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GUIDELINES

International experts consensus guidelines on robotic liver resection in 2023

Rong Liu, Mohammed Abu Hilal, Go Wakabayashi, Ho-Seong Han, Chinnusamy Palanivelu, Ugo Boggi, Thilo Hackert, Hong-Jin Kim, Xiao-Ying Wang, Ming-Gen Hu, Gi Hong Choi, Fabrizio Panaro, Jin He, Mikhail Efanov, Xiao-Yu Yin, Roland S Croner, Yu-Man Fong, Ji-Ye Zhu, Zheng Wu, Chuan-Dong Sun, Jae Hoon Lee, Marco V Marino, Iyer Shridhar Ganpati, Peng Zhu, Zi-Zheng Wang, Ke-Hu Yang, Jia Fan, Xiao-Ping Chen, Wan Yee Lau

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Abstract

The robotic liver resection (RLR) has been increasingly applied in recent years and its benefits shown in some aspects owing to the technical advancement of robotic surgical system, however, controversies still exist. Based on the foundation of the previous consensus statement, this new consensus document aimed to update clinical recommendations and provide guidance to improve the outcomes of RLR clinical practice. The guideline steering group and guideline expert group were formed by 29 international experts of liver surgery and evidence-based medicine (EBM). Relevant literature was reviewed and analyzed by the evidence evaluation group. According to the WHO Handbook for Guideline Development, the Guidance Principles of Development and Amendment of the Guidelines for Clinical Diagnosis and Treatment in China 2022, a total of 14 recommendations were generated. Among them were 8 recommendations formulated by the GRADE method, and the remaining 6 recommendations were formulated based on literature review and experts' opinion due to insufficient EBM results. This international experts consensus guideline offered guidance for the safe and effective clinical practice and the research direction of RLR in future.

Key Words: Robotic liver resection; Laparoscopic liver resection; Guidelines; Expert consensus

Core Tip: The robotic liver resection (RLR) has been increasingly applied in recent years. Based on the foundation of the previous consensus statement, this new consensus guideline document aimed to update clinical recommendations and provide guidance to improve the outcomes of RLR clinical practice. The guideline steering group and guideline expert group were formed by 29 international experts of liver surgery and evidence-based medicine. Relevant literature was reviewed and analyzed by the evidence evaluation group. According to the WHO Handbook for Guideline Development, the Guidance Principles of Development and Amendment of the Guidelines for Clinical Diagnosis and Treatment in China 2022, a total of 14 recommendations were generated.

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INTRODUCTION

Laparoscopic liver resection (LLR) was first introduced in the 1990s for treatment of benign and malignant tumors[1,2]. Over the past few decades, laparoscopic hepatectomy has developed rapidly and gained widespread acceptance around the world. In 2008, the Louisville Statement indicated the feasibility of laparoscopic hepatectomy[3], and the second international consensus conference in Morioka recommended LLR as a standard practice in specific types of hepatectomy [4]. After that, the Southampton consensus guidelines promoted the safe expansion of LLR and improved patient care after LLR[5]. Since then, laparoscopic techniques have occupied an important place in hepatobiliary surgery. At the same time, with the approval of clinical application and technique developments of robotic surgery systems, robotic hepatectomy has gradually been used in clinical practice. Experience in robotic liver resection (RLR) has been reported in many parts of the world and its feasibility has gradually been identified by clinical studies[6-8]. The robotic system is superior in providing three-dimensional magnified field of vision and robotic arms offer flexibility and tremor filter to surgeons to overcome the shortages of conventional laparoscopic hepatectomy. However, the high cost of the mainstream models and the lack of surgical instruments and experience limit the application of RLR.

With the advent of advanced technique and with experience accumulation, the indications of RLR have been expanded. The robotic system can be used in almost all types of liver resection, including minor hepatectomy, major hepatectomy, donor hepatectomy, and complex liver resections[9-16]. In 2018, an international consensus statement on robotic hepatectomy provided recommendations for RLR based on relevant research and experts' opinions, which contribute to standardization of robotic hepatectomy[17]. However, the recommendation grades were relatively low due to the lack of high-quality evidence. Furthermore, some important issues, like cost-effectiveness of RLR, learning curve of RLR, and outcomes of RLR for difficult liver segments, remain controversial. Available data derive from case series, case-comparative studies, reviews and meta-analyses have been published in recent years, and some new theories also provide new views and perspectives on RLR[18-25].

Based on the foundation of the previous statement, the 2023 international consensus statement on RLR aimed to update clinical recommendations and provide guidance to improve the outcomes of RLR clinical practice. We invited a team of robotic surgeon experts to provide their views related to RLR. Based on the related topics, we searched the online databases for RLR studies and the GRADE system was used to grade the evidence. The final consensus was reached by using a combination of clinical evidence and experts' opinions with the aim to improve the clinical outcome of RLR.

METHODS

This international evidence-based guideline is based on the WHO Handbook for Guideline Development and refers to the Guidance Principles of Development and Amendment of the Guidelines for Clinical Diagnosis and Treatment in China 2022. Reporting of the guideline follows the Appraisal of Guidelines, Research, and Evaluation (AGREE II)[26]. A flow chart describes the process and steps of the guideline development (Figure 1).

The guideline development group included the Steering Committee, Consensus Expert Group, and Evidence Evaluation Group. The Steering Committee and Consensus Expert Group consisted of 29 members from different countries or regions. Literature review, questionnaires, and expert discussions were implemented to initially identify clinical questions. All members of the Steering Committee and Consensus Expert Group were encouraged to submit suggestions and add potential questions. Each question was then evaluated and confirmed by the Steering Committee through meetings or emails. The Evidence Evaluation Group performed a literature search, literature screening, method quality evaluation, and data extraction, and thus formed the body of evidence.

PubMed, Cochrane Library, Web of Science, Embase, Scottish Intercollegiate Guidelines Network, WHO, National Guideline Clearing-house, Guidelines International Network, and National Institute for Health and Care Excellence

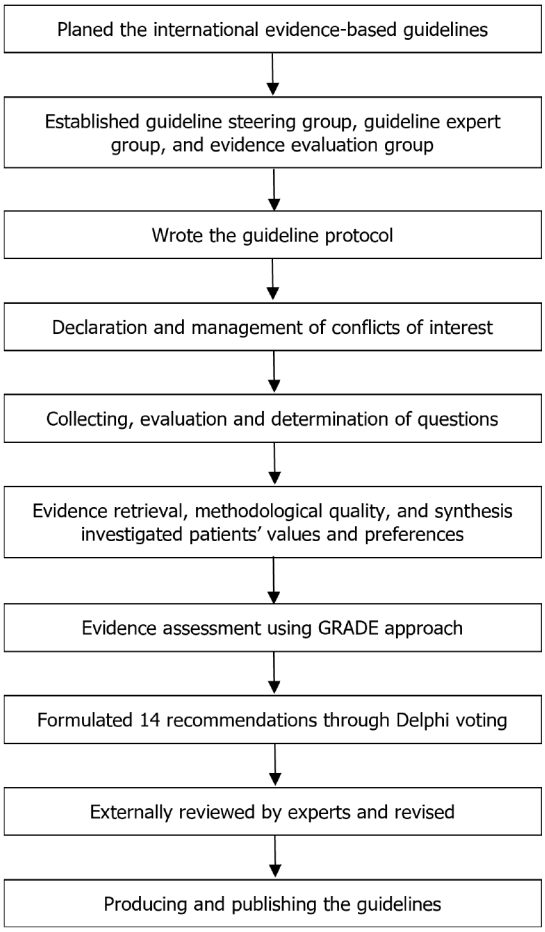

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Figure 1 The flow chart illustrates the process of guideline development.

databases were systematically searched from inception to September 5, 2022, to retrieval potential eligible studies. There were no regional and language restrictions, but it was limited to human studies. The Evidence Evaluation Group evaluated titles, abstracts, and full texts to identify the eligible publications based on inclusion and exclusion criteria approved by the Steering Committee. The eligible literature included clinical guidelines, systematic reviews and meta-analyses, randomized controlled trials (RCT), non-RCT, cohort studies, case-comparative studies, and case reports or case series that were considered for inclusion when necessary. Information on the included studies was extracted according to a pre-designed extraction form. A Measure Tool to Assess Systematic Reviews (AMSTAR II) was used to evaluate the methodological quality of the included systematic reviews and meta-analyses[27]. The revised Cochrane Risk of Bias Tool and the Newcastle-Ottawa Scale were performed to assess the methodological quality of their corresponding types of clinical studies[28,29]. All the above processes were completed by two independent terms of the Evidence Evaluation Group. If there are differences, they will be resolved by discussing or consulting third parties.

According to the WHO Handbook for Guideline Development, the Evidence Evaluation Group evaluated the need for an update or rapid development of systematic review[30]. The method of GRADE was used to assess the quality of the evidence[31]. Based on the GRADE approach, clinical outcomes, patients' benefit, and economic evaluation, the Steering Committee and Consensus Expert Group used the GRADE decision form and Delphi voting to formulate the strength of recommendations. The consensus was considered to have been reached when 80% of the experts approved a proposal. Before submitting the manuscript for publication, the final draft was reviewed and approved by all members of the guideline development group. This evidence-based guideline is expected to be updated again in 2026.

RESULTS

Question 1: What are the indications of RLR?

In general, the indications of the RLR are similar to those of LLR and open liver resection (OLR). RLR can be applied for treating various liver diseases, including hepatocellular carcinoma (HCC), intrahepatic cholangiocarcinoma (ICC), colorectal liver metastases (CRLM), benign tumors of liver, and living donor hepatectomy. RLR is associated with the following advantages over OLR and LLR in the majority of the currently available studies: less intraoperative blood loss, shorter postoperative hospital stay, less overall complications, and lower pain intensity after surgery[21,22,32-45].

However, in complex hepatectomies such as large tumor size or proximity of tumor to vital vascular structures, RLR should be performed with caution and by highly experienced surgeons (clinical recommendation: Expert agreement 100%).

In a prospective study using propensity score matching, Zhu *et al*[21] compared the short-term perioperative outcomes and long-term survival outcomes of RLR, LLR, and OLR in patients with BCLC stage 0-A hepatocellular carcinoma. The results showed that there were no significant differences among the three interventions except for a shorter hospital stay in the minimally invasive group (RLR and LLR)[21]. Hu *et al*[33] evaluated the clinical efficacy of robotic, laparoscopic, and open liver resection for giant liver haemangiomas (> 10 cm in diameter). They reported robotic hemihepatectomy to be associated with less intraoperative blood loss, better postoperative recovery and lower pain score than OLR. When compared with laparoscopic hemihepatectomy, robotic hemihepatectomy was associated with significantly less intraoperative blood loss and a shorter operative time[33]. Masetti *et al*[46] analyzed the short-term outcomes of patients with CRLM comparing from a multicenter study who underwent RLR *vs* LLR. The results showed RLR and LLR to be comparable in postoperative overall complication rates, intraoperative blood loss, conversion rates, operation time, and hospital stay, but RLR showed a reduced rate of R1 resection margins compared with LLR. Masetti *et al*[46] found the effect size of RLR to be increased for posterosuperior lesions and difficult procedures, which may be due to RLR being able to offer some technical advantages over conventional laparoscopy to improve the short-term outcomes of these patients.

Question 2: Is RLR safe and effective in patients with HCC?

Recommendation: RLR is safe and feasible for HCC, as it is associated with lower overall complication rates than LLR and OLR and a shorter hospital stay than OLR, although it has a longer operative time than LLR and OLR. Other perioperative outcomes are comparable among the three interventions. Regarding oncologic outcomes, limited evidence suggested there is also no significant difference.

Level of evidence: Low level of recommendation: Weak (Grade 2C). Expert agreement: 96.55%.

Two systematic reviews[47,48] and 15 publications[21,35,37,43,49-59] were included to evaluate the perioperative and oncologic outcomes of robotic *vs* laparoscopic or open hepatectomy for treatment of HCC patients, respectively. When compared with LLR for HCC patients, the pooled results indicated that RLR had a lower overall complications rate (RR = 0.72, 95%CI: 0.57 to 0.90; P = 0.003), lower minor complication (Clavien-Dindo I-II) rate (RR = 0.72, 95%CI: 0.54 to 0.96; P = 0.030), and longer operative time (SMD = 0.66, 95%CI: 0.22 to 1.10; P = 0.003)[21,50,52-57]. When compared with OLR for HCC patients, RLR was associated with a shorter hospital stay (SMD = -0.42, 95%CI: -0.57 to -0.28; P < 0.00001), lower overall complication rate (RR = 0.61, 95%CI: 0.45 to 0.84; P = 0.002), lower minor complication rate (RR = 0.59, 95%CI: 0.42 to 0.84; P = 0.003), and longer operative time (SMD = 0.82, 95%CI: 0.18 to 1.46; P = 0.01)[21,35,37,43,49,51,58,59]. For other outcomes, RLR showed similar results when compared with LLR or OLR, including estimated blood loss, transfusion rate, severe complication (Clavien-Dindo III-IV), and 90-d mortality. Thus, RLR was comparable to OLR and LLR in feasibility and safety for patients with HCC.

Regarding oncologic outcomes, the three interventions showed comparable R0 resection rates and similar short- and long-term oncological outcomes[21,35,37,43,49,51,53-56,58,59]. Also, a recently published study with a large sample size suggested that robotics may be associated with improved overall survival[54]. The study reported that the RLR group had significantly higher 1- , 3- , and 5- year overall survival rates of 92%, 75%, and 63% compared with the LLR group of 86%, 60%, and 45%, respectively (all P < 0.01)[54]. Based on the currently available studies, the implementation of RLR is feasible and safe and is associated with similar oncologic outcomes. Prospective studies are recommended to further evaluate whether the safety and efficacy of RLR can be affected by tumor size, resection complexity, and the quality of the underlying liver parenchyma.

Question 3: Is RLR safe and effective in patients with ICC?

Recommendation: Currently, there is insufficient evidence to compare the safety and feasibility between RLR and LLR for treatment of ICC. Limited evidence suggests that RLR has less intraoperative blood loss, shorter hospital stay, and better overall survival than OLR.

Level of evidence: Very low. Level of recommendation: Weak (Grade 2D). Expert agreement: 86.21%.

Two comparative studies were reported to evaluate the safety and feasibility of robotic *vs* open hepatectomy for ICC [36,60]. The pooled results suggested that RLR had less intraoperative blood loss (SMD = -0.75, 95%CI: -1.46 to -0.05; P = 0.04) and shorter hospital stay (SMD = -0.31, 95%CI: -0.54 to -0.09; P = 0.006) than OLR[36,60]. There were no significant differences in other perioperative outcomes between the two groups. Limited evidence suggests RLR may improve the outcomes of ICC when compared to OLR, but we need to carefully interpret these findings. Shapera *et al*[60] suggested that patients after RLR or OLR had similar resection margins, and median overall survival was similar in patients with any resection margin distance. Hamad *et al*[36] compared short- and long-term outcomes between RLR and OLR for patients with ICC, and they reported that patients who underwent RLR had shorter hospital stays and similar long-term risk of death between the two groups. These results suggested RLR for ICC could shorten hospital stay without compromising oncological outcomes such as negative margins, postoperative mortality, and long-term survival[36]. As there have been few comparative studies focusing on ICC and there is no meta-analysis on the short- and long-term clinical outcomes of RLR *vs* LLR or OLR for ICC, higher levels of evidence are urgently needed to answer this question.

Question 4: Is RLR safe and effective in patients with CRLM?

Recommendation: RLR is safe and feasible for patients with CRLM, since it is associated with a lower conversion rate but

longer hospital stay than that of LLR. Limited evidence suggests no significant difference in all the perioperative outcomes between RLR and OLR in patients with CRLM. Oncologic outcomes with limited evidence suggested there was also no significant difference between RLR *vs* LLR and RLR *vs* OLR.

Level of evidence: Very low. Level of recommendation: Weak (Grade 2D). Expert agreement: 89.66%.

Studies on RLR *vs* LLR or OLR for CRLM have been extensively published in the past three years[45,46,61-63]. The evidence suggested RLR to have a lower conversion rate (RR = 0.42, 95%CI: 0.23 to 0.77; P = 0.005) and longer hospital stays (SMD = 0.19, 95%CI: 0.03 to 0.35; P = 0.020) than LLR for patients with CRLM, and there were no significant difference in operation time, estimated blood loss, intraoperative blood transfusion rates, R0 resection, postoperative morbidity, and overall survival[45,46,61,62]. In addition, limited evidence showed there were no significant differences in operation time, estimated blood loss, R0 resection, hospital stays, overall complications, minor and major complications, postoperative 30-d mortality, and survival outcomes between RLR and OLR for patients with CRLM[61,63]. With appropriate expertise and experience, robotic-assisted surgery can be considered to be an alternative minimally invasive approach to CRLM resections[64,65].

Currently, there is insufficient evidence on patients who underwent simultaneous robotic-assisted resections of CRLM and the primary tumor. A conference abstract on one RCT in comparing RLR and OLR for simultaneous resections suggested a longer operating time in the robotic arm, and patients who underwent RLR had less blood loss, less Clavien-Dindo III-IV complications, a shorter time to pass first flatus, and a shorter hospital stay when compared to open surgery [40]. A recent systematic review reported that all patients who underwent robot-assisted R0 resection had no perioperative deaths[65]. However, when patients who underwent simultaneous major hepatectomy combined with complex colorectal surgery, the operative risks were increased, leading to poorer perioperative outcomes with increased length of stay, morbidity, and mortality, and unfavorable patient recovery due to delay in the initiation of subsequent adjuvant therapy[5,65].

Question 5: Is robot approach safe and feasible for living donor hepatectomy?

Recommendation: Robotic living donor hepatectomy can be a safe and feasible alternative to open and laparoscopic approach. Robotic living donor hepatectomy has a longer operative time than that of OLR and LLR, but a shorter hospital stay compared with OLR. The other donor and recipient outcomes were reported to be comparable among the three interventions.

Level of evidence: Very low. Level of recommendation: Weak (Grade 2D). Expert agreement: 96.55%.

We systematically reviewed 4 studies that investigated the safety and feasibility of robotic and open living donor hepatectomy, with a total of 972 patients being included in the meta-analysis[41,66-68]. The pooled results suggested that RLR had lower postoperative peak serum bilirubin (SMD = -0.59, 95%CI: -0.81 to -0.37; P < 0.0001), shorter postoperative hospital stays (SMD = -0.53, 95%CI: -0.90 to -0.17; P = 0.004) and a longer operative time (SMD = 1.45, 95%CI: 0.66 to 2.25; P = 0.003) compared to the open group[41,66-68]. There were no significant differences in terms of other donor and recipient outcomes. Rho *et al*[41] evaluated the clinical and perioperative outcomes of robotic living donor right hepatectomy from carried out on 52 consecutive cases patients and compared with patients who underwent comparison with open (n = 62) and laparoscopic (n = 118) donor hepatectomy. They reported that although RLR was associated with a longer operation time, the mean estimated blood loss was significantly lower compared with LLR and OLR, and donor satisfaction (body image and cosmetic appearance scores) was higher in RLR than that of LLR[41].

Large incisions resulting in large scars are an ongoing concern for surgeons performing open living donor hepatectomy. For robotic living donor hepatectomy, there is a better body image, improved cosmetic appearance, and fewer wound-related complications[41]. Living donor hepatectomy is considered to be the pinnacle of hepatobiliary surgery, which requires assurance of donor survival, good graft status and minimization of associated complications[66]. Based on the currently available studies, RLR reduced postoperative pain, resulted in rapid donor recovery and with similar postoperative complications[41,66-68]. However, studies comparing RLR with LLR are insufficient[41]. In summary, RLR for living donor hepatectomy is feasible and safe when performed by surgeons with both excellent knowledge of liver anatomy and long experience of open living donor hepatectomy[41,66]. However, biliary complications are a problem that should never be ignored. The combination of robotic-assisted procedures and indocyanine green (ICG) fluoroscopy is recommended for precise division and fine suturing of the divided bile ducts[41,66].

Question 6: Is robotic approach safe and feasible for minor hepatectomy?

Recommendation: For minor hepatectomy, the safety and feasibility of RLR are comparable to that of LLR and OLR. Robotic minor hepatectomy was reported to have a longer operative time than LLR, but there was less overall complication. RLR resulted in a shorten hospital stay and decreased overall morbidity compared to the open approach. The other perioperative outcomes were comparable among the three interventions.

Level of evidence: Low. Level of recommendation: Weak (Grade 2C). Expert agreement: 96.55%.

Minor hepatectomy with fewer than 3 adjacent hepatic segmental resections is the most commonly carried out procedure in RLR. As a minimally invasive surgery, the benefits of the robotic approach for minor hepatectomy have been controversial[36,37,49,51,69-72]. An updated meta-analysis based on new evidence was conducted by the Evidence Evaluation Group on 13 retrospective cohort studies with 735 robotic and 1362 laparoscopic minor hepatectomies[12,22,34,53,73-81]. The results showed that the operative time was significantly longer (SMD = 0.44, 95%CI: 0.19 to 0.69; P = 0.0006) with the robotic approach, but it had less overall complication (RR = 0.76, 95%CI: 0.61 to 0.95; P = 0.01) than LLR. No statistically significant differences were observed in estimated blood loss, conversion rate, blood transfusion rate,

postoperative hospital stays, 90-d mortality, and R0 resection. Based on the currently available studies and expert opinions, RLH was associated with a longer operation time when compared with LLH because of the additional time required to dock and undock the robot, and the large extent to exchange instruments required by the robot-assisted procedures[34,69,70].

We also systematically reviewed 6 studies to investigate the safety and feasibility between RLR and OLR[9,38,71,73,82,83]. The results showed that robotic minor hepatectomy had a shorter postoperative hospital stay (SMD = -0.91, 95%CI: -1.27 to -0.55; P < 0.00001), less overall complications (RR = 0.39, 95%CI: 0.23 to 0.64; P = 0.0002), and less severe complications (RR = 0.28, 95%CI: 0.08 to 0.97; P = 0.04) when compared to open surgery. There were no significant differences in terms of operation time, estimated blood loss, intra-operative blood transfusion rate, and readmission rate, and R0 resection for malignancy between robotic and open minor hepatectomies. Liver resections for difficult-located segments (1, 4a, 7, and 8) are recommended to be defined as technical major hepatectomy.

Question 7: Is robotic approach safe and feasible for major hepatectomy?

Recommendation: For major hepatectomy, robotic hepatectomy is as safe and feasible as laparoscopic and open hepatectomy. Compared with LLR, RLR was significantly better in estimated blood loss and conversion rate. In comparison with OLR, the estimated blood loss and hospital stay of RLR are significantly better than those of OLR, but there is a longer operation time in the RLR group.

Level of evidence: Low. Level of recommendation: Weak (Grade 2C). Expert agreement: 93.10%.

Chong *et al*[32] compared the efficacy and safety of 989 individuals, including 220 who underwent robotic and 769 who underwent laparoscopic right and extended right hepatectomy (RH/ERH). They reported a lower open conversion rate and a shorter postoperative hospital stay for robotic RH/ERH. We systematically reviewed 10 studies that investigated the safety and feasibility of robotic and laparoscopic major hepatectomy[22,32,33,39,84-89]. The results showed that robotic major hepatectomy resulted in less estimated blood loss (SMD = -0.52, 95%CI: -0.88 to -0.16; P = 0.005) and a lower conversion rate (RR = 0.44, 95%CI: 0.29 to 0.67; P = 0.0001) compared to laparoscopic surgery. There were no significant differences between robotic and laparoscopic major hepatectomies in transfusion rate, R0 resection, readmission, mortality within 90 d, overall complications, mild/severe complication, bile leakage and liver failure rates.

Hamad *et al*[36] investigated 1876 patients who underwent open (n = 1804) and robotic assisted (n = 72) resection between 2004 and 2017. The results showed that the patients who underwent RLR had a shorter length of hospital stays yet there was no difference in 30-d readmission or 90-d mortality[36]. Lee *et al*[43] matched 36 patients each in the robotic and open group. They found that operative time was significantly longer but the postoperative hospital stays was significantly shorter in the robotic group[43]. An updated meta-analysis based on the latest evidence was conducted by the Evidence Evaluation Group on 8 retrospective cohort studies. The pooled results suggested the estimated blood loss and hospital stay of the robotic group to be significantly shorter than the open group, but there was a longer operation time in the robotic group[22,33,37,43,49,51,71,72].

We also focused on the safety and feasibility of RLR in technically "major" resections (segment 1, 4a, 7, and 8). Liver resection on segments 1, 4a, 7, and 8 is relatively more technically difficult owing to a large parenchymal transection plane with proximity to critical structures and major vessels[90-92]. RLR on difficult liver segments has been reported to be a safe and feasible procedure, and many experienced surgeons tend to perform robotic resections on these selected patients. In a recent international multicenter retrospective study comparing robotic *vs* laparoscopic right posterior sectionectomy, the author reported RLR to be associated with reduced blood loss and lower open conversion rates than LLR, and suggested that RLR and LLR could be performed in expert centers with good outcomes in well selected patients [93]. A study reported a patient with hepatocellular carcinoma which involved segments 4 and 8 to undergo robotic central bisectionectomy. The outcomes indicated that robotic central bisectionectomy could be performed safely using proper exposure techniques and an appropriate combination of several useful technical tips[16]. A multicenter study reported that robotic right posterior sectionectomy could be performed in expert centers with less blood loss and lower conversion rates in well-selected patients[93,94]. Another study indicated that RLR on segments 1, 4a, 7, and 8 showed similar surgical outcomes, including blood loss, hospital stay, R0 negative margin rate, and morbidity, when compared with laparoscopic liver resection[95]. The application of robotic approach has also been reported for caudate lobectomy, including Spiegelian lobectomy, isolated partial and complete caudate lobectomy[96,97]. However, most studies reported only a few cases with the potential of selection bias, thus limiting the application of the results. It is necessary to conduct high-quality clinical studies in the future to further clarify the impact of robotic hepatectomy on difficult liver segments.

Question 8: Is RLR more cost-effective than LLR and OLR?

Recommendation: As the policy on medical expense and definition of cost are different in the literature, the real cost of three interventions should be calculated and compared based on a standard method in the future. Limited evidence suggests the total cost of RLR to be higher than LLR, but there were no significant differences between RLR and OLR. The cost-effectiveness of the three interventions should be synthetically evaluated based on many factors, including direct and indirect costs, hidden benefits from favorable clinical outcomes and local social and economic situations.

Level of evidence: Very low. Level of recommendation: Weak (Grade 2D). Expert agreement: 89.66%.

The most common concern about cost-effectiveness analysis of RLR is simply on the total cost, hospitalization costs, and readmission costs. One systematic review and meta-analysis and nine additional updated original studies were included to answer this question[9,34,42,84,98-103]. The resynthesized evidence suggests that the total costs (SMD = 1.15, 95%CI: 0.24 to 2.07; P = 0.01) and hospitalization costs (SMD = 0.96, 95%CI: 0.50 to 1.41; P < 0.0001) of RLR is higher compared to LLR[34,42,75,78,104]. However, no statistically significant differences were observed regarding total costs,

hospitalization costs, and readmission costs between RLR and OLR[9,42,44,68,101]. In addition, total costs were assessed for different type of liver resection. For the included studies which investigated RLR *vs* LLR, these studies were more focused on the cost-effectiveness in minor liver resections, and the results showed that RLR had higher total costs than LLR (SMD = 1.22, 95%CI: 0.60 to 1.83; $P < 0.0001$)[34,75,78]. When various types of pathologies in patients were included in the comparison between RLR and OLR, including complex hepatolithiasis, donor right hepatectomy, minor and major resections, these confounders were introduced to become an important reason to decrease the level of evidence[9,44,68,103]. According to the currently available studies, even though the higher total costs of RLR were closely associated with more operating room supplies[34,68,73,79,80,84], the higher total costs were mainly due to longer hospital stays and more treatment of complications in the laparoscopic and open group than the robotic group[9,71,100,105,106]. The favorable perioperative outcomes in RLR should be weighed against the higher operative costs when compared with laparoscopic or open approaches[98].

Robotic surgical systems have evolved rapidly in recent years, and the trends in the total cost and operative cost are worth future investigating. Although the costs of RLR may differ among countries or regions due to differences in medical policy or insurance premium, a serious obstacle to the widespread use of robotic surgical system is the large indirect cost[34,78-80]. Furthermore, a comprehensive evaluation of the cost-effectiveness should also consider the hidden benefits from favorable clinical outcomes associated with robotic hepatectomy to include patient's psychological benefits and social benefits.

Question 9: What is the role of RLR for cirrhotic patients?

In the setting of cirrhotic patients, similar to LLR, RLR could also be performed in to selected patients. Currently, there are insufficient studies focusing on the application of RLR on cirrhotic patients (clinical recommendation: Expert agreement 100%).

The improvement of surgical techniques, perioperative management, and patient selection improved the surgical outcome of patients with cirrhosis, and the overall improvement of surgical techniques as represented by the minimally invasive approach improved the outcomes of perioperative management in patients with cirrhosis[107-109]. Minimally invasive hepatectomy for cirrhotic patients has been reported to be associated with less complications, shorter postoperative hospital stay, and similar outcomes in operative time, estimated blood loss and postoperative complications when compared with non-cirrhotic patients, and laparoscopic surgery may provide potential benefits in reducing the incidence of postoperative ascites and liver failure[5,110]. Only a few patients were included in these studies and the outcome of robotic hepatectomy in patients with cirrhosis were not evaluated separately[47]. It is necessary to conduct more research on RLR for liver cancer patients with cirrhosis to further evaluate the safety and efficacy of the robotic approach.

Question 10: What is the role of RLR for lesions located close to major vascular and biliary structures?

For lesions located close to major vascular and biliary structures, especially for deeply located lesions, parenchyma-sparing liver resection should be performed by using the robotic approach to rely on the delicate dissection offered by the stable and flexible movements of the robotic arms, as an alternative approach to major liver resection. Compared to robotic major liver resection, robotic parenchyma-sparing liver resection could potentially increase resectability of these lesions. However, as this is a technically demanding procedure, it should be performed by experienced surgeons on well-selected patients (clinical recommendation: Expert agreement 100%).

For malignant liver lesion with a small tumor size but in close proximity to important vessels such as the hepatocaval confluence, or the bifurcation of the primary or secondary Glissonean pedicles, the conventional approach is to use major resection to remove the vessel in its entirety along with the liver tissues it supplies or drains. However, in patients with a poor liver function and insufficient liver remnant volume, the safety of major resection is difficult to ensure, resulting in a reduced operative resection rate. Parenchyma-sparing liver resection addresses this dilemma to some extent. Removal of the tumor off the adjacent vascular structures or combined vascular resection and reconstruction could increase the resection rate of the tumor. In contrast to the laparoscopic approach with its intrinsic drawbacks including the fulcrum effect and rigid instruments, the robotic system could offer stable and flexible movements of the instruments, which facilitate the operative procedures to achieve a higher level of difficulty. RLR for treatment of centrally located lesions has been reported and shown to be safe and feasible in small case series[111,112]. In addition, with the help of intraoperative ultrasound, it has been shown that the robotic approach allowed optimal access to all liver segments and facilitated parenchymal-sparing surgery for lesions located in the posterosuperior segments or in contact with main liver vessels, not only for colorectal liver metastasis but also for HCC[113]. Robotic parenchyma-sparing liver resection for lesions located close to major vascular and biliary structures allows for sufficient liver remnant to be retained, thus allowing patients to start their postoperative adjuvant therapy early, which is important in improving patients' survival in today's rapidly evolving of comprehensive treatment.

Question 11: What is the role of robotic approach for associating liver partition and portal vein ligation for staged hepatectomy?

Robotic first- or second-stage associating liver partition and portal vein ligation for staged hepatectomy (ALPPS) is an optional strategy for treatment of primary and metastatic liver cancer in patients with insufficient residual liver volume. Due to the complexity of ALPPS surgery and the high morbidity rate, the benefit of robotic ALPPS is unclear on the curative effect of the initially unresectable liver cancer, as there have been rapidly evolving developments in locoregional and systemic therapies. Robotic ALPPS must be evaluated with caution before operation and should only be performed in highly selected patients (clinical recommendation: Expert agreement 100%).

ALPPS has been introduced to treat patients with advanced liver tumors and insufficient residual liver volumes for more than 10 years. However, there is still a lot of controversies on the value of this approach due to its high mortality rate[114,115]. Minimally invasive approach for ALPPS has been applied for the purpose of reducing mortality and improving recovery of patients. Most of the ALPPS procedures were used for the first-stage[116]. A systematic review of 27 patients who underwent ALPPS reported the potential benefit of using the minimally invasive ALPPS in reducing morbidity and mortality[117]. However, there was selective bias in this study and the sample size was small, which compromised the robustness of the conclusions. Previous studies with small case numbers showed that the robotic approach could be used for the first-stage ALPPS for HCC, CRLM and ICC[118-120]. Complex robotic ALPPS procedures (*e.g.*, robotic ALPPS for patients with portal vein thrombosis, robotic ALPPS with simultaneous left colectomy) have also been reported to be safe and feasible carried out by experienced surgeons[121-124]. After the long period of development of ALPPS and its modified approaches, the role of ALPPS should be revisited due to its complexity in treating patients with advanced stage of malignancy and the high morbidity rate, especially when facing the major developments in locoregional and systemic therapies[125-128]. Although the robotic ALPPS is technically feasible, the oncological and health economic benefits are still unclear. The efficacy of robotic ALPPS still needs to be evaluated through high-quality clinical researches.

Question 12: Could the robotic approach shorten the learning curve of liver resection?

The case number required to surmount the learning curve for RLR has been reported to be lower than that for LLR. The case number required to surmount the learning curve of RLR varied among different studies[12,32,129-134]. The surgeons' experience in LLR could have a significant influence on the learning curve of RLR. About 25 consecutive cases are needed for an experienced surgeon to surmount the learning curve of major RLR and 15 cases for minor RLR (clinical recommendation: Expert agreement 100%).

Because of the enhanced surgical dexterity offered by the robotic system, robotic hepatectomy may have a shorter learning curve compared to laparoscopic hepatectomy. However, the large experience in LLR surgeons had before embarking on robotic liver surgery could have an impact on the learning curve[100,135]. Chua *et al*[136] reviewed the literature reported up to July 2019 pertaining to the learning curves in minimally invasive hepatectomy (MIH) by searching PubMed and Scopus databases. Forty studies were included to explore quantitatively the learning curve for MIH. They reported the case number required to surmount the learning curve for RLR was 25 consecutive cases (range: 16-50)[136].

Liu *et al*[131], by analyzing the learning curve of 100 robotic left and right hemihepatectomy (RLH/RRH) in terms of operative time, reported that the learning process was completed in the RLH group after an initial phase of 35 cases, which was shorter than the RRH group (n = 45). Given that, mobilization and resection of the left liver are easier to perform than the right liver, RLH might be the first choice for beginners.

Question 13: Which difficulty scoring systems should be used for RLR?

Ban and Iwate reported on the difficulty scoring system for LLR which was externally validated for RLR. The two difficulty scoring systems are currently recommended. A difficulty scoring system exclusively for RLR should be established by further studies (clinical recommendation: Expert agreement 100%).

By predicting the complexity of different surgeries, the difficulty scoring system (DSS) can recommend suitable patients to surgeons at their corresponding learning stages. Current DSS scores on the basis of the weighted combination of predicted factors that affect the surgeries' complexity include tumor size, position, number of the tumor, extent of liver resection and liver function[137,138].

However, the scoring system for RLR remains to be designed. Linn *et al*[137] summarized 11 types of DSS for LLR based on previously reported studies and conducted a meta-analysis. The results showed that 5 DSS (including Ban DSS, Iwate DSS, Hasegawa DSS, IMM DSS, and Southampton DSS) could be used for predicting the difficulty of LLR surgeries [138-141]. Though no DSS has significant advantages, Ban and Iwate DSS were externally validated for RLR[137,138].

Question 14: What is the role of intraoperative navigation techniques in RLR?

Intraoperative ultrasonography (IOUS) and indocyanine green (ICG) imaging has been used for tumor locating and surgical margin delineation in RLR. Surgeons are supposed to master these techniques and choose the suitable navigation tools to increase the safety of RLR (clinical recommendation: Expert agreement 100%).

Studies have supported the importance of IOUS in liver operations[142,143]. Based on the experience of IOUS in 110 consecutive patients, Zhu *et al*[144] put forward a standardized 4-step IOUS agreement to ensure the safety of RLR operation. Moreover, when using IOUS to examine all the patients, they found 11 patients (10%) had extra lesions, and 7 of these patients (63.64%) underwent improved surgical strategies[144]. However, IOUS has the limitations to accurately show irregular hepatic segmental demarcation and anatomical structure, while ICG imaging has become a supplementary tool for IOUS due to its ability on real-time 3D recognition of tumor margins and to guide liver transection plane. Wakabayashi *et al*[145] reviewed articles related to ICG imaging in liver resection and showed that the combination of IOUS and ICG imaging can increase the safety of liver resection, but the timing and dose administration of ICG remain uncertain. Furthermore, Liu and his team reported the application of ICG using "four-zone three-phase" fluorescence imaging in robot-assisted anatomical hepatectomy in which the liver was divided into 4 anatomical zones include the "tumor zone", "peritumor zone", "ischemia zone", and "reserved liver zone"[146]. The ICG "four-zone three-phase" fluorescence imaging could accurately locate most tumors, clearly display the liver resection plane in a real-time manner and achieved the precision and standardization of anatomical hepatectomy.

The application of augmented reality (AR) technology can increase the accuracy of tumor positioning and surgical margin delineation. At present, only a few cases have been reported on the application of AR technology in RLR. Further technological advance and studies on evaluation of AR technology in clinical applications remain to be done.

CONCLUSION

This international experts consensus guideline offered guidance for the safe and effective clinical practice and the research direction of RLR in future. This evidence-based guideline is expected to be updated again in 2026 and further randomized controlled trials are needed to validate these recommendations.

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FOOTNOTES

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REVIEW

Non-alcoholic fatty liver disease: Immunological mechanisms and current treatments

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Abstract

Non-alcoholic fatty liver disease (NAFLD) causes significant global disease burden and is a leading cause of mortality. NAFLD induces a myriad of aberrant changes in hepatocytes at both the cellular and molecular level. Although the disease spectrum of NAFLD is widely recognised, the precise triggers for disease progression are still to be fully elucidated. Furthermore, the propagation to cirrhosis is poorly understood. Whilst some progress in terms of treatment options have been explored, an incomplete understanding of the hepatic cellular and molecular alterations limits their clinical utility. We have therefore reviewed some of the key pathways responsible for the pathogenesis of NAFLD such as innate and adaptative immunity, lipotoxicity and fibrogenesis, and highlighted current trials and treatment options for NAFLD patients.

Key Words: Liver; Fat; Inflammation; Mitochondria; Immune system

Core Tip: Non-alcoholic fatty liver disease (NAFLD) is a significant global disease burden and a leading cause of mortality causing aberrant changes in hepatocytes. Although the disease spectrum is widely recognised, precise triggers for disease progression remain poorly understood. Whilst some progress has evolved in terms of treatment, there are still no approved pharmacological therapies for NAFLD treatment due to the incomplete understanding of the hepatic cellular and molecular alterations in disease pathogenesis.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFL) (NAFLD) is the most common cause of liver disease globally which occurs due to an excessive accumulation of fat in the liver in the absence of secondary causes or other liver disease aetiologies[1]. NAFLD encompasses the spectrum of disease from simple NAFL, non-alcoholic steatohepatitis (NASH), fibrosis, cirrhosis, and in many cases hepatocellular carcinoma (HCC)[1]. Due to increasing trends of sedentary lifestyles and dietary choices the prevalence of NAFLD continues to rise[1].

NAFLD has a significant global disease burden (882 million cases in 2017)[2], with cases likely to be higher due to poor screening of high risk asymptomatic populations. Furthermore, inaccurate disease progression predication markers and a lack of available licenced therapeutics hinders the treatment of NAFLD. Management of NAFLD is largely based on lifestyle modifications, but many newer therapies are being evaluated (discussed later). Further understanding into the mechanisms of disease progression, diagnostic biomarkers and therapeutic intervention should help mitigate burden of disease. Finally, liver disease combination therapies are becoming increasingly popular. This review will highlight some of the recent developments in NAFLD and aims to define the pathological features associated with disease progression including the immunological mechanisms as well as the current therapeutic interventions used for NAFLD.

Epidemiology and prevalence

Global reports relating to the epidemiology of NAFLD have estimated a global prevalence of NAFLD between 25% and 35%[3-5], with Europe as high as 30%[6], 35% in South American countries[6,7], and 35% in North America[6]. During the period of 1991 to 2019, trend analysis shown that increasing global prevalence increased yearly by 0.7%, rising from 21.9% in 1991 to 37.3% in 2019[5].

NAFLD has a global impact on health care systems due to its high rates of morbidity and mortality[8]. NASH is a particularly prevalent cause of chronic liver disease which in turn leads to cirrhosis and HCC, whereas NAFLD has been noted as the biggest cause of HCC in the United States, France, and the United Kingdom, with NAFLD-related HCC predicted to increase globally alongside the rise in obesity[3]. In the United States, by 2030, almost 49% of the total population is projected to be obese[3].

Globally, the phenotype of patients with NAFLD appears to be men of a mean age of 51.7 years old, with obesity and/or type 2 diabetes[6]. A linear increase prevalence of NAFLD, diabetes and metabolic disorders have also been documented[9,10], and especially occurs in those with central obesity, diabetes and metabolic syndrome[11,12]. Metabolic comorbidities include hypertension (37%) and metabolic syndrome (40%)[6]. NAFLD prevalence in type-2 diabetes mellitus (T2DM) is as high as 70%[13], and patients with T2DM also have a twofold increased risk of all-cause mortality [4,14]. Prevalence of NAFLD in patients with morbid obesity also rises to 90%[4]. Therefore, prevalence of NAFLD poses a significant global health burden requiring clinical attention.

Histopathology and disease spectrum

NAFLD is the most common cause of liver dysfunction with a high association for obesity and insulin resistance (IR). The spectrum of NAFLD can lead to progressive NASH, fibrosis, and lastly HCC and liver failure. NAFLD is a complex disease which results from environmental causes as well as polygenic background and risk factors (Figure 1). Although the molecular mechanisms underlying disease progression are complex, the histological spectrum of disease has been well described[15].

NAFLD is defined as a disease of the liver which is characterised by macrovesicular fat deposition and storage (> 5% of the hepatocytes) due to dysregulation in the mechanisms of fat synthesis and utilisation by the liver[7,16]. Steatosis is predominantly graded on a four-step scale, from 0 to 3. Grade 0 is defined as a normal liver containing fat in < 5% of hepatocytes[17,18]; grade 1 occurs when fat deposits occur in < 33% of hepatocytes, and grade 2 when fat occurs in 33%-66%[17,18]; grade 3 is the final stage in the spectrum of steatosis which occurs when > 66% hepatocytes contain fat[17,18]. The most important histological feature of NASH is hepatocyte ballooning and lobular inflammation as well as a steatotic liver. Hepatocyte ballooning is the second histological feature of NASH. Hepatocyte ballooning is defined by a clear, flocculent, not vacuolar cytoplasm with a ballooned shape. Inflammation occurs in a lobular pattern in NASH, containing Kupffer cells (KC), aggregates of neutrophils, and Mallory-Denk bodies[15]. NASH is defined as a chronic state of inflammation whereby hepatic stellate cells (HSCs) transform into myofibroblasts[19]. These transformed cells produce extracellular collagen matrix[19]. Normally fibrogenesis is a wound healing process. However, in NAFLD, sustained and progressive insults occurring over many years causes unregulated fibrogenesis[19]. Initially collagen deposits form in the perisinusoidal space. As collagen bundles form, architectural remodelling occurs which can lead to cirrhosis and HCC. Fibrosis stage 1 (F1) occurs when mild perisinusoidal/pericellular fibrosis is documented without septa[20]. Stage two occurs when fibrosis occurs in perisinusoidal/pericellular and portal/periportal regions with few septa (F2)[20]. Stage three (F3) is seen when numerous septa are documented, also known as bridging fibrosis[20]. Lastly, stage four is a cirrhotic liver[20]. An overview of NAFLD progression is shown in Figure 1.

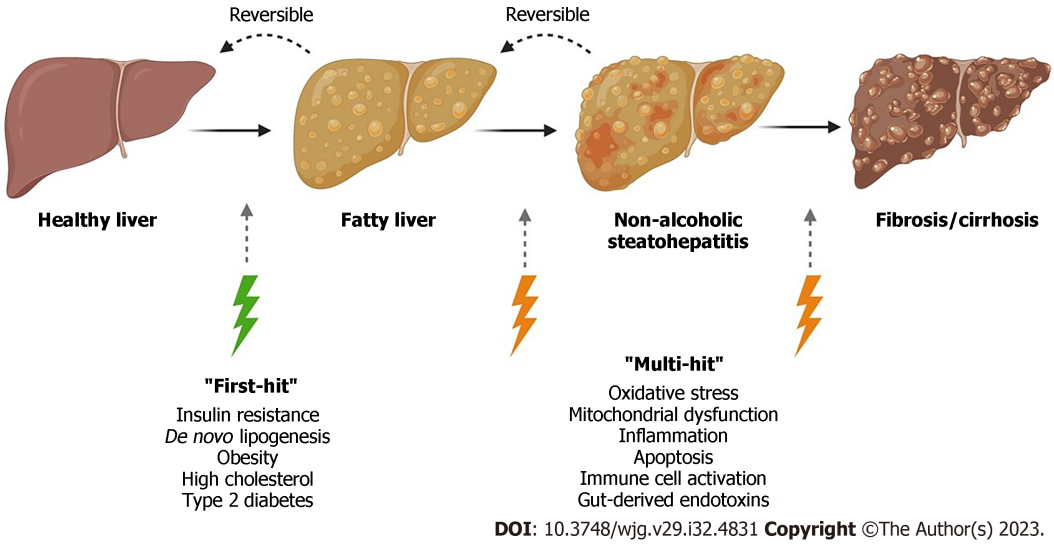

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Figure 1 Overview of the current understanding of non-alcoholic fatty liver disease progression. Non-alcoholic fatty liver disease (NAFLD) encompasses a range of liver damage, from simple accumulation of fat in liver cells, named steatosis, to more severe forms of the diseases such as steatohepatitis, involving inflammation which can lead to fibrosis and cirrhosis. In the "multiple-hit" theory of progression, the first cause or "first-hit" in NAFLD is insulin resistance, obesity, type 2 diabetes, and metabolic syndrome. As the first hit occurs, free fatty acids are stored in the liver as triglycerides, resulting in simple steatosis. Disease progresses when multiple factors, or "multi-hits", such as oxidative stress, inflammatory mediators, apoptosis, and mitochondrial dysfunction cause liver damage (created with BioRender.com).

Progression of the fibrosis stage to a cirrhotic scarred liver can vary between individuals, with cirrhosis occurring up to 15 to 20 years after initial diagnosis[19,21]. The median survival rate for patients with compensated cirrhosis is approximately 9 to 12 years, however, patients with decompensated cirrhosis have a significantly lower median survival rate of approximately 2 years[22]. Patients in the compensated stage are frequently asymptomatic, and often remain undiagnosed[22]. Therefore, early detection of cirrhotic patients who are still in the compensated stage is crucial, as early diagnosis could prevent or slow down disease progression. The onset of decompensated cirrhosis occurs when symptoms such as ascites, hepatic encephalopathy, and/or gastroesophageal variceal haemorrhage are found[22]. Histologically, cirrhosis is diagnosed when a disconnection of the hepatocytes from the central vein occurs as well as capillarisation of the liver sinusoids[19]. These modifications that occur to the liver structure and integrity lead to elevated intravascular resistance within the portal system and decreased hepatic perfusion, ultimately causing a loss of liver function[19]. Hence, timely diagnosis and management of cirrhosis is required to improve patient outcomes.

IR in NAFLD

Many studies have shown that metabolic dysfunction and IR play a significant role in the development of NAFLD. Various animal and clinical studies suggest that chronic low grade inflammation may play a role in the development of IR and NAFLD as well as extrahepatic complications such as cardiovascular diseases, T2DM, and renal dysfunction[23]. IR is a complex state by which the skeletal muscle, liver, and adipose tissue become less sensitive to insulin and its metabolic effects[24]. IR is related to obesity, hypertension, hyperglycaemia and metabolic syndrome. During a carbohydrate-rich diet, excess glucose is converted into fatty acids *via* lipogenesis, using acetyl-CoA which is generated from glycolysis-driven pyruvate[25]. These fatty acids are then incorporated into very low-density lipoproteins (VLDL) for transport to white adipose tissue for storage[25]. Accumulation of lipids in adipocytes trigger downstream signalling of pathways including c-Jun N-terminal kinase (JNK) and nuclear factor-kappa B (NF-κB), leading to production of proinflammatory cytokines, such as tumour necrosis factor-alpha (TNF-α) and interleukin (IL)-6[26,27]. JNK regulates the production of proinflammatory cytokines, karyomitosis, and cellular apoptosis, and therefore, contributes to inflammation and IR. Research has proposed activation of JNK also accelerates lipid accumulation, thus, exacerbating liver injury. JNK-1 deficiency in adipose tissue shows protection from development of hepatic steatosis and promotes glucose intolerance, insulin clearance, IR, and hepatic steatosis[24]. Kluwe *et al*[28] also showed that in a mouse model, inhibition of JNK lead to modulation of fibrosis in hepatocytes. In a high fat diet (HFD) model of NAFLD, IR, liver injury and increased autophagy were documented[29]. However, JNK inhibition decreased autophagy and IR[29]. Therefore, JNK signalling plays a significant role in NAFLD progression.

The hormone adiponectin is secreted by adipocytes and is associated with improved insulin sensitivity. Adiponectin-mediated signalling is also able to stimulate fatty acid β-oxidation, glucose utilisation and uptake, as well as suppression of fatty acid synthesis[30]. Adiponectin can also inhibit the glycerol 3 phosphate (G3P) pathway; however, research has shown that during NAFLD serum levels of adiponectin are reduced[30]. Another metabolite of the G3P pathway is fatty acyl-CoA which is involved in mitochondrial β-oxidation. In patients with NAFLD, an increase in mitochondrial β-oxidation can lead to a state of oxidative stress due to increased substrate delivery to the mitochondrial electron transport chain (ETC), increasing reactive oxygen species (ROS) and damaging mitochondrial DNA[30,31]. Although β-oxidation is upregulated, ATP levels are decreased in NAFLD due to reduced activity of ETC complexes I and IV[32], and have been

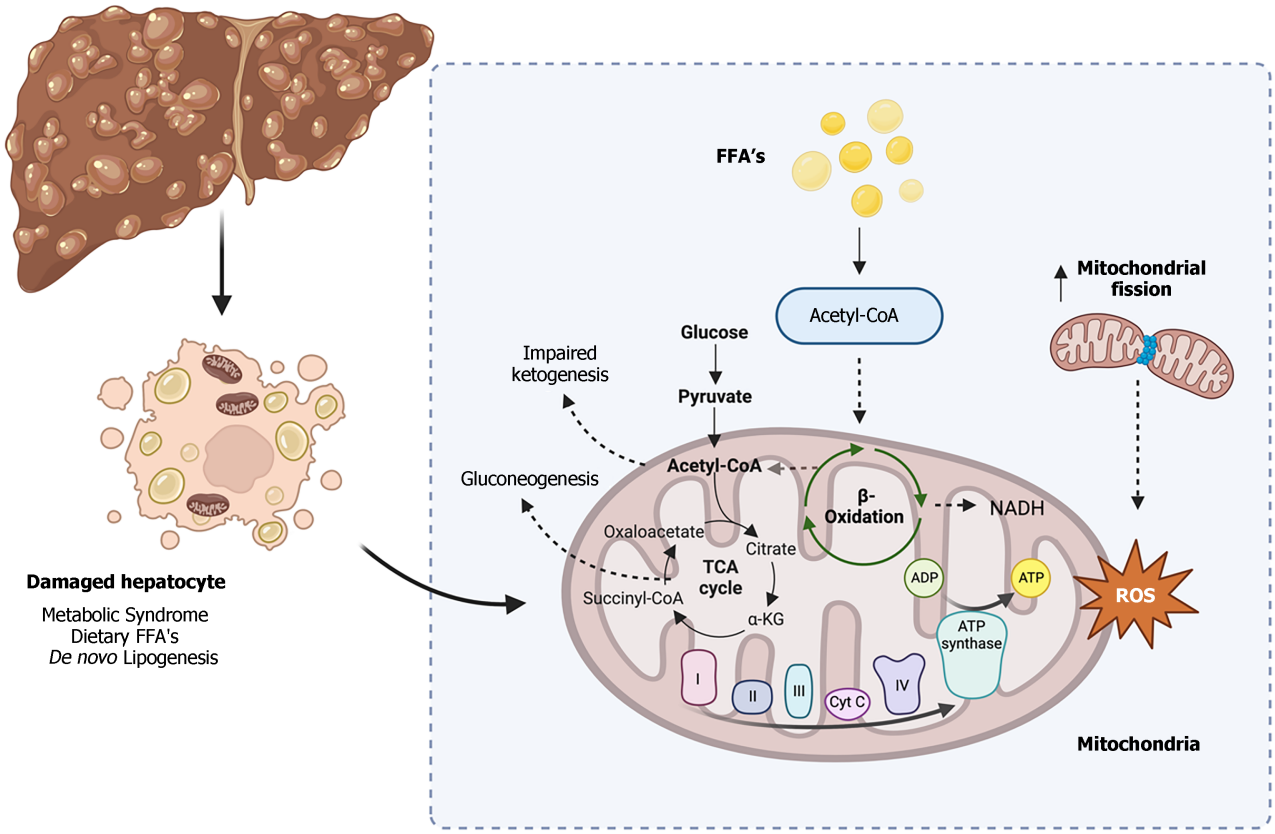

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Figure 2 Overview of mitochondrial dynamics in non-alcoholic fatty liver disease. The accumulation of triglycerides and free fatty acids (FFAs) in the liver are converted to fatty acyl-CoA, which is then transported to the mitochondria for β-oxidation, generating acetyl-CoA. However, increased accumulation of FFA can cause insufficient hepatic β-oxidation, triggering inflammation and oxidative stress. Acetyl-CoA enters the mitochondrial tricarboxylic acid cycle allowing continuation of gluconeogenesis. The by-product nicotinamide adenine dinucleotide, produced though β-oxidation are transported to the electron transport chain (ETC) for oxidative phosphorylation. Disruption and dysfunction of the ETC can cause electron leakage and hepatocyte damage leading to reactive oxygen species production and oxidative stress. Accumulation of FFAs can cause mitochondrial damage, resulting in increased mitochondrial fission and degradation *via* mitophagy processes (created with BioRender.com). ROS: Reactive oxygen species; NADH: Nicotinamide adenine dinucleotide; TCA: Tricarboxylic acid; ADP: Adenosine diphosphate; ATP: Adenosine triphosphate.

correlated to levels of disease progression. Therefore, modifications to mitochondrial function can exacerbate disease progression in NAFLD. Hyperinsulinemia alongside increased levels of ROS also leads to an imbalance between mitochondrial fission and fusion proteins such as dynamin-related protein 1 (Drp1) and mitofusin-2 proteins (Mfn2)[33]. It is possible that an excessive mitochondrial fission in the liver plays an important role in NAFLD progression. Increases in the Drp1-to-Mfn2 ratio causes enhanced mitochondrial fission which in turn causes reduced endoplasmic reticulum association, decreases oxidative phosphorylation capacity, and elevated mitochondrial ROS production[33], which can exacerbate disease state. Therefore, it is plausible that a reduction in mitochondrial oxidative capacity as well as the impairment of metabolic fuels provides a plausible relationship between mitochondrial dysfunction, lipotoxicity and IR. Therefore, metabolic health and mitochondrial function are fundamental to progression of NAFLD (Figure 2).

The inflammasome pathway and macrophages are known to play a significant role in the development of IR. Production of IL-1β and IL-18 can be regulated by the inflammasome pathway, which contains complexes including nucleotide-binding oligomerization domain (NOD)-, leucine-rich repeats-and pyrin domain-containing protein 3 (NLRP3) and activation of caspase-1[34]. In NAFLD, inflammasome formation can be activated upon a variety of stress stimuli such as damage-associated molecular patterns, pathogen-associated molecular patterns, for example, lipopolysaccharide (LPS) from the gut-liver axis, mitochondrial ROS, endoplasmic reticulum stress, and ROS[34-36]. Activation of the NLPR3 inflammasome has shown to contribute to disease progression from steatosis to NASH[34,37]. MRNA levels of NLRP3 inflammasome components such as NLRP3, caspase-1, IL-1β, and IL-18 were also found to be significantly upregulated in NAFLD patients[37-39]. Therefore, targeted modulation of the inflammasome will lead to improved insulin signalling and mitochondrial function.

Immunological Mechanisms

In chronic liver disease, the activation of the immune response can both restore tissue function as well as cause tissue injury. Therefore, an amplified immune response may lead to organ dysfunction. The following section describes the involvement of various immune cell types in the pathogenesis of NAFLD (Figure 3).

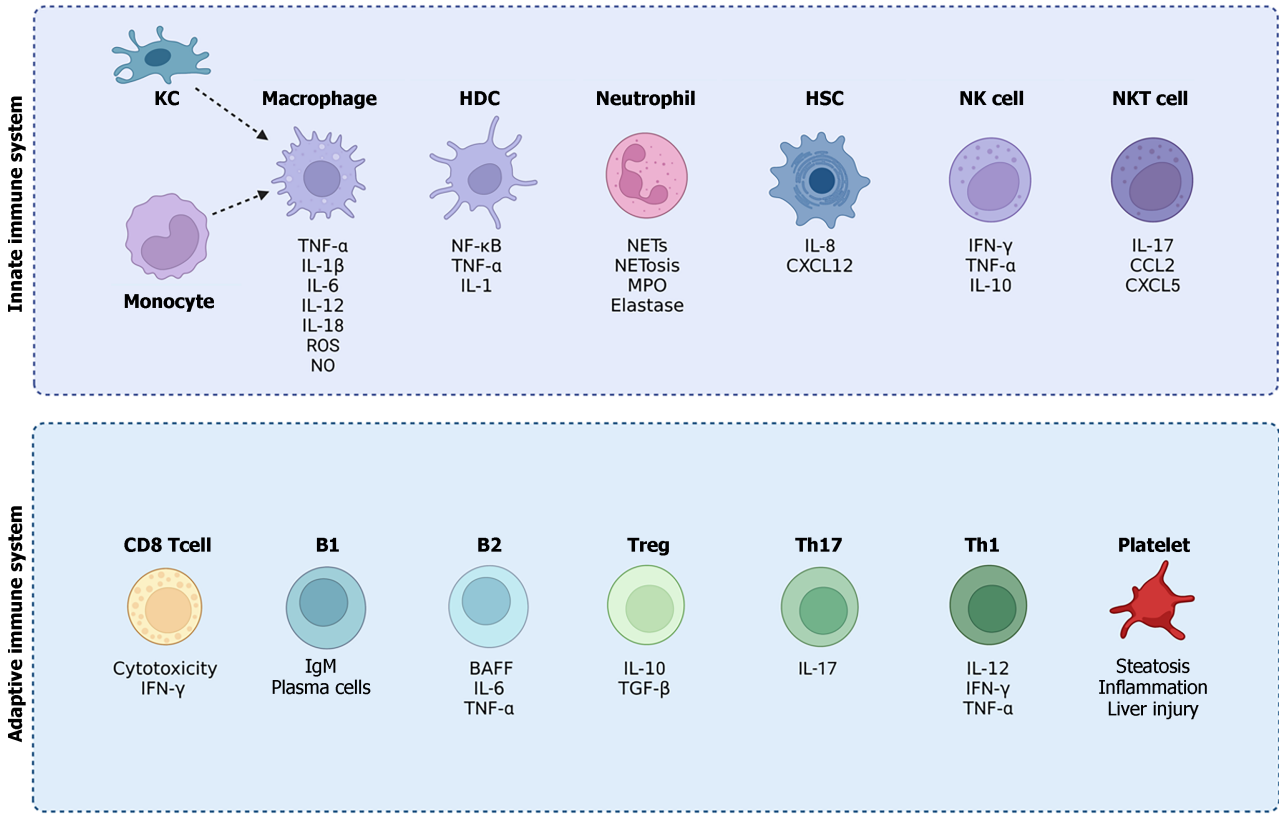

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Figure 3 Overview of innate and adaptive immune cells involved in non-alcoholic fatty liver disease (NAFLD). Activation of immune cells can promote inflammation and liver injury in NAFLD. The innate immune system cells include Kupffer cells, monocytes, and macrophages as well as hepatic dendritic cells, neutrophils and natural killer cells. Natural killer T cells provide a bridge between both the innate and adaptive immune system. The adaptive immune system includes T cells such as CD8 T cells and CD4 T cells (Th1, Th17 and regulatory T cells). The adaptive immune system also comprises of B1 and B2 cells as well as platelets. Although the contribution of immune cells to the development of NAFLD has been explored, the crosstalk between the distinctive immune cell subsets in the pathogenesis of NAFLD requires further investigation (created with BioRender.com). KC: Kupffer cells; TNF-α: Tumour necrosis factor-alpha; IFN-γ: Interferon-γ; IL: Interleukin; ROS: Reactive oxygen species; NO: Nitric oxide; NETs: Neutrophil extracellular traps; NF-κB: Nuclear factor-kappa B; HDC: Hepatic dendritic cells; MPO: Myeloperoxidase; HSC: Hepatic stellate cells ; CXCL: C-X-C motif chemokine ligand; BAFF: B cell activating factor; TGF-β: Transforming growth factor-β; NK: Natural killer; NKT: Natural killer T cells; Treg: Regulatory T cells.

Innate immune system in NAFLD: During NAFLD, the innate immune system is involved in the activation of resident KCs, with recruitment of innate immune cells such as neutrophils, monocytes, natural killer (NK) cells and natural killer T (NKT) cells[40], and inflammation is further exacerbated *via* the production of cytokines, (*e.g.* TNF-α, IL-1β, IL-6, IL-12, IL-18), chemokines, nitric oxide (NO) and ROS[40-42].

Macrophages: The main role of macrophages in the liver is predominantly for immunoregulatory and detoxifying functions, however, alterations in macrophage phenotypes and dynamics have been reported to be involved in the pathogenesis of NAFLD[43,44]. Liver resident KCs are a large population of macrophages as well as other subsets of macrophages such as monocyte-derived macrophages and liver capsular macrophages[45]. M1 macrophages have a pro-inflammatory phenotype and are induced by mediators such as LPS and interferon-γ (IFN-γ) and their activation causes secretion of pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α[46,47]. On the other hand, M2 macrophages have an anti-inflammatory phenotype and are induced by Th2 cytokines such as IL-4 and IL-13, causing secretion of anti-inflammatory factors such as IL-10 and transforming growth factor-β (TGF-β)[46,47]. M2-type macrophages have also been reported to induce M1-type KCs apoptosis decreasing disease progression[48]. Therefore, the balance between M1 and M2 macrophages is important for homeostasis in the liver.

During NAFLD, macrophages can recognise and respond to stimuli through pattern recognition receptors such as membrane-bound toll-like receptors (TLRs), such as TLR4 and TLR9, and cytoplasmic NOD-like receptors [43,45,49]. LPS translocated from the gut can activate TRL4 and TLR9, and this recognition of stimuli can then result in inflammation *via* secretion of proinflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-12, and IL-18, as well as molecules such as ROS and NO. Activation of TLR9 can induce the release of IL-1β from KCs, which occurs during processes such as lipid accumulation, fibrinogenesis and cell death[50]. In NAFLD patients, TLR4 expression is often correlated with hepatic inflammation and fibrosis[51]. There are also many advantages to the activation of macrophages due to their immunosuppressive effects and secretion of anti-inflammatory cytokines, however, they also have pro-fibrinogenic effects. Activation of M2-type macrophages can release TGF-β1 and IL-13, leading to a fibrotic response of liver remodelling and tissue repair[52].

During IR, circulating free fatty acids (FFA) can also directly activate KCs causing activation of the stress-response kinases (JNK1 and JNK2) producing pro-inflammatory cytokines and extracellular vesicles (EVs), stimulating macrophage activation. EVs have been studied in their contribution to pathobiology of NAFLD, and have been found to mediate inflammation[53,54]. Hepatocytes can release EVs containing cargoes which activate signalling pathways[53,54]. Some EVs can carry mitochondrial DNA, which in mice and humans have been shown to activate macrophages *via* TLR9 [53,54]. Activation of TLR9 then causes inflammation though resultant downstream activation of NF-κB-dependent pro-inflammatory cytokines in macrophages[53,54]. Macrophage activation and inflammation may also occur *via* EV formation in response to lipid-associated toxicity[55]. Therefore, both harmful and beneficial macrophage phenotypes can co-exist during NAFLD, and the balance between these phenotypes must be considered for therapeutic strategies. Both cellular and molecular macrophage targets for therapy may provide a new perspective as well as the adoptive transfer therapies.

Neutrophils: Neutrophil infiltration during NASH has been documented in both patients and in mouse models. In the early stages of NAFLD, recruitment of neutrophils occurs *via* chemokines such as C-X-C motif chemokine ligand (CXCL) 1, IL-8, and CXCL2[56]. Research has shown that various neutrophil specific components are released in NAFLD. Neutrophil elastase is a major inflammatory protease which can be released by neutrophils. In a mouse model of NAFLD, elastase suppression has been shown to improve disease severity[57]. As well as elastase, neutrophil proteinase-3 has also been reported as elevated in NASH and both the levels of proteinase-3 and elastase were correlated with liver fibrosis [58]. Elastase has also been shown to play a role in metabolic dysfunction and IR, and therefore, targeting or improving IR and metabolic disease may improve inflammation in NAFLD. Myeloperoxidase (MPO), a neutrophil derived enzyme has been shown to become increased in NASH. It is thought that both neutrophils and MPO may cause activation of HSCs inducing liver fibrosis and inflammation in NASH[59,60]. Plasma levels of MPO have been found to be correlated with fibrosis in NAFLD patients[61]. More research is required to determine how neutrophils contribute to disease pathogenesis in NAFLD.

NKT cells: NKT cells provide a bridge between the innate and adaptive immune system and express both NK cell and T cell markers[40]. NKT cells become activated upon antigen presentation by CD1d which can be expressed by a variety of cells such as hepatocytes, macrophages, dendritic cells, and B cells[40]. Pro-inflammatory cytokines such as IL-4 and IFN-γ are secreted after NKT activation causing further tissue injury. Although there is conflicting evidence, during steatosis levels of NKT cells become reduced, whereas in NASH and fibrosis they are increased[40]. This has been shown by in vivo studies where NKT cells were elevated in the blood and liver of patients with moderate-to-severe steatosis[62]. During early-stage NAFLD such at fatty liver, the phenotype of NKT cells demonstrate a pro-inflammatory Th1 cytokines profile such as IFN-γ, whereas, in advanced end stage disease, they have a profibrotic role[40].

NK cells: NK cells have a cytotoxic role and can attack cells *via* perforin-mediated pathways or cell-cell interactions, for example Fas. NK cells can also act as regulatory cells by releasing various cytokines and chemokines, such as IFN-γ, TNF-α, and IL-10 as well as growth factors[40]. NK cell-associated cytotoxic ligands, such as TNF-related apoptosis-inducing ligand (TRAIL), NK group 2 member D, and major histocompatibility (MHC) class I chain-related protein A and B mRNAs, have been reported to be elevated in obese NASH patients[63]. NK cells therefore may possibly promote inflammation and hepatocyte apoptosis *via* TRAIL secretion[63], leading to progression of fibrosis.

Dendritic cells**:** Hepatic dendritic cells (HDCs), which highly express MHC-class II molecules and CD45 are important immune cells in the liver which have migratory capabilities as well as production of cytokines such as TNF-α, IFN-γ, IL-2, IL-4, and IL-6[64]. Three distinct subsets of HDCs have been described in experimental models of HDCs: Lymphoid, myeloid and plasmacytoid[64]. They play a crucial role in the progression of metabolic steatohepatitis bridging lipid metabolism and inflammation. HDCs can shift from a tolerant state to an active state, triggering an inflammatory process [65]. In an immune tolerant state, immature HDCs secrete IL-10, and TGF-β, as well as limit T cell expansion[64]. In a tolerant state they supress inflammasome activation, maintaining homeostasis in the liver. This regulatory role of HDC in NASH can therefore restrict inflammation as well as clear apoptotic cells and necrotic debris[65]. Additionally, they are involved in lipid storage within the liver and are important for antigen presentation and induction of inflammatory pathways[64]. Recent studies suggest that HDCs have antifibrogenic effects by activating metalloproteinases, such as matrix metallopeptidase 9 which has been found to be involved in regression of fibrosis as well as remodelling of the extracellular matrix.

On the other hand, in an active state HDCs cause inflammation and liver damage as well as contribute to fibrosis. In an active or inflammatory state, mature recruit macrophages to the liver and can activate the NF-κB pathway as well as produce pro-inflammatory cytokines such as TNF-α and IL-1, contributing to the inflammatory microenvironment[64]. In humans, a subset of HDCs (CD11C + cDC2) have been shown to play an important role in development of fibrosis and have been positively correlated with metabolic steatohepatitis[64]. HDC's in a mouse model of metabolic associated fatty liver disease (MAFLD) have shown that in a matured form they produce more inflammatory cytokines and may be responsible for proinflammatory responses[65]. HDC may therefore play dual roles in both the suppression and progression of disease state.

Adaptive immune system in NAFLD: The adaptive immune system is defined by antigenic specificity and immunological memory and includes predominantly B and T lymphocytes. Experimental research has suggested that sustained immune responses activated by oxidative stress-related antigens can affect the pathogenesis of disease *via* activation of CD4+ T-cells, which, in turn, stimulate macrophage M1 responses and liver CD8+ T- and NKT cell recruitment[66]. Experimental in vitro data as well as in vivo data supports adaptive immunity in NAFLD disease progression.

T cells: Conventional T cells have been well studied in their involvement in the pathogenesis of NAFLD. The main CD4+ T-cell are divided into Th1, Th2, and Th17 populations characterised by production of specific cytokines. Recruitment of CD4+ T-cell in the liver have been reported to be increased in individuals diagnosed with NASH[67-71],

as well as in animal models fed a high calorie diet[72]. Upon presentation to inflammatory stimuli, CD4+ T cells can differentiate into Th17 cells[73], a subset of pro-inflammatory T helper cells defined by their ability to produce IL-17. In animal models of NAFLD, in the liver it has been documented that the Th-17 phenotype was favoured, promoting inflammation[74]. Human studies in obese and overweight patients have also shown an increase in the Th17 population[74]. The IL-17 family of cytokines have been implicated in the progression of fatty liver disease through interference of the insulin signalling pathway[75]. In mouse models, deficiency of IL-17A, IL-17F or IL-17A receptor (IL-17RA) results in increased steatosis but reduced steatohepatitis[76,77]. In HFD mouse model, neutralisation or IL-17RA deficiency as well as treatment with anti-IL-17mAb therapy has shown to protect mice from diet-induced liver injury *via* improvement of lipid accumulation, suppressing KCs activation, decreased pro-inflammatory cytokines levels and inhibition downstream of NF-κB signalling[40,78]. IL-17 has also been shown to activate the signal transducer and activator of transcription 3 pathway in HSCs causing progression of fibrosis in the liver[79]. These studies indicate the involvement of IL-17 in the pathogenesis of NAFLD.

Regulatory T cells (Tregs), a subset of cells which promote immune tolerance and facilitate tissue repair and express the transcription factor forkhead box P3. Although there is limited data available, the number of hepatic Tregs have been documented to be decreased in in animal models of NAFLD[80]. In humans, the levels of Tregs in the liver and the circulation of patients with NAFLD is also reported to be decreased[80]. Also, in a NASH mouse model, induced by a HFD and endotoxin challenge, a decrease in liver Tregs was documented, however, transfer of Tregs into mice showed reduced liver injury and inflammation, through decreased expression of TNF-α[81].

B cells: Until recently, the role of B cells is the pathogenesis of NAFLD was less understood. B cells are highly specific antibody producing cells of the adaptive immune system. B cells contribute to approximately 6% of intrahepatic cells[40,82]. B cells produce the B cell activating factor (BAFF), a cytokine controlling the process of B cell survival and maturation [83,84]. Liver biopsies from patients with NASH have been shown to contain both B and T cells in the inflammatory infiltrates [73,85,86].

In mouse models, B cells have been shown to become activated in NASH, concomitantly with the onset of steatohepatitis and thus, maturing to plasma blasts and plasma cells[73,86]. Another study has also shown that BAFF signalling increased IR in an NAFLD model as well as promoting fatty liver[87]. Whereas, in mice models of NASH, B2 cell responses upregulate BAFF[73]. Levels of BAFF in patients with NASH appear to occur at a higher level than with patients with fatty liver, therefore it has been proposed BAFF levels correlate with severity of steatohepatitis fibrosis[88]. BAFF receptor-deficient mice showed an improvement in HFD-induced obesity and IR, as well as a reduction in the number of B cells and a decrease in serum immunoglobulin G level[40]. The involvement of B cells in the progression of liver disease can also be due to production of pro-inflammatory mediators and antigen-presentation[89,90]. In patients with NASH, MHC class II molecules become upregulated causing inflammatory infiltration and recruitment of CD4+ and CD8+ T cells to the liver[73], whereby Th1 CD4 + T cell activation and IFN-γ production occurs[86]. Therefore, this research suggests B cells and BAFF play an important role in NAFLD pathogenesis and can contribute to pathogenesis of NAFLD *via* autoimmune hepatitis and liver fibrogenesis [87,90,91].

Serum levels of IgA has been found to be associated with patients with NASH and levels of IgA can predict advanced liver disease progression[92]. Patients with HCC have also been documented to have higher serum IgA[93-95]. In both mouse models and human studies, liver resident IgA accumulation in hepatic cells has been associated with chronic inflammation and fibrosis[93]. Regression of HCC has also been documented when IgA is inhibited, causing reactivation of CD8+ T cell function[93,94].

B cell deficiency also has been shown to improve development of NASH. The mechanisms and signalling of B cell activation in NASH is *via* myeloid differentiation early response protein 88[40,96]. Studies have shown that elimination of myeloid differentiation early response protein 88 on B cells reduced hepatic T cell-mediated inflammation and fibrosis, but had no effects on steatosis[40,96]. Therefore, B cells show involvement in the pathogenesis of NAFLD, and manipulation on B cells may provide a therapeutic opportunity for NAFLD treatment. An overview of innate and adaptive immune cells involved in NAFLD is shown in Figure 3.

Diagnostic procedures

Most commonly a combination of clinical history, laboratory findings, biopsies and radiological testing are used in the diagnosis of NAFLD. The clinical diagnosis of NAFLD is usually considered when aminotransferases become elevated or imaging on the liver detects high hepatic fat[97]. Both the American and European guidelines agree that suspicion of fibrosis warrants a confirmatory liver biopsy[98].

Current biomarkers: Liver function tests, particularly alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels, can indicate liver damage in NAFLD/NASH[12,99]. These levels may be elevated by two to four times the normal limit. Mild elevations in aminotransferase levels may be present in lower stages of the disease, but routine tests may appear normal. Alkaline phosphatase and gamma-glutamyl transferase (GGT) levels may be up to three times the upper limit even in the absence of advanced disease[100]. A serum GGT cut-off value of 96.5 can predict advanced fibrosis with a sensitivity of 83% and specificity of 69%[101].

Several different biomarker panels have been described in their use to assess liver fat. These include the liver fat score (LFS) (sensitivity 86%, specificity 71%), hepatic steatosis index (sensitivity 92.5%, specificity 92.4%), fatty liver index (FLI) (sensitivity 87%, specificity 86%) and the steatotest (sensitivity 95.5%, positive predictive value 97.0%)[102]. LFS is calculated using a patients serum AST/ALT ratio, fasting serum AST level, fasting serum insulin level, any presence of metabolic syndrome and diagnosed diabetes mellitus. Studies have shown that a score greater than -0.640 can predict NAFLD with a sensitivity of 86% and specificity of 71%. The FLI uses waist circumference, body mass index (BMI), triglyceride, and GGT and this was initially developed to detect fatty liver in western countries.

Screening tools: The fibrosis-4 scoring system uses a patients age, platelet count, AST, and ALT to determine a liver fibrosis score and can help to predict advanced fibrosis. A score < 1.45 defines a negative predictive value or low probability of advanced liver fibrosis[103]. Scores of A score > 3.25 indicated a higher likelihood and has a positive predictive value of 65%, and specificity of 97% at predicting advanced fibrosis[103]. Recently, a new FAST™ screening tool has been used which compromises a combination of FibroScan® parameters such as liver stiffness measurement and controlled attenuation parameter as well as AST. This screening tool has been shown to have diagnostic accuracy when predicting those at risk of NASH. The FAST™ screening tool may provide a better non-invasive algorithm for diagnosis of NASH. FAST™ performed better than other non-invasive algorithms for the diagnosis of at-risk NASH[104,105]. Results have shown that the FAST score had the highest area under curve for the most high-risk NASH criteria as well as liver stiffness showing a consistently acceptable performance in predicting NASH[106].

Emerging biomarkers: Various non-invasive biomarkers for NAFLD have been developed and validated over the last 20 years, however, there is a need to develop and validate new biomarkers for NAFLD which encompass IR, inflammation and fibrogenesis[107-109].

Cell Death**:** As well as current markers such as cytokeratin 18, necroptosis is a form of cell death which is characterised by changes such as organelle swelling, plasma membrane damage, and release of cellular contents[110]. Proteins such as receptor-interacting protein kinase (RIPK) family members such as RIPK1 and RIPK3, and mixed lineage kinase domain-like (MLKL) are involved in the process[111]. During NAFLD, TLR4 ligands such as LPS cause activation of the TLR4 receptor causing activation of proteins such as RIPK3 and MLKL leading to necroptosis[112]. In patients with MAFLD, necroptosis components such as RIPK3 have been shown to be upregulated, therefore, it may be important to consider markers of necroptosis in NAFLD.

MicroRNAs (miRNAs): miRNAs are non-coding RNAs which play a role in gene expression regulation[113] and have been implicated as potential biomarkers in NAFLD. Research has shown that miRNAS such as miR-21, miR-34a, miR-122, and miR-451 have been found to be upregulated in NAFLD patients[114-117]. Various studies have also shown that profiles of miRNAs in NAFLD patients can differentiate between disease stage. In NAFLD and NASH, miR-122, miR-192, miR-19a, miR-19b, miR-125b, and miR-375 have been found to be increased greater than 2-fold, whereas continuous upregulated expression of miR-122, miR-192, and miR-375 was found in NASH patients[118,119]. Furthermore, higher expression of miR-122 and miR-21 have been reported in people with high fasting blood glucose, obesity and fatty liver infiltrations[119,120]. Therefore, these findings suggest that miRNAs may be promising biomarkers for NAFLD and metabolic disease, in particular miR-122, miR-192, and miR-34a, which have been shown to be correlated with severity of NAFLD.

EVs**:** Growing evidence suggests that EVs play a significant role pathological disease including those associated with obesity and metabolic syndrome. Obesity, IR, T2DM and NAFLD have all been linked to changes in the abundance and phenotype of circulating EVs[121] and therefore circulating EVs and their composition may be a candidate biomarker for NAFLD. EVs contain substances such as genetic material including miRNAs[122]. It has been documented that in humans, the number of AD-EVs is correlated with IR in overweight people[123], as well as visceral AD-EV number correlated with liver injury measured by ALT and AST and metabolic syndrome[124]. Circulating EVs have also been shown to become elevated during the progression of NASH and have reported to be correlated with histological findings [125]. Research has also shown that EVs from a NAFLD model in mice contained miR-122 and miR-192[125]. In cirrhotic patients, plasma hepatocyte-EVs were found to contain elevated levels of CK-18[126]. EVs from visceral adipose tissue can exacerbate disease in NAFLD by causing further inflammation, fibrosis and IR. Both pro- and anti-fibrotic EVs have been documented in the liver and therefore it is plausible to investigate the differences in the pro- and anti-fibrotic phenotypes to track disease progression and likelihood of fibrosis development. Future research should consider the use of EVs in monitoring metabolic dysfunction and IR in NAFLD, with focus on EV phenotypes, cargo and cell specific markers.

Therapeutic interventions

Despite NAFLD being an extremely prevalent liver disease, no specific pharmacological interventions are currently Food and Drug Administration approved for treatment. Some therapeutic agents used as anti-diabetics, antilipidemic and natural bile treatments have previously been evaluated in their ability to treat liver disease, although they have limitations. Predominantly, lifestyle interventions including diet and exercise are most commonly used to treat NAFLD. The current therapeutic interventions used for NAFLD are outlined below including future potential agents such as sirtuins, antioxidants and vitamins.

Lifestyle modifications: A substantial amount of research indicated that changes to lifestyle are a primary approach for the treatment of NAFLD[127]. Diet changes and weight loss can reverse liver disease. Weight loss in the range of 5%-10% can provide beneficial effects to NAFLD patients *via* a reduction in NAFLD activity score (NAS)[128,129]. A weight reduction greater than 10% has also been shown to reduce the severity of fibrosis as well as resolution of NASH[12,129,130].

European Association for the Study of the Liver (EASL), National Institute for Health and Care Excellence and American Association for the Study of Liver Diseases guidelines provide recommendations in terms of diet and physical activity for the management of NAFLD. These bodies recommend caloric restriction with a calorie deficit of 500-1000 kcal a day, as well as limiting the consumption of alcohol, fats and coffee. EASL also favours the Mediterranean diet, with studies indicating a reduction in liver fat in NAFLD patients[131]. Lifestyle modifications in terms of physical exercise can also positively effect liver fat content. Both resistance/weight training, high-intensity interval training and aerobic exercise have all equally been shown to reduce liver fat content; however, in men with NAFLD, high-intensity interval

training was the most effective in restoring hepatic fat, reducing hepatic stiffness and improving Kupffer cell function; key features of NASH[132].

Evidence has suggested that the ketogenic diet (KD) is an effective treatment for NAFLD. Ketogenesis is a metabolic process resulting in the production of ketone bodies, namely acetoacetate, beta-hydroxybutyrate (BHB), and acetone, which act as alternative energy sources[33,133]. The KD has been shown to change hepatic mitochondrial fluxes and redox state as well as significantly reducing liver fat content and hepatic IR[134]. These changes were found to be accompanied by an increase in the net hydrolysis of liver triglycerides, a decrease in endogenous glucose production, and lower serum insulin levels[134]. BHB has also been shown to interact with inflammasomes[135,136] and neutralise ROS [137], leading to a reduction in inflammatory cytokines and oxidative damage *via* its antioxidant capacity. These findings suggest a KD can contribute to the reversal of NAFLD through improvement of IR and cellular redox function.

Bariatric surgery: Bariatric surgery been shown to be an effective treatment for NAFLD improving overall liver health *via* facilitating weight loss, improving insulin sensitivity, and subsiding inflammation[138,139]. Roux-en-Y gastric bypass (RYGB) and laparoscopic sleeve gastrectomy (LSG) are the most prevalent bariatric procedures, which lead to significant weight loss and metabolic health improvements[140].

Several research studies have investigated the impact of bariatric surgery in NAFLD and have shown positive results. Studies have indicated that bariatric surgery resulted in significant improvements in liver enzymes and histology, with a decrease in liver fat and fibrosis. Results from two meta-analyses have shown that treatment of NAFLD using bariatric surgery resulted in a biopsy-confirmed resolution of steatosis (56%-66%), inflammation (45%-50%), ballooning degeneration (49%-76%), and fibrosis (25%-40%), as well as showing a decrease in NAS scoring[140-142]. Bariatric surgery has therefore proven to be effective in ameliorating NAFLD, however, it is important to clarify which type of surgery is most effective. A study by Baldwin *et al*[143] compared RYGB and LSG against its effectiveness at improving AST and ALT concentration, NAS and NAFLD fibrosis score. Overall, both procedures reduce AST and ALT levels, however, LSG showed slightly more favourable results[139]. Another study has shown NAS scoring reduced significantly in patients who underwent both surgery types 12-mo after the surgery[139]. RYGB patients had a more significantly decreased steatosis and superior improvement in plasma lipid profile[144]. Furthermore, bariatric surgery has been demonstrated to lower the risk of liver-related complications and death in individuals with NAFLD. Bariatric surgery can therefore be considered as a promising treatment option for those with NAFLD who are overweight or obese.

Insulin sensitising agents: IR is believed to play an essential role in the development and progression of NAFLD. In recent years, various insulin sensitizers such as biguanides, thiazolidinediones (TZDs), glucagon-like peptide-1 receptor agonists (GLP-1), and dipeptidyl peptidase 4 inhibitors have been investigated as potential therapeutic targets for NAFLD[145]. However, there are safety concerns associated with long-term use of these targets[145].

Biguanides*:* As the development of NAFLD is closely associated with IR, diabetes, hyperglycaemia and hyperlipidaemia, antidiabetic drugs are often utilised in the treatment of NAFLD[145]. Metformin is considered the first-line treatment for T2DM due to its ability to improve insulin sensitivity and promote weight loss without causing hypoglycaemia[145,146]. Although its mechanisms of action are not fully understood, it works by lowering hepatic glucose production[145]. Previous open-label studies have suggested that metformin may have a positive impact on hepatic steatosis and necroinflammation, although excessive weight loss may have confounded these results[30]. However, some studies have shown that metformin does not significantly improve the histological response in NAFLD [30,147], but improves liver function and BMI[148]. In a mouse model, metformin treatment showed improvements to the gut-liver axis *via* attenuation of the loss of tight junction proteins in the small intestine as well as reducing the increase of endotoxin levels in the portal circulation[149].

GLP-1: GLP-1 receptor agonists can alter IR by promoting weight loss *via* suppressing appetite and delaying gastric emptying[150]. In NAFLD patients, research has shown GLP-1 receptor agonists improve hepatic and adipose tissue IR, suppress de novo lipogenesis and oxidative stress as well as increased clearance of VLDL[30,150]. GLP-1 may also modulate the immune system *via* the reprogramming of macrophages to the M2 phenotype. In the exenatide study of cardiovascular event lowering trial, GLP-1 receptor agonists reduced cardiovascular risk[151] and visceral fat, improved glucose tolerance, body fat percentage and resting energy rate (NCT01144338). The trial semaglutide unabated sustainability in treatment of type 2 diabetes (NCT02054897) are a series of phase III clinical trials which suggest treatment with semaglutide has a higher effectiveness than other GLP-1 therapeutics in the reduction of HbA1c in patients with T2DM. In a phase 2 trial with patients diagnosed with NASH receiving semaglutide treatment of 0.1 mg (80 patients), 0.2 mg (78 patients), or 0.4 mg (82 patients), patients resulted in a significantly higher NASH resolution when compared to the placebo (NCT02970942)[152]. Although NASH resolution was significantly improved, no significant difference was shown in the fibrosis stage[152].

Fibroblast growth factor (FGF) based therapeutics: FGF analogs have been proposed to target steps of disease pathogenesis. FGF-21 treatments are currently in clinical development for the treatment of NASH[153,154] and data suggests that FGF21 is anti-fibrotic and has the potential to improve the metabolic syndrome and is effective in treating NASH. The BALANCED trial (NCT03976401) evaluated the effects of efruxifermin, a long-acting Fc-FGF21 fusion protein [155]. In this study, 80 patients were given either placebo (n = 21) or efruxifermin 28 mg (n = 19), efruxifermin 50 mg (n = 20) or efruxifermin 70 mg (n = 20) *via* weekly subcutaneous injection for 16 wk[155]. Treatment with efruxifermin was found to significantly reduced hepatic fat fraction (HFF) in patients with F1-F3 stage NASH[155]. FGF based compounds which are currently in phase II are efruxifermin (FGF-21), pegbelfirmin (FGF-21), aldafermin (FGF-19), pegozafermin (FGF-21) and BFK8588A (FGF-21), which have been shown to achieve a reduction in ALT levels[109].

Thyroid hormone receptor-β (THRβ) 1 agonists: Currently, several different THRβ specific agonists have been shown to produce positive therapeutic effects in both animal models and clinical trials for treatment of NAFLD. Treatment with

TG68, a novel THRβ agonist, has positive effects on resolution of NAFLD *via* reduction in liver weight, hepatic steatosis, serum transaminases, and circulating triglycerides in a NAFLD model[156]. Resmetirom (MGL-3196) has also been found to significantly reduce hepatic lipid content and improve liver enzyme levels and plasma lipid levels in NASH patients [157], however, glucose or insulin levels remained unchanged. Another THRβ agonist, VK2809 has been shown to reduce hepatic steatosis in a mouse model and in a phase II clinical trial reduced liver lipid content[158,159].

Currently there are many ongoing clinical trials studying the effects of THRβ agonists in NAFLD. The VOYAGE study is assessing VK2809 to determine its efficacy in the resolution of biopsy proven NASH (NCT04173065). The DUET study is also assessing the effects of orally administered TERN-501 and TERN-101 in presumed NASH (NCT05415722), whilst the LIFT study is assessing TERN-101 alone in NASH patients (NCT04328077)[160]. In a NASH mouse model, TERN-501 reduced steatosis as well as reducing serum total cholesterol, triglyceride and ALT levels[161].

Peroxisome proliferator-activated receptor-γ (PPAR-γ) agonists: PPAR-γ is a ligand-activated nuclear receptor and its activation causes insulin sensitization and enhances glucose metabolism. TZDs are a group of insulin sensitisers used to treat T2DM by action on PPAR-γ. PPAR agonists have been shown to modulate the innate immune response. PPAR-δ can promote anti-inflammatory polarization of macrophages and modulate their activation[162], whilst PPAR-γ ligands can inhibit the activation of macrophages and cytokine production (TNF-α, IL-6, and IL-1β), thus, reducing inflammation [163]. Activated PPARs can also regulate immune cells (macrophages, DCs, T cells, and B cells) as well as decrease inflammatory cytokine production[163].

Pioglitazone, a TZD, has been used for treatment of NASH with its effects improving steatohepatitis, ballooning degeneration and lobular inflammation[164]. Pioglitazone (45 mg/d) for 6 mo has been shown in patients with prediabetes or T2DM to improve the fibrosis stage (NCT00227110)[165]. Elafibranor is a dual PPAR-α/δ agonist. PPAR-δ functions to regulates peroxisomal β-oxidation of FFA as well as improve insulin sensitivity, lipid and glucose homeostasis[30]. Several clinical trials have assessed the effects of elafibronor in improving histology in NASH patients such as RESOLVE-IT (NCT02704403), and the GOLDEN trial[166]. In both trials elafibranor showed no effect.

Sodium glucose cotransporter 2 (SGLT2) Inhibitors: Another class of drugs lower serum glucose levels *via* inhibition of the SGLT2 and promote weight loss. Studies have shown that SGLT2 inhibitors reduce ALT levels correlating with changes in bodyweight and glycaemic control[30], although further studies are required to identify whether SGLT2 inhibitors can prevent progression of NAFLD/NASH.

Current clinical trials are underway to assess the efficacy and effectiveness of SGLT2 inhibitors. A randomised clinical trial aims to compare the effect of the pioglitazone and empagliflozin combination on liver fat mass (NCT04976283). Another trial is investigating dapagliflozin in NASH (NCT05254626).

Farnesoid X receptor (FXR) agonists: The FXR is a nuclear receptor which is activated by bile acids. FXR inhibits the expression sterol regulatory element binding protein-1 and carbohydrate-responsive element-binding protein. FXR also enhances the clearance of high-density lipoprotein and VLDL in the liver and well as promoting hepatic regeneration. However, little is documented regarding the immune modulation in this class of drugs.

Obeticholic acid (OCA) is a synthetic bile acid which acts as a FXR agonist. The use of OCA for NASH is still under investigation, due to reported side effects. The FLINT trial (NCT01265498) was a multicentre, randomized, double-blind, placebo-controlled phase IIb study assessing the effects of 25 mg of OCA for 72 wk. Treatment with OCA was linked with a significant improvement fibrosis stage in the treated group (35% *vs*. 19%; $P = 0.004$), although there was no difference in rates of NASH resolution. A phase III trial (REGENERATE) is currently active to further assess the treatment of OCA in patients with NASH (NCT02548351).

Other FXR agonists, such as tropifexor, have been studied in the treatment of NASH in the FLIGHT-FXR study (NCT02855164). This 48-wk study using tropifexor found sustained decreases in ALT and liver fat content (measured by HFF using magnetic resonance imaging-estimated proton density fat fraction) during the therapy duration[167]. A Phase IIa (LIFT trial) studying TERN-101, a FXR agonist, showed that in a 12-wk controlled trial, significant improvements in cT1, a marker of fibro-inflammation was observed[160].

Other treatment approaches: Modulation of the gut microbiome: Gut dysbiosis is a common feature of NAFLD[168]. Whilst pre- and pro- biotics are being evaluated, faecal microbiota transplant (FMT) may provide an alternative approach. HFD-fed mice which received FMT from healthy donors showed a significant reduction in intracellular hepatic lipid and proinflammatory cytokines concentration (IFN-γ and IL-17)[169]. Small intestinal microbiota transplants from healthy lean individuals to obese individuals have reported improvements in insulin sensitivity in those patients with metabolic syndrome[170]. A phase I pilot study is currently underway to study FMT in patients with NASH (NCT02469272). FMT as a treatment for NASH patients seems to be both a safe and efficient treatment, although, more high quality studies, trials and follow-ups are required to verify its therapeutic potential[168].

Modulation of the immune system: Methods for modulating the immune system as potential therapies for NAFLD are currently under investigation. Various pleiotropic effects of platelets have recently been discovered in liver homeostasis and disease as platelets are also involved in inflammatory regulation. Anti-platelet therapy (APT) has been shown to reduce NASH pathogenesis in rats[171]. Evidence has shown that APT may have a protective effect in patients with NAFLD[172]. In the liver, platelet and neutrophils can interact leading to neutrophil extracellular trap formation[173]. APT has been shown to reduce both NASH and HCC development[174]. It has been observed that APT reduced platelet accumulation in hepatocytes as well as reducing immune cell interaction, which led to decreases in cytokine and chemokine release, thus attenuating macrovesicular steatosis and liver damage[174].

It is well documented that NASH can progress to HCC. Immunotherapy, including programmed cell death 1 (PD-1) treatment, has been approved for the treatment of HCC[175]. PD-1 can interfere with the immune response and contributes to the growth and expansion of cancer. It has been documented that in NASH there is a progressive accumulation of exhausted, unconventionally activated CD8+ PD1 + T cells[175] and data has shown that in HCC, immune

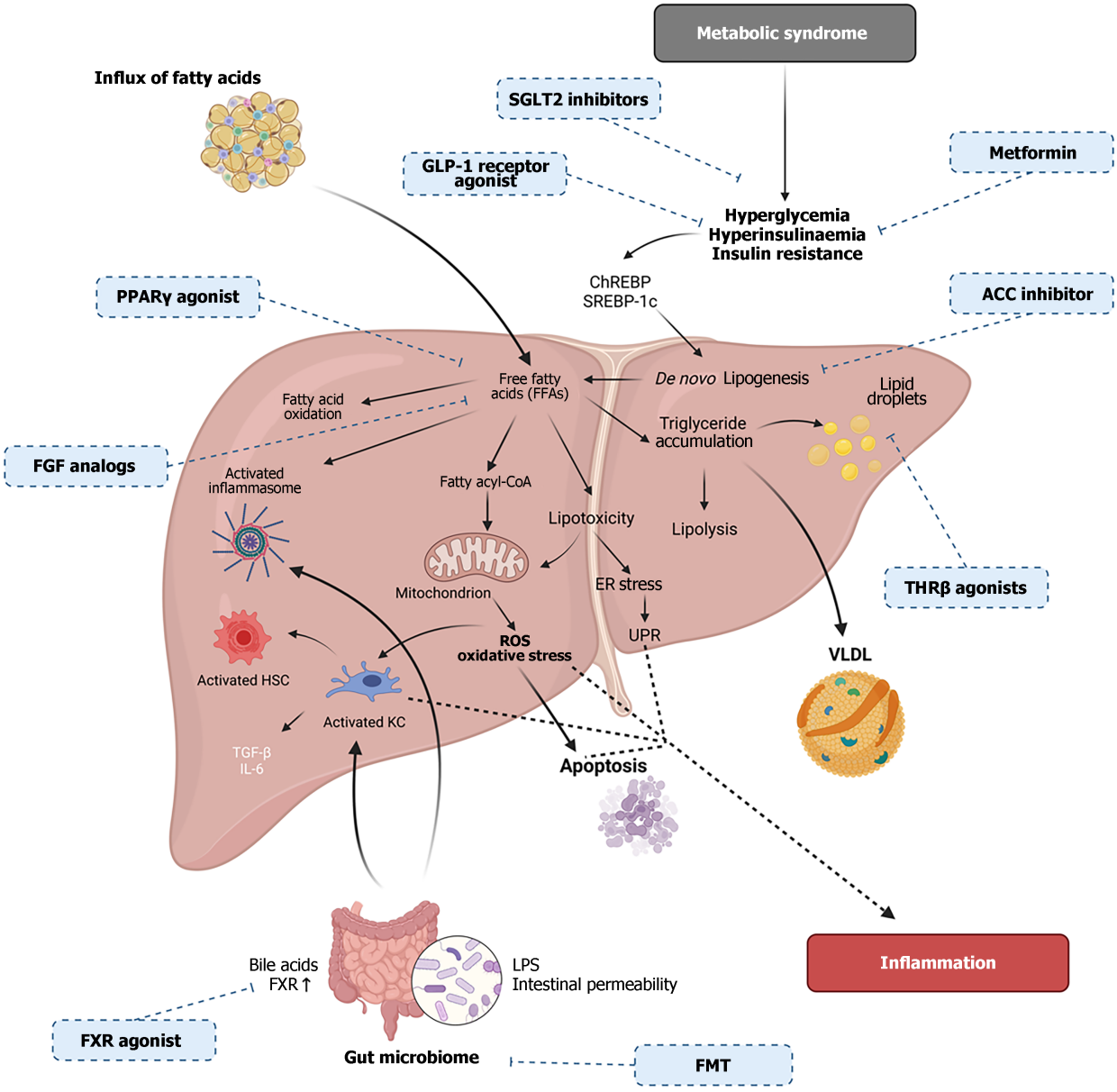

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Figure 4 Overview of the metabolic and molecular mechanisms occurring in non-alcoholic fatty liver disease pathogenesis with therapeutic targets. Non-alcoholic fatty liver disease (NAFLD) is a multifactorial disease, including metabolic syndrome, insulin resistance (IR) and gut microbiome changes. IR and metabolic syndrome cause increased free fatty acids (FFAs) which lead to endoplasmic reticulum stress, reactive oxygen species accumulation, oxidative stress, apoptosis, immune cell activation and inflammasome activation. Changes in the gut barrier can cause changes to bile acids and intestinal permeability causing lipopolysaccharides translocation from the gut causing immune activation, thus, inducing inflammation. FFA's can also activate transcription factors such as sterol regulatory binding protein-1c and carbohydrate response element binding protein causing the storage of FFAs as triglycerides in lipid droplets. Accumulated triglycerides can be exported as very low-density lipoprotein or oxidized by mitochondrial β-oxidation. Pharmacologic treatments for NAFLD are identified with corresponding drug mechanism of action (created with BioRender.com). ROS: Reactive oxygen species; LPS: Lipopolysaccharide; UPR: Unfolded protein response; FMT: Faecal microbiota transplant; VLDL: Very low-density lipoproteins; PPAR-γ: Peroxisome proliferator-activated receptor-γ; KC: Kupffer cells; HSC: Hepatic stellate cells; FXR: Farnesoid X Receptor; SGLT2: Sodium-glucose cotransporter 2; SREBP-1c: Sterol regulatory binding protein-1c; ChREBP: Carbohydrate response element binding protein.

surveillance was impaired[175]. Several pharmaceutical agents have been developed to target PD-1 receptors. Pembrolizumab has shown significant enhancement in overall survival and progression-free survival although statistical significance was not met[176]. However, it is also thought that NASH-derived HCC may be less responsive to immune modulated therapy due to NASH-related aberrant T cell activation, causing damage to tissues[175,177,178]. A summary of current clinical trials is shown in Table 1; and an overview of the metabolic and molecular mechanisms occurring in NAFLD pathogenesis with therapeutic targets in Figure 4.

Table 1 Current active and recruiting clinical trials assessing therapeutics for non-alcoholic fatty liver disease

Drug name	Condition	Target	Clinical trial Number	Phase	Status	Primary endpoint
GH509	NASH NAFLD		NCT05784779	Phase Ib/II	Recruiting	Change in liver fat content assessed by MRI-PDFF
LUM-201	NASH NAFLD	Lipid accumulation	NCT05364684	Phase II	Recruiting	Change in intrahepatic lipid content measured by proton magnetic resonance spectroscopy
Choline	NAFLD	Choline deficiency	NCT05200156	N/A	Recruiting	Change in Thiobarbituric acid reactive substances serum level
Dasatinib and quercetin	Fibrosis	Senescence	NCT05506488	Phase I	Recruiting	Improvement of fibrosis NAFLD score based on histology after 21 wk
GSK4532990	NAFLD fibrosis	17 β-HSD	NCT05583344	Phase II	Recruiting	Improvement of fibrosis measured by clinical research network scoring
Lisinopril	NASH HCC	ACE inhibitor	NCT04550481	Phase II	Recruiting	Changes in fibrosis marker PRO-C3
Rencofilstat	NASH Fibrosis NAFLD	Cyclophilin inhibitor	NCT05402371	Phase II	Recruiting	Improvement in fibrosis score CRN or NASH resolution
Lactobacillus reuteri GMNL-263 and GMNL-89 and lactobacillus rhamnosus GMNL-74	NAFLD	Gut microbiome	NCT05402449	N/A	Recruiting	Changes in serum ALT levels
Lubiprostone	NAFLD	Type 2 chloride channel activator	NCT05768334	Phase III	Recruiting	Changes in liver fat by measured by MRI-PDFF
Bacillus coagulans TCI711	NAFLD	Gut microbiome	NCT05635474	N/A	Recruiting	Changes measured by fibroscan
TVB-2640	NAFLD	FASN inhibitor	NCT04906421	Phase III	Active, not recruiting	Improvement in NAS and CRN scoring
ASC41		THRβ agonist	NCT05462353	Phase II	Recruiting	Improvement in NAS score
Ketohexokinase inhibition	NAFLD		NCT05463575	Phase II	Recruiting	Insulin-mediated suppression of endogenous glucose production
PF-06865571/ PF-05221304	NAFLD NASH with fibrosis	DGAT2 inhibitor/ACC inhibitor	NCT04321031	Phase II	Active, not recruiting	Resolution of NASH
ZED1227		TG2 inhibitor	NCT05305599	Phase II	Recruiting	Relative change of serum PRO-C3 levels
MK-3655	NASH	FGF21 agonist	NCT04583423	Phase II	Active, not recruiting	Resolution of NASH
MXP22 (probiotic and antioxidant capsule)	NAFLD	Gut microbiome	NCT05808049	N/A	Recruiting	Changes in steatosis measure by fibroscan
TERN 501/TERN-101	NASH	THRβ agonist/ FXR agonist	NCT05415722	Phase II	Active, not recruiting	Relative change in liver fat content (MRI-PDFF)
MET642	NASH	FXR agonist	NCT04773964	Phase II	Active, not recruiting	Safety study to measure adverse events
VK2809	NASH	THRβ agonist	NCT04173065	Phase II	Recruiting	Relative change in liver fat content (MRI-PDFF)

MRI-PDFF: Magnetic resonance imaging-estimated proton density fat fraction; NASH: Non-alcoholic steatohepatitis; FXR: Farnesoid X receptor; NAFLD: Non-alcoholic fatty liver disease; THRβ: Thyroid hormone receptor-β; NAS: Non-alcoholic fatty liver disease activity score; HCC: Hepatocellular carcinoma; ALT: Alanine aminotransferase; FASN: Fatty acid synthase; HSD: High-salt diet; CRN: Clinical research network; N/A: Not applicable.

CONCLUSION

NAFLD is a prevalent and progressive disease that can lead to liver damage and is strongly associated with obesity, IR,

and metabolic syndrome. Current treatments for NAFLD include lifestyle modifications such as diet and exercise, surgery, as well as medications to sensitise insulin. However, there is a need for effective and safe interventions that can directly target the underlying mechanisms of NASH/NAFLD in relation to IR, the gut microbiome and immunological mechanisms.

There is a growing body of evidence that NAFLD and metabolic syndrome are closely linked, and it is crucial for future research to prospectively evaluate interventions and therapeutics which both target improvement to liver outcomes as well as comorbidities associated with NAFLD (including cardiovascular diseases, T2DM, renal dysfunction). It is also essential to develop more refined and early risk stratification tools and biomarkers to identify individuals at the highest risk for NAFLD, considering that this condition affects a substantial portion of the world. Understanding the implications of metabolic signatures, chronic insulin signalling, mitochondrial dysfunction and cellular redox, is important for accurate prognosis and potential therapeutics.

Significant progress in the field has been made using bioinformatics to integrate intra- and extra-hepatic signals including gut and adipose interactions as well as patient information regarding lifestyle, nutrition, and comorbidities. The interplay between this multitude of factors provides promise for advancing therapeutics targeting immune regulation and mitochondrial function during progression of NAFLD. Advancements to the field should consider multidisciplinary approaches for the prevention, diagnosis, treatment, and care of patients with NAFLD.

FOOTNOTES

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MINIREVIEWS

Role of non-*Helicobacter pylori* gastric Helicobacters in *helicobacter pylori*-negative gastric mucosa-associated lymphoid tissue lymphoma

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Abstract

Marginal zone lymphomas rank as the third most prevalent form of non-Hodgkin B-cell lymphoma, trailing behind diffuse large B-cell lymphoma and follicular lymphoma. Gastric mucosa-associated lymphoid tissue lymphoma (GML) is a low-grade B-cell neoplasia frequently correlated with *Helicobacter pylori* (*H. pylori*)-induced chronic gastritis. On the other hand, a specific subset of individuals diagnosed with GML does not exhibit *H. pylori* infection. In contrast to its *H. pylori*-positive counterpart, it was previously believed that *H. pylori*-negative GML was less likely to respond to antimicrobial therapy. Despite this, surprisingly, increasing evidence supports that a considerable proportion of patients with *H. pylori*-negative GML show complete histopathological remission after bacterial eradication therapy. Nonetheless, the precise mechanisms underlying this treatment responsiveness are not yet fully comprehended. In recent years, there has been growing interest in investigating the role of non-*H. pylori* gastric helicobacters (NHPHs) in the pathogenesis of *H. pylori*-negative GML. However, additional research is required to establish the causal relationship between NHPHs and GML. In this minireview, we examined the current understanding and proposed prospects on the involvement of NHPHs in *H. pylori*-negative GML, as well as their potential response to bacterial eradication therapy.

Key Words: Lymphoma; B cell; Marginal zone; Gastric mucosa-associated lymphoid tissue lymphoma; *Helicobacter pylori*; Non-*Helicobacter pylori* gastric helicobacters; *Helicobacter heilmannii*; *Helicobacter suis*

Core Tip: Gastric mucosa-associated lymphoid tissue lymphoma (GML) is a type of non-hodgkin lymphoma that arises in the stomach. It has been well-established that *Helicobacter pylori* (*H. pylori*) infection plays a crucial role in the development of GML. However, a subset of patients diagnosed with GML are negative for *H. pylori.* In recent years, there has been growing interest in investigating the role of non-*H. pylori* gastric helicobacters (NHPHs) in the pathogenesis of *H. pylori*-negative GML. This minireview aims to explore the current understanding of the involvement of NHPHs in the development of GML and its potential responsiveness to bacterial eradication therapy.

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INTRODUCTION

Marginal zone lymphomas (MZLs) rank as the third most prevalent form of non-hodgkin B-cell lymphoma, trailing behind diffuse large B-cell lymphoma and follicular lymphoma[1]. The 5th edition of the World Health Organization Classification of Hematolymphoid Tumors-Lymphoid Neoplasms further categorizes MZL into four subtypes: Extranodal MZL of mucosa-associated lymphoid tissue (MALT), primary cutaneous MZL, nodal MZL, and pediatric MZL[2].

Gastric MALT lymphoma (GML) is a low-grade B-cell neoplasia often correlated with *Helicobacter pylori* (*H. pylori*)-induced chronic gastritis[3]. Although the normal gastric mucosa lacks lymphoid follicles, chronic inflammation can lead to the formation of MALT. Continuous antigenic stimulation fosters the clonal expansion of B cells within the MALT, supported by specific T helper cells, which may lead to malignant transformation[4,5]. As GML progresses, genetic and epigenetic alterations occur in both oncogenes and tumour suppressor genes, resulting in dysregulated cell growth and survival. Common genetic alterations seen in MALT lymphoma include chromosomal translocations involving the *API2-MALT1* gene fusion and mutations in genes such as *TP53* and *MYD88*[5-7].

The current clinical guidelines advocate for the use of *H. pylori* eradication therapy as the primary treatment approach for localized GML[8-10]. In a recent systematic review conducted by our group, including meta-analyses, it was highlighted that bacterial eradication treatment resulted in the disappearance of lymphoma in over 75% of patients with low-grade, *H. pylori*-positive GML[11]. Hence, our results ratified that bacterial eradication is effective as the sole initial therapy for early-stage GML.

On the other hand, a specific subset of individuals diagnosed with GML does not exhibit *H. pylori* infection[12-15]. Consequently, it was assumed that these patients might not respond favorably to bacterial eradication therapy. However, another meta-analysis conducted by Jung *et al*[16] showed that 29.3% (95% confidence interval: 22.2%-37.4%, I^2 = 41.5%) of *H. pylori*-negative GML patients experienced complete histopathological remission after eradication therapy. Nonetheless, the underlying mechanisms for this responsiveness remain unclear[16].

There has been a growing interest in exploring the involvement of species of non-*H. pylori* gastric helicobacters (NHPHs) in the development of *H. pylori*-negative GML and its responsiveness to bacterial eradication therapy[17-20]. NHPHs represent a group of bacterial species that colonize the stomach but differ genetically and phenotypically from *H. pylori*[21-24]. These differences include variances in flagella, urease activity, and other virulence factors[25,26]. While NHPHs have been detected in some patients with gastritis and peptic ulcers, their precise role and contribution to disease progression are not yet fully understood[27].

Some studies have indeed suggested an association between specific NHPH species and the development of GML, particularly in *H. pylori*-negative cases[28]. However, further research is required to establish a definitive causal relationship between NHPHs and GML. This article aims to explore the current understanding and propose prospects on the role of NHPHS in *H. pylori*-negative GML and its potential responsiveness to bacterial eradication therapy.

H. pylori-negative GML

H. pylori-negative GML accounts for around 10% of all GML cases[29-31]. The cause of *H. pylori*-negative GML is not fully understood, and ongoing research aims to uncover the underlying factors contributing to its development. Symptoms of this type of lymphoma, such as abdominal pain, indigestion, bloating, nausea, vomiting, and weight loss, are similar to other gastric lymphomas but are nonspecific and can be caused by various conditions[32]. Diagnosis is made based on morphologic, immunophenotypic, and genetic analysis of biopsy material. Once the diagnosis is confirmed, a staging procedure to evaluate the extent of lymphoma dissemination is imperative[33].

In contrast to its *H. pylori*-positive counterpart, *H. pylori*-negative GML was previously believed to have a reduced likelihood of responding to antimicrobial therapy. In this context, treatment options may involve watchful waiting, radiation therapy (RT), chemotherapy (ChT), and immunotherapy[9]. Watchful waiting is suitable for slow-growing

lymphomas without significant symptoms and with regular monitoring[33]. However, RT is the preferred treatment for localized disease in the management of *H. pylori*-negative GML. Several series have reported excellent disease control using RT alone, highlighting the efficacy of moderate-dose involved-field RT. Typically, a dose of 24-30 Gy is delivered to the stomach and perigastric nodes throughout 3-4 wk. To achieve optimal outcomes in gastric extranodal MZL[34,35]. Systemic treatment with ChT, immunotherapy, or a combination of both (chemoimmunotherapy) is recommended for patients with symptomatic systemic disease, contraindications to RT, treatment failure following antibiotic therapy or local treatments (such as RT or surgery), and those with histological transformation[36].

Despite this, surprisingly, increasing evidence supports that a considerable proportion of patients with *H. pylori*-negative GML show complete histopathological remission after bacterial eradication therapy[16,28,37]. Nonetheless, the precise mechanisms underlying this treatment responsiveness are not yet fully comprehended. Initially, it was attributed to false-negative tests for *H. pylori*[8,37]. However, more recently, the infection with other *Helicobacter* species (NHPHs) is acknowledged as a potential explanation for this phenomenon.

NHPHs

The *Helicobacter* genus includes gram-negative, microaerophilic, spiral, helical, curved, or fusiform rod-shaped bacteria that inhabit the gastrointestinal tract of several animals, such as humans, cats, dogs, pigs, and mice[38,39]. Currently, 53 species with validly published names comprise this genus[40], with *H. pylori* being the most prevalent in humans and well-known to be related to the development of chronic gastritis, peptic ulcer, and gastric cancer[41-43]. However, emerging evidence has highlighted the potential role of NHPHs in the progression of these diseases, including GML[24,44-46].

Among the NHPHs, *H. suis*, *H. heilmannii*, *H. felis*, *H. salomonis* and *H. bizzozeronii* are the most common species associated with human infection[47,48]. According to Yakoob *et al*[49], the prevalence of *H. heilmannii* and *H. felis* among patients with dyspepsia was 6% and 4%, respectively. On the other hand, Øverby *et al*[48] revealed a prevalence of gastric NHPH in Japanese patients of 6.1% and within this group, *H. suis* was the most prevalent, followed by *H. heilmannii*. This latter finding agrees with Nakamura *et al*[50], who found a prevalence of NHPHs of 20.8% in gastric mucosal samples of *H. pylori*-negative gastric disease patients, with *H. suis* and *H. heilmannii* also as the most prevalent species. However, it is important to note that the current diagnostic methods available, such as polymerase chain reaction (PCR) and immunohistochemistry, have limited accuracy in detecting NHPHs infections[26]. In a specific study, researchers faced difficulties in identifying the species associated with the infection in approximately 50% of the cases[50]. This challenge can be attributed to several factors, including the high genetic similarity between different NHPH species, significant genetic variation within a single species, limitations imposed by identification methods, and the concurrent presence of multiple NHPH species[51-53]. As a result, there is a concern that the actual prevalence of NHPHs infections among patients with dyspepsia may be underestimated.

Regarding the association of NHPHs with GML, some studies have evaluated the prevalence of infections by these species and its correlation with the complete remission of *H. pylori*-negative GML through eradication therapy. In this regard, Takigawa *et al*[54] report that the rate of complete remission in NHPH positive group of patients was significantly higher (75%) when compared to the negative cases (23%) of *H. pylori*-negative GML, which suggests a potential role for NHPHs in the pathogenesis of GML and the treatment effectiveness of *H. pylori*-negative GML. Such data are corroborated by Morgner *et al*[17], which advocate that *H. heilmannii* infection might be a causative factor in GML and that the current eradication therapy employed for *H. pylori* (standard antibiotics combined with proton pump inhibitors) is effective and results in complete remission of the lymphoma[17]. Nevertheless, upon confirming the presence of NHPH infection, it is strongly advised to implement a therapeutic approach that is tailored to the susceptibility profile of the individual bacterium.

Pathogenesis of GML

The pathogenesis of GML is a complex event that involves antigen-induced transformation of normal marginal-zone B-cells into malignant cells[55]. In contrast to MALT lymphomas observed in various locations, GML is distinguished by its association with specific microbial species: Primarily, *H. pylori*, and to a lesser extent, *Helicobacter heilmannii*[17,54,56]. Under normal physiological conditions, the stomach does not possess MALT. However, in the presence of chronic antigenic stimulation, gastric mucosal cells produce proinflammatory cytokines (such as lymphotoxin beta) and B-cell homing factors (*e.g.*, bicinchoninic acid-1), leading to the infiltration of lymphoid cells into the gastric tissue. This cascade of events leads to the development of MALT[32,57,58] (Figure 1).

Regarding *H. pylori* infection, it is well-established that certain T helper cells target specific epitopes of the bacterium and support polyclonal B cells[59,60]. These B cells possess receptors that are able recognize autoantigens found in the gastric mucosa due to cross-reactivity. Consequently, the polyclonal B cell population undergoes expansion and a selection process, resulting in the emergence of an antigen-dependent MZL clone[61,62].

Sustained antigenic exposure not only stimulates the proliferation of a diverse array of B cells but also attracts neutrophils to the site of inflammation. The inflammatory process initiates the release of reactive oxygen species, leading to the occurrence of various genetic abnormalities[55,63,64]. Furthermore, the persistent proliferation of B cells during chronic inflammation increases the risk of double-stranded DNA breaks and translocations[5] (Figure 2).

Likewise, the involvement of NHPHs in *H. pylori*-negative GML could also be attributed to the induction of chronic inflammation, resulting in the local aggregation and proliferation of antigen-dependent B cells and T cells. Indeed, the infection of mice with NHPHs species, including *H. felis*, *H. suis* and *H. heilmannii*, also leads to a similar process of chronic gastritis and GML development with similarities to the human disease[65-69]. Possibly, the inflammatory microenvironment associated with NHPH-induced gastritis also facilitates the acquisition of genetic abnormalities by B cell clones. Nevertheless, further studies are required to construct a more comprehensive pathogenesis model.

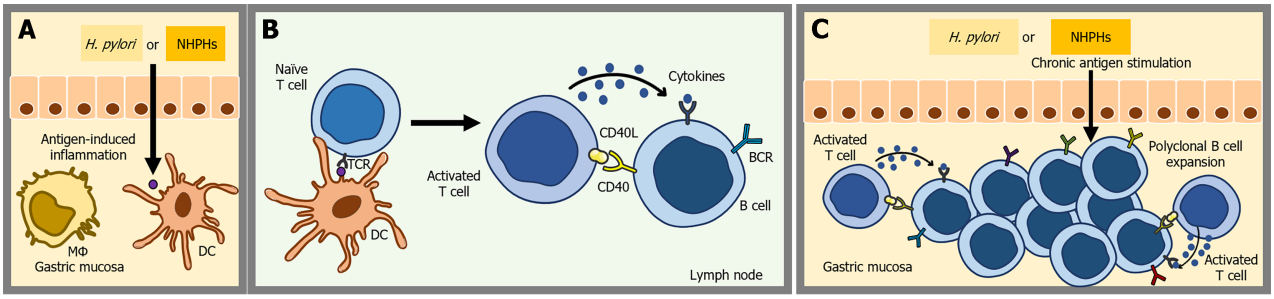

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Figure 1 Antigen-induced acquisition of gastric mucosa-associated lymphoid tissue. A: Antigen-induced inflammation; B: Clonal expansion of B cells supported by specific T helper cells; C: Acquisition of mucosa-associated lymphoid tissue (MALT). In the presence of chronic antigenic stimulation, gastric mucosal cells undergo activation and produce proinflammatory cytokines. These molecular mediators play a crucial role in initiating and perpetuating an immune response within the gastric tissue. As a consequence, lymphoid cells are recruited and infiltrate the gastric tissue. This cascade of events ultimately culminates in the development of MALT. *H. pylori: Helicobacter pylori*; NHPHs: Non-*Helicobacter pylori* gastric helicobacters; DC: Dendritic cell; MΦ: Macrophage; TCR: T cell receptor; CD40: Cluster of differentiation 40; CD40L: Cluster of differentiation 40 Ligand; BCR: B cell receptor.

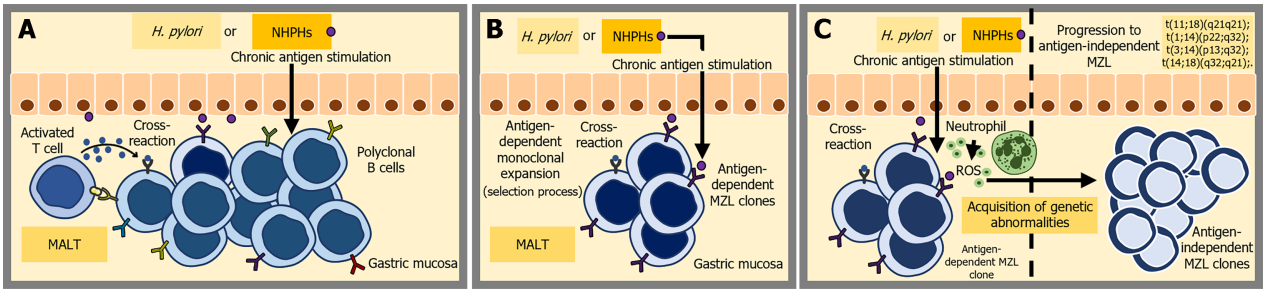

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Figure 2 Simplified scheme of antigen-induced transformation of normal marginal-zone B-cells into malignant cells. A: Polyclonal B cell expansion and a selection process; B: Antigen-dependent monoclonal expansion; C: Acquisition of genetic abnormalities and antigen-independent lymphomagenesis. The proliferation of B cells is primarily induced by the interaction between CD40 and CD40 Ligand, facilitated by antigen-activated reactive T cells. Additionally, cytokines play a role in driving this B-cell proliferation. The persistent proliferative state of these B cells, along with chronic inflammation, triggers additional oncogenic events. Ultimately, these events lead to the development of antigen-independent lymphoproliferation. NHPHs: Non-*Helicobacter pylori* gastric helicobacters; ROS: Reactive oxygen species; MZL: Marginal zone lymphoma; MALT: Mucosa-associated lymphoid tissue; *H. pylori: Helicobacter pylori*.

Irrespective of etiology, progression towards antigen-independent MZL is associated with genetic events, while the role of direct antigenic stimulation gradually decreases in the development of GML[5,70] (Figure 2). Four recurrent chromosomal translocations have been found in MZL: *t* (1; 14) (p22; q32), *t* (11; 18) (q21; q21), *t* (14; 18) (q32; q21), and *t* (3; 14) (p14.1; q32)[71-73]. In GML, the translocation *t* (11; 18) (q21; q21) is the prominent structural chromosomal abnormality, occurring in approximately 10%-50% of cases[74-76]. This translocation results in the activation of NF-kappaB, which is a downstream target of B-cell receptor (BCR) signaling, independent of BCR signaling itself. The activation is mediated by the disruption of a signalosome complex involving CARD11, BCL10, and MALT1. Within this context, the presence of the MALT1 fusion protein is notably linked to more advanced stages of MALT lymphoma[77-80].

Indeed, numerous studies have demonstrated that GMLs harboring the *t* (11; 18) (q21; q21) translocation are frequently resistant to *H. pylori* eradication treatment compared to tumors that do not possess this specific translocation[11,81,82]. The decrease in the rate of complete histopathological remission following eradication therapy was also observed in *H. pylori*-negative GML cases; however, its influence on the treatment of NHPH-positive GML is still unclear[16].

Clinical implications and research prospects

Given the limited regression observed in *H. pylori*-negative GML after antibiotic treatment, clinical guidelines previously advised prompt initiation of targeted anti-lymphoma treatments[8,83]. Currently, the European Society for Medical Oncology Guidelines Committee suggests that a trial of anti-*Helicobacter* therapy may be worthwhile in *H. pylori*-negative early-stage GML (stages I and II_1)[9]. This recommendation presents new opportunities for research in this field. Specifically, future studies could focus on investigating the mechanisms underlying the response to this therapy and further exploring the involvement of other *Helicobacter* species (NHPHs) in the development of *H. pylori*-negative GML. Additionally, it is crucial to investigate the long-term outcomes and assess the effects of early intervention with targeted anti-lymphoma treatments on patient prognosis.

In this context, accurate detection of NHPHs is vital for precise clinical diagnosis and targeted treatment strategies. However, current diagnostic methods primarily focus on *H. pylori*, leaving a gap in the detection of NHPHs infections. Goji *et al*[26] conducted a review of 26 articles and determined that the sensitivities of diagnostic methods for *H. pylori* infection, such as the rapid urease test, urea breath test, blood antibody analysis, immunohistochemical analysis, and

stool antigen analysis, were low for NHPHs. The calculated sensitivities were only 40.0%, 14.8%, 23.1%, 40.0%, and 0%, respectively[26]. Therefore, at present, the most effective diagnostic tools for identifying NHPH infections are histological techniques and genetic diagnosis based on PCR, which hinders the clinical diagnosis of NHPHs infection, both due to the inflated cost and the dependence on laboratory apparatus. To address this, the development of tests that possess sensitivity, specificity, and the ability to detect different strains of NHPHs is crucial. The availability of reliable diagnostic methods for NHPHs will not only enable timely diagnosis and treatment for *H. pylori*-negative GML, but also contribute to a better understanding of their epidemiology and impact on human health.

When it comes to comprehending the pathogenesis of NHPH-positive GML, the significance of molecular and immunological studies cannot be overstated. These investigations should encompass the analysis of gene expression profiles in affected tissues, identification of pertinent genetic mutations, and study of cellular signaling pathways involved in the development and progression of the lymphoma. Additionally, it would also be interesting to analyze cytokine profiles, characterize immune cells infiltrating the affected gastric tissue, and conduct studies on the interaction between NHPHs and the host immune system. This deeper understanding might open doors to the development of targeted therapeutic strategies and hold promise for improved clinical outcomes in patients with NHPH-positive GML.

CONCLUSION

While *H. pylori* remains the primary pathogenic factor in the development of GML, the role of NHPHs in *H. pylori*-negative cases is an emerging area of research. It is crucial to identify these alternative pathogens and understand their mechanisms of pathogenesis to improve diagnostic accuracy and guide appropriate treatment strategies for patients with *H. pylori*-negative GML. Further research is warranted to elucidate the complex interplay between these bacteria, the host immune system, and the gastric microenvironment, which may lead to the development of novel therapeutic interventions and personalized approaches for this subset of patients.

FOOTNOTES

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ORIGINAL ARTICLE

Basic Study

Linolenic acid-metronidazole inhibits the growth of *Helicobacter pylori* through oxidation

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Abstract

BACKGROUND

Resistance to antibiotics is one the main factors constraining the treatment and control of *Helicobacter pylori* (*H. pylori*) infections. Therefore, there is an urgent need to develop new antimicrobial agents to replace antibiotics. Our previous study found that linolenic acid-metronidazole (Lla-Met) has a good antibacterial effect against *H. pylori,* both antibiotic-resistant and sensitive *H. pylori*. Also, *H. pylori* does not develop resistance to Lla-Met. Therefore, it could be used for preparing broad-spectrum antibacterial agents. However, since the antibacterial mechanism of Lla-Met is not well understood, we explored this phenomenon in the present study.

AIM
To understand the antimicrobial effect of Lla-Met and how this could be applied in treating corresponding infections.

METHODS
H. pylori cells were treated with the Lla-Met compound, and the effect of the compound on the cell morphology, cell membrane permeability, and oxidation of the bacteria cell was assessed. Meanwhile, the differently expressed genes in *H. pylori* in response to Lla-Met treatment were identified.

RESULTS
Lla-Met treatment induced several changes in *H. pylori* cells, including roughening and swelling. *In vivo* experiments revealed that Lla-Met induced oxidation, DNA fragmentation, and phosphatidylserine ectropionation in *H. pylori* cells. Inhibiting Lla-Met with L-cysteine abrogated the above phenomena. Transcriptome analysis revealed that Lla-Met treatment up-regulated the expression of superoxide dismutase *SodB* and *MdaB* genes, both anti-oxidation-related genes.

CONCLUSION
Lla-Met kills *H. pylori* mainly by inducing oxidative stress, DNA damage, phosphatidylserine ectropionation, and changes on cell morphology.

Key Words: *Helicobacter pylori*; Oxidation; Superoxide dismutase; *SodB* genes; *MdaB* genes

Core Tip: The clarithromycin resistant *Helicobacter pylori (H. pylori)* is listed by the World Health Organization as the priority bacteria in urgent need of developing new antibiotics. Our previous research found that linolenic acid-metronidazole has a good antibacterial effect on *H. pylori* and is not easy to develop drug resistance. Therefore, we further explored its antibacterial mechanism against *H. pylori*. It was found that it mainly kills *H. pylori* by inducing oxidative stress, DNA damage, phosphatidylserine ectropionation, and changes on cell morphology. This study may provide a theoretical basis for the development and application of new anti *H. pylori* lead compound.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is the main pathogen that causes upper digestive diseases, such as chronic gastritis, peptic ulcer, and gastric cancer[1-4]. At present, the treatment options for *H. pylori* infections include standard triple therapy, bismuth-containing quadruple therapy, and sequential therapy[5,6]. Due to the overuse and misuse of antibiotics, the drug resistance rate of *H. pylori*, including multi-drug resistance, is gradually increasing, negatively impacting the control and treatment of *H. pylori* infections[7-10]. Therefore, there is an urgent need to develop new anti-*H. pylori* agents[11].

Due to the long period and significant investment required for developing new antibiotics, the transformation or modification of the existing drugs is more efficient in shortening the drug research and development cycle. Modifying existing drugs could improve their efficacy while reducing the development of antimicrobial resistance. Zinc linolenic acid and liposome linolenic acid are linolenic acid derivatives effective at increasing the sensitivity of drug-resistant *H. pylori*. Resistance against zinc linolenic acid and liposome linolenic acid is minimal[12,13]. Although metronidazole is a widely used and cost-effective drug, its clinical application for *H. pylori* infection treatment is limited by resistance development.

Our previous study found that the minimum inhibitory concentration (MIC) of linolenic acid-metronidazole (Lla-Met) against six strains of the drug-resistant *H. pylori* was 2-4 μg/mL. Additionally, the *H. pylori* strains did not develop resistance against this compound. Therefore, Lla-Met would serve as promising antibiotics. However, its antibacterial mechanism is poorly understood[14], this study explored this mechanism.

MATERIALS AND METHODS

Materials

H. pylori strain G27 (Courtesy of Prof. Bi Hongkai, Nanjing Medical University), calf serum, a Columbia blood agar base, a brain heart infusion (BHI,OXOID) medium, L-cysteine (L-cys) (AR 99%, MACKLIN), a fluorescence orthomicroscope (OLYMPUS, Tokyo, Japan), reactive oxygen species (ROS) detection kits (Beyotime), cell apoptosis 4',6-diamidino-2-phenylindole (DAPI) detection kits (Beyotime), apoptosis detection kits (Beyotime), reverse transcription kits (Monad), reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) kits (Monad), a Lightcycler96 fluorescence ration PCR instrument (Roche, Germany), and a scanning electron microscope were used in the present study.

Thawing and culture of H. pylori strain

Standard *H. pylori* strain G27 stored at -80 °C were thawed and centrifuged to remove the preservation solution (Glycerin:BHI:serum = 3:6:1). The bacteria were inoculated on a Columbia agar medium, or a brain heart infusion medium supplemented with 10% calf serum and cultured in a microaerophilic environment.

Cell morphology assay

The effect of the Lla-Met on *H. pylori* morphology was observed by scanning electron microscopy[15-18]. *H. pylori* was treated with 4 and 8 μg/mL of Lla-Met and incubated for 24 h in a three-gas incubator. The bacteria were pelleted by centrifugation and fixed overnight with 2.5% glutaraldehyde. The bacteria suspension was centrifuged to remove glutaraldehyde before dehydration with 30%, 50%, 70%, 90%, and 100% ethanol. The pellet was dried through refrigeration in a vacuum. After that, the *H. pylori* morphology was observed and photographed under a KYKY-EM8100 scanning electron microscope (KYKY, Beijing).

Membrane damage assay

The *H. pylori* cells were stained as previously described by Hwang *et al*[19]. Briefly, the G27 bacterial suspension (1×10^8 CFU/mL) at the logarithmic phase was treated with Lla-Met for 2 h at the rate of 16 μg/mL. The cell suspension was centrifuged at 12000 r/min for 2 min to pellet the cells. The medium was poured out, and the harvested cells were suspended in phosphate buffered saline (PBS). The cells were stained with a propidium iodide solution (PI, 10 μg/mL, Thermo Fisher) at 37 °C protected from light 30 min and thereafter centrifuged at 12000 r for 5 min. The dye unbound to the harvested cells was washed away with sterile PBS. Thereafter, the cells were suspended in PBS and immediately observed under a fluorescence microscope (Olympus Corporation, Tokyo, Japan).

Cell membrane pore size assay

FITC-FD was mainly used to evaluate the degree of *H. pylori* cell membrane damage after treatment with Lla-Met. The process was performed as previously described[20]. Briefly, G27 bacterial suspension (1×10^8 CFU/mL) at the logarithmic phase was incubated with Lla-Met (16 μg/ML) for 2 h, centrifuged, and the pellet was suspended in sterile PBS. The cell suspension was protected from light with FIFC-labeled glucan FD4, about 4.0 kDa, with a diameter of 1.4 nm (Sigma, United States) and FD10, about 10.1 kDa, with a diameter of 2.3 nm (Sigma, United States), both at a final concentration of 100 μg/mL. After 30 min of incubation at 37 °C, the unbound fluorescent dye was washed off with sterile PBS. The cells were suspended in sterile PBS. Finally, the fluorescence influx of FD4 and FD10 was detected at the excitation and emission wavelengths of 495 nm and 520 nm, respectively, by a multimode reader (BioTek, America).

Ion channels assay

The G27 bacterial suspension (1×10^8 CFU/mL) at the logarithmic phase was incubated with Lla-Met (16 μg/mL) for 2 h and centrifuged at 12000 r/min for 2 min. The changes in concentrations of extracellular K^+, Na^+, Cl^-, and Ca^{2+} were determined by ion-selective electrodes. Three biological repeats were performed for each experiment.

Intracellular reactive oxygen assay

The level of intracellular ROS in the *H. pylori* cell was detected by the DC-FDH probe, as described by Akhtar *et al*[21-22]. Briefly, G27 bacterial suspension (1×10^8 CFU/mL) was treated with Lla-Met (8 μg/mL and 16 μg/mL) for 2 h and centrifuged to remove the supernatant. The harvested cells were protected from light DCF-DA (10 μM) for 30 min and centrifuged at 12000 r for 2 min. The excess probe was washed off with sterile PBS. Finally, the cells were resuspended in PBS solution, and the fluorescence intensity was analyzed using a multifunctional microplate reader (BioTek, America) at the excitation and emission wavelengths of 485 nm and 520 nm, respectively. The fluorescence intensity was also analyzed using a fluorescent microscope (OLYMPUS, Tokyo, Japan). PBS and polymyxin were the controls.

Validation of how Lla-Met kills H. pylori

The G27 bacterial suspension was incubated with L-cys, a ROS scavenger, to evaluate the effect of ROS on the viability of G27 bacteria. Briefly, G27 bacterial suspension (1×10^8 CFU/mL) at the logarithmic phase was incubated with or without 40 mmol/L L-cys (which did not affect the viability of G27 cells) for 1 h and thereafter with Lla-Met (16 μg/mL) for 8 h. The optical density values were measured at $OD_{600\ nm}$ using a multifunctional microplate reader (BioTek, America). In addition, the fluorescence intensity was analyzed using fluorescence microscopy (OLYMPUS, Tokyo, Japan). G27 bacterial suspension treated with or without 40 mmol/L L-cys was incubated for 1 h. The suspension was treated with

Lla-Met (16 μg/mL) for 2 h and centrifuged to remove the supernatant. The harvested bacteria were incubated with DCF-DA (10 μM) for 30 min and washed with sterile PBS.

DNA fragmentation assay

When DAPI passes through the intact cell membrane, it binds to bacterial DNA. The bacterial DNA thus stains blue. Damaged DNA appears as dots. Therefore, the fragmentation of *H. pylori* DNA after Lla-Met treatment was detected using the DAPI staining[23]. Briefly, the G27 bacterial suspension (1×10^8 CFU/mL) was incubated with or without 40 mmol/L L-cys for 1 h and thereafter with Lla-Met (16 μg/mL) for 2 h. Thereafter, the suspension was centrifuged to remove the supernatant and treated with DAPI (1 μg/mL) for 30 min. The unbound dye was washed off with PBS, and the cells were resuspended in PBS. Fluorescence intensity was determined at excitation and emission wavelengths of 358 nm and 460 nm, respectively (OLYMPUS, Tokyo, Japan), with PBS as a control.

Phosphatidylserine ectropion assay

Phosphatidylserine (PS) is usually located on the inner side of the cell membrane. At the early stage of apoptosis, PS is translocated to the cell surface. Annexin-V, a $Ca2^+$-dependent phospholipid binding protein, bind to PS with high affinity [24]. The G27 bacterial suspension in the logarithmic phase (1×10^8 CFU/mL) was incubated with or without 40 mmol/L L-cys for 1 h and thereafter with Lla-Met (16 μg/mL) for 2 h. Thereafter, the suspension was centrifuged to remove the supernatant, and the pellet was incubated with Annexin-V and incubated for 30 min. The unbound dye was washed off with PBS, and the cells were resuspended in PBS. The fluorescence intensity was analyzed at excitation and emission wavelengths of 490 nm and 520 nm, respectively, using a multifunctional microplate reader (BioTek, America) and a fluorescence microscope (OLYMPUS, Tokyo, Japan), with PBS as a control.

Transcriptome sequencing

G27 bacterial suspension (1×10^8 CFU/mL) (OD_{600} = 0.3) was incubated with 2, 4, 8, and 10 μg/mL Lla-Met for 0 h, 2 h, and 8 h, and the ODs were measured at 600 nm. Three biological repeats were performed for each experiment. When the OD remained constant (0.3), the bacterial RNA was extracted for transcriptome sequencing, which was performed by Nanjing Fengzi Bio-pharm Technology. Three biological repeats were performed for each experiment.

The sequencing was performed using Illumina PE150 technology. The alignment and transcript assembly were performed using Boetie2 and the Rockhhoper software. All genes were quantitatively analyzed, and the differentially expressed genes (DEGs) were identified. The biological processes and pathways regulated by the DEGs were then identified. Principal component analysis (PCA) demonstrates principal component analysis, analyzing the composition of different samples can respond to the differences and distances between samples, the more similar the sample composition, the closer the distance in the PCA graph.

Validation of differential gene expression

Total bacterial RNA was extracted using a Novizan RNA kit, and the expression of mRNA was analyzed by a real-time fluorescent quantitative PCR instrument (Lightcycler96 fluorescent quantitative PCR instrument, Roche, Germany). The 16s was used as the reference gene. The sequences of primers used in this study are shown Table 1. Three biological repeats were performed for each experiment.

Statistical analysis

Statistical analysis was performed using the SPSS software, Version 26.0. Continuous data were expressed as mean ± SD. Differences between groups were analyzed using the *t*-test, while multiple groups were compared using the single factor variance analysis. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of Lla-Met on H. pylori morphology

The impact of the Lla-Met compound on the morphology of *H. pylori* was observed using scanning electron microscopy. *H. pylori* in the control group was found to have a smooth and homogenous cell surface (Figures 1A and D). The surface of *H. pylori* in the treatment group (4 μg/mL and 8 μg/mL) was rough and swollen, and the cell damage worsened with the Lla-Met concentration (Figures 1B, C, E, and F).

Lla-Met impact of cell membrane integrity

PI penetrates through a damaged cell membrane, where it binds and stains the DNA. Therefore, an influx of intracellular PI represents the integrity of the bacterial cell membrane. The fluorescence intensity of PI in the G27 treatment group (16 μg/mL Lla-Met) was weaker than in the control group, though statistically insignificant (Figure 2A).

FITC-labelled glucans of different pore sizes (FD4 and FD10) were detected using a multifunctional microplate detector to examine damage to the *H. pylori* cell membrane after treatment with Lla-Met. Linolenic acid treatment had no significant effect on the permeability of *H. pylori* cells (Figures 2B and C).

Table 1 List of primers used in this study

Name	Forward primers	Reverse primers
16s	AGGATCAAGGTTTAAGGATT	CTGGAGACTAAGCCCTCC
MdaB	AGGCTATGAACACGCTCAAGAAGTG	TTTCACAATCCAAGGCTCTCCCATC
SodB	AAGCGACTGCCTTAAGCGATGAG	TCCAGCCAGAGCCAAACAAAGTG

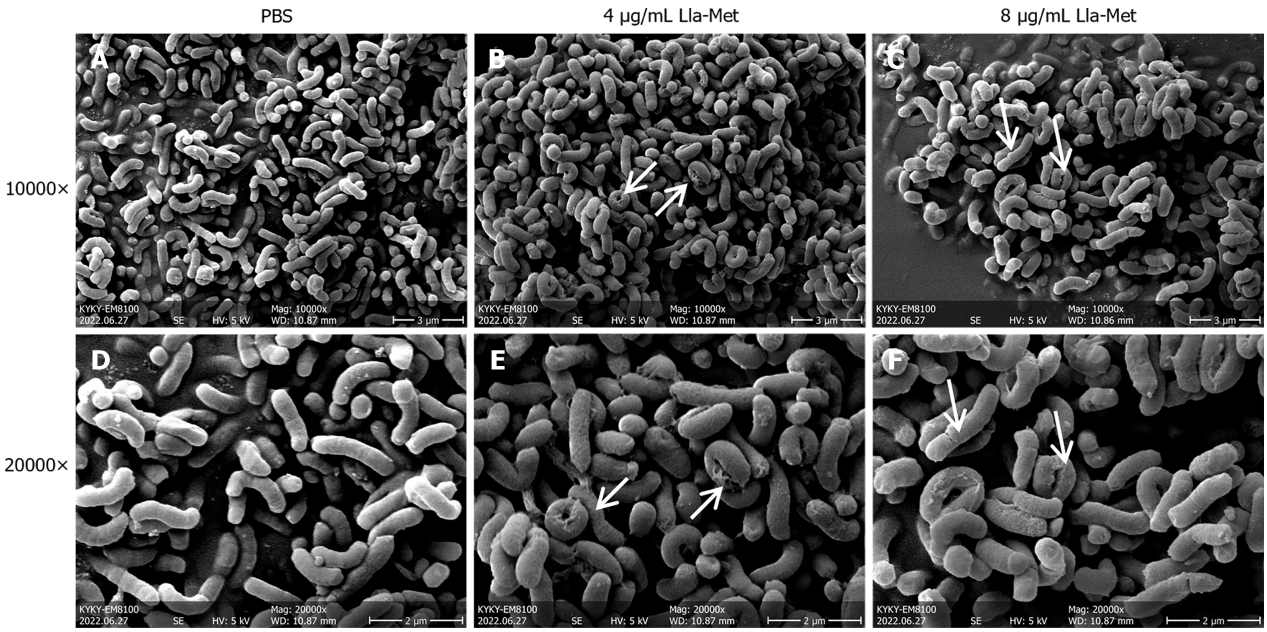

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Figure 1 The effect of linolenic acid-metronidazole on *Helicobacter pylori* morphology. A: The control group shows the cell morphology at × 10000 magnification; B: The appearance of *Helicobacter pylori* (*H. pylori*) in linolenic acid-metronidazole concentrations of 4 μg/mL shows the cell morphology at × 10000 magnification; C: The appearance of *H. pylori* in linolenic acid-metronidazole concentrations of 8 μg/mL shows the cell morphology at × 10000 magnification; D: The control group shows the cell morphology at × 20000 magnification; E: The appearance of *H. pylori* in linolenic acid-metronidazole concentrations of 4 μg/mL shows the cell morphology at × 20000 magnification; F: The appearance of *H. pylori* in linolenic acid-metronidazole concentrations of 8 μg/mL shows the cell morphology at × 20000 magnification. The arrow points to the cell damage. Roughness, swelling, breakages on the cell surface, *etc.*, are shown. PBS: Phosphate buffered saline; Lla-Met: Linolenic acid-metronidazole.

Lla-Met impact on H. pylori ion channels

To further investigate whether Lla-Met compound penetrated the cells *via* ion channels, the concentrations of K^+, Na^+, Cl^-, and Ca^{2+} ions in the supernatant were measured after treating *H. pylori* with Lla-Met. There was no statistically significant difference in the concentrations of the aforementioned ions between the treatment and the control group after G27 was applied with 16 μg/mL compound (Figures 3A and B).

Lla-Met impact on the intracellular ROS

DC-FDA fluorescent probes can be used to detect whether Lla-Met compounds can accelerate intracellular oxidation reactions. Compared with the control group, 8 μg/mL and 16 μg/mL Lla-Met compound increased the intracellular oxidation in *H. pylori* cell. Moreover, 8 μg/mL Lla-Met was more potent than 16 μg/mL metronidazole (Figure 4A); The relative fluorescence intensity of *H. pylori* treated with 16 μg/mL Lla-Met was stronger than that treated with 40 μg/mL polymyxin B ($P < 0.01$, Figure 4D). However, L-cys treatment abrogated the effect of Lla-Met (Figures 4B and D, $P < 0.01$), implying that L-cys abolished ROS generated by Lla-Met in *H. pylori*. In addition, L-cys pretreatment increased the cell viability from 20.5% to 57.7% (Figure 4C).

Lla-Met induced DNA fragmentation and apoptosis

In the early stage of apoptosis, PS translocates to the cell surface, where it could be bound by Ca^{2+}-dependent phospholipid-binding protein. Thus, the phospholipid-binding protein could be used for analyzing cell apoptosis in prokaryotes. In the present study, we found that the fluorescence intensity of *H. pylori* treated with 16 μg/mL Lla-Met was higher than that of the untreated group and the L-cys pretreatment group (Figure 5A). The multifunctional microplate labelling instrument results showed that the relative fluorescence intensity of Lla-Met-compound treatment group was significantly stronger than that of the PBS group and L-cys pretreatment group (Figure 5C, $P < 0.0001$). These results indicated that linolenic-acid-metronidazole caused PS eversion, but L-cys pretreatment inhibited this

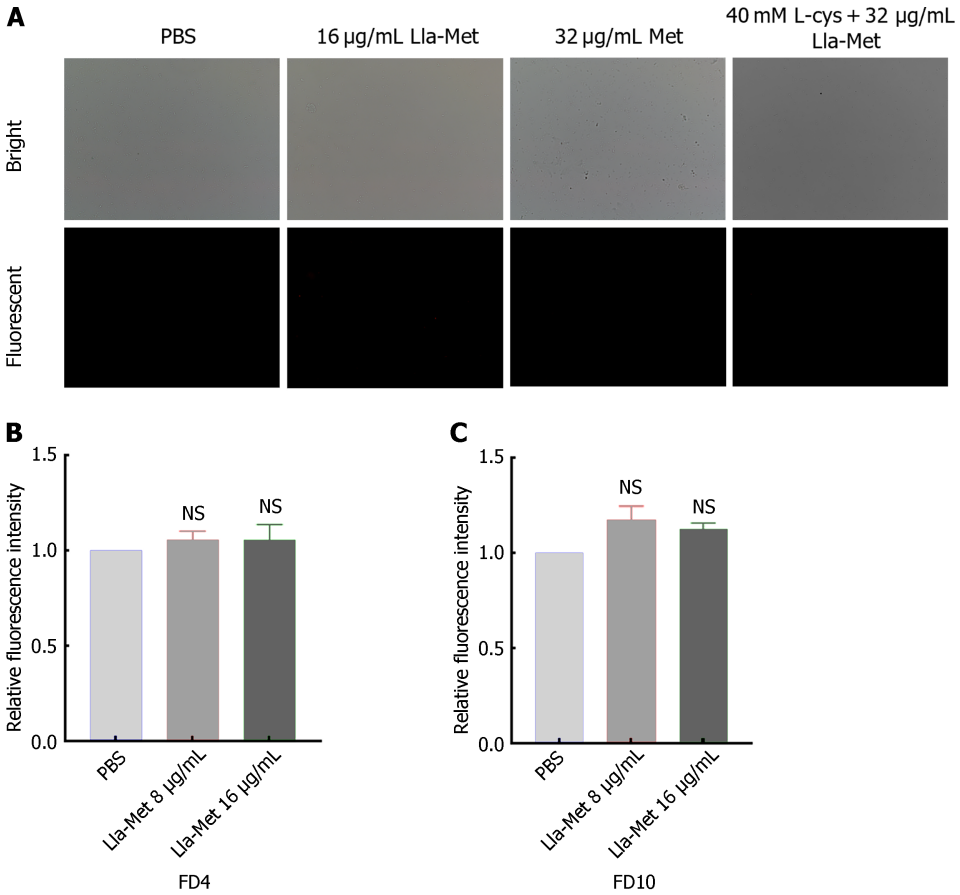

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Figure 2 The effect of linolenic acid-metronidazole on cell membrane permeability. A: Linolenic acid-metronidazole (Lla-met) induced membrane damages of *Helicobacter pylori* (*H. pylori*) using PI staining; B: Lla-met induced membrance pore size damage of *H. pylori* using FD4; C: Lla-met induced membrance pore size damage of *H. pylori* using FD10. PI, FD4 and FD10 can't pass through the intact cell membrane. NS: Not significant; PBS: Phosphate buffered saline; Lla-Met: Linolenic acid-metronidazole.

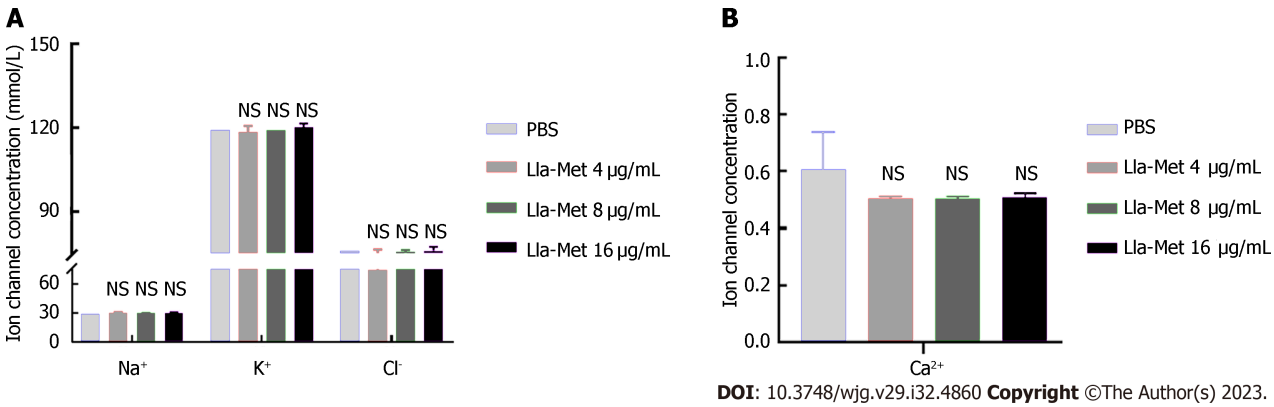

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Figure 3 The effect of the linolenic acid-metronidazole compound on *Helicobacter pylori* ion channels. A: K^+, Na^+, Cl^-, and Ca^{2+} ion channels; B: Ca^{2+} ion channels. NS: Not significant; PBS: Phosphate buffered saline; Lla-Met: Linolenic acid-metronidazole.

phenomenon.

Subsequently, cellular DNA fragmentation serves as a marker of late apoptosis. The DAPI staining evaluated whether Lla-Met compound could cause the fragmentation of *H. pylori* DNA. The results showed that 16 μg/mL Lla-Met caused the fragmentation of bacterial *H. pylori* DNA (Figure 5B red circles represent the fragmented DNA). However, DNA fragmentation was inhibited in the L-cys treatment group (Figure 5D, $P < 0.0001$). These findings suggested that Lla-Met caused the fragmentation of *H. pylori* DNA by inducing the accumulation of ROS.

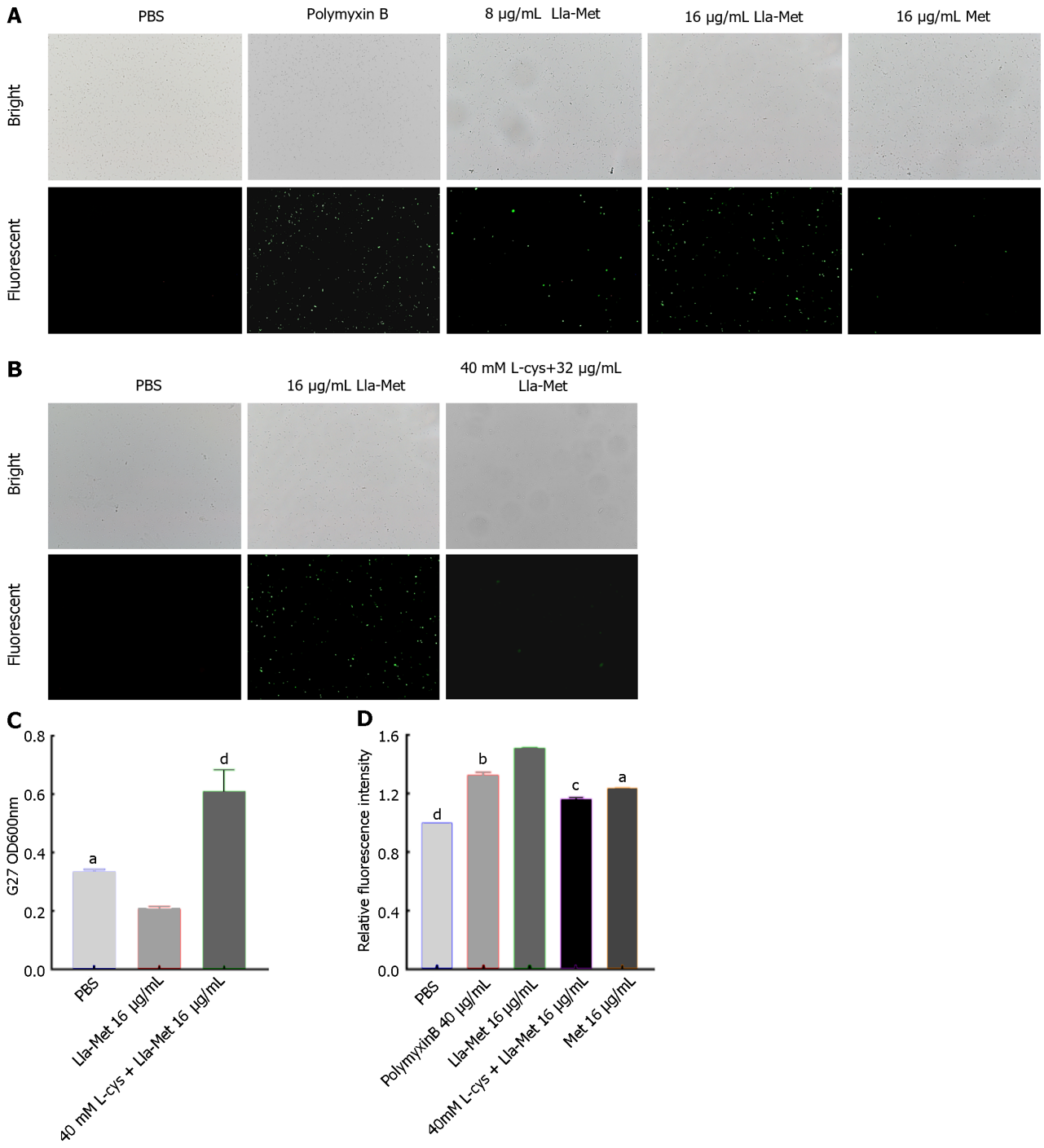

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Figure 4 The effect of linolenic acid-metronidazole on the intracellular reactive oxygen species content in *Helicobacter pylori*. A: Fluorescent microscopy for the effect of linolenic acid-metronidazole on the production of reactive oxygen species (ROS); B: The effect of L-cysteine (L-Cys) on intracellular production of ROS; C: The effect of L-cys on cell viability; D: Effect of L-cys on ROS. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$; $^{d}P < 0.0001$. PBS: Phosphate buffered saline; Lla-Met: Linolenic acid-metronidazole; L-Cys: L-cysteine.

Lla-Met upregulates MdaB, SodB expression

The half inhibitory concentration of Lla-Met was used for the oxidation analysis (Figure 6A). The OD values were unchanged after *H. pylori* was dosed with 8 μg/mL Lla-Met compound for 0, 4, and 8 h. The RNA-seq data for *H. pylori* in different treatment groups (Figure 6B). The closer the Pearson correlation coefficient approaches 1, the higher the similarity of events. The PCA is in Figure 6C. The difference and distance between samples are illustrated. The closer the similarity between samples, the closer the distance in the PCA diagram. The Venn diagram, which shows the DEGs in each group. A total of 1130 DEGs were detected between A_M_1 and A_M_2, of which 575 were up-regulated and 555 were down-regulated. A total of 1016 DEGs were detected between A_M_1 and A_M_3, of which 488 were up-regulated, and 528 were down-regulated. A total of 533 DEGs were detected between A_M_2 and A_M_3, including 265 up-regulated genes and 268 down-regulated genes. Among them, 344 genes were co-expressed in A_M_1, A_M_2, and

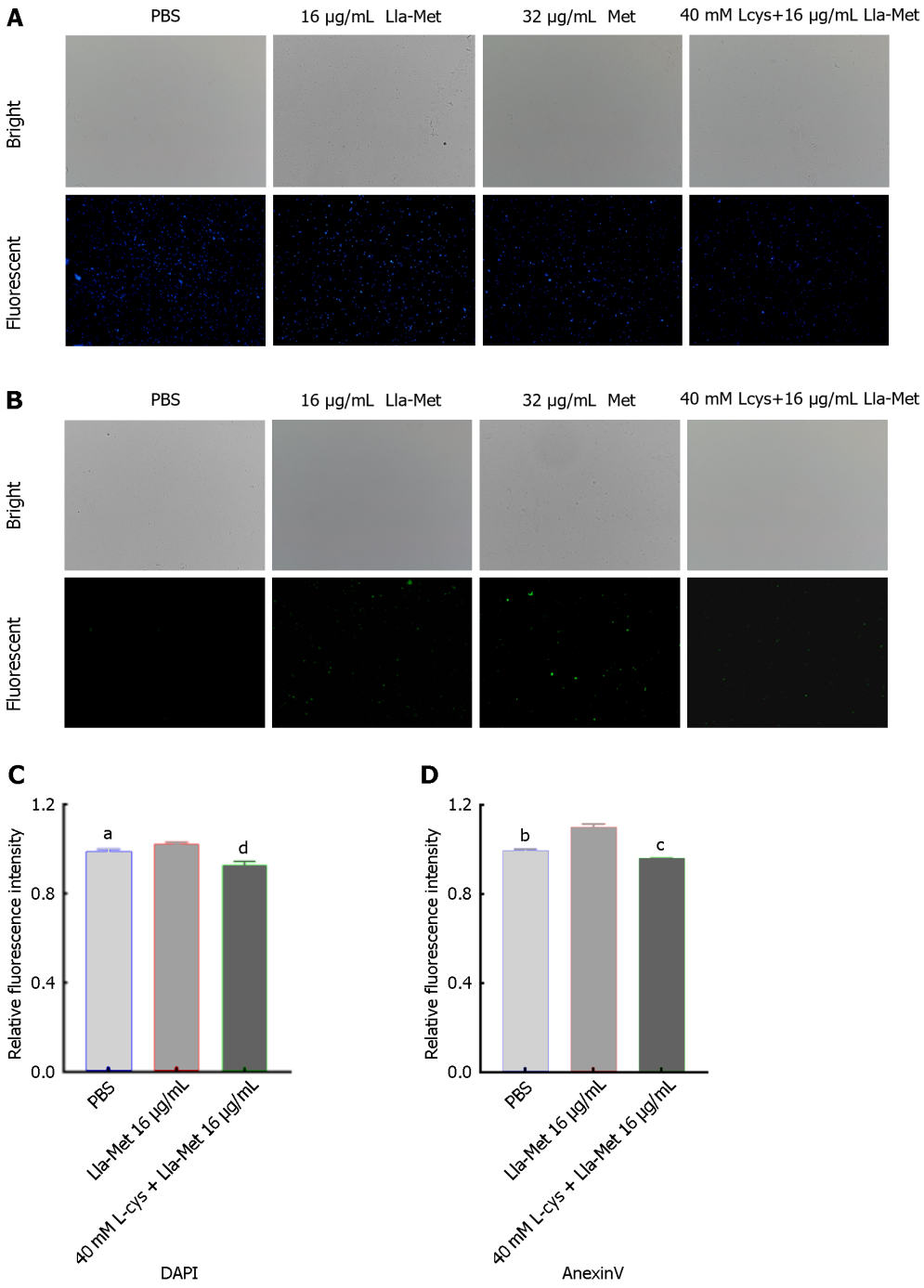

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Figure 5 Detection of phosphatidylserine ectropion and DNA fragmentation of *Helicobacter pylori*. A and C: Fluorescence microscope detection of phosphatidylserine ectropion; B and D: Multifunctional enzyme labeler detection of bacterial DNA fragmentation. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$; $^{d}P < 0.0001$. PBS: Phosphate buffered saline; Lla-Met: Linolenic acid-metronidazole; L-Cys: L-cysteine.

A_M_3 (Figure 6D). The gene set enrichment analysis and Gene Ontology of the DEGs. The DEGs were divided into three main categories: Those that regulate biological processes, secretion of cellular components, and molecular function. The differential genes between the groups are primarily concentrated in tRedox pathways, metabolic processes and other pathways. Gene set enrichment analysis revealed that the DEGs regulated the REDOX and the metabolism pathway (Figure 6E). Lla-Met is inducing production of ROS in *H. pylori*, and therefore an increased expression of MdaB and SodB, both of which are associated with protection against the oxidative stress (Table 2). RT-qPCR and transcriptome sequence analyses revealed comparable findings (Figure 6F).

Table 2 Gene affected by linolenic acid-metronidazole treatment

Gene	Name	Log_2 fold change	Description	Enrichment pathway
MdaB	*HPG27_RS03065*	4.962	Flavodoxin family protein	Oxidoreductase activity
SodB	*HPG27_RS05265*	4.2287	Superoxide dismutase	Oxidoreductase activity

DISCUSSION

Lla-Met compound is synthesized from linolenic acid and metronidazole. Linolenic acid is an essential fatty acid with broad-spectrum antibacterial spectrum and antioxidant activities and capability to overcome *H. pylori* resistance to antibiotic treatment. In addition, with more functional groups, it can react with various substances to form related derivatives, which are widely used in the anti-infection treatment. Obonyo *et al*[25] suggested that the antibacterial mechanism of linolenic acid liposome against *H. pylori* is mainly to cause damage to the bacterial cell membrane. Huang *et al*[26] used linolenic acid and zinc to synthesize zinc linolenic acid with an MIC of 4-8 μg/mL to drug-resistant *H. pylori* strains. Its antibacterial mechanism is mainly to destroy cell membrane and cause accumulation of ROS, which finally leads to the death of bacteria. In this experiment, the damage caused by Lla-Met compound to the cell membrane was detected by PI, FD4, FD10 and lactate dehydrogenase activity determination. There was no change in cell membrane permeability after *H. pylori* was treated with 16 μg/mL Lla-Met compound. This result suggested that Lla-Met compound did not inhibit *H. pylori* by damaging the cell membrane.

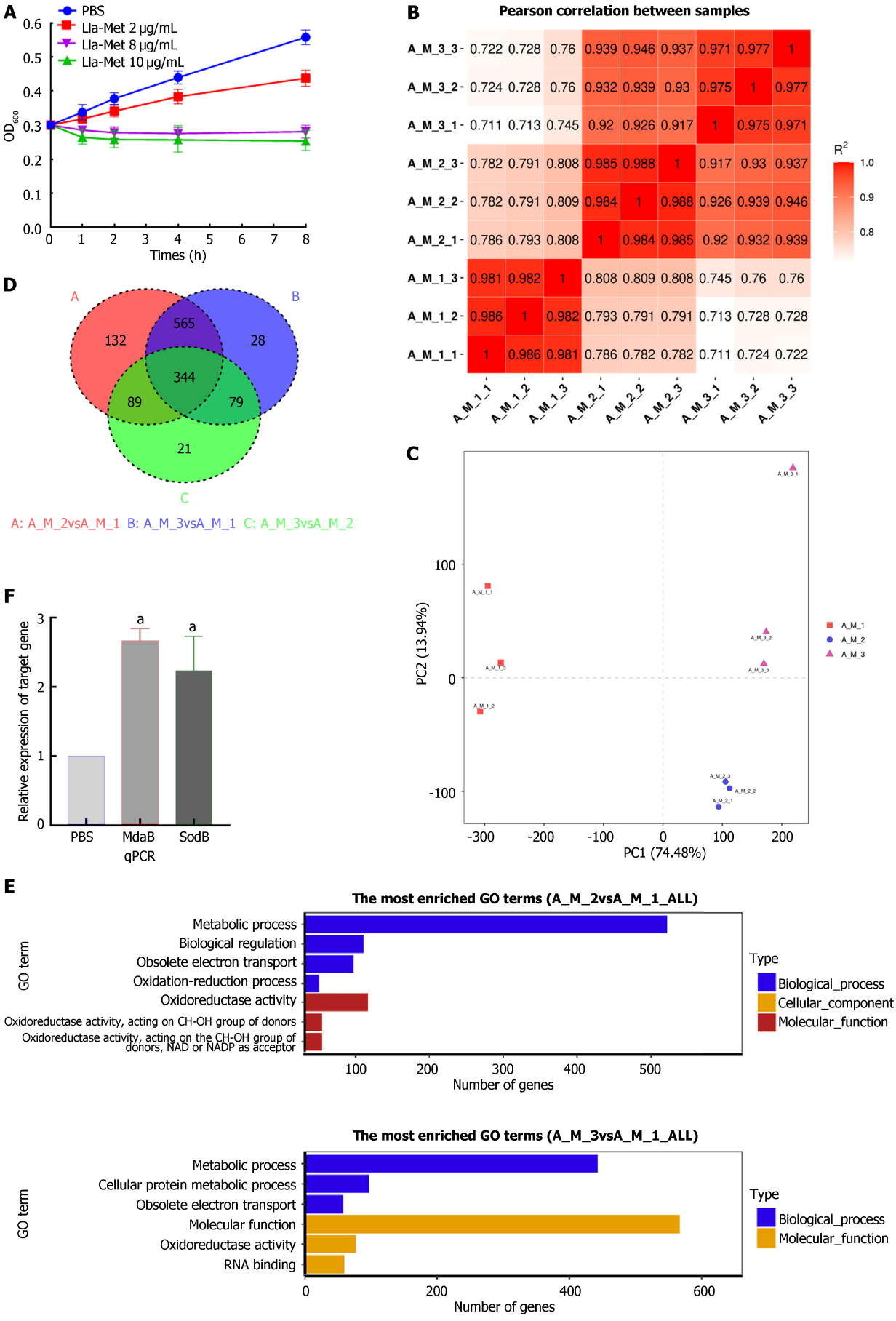
The accumulation of intracellular ROS activates eukaryotic cell apoptosis[27]. This process produces dying cells with typical morphological features, including cell shrinkage, membrane blistering, chromatin condensation, DNA fragmentation, and PS ectropion[28]. Studies have shown that apoptosis also occurs in prokaryotic cells, and is characterized with similar morphological characteristics as those in eukaryotes, such as the destruction of bacterial membrane integrity, DNA fragmentation, and PS ectropion[22,29]. Therefore, in the present study, we investigated whether Lla-Met compound could inhibit *H. pylori* growth by causing oxidative damage. In the experiment, 8 μg/mL Lla-Met compound was found to produce a stronger fluorescence signal compared with the control group. In addition, at a higher dose of 16 μg/mL Lla-Met, *H. pylori* produced a stronger fluorescence signal compared with the positive control group. Indicated that Lla-Met compound could increase accumulation of ROS in *H. pylori* in a dose-and time-dependent manner. This experiment also investigated whether intracellular ROS accumulation could affect *H. pylori* viability. The results showed that excessive accumulation of ROS could affect viability of *H. pylori* by reducing it to 20.5%, which increased to 57.7% when *H. pylori* was treated with 40 mmol/L L-cys. Interestingly, the accumulation of intracellular ROS was also found to significantly decrease after *H. pylori* was treated with L-cys. This result suggested that Lla-Met compound could cause excessive accumulation of intracellular ROS, leading to a decrease in cell viability, and that ROS accumulation could be reversed by L-cys treatment. In addition, after treatment with Lla-Met compound at different concentrations for 24 h, *H. pylori* surface became rough and swollen compared with the control group. As previously demonstrated, Lla-Met compound caused no damage to the cell membrane of *H. pylori*. This suggested that the death of *H. pylori* was due to the accumulation of intracellular ROS caused by Lla-Met compound.

Oxidation can cause both prokaryotic and eukaryotic cell death. In the present study, treatment with 16 μg/mL Lla-Met for 2 h was found to cause *H. pylori* DNA fragmentation and PS ectropion. Meanwhile, these effects were found to be reversed after *H. pylori* was treated with L-cys. DNA damage and membrane depolarization are characteristic changes in eukaryotic cell apoptosis[30]. Our experimental results showed that *H. pylori* cell death is similar to eukaryotic apoptosis, and ROS accumulation could induce prokaryotic cell-like death. However, compared with other studies, significant damage to the cell membrane was not found in the present study. This result indicated that damage to the integrity of the cell membrane might not be as necessary in the apoptosis of prokaryotic cells as slight DNA fragmentation and PS ectropion.

These results indicated that Lla-Met compound can promote intracellular ROS-generation reaction in *H. pylori* and effectively inhibit its growth. However, since several enzymes are involved in ROS-generating reaction, we used RT-qRCR to identify and verify key enzymes involved in this process and detect transcriptome changes. The results revealed that superoxide dismutase *MdaB* and *SodB* genes were found to play an important role. Under normal circumstances, the intracellular oxidative system and antioxidant system are in a dynamic balance. However, after treatment of with linolonic acid-metronidazole compound, superoxide dismutase *MdaB* and *SodB* genes were found to be highly expressed in *H. pylori*, and intracellular ROS was found to accumulate excessively, thereby damaging DNA and causing PS ectropion.

CONCLUSION

In this paper, the mechanism of linoleic-metronidazole compound was demonstrated to involve inhibiting *H. pylori* growth by inducing excessive ROS accumulation, resulting in excessive superoxide dismutase *MdaB* and *SodB* genes expression (Figure 7). Besides, this study further proves the antibacterial effect of Lla-Met on *H. pylori* at the molecular level, providing theoretical support for further research and development of Lla-Met as an anti-*H. pylori* drug to help overcome *H. pylori* resistance to current antibiotic drugs.


A
PBS
Lla-Met 2 μg/mL
Lla-Met 8 μg/mL
Lla-Met 10 μg/mL
OD600
Times (h)
B
Pearson correlation between samples
A_M_3_3 0.722 0.728 0.76 0.939 0.946 0.937 0.971 0.977 1
A_M_3_2 0.724 0.728 0.76 0.932 0.939 0.93 0.975 1 0.977
A_M_3_1 0.711 0.713 0.745 0.92 0.926 0.917 1 0.975 0.971
A_M_2_3 0.782 0.791 0.808 0.985 0.988 1 0.917 0.93 0.937
A_M_2_2 0.782 0.791 0.809 0.984 1 0.988 0.926 0.939 0.946
A_M_2_1 0.786 0.793 0.808 1 0.984 0.985 0.92 0.932 0.939
A_M_1_3 0.981 0.982 1 0.808 0.809 0.808 0.745 0.76 0.76
A_M_1_2 0.986 1 0.982 0.793 0.791 0.791 0.713 0.728 0.728
A_M_1_1 1 0.986 0.981 0.786 0.782 0.782 0.711 0.724 0.722
R2
D
A
B
C
132
565
28
344
89
79
21
A: A_M_2vsA_M_1 B: A_M_3vsA_M_1 C: A_M_3vsA_M_2
C
PC2 (13.94%)
PC1 (74.48%)
A_M_1
A_M_2
A_M_3
F
Relative expression of target gene
a
a
PBS
MdaB
SodB
qPCR
E
The most enriched GO terms (A_M_2vsA_M_1_ALL)
GO term
Metabolic process
Biological regulation
Obsolete electron transport
Oxidation-reduction process
Oxidoreductase activity
Oxidoreductase activity, acting on CH-OH group of donors
Oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor
Number of genes
Type
Biological_process
Cellular_component
Molecular_function
The most enriched GO terms (A_M_3vsA_M_1_ALL)
GO term
Metabolic process
Cellular protein metabolic process
Obsolete electron transport
Molecular function
Oxidoreductase activity
RNA binding
Number of genes
Type
Biological_process
Molecular_function

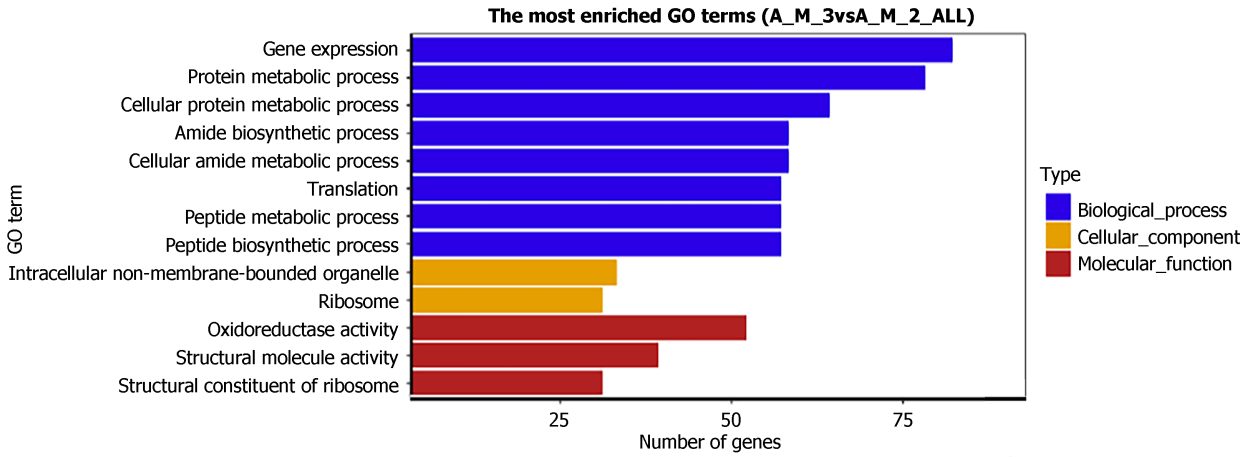


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Figure 6 Linolenic acid-metronidazole up-regulates the expression of superoxide dismutase. A: The half inhibitory concentration of linolenic acid-metronidazole; B: RNA-seq quality data; C: The principal component analysis; D: Venn diagram; E: Gene set enrichment analysis; F: Up-regulates the expression of genes. $^{a}P < 0.05$. PBS: Phosphate buffered saline; Lla-Met: Linolenic acid-metronidazole; qPCR: Quantitative polymerase chain reaction; GO: Gene Ontology.

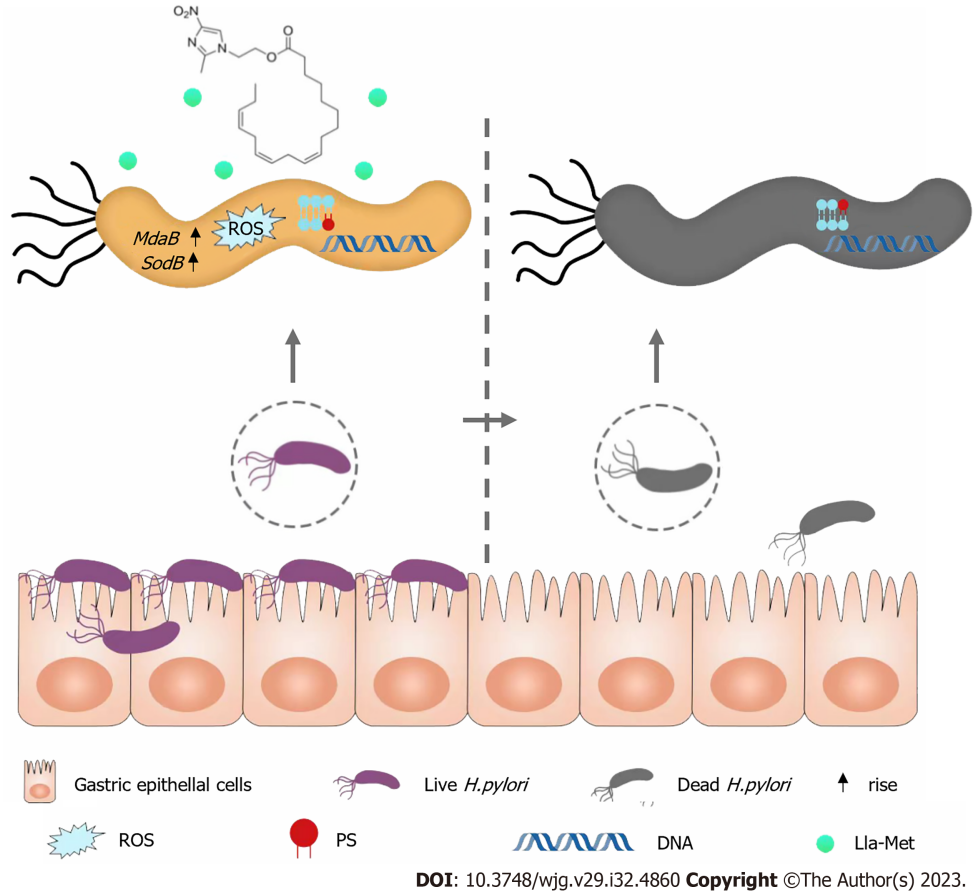

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Figure 7 Hypothesized model of the mechanism of linolenic acid-metronidazole against *Helicobacter pylori*. ROS: Reactive oxygen species; PS: Phosphatidylserine; Lla-Met: Linolenic acid-metronidazole; *H. pylori*: *Helicobacter pylori*.

ARTICLE HIGHLIGHTS

Research background

Helicobacter pylori (*H. pylori*) is recognized as an important human pathogen associated with superficial gastritis, atrophic gastritis, gastric cancer, *etc.*, each of which has become a serious threat to human health and survival. The rate of drug resistance is increasing due to the wide use of antibiotics and high rates of resistance to clarithromycin, metronidazole, and levofloxacin are associated with the failure of *H. pylori* eradication. At present, the mechanism of antibiotic resistance

of *H. pylori* is not completely understood. It is very difficult to prevent drug resistance and improve the rate of eradication of the target, thus warranting exploration of the mechanism of drug resistance to *H. pylori*, and provision of an experimental basis for the prevention and treatment of drug resistance.

Research motivation

Currently, there is a serious drug resistance situation in *H. pylori* and new antibiotics are urgently needed; however, antibiotic research and development are very difficult. If we can understand the antibacterial mechanism of linolenic acid-metronidazole (Lla-Met), we can better apply it to antimicrobial treatment and solve the problem of antibiotic resistance.

Research objectives

The objectives of this study were to confirm the antibacterial effect of Lla-Met on *H. pylori*, and to provide theoretical support for further research and development of Lla-Met as an anti-*H. pylori* drug, and to help overcome the resistance of *H. pylori* to existing antibiotic drugs.

Research methods

H. pylori cells were treated with the Lla-Met compound, and the effect of the compound on the cell morphology, cell membrane permeability, and oxidation of the bacteria cell was assessed by scanning electron microscope, propidium iodide staining, FIFC-FD, detection of ion channels, detection of intracellular reactive oxygen species, and detection of phosphatidylserine ectropion. Meanwhile, the differently expressed genes in H. pylori in response to Lla-Met treatment were identified by transcriptome sequencing and quantitative real-time polymerase chain reaction.

Research results

The expression of both *SodB* and *MdaB* genes was up-regulated after treatment with Lla-Met, and both genes are associated with antioxidants. Lla-Met inhibits the growth of *H. pylori* through oxidation.

Research conclusions

The mechanism of linoleic-metronidazole compound was demonstrated to involve inhibiting *H. pylori* growth by inducing excessive reactive oxygen species accumulation, resulting in excessive superoxide dismutase *MdaB* and *SodB* genes expression.

Research perspectives

This study proves the antibacterial effect of Lla-Met on *H. pylori* at the molecular level, providing theoretical support for further research and development of Lla-Met as an anti-*H. pylori* drug to help overcome *H. pylori* resistance to current antibiotic drugs.

FOOTNOTES

Author contributions: Zhou WT and Dai YY consulted literature, performed experiments, collected and analyzed data and wrote the first draft, making equal contributions to this work; Liao LJ, Yang SX, and Chen H corrected it; Zhao LJ, Huang YQ, and Huang L designed, checked, modified, and completed the manuscript, making equal contributions to this work as co-corresponding authors; Huang L is the first corresponding author; and all authors approved the final version of the article.

Institutional review board statement: The study was reviewed and approved by the Institutional Review Board at Youjiang Medical University for Nationalities.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

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Country/Territory of origin: China

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ORIGINAL ARTICLE

Retrospective Cohort Study

Validation of the albumin-bilirubin score for identifying decompensation risk in patients with compensated cirrhosis

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Abstract

BACKGROUND

The albumin-bilirubin (ALBI) score is an index of liver function recently developed to assess prognosis in patients with hepatocellular carcinoma (HCC). It can detect small changes in liver dysfunction and has been successfully applied to the prediction of survival in patients with non-malignant liver diseases of various etiologies.

AIM

To investigate the ALBI score for identifying decompensation risk at the 3-year follow-up in patients with compensated cirrhosis.

METHODS

One-hundred and twenty-three patients with compensated cirrhosis without HCC in King Chulalongkorn Memorial Hospital diagnosed by imaging were retrospectively enrolled from January 2016 to December 2020. A total of 113 patients (91.9%) had Child A cirrhosis with a median model for end-stage liver disease (MELD) score of less than 9. Baseline clinical and laboratory variables and decompensation events were collected. The ALBI score was calculated and validated to classify decompensation risk into low-, middle-, and high-risk groups using three ALBI grade ranges (ALBI grade 1: ≤ -2.60; grade 2: > -2.60 but ≤ -1.39; grade 3: > -1.39). Decompensation events were defined as ascites development, variceal bleeding, or grade 3 or 4 hepatic encephalopathy.

RESULTS

Among 123 cirrhotic patients enrolled, 13.8% (n = 17) developed decompensating events at a median time of 25 [95% confidence interval (CI): 17-31] mo. Median baseline ALBI score in compensated cirrhosis was significantly lower than that of patients who developed decompensation events [-2.768 (-2.956 to -2.453) *vs* -2.007 (-2.533 to -1.537); P = 0.01]. Analysis of decompensation risk at 3 years showed that ALBI score had a time-dependent area under the curve (tAUC) of 0.86 (95%CI: 0.78-0.92), which was significantly better than that of ALBI-Fibrosis-4 (ALBI-FIB4) score (tAUC = 0.77), MELD score (tAUC = 0.66), Child-Pugh score (tAUC = 0.65), and FIB-4 score (tAUC = 0.48) (P < 0.05 for all). The 3-year cumulative incidence of decompensation was 3.1%, 22.6%, and 50% in the low-, middle-, and high-risk groups, respectively (P < 0.001). The odds ratio for decompensation in patients of the high-risk group was 23.33 (95%CI: 3.88-140.12, P = 0.001).

CONCLUSION

The ALBI score accurately identifies decompensation risk at the 3-year follow-up in patients with compensated cirrhosis. Those cirrhotic patients with a high-risk grade of ALBI score showed a 23 times greater odds of decompensation.

Key Words: Albumin-bilirubin score; Compensated cirrhosis; Hepatic decompensation risk

Core Tip: The albumin-bilirubin (ALBI) score has been successfully applied to the prediction of survival in patients with non-malignant liver diseases of various etiologies. This study demonstrated that the ALBI score can accurately identify decompensation risk at the 3-year follow-up in patients with compensated cirrhosis. The ALBI score is a simple and ready-to-use tool to help clinicians monitor and make appropriate treatment strategies in patients with compensated cirrhosis.

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INTRODUCTION

Cirrhosis is the end stage of chronic liver disease which is currently the 11th leading cause of death and 15th leading cause of morbidity across the world, accounting for 2.2% of deaths and 1.5% of disability-adjusted life years worldwide[1]. The disease evolves from an asymptomatic phase as compensated cirrhosis to a symptomatic phase as decompensated cirrhosis[2]. Decompensated cirrhosis is defined by the presence of variceal bleeding, encephalopathy, ascites, hepatorenal syndrome, and/or jaundice[3]. Transition from a compensated to a decompensated stage occurs at a rate of 5%-7% per year[4]. Median survival of patients with compensated cirrhosis is 12 years, while that of decompensated patients is less than 2 years[5]. Once decompensation has occurred, mortality without transplant is as high as 85% over 5 years[4].

For over 60 years, the best predictor of decompensation in cirrhotic patients has been the hepatic venous pressure gradient (HVPG)[6]. HVPG has a greater discriminative ability to predict clinical decompensation in patients with compensated cirrhosis than either the model for end-stage liver disease (MELD) or Child-Pugh score. Research shows that patients with a HVPG < 10 mmHg have a 90% probability of not developing clinical decompensation in a median follow-up of 4 years[6]. However, HVPG measurement is invasive, requires specialized healthcare personnel, and is often unavailable in many healthcare systems. The appearance of noninvasive tests, most notably, transient elastography, has provided a staging tool for prognostic markers of portal hypertension[7]. Recently, Baveno VII criteria were developed using transient elastography for liver stiffness measurements and platelet counts to define clinically significant portal hypertension and prognosis, risk stratification, and indication to start beta-blocker therapy in compensated advanced chronic liver disease and compensated cirrhosis patients[8]. Within a median follow-up of 40 mo, 7.2% of the 1159 compensated advanced chronic liver disease and compensated cirrhosis patients developed an initial decompensation event[8].

Well-known prognostic scoring systems that are currently used such as the MELD score and Child-Pugh score were primarily established to predict mortality in patients with cirrhosis. The Child-Pugh score was originally developed to assess the survival of cirrhotic patients undergoing shunt surgery to relieve portal hypertension in order to treat variceal bleeding[9,10]. The MELD score was developed to more precisely evaluate 3-mo mortality for patients with cirrhosis in order to prioritize liver donor allocation[11]. The MELD score is considered more reproducible than the Child-Pugh score because it does not include subjective variables such as ascites and encephalopathy. However, the MELD score has not been shown to be superior to the Child-Pugh score in terms of predictive accuracy in different cirrhotic populations[12].

Many studies have attempted to evaluate or develop a prognostic scoring system for predicting the risk of decompensation in a patient with compensated cirrhosis based on clinical and laboratory parameters. Well-known scoring systems such as MELD, Child-Pugh, Fibrosis-4 (FIB-4) scores and newly developed scoring system such as albumin-bilirubin FIB-4 (ALBI-FIB4) score which combine the ALBI score and FIB-4 score were evaluated for predicting the risk of decompensation in a patient with compensated cirrhosis. Prior studies show that the ALBI-FIB4 score can identify a high-risk patient more accurately than MELD, Child-Pugh and FIB-4 scores[13-15].

The ALBI score was recently created and validated to specifically assess hepatocellular carcinoma (HCC) liver functional reserve for predicting survival of HCC patients receiving various treatment modalities[16]. The ALBI grade was calculated using albumin and bilirubin levels. Its application has been increasingly expanded to chronic liver disease in general and has proven remarkably accurate in terms of prognosis[17]. Many publications have shown that the ALBI score is highly prognostic in cirrhotic patients and has shown the ability to correlate to HVPG levels[18], predicting the presence of gastroesophageal varices and stratