD because it is a multifactorial disease resulting from the interaction between the genetic background and environmental factors (4). Obesity, type 2 diabetes, reduced physical activity, and genetic variants are the most important risk factors; however, it is unclear which of them plays a decisive role in the disease promotion and progression. Therefore, in clinical practice, it is unknown which patients have the greatest risk of suffering from liver damage or developing liver cirrhosis (4).

The genetic contribution to NAFLD development has been previously studied and up to now the genome-wide association studies (GWASs) have greatly contributed to understanding the role of the genetic factors in NAFLD pathogenesis and variability of prognosis (5). For example, the single nucleotide polymorphisms (SNPs) in phospholipase domain-containing 3 (*PNPLA3*) and transmembrane 6 superfamily member 2 (*TM6SF2*) gene have been recently associated with the development of steatosis, NAFLD-related hepatocellular carcinoma (HCC), and the stage of liver fibrosis (6–8). On the other hand, type 2 diabetes is considered *per se* an independent risk factor for liver disease progression (9); however, it is not known whether differences in the genetic background of the diabetic population may account for promoting metabolic liver disease or have a minor role because metabolic profile plays a key role in the disease progression. Only few studies have addressed this topic and, therefore, data are currently limited and inconclusive.

Kogiso et al. studied 272 Japanese patients with diabetes and found that the frequency of the risk allele G of *PNPLA3* gene was not different between the diabetic and non-diabetic subjects even if the diabetic patients with the GG genotype have a lower reduction in hemoglobin A1c (HbA1c) level after starting the antidiabetic treatment (10). Luukkonen et al. studied a large population of the obese individuals from Finland carrying the *PNPLA3 I148M* variant and found that the GG genotype was associated with an antiatherogenic lipid profile, decreased very-low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) particles, but increased liver fat content due to a loss-of-function mutation leading to impaired hepatocellular triglyceride hydrolysis (11). Rüschenbaum et al. confirmed these results in a German population; however, only a small number of the patients with diabetes were included in the study and the results were not definitive (12).

It much still needs to be done to understand the potential role of specific genetic background on the development of metabolic liver disease because there are several confounding factors.

This study presents the results of our experience with an Italian cohort of the diabetic patients from Southern Italy and discusses the potential utility of the polymorphisms in clinical practice.

METHODS

Study Population

We conducted a retrospective cohort study using data from 454 patients who were referred to the Ultrasound Clinic of the University Centre for Liver Disease Research and Treatment of the University of Foggia between March 2016 and March 2020. The study population included patients with the type 2 diabetes ($N = 260$) and non-diabetic individuals ($N = 194$).

At the recruitment, anthropometric, clinical, and biochemical data were recorded.

Moreover, all the patients underwent medical history, abdominal B-mode ultrasound (US), and transient elastography at baseline.

Ultrasound examination was performed by using EPIQ7 US system (Philips Medical Systems International, Best, Netherlands) and a C5-C1 convex probe. The diagnosis of fatty liver was based on the brightness of the liver tissue on US compared with the kidney, vascular blurring of the hepatic vein trunk, and deep attenuation in the right hepatic lobe. The severity of fatty liver change was classified as absent; mild fatty liver in case of a mild increase in hepatic echogenicity with normal visualization of the portal vessels and diaphragm; moderate fatty liver in case of moderate hepatic echogenicity, reduced visualization of the portal vessels, and diaphragm; and, finally, severe fatty liver when a marked increase in echoes was detected in the parenchyma with poor or no visualization of diaphragm.

The liver stiffness evaluation was performed by FibroScan (Echosens, Paris, France) and expressed as KPa. The criteria for a valid examination were at least 10 valid measurements, a ratio of the valid measurements to the total number of the valid and invalid measurements (success rate) $>$ 60% and interquartile range (IQR) $<$30% of the median value. Liver stiffness $\geq$ 7.8 KPa was used as a cutoff value for F2 stage of liver fibrosis and considered as clinically significant (13).

Patients with liver stiffness $\geq$ 10.5 KPa underwent liver biopsy to avoid misclassification of fibrosis stage (13, 14).

The criteria for the definition of liver cirrhosis were: (a) biopsy-proven presence of the regenerative nodules and fibrous tissue (histological criteria) and (b) presence of the clinical signs of decompensated disease (ascites, jaundice, and variceal bleeding) and/or portal hypertension (splenomegaly and esophageal varices) (clinical criteria).

Inclusion criteria were:

- Diagnosis of NAFLD $\pm$ Type 2 diabetes

Non-alcoholic fatty liver disease was defined by the excessive hepatic fat accumulation detected by ultrasonography or liver biopsy in the absence of the secondary causes of fatty liver.

Secondary causes of fatty liver were:

- Alcohol use disorders defined according to the European Association for the Study of the Liver (EASL) guidelines (15)
- Hepatitis C virus-associated fatty liver
- Autoimmune hepatitis
- Inherited liver disorders (hemochromatosis, Wilson disease, celiac disease, and abeta-/hypobetalipoproteinemia)
- Drug-induced liver injury

Patients aged $<$ 18 years with type 1 diabetes mellitus (T1DM), hepatitis B virus (HBV), or HIV infection were also excluded from the final analysis.

Patients with diabetes received one of the following antidiabetic regimens:

(a) Non-insulin therapy (use of the oral antidiabetic agents)

(b) Basal regimen (use of the basal insulin ± oral antidiabetic agents)

(c) Basal-bolus regimen (combination of basal insulin with a rapid-acting insulin at mealtimes).

Glycated hemoglobin was assessed from 8 weeks to 12 weeks after changing the antidiabetic medications.

Dyslipidemia was defined as the serum triacylglycerols > 150 mg/dl or total cholesterol level ≥ 200 mg/dl or LDL level ≥ 150 mg/dl or lipid-lowering treatment.

All the patients provided a signed informed consent and the study protocol was conducted according to the principles reported in the Declaration of Helsinki. The protocol was approved by the local Institutional Review Board. All the data generated or analyzed during this study are included in this published article.

Genetic Analysis

The *PNPLA3 rs738409* C→ G, *LPIN1 rs13412852* T→ C, *KLF6 rs3750861* T→ C, *SOD2 rs4880* C→ T, *TM6SF2 rs58542926* C→ T, and *ZNF624 rs12603226* C→ T SNPs were genotyped.

Genomic DNA was extracted from whole blood using the MagCore® Nucleic Acid Extractor, Bioscience, Taiwan.

The polymorphism analysis was done by real-time polymerase chain reaction (rt-PCR) by using a commercial kit (FLT PLUS Fatty Liver Test; Orga Bio Human, Rome, Italy) as instructed by the manufacturer.

The distribution of the allelic frequencies was studied in the overall population and the subgroups to show the potential deviations from the Hardy–Weinberg equilibrium (HWE).

Statistical Analysis

Statistical analysis was performed by using the SPSS (Statistical Package for the Social Sciences, version 20, Armonk, New York, USA) and GraphPad Prism version 8 (GraphPad Software, La Jolla, California, United States of America). Categorial data were reported as the absolute numbers (percentages) and the continuous variables as mean ± SD. The comparisons between the groups were performed by using ANOVA, Kruskal–Wallis test, chi-squared test, or Fisher's exact test where appropriate. Two-tailed $p < 0.05$ were considered as statistically significant. Sample size was not calculated due to the exploratory design of the study.

RESULTS

Study Population

Baseline characteristics of the study population are reported in **Table 1**. There was a statistically significant difference between the subgroups by age; therefore, age-adjusted results are shown below.

The prevalence of NAFLD was significantly higher in the diabetic population than in non-diabetic group (78.8 vs. 49.5%; $p < 0.001$). Twenty-three patients with diabetes and NAFLD had liver cirrhosis, whereas no patients had advanced liver fibrosis in the control group. As expected, there was a notably higher prevalence of dyslipidemia in the diabetic patients than in control group (75.8 vs. 23.7%; $p < 0.001$). In subgroup analysis by the antidiabetic treatment, the patients treated with non-insulin therapy had significantly lower HbA1c levels (mean 6.9 ± 1.36%) compared with the patients treated with basal regimen (mean 8.06 ± 1.44%), whereas the patients treated with basal-bolus therapy had a mean HbA1c of 8.37 ± 1.08% (non-insulin therapy group vs. basal regimen group $p < 0.001$; non-insulin therapy group vs. basal-bolus regimen group $p < 0.001$; and basal regimen group vs. basal-bolus regimen group $p = 0.16$).

Frequency of SNPs in the Study Population

Figure 1 shows the overall distribution of *PNPLA3 rs738409* (**Figure 1A**), *KLF6 rs3750861* (**Figure 1B**), *LPIN1 rs13412852* (**Figure 1C**), and *SOD2 rs4880* SNPs (**Figure 1D**). No differences were observed in the distribution of these polymorphisms between the diabetic and non-diabetic patients. Similarly, *TM6SF2 rs58542926* and *ZNF624 rs12603226* SNPs did not show the different distribution between the two groups (data not shown). **Table 2** shows the genotype distribution that was in HWE. The risk allele frequency, which is also reported in **Table 2**, was not statistically different between the diabetic and non-diabetic patients.

Single Nucleotide Polymorphisms and Prevalence of NAFLD in the Diabetic Population

Figure 2 shows the distribution of genotypes in the diabetic and non-diabetic patients according to the presence of NAFLD. Both in the diabetic and non-diabetic patients, we observed a higher frequency of risk allele for *PNPLA3 rs738409* ($p = 0.04$) (**Figures 2A,B**) and *LPIN1 rs13412852* ($p = 0.05$) SNPs (**Figures 2C,D**). Particularly, both the patients with diabetes and non-diabetic ones had a higher frequency of *PNPLA3 rs738409*G allele in NAFLD group (GG genotype prevalence was 11% in the NAFLD patients vs. 4% in control group and GC genotype prevalence was 45% in NAFLD group vs. 40% in the individuals without liver steatosis). The mean age of the patients was not different in the subgroups by *PNPLA3 rs738409* SNPs (the diabetic patients CC genotype 62.7 ± 11.2 years vs. CG genotype 64.8 ± 8.6 years vs. GG genotype 63.6 ± 9.3 years and the non-diabetic patients CC genotype 58.4 ± 10.2 years vs. CG genotype 57.8 ± 12.3 years vs. GG genotype 59.3 ± 13.9 years).

Regarding *LPIN1 rs13412852* SNP distribution, the risk allele C was more frequently observed in the patients with hepatic steatosis irrespective of the history of diabetes. The frequency of the different genotypes was similar in the diabetic vs. control group for *KLF6 rs3750861*, *SOD2 rs4880*, *TM6SF2 rs58542926*, and *ZNF624 rs12603226* SNPs.

Single Nucleotide Polymorphisms and Lipid Profile

We analyzed the lipid profile of the diabetic patients according to the genotypes. In the subgroup analysis by *PNPLA3 rs738409* genetic variants, no differences were observed between the

TABLE 1 | Baseline characteristics of study population.

	All (*N* = 454)	DM+ (*N* = 260)	DM– (*N* = 194)	*p*
Age (years)	58.5 (13.6)	63.8 (9.7)	58.6 (13.4)	<0.001
Sex (M/F)-*N* (%)	244 (53.7%)/210 (46.3%)	142 (54.6%)/118 (45.4%)	102 (52.6%)/92 (47.4%)	0.66
BMI (Kg/m^2)	29.4 (5.6)	30.5 (5.5)	27.6 (5.2)	<0.001
Waist circumference (cm)	101.2 (14.3)	105.5 (13)	93.3 (12.3)	<0.001
NAFLD-*N* (%)	301 (62.3%)	205 (78.8%)	96 (49.5%)	<0.001
Cirrhosis-*N* (%)	23 (5%)	23 (8.8%)	–	<0.001
Dyslipidemia-*N* (%)	243 (53.5%)	197 (75.8%)	46 (23.7%)	<0.001

Age, BMI, and waist circumference are expressed as mean ± SD.
BMI, body mass index; NAFLD, non-alcoholic fatty liver disease.

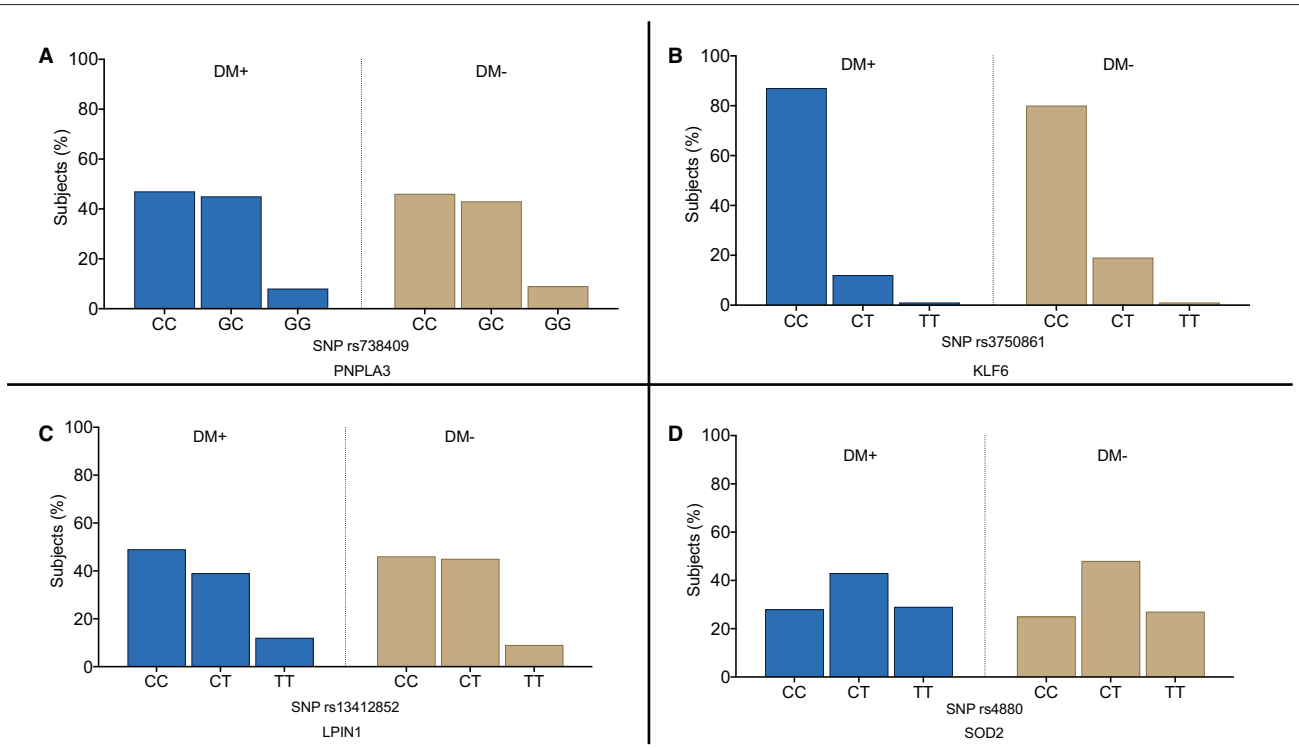

FIGURE 1 | Overall distribution of *PNPLA3 rs738409* **(A)**, *KLF6 rs3750861* **(B)**, *LPIN1 rs13412852* **(C)**, and *SOD2 rs4880* **(D)** single nucleotide polymorphisms (SNPs) in our study population. DM+, patients with type 2 diabetes; DM–, non-diabetic individualsof.

different genotypes in the serum total cholesterol and HDL levels, whereas the patients with the risk allele G showed a lower mean value of serum triglycerides (172.5 mg/dl for CC genotype, 121 mg/dl for CG genotype, and 117 mg/dl for GG genotype; CC vs. CG $p < 0.001$; CC vs. GG $p < 0.001$). **Table 3** shows no differences that were found in total cholesterol, HDL, and triglycerides serum levels in the patients according to *LPIN1 rs13412852*, *KLF6 rs3750861*, and *SOD2 rs4880 SNPs*. Similarly, no differences in lipid profile were observed in the diabetic patients according to *TM6SF2 rs58542926* and *ZNF624 rs12603226* SNPs (data not shown).

Single Nucleotide Polymorphisms and Kidney Function

We analyzed the serum creatinine and estimated glomerular filtration rate (eGFR) values in the subgroups by the genotypes. **Figures 3A,B** shows that there was no association between kidney function and *PNPLA3 rs738409* and *LPIN1 rs13412852* SNPs. On the other hand, there was a statistically significant reduction in eGFR value for *SOD2 rs4880* (**Figure 3C**) and *KLF6 rs3750861* SNPs (**Figure 3D**). Particularly, the patients who carried the risk allele T for *SOD2 rs4880* SNP showed a reduction in eGFR value (CC genotype 96.2 ± 24.4 ml/min/1.73 m^2; CT genotype 88.3 ± 29 ml/min/1.73 m^2; TT genotype 85.1 ± 23.2

TABLE 2 | Frequency of *PNPLA3 rs738409*, *LPIN1 rs13412852*, *SOD2 rs4880*, *KLF6 rs3750861*, and *TM6SF2 rs58542926* risk alleles in the study population.

	HWE *p*	Minor allele	Risk allele	Risk allele frequency overall	Risk allele frequency diabetic patients	Risk allele frequency diabetic patients with NAFLD	Risk allele frequency Non-diabetic patients
PNPLA3 rs738409	0.968	G	G	0.311	0.308	0.343	0.317
LPIN1 rs 13445678	0.850	T	C	0.665	0.687	0.673	0.678
SOD2 rs 4880	0.486	T	T	0.504	0.503	0.517	0.505
KLF6 rs 3750861	0.992	T	C	0.918	0.934	0.923	0.895
TM6SF2 rs 58542926	0.998	T	C	0.958	0.973	0.968	0.935

HWE, Hardy–Weinberg equilibrium; NAFLD, non-alcoholic fatty liver disease.

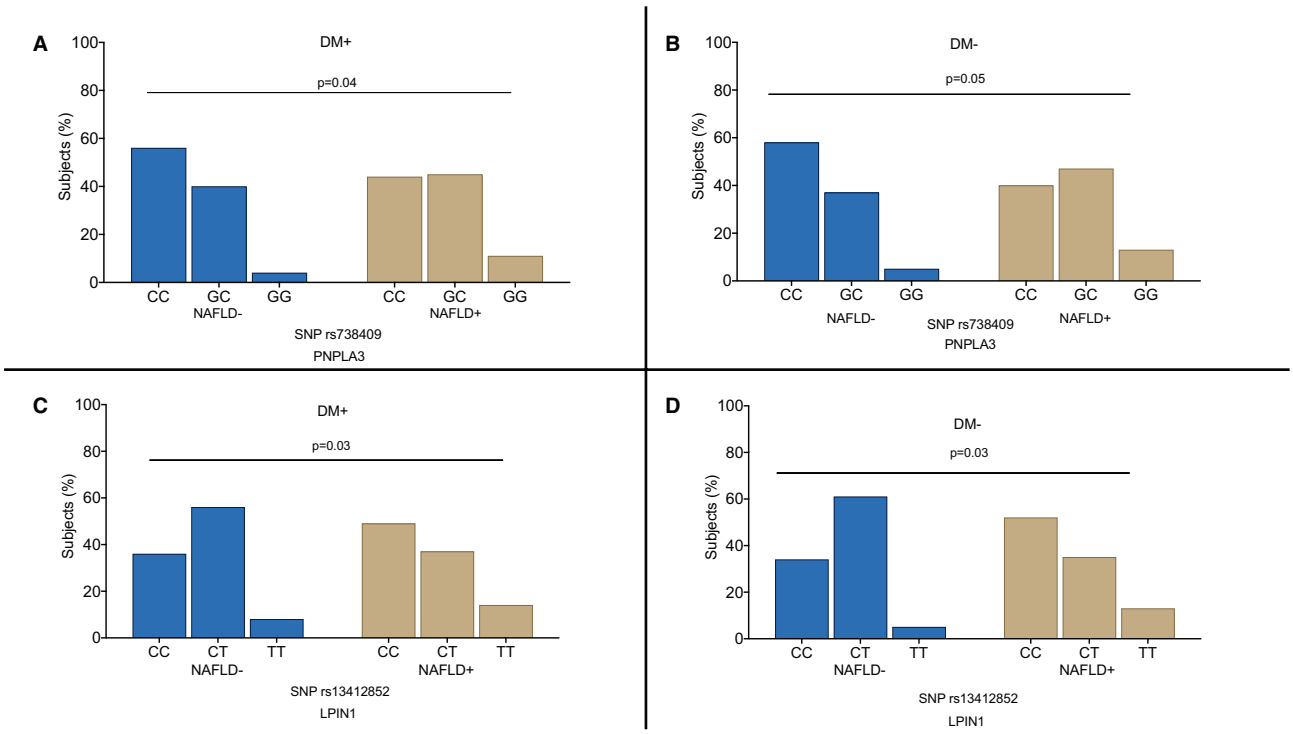

FIGURE 2 | Distribution of the genotypes in the diabetic patients **(A–C)** and non-diabetic individuals **(B–D)** according to the presence of non-alcoholic fatty liver disease (NAFLD). DM+: patients with type 2 diabetes; DM−: non-diabetic individuals.

ml/min/1.73 m^2; CC vs. TT genotype $p = 0.008$). Patients who carried allele T for *SOD2 rs4880* SNP had an odds ratio (OR) of 1.62 (95% CI: 1.06–2.56) for eGFR value < 90 ml/min/1.73 m^2.

The risk allele C for *KLF6 rs3750861* polymorphism was also associated with a significant reduction in eGFR value [CT genotype 97.5 ± 25.6 ml/min/1.73 m^2 vs. TT genotype 89.2 ± 26.3 ml/min/1.73 m^2 ($p = 0.02$)]. Serum creatinine values were not different in the subgroups by the genotypes for all the available SNPs. The OR for eGFR value < 90 ml/min/1.73 m^2 in the individuals who carried allele C was 1.95 (95% CI: 1.21–3.15).

Single Nucleotide Polymorphisms and Liver Fibrosis

Figure 4 shows liver stiffness values for the diabetic patients according to genotype. Genotype GG for *PNPLA3 rs738409* SNP was associated with significantly higher liver stiffness values compared with the CC and CG genotypes (GG: 13.9 ± 8.2 KPa; CG: 7.4 ± 5.1 KPa; CC: 7 ± 3.4 KPa; CC vs. CG genotype $p < 0.001$; CC vs. GG genotype $p < 0.001$) (**Figure 4A**). Patients who carried the *PNPLA3 rs738409* allele had a GWAS that was associated with an age-adjusted OR for significant fibrosis of 1.44 (95% CI: 1.03–2.26).

Similar results were observed for the patients with *KLF6 rs3750861* CC genotype who had a higher liver stiffness (8.2 ± 4 KPa) compared with the patients with CT genotype (5.6 ± 3.3 KPa; $p = 0.03$) (**Figure 4D**).

Patients who carried *KLF6 rs3750861* allele C had an OR of 1.98 (95% CI: 1.13–5.51) for significant fibrosis.

Our cohort of the patients included 23 patients with the diabetes and biopsy-proven metabolic-related cirrhosis.

TABLE 3 | Lipid profile in the diabetic patients according to the SNPs and genotypes.

SNP	Genotype	Total cholesterol (mg/dl)	LDL cholesterol (mg/dl)	HDL cholesterol (mg/dl)	Triglycerides (mg/dl)
PNPLA3 rs738409	C/C	178.9 ±43.1	112.2 ±35.5	46.8 ±11.4	172.5 ±68.8
	C/G	178.5 ±43.8	115.6 ±33.3	47.7 ±17.9	121 ±73.1*
	G/G	162.7 ±46.7	110.8 ±28.6	43.8 ±9.1	117.4 ±63.1§
LPIN1 rs13445678	C/C	183.7 ±52.5	118.4 ±31.6	49.5 ±16.7	166.3 ±68.8
	C/T	168.9 ±45.1	117.1 ±34.3	46.1 ±12.9	146.2 ±78.7
	T/T	169.2 ±33.9	169.2 ±33.9	45 ±11.1	162.1 ±97.4
SOD2 rs4880	C/C	186.2 ±44.5	118.1 ±36.7	48.6 ±12.9	167.6 ±90.7
	C/T	179.4 ±48.8	108.9 ±27.6	46.6 ±17	166.3 ±110.3
	T/T	176.9 ±46.7	113.2 ±35.2	48.3 ±13.3	157.9 ±69.4
KLF6 rs3750861	C/C	176.2 ±42.7	111.2 ±31.9	47.3 ±12.1	154.2 ±92.8
	C/T	184.3 ±51.2	122.5 ±36.3	48.7 ±27	179.1 ±114.9

*SNPs, single nucleotide polymorphisms; LDL, low-density lipoprotein; HDL, high-density lipoprotein. *p < 0.001 for comparison genotype CC vs CG. §p < 0.001 for comparison genotype CC vs. GG.*

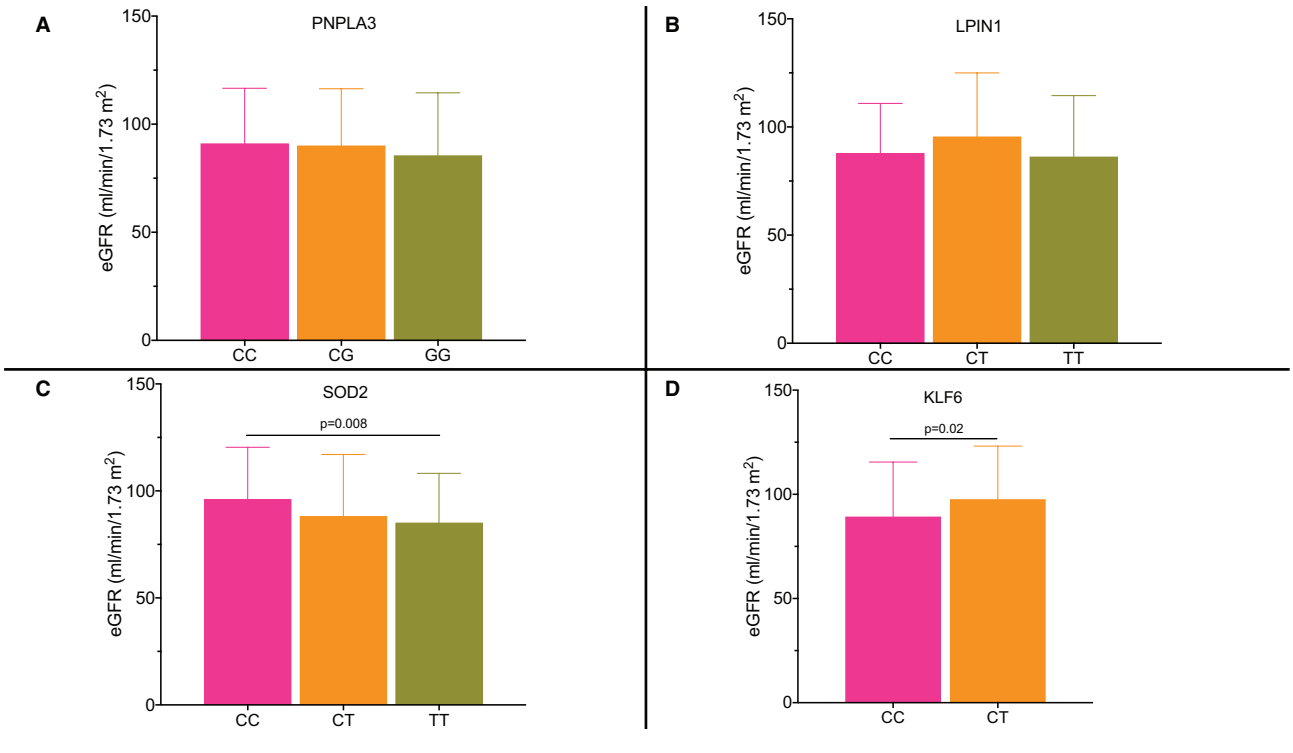

FIGURE 3 | (A–D) Estimated glomerular filtration rate (eGFR) according to the single nucleotide polymorphisms and their genotypes.

We analyzed their genotypes and found that all the patients except for four had GG genotype for *PNPLA3 rs738409* SNP, whereas all the patients except for one had at least one allele risk for *LPIN1 rs13412852* SNP. All the patients with cirrhosis had at least one risk allele for *SOD2 rs4880* SNP too. Patients included in the non-diabetic group did not show different stiffness values by the genotypes for all the studied SNPs (data not shown).

Single Nucleotide Polymorphisms and Glucose Control

Glucose level and HbA1c were analyzed in the subgroups by the SNPs and, for each polymorphism, data were analyzed by antidiabetic therapy (non-insulin treatment vs. insulin therapy groups). The mean values of glycated hemoglobin in the patients by *PNPLA3 rs738409* SNP genotype were not statistically different even if a trend toward increased levels was observed in the patients with the GG genotype

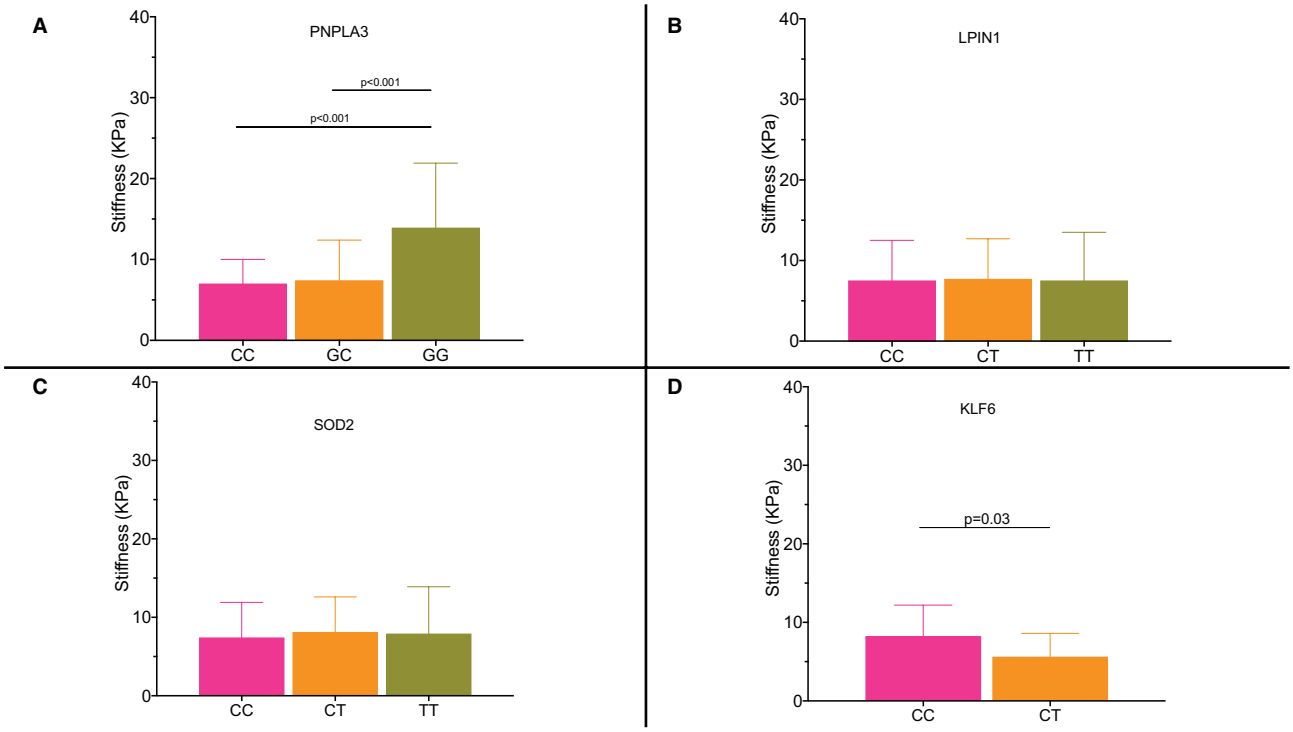

FIGURE 4 | (A–D) Liver stiffness expressed as KPa according to the single nucleotide polymorphisms and their genotypes.

both in the patients treated with insulin and in non-insulin-treated subjects. Similarly, no significant results were observed for the HbA1c levels by *LPIN1 rs13412852*, *KLF6 rs3750861*, *SOD2 rs4880*, *TM6SF2 rs58542926*, and *ZNF624 rs12603226* SNPs. No differences in the glucose levels were observed in all the subgroups by the SNPs and antidiabetic treatment.

DISCUSSION

Non-alcoholic fatty liver disease affects almost two billion people globally and its burden is expected to grow in the coming decades. The prevalence estimates indicate that it is the most prevalent liver disease in human history (16). NAFLD is becoming an established risk factor for the cardiovascular disease and type 2 diabetes, which are currently the leading causes of death and disability (16).

The identification of familiar clustering and interethnic differences in susceptibility has suggested that a significant heritable component may be involved in the development and progression of NAFLD (17, 18). The availability of the GWASs in large cohorts of the patients with NAFLD has enabled the researchers to identify the potential genetic factors that could predispose the individuals to the clinically relevant consequences such as liver cirrhosis (17). However, although several studies have improved our understanding of the role of genetic background on NAFLD pathogenesis and the potential mechanisms underlying fat accumulation and fibrosis development, at present, the predictive power of the genetic factors is still too limited to support their daily use in clinical practice.

A large number of studies have found that the several gene variants for *PNPLA3*, *TM6SF2*, glucokinase regulatory protein (*GCKR*), lysophospholipase-like 1 (*LYPLAL1*), or membrane-bound O-acyltransferase domain-containing 7 (*MBOAT7*) significantly increase the risk of NAFLD (19). Some of them are associated with an increase in the risk of diabetes and liver disease and others are involved only in NAFLD development. For example, *PNPLA3 rs738409* GG genotype carriers have a higher risk of developing fatty liver (73%) than non-carriers (20%) but a small increase in the risk of type 2 diabetes (21), whereas a *TM6SF2* gene variant is associated with a 2.1-fold increase in the risk of NAFLD and a 40% increase in the incidence of diabetes (19, 22). Most available data have been obtained from the international studies and, currently, few studies are available concerning the Italian population.

Bellan et al. studied the impact of *PNPLA3 rs738409* SNPs in the prediction of hepatic steatosis and fibrosis in a cohort of 328 patients from the Northern Italy and found that the carriage of the G allele was associated with higher liver stiffness values (5.9 kPa in CC homozygotes, 6.1 kPa in CG heterozygotes, and 6.8 kPa in GG homozygotes; $p = 0.01$), whereas no differences were found in glycated hemoglobin in the subgroups by the genotypes (23). The impact of *PNPLA3* polymorphisms on the onset and severity of liver disease in the subgroup of the patients with diabetes was also investigated; however, the strength of

this association was irrelevant and the authors concluded that confounding factors, such as body mass index (BMI), could play a major role in the development of liver disease in the diabetic population (23).

Grimaudo et al. studied the impact of genetic background on the development of liver disease in a large population from the Southern Italy; however, only 46% had diabetes and the study population included only the patients with NAFLD-related cirrhosis (24).

In this study, we found that the genotype distribution of *PNPLA3 rs738409*, *TM6SF2 rs58542926*, *LPIN1 rs13412852*, *KLF6 rs3750861*, *SOD2 rs4880*, and *ZNF624 rs12603226* SNPs was not different in the diabetic vs. non-diabetic population, whereas their distribution was different between the patients with NAFLD and the patients without NAFLD irrespective of presence of diabetes. The SNPs seemed not to be involved in the pathogenesis of diabetes but rather involved in the hepatic changes regardless of glucose levels.

Our analysis of lipid profile in the diabetic patients by the SNPs did not show any changes in the total cholesterol and LDL cholesterol levels, whereas the serum triglyceride levels were significantly lower in *PNPLA3 rs738409* G allele carriers. *PNPLA3*, also known as adiponutrin, is a member of the patatin-like phospholipase family and *PNPLA3* gene encodes a membrane-bound triacylglycerol lipase that mediates triacylglycerol hydrolysis and its polymorphisms have been widely associated with the progression of liver fibrosis and development of HCC (20, 25–28). The isoleucine-to-methionine substitution at residue 148 variant of *PNPLA3* gene (allele G) is due to a loss-of-function mutation, which leads to impaired hepatocellular triglyceride hydrolysis and, finally, triglyceride accumulation in the hepatocytes (11). Our findings are in accordance with the results reported by Palmer et al. who observed in two large populations including obese patients, paradoxical lower serum triglyceride levels in *PNPLA3 rs738409* G allele carriers in accordance with the impaired hepatic secretion of the triglycerides.

In this study population, we observed the higher stiffness values in the patients with diabetes who carried the GG genotype. These results confirmed the role of *PNPLA3 rs738409* SNPs in the prediction of liver fibrosis in a population of the patients with diabetes from the Southern Italy and suggested that its use in routine clinical practice improves the identification of the patients who are at a higher risk of fibrosis development and progression. Moreover, concordantly with the previously reported data (29, 30), we observed a significant increase in liver stiffness values in the patients with the CC genotype for *KLF6 rs3750861* SNP. *KLF6* is a ubiquitously expressed transcription factor, which is involved in the cell proliferation, differentiation, and cell death (31). It is early expressed in activated hepatic stellate cells (HSCs) after liver injury, suggesting a potential role in the process of liver fibrogenesis (32). Vespasiani-Gentilucci et al. reported a prevalence of CC, CT, and TT genotypes, which are very similar to our population; however, this study population included 290 patients and prevalence of diabetes was only 40%. In this study, the authors did not find a significant difference in the stage of liver fibrosis between CC and TT subgroups (33).

The impact of the SNPs on glucose metabolism of the patients with NAFLD is also a hot topic in hepatology because evidence is inconsistent. Machado et al. reported an unexpected better fasting plasma glucose control in the patients with *PNPLA3 rs738409* GG genotype (34), whereas Petit et al. did not report any differences in fasting plasma glucose and the HbA1c levels by *PNPLA3 rs738409* SNPs (35).

As suggested by Mantovani et al. (36), given that the risk allele G of *PNPLA3 rs738409* is linked to an increased risk of the fibrosis development and progression and that the severity of NAFLD is associated with a worse glycemic control (37, 38), we should not expect the lower glucose and HbA1c levels in the patients who carry the risk allele G of *PNPLA3 rs738409*.

Concordantly, we found no differences in the fasting plasma glucose and HbA1c levels in the patients with the GG genotype vs. CG or CC genotype, showing that our data are consistent with the results previously reported by Mantovani et al. (37). Concerning the potential role of *LPIN1 rs13412852*, *KLF6 rs3750861*, *SOD2 rs4880*, *TM6SF2 rs58542926*, and *ZNF624 rs12603226* SNPs in glycemic control of the patients with diabetes, data are also limited. *LPIN1* is a magnesium-dependent phosphatase responsible for catalyzing the penultimate step in triacylglycerol synthesis and, in addition, it is a transcriptional coactivator that interacts with the nuclear receptor peroxisome proliferator-activated receptor-α (PPARα) and PPARγ coactivator 1α (PPARGC1A) to regulate fatty acid oxidation gene expression (39). Body fat accumulation is a major regulator of *LPIN1* gene expression and this is strongly associated with insulin-mediated subcutaneous adipocyte glucose transport (40). Some authors have investigated a potential role of *LPIN1* SNPs in the phenotype of the patients with metabolic alteration; however, a large number of SNPs have been studied and all of them have confirmed that there are no differences in fasting plasma glucose by *LPIN1* SNPs (39, 41). These results are consistent with our findings. Data on the impact of *KLF6* mutation on the fasting glucose control and insulin level are available for *KLF6 rs3750861* G>A polymorphism, whereas data concerning the potential role of *KLF6 rs3750861* T>C polymorphism on glucose control are very limited (29, 42). Hepatocyte expression of *KLF6* regulates hepatic fatty acid and glucose metabolism via transcriptional activation of liver glucokinase and posttranscriptional regulation of PPARα (43). *KLF6 rs3750861* polymorphisms are associated with the novel binding sites and promotion of alternative splicing of *KLF6* into the truncated isoforms (42). We reported for the first time in a population of the patients with diabetes that *KLF6 rs3750861* T>C polymorphism is not associated with significant differences in fasting plasma glucose and HbA1c levels; however, because of the limited sample size, further confirmation is needed. We found a similar result for *TM6SF2 rs58542926* SNP, which has been previously linked to an increased hazard of developing diabetes (22) and that, on the contrary, did not show a significant impact on glucose control in our study population.

We analyzed the association between the SNPs and kidney function. Some authors have recently suggested a significant association between the SNPs and risk of chronic kidney disease (CKD), but the mechanisms supporting this association are

poorly understood. Mantovani et al. have observed in a cohort of diabetic patients ($N = 112$) that the patients homozygous for *PNPLA3 rs738409 I148M* variant (GG genotype) had lower eGFR values and a higher prevalence of CKD independently from liver disease (44). These results were confirmed by the same authors in a group of 101 women with diabetes (37). Sun et al. showed that the patients with NAFLD, normal alanine aminotransferase (ALT) levels, and carriage of *PNPLA3 rs738409* G allele were at higher risk of early glomerular and tubular damage (45).

In our cohort, we found a trend toward the lower eGFR values in the patients with the GG genotype for *PNPLA3 rs738409* polymorphisms without reaching statistical significance. This result could be affected by the small number of the patients included in the GG genotype group. Conversely, we found a significant difference in mean eGFR value for the diabetic patients by *SOD2* and *KLF6* genetic variants.

Several authors have studied the impact of polymorphism T>C of *SOD2 rs4880* on kidney function in the diabetic patients (46). The gene is coded in the nuclear DNA, therefore the enzyme translocates to the mitochondria after translation of the protein in the cytosol. The valine-to-alanine substitution induces the conformational changes with a less efficient transport of *SOD2* into the mitochondrial matrix and, finally, lowers the ability to neutralize the superoxide radicals, which are largely produced in the mitochondria of the patients with hyperglycemia (47).

Mollsten et al. found a significant association between *SOD2* polymorphisms and diabetic nephropathy in the patients with type 1 diabetes (46).

Nomiyama et al. observed a strong association between the CT or CC genotype and risk of nephropathy in a large population ($N = 478$) of the Japanese diabetic patients. Data on the correlation between *SOD2* genotype and risk of CKD are not available for the Italian population and we confirmed for the first time these results in the Italian patients.

Limitations of our study are the sample size, the use of ultrasonography for the detection of fatty liver, and the lack of follow-up data. Long-term follow-up data are needed to understand whether the SNPs can be associated with pivotal clinical outcomes such as the rate of disease progression, the risk of HCC, or the response to a specific class of the antidiabetic drugs.

These clinical issues should be investigated in the future studies to reach one of the most important challenges of the modern medicine, which is the tailoring of medical treatment to the individual characteristics of each patient.

In conclusion, our findings suggest that in diabetic population, the GG genotype of *PNPLA3 rs738409* and the CC genotype of *KLF6 rs3750861* SNPs are associated with the higher stiffness values and the risk of developing liver fibrosis, whereas the CC genotype of *SOD2 rs4880* and the CT genotype of *KLF6 rs3750861* SNPs are associated with the lower eGFR values. Their use in clinical practice may help the clinicians to select the patients with diabetes who require a strict follow-up program.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional review Board (University of Foggia). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RV and GS contribute to the concept and design of study. GPM, GDG, MS, and ADR contribute to the acquisition of data. RV and GS contribute to the statistical analysis. RV, TC, and GS contribute to the analysis and interpretation of data and contribute to the drafting of the manuscript. RV, GS, GPM, GDG, MS, and ADR contribute to the critical revision of the manuscript. All authors contributed to the article and approved the submitted version.

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Role of Galectins in the Liver Diseases: A Systematic Review and Meta-Analysis

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Background: Galectins, a family of β-galactoside-binding proteins, are related to the development and progression of various human diseases such as cancer, heart failure, and chronic kidney disease. However, its role in liver diseases is unclear.

Methods: The PubMed, Embase, and Cochrane Library databases were searched. Hazard ratios (HRs), odds ratios (ORs), and mean differences (MDs) with 95% CIs were pooled to evaluate the association of the galectins with the outcomes and risk of liver diseases by a random effects model.

Results: Thirty three studies involving 43 cohorts and 4,168 patients with liver diseases were included. In the patients with hepatocellular carcinoma (HCC), high expression of galectin-1 and -3 in the tissues was significantly associated with worse overall survival (galectin-1: HR = 1.94, 95% CI = 1.61–2.34, $p < 0.001$; galectin-3: HR = 3.29, 95% CI = 1.62–6.68, $p < 0.001$) and positive vascular invasion (galectin-1: OR = 1.74, 95% CI = 1.18–2.58, $p = 0.005$; galectin-3: OR = 2.98, 95% CI = 1.58–5.60, $p = 0.001$); but, high expression of galectin-4 and −9 in the tissues was significantly associated with better overall survival (galectin-4: HR = 0.53, 95% CI = 0.36–0.79, $p = 0.002$; galectin-9: HR = 0.56, 95% CI = 0.44–0.71, $p < 0.001$) and negative vascular invasion (galectin-4: OR = 0.36, 95% CI = 0.19–0.72, $p = 0.003$; galectin-9: OR = 0.60, 95% CI = 0.37–0.97, $p = 0.037$). Serum galectin-3 level was significantly higher in HCC (MD = 3.06, 95% CI = 1.79–4.32, $p < 0.001$), liver failure (MD = 0.44, 95% CI = 0.23–0.66, $p < 0.001$), liver cirrhosis (MD = 1.83, 95% CI = 1.15–2.51, $p < 0.001$), and chronic active hepatitis B (MD = 18.95, 95% CI = 10.91–27.00, $p < 0.001$); serum galectin-9 level was significantly higher in HCC (MD = 3.74, 95% CI = 2.57–4.91, $p < 0.001$) and autoimmune hepatitis (MD = 8.80, 95% CI = 7.61–9.99, $p < 0.001$).

Conclusion: High galectin-1 and -3 and low galectin-4 and -9 expression indicate worse outcomes of patients with HCC. Serum galectin-3 and -9 levels are positively associated with the risk of chronic liver diseases.

Keywords: galectins, hepatocellular carcinoma, cirrhosis, hepatitis, fibrosis

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INTRODUCTION

Liver diseases, including chronic hepatitis, liver fibrosis or cirrhosis, acute liver injury or liver failure, and hepatocellular carcinoma (HCC), are a major global health burden. They are often subtle, but potentially lethal (1). According to the report of the Global Burden of Disease Study 2019, there are 79,200 deaths from acute hepatitis (2), 1,470,000 deaths from liver cirrhosis and other chronic liver diseases (3), and 485,000 deaths from HCC (4) in the world. Early assessment and identification of liver diseases by molecular biomarkers are clinically important.

Galectins are a family of lectins composed of one or two carbohydrate recognition domains (CRDs) that bind to the β-galactoside-containing glycans (5). Galectins are classified into three groups according to their molecular-structural characteristics: "prototype" galectins with a single CRD (i.e., galectin-1,-2,-5,-7,-10,-11,-13,-14,-15, and -16); "chimeric-type" galectins (i.e., galectin-3) with the tandem repeats of proline- and glycine-rich short stretches fused onto the CRD; and "tandem repeat"-type galectins with two distinct CRDs (i.e., galectin-4,-6,-8,-9, and -12) (6). Galectins are responsible for the regulation of premessenger RNA (mRNA) splicing, cell cycle, cell growth, and cell apoptosis (7), and the development and/or progression of many human diseases, including cancer, heart failure, and chronic kidney disease (8).

Galectins play a regulatory role in liver diseases by binding their CRDs to the glycoconjugates expressed in the hepatocytes (9). Abnormal expression of the galectins may be related to the development of hepatitis and liver fibrosis/cirrhosis and the progression of HCC (10). In this study, we conducted a systematic review and meta-analysis to evaluate the role of galectins in various liver diseases.

METHODS

This meta-analysis was performed in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement (11).

Registration

The registration number was CRD42020210038 in the PROSPERO.

Literature Search

The literature was searched via the PubMed, Embase, and Cochrane Library databases from the earliest available publication until September 18, 2020. Search items were as follows: "(galectin)" and "(liver)" or "(hepatic)" or "(hepatitis)" or "(hepatocellular)" or "(fibrosis)" or "(failure)." There was no language restriction.

Selection Criteria

The inclusion criteria were as follows: (1) study population should be the patients diagnosed with liver diseases and (2) galectin expression or level was detected in patients with liver diseases. The exclusion criteria were as follows: (1) duplicate papers; (2) reviews, meta-analyses, or case reports; (3) notes, conferences, corrections, editorials, comments, or letters; (4) experimental or animal studies; and (5) studies which were lacking of detailed data regarding galectin expression or level.

Data Extraction

We extracted the following data from each study, including first author, publication year, country, study design, enrollment period, sample size, subtypes of the galectins, and methods to detect the galectins. As for the studies regarding the clinicopathological features and the outcomes of HCC, we specifically extracted the data as follows: galectin expression and its grouping; clinicopathological features including tumor size, tumor-node-metastasis (TNM) stage, differentiation grade, and vascular invasion; and outcomes, which include overall survival (OS), disease-free survival (DFS), and relapse-free survival (RFS). As for the studies regarding the risk of liver diseases, we specifically extracted the data regarding the type of liver diseases, the Child–Pugh class, and the level of serum galectins.

As for the survival data, we directly extracted or indirectly estimated the hazard ratio (HR) and 95% CI. If a study did not give the HR and 95% CI, but only reported the Kaplan–Meier curves, we would employ the Engauge Digitizer 4.1 software (Linux, Mac OSX, and Windows Slashdot Media, CA, USA) to extract the survival rate at the different time points from the Kaplan–Meier curves and then utilize Tierney's table (12) to estimate its correlative HR with 95% CI.

Study Quality Assessment

Quality of the case–control and cohort studies were evaluated by the Newcastle–Ottawa Scale (NOS), which included the three parts (i.e., selection, comparability, and outcomes) and eight questions (13). The highest NOS score was nine points. High quality was considered if the NOS score was more than six points.

Statistical Analysis

The Stata version 12.0 (Stata Corporation, College Station, Texas, USA) was employed for the statistical analysis. Only a random effects model was implemented. HRs, odds ratios (ORs), and mean differences (MDs) with 95% CIs were pooled. A two-sided $p < 0.05$ was considered as statistically significant. If the data were expressed as median with range, mean with SD would be estimated (14). Heterogeneity was evaluated by the I^2 statistics and the Cochran's Q test. $I^2 > 50\%$ or $p < 0.1$ was considered as a statistically significant heterogeneity. Sensitivity analysis was performed after omitting one study at a time in order to check the consistency to estimate the overall effect. Publication bias was assessed by Egger's test (15) and $p < 0.1$ was considered to imply a significant publication bias.

RESULTS

Study Selection and Characteristics

Among the 4,005 papers initially retrieved, 33 papers were eligible (**Figure 1**). They were published from 2008 to 2020 (16–48). Members of the galectins evaluated included galectin-1,-3,-4, and -9. The sample size ranged from 10 to 386; 25 studies came from Asia (16–20, 22, 25–39, 42, 44, 46, 47), six studies came

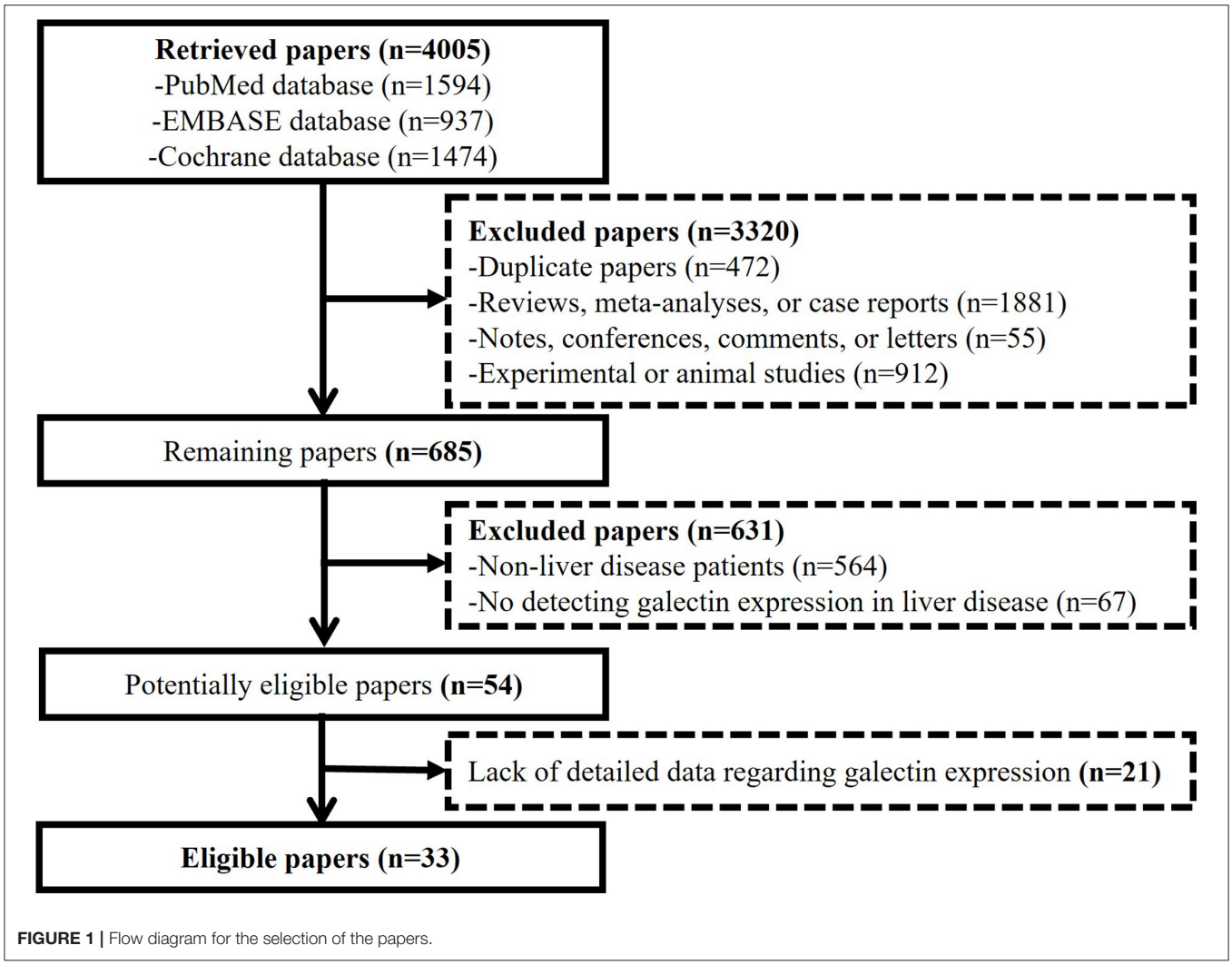

FIGURE 1 | Flow diagram for the selection of the papers.

from Europe (21, 40, 41, 43, 45, 48), and two studies came from Oceania (23, 24); five studies were published as the abstracts (27, 28, 35, 37, 42) and 28 studies were published as the full texts (16–26, 29–34, 36, 38–41, 43–48); and 29 studies were of high quality (16–26, 29, 30, 32–39, 41–48), but four studies were of low quality (27, 28, 31, 40).

Meta-Analyses Regarding the Galectins With Prognosis and Clinicopathological Features of the Hepatocellular Carcinoma

Seventeen studies involving 19 cohorts and 3,120 patients focused on the relationship of the galectins expressed in the tissues with prognosis and clinicopathological features of HCC (16–32) (**Table 1**). Among them, five study cohorts focused on galectin-1 (17–21), seven study cohorts focused on galectin-3 (22, 25–29, 31), one study cohort focused on galectin-4 (32), and six study cohorts focused on galectin-9 (16, 23, 24, 26, 30). Results of the meta-analyses are shown in **Table 2**.

Overall Survival

The relationship between the galectins and OS was explored in 17 study cohorts (16–20, 22–30, 32).

High galectin-1 expression was significantly correlated with worse OS in the patients with HCC (HR = 1.94, 95% CI = 1.61–2.34, $p < 0.001$) without significant heterogeneity ($I^2 = 0.0\%$, $p = 0.739$).

High galectin-3 expression was significantly correlated with worse OS in the patients with HCC (HR = 3.29, 95% CI = 1.62–6.68, $p = 0.001$) with a significant heterogeneity ($I^2 = 90.00\%$, $p = 0.008$). Sensitivity analysis illustrated that the study by Song et al. (22) displayed an apparent influence on the overall result of the meta-analysis (**Supplementary Figure 1**). After the exclusion of this study, the pooled HR was similar (HR = 2.51, 95% CI = 1.51–4.16, $p < 0.001$), but with a mild reduction in heterogeneity ($I^2 = 71.10\%$, $p = 0.008$).

High galectin-4 expression was significantly correlated with better OS in the patients with HCC (HR = 0.53, 95% CI = 0.36–0.79, $p = 0.002$).

TABLE 1 | Characteristics of the included studies regarding the galectins with the prognosis and clinicopathological features of HCC in the tissues.

References	Country	Type of publication	Enrollment period	No. total pts.	Galectin subtypes	No. high expression	Pathological stage	IHC positive	Outcomes	Clinicopathologic features	HR with 95% CI	NOS score
Matsuda et al. (25)	Japan	Full text	1994–2003	52	Galectin-3	34	TNMII-IV	NA	OS	Report	Survival curve	8
Spano et al. (21)	Italy	Full text	1988–2007	197	Galectin-1	44	TNMII-IV	Score>2	NA	Report	NA	7
Fang et al. (31)	China	Full text	2001–2007	46	Galectin-3	36	TNMI-III	Score>2	NA	Report	NA	5
Zhang et al. (16)	China	Full text	1995–2005	200	Galectin-9	113	TNMI-IV	Score>2	OS	Report	Survival curve	7
Wu et al. (20)	China	Full text	Up to 2011 3/15	386	Galectin-1	189	TNMI-IV	NA	OS, RFS	Report	Report	6
Gu et al. (30)	China	Full text	2006.06–2008.08	147	Galectin-9	68	TNMI-IV	NA	OS, RFS	Report	Survival curve	8
Jiang et al. (29)	China	Full text	2001–2004	165	Galectin-3	135	NA	2+ or 3+	OS	Report	Report	7
Cai et al. (32)	China	Full text	2005–2011	201	Galectin-4	89	TNMI-IV	2+ or 3+	OS, RFS	Report	Report	7
Kong et al. (26)	China	Abstract	NA	110	Galectin-3	52	NA	NA	OS	NA	Report	5
Kong et al. (28)	China	Full text	2008.10–2012.09	197	Galectin-9	106	TNMI-III	Score>100	OS	Report	Report	8
				197	Galectin-3	77					Report	
Yeh et al. (19)	China	Full text	2007–2012	91	Galectin-1	52	NA	2+ or 3+	OS	NA	Survival curve	8
Zhang et al. (17)	China	Full text	NA	209	Galectin-1	128	TNMI-IV	ICH>20%	OS	NA	Survival curve	6
You et al. (18)	China	Full text	2009–2011	162	Galectin-1	105	TNMI-IV	2+ or 3+	OS	Report	Report	7
Kong et al. (17)	China	Abstract	NA	247	Galectin-3	116	NA	NA	OS	NA	Report	5
Sideras et al. (24)	Netherlands	Full text	2001.06–2014.06	60	Galectin-9	46	TNMI-III	2+ or 3+	OS	NA	Survival curve	7
				94	Galectin-9	73					Report	
Sideras et al. (23)	Netherlands	Full text	2007.01–2013.03	81	Galectin-9	65	TNMI-III	NA	OS	NA	Report	6
Song et al. (22)	China	Full text	2005–2008	278	Galectin-3	135	TNMI-III	2+ or 3+	OS	Report	Report	7

HCC, hepatocellular carcinoma; Pts., number of patients; NA, not available; IHC, immunohistochemistry; NOS, Newcastle–Ottawa Scale; OS, overall survival; RFS, relapse-free survival; HR, hazard ratio.

TABLE 2 | Galectins with the prognosis and clinicopathological features of HCC: results of the meta-analyses.

Groups	No. studies	Pooled proportion using random-effects mode	*P*-value	Heterogeneity	
				I^2	*P*-value
OS					
Galectin-1	4	HR = 1.94 (95% CI = 1.61–2.34)	**<0.001**	0.0%	0.739
Galectin-3	6	HR = 3.29 (95% CI = 1.62–6.68)	**0.001**	90.0%	**0.008**
Galectin-4	1	HR = 0.53 (95% CI = 0.36–0.79)	**0.002**	–	–
Galectin-9	6	HR = 0.56 (95% CI = 0.44–0.71)	**<0.001**	3.7%	0.393
RFS					
Galectin-1	1	HR = 1.62 (95% CI = 1.26–2.08)	**<0.001**	–	–
Galectin-4	1	HR = 0.65 (95% CI = 0.47–0.89)	**0.008**	–	–
Galectin-9	1	HR = 0.46 (95% CI = 0.26–0.82)	**0.009**	–	–
Tumor size					
Galectin-1	2	OR = 1.59 (95% CI = 0.74–3.41)	0.238	75.8%	**0.042**
Galectin-3	4	OR = 1.69 (95% CI = 1.01–2.84)	**0.046**	48.8%	0.119
Galectin-4	1	OR = 0.43 (95% CI = 0.20–0.91)	**0.027**	–	–
Galectin-9	3	OR = 0.98 (95% CI = 0.70–1.39)	0.924	0.0%	0.394
TNM stage					
Galectin-1	2	OR = 2.53 (95% CI = 1.31–4.87)	**0.006**	41.9%	0.189
Galectin-3	4	OR = 2.06 (95% CI = 0.82–5.16)	0.122	66.6%	**0.030**
Galectin-4	1	OR = 0.49 (95% CI = 0.28–0.86)	**0.013**	–	–
Galectin-9	1	OR = 0.44 (95% CI = 0.20–0.98)	**0.044**	–	–
Differentiation grade					
Galectin-1	3	OR = 0.96 (95% CI = 0.70–1.32)	0.795	0.0%	0.830
Galectin-3	4	OR = 2.13 (95% CI = 0.97–4.69)	0.061	65.6%	**0.033**
Galectin-4	1	OR = 0.35 (95% CI = 0.16–0.78)	**0.010**	–	–
Galectin-9	3	OR = 0.70 (95% CI = 0.34–1.47)	0.348	70.2%	**0.035**
Vascular invasion					
Galectin-1	2	OR = 1.74 (95% CI = 1.18–2.58)	**0.005**	0.0%	0.679
Galectin-3	2	OR = 2.98 (95% CI = 1.58–5.60)	**0.001**	0.0%	0.421
Galectin-4	1	OR = 0.36 (95% CI = 0.19–0.72)	**0.003**	–	–
Galectin-9	2	OR = 0.60 (95% CI = 0.37–0.97)	**0.037**	2.8%	0.311

HCC, hepatocellular carcinoma; OS, overall survival; RFS, relapse-free survival; HR, hazard ratio; OR, odds ratio. The values in bold is defined as being statistically significant.

High galectin-9 expression was significantly correlated with better OS in the patients with HCC (HR = 0.56, 95% CI = 0.44–0.71, $p < 0.001$) without significant heterogeneity ($I^2 = 3.7\%$, $p = 0.393$).

Relapse-Free Survival

The relationship between the galectins and RFS was explored in three study cohorts (20, 30, 32).

High galectin-1 expression was significantly correlated with worse RFS in the patients with HCC (HR = 1.62, 95% CI = 1.26–2.08, $p < 0.001$).

High galectin-4 (HR = 0.65, 95% CI = 0.47–0.89, $p = 0.008$) and galectin-9 (HR = 0.46, 95% CI = 0.26–0.82, $p = 0.009$) expression were significantly correlated with better RFS in the patients with HCC.

Tumor Size

The relationship between the galectins and tumor size was explored in 10 study cohorts (16, 18, 20, 22, 25, 26, 29, 30, 32).

High galectin-1 expression was not significantly associated with tumor size (OR = 1.59, 95% CI = 0.74–3.41, $p = 0.238$) with a significant heterogeneity ($I^2 = 75.8\%$, $p = 0.042$).

High galectin-3 expression was significantly associated with bigger tumor size (OR = 1.69, 95% CI = 1.01–2.84, $p = 0.046$) without significant heterogeneity ($I^2 = 48.8\%$, $p = 0.119$).

High galectin-4 expression was significantly associated with smaller tumor size (OR = 0.43, 95% CI = 0.2–0.91, $p = 0.027$); by contrary, high galectin-9 expression was not significantly associated with tumor size (OR = 0.98, 95% CI = 0.7–1.39, $p = 0.924$) without significant heterogeneity ($I^2 = 0.0\%$, $p = 0.394$).

Tumor-Node-Metastasis Stage

The relationship between the galectins and TNM stage was explored in eight study cohorts (18, 21, 22, 25, 26, 31, 32).

High galectin-1 expression was significantly associated with advanced TNM stage (OR = 2.53, 95% CI = 1.31–4.87, $p = 0.006$) without significant heterogeneity ($I^2 = 41.9\%$, $p = 0.189$).

High galectin-3 expression was not significantly associated with TNM stage (OR = 2.06, 95% CI = 0.82–5.16, $p = 0.122$) with a significant heterogeneity ($I^2 = 66.6\%$, $p = 0.030$). Sensitivity analysis illustrated that the study by Kong et al. (26) displayed an apparent influence on the overall result of the meta-analysis (**Supplementary Figure 2**). After the exclusion of this study, the pooled OR was similar (OR = 2.90, 95% CI = 1.84–4.56, $p = 0.044$), but the heterogeneity was statistically insignificant ($I^2 = 0.0\%$, $p = 0.731$).

High galectin-4 (OR = 0.49, 95% CI = 0.28–0.86, $p = 0.013$) and galectin-9 (OR = 0.44, 95% CI = 0.20–0.98, $p = 0.044$) expression were significantly associated with early TNM stage.

Differentiation Grade

The relationship between the galectins and tumor differentiation grade was explored in 11 study cohorts (16, 18, 20–22, 26, 29–32).

High galectin-1 expression was not significantly associated with differentiation grade (OR = 0.96, 95% CI = 0.7–1.32, $p = 0.795$) without significant heterogeneity ($I^2 = 0.0\%$, $p = 0.830$).

High galectin-3 expression was not significantly associated with differentiation grade (OR = 2.13, 95% CI = 0.97–4.69, $p = 0.061$) with a significant heterogeneity ($I^2 = 65.6\%$, $p = 0.033$). Sensitivity analysis demonstrated that the study by Fang et al. (31) displayed an apparent influence on the overall result of the meta-analysis (**Supplementary Figure 3**). After the exclusion of this study, the pooled OR was similar (OR = 1.65, 95% CI = 1.01–2.69, $p = 0.044$), but the heterogeneity was statistically insignificant ($I^2 = 18.5\%$, $p = 0.293$).

High galectin-4 expression was significantly associated with well-differentiation grade (OR = 0.35, 95% CI = 0.16–0.78, $p = 0.010$).

High galectin-9 expression was not significantly associated with tumor differentiation grade (OR = 0.70, 95% CI = 0.34–1.47, $p = 0.348$) with a significant heterogeneity ($I^2 = 70.2\%$, $p = 0.035$). Sensitivity analysis illustrated that the study by Gu

TABLE 3 | Characteristics of the included studies regarding the galectins with the risk of different liver diseases.

References	Country	Study design	Type of publication	Enrollment period	Target population	No. total pts.	Child-Pugh A/B/C	Galectin subtypes	Measure-ment	NOS score
Matsuda et al. (25)	Japan	Retrospective case control	Full text	2005.06–2008.02	HCC	51	38/12/1	Galectin-3	ELISA	8
					LC	16	12/2/2			
					Hepatitis	23	23/0/0			
Honsawek et al. (47)	Thailand	Retrospective case control	Full text	NA	Biliary Atresia	58	NA	Galectin-3	ELISA	6
Yilmaz et al. (34)	Turkey	Retrospective case control	Full text	NA	NAFLD	71	NA	Galectin-3	ELISA	7
Giebultowicz et al. (43)	Poland	Retrospective case control	Full text	NA	HCC	10	NA	Galectin-3	ELISA	6
Gu et al. (30)	China	Prospective cohort	Full text	2006.06–2008.08	HCC	31	NA	Galectin-9	ELISA	8
Kamada et al. (46)	Japan	Retrospective cohort	Full text	NA	NASH	127	NA	Galectin-3	ELISA	6
Yang et al. (35)	China	Prospective cohort	Abstract	NA	Liver Failure	55	NA	Galectin-3	ELISA	6
Zheng et al. (33)	China	Retrospective case control	Full text	2010.01–2011.12	Liver Failure	55	NA	Galectin-3	ELISA	8
Eisa et al. (41)	Egypt	Retrospective case control	Full text	2012.03–2012.09	HCC	50	21/18/11	Galectin-3	ELISA	8
Ulu et al. (36)	Turkey	Retrospective case control	Full text	2009–2011	HCC	19	NA	Galectin-3	ELISA	6
					LC	22				
Akyuz et al. (42)	Turkey	Retrospective case control	Abstract	NA	HCC	60	37/21/2	Galectin-3	ELISA	6
Gudowska et al. (40)	Poland	Retrospective case control	Full text	NA	LC	57	NA	Galectin-3	CMIA	5
Uluca et al. (44)	Turkey	Retrospective case control	Full text	NA	CAHB	32	NA	Galectin-3	ELISA	6
					IHB	30				
Abbas et al. (48)	Egypt	Retrospective case control	Full text	2015.08–2015.11	LC with ascites	25	0/8/17	Galectin-3	ELISA	7
					LC without ascites	26	18/8/0			
Tekin et al. (37)	Turkey	Prospective case control	Abstract	NA	CAHB	56	NA	Galectin-3	ELISA	6
					IHB	57				
Lukic et al. (45)	Bosnia and Herzegovina	Retrospective case control	Full text	NA	Hepatitis C	20	NA	Galectin-3	ELISA	8
Moon et al. (38)	Korea	Retrospective case control	Full text	2016.10–2017.02	LC	28	NA	Galectin-3	ELISA	7
Matsuoka et al. (39)	Japan	Retrospective case control	Full text	NA	AIH	77	NA	Galectin-9	ELISA	6

Pts., number of patients; NA, not available; NOS, Newcastle–Ottawa Scale; AIH, autoimmune hepatitis; LC, liver cirrhosis; CAHB, chronic active hepatitis B; IHB, inactive hepatitis B; HCC, hepatocellular carcinoma; NAFLD, nonalcoholic fatty liver disease; LF, liver failure.

et al. (30) displayed an apparent influence on the overall result of the meta-analysis (**Supplementary Figure 4**). After the exclusion of this study, the pooled OR was similar (OR = 0.51, 95% CI = 0.28–0.95, $p = 0.034$), but the heterogeneity was statistically insignificant ($I^2 = 35.0\%$, $p = 0.215$).

Vascular Invasion

The relationship between the galectins and vascular invasion was explored in seven study cohorts (16, 20–22, 25, 30, 32).

High galectin-1 expression was significantly associated with positive vascular invasion (OR = 1.74, 95% CI = 1.18–2.58, $p = 0.005$) without significant heterogeneity ($I^2 = 0.0\%$, $p = 0.679$).

High galectin-3 expression was significantly associated with positive vascular invasion (OR = 2.98, 95% CI = 1.58–5.60, $p = 0.001$) without significant heterogeneity ($I^2 = 0.0\%$, $p = 0.421$).

High galectin-4 expression was significantly associated with negative vascular invasion (OR = 0.36, 95% CI = 0.19–0.72, $p = 0.003$).

High galectin-9 expression was significantly associated with negative vascular invasion (OR = 0.60, 95% CI = 0.37–0.97, $p = 0.037$) without significant heterogeneity ($I^2 = 2.8\%$, $p = 0.311$).

Meta-Analyses Regarding the Galectins With the Risk of Different Liver Diseases

About 18 studies involving 24 cohorts and 1,048 patients focused on the relationship between the serum galectin levels and the risk of different liver diseases (25, 30, 33–48) (**Table 3**). Among them, 16 studies focused on galectin-3 (25, 33–38, 40–48), and two studies focused on galectin-9 (30, 39). Results of the meta-analyses are shown in **Table 4**.

Hepatocellular Carcinoma

The relationship between the galectins and the risk of HCC was explored in six study cohorts (25, 30, 36, 41–43). Among them, five study cohorts selected the healthy volunteers as the control subjects, and one study cohort selected the patients with chronic hepatitis as the control subjects.

Serum galectin-3 level was significantly higher in the patients with HCC compared to the healthy volunteers or the patients with chronic hepatitis (MD = 2.71, 95% CI = 1.56–3.85, $p < 0.001$) with a significant heterogeneity ($I^2 = 86.9\%$, $p < 0.001$). Sensitivity analysis illustrated that the study by Akyuz et al. (42) displayed an apparent influence on the overall result of the meta-analysis (**Supplementary Figure 5**). After the exclusion of this study, the pooled MD was similar (MD = 2.28, 95% CI = 2.07–2.50, $p < 0.001$), but the heterogeneity was statistically insignificant ($I^2 = 0.6\%$, $p = 0.389$).

Serum galectin-9 level was significantly higher in the patients with HCC compared to the healthy volunteers (MD = 3.74, 95% CI = 2.57–4.91, $p < 0.001$).

Liver Failure

The relationship between galectin-3 and the risk of liver failure was explored in two study cohorts, both of which selected the healthy volunteers as the control subjects (33, 35).

Serum galectin-3 level was significantly higher in the patients with liver failure compared to the healthy volunteers (MD = 0.44, 95% CI = 0.23–0.66, $p < 0.001$) with a significant heterogeneity ($I^2 = 97.8\%$, $p < 0.001$).

Liver Cirrhosis

The relationship between galectin-3 and the risk of liver cirrhosis was explored in six study cohorts, all of which selected healthy volunteers as the control subjects (25, 36, 38, 40, 48).

Serum galectin-3 level was significantly higher in the patients with liver cirrhosis compared to the healthy volunteers (MD = 1.83, 95% CI = 1.15–2.51, $p < 0.001$) with a significant heterogeneity ($I^2 = 98.3\%$, $p < 0.001$). Sensitivity analysis did not find any source of heterogeneity.

Other Chronic Liver Diseases

The relationship between the galectins and the risk of other chronic liver diseases, including inactive hepatitis B, chronic active hepatitis B, non-alcoholic steatohepatitis, hepatitis C, autoimmune hepatitis, non-alcoholic fatty liver disease, and biliary atresia, was explored in 10 study cohorts. All of them selected healthy volunteers as the control subjects (25, 34, 37, 39, 44–47).

In comparison to the healthy volunteers, serum galectin-3 level was significantly higher in chronic active hepatitis B (MD = 18.95, 95% CI = 10.91–27.00, $p < 0.001$) and biliary atresia (MD = 1.30, 95% CI = 1.11–1.49, $p < 0.001$), but not inactive hepatitis B (MD = 1.29, 95% CI = 1.40–3.97, $p = 0.347$), non-alcoholic steatohepatitis (MD = 0.48, 95% CI = 0.77–1.73, $p = 0.452$), hepatitis (MD = 0.37, 95% CI = 0.65–1.39, $p = 0.479$), or non-alcoholic fatty liver disease (MD = 0.10, 95% CI = 0.30–0.50, $p = 0.485$); on the contrary, serum galectin-3 level was significantly lower in hepatitis C (MD = 0.27, 95% CI = 0.34–0.20, $p < 0.001$) (**Figure 2**).

Serum galectin-9 level was significantly higher in the patients with autoimmune hepatitis compared to the healthy volunteers (MD = 8.80, 95% CI = 7.61–9.99, $p < 0.001$).

TABLE 4 | Galectins with the risk of different liver diseases: results of the meta-analyses.

Groups	No. studies	Pooled proportion using random-effects mode	*P*-value	Heterogeneity I^2	Heterogeneity *P*-value
HCC					
Galectin-3	5	MD = 2.71 (95% CI = 1.56–3.85)	**<0.001**	86.9%	**<0.001**
Galectin-9	1	MD = 3.74 (95% CI = 2.57–4.91)	**<0.001**	–	–
Liver failure					
Galectin-3	2	MD = 0.44 (95% CI = 0.23–0.66)	**<0.001**	97.8%	**<0.001**
Liver cirrhosis					
Galectin-3	6	MD = 1.83 (95% CI = 1.15–2.51)	**<0.001**	98.7%	**<0.001**
Chronic liver diseases					
Galectin-3 in CAHB	2	MD = 18.95 (95% CI = 10.91–27.00)	**<0.001**	73.1%	**0.054**
Galectin-3 in IHB	2	MD = 1.29 (95% CI = −1.40–3.97)	0.347	58.9%	0.119
Galectin-3 in NASH	1	MD = 0.48 (95% CI = −0.77–1.73)	0.452	–	–
Galectin-3 in Hepatitis	1	MD = 0.37 (95% CI = −0.65–1.39)	0.479	–	–
Galectin-3 in Hepatitis C	1	MD = −0.27 (95% CI = −0.34 to−0.20)	**<0.001**	–	–
Galectin-3 in NAFLD	1	MD = 0.10 (95% CI = −0.30–0.50)	0.485	–	–
Galectin-3 in BA	1	MD = 1.30 (95% CI = 1.11–1.49)	**<0.001**	–	–
Galectin-9 in AIH	1	MD = 8.80 (95% CI = 7.61–9.99)	**<0.001**	–	–

HCC, hepatocellular carcinoma; MD, mean difference; NA, not available; CAHB, chronic active hepatitis B; IHB, inactive hepatitis B; NASH, nonalcoholic steatohepatitis; NAFLD, nonalcoholic fatty liver disease; BA, biliary atresia; AIH, autoimmune hepatitis. The value in bold is defined as being statistically significant.

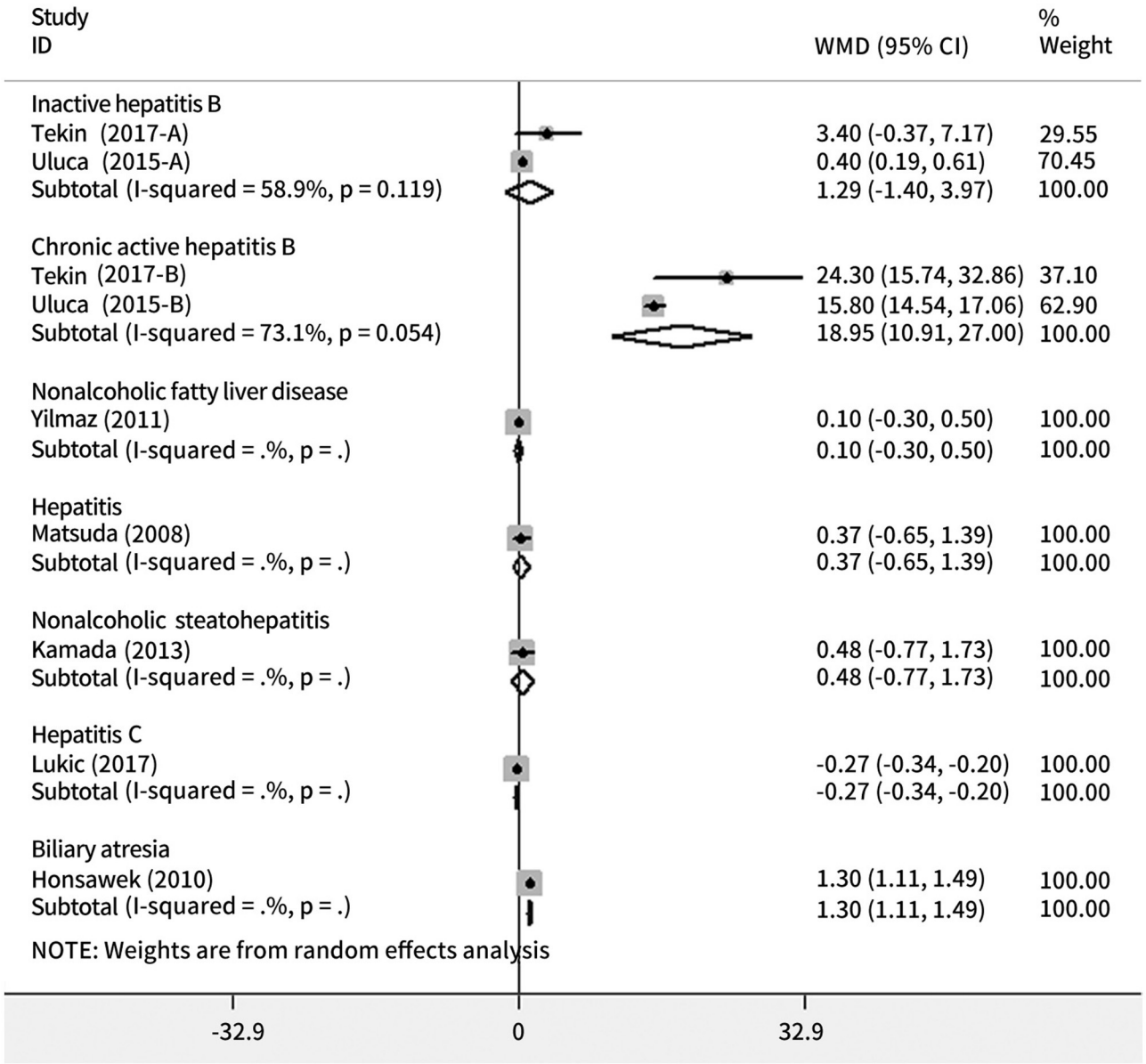

FIGURE 2 | Forest plots showing the association between the risk of chronic liver diseases and galectin-3.

Publication Bias

Publication bias is reported in **Supplementary Table 1**.

DISCUSSION

Until now, 11 subtypes of galectins family have been identified in humans, of which galectin-1,-3, and -9 are the most commonly studied in various diseases (49). According to the current systematic analyses, the role of galectin-1,-3,-4, and -9 was studied in patients with liver diseases.

Patients with HCC have a 5-year survival rate of <12% (50). Therefore, it is vital to identify the biomarkers to predict the prognosis of HCC (51). This study found that the higher serum galectin-3 and -9 levels were associated with an increased risk of HCC and high galectin-1 and -3 and low galectin-4 and -9 expression were significantly associated with worse OS and positive vascular invasion in HCC. Indeed, experimental studies have also suggested the potential mechanisms of galectin-1,-3, and -9 expression in the development and progression of HCC. First, galectin-1 can induce the epithelial–mesenchymal transition (EMT), which is a major process during the progression of cancer in the HCC cells of humans (52). Galectin-1 inhibitor combined with sorafenib can further decrease the tumor size (53). Second, galectin-3 can inhibit the tumor-reactive

T cells and promote tumor growth in the mice receiving the tumor-reactive $CD8^+$ T cells (54). Silencing of galectin-3 can significantly reduce the mRNA and protein levels of urokinase-type plasminogen activator receptor (uPAR) and downstream signaling transduction pathway of uPARs in the HCC cells by inhibiting the MEK/ERK signaling pathway, further influencing the proliferation, migration, and invasion of the HCC cells (55). Third, galectin-9 can inhibit the growth of the HCC cell lines by inducing cell apoptosis (56). Galectin-9 also increases the number of $Tim\text{-}3^+$ dendritic cells and $CD8^+$ T cells and enhances antitumor immunity through the interaction of galectin-9 with Tim-3 (57). By comparison, blockade of the Tim-3/galectin-9 signaling pathway importantly increases the functionality of tumor-infiltrating $Tim\text{-}3^+$ T cells and is negatively associated with the survival of patients with HCC (58).

Another major finding of this study was that higher serum galectin-3 level was associated with an increased risk of liver failure, liver cirrhosis, and chronic active hepatitis B. Other evidence was also in favor of the importance of galectin-3 in these liver diseases. First, if the patients with acute-on-chronic liver failure related to hepatitis B had galectin-3 methylated promoter, they would have shorter survival time, higher 3-month mortality, and higher model for end-stage liver disease (MELD) score (59). Second, galectin-3 modulates the phagocytosis-induced hepatic stellate cell activation and liver fibrosis *in vivo* (60). Galectin-3 level is significantly higher in the Child–Pugh class C and positively correlates with the MELD score, suggesting the association of galectin-3 level with hepatic decompensation (61). By comparison, the galectin-3 inhibitor can reduce the hepatic venous pressure gradient in patients with esophageal varices (62). Third, galectin-3 deficiency can lead to a significant reduction in the incidence of concanavalin A-induced hepatitis in mice by inhibiting inflammation (63).

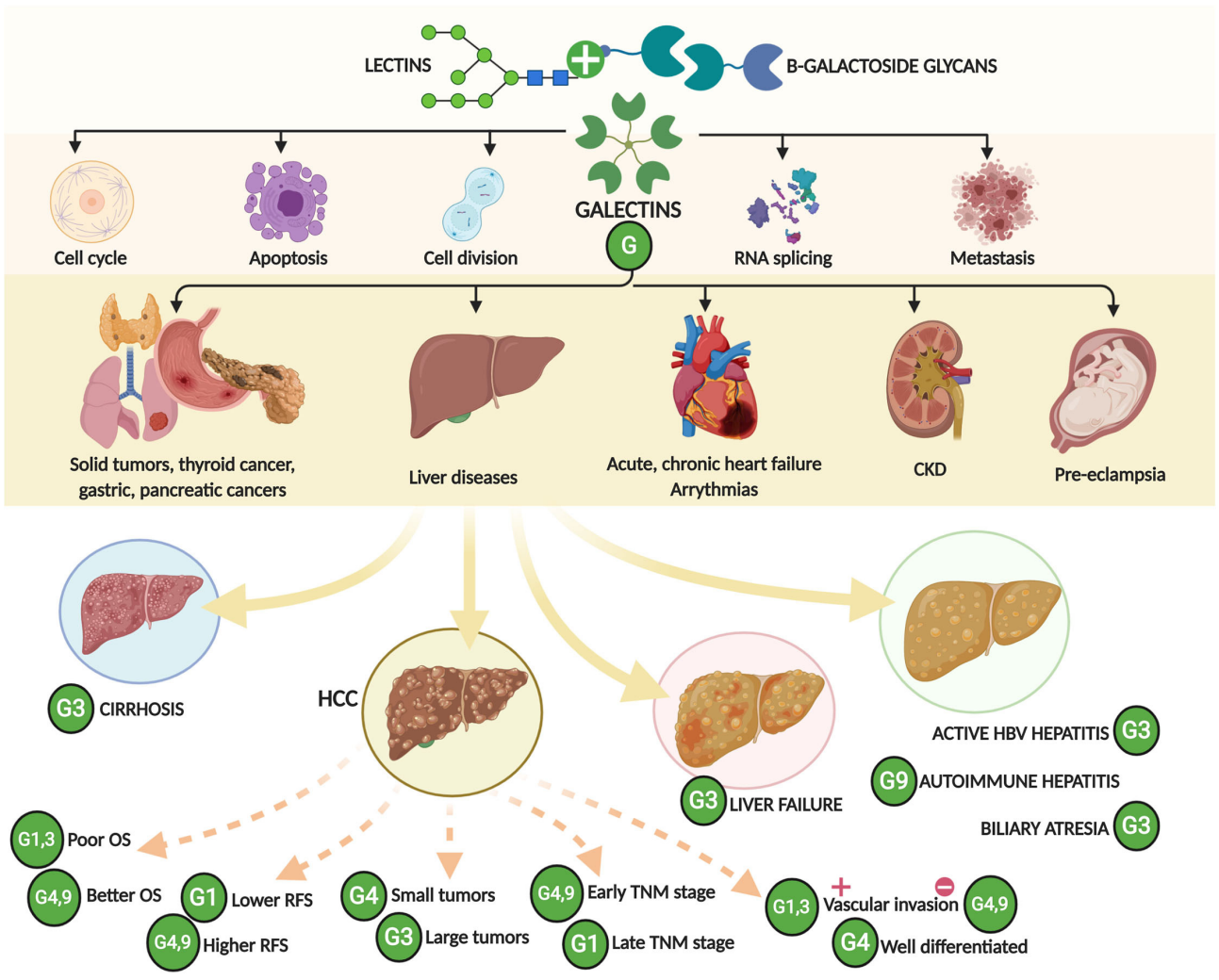

FIGURE 3 | A schematic drawing showing the major findings of this study. CKD, chronic kidney disease; OS, overall survival; RFS, relapse-free survival; HCC, hepatocellular carcinoma; TNM, tumor-node-metastasis.

This study did not find any significant association of serum galectin-3 level with inactive hepatitis B, non-alcoholic steatohepatitis, or non-alcoholic fatty liver disease. This illustrated that the impact of galectin-3 level on chronic liver diseases might be dependent upon the severity and stage of liver damage (40). Indeed, the evidence regarding the role of galectin-3 in non-alcoholic fatty liver disease and non-alcoholic steatohepatitis is also controversial. Some studies have shown that galectin-3 deficiency in male mice can spontaneously develop non-alcoholic fatty liver disease and more severe hepatic injury (64, 65). In contrast, other studies have reported that galectin-3 ablation protected the mice from the diet-induced non-alcoholic steatohepatitis (66).

There were several limitations in this study. First, this meta-analysis contained a relatively small number of studies, which might lead to insufficient statistical power. Second, the cutoff values of high galectin expression were heterogeneous among the studies. Third, HR values were not directly reported in the six included studies, where their survival data were extracted from the Kaplan–Meier curves by the Engauge Digitizer 4.1 software. Fourth, most of the included studies were from Asia. Our findings are not a global representation.

In conclusion, based on this systematic review and meta-analysis, both high galectin-1 and -3 and low galectin-4 and -9 expression in the tissues were significantly related to worse prognosis and positive vascular invasion in patients with HCC and serum galectin-3 level was associated with the risk of HCC, liver failure, liver cirrhosis, and chronic active hepatitis B (**Figure 3**). Further studies are needed to explore the role of galectins as a potential therapeutic target and biomarker for liver diseases.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

XQ contributed to the conceptualization, supervision, and project administration. YA, SX, YL, XX, and XQ contributed to the methodology, formal analysis, data curation, and writing the original draft. YA, SX, YL, XX, CP, JC, NM-S, XG, and XQ contributed to the validation, writing, review, and editing. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.744518/full#supplementary-material

Supplementary Figure 1 | Sensitivity analysis of galectin-3 expression with overall survival (OS) in HCC.

Supplementary Figure 2 | Sensitivity analysis of galectin-3 expression with TNM stage in HCC.

Supplementary Figure 3 | Sensitivity analysis of galectin-3 expression with the differentiation grade in HCC.

Supplementary Figure 4 | Sensitivity analysis of galectin-9 expression with the differentiation grade in HCC.

Supplementary Figure 5 | Sensitivity analysis of serum galectin-3 level with the risk of HCC.

Supplementary Table 1 | Publication bias.

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Combined Estrogen Alpha and Beta Receptor Expression Has a Prognostic Significance for Colorectal Cancer Patients

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We reported that high estrogen receptor beta (ERβ) expression is independently associated with better prognosis in female colorectal cancer (CRC) patients. However, estrogen receptor alpha (ERα) is expressed at very low levels in normal colon mucosa, and its prognostic role in CRC has not been explored. Herein, we investigated the combined role of ERα and ERβ expression in the prognosis of female patients with CRC, which, to the best of our knowledge, is the first study to investigate this topic. A total number of 306 primary CRCs were immunostained for ERα and ERβ expression. A Cox regression model was used to evaluate overall survival (OS) and disease-free survival (DFS). The combined expression of high ERβ + negative ERα correlates with longer OS (HR = 0.23; 95% CI: 0.11–0.45, P <0.0001) and DFS (HR = 0.10; 95% CI: 0.03–0.26, P < 0.0001) and a more favorable tumor outcome, as well as significantly higher expression of antitumorigenic proteins than combined expression of low ERβ + positive ERα. Importantly, we found that low ERβ expression was associated with local recurrence of CRC, whereas ERα expression was correlated with liver metastasis. Overall, our results show that the combined high ERβ + negative ERα expression correlated with a better prognosis for CRC patients. Our results suggest that the combined expression of ERα and ERβ could be used as a predictive combination marker for CRC patients, especially for predicting DFS.

Keywords: estrogen receptor beta, estrogen receptor alpha, colorectal cancer, CRC disease-free survival, CRC overall survival

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INTRODUCTION

The physiological effects of estrogens are mediated by two main receptors, estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ), which belong to the nuclear receptor family and are encoded by two different genes, *ESR1* (ERα) and *ESR2* (ERβ) (1, 2). These receptors are implicated in different types of cancer, including colorectal cancer (CRC) (1–3).

ERβ is the predominant ER in normal colon mucosa, and its expression is reduced during tumor progression (4). Previous research has reported association of ERβ expression with CRC survival (5, 6). We recently reported that high nuclear ERβ expression is independently associated with

better prognosis in female CRC patients and associated with hormonal status but not with lifestyle indicators (7). Furthermore, we investigated the antitumor effects of ERβ induction in colon cancer cells and in an *in vivo* zebrafish xenograft model (8). On the other hand, ERα is expressed at very low levels in normal colon mucosa (1, 2), and few studies have reported its prognostic role in CRC survival (9–11). Evidence shows that the manipulation of estrogen signaling to inhibit ERα and stimulate ERβ may have preventive and therapeutic effects for obesity-associated colon cancer (12, 13). However, the relationships among estrogen hormones, reproductive factors, and CRC remain unclear and await further investigation (14).

Many mutations and proteins have been implicated in CRC progression. *KRAS* mutation status is reported to be an important prognostic and treatment marker in CRC, and screening for *KRAS* mutations is now mandatory for metastatic colon cancer before treatment with therapies that target the EGFR pathway (15–17). Furthermore, the activation of the Wnt/β-catenin pathway plays a crucial role in CRC development and progression (18). In addition, high cyclooxygenase-2 (COX-2) expression in CRC correlates with poor prognosis *via* the effect of prostaglandin E_2 (PGE_2) (19). 15-Hydroxyprostaglandin dehydrogenase (15-PGDH) is the key enzyme in PGE_2 catabolism and is often downregulated in CRC, while its upregulation has been shown to lead to a better prognosis in CRC (20–22). The G protein-coupled receptors cysteinyl leukotriene receptors 1 and 2 ($CysLT_1R$ and $CysLT_2R$, the receptor for LTD_4 respectively LTC_4) are implicated in the prognosis of CRC (23). Patients with low $CysLT_1R$ and high $CysLT_2R$ expression levels have better survival than those with high $CysLT_1R$ and low $CysLT_2R$ expression levels (23).

In this study, we aimed to investigate the prognostic significance of the combined expression of ERα and ERβ in female CRC patients and to explore their correlations with other tumor promoter or suppressor proteins and hormonal status.

MATERIALS AND METHODS

Study Populations

The study included a cohort of female patients who were diagnosed with CRC and operated between January 1, 2008, and June 30, 2012. This investigation included 269 patients with available data on clinical information, tumor characteristics, hormonal status as well as ER, ER, KRAS, $CysLT_1R$, $CysLT_2R$, COX-2, 15-PGDH, β-catenin, Mucin-2 and PGD2 synthase expression in CRC tissue. The study population is briefly described in the **Supplementary Materials**. Details about the study design, patient follow-up and data collection are provided elsewhere (7).

Immunohistochemistry (IHC)

Tumor samples were retrieved and incorporated into tissue microarray (TMA) blocks based on the protocol described earlier (7). The tissues were stained with specific antibodies for the expression of ERα ERβ and other proteins of interest (**Supplementary Material**). Two independent investigators (GT and RE), blinded to the patient and tumor characteristics, evaluated the staining immunoreactivity using the immunoreactive score (IRS) with a range 0–9, which was calculated as a multiplication of staining intensity (0 = negative, 1 = weak, 2 = moderate and 3 = strong) with percentage of positive stained cells (1 = <10%, 2 = 11–50% and 3 = >50%) (7). The staining intensity was determined based on the criteria of Konstantinopoulos et al. (4), which are described in the **Supplementary Materials**. For ERα and ERβ expression, only the nuclear staining intensity was taken into consideration, based on which they were also scored as categorical variables, respectively low/high and negative/positive expression (**Figure 2A**). Briefly, negative and weak ERβ staining were grouped as low expression and moderate and strong ERβ staining as high expression (7). Because ERα is very little expressed in the normal colonic mucosa (1, 2), we defined its expression as positive if more than 10% of the nuclei were stained, regardless the staining intensity. All the other tumor samples that had <10% of the nuclei stained, regardless the staining intensity, were considered to have negative ERα expression. Each tumor sample was in duplicate. Cores with loss of tissue or with only stromal tissue were excluded from the analysis.

Acquisition of Gene Expression and Clinical Data From the Cancer Genome Atlas (TCGA) Dataset

Normalized RNA sequencing data in transcripts per million (TPM), reverse phase protein array (RPPA) data, and the associated clinical information of the colon adenocarcinoma (COAD) samples were downloaded from the TCGA dataset (https://portal.gdc.cancer.gov/; https://tcpaportal.org/tcpa/; ≤June 20, 2020). Out of 361 patients, 12 patients missing pathological information, 16 patients with a follow-up period of ≤30 days, and 52 patients with metastasis (stage IV) were eliminated. Thus, 282 patients with clinical information were included in the study. Normalized gene expression and protein expression data from the TCGA-COAD dataset were log2-transformed for further analysis.

Identification of Independent Prognostic Parameters of Colon Cancer

To identify independent prognostic parameters and to validate the independent prognostic value of ERα and ERβ, univariate and multivariate Cox regression analyses were performed in the TCGA-COAD dataset on the ERα and ERβ gene and protein signature and clinicopathological parameters. Parameters with $P < 0.05$ in the univariate analysis were further included in the multivariate Cox regression analysis. The TCGA samples were divided into high- and low-risk groups according to the optimal cutoffs determined by the Youden Index association criteria and analyzed using Circos visualization package (24).

Abbreviations: ERβ, estrogen receptor beta; ERα estrogen receptor alpha; CC, colon cancer; CRC, colorectal cancer; DFS, disease-free survival; OS, overall survival.

Statistical Analysis

The variables were compared between the group of interest using Pearson's χ^2 test or Fisher's exact test for categorical variables and the Mann-Whitney *U* test or *t*-test for continuous variables. Survival curves, generated *via* the Kaplan-Meier method, were compared between the groups using the log-rank test. Univariate and multivariate Cox proportional hazards regression models were applied, and hazard ratios (HRs) together with 95% confidence intervals (CIs) were calculated to determine the risk of death or cancer recurrence. Receiver operating characteristic (ROC) curves were used to calculate the area under the curve (AUC) to determine the predictive ability of the final model with combined ERβ + ERα expression compared to models with only one ER expression or the basic model. Binary logistic regression model was used to determine the odds ratios (ORs) of having a metastatic event for each unit increase in ERα and ERβ intensity. The estimates with their corresponding 95% CIs were used to build forest plots by the ggplot2 package in R. Statistical analyses were performed using SPSS version 23.0 (SPSS, IBM, Armonk, NY, USA) and GraphPad Prism version 8.0a (GraphPad Software, Inc., San Diego, CA, USA). A two-sided $P < 0.05$ was considered statistically significant.

RESULTS

Evaluation of ERα and ERβ Expression in Female CRC Patients

We had 306 primary CRC samples available for the evaluation of ERα and ERβ expression. Fourteen patients, who were previously operated and treated for breast cancer, were excluded from the study due to the risk of ERα alterations from the anti-estrogen therapies (**Figure 1**). We successfully evaluated ERβ in 300 CRC patients and ERα in 270 CRC patients. Based on the staining intensity assessed with IHC, ERβ expression was categorized as low and high, while ERα expression was categorized as negative and positive (**Figure 2A**). We next compared the expression of these receptors between normal and matched cancer tissues and found that compared to ERα expression levels, ERβ expression levels were higher in both normal and cancer tissues (**Figure 2B**). However, compared to normal tissues, a downregulation of ERβ and an upregulation of ERα were observed in the matched CRC tissues (**Figure 2B**, see violin bar graph). Since we previously reported that high ERβ expression correlated with better prognosis in CRC (7), we investigated the distribution of ERα expression in patients with low and high ERβ expression. We grouped the patients into four categories based on ERα and ERβ expression (**Figure 2C**). We found that 79% of patients with high ERβ expression had also negative ERα expression compared with 63% in the low ERβ group (**Figure 2D**). Likewise, the percentage of patients with positive ERα expression was higher in the low ERβ expression group (37%) than in the high ERβ expression group (21%) (**Figure 2D**). For representative IHC images of matched pairs of patients for both ERα and ERβ expression, see **Supplementary Figure 1A**.

Next, we used *ESR1* (ERα) and *ESR2* (ERβ) mRNA levels from the TCGA-COAD database to investigate the differential expression of ERα and ERβ in CRC patients with TNM stage I disease and TNM stage IIIc+IV disease. Compared to those with stage I disease, a smaller percentage of patients with stage IIIc+IV disease had upregulated *ESR2* mRNA levels (**Figure 2E**). Additionally, *ESR2* levels were lower in patients with stage IIIc+IV disease than in those with stage I disease (**Figure 2E**). Furthermore, *ESR1* mRNA levels were obviously higher in patients with stage IIIc+IV disease than in those with stage I disease (**Figure 2E**).

The Specificity of the ERα Antibody

Because the role of ERα expression in CRC is very little studied and all our results are based on antibody staining, we tested the specificity of the antibodies we used, in order to validate the antibodies. First, we stained the normal breast tissue, which is known to abundantly express ERα (positive control), and normal kidney, prostate, and skin tissues, which are known to lack ERα expression (negative controls, **Supplementary Figure 1B**) (25–27). Next, the same tissues were also stained with another anti-ERα antibody, D12 (**Supplementary Figure 1C**), which is widely used for the detection of ERα expression (28–30). We randomly stained 59 patients from the Female cohort with the D12 antibody. As shown in **Supplementary Figure 1D** the distribution of the IRS for nuclear ERα expression for each patient ($n = 59$) was the same for both antibodies. Likewise, when the patients were grouped as positive and negative nuclear ERα expression, no significant difference was observed between the two antibodies ($P = 0.11$, **Supplementary Figure 1E**). Out of 59 patients randomly stained with D12 antibody, 13 patients (22%) were positive for ERα expression, while 19 patients (32%) were detected as positive using the cocktail antibody (**Supplementary Figure 1E**). This could be explained by the fact that the cocktail antibody 1D5 + 6F11 was created by mixing two monoclonal antibodies that detect two different epitopes (31, 32). Representative IHC images of matched-pair CRC tissues for both antibodies are shown in the **Supplementary Figure 1F**.

Correlation of ERα and ERβ Expression With KRAS Mutation Status

Out of 252 patients with successful staining for the KRAS mutation, only 31 (12.3%) had positive staining (**Figure 2F**). Patients with a KRAS mutation had a significantly higher intensity of ERα expression ($P < 0.05$) and a tendency to have lower ERβ expression ($P = 0.06$) than patients with wild-type (WT) KRAS (**Figure 2F**). Additionally, we observed that 19% of patients with positive ERα expression had KRAS mutations, while 9% of patients with negative ERα expression had KRAS mutations (**Figure 2G**). An opposite tendency was observed when looking at the distribution of KRAS mutations in patients with low and high ERβ expression. While 15% of patients with low ERβ expression had KRAS mutations, only 7% of patients with high ERβ expression had KRAS mutations (**Figure 1G**). However, no statistical significance was reached. To further validate these findings, we used mRNA data from the TCGA-COAD public database and found a strong and significant positive correlation between the mRNA levels of *ESR1* (ERα)

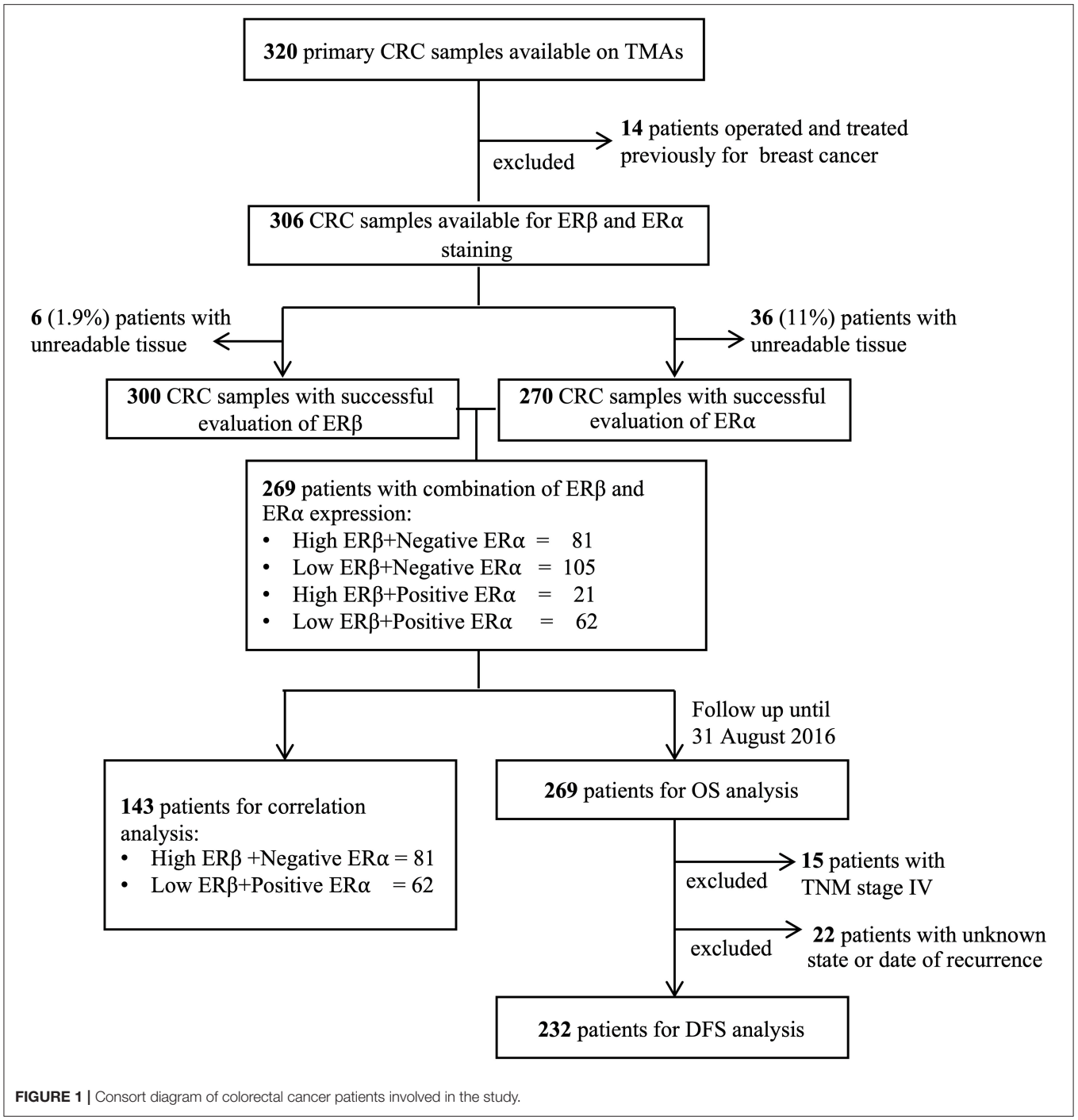


FIGURE 1 | Consort diagram of colorectal cancer patients involved in the study.

and *KRAS* mutations, while no correlation was found with *ESR2* mRNA levels (ERβ) (**Figure 2H**).

Evaluation of the Prognostic Relevance of ERα and ERβ Expression in CRC Patients

Previously we reported that high nuclear ERβ expression is independently associated with better OS and DFS in female CRC patients (7). Herein, we report that CRC patients with negative nuclear ERα expression have 19% lower risk for 5-years overall mortality (HR = 0.81; 95% CI, 0.68-0.94; P = 0.042, **Figure 3A**). Likewise, in the TCGA-COAD cohort, low ERα protein expression (HR = 0.73; 95% CI, 0.62-0.92; P = 0.035, **Figure 3B**) and high ERβ protein expression (HR = 0.78; 95% CI, 0.68-0.89; P = 0.001, **Figure 3C**) are associated with better prognosis of CRC patients. Additionally, we investigated the predicting ability of ERα and ERβ expression in our female patient's cohort calculating the ROC curves. We found that

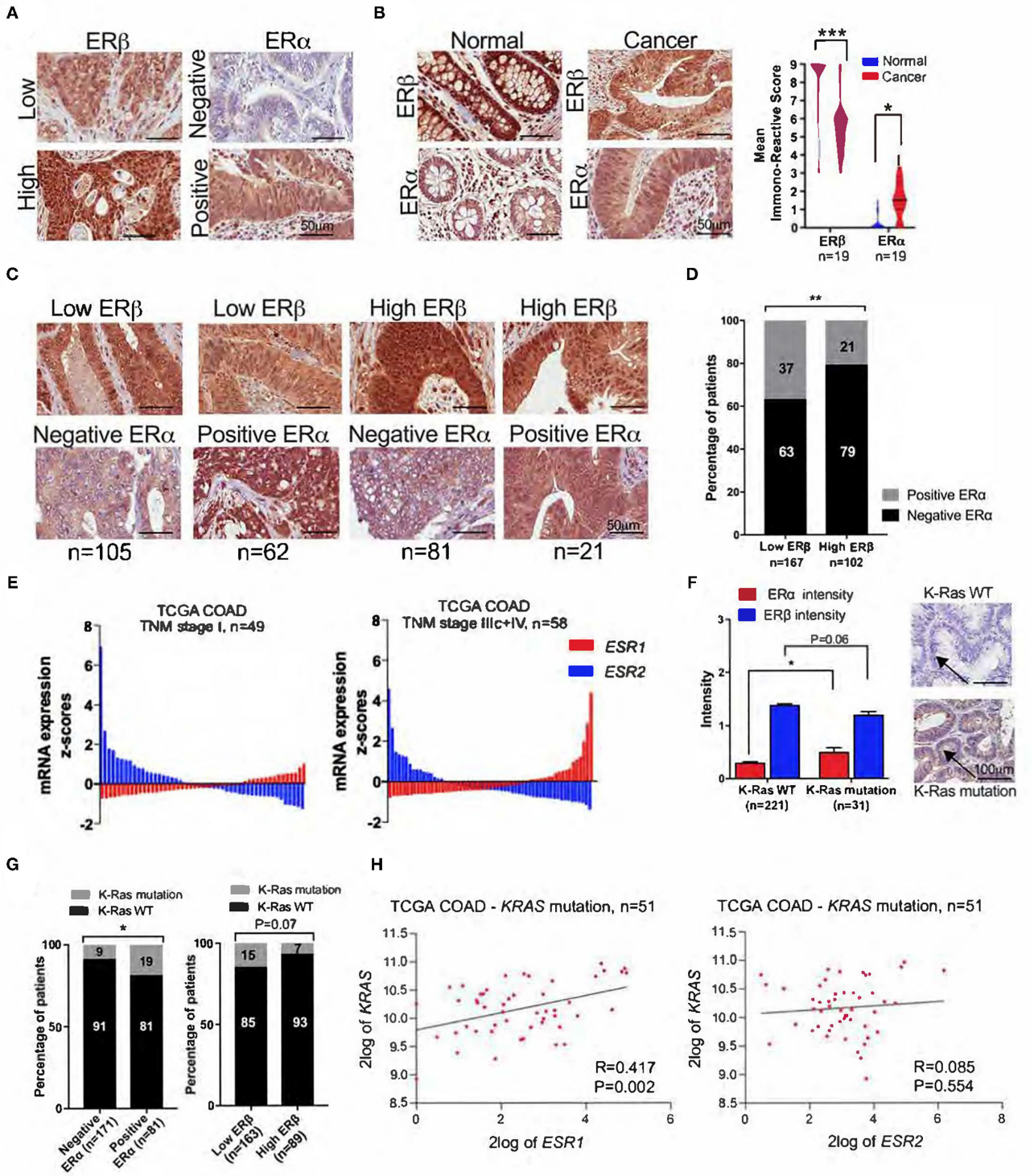


FIGURE 2 | Expression levels of ERα and ERβ in CRC tissue. **(A)** Representative IHC images showing the nuclear expression of ERα and ERβ in CRC tissue. **(B)** Representative IHC images of ERα and ERβ expression in normal and matched cancer tissues, and violin plots showing the distribution of IRSs for ERα and ERβ expression in normal and matched cancer tissues. **(C)** IHC images of CRC tissue in four subgroups of patients with combined ERα and ERβ expression levels. **(D)** The percentage of CRC patients with negative and positive ERα expression according to low and high ERβ expression. **(E)** Waterfall plots of the mRNA expression levels of ESR1 (ERα) and ESR2 (ERβ) in the subgroups of CRC patients with TNM stage I ($n = 49$) and TNM stage IIIc + IV ($n = 58$) from the TCGA-COAD public database. **(F)** Intensity of ERα and ERβ expression in patients with wild-type (WT) and KRAS mutations, together with representative IHC images for KRAS status. The arrows

(Continued)

FIGURE 2 | indicate negative and positive staining. **(G)** The percentage of CRC patients with KRAS mutations and KRAS WT according to ERα and ERβ expression. **(H)** XY scatter plot of the mRNA levels of ESR1 (ERα), ESR2 (ERβ), and *KRAS* mutations from the TCGA-COAD database with 62 CRC patients. The data are presented as the mean ± SEM **(C,F)** or as the percentage **(E,G)**. The scale bar is 50 μm **(A–C)** and 100 μm **(F)**. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, paired t-test **(B)**, Mann-Whitney test **(F)** and χ_2 test **(D,G)**.

ERα expression predicts the 5-years OS with higher specificity (AUC = 0.720, Sensitivity = 65.22 and Specificity = 79.37, **Figure 3D**), while ERβ expression with higher sensitivity (AUC = 0.674, Sensitivity = 71.05 and Specificity = 49.42, **Figure 3E**). When we combined the ERα and ERβ expression, the predicting ability for 5-years OS in CRC patients was significantly improved with higher sensitivity and higher specificity (AUC = 0.842, Sensitivity = 71.53 and Specificity = 82.90, **Figure 3F**). Next, we looked at the risk score profile with TNM-stage and 5-years OS event by combining the ERα and ERβ expression in four groups as described above (**Figure 2C**). As shown in **Figure 3G**, the subgroups with positive ERα expression had the highest risk score profile, while the patients with negative ERα expression had the lowest risk score profile, despite the ERβ expression levels.

Association of Combined ERα and ERβ Expression With OS and DFS in CRC Patients

Next, we investigated the combined role of ERα and ERβ expression in CRC OS and DFS (**Figure 4**). The Cox regression analysis showed that patients with combined high ERβ + negative ERα expression were independently associated with better OS and had a 77% reduction in overall mortality (**Figures 4A,B**, **Supplementary Table 1**), as well as better DFS with a 90% reduction in cancer recurrence (**Figures 4C,D**, **Supplementary Table 1**) after adjustment for age, TNM stage and tumor vascular invasion, compared to patients with combined low ERβ + positive ERα expression, which were taken as the reference group. This finding was consistent even for the subgroups of patients with stage I-III cancer (**Figure 4E**), patients with colon cancer (**Supplementary Figures 2A,B**) and patients who did not receive adjuvant treatment (**Figure 4F** and **Supplementary Figure 2C**). In the second group of patients with low ERβ expression, even though the expression of ERα remained negative, the risk was increased by 14% for overall mortality and 33% for cancer recurrence compared to patients with combined high ERβ negative ERα expression (**Supplementary Table 1**). In addition, in the third group of patients with positive ERα expression, even though the expression of ERβ was high, the increase in the risks of overall mortality and cancer recurrence was much lower than that in the first group with combined high ERβ + negative ERα expression (3 and 22% lower, respectively: **Supplementary Table 1**, multivariate analysis). It is difficult to draw any conclusions about the subgroup of patients with rectal cancer due to the very small number of patients in each category, especially the category with combined high ERβ + positive ERα expression that has only one patient, $n = 1$ (**Supplementary Table 1**, **Supplementary Figures 2D,E**). These results clearly show that CRC patients with combined high ERβ + negative ERα expression have the best prognosis and that the subgroup with combined low ERβ + positive ERα expression has the worst prognosis.

Predictive Ability of Combined ERα and ERβ Expression

To further investigate the role of the combined ERα and ERβ expressions in predicting CRC prognosis, we evaluated the ROC curves for the basic model (adjusted for age, TNM stage and tumor vascular invasion), the model extended with only ERβ expression, the model extended with only ERα expression, and the model that included the combined ERβ + ERα expressions. As shown in **Figures 4G,H**, the AUC was significantly higher for the model with the combined ERβ + ERα expressions than for all the other models for both OS and DFS. However, the predictive ability of the combined ERβ + ERα extended model was higher for DFS (AUC = 0.812, **Figure 4H'**) than for OS (AUC = 0.801, **Figure 4G'**). The same results were obtained using the TCGA-COAD external cohort, where the combined expression of ERs had the best predictive ability for DFS compared with the other models (**Figures 4I,I'**). These results clearly show that the combined expression of ERα and ERβ plays an important role in predicting the prognosis of CRC patients.

Distribution of Clinical Parameters and Tumor Characteristics in Patients With Combined High ERβ + Negative ERα Expression VS. Patients With Combined Low ERβ + Positive ERα Expression

We aimed to evaluate the distribution of clinical parameters and tumor characteristics between patients with combined high ERβ + negative ERα expression, considered to be the best prognostic group, and those with combined low ERβ + positive ERα expression, considered to be the worst prognostic group. As shown in **Table 1**, patients with combined high ERβ + negative ERα expression had a significantly lower number of overall deaths and cancer recurrence events, smaller tumor extent, fewer tumor metastases in the regional lymph nodes and distant organs, predominantly stage I and II disease, and were less likely to receive adjuvant treatment after the operation. Additionally, tumors with combined high ERβ + negative ERα expression had a higher frequency of the mucinous type of COAD and a never smoking status (**Table 1**).

Correlation of Combined ERα and ERβ Expression With Hormonal Characteristics in Female Patients With CRC

We explored the hormonal characteristics of CRC female patients in relation to the combined ERα and ERβ expression. We found that female patients with combined high ERβ +

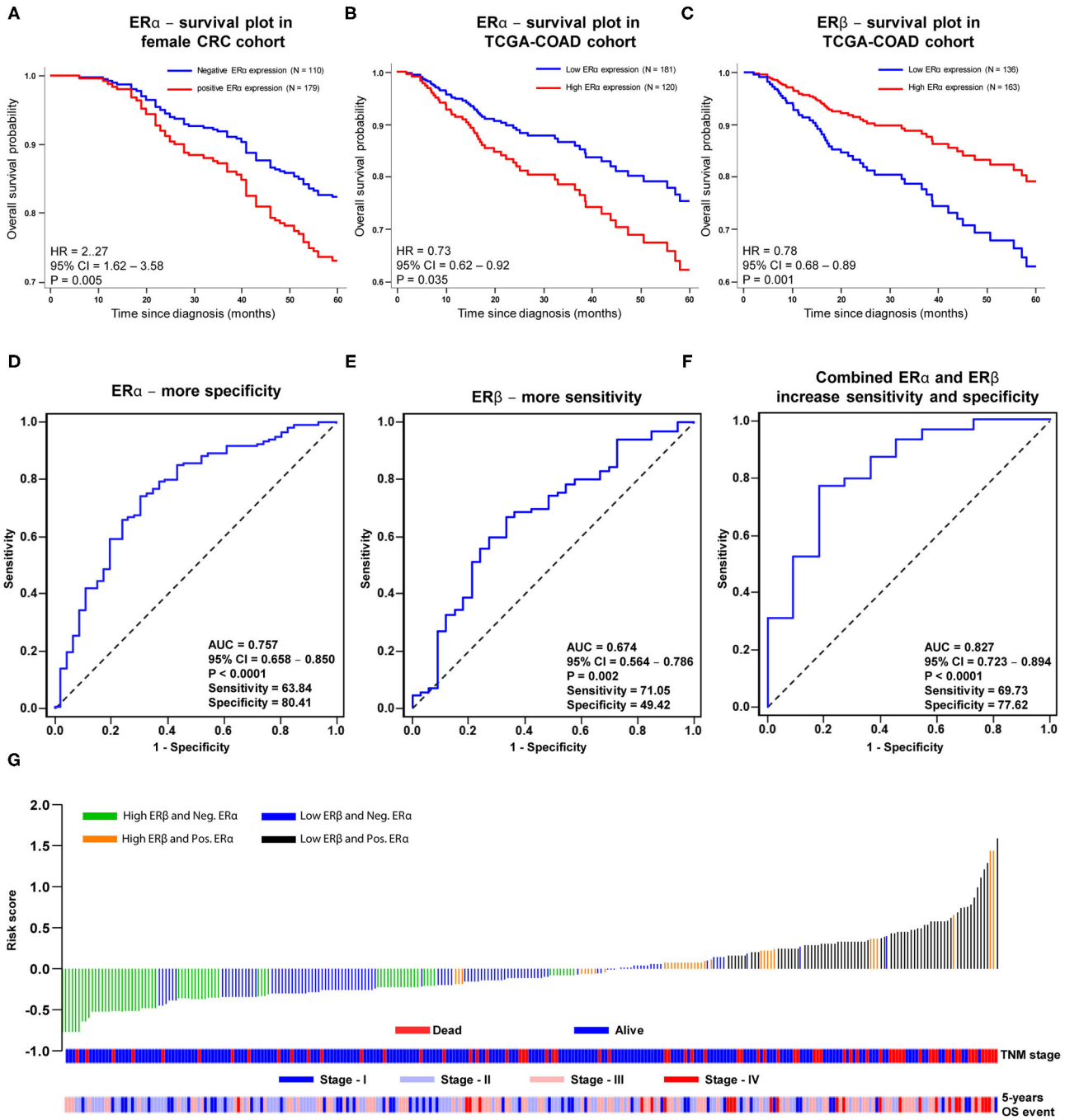
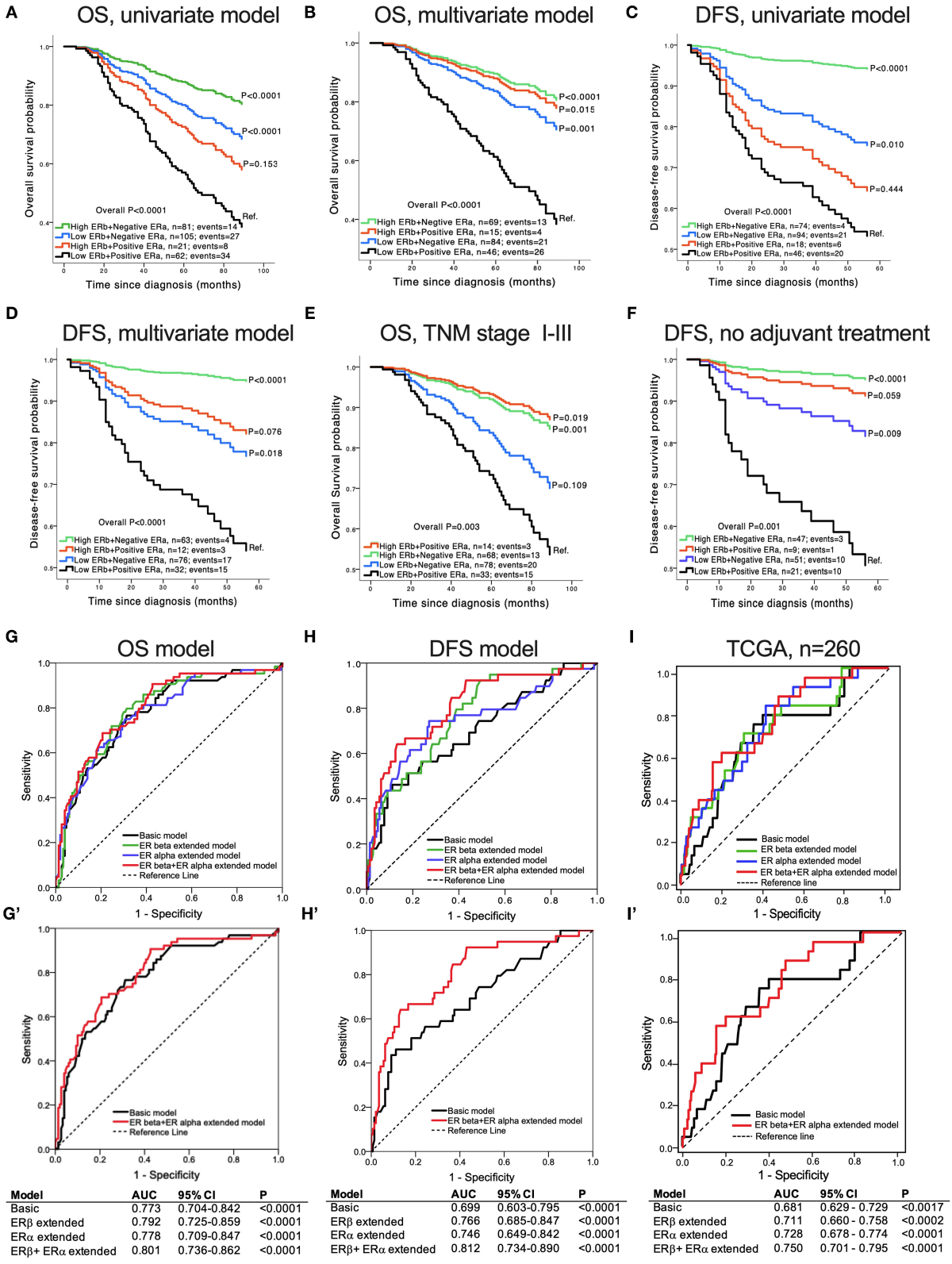


FIGURE 3 | Prognostic assessment with sensitivity and specificity estimation for only ERα, ERβ and combined ERα – ERβ protein expression without clinical factors in female CRC and TCGA-COAD cohorts. Kaplan-Meier survival curves for: **(A)** ERα expression in female CRC cohort, **(B)** ERα and **(C)** ERβ expressions in TCGA-COAD cohort with cancer stage I-III. ROC curve, sensitivity and specificity analysis for the univariate model for **(D)** ERα, **(E)** ERβ and **(F)** combined ERα – ERβ protein expressions in female CRC cohort for 5-years OS. **(G)** Water fall plot for estimated risk score profile for combined ERα - ERβ protein expressions in four patients'groups in female CRC cohort with stage and event information (cutoff based on Youden's index association criteria with OS). *P*-values according to the log-rank test.

negative ERα expression had a lower number of pregnancies (mean ± standard error of the mean, 1.8 ± 0.13, $P = 0.04$; **Figure 5A**) and shorter breastfeeding times (calculated as the total breastfeeding months for all the children a woman had; 8.2 ± 0.95, $P = 0.08$; **Figure 5B**) than female patients with combined low ERβ + positive ERα expression (2.2 ± 0.14 and 10.8 ± 1.2, respectively). No significant differences were observed between the two groups regarding the age of menopause and



Model	AUC	95% CI	P
Basic	0.773	0.704-0.842	<0.0001
ERβ extended	0.792	0.725-0.859	<0.0001
ERα extended	0.778	0.709-0.847	<0.0001
ERβ+ ERα extended	0.801	0.736-0.862	<0.0001

Model	AUC	95% CI	P
Basic	0.699	0.603-0.795	<0.0001
ERβ extended	0.766	0.685-0.847	<0.0001
ERα extended	0.746	0.649-0.842	<0.0001
ERβ+ ERα extended	0.812	0.734-0.890	<0.0001

Model	AUC	95% CI	P
Basic	0.681	0.629 - 0.729	<0.0017
ERβ extended	0.711	0.660 - 0.758	<0.0002
ERα extended	0.728	0.678 - 0.774	<0.0001
ERβ+ ERα extended	0.750	0.701 - 0.795	<0.0001

FIGURE 4 | Association of concomitant ERβ and ERα expression with CRC patient survival. Kaplan-Meier survival curves for OS: **(A)** univariate model, $n = 269$; **(B)** multivariate model adjusted for age, TNM stage and tumor vascular invasion, $n = 214$; **(C)** multivariate model for patients with stage I-III cancer, $n = 180$.

(Continued)

FIGURE 4 | Kaplan-Meier survival curves for DFS: **(D)** univariate model, $n = 232$; **(E)** multivariate model adjusted for age, TNM stage and tumor vascular invasion, $n = 183$; **(F)** multivariate model for patients who did not receive adjuvant treatment after surgery, n =128. **(G–I)** ROC curves comparing the basic model (adjusted for age, TNM stage and tumor vascular invasion), the extended model including only ERβ expression, the extended model including only ERα expression, and the extended model with combined ERβ and ERα expression for OS **(G)** and DFS **(H)**. **(I)** ROC curves from the TCGA-COAD database for stage I-III colon cancer, comparing the basic model (adjusted for age, TNM stage and tumor vascular invasion), the extended model including only ERβ expression, the extended model including only ERα expression, and the extended model with combined ERβ + ERα expression for DFS**. (G'–I')** ROC curves comparing the basic model with the model including the combined ERβ and ERα protein expression for OS **(G')**, DFS **(H')** and DFS from the TCGA-COAD database **(I')**. The tables show the values of the area under the curve (AUC) for each of the corresponding models. *P*-values according to the log-rank test.

age of menarche (**Figures 5C,D**). Next, we examined how the use of hormonal contraception (HC) differed between the two groups. We found that most of the female patients with combined high ERβ + negative ERα expression never used HC compared with women with combined low ERβ + positive ERα expression (63% vs. 37%, $P = 0.02$, **Figure 5E**). When we looked at the type of HC, we found that 61% of female patients with combined high ERβ + negative ERα expression had never used combined (estrogen and progesterone) HC and 48% of them had used combined HC. In the subgroup of women with combined low ERβ + positive ERα expression 39% had never used combined HC and 52% had used combined HC ($P = 0.07$, **Figure 5F**). However, no difference was observed between the two groups regarding the use of progesterone HC (**Figure 5G**). We also looked at the use of hormone replacement therapy (HRT) and found that most of the female patients with combined high ERβ + negative ERα expression had used HRT for more than 5 years, while very few female patients with combined low ERβ + positive ERα expression had used HRT for a long time (71 and 29%, respectively, $P = 0.02$, **Figure 5H**). All the female patients who had used combined (estrogen and progesterone) HRT had combined high ERβ + negative ERα expression ($P < 0.0001$; **Figure 5I**). No significant results were found regarding the use of estrogen HRT (**Figure 5J**).

Correlation of Combined ERα and ERβ Expression With Proteins Important for CRC Progression and Development

To further explore the prognostic role of combined ERα and ERβ expression in CRC patients, we correlated the patient with combined high ERβ + negative ERα expression or combined low ERβ + positive ERα expression with proteins important in CRC development and progression (**Figure 6A**). We noticed that patients with combined low ERβ + positive ERα expression had lower IRSs for $CysLT_1R$ ($P < 0.01$), COX-2 ($P < 0.001$) and nuclear β-catenin ($P < 0.001$), which are connected to enhanced cell proliferation and poor patient outcome (18, 19, 23), compared to patients with combined high ERβ + negative ERα expression (**Figure 6A**, **Supplementary Figure 3** for IHC images). On the other hand, patients with combined high ERβ + negative ERα expression had higher IRSs for $CysLT_2R$ ($P < 0.001$), membrane β-catenin ($P < 0.001$), 15-PGDH ($P < 0.01$) and PGD2 synthase ($P < 0.001$), which are associated with a better outcome in CRC (20, 23, 33, 34) (**Figure 6A**, **Supplementary Figure 3**). Since we observed a higher frequency of mucinous adenocarcinomas in the group of patients with combined high ERβ + negative ERα expression, we investigated the association with Mucin-2 expression known to be reduced in CRC tissues compared to the normal mucosa (35, 36). We found that patients with combined high ERβ + negative ERα expression had significantly higher IRSs for Mucin-2 expression levels ($P < 0.05$) than patients with combined low ERβ + positive ERα expression (**Figure 6A**, **Supplementary Figure 3**). In the TCGA-COAD cohort, the same correlations were observed between the combined protein expression of ERs and $CysLT_1R$, COX-2, $CysLT_2R$ and PGD2 synthase, whereas no correlation was found for combined ERs expression with 15-PGDH and Mucin-2 expression levels (**Figure 6B**).

Association of ERα and ERβ Expression With Metastasis in Patients With CRC

We investigated the risk of having a metastatic event for each unit increase in the ERβ and ERα staining intensity, evaluated by IHC. We found that for each unit increase in the ERβ intensity, the risk of having a metastatic event were significantly and independently decreased by 60% after adjustment for age, TNM stage and tumor vascular invasion (OR = 0.40; 95% CI: 0.19–0.82; $P = 0.012$; **Figure 7A**). In addition, for each unit increase in the ERα intensity, the risk of having a metastatic event increased almost 2.5-fold (OR = 2.47; 95% CI: 1.15–5.32; $P = 0.021$; **Figures 7A,B**). The ERα intensity was strongly associated with liver metastasis, where for each unit increase in the ERα intensity, the risk of liver metastasis independently increased almost 4-fold (OR = 3.72; 95% CI: 1.36–10.17; $P = 0.01$; **Figures 7A,B**). However, no role of ERβ was found in lung metastasis and the promoting effect of increased ERα staining intensity (OR = 3.48; 95% CI: 1.38–8.77; $P = 0.008$) disappeared after adjustment for other confounding factors (OR = 3.05; 95% CI: 0.99–9.42; $P = 0.052$; **Figure 7A**). Importantly, each unit increase in the ERβ intensity significantly and independently decreased the risk of local recurrence and abdominal metastasis by 79% (OR = 0.21; 95% CI: 0.06–0.67; $P = 0.009$; **Figures 7A,B**). These results were summarized graphically using the forest plots, where the increased risk is shown in red, and the decreased risk is shown in blue (**Figure 7B**).

DISCUSSION

CRC is one of the most common malignancies worldwide. Despite the current technologies for early detection and targeted therapies, the risk of recurrence in patients with stage II and III cancer remains high (37). Prognostic markers are needed to predict the recurrence risk with higher precision. Herein, we demonstrate the prognostic significance of the combined

TABLE 1 | Distribution of clinical parameters and tumor characteristics in 143 CRC patients according to subgroups with combined high ER&-negative ERa and combined low ERB-positive ERa expressions.

	Total	High ERß Negative ERα	Low ERß Positive ERα	
Characteristics	**N (%)**	**N (%)**	**N (%)**	***P***
Patients no.	143 (100)	81 (56)	62 (44)	
Deaths	48 (34)	14 (29)	34 (71)	<0.0001[a]
DFS events*	24 (19)	4 (17)	20 (83)	<0.0001[a]
Age (mean, years)	70.9	71.8	69.8	0.198[b]
BMI (mean, kg/m^2)	26.1	25.9	26.2	0.931[b]
Tumor extent	41 (29)	30 (73)	11 (27)	0.011[a]
≤T2	102	51 (50)	51 (50)	
>T2	(71)			
Lymph node metastasis	90 (63)	60 (67)	30 (33)	0.002[a]
N0	53 (37)	21 (40)	32 (60)	
N1/N2				
Distant metastasis at	128 (89)	80 (63)	48 (37)	<0.0001[a]
diagnosis	15 (11)	1 (7)	14 (93)	
M0				
M1				
TNM stage	30 (21)	21 (70)	9 (30)	<0.0001[a]
I	55 (39)	38 (69)	17 (31)	
II	42 (29)	20 (48)	22 (52)	
III	15 (11)	1 (7)	14 (93)	
IV	1			
Missing				
Tumor intravascular invasion	83 (72)	53 (64)	30 (36)	0.174[a]
No	32 (28)	16 (50)	16 (50)	
Yes	28			
Missing				
Tumor differentiation	21 (15)	14 (67)	7 (33)	0.354[a]
Low	120	67 (56)	53 (44)	
Moderate/High	(85) 2			
Missing				
Tumor localization	106 (74)	58 (55)	48 (45)	0.431[a]
Colon	37 (26)	23 (62)	14 (38)	
Rectum				
Tumor histological type	110 (77)	57 (52)	53 (48)	0.079[a]
Non-mucinous AC[†]	22 (15)	15 68)	7 (32) 2	
Partly Mucinous AC	11 (8)	9 (82)	(18)	
Mucinous AC				
Neoadjuvant treatment	124 (87)	70 (57)	54 (43)	0.906[a]
No	19 (13)	11 (58)	8 (42)	
Yes				
Adjuvant treatment	99 (71)	63 (64)	36 (36)	0.016[a]
No	41 (29)	17 (42)	24 (58)	
Yes	3			
Missing				
Smoking status	5 (11)	1 (20)	4 (80)	0.059[a]
Ever smokers	39 (89)	25 (64)	14 (36)	
Never smokers	99			
Missing				
Alcohol use	19 (43)	9 (47)	10 (53)	0.168[a]
Yes	25 (57)	17 (68)	8 (32)	
No	99			
Missing				

**Patients with TNM stage IV are excluded.* [a]*Pearson chi-square test.* [b]*Mann-Whitney U test.*
[†]*AC, Adenocarcinoma; BMI, Body Mass Index.*

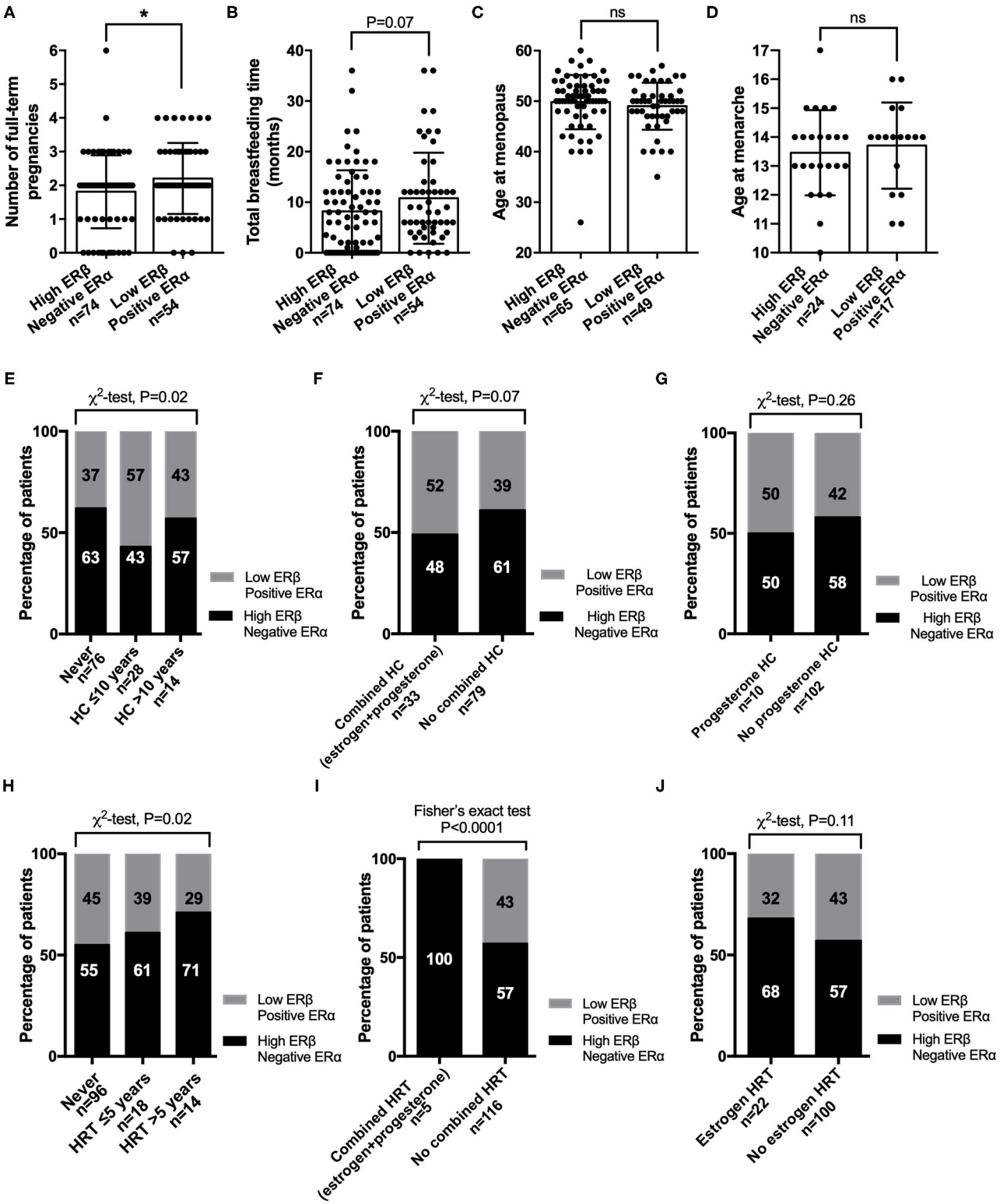


FIGURE 5 | Correlation of hormonal status with subgroups of female CRC patients with both ERβ and ERα expression. Hormonal characteristics for **(A)** number of full-term pregnancies, where 0 refers to women who never had children; **(B)** total breastfeeding time for all the children a woman had, where 0 refers to women who never breastfed; **(C)** age at menopause; and **(D)** age at menarche. Percentage of female CRC patients with combined high ERβ + negative ERα expression or combined low ERβ + positive ERα expression who never or ever used **(E)** hormonal contraception (HC); **(F)** combined (estrogen and progesterone) HC; **(G)** progesterone HC; **(H)** hormonal replacement therapy (HRT); **(I)** combined (estrogen and progesterone) HRT; or **(J)** estrogen HRT. The data are presented as the mean ± SEM **(A–D)**. $^{*}P < 0.05$, unpaired *t*-test; χ_2 test or Fisher's exact test as indicated.

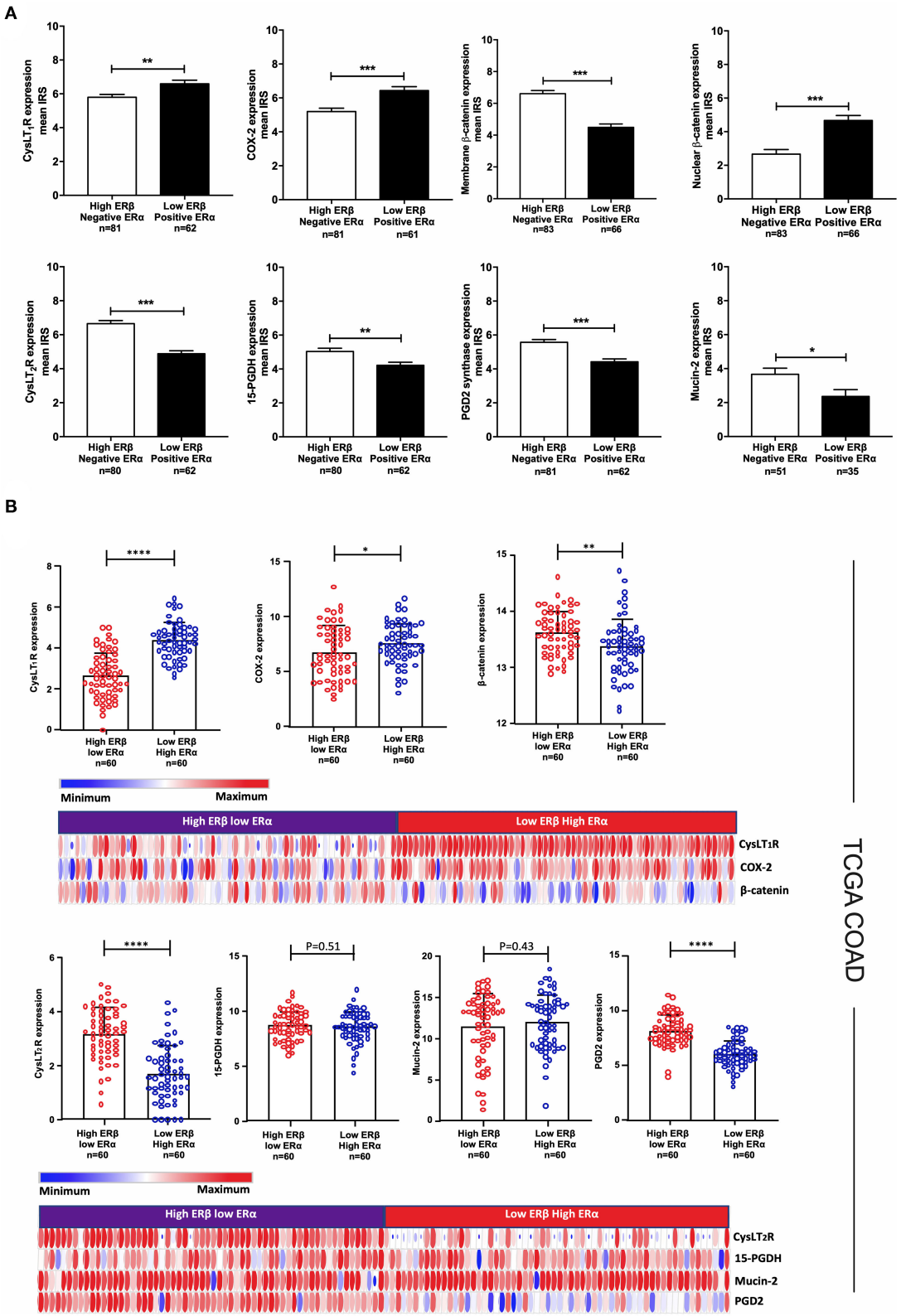


FIGURE 6 | Correlation of subgroups of patients with ERβ and ERα expression with proteins important for CRC progression and development. **(A)** Mean IRS for $CysLT_1R$, COX-2, membrane and nuclear β-catenin, $CysLT_2R$, 15-PGDH, Mucin-2, and PGD2 synthase expression levels evaluated with IHC in subgroups of CRC

(Continued)

FIGURE 6 | patients with combined high ERβ + negative ERα expression ($n = 81$) or combined low ERβ + positive ERα expression ($n = 62$). **(B)** Expression of the indicated proteins ($CysLT_1R$, COX-2, β-catenin, $CysLT_2R$, 15-PGDH, Mucin-2 and PGD2 synthase) in the TCGA-COAD patients with combined high ERβ + low ERα expression ($n = 60$) or combined low ERβ + high ERα expression ($n = 60$) together with the corresponding heat maps. The data are presented as the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, Mann-Whitney test.

ERα and ERβ expression in female patients with CRC and explore their correlations with other prognostic markers and hormonal status.

We found that in cancer tissues, ERβ expression was downregulated while ERα expression upregulated, compared to the normal matched pair tissues (**Figure 2B**). We previously reported that high ERβ expression is associated with better OS and DFS (7), and in this investigation we showed that most of the patients with high ERβ expression were negative for ERα expression, while the majority of patients with low ERβ expression were positive for ERα expression. Many have reported the downregulation of ERβ during tumor progression (2–4, 7), while others have shown that ERα protein levels significantly increase in men but not in women with CRC (38). Herein, we showed that ERα expression levels are increased in cancer tissues compared to matched normal tissues in females with CRC. A previous report detected ERα and ERβ protein levels in CRC and they found no significant difference of ERβ expression levels between normal and cancer colon tissues (39). Another report showed that ERα expression is rare in CRC tissue and its expression does not correlate with colon carcinogenesis, while ERβ expression was upregulated in CRC tissues and correlated with poor DFS (40). It is worth noting that both studies had a small number of patients and included in their studies even colon adenomas (41). Moreover, both studies used polyclonal antibodies and the antibody used from Grivas et at., recognizes only the β1 isoform (40).

Furthermore, we investigated the correlation of ERα and ERβ expression with KRAS mutation, which plays an important role in the prognosis and treatment of CRC (15). In 4,411 CRC patients, *KRAS* mutations were independently associated with shorter relapse times, survival after recurrence and OS in patients with MSS but not MSI tumors (16). Additionally, treatment with anti-EGFR is ineffective in CRC patients with *KRAS* mutations (17). Interestingly, we found that patients with positive ERα expression, which were associated with shorter OS (**Figures 3A,B**), had a higher frequency of KRAS mutations than patients with negative ERα expression. This result was further supported by mRNA data from the TCGA-COAD cohort, where we found a significant positive correlation between the mRNA levels of *ESR1* (ERα) and *KRAS* mutations. This finding can provide new opportunities for patients with *KRAS* mutations, where ERα-selective antagonists might be an alternative to improve their prognosis. No correlations were observed between KRAS status and ERβ expression at either expression level detected by IHC or mRNA levels from the TCGA-COAD cohort.

Next, we evaluated the prognostic role of the combined ERα and ERβ expression in CRC patient survival. Patients with combined high ERβ + negative ERα expression had the best OS and DFS, with a reduction in overall mortality by 77% and cancer recurrence by 90%. Patients with combined low ERβ + positive ERα expression, taken as the reference category, had the worst OS and DFS. The model with the combined expression of ERs had the highest predicting ability compared to all the other models taken into consideration. Moreover, we found that each unit increase in the ERα intensity independently increased the risk of liver metastasis almost 4-fold, while each unit increase in the ERβ intensity reduced the risk of local recurrence and abdominal metastasis by 79%. These results imply an important role of the combined ERα and ERβ expression as a future prognostic marker in patients with CRC. Reports show that $CysLT_1R$, $CysLT_2R$, COX-2 and β-catenin expression levels are linked to CRC development and prognosis (42). High levels of 15-PGDH and PGD2 synthase in CRC are reported to have antitumor properties (20–22, 33, 34). We found that patients with combined high ERβ + negative ERα expression had significantly lower IRSs of tumor-promoting proteins, such as $CysLT_1R$, COX-2 and nuclear β-catenin, and higher IRSs of anti-tumorigenic proteins such as $CysLT_2R$, membrane β-catenin, 15-PGDH and PGD2 synthase, compared to patients with combined low ERβ + positive ERα expression. To validate our findings, we used protein data from the TCGA-COAD cohort and found that compared to patients with combined low ERβ + high ERα expression, patients with combined high ERβ + low ERα expression had a better tumor profile and a more favorable prognosis (**Figure 7C**).

Interestingly, we found that patients with combined high ERβ + negative ERα expression had significantly smaller tumors, fewer regional and distant metastases, predominantly TNM stage I and II and were less likely to receive adjuvant treatment. In addition, patients with combined high ERβ + negative ERα expression were more likely to have a never smoking status, which is an established risk factor for CRC (43), and a higher frequency of mucinous adenocarcinoma, which also correlated with higher IRS for Mucin-2 expression. High Mucin-2 levels are linked to colon cell differentiation (36, 44). Previous studies have shown that ERs are implicated in the obesity-associated CRC (12, 13), however we found no correlation between BMI and the combined ERα and β expression.

We previously found that high ERβ expression in female CRC patients was associated with a lower number of pregnancies, shorter breastfeeding times, a longer time of combined HC use, and a longer time of HRT use (7). Many studies have suggested a lower risk of CRC incidence among women who use HRT (45). However, none of them took into consideration the combined expression of ERα and ERβ in CRC tissue. Herein, we showed that in female CRC patients, combined high ERβ + negative ERα expression correlated with lower pregnancy number, shorter breastfeeding times, non-use of HC and long-term use of HRT, both estrogen monotherapy and combined HRT.

A

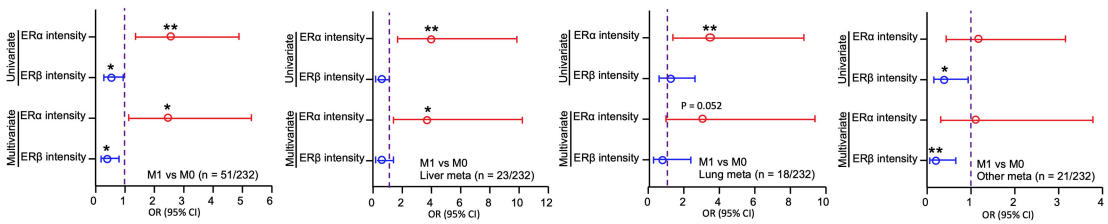
Disease-free survival events (M1)

	M1 vs M0 n=51/232			M1 vs M0 Liver meta, n=23/232		
	OR	**95% CI**	**P**	**OR**	**95% CI**	**P**
Univariate model						
ERβ intensity	0.58	0.34-0.99	**0.046**	0.55	0.26-1.16	0.116
ERα intensity	2.58	1.36-4.89	**0.004**	4.01	1.64-9.83	**0.002**
Multivariate model (adjusted for age, TNM stage and tumor intravascular invasion)						
ERβ intensity	0.40	0.19-0.82	**0.012**	0.56	0.23-1-35	0.197
ERα intensity	2.47	1.15-5.32	**0.021**	3.72	1.36-10.17	**0.010**

	M1 vs M0 Lung meta, n=18/232			M1 vs M0 Other meta*, n=21/232		
	OR	**95% CI**	**P**	**OR**	**95% CI**	**P**
Univariate model						
ERβ intensity	1.22	0.57-2.62	0.602	0.40	0.17-0.95	**0.037**
ERα intensity	3.48	1.38-8.77	**0.008**	1.17	0.44-3.15	0.749
Multivariate model (adjusted for age, TNM stage and tumor intravascular invasion)						
ERβ intensity	0.81	0.27-2.41	0.702	0.21	0.06-0.67	**0.009**
ERα intensity	3.05	0.99-9.42	0.052	1.10	0.32-3.79	0.875

**Other metastases include local recurrence (n=12), abdominal cavity (n=8) and bone metastases (n=1). Significant P-values are shown in bold.*

B



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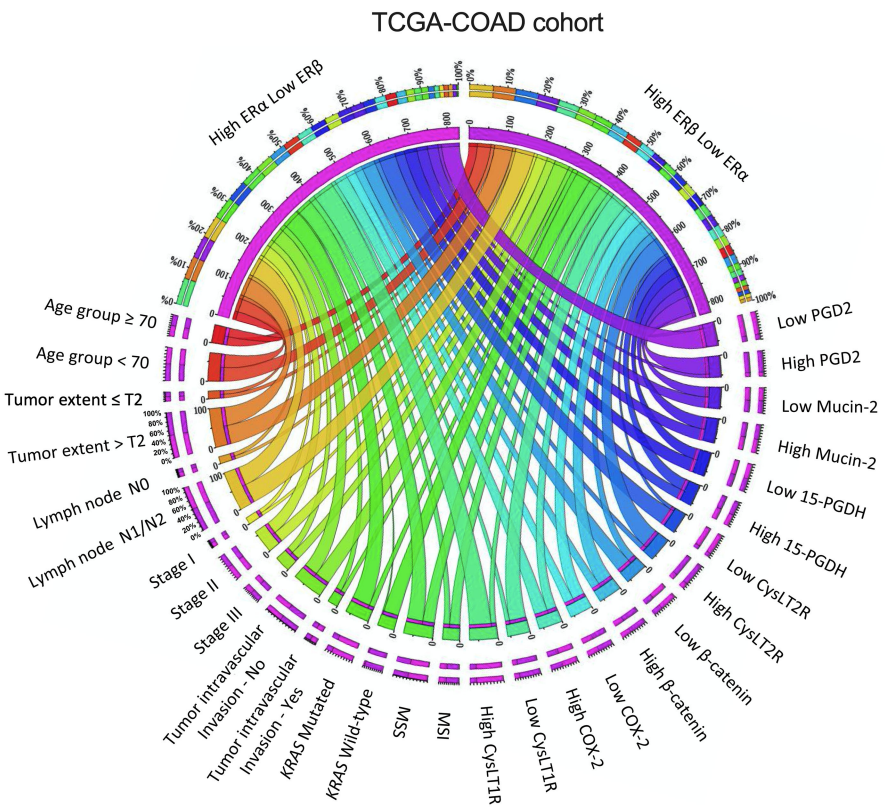

FIGURE 7 | Correlation of ERβ and ERα expression with CRC metastasis. **(A)** Binary logistic regression model showing the odds ratios (ORs) and 95% confidence intervals (CIs) for total metastatic events; liver metastasis; lung metastasis; other metastases; ocal recurrences; abdominal metastasis and bone metastasis. **(B)** Forest plots showing the respective estimates for the corresponding metastatic events for the patients included in the study. **(C)** Distributions of each clinical factor and associated protein expression pattern in the combined high ERβ + low ERα or combined low ERβ + high ERα expression groups in the TCGA-COAD cohort. The data were visualized *via* Circos software. The area of each colored ribbon depicts the frequency of the samples. **P* < 0.05, ***P* < 0.01.

An important issue to address is the antibody used in IHC. The use of TMAs in cancer research raises the concern whether the chosen core tissue is representative of the whole tumor. However, the use of two cores to represent the tumor has shown sufficient concordance for many cancer types, including CRC (46). The clone 14C8 of the anti-ERβ antibody that we used, recognizes most of ERβ variants including ERβ wild-type, and is shown to be useful for the assessment of ERβ expression in paraffin-embedded tissues (47). In a recent publication for the validation of ERβ antibodies in 44 different tissues, 14C8 antibody showed in CRC IHC the same intensity band as PPZ0506, which was reported to be the most specific anti- ERβ antibody, and that correlated with ERβ mRNA levels detected in the CRC tissue [**Figure 3**, see reference (48)]. Because ERα is low expressed in the colon tissue, we used a cocktail antibody (1D5 + 6F11) created by mixing two monoclonal antibodies that target ERα. Human normal tissues verified for ERα expression levels were used as positive and negative controls to test the antibody specificity (25–27). To validate the IHC staining, 59 randomly selected patients from the cohort were stained with another ERα monoclonal antibody D12, widely used for the detection of ERα (28–30). The same control tissues that were stained positive for ERα expression using the cocktail antibody, were also stained positive with D12 antibody but the staining intensity was weaker. This was the reason that we identified more patients with positive ERα expression using the cocktail antibody, which might be missed using the monoclonal D12 antibody (32). It is important to highlight that we validated our findings by using protein expression data from the TCGA-COAD cohort, which was used as an external cohort and includes both female and male patients.

To the best of our knowledge, this is the first study to investigate the prognostic significance of combined ERα and ERβ expressions in CRC patients. Our results suggest that patients with combined high ERβ + negative ERα expression have a better outcome with longer OS and DFS. Interestingly, ERβ intensity was important for the local recurrence of CRC, while the ERα intensity was important for the liver metastasis. ERβ expression levels are found significantly decreased in CC tissues of both males and females compared to the matched normal mucosa, and ERα/ERβ protein ratio are altered in both male and female CRC tissues (38). Therefore, we believe that our results are applicable to both female and male CRC patients. In summary, our results highlight the role of combined expression of ERα and ERβ as important prognostic and treatment markers in CRC patients.

DATA AVAILABILITY STATEMENT

The datasets used and analyzed in the current study are available from the corresponding author upon request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Lund University Ethical Committee Approval 3/2006. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

GT and AS: conception and design. GT, SG, and M-LL: development of methodology. GT, SG, RE, and AS: analysis and interpretation of data. RE and ML-L: administrative and/or material support. GT, AS, SG, and SS: writing and review of the manuscript. All authors have read, reviewed, and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.739620/full#supplementary-material

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An Assessment of Physicians' Recommendations for Colorectal Cancer Screening and International Guidelines Awareness and Adherence: Results From a Thai National Survey

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Background: Colorectal cancer (CRC) screening uptake is generally low in the Asia Pacific and physicians' recommendations affect the screening participation.

Objective: The study aimed to assess Thai physicians' recommendations for CRC screening, and the awareness of and adherence to international guidelines.

Methods: A survey containing questions assessing physicians' demographic data, screening recommendations, and awareness of the international CRC screening guidelines assessed by clinical vignettes. Independent predictors of physicians' recommendations for CRC screening were determined by logistic regression analysis.

Results: Five hundred and eighty-sixth of 1,286 (46%) physicians completed the survey, and 58% of them offered CRC screening. The majority of colorectal surgeons (91%) and gastroenterologists (86%) endorsed screening, whereas 35% of primary care physicians recommended screening. The patient's age was the only factor influencing the physician's decision to offer CRC screening (OR, 2.75: 95% CI, 1.61–4.67). Colonoscopy was the most recommended modality among specialists, whereas 60% of primary care physicians offered fecal occult blood tests (FOBTs). The guidelines awareness was noted in 81% of participants, with the highest rates among gastroenterologists and colorectal surgeons. Gastroenterologists were more likely to adhere to the guidelines than surgeons, but both recommended shorter interval surveillance colonoscopy than guidelines recommendations in cases of small hyperplastic rectosigmoid polyps.

Conclusions: Recommendations for CRC screening and awareness of guidelines vary among different specialties. A low proportion of primary care physicians recommended screening and colorectal surgeons and gastroenterologists recommended shorter intervals for surveillance of small hyperplastic polyp than suggested by guidelines.

Keywords: colorectal cancer, screening, physician, recommendation, awareness, adherence

INTRODUCTION

Colorectal cancer (CRC) is the third most prevalent cancer worldwide, with wide geographical variation in incidence and mortality rates (1). CRC screening is recognized as a proven strategy to improve prognosis and survival given the nature of the extended precancerous phase, and the ability to cure precancerous lesions (1–3). The optimal screening approach for each region may differ according to resources, availability of tests and healthcare providers, procedural risks, costs, personal beliefs, and cultural barriers.

Several international guidelines have been published to provide evidence-based recommendations for CRC screening programs. Most guidelines recommend screening for average-risk individuals at 45–50 years of age (4–8). Despite the evident benefits of CRC screening, the participation rate in the Asia Pacific region is generally low, ranging from 1.0 to 49.0% (9–13). In contrast, the United States reported a CRC screening uptake rate of 65.5% (14). A systematic review of screening in the United States showed that facilitator factors (physician recommendation, public education, social network, and self-motivation) and barriers (fear, fatalism, aversion, and cultural barriers) influenced CRC screening uptake rates (15). A multicenter, international study involving 14 countries in the Asia-Pacific area surveyed the population's attitudes and barriers to CRC screening. The investigators found that physicians' recommendations and knowledge of screening tests were significant factors in CRC screening uptake (12). The authors stated that promoting physicians' roles in improving awareness of CRC is essential to implementing a mass screening program to increase screening participation rates.

In Thailand, CRC remains one of the major unsolved national healthcare issues. Colon cancer is the third most frequent cancer in men and the fourth most common cancer in women (16). Furthermore, it is only cancer with increased incidence rates in both sexes (17). The majority of patients with CRC have nodal or distant metastases at their presentations (18, 19). Therefore, a mass screening program is required nationwide to reduce cancer incidence and allow early detection. A national survey study among colorectal surgeons showed that 84% of them offered CRC screening to the average-risk population, using a variety of screening modalities (20). Nonetheless, recommendations from physicians other than colorectal surgeons have not been evaluated.

The primary objective of this study was to evaluate physicians' recommendations for CRC screening across the country. Also, the present study aimed to determine the proportion of physicians who are aware of the international CRC screening guidelines, and to assess the adherence to guidelines among those with awareness.

MATERIALS AND METHODS

Subjects

A questionnaire-based survey study was conducted. In the context of this study, physicians included resident physicians, primary care physicians, internists, gastroenterologists, and surgeons. The questionnaires were sent to practicing physicians nationwide, including members of the following organizations: (1) the Royal College of Thai Physicians (RCTP); primary care physicians, internists, and resident physicians in internal medicine, (2) the Gastroenterological Association of Thailand (GAT); gastroenterologists, and (3) the Thai Royal College of Surgery (TRCS); general surgeons, colorectal surgeons, and resident physicians in surgery.

Questionnaire

Content experts in gastroenterology, general surgery, and colorectal surgery were invited to participate in the questionnaire development. The experts reviewed the current CRC screening guidelines, including the 2008 American Gastroenterological Association CRC screening and surveillance guidelines (21). A face-to-face meeting was held to ensure that the questions captured the topic of interest during the questionnaire development. The completed questionnaire was tested with colleagues in both surgical and gastroenterological fields, trainees included, *via* a web-based survey monkey for feedback. A minor revision was made to finalize the questionnaire (**Supplementary Material 1**). The paper-and-pencil self-administered questionnaires were mailed to participants. In addition, the web-based online survey was done *via* SurveyMonkey.[1] The two versions of the questionnaire were identical in terms of the questions asked, their wording, and their order of presentation in the survey. The questionnaire contains three domains, including (1) physician demographics, (2) physician's practice in CRC screening and the awareness of CRC screening guidelines, and (3) physician's adherence to CRC screening guidelines.

The first domain of the questionnaire assesses the following demographic data and general information: age, sex, area of specialty (general practice, resident physician, internal medicine, general surgery, colorectal surgery, and gastroenterology), years of practice, number of patients seen per week, place of employment (academic center, community hospital, tertiary care center, and private hospital) and geographic location of the workplace (central, north, northeast, east, and south region of the country).

The second domain of the questionnaire was designed to provide insight into physicians' CRC screening practices and their awareness of guidelines. Participants were asked specific questions about their rate of CRC screening offers, their impression of the appropriate age at which screenings should be initiated and discontinued, factors influencing their decision to recommend CRC screening (age, sex, family history of CRC, comorbidity, reimbursement, and the availability of CRC screening), and questions regarding the modalities of CRC screening [fecal occult blood test (FOBT), including guaiac fecal occult blood test (gFOBT) and immunochemical fecal occult blood test (iFOBT) or fecal immunochemical testing (FIT), barium enema, computed tomography (CT)

[1] www.surveymonkey.com

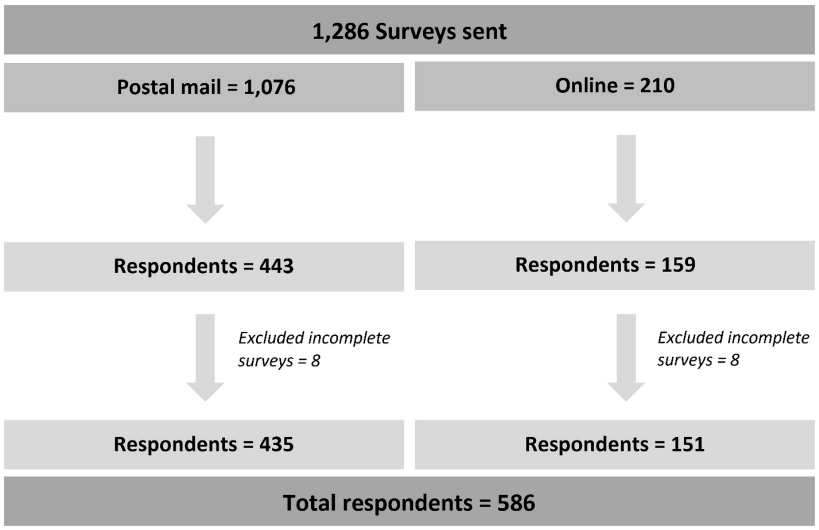

FIGURE 1 | Study flow chart.

colonography, flexible sigmoidoscopy, colonoscopy, and blood test for carcinoembryonic antigen (CEA) level].

The last domain of the questionnaire asked gastroenterologists and surgeons participants to rate their own adherence to CRC surveillance guidelines using four clinical vignettes. The following are the four questions that were posed: (1) When do you recommend surveillance in a patient without a family history of CRC who had a normal screening colonoscopy?, (2) When do you recommend surveillance in a patient with a family history of CRC who had normal screening colonoscopy?, (3) When do you recommend surveillance for a patient who had complete polypectomy of villous or tubulovillous adenoma of larger than 1 cm?, and (4) When do you recommend surveillance for a patient who had multiple (<10 polyps) hyperplastic polyps of less than 1 cm at the sigmoid colon and rectum?

Ethics

The study protocol conformed to the ethical guidelines of the 1975 Helsinki Declaration and has been approved by the Siriraj Institutional Review Board (COA no. Si448/2015). RCTP, GAT, and TRCS did not receive any compensation for their participation in this survey, which was completely voluntary. The questionnaire was anonymous, and no personal information about the respondents was gathered. Participants gave their informed consent to take part in the study.

Statistical Analysis

Data were summarized using descriptive statistics. Categorical variables were compared using the χ^2 test. Variables that might influence CRC screening recommendations were identified using logistic regression analysis and summarized with odds ratio (OR) and 95% confidence interval (CI). All statistical testing was performed at the conventional 2-tailed α level of 0.05. SPSS 18.0 software (SPSS, Inc., Chicago, IL, United States) was used to perform all statistical analyses.

RESULTS

Demographic Data

The questionnaires were distributed to 1,286 physicians nationwide (postal mail = 1,076 and online = 210). A total of 586 respondents were included in the study indicating a response rate of 46% (**Figure 1**). The mean age of respondents was 34.0 ± 11.4 years (range: 24–78), and males accounted for 59.7%. Of them, 29.5% were internists, 17.9% were resident physicians in internal medicine, 17.4% were general surgeons, 13.1% were primary care physicians, 13.0% were gastroenterologists, 5.1% were resident physicians in surgery, and 3.9% were colorectal surgeons. Most respondents graduated 6–10 years ago from medical schools (35.2%), 22.0% graduated less than five years ago, and 21.5% graduated more than 20 years ago. One-third of respondents encountered 20–49 patients per week, and 32.6% encountered 50–100 patients per week in the clinic. Thirty percent worked at academic centers and 25.6% in tertiary care hospitals. Regarding workplaces' geographical location in Thailand, 52.4% lived in the central area, 18.4% in the south, and 15.5% in the north. Characteristics of respondents are shown in **Table 1**.

Rate of Colorectal Cancer Screening Recommendation

Overall, 58.2% of the respondents offered CRC screening with no variation among different geographic areas and years of practice. Both colorectal surgeons (91.3%) and gastroenterologists (86.8%) were most likely to offer CRC screening, followed by internal medicine residents (65.9%), general surgeons (58.8%), surgical residents (50.4%), and internists (49.7%). In contrast, primary care physicians recommended screening at the lowest rate of 35.1%. Physicians in the community were less likely than specialists to offer screening.

TABLE 1 | Baseline characteristics of respondents.

Baseline characteristics	Responders (*N* = 586)
Age, year	34.0 ± 11.4
Male gender, *N* (%)	350 (59.7)
Specialty, *N* (%)	
Internists	173 (29.5)
Resident physicians in internal medicine	105 (17.9)
General surgeons	102 (17.4)
Primary care physicians	77 (13.1)
Gastroenterologists	76 (13.0)
Resident physicians in surgery	30 (5.1)
Colorectal surgeons	23 (3.9)
Years of practice, *N* (%)	
≤5 years	129 (22.0)
6–10 years	206 (35.2)
11–15 years	69 (11.8)
16–20 years	56 (9.6)
>20 years	126 (21.5)
Patients seen per week, *N* (%)	
≤20	49 (8.4)
21–50	192 (32.8)
51–100	191 (32.6)
>100	154 (26.3)
Distribution of workplace, *N* (%)	
Academic centers	176 (30.0)
Tertiary care centers	150 (25.6)
Private hospitals	92 (15.7)
Provincial hospitals	80 (13.7)
Community hospitals	77 (13.1)
Private clinics	11 (1.9)
Geographic distribution, *N* (%)	
Central	307 (52.4)
South	108 (18.4)
North	91 (15.5)
Northeast	58 (9.9)
East	22 (3.8)

Data presented as mean ± standard deviation or number and percentage.

Factors Influencing Physicians' Recommendations for Colorectal Cancer Screening

We performed a univariate analysis and determined physicians' specialty and type of hospital were associated with recommendations for CRC screening. To account for potential confounding factors, physician's specialty, years of practice, number of patients seen per week, type of hospital, and geographic distribution were included in the multivariate regression model for assessing physician-related factors associated with CRC screening recommendations. We found that colorectal surgeons, gastroenterologists, resident physicians in internal medicine, and general surgeons were more likely to offer screening than primary care physicians. Furthermore, physicians working in academic centers, tertiary care hospitals, and private hospitals were more likely to recommend CRC screening than those working in community hospitals or private clinics (**Table 2**).

The patient's age was the only significant patient factor influencing the physician's decision to offer CRC screening (OR, 2.75: 95% CI, 1.61–4.67). Physicians' intention to recommend CRC screening was not affected by other patient-related factors (such as gender, family history of CRC, and comorbidities), reimbursement policies, or hospital facility, as shown in **Table 3**.

Screening Modalities

Almost all respondents' workplaces (96.9%) had a tool to perform CRC screening. FOBT was the most commonly accessible option for 88.6% of respondents. Colonoscopy was the second most prevalent test, accounting for 81.9%, followed by barium enema (68.4%). Colonoscopy was advised by the majority of respondents (68.9%), while FOBT was offered by 45.1%. Other screening modalities, such as flexible sigmoidoscopy combined with barium enema, flexible sigmoidoscopy alone, CT colonoscopy, and serum CEA, were all recommended at the rates of 5–10%. Colonoscopy was the test of choice recommended by gastroenterologists and colorectal surgeons, whereas primary care physicians preferred FOBT. Colonoscopy was offered as a screening test by 96.1% of gastroenterologists and 91.3% of colorectal surgeons. The screening methods offered by each specialty and the availability of each method in their facility are shown in **Table 4**.

Adherence to Colorectal Cancer Screening Guideline

Overall awareness of the CRC guidelines was 81.1%, with the highest rates noted among gastroenterologists and colorectal surgeons. Three hundred and twenty-nine (56.1%) of 586 respondents began screening average-risk people at age 50 as recommended by the international guidelines. Approximately 90% of gastroenterologists and colorectal surgeons adhered to the guideline recommendation regarding the start age for screening. The decision to discontinue screening at the age of 80 was noted in 44.0% of respondents. Only 29.0% agreed to cease screening at the age of 75, as recommended by the guidelines. Notably, 77.0% of respondents would continue to offer screening if the government covered the cost.

The clinical vignettes model around the CRC screening consensus guidelines were applied exclusively to gastroenterologists, general surgeons, and colorectal surgeons (**Table 5**). Gastroenterologists were more likely to adhere to the guidelines than surgeons, but both specialists recommended shorter interval surveillance colonoscopy than recommended by guidelines in small hyperplastic rectal polyps.

DISCUSSION

This study revealed that recommendations for CRC screening and awareness of guidelines varied across different groups of physicians. Physicians in the community were less likely to offer screening than specialists practicing in the

TABLE 2 | Physician-related factors associated with colorectal cancer screening recommendations.

Factors	Univariate analysis unadjusted odds ratio (95% CI)	*P*-value	Multivariate analysis adjusted odds ratio (95% CI)	*P*-value
Specialty		<0.001		<0.001
Primary care physicians	Reference		Reference	
Colorectal surgeons	20.05 (4.30–93.45)		13.18 (2.56–67.86)	
Gastroenterologists	11.28 (4.96–25.65)		7.72 (3.08–19.39)	
Resident physicians	3.14 (1.69–5.56)		2.22 (1.02–4.84)	
General surgeons	2.62 (1.37–5.01)		2.09 (0.97–4.50)	
Years of practice		0.056		0.787
<10 years	Reference		Reference	
15–20 years	1.64 (1.07–2.52)		1.09 (0.65–1.86)	
>20 years	1.34 (0.88–2.03)		0.88 (0.50–1.54)	
Number of patients seen per week		0.084		0.296
<20 patients/week	Reference		Reference	
21–50 patients/week	1.34 (0.68–2.64)		1.58 (0.77–3.24)	
51–100 patients/week	0.92 (0.47–1.80)		1.03 (0.49–2.15)	
>100 patients/weeks	0.78 (0.39–1.53)		1.26 (0.58–2.45)	
Type of hospital		0.008		0.010
Primary/private clinics	Reference		Reference	
Private hospitals	6.67 (3.37–13.18)		4.43 (1.99–9.86)	
Academic centers	3.67 (2.09–6.45)		1.93 (0.92–4.06)	
Tertiary care centers	2.23 (1.27–3.94)		1.80 (0.93–3.46)	
Geographic distribution		0.600		0.764
North	Reference		Reference	
Northeast	0.84 (0.43–1.61)		1.34 (0.64–2.79)	
East	1.29 (0.50–3.32)		1.04 (0.37–2.94)	
Center	1.67 (1.04–2.69)		1.36 (0.79–2.33)	
South	0.83 (0.48–1.45)		1.06 (0.57–1.96)	

Parameters included for multivariate analysis model: physicians' specialty, years of practice, number of patients seen per week, type of hospital and geographic distribution.

TABLE 3 | Univariate analysis of patient-related factors associated with colorectal cancer screening recommendations.

Factor influencing the decision to offer CRC screening	Odds Ratio (95% CI)	*P*-value
Age (yes vs. no)	2.75 (1.61–4.67)	<0.001
Gender (yes vs. no)	0.83 (0.54–1.23)	0.407
Family history of CRC (yes vs. no)	1.40 (0.40–4.91)	0.749
Comorbidities (yes vs. no)	0.89 (0.60–1.33)	0.579
Reimbursement policies (yes vs. no)	1.37 (0.84–2.22)	0.209
Hospital facility (yes vs. no)	1.08 (0.75–1.57)	0.654

CRC, colorectal cancer.

referral or private hospitals. The main factor determining physicians' recommendations was the patient's age. Increasing screening participation rates requires promoting physicians' recommendations and improving physician awareness and adherence to guidelines.

A population-based study in Thais demonstrated that the screening participation rate increased to 63.0% when the primary care providers were required to offer FOBT for CRC screening in their practice (22). The overall rate of physicians' recommendations for CRC screening in our study was 58.2%, increasing from a previous report of 32.7% in 2007 (12). However, the distribution of respondents' area of expertise, workplace, and geography may affect the rate of CRC recommendations in this study. For example, primary care physicians accounted for 13.0% of respondents; therefore, the high rate of screening recommendations may not reflect the practice of the front liners responsible for providing health promotion and screening services. It is worth noting that only about one-third of primary care physicians recommended colon cancer screening even though ∼85.0% of them had access to CRC screening modalities. The study was not designed to explore the reason for this observation, but we hypothesized that the lack of guidelines awareness might be partly responsible for it. Furthermore, the present study showed that 91.0% of colorectal surgeons and 96.0% of gastroenterologists offered screening colonoscopy, a higher than reported number in a previous study (68.0%) (23). Forty-three percent of primary care physicians recommended a colonoscopy, and 60.0% offered FOBT. The data showed that FOBT was the most accessible screening tool for primary care physicians; therefore, it is possible that access to screening tools influenced the physicians' selection of screening methods.

There is a broad variety of options for CRC screening in the average risk population, including stool tests, such as different types of FOBT, fecal DNA testing, double-contrast barium enema, virtual colonoscopy, and endoscopic procedures. Based on a

TABLE 4 | Colorectal cancer screening method by each specialty.

Factors	Primary care physician (*N* = 77)	Internist (*N* = 173)	Resident physician (*N* = 135)	General Surgeon (*N* = 102)	Colorectal Surgeon (*N* = 23)	Gastroent-erologist (*N* = 76)	*P*-value
Screening method, *N* (%)							
FOBT	45 (58.4)	86 (49.7)	57 (42.2)	42 (41.2)	7 (30.4)	27 (35.5)	0.020
CEA	8.4 (10.9)	29 (16.8)	4 (3.0)	17 (16.7)	2 (8.7)	5 (6.6)	0.001
CT colonography	0 (0.0)	8 (4.6)	6 (4.4)	5 (4.9)	5 (21.7)	7 (9.2)	0.003
Sigmoidoscopy and barium enema	12 (1.6)	8 (4.6)	15 (11.1)	26 (25.5)	2 (8.7)	7 (9.2)	<0.001
Sigmoidoscopy	12 (1.6)	9 (5.2)	5 (3.7)	8 (7.8)	1 (4.1)	2 (2.6)	0.420
Colonoscopy	33 (42.9)	100 (57.8)	100 (74.1)	77 (75.5)	21 (91.3)	73 (96.1)	<0.001
Available method, *N* (%)							
No screening tool	11 (14.3)	5 (2.9)	0 (0.0)	2 (2.0)	0 (0.0)	0 (0.0)	<0.001
FOBT	59 (76.6)	156 (90.2)	127 (94.1)	86 (84.3)	20 (87.0)	71 (93.4)	0.003
Sigmoidoscopy	16 (20.8)	78 (45.1)	98 (72.6)	61 (59.8)	15 (65.2)	41 (54.0)	<0.001
Barium enema	31 (40.3)	113 (65.3)	105 (77.8)	75 (73.5)	19 (82.6)	58 (76.3)	<0.001
Colonoscopy	39 (50.7)	133 (76.9)	126 (93.3)	87 (85.3)	23 (100.0)	72 (94.7)	<0.001
CT colonoscopy	16 (20.8)	53 (30.6)	70 (51.9)	33 (32.4)	15 (65.2)	45 (59.2)	<0.001

Data presented as percentage of screening method by each specialty.
FOBT, fecal occult blood test; CEA, carcinoembryonic antigen; CT; computed tomography.

balance between availability, cost, and potential benefits and safety profile, FOBT is the simplest, most non-invasive, and least expensive screening method. Direct comparison of FOBT showed that people were more likely to pursue an iFOBT than a gFOBT (52.7 vs. 43.9%) because iFOBT was easier to use without diet restriction. The study also showed that iFOBT was more superior to gFOBT in detecting advanced neoplasms. The advanced adenoma detection rate of iFOBT was 1.4% and gFOBT was 0.5%. The iFOBT had a sensitivity of 67.0% and a specificity of 85.0%, whereas gFOBT had a sensitivity of 54.0% and a specificity of 80.0% (24). Therefore, the iFOBT is now recommended as the first-option FOBT for CRC screening. For the present study, both FOBT methods were included because both tests were available and were used in our country when the study was conducted.

A meta-analysis showed that FOBT, flexible sigmoidoscopy, and colonoscopy had no differences in all-cause mortality (25). Studies comparing the efficacy between iFOBT and colonoscopy showed that both methods were comparable in terms of CRC detection, but iFOBT was inferior to colonoscopy in detecting non-advanced adenoma and advanced adenoma. However, iFOBT had higher participation compared to colonoscopy and higher acceptance may counter balance its lower detection ability (26, 27). The cost-effectiveness and budget impact analyses revealed that colonoscopy was more cost-effective in a low-and middle-income country, with an Incremental Cost-Effectiveness Ratio (ICER) of United States Dollars (USD) 646.5/Quality-Adjusted Life Year (QALY) gained (28). However, providing a nationwide screening colonoscopy can be a challenge in Thailand due to low numbers of endoscopists, less than 1,000 for an estimated number of cases requiring CRC screening of 14 million (29). Hence, a population-based one-step screening colonoscopy program is less likely to succeed in resource-limited countries with a shortage of endoscopists. A two-step approach employing FOBT to select average-risk people for colonoscopy is another appropriate CRC screening strategy. The usage of FOBT is encouraging since a screening uptake study revealed that the rural population has easier access to non-invasive screening tests through their primary care physicians (30). However, a population-based study on CRC screening strategy showed that 28.0% of patients with positive FOBT did not undergo colonoscopy (22).

TABLE 5 | Comparison of appropriate responses to clinical vignettes between the gastroenterologists and the colorectal surgeons based on guidelines adherence.

Clinical vignette question	Gastroenterologist *N* (%)	Colorectal Surgeon *N* (%)	*P*-value
Surveillance interval following normal colonoscopy in average-risk patients	48/75 (64.0)	36/112 (32.1)	<0.001
Surveillance interval following normal colonoscopy in high-risk patients	50/74 (67.6)	43/112 (38.4)	<0.001
Surveillance interval following complete endoscopic resection of villous adenoma or tubular adenoma (larger than 1 cm)	40/74 (54.1)	30/112 (26.8)	<0.001
Surveillance interval following resection of hyperplastic polyps in recto-sigmoid region (smaller than 1 cm)	15/75 (20.0)	5/112 (4.5)	0.001

Data presented as a percentage of appropriate response among responders of each specialty.

Raising public awareness of CRC and altering people's attitudes and beliefs is crucial to impact the local community in this major health issue. Participation rates in CRC screening were greater when educational pamphlets were distributed to participants (31–33). Another strategy for increasing CRC

screening rates is for clinicians to offer either FOBT or endoscopic procedures if the patient prefers one type of test over the others (34–36). This finding underscores the necessity of providing various screening options whenever available to reach the greatest number of patients.

One pitfall in the choice of the modality of CRC screening was that 10.0–16.0% of primary care physicians, internists, and general surgeons advocated blood CEA level as a screening method. Serum CEA has low sensitivity for diagnosing and screening CRC; therefore, it should not be recommended as a screening tool. Also, serum CEA levels can be elevated in various benign conditions and most types of adenocarcinoma, including breast, gastric, lung, and pancreatic cancers (37–39). This finding is a setback that would hinder Thailand's efforts to improve CRC screening programs.

Several studies have explored the factors that influence physicians' recommendations and screening adherence. Patient-related factors such as age, race, and sex have been demonstrated to affect the selection of screening modalities (e.g., colonoscopy vs. FOBT) (40–43). Physicians were less likely to offer screening to patients with chronic conditions, low levels of education, and poor socioeconomic status (40–44). The present study showed that the only patient-related factor influencing the physicians' decision to offer CRC screening was age. Physicians' implementation of CRC screening was unaffected by other demographics, family history of CRC, or comorbid illnesses.

The physicians' adherence to the practice guidelines was also assessed. For interval surveillance colonoscopy following colonoscopy with polypectomy (45, 46), gastroenterologists were more likely to adhere to the guidelines than colorectal surgeons. Both specialties preferred early surveillance colonoscopy for small hyperplastic polyps in the recto-sigmoid region. Shorter interval surveillance colonoscopy may result in unnecessary colonoscopies and increased healthcare costs. This finding reinforces the need to tailor surveillance colonoscopy in order to promote optimal CRC screening utilization.

A strength of our study is that it was a nationwide survey that included all specialties who participate in the national CRC screening program. Also, large samples (586 physicians) responded. The outcomes provided many instances of the drawbacks mentioned earlier or inadequacies, which could be eliminated to help improve the national CRC screening strategy. However, a known limitation of the survey study design is that our survey responses may not reflect the respondents' actual practices or attitudes. More than half of the respondents worked in the central part of the country, indicating that these practitioners were representative of urban rather than rural area healthcare professionals. Also, the question concerning when to start screening (Item 2.3) can be difficult to answer directly because the information about family history of CRC was not provided. Asymptomatic individuals who have a first-degree relative with CRC diagnosed at a young age would require earlier surveillance. Lastly, the questionnaire did not differentiate iFOBT from gFOBT to determine whether the type of FOBT influenced the decision to offer CRC screening.

In conclusion, recommendations for CRC screening and awareness of the guidelines varied among different specialists. The necessity for CRC screening should be emphasized among primary care physicians. The factors that influence colorectal surgeons' and gastroenterologists' lack of adherence to CRC guidelines in small hyperplastic rectal polyps should be explored, and the need for proper interval surveillance colonoscopy should be underscored.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The study protocol conformed to the Ethical Guidelines of the 1975 Helsinki Declaration, and it was approved by the Siriraj Institutional Review Board (COA no. Si448/2015). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

NP: conception and supervision. NP and PC: methodology and writing – review and editing. NP, PT, TG, and PC: formal analysis. PT: data curation. PT and TG: writing – original draft preparation. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.847361/full#supplementary-material

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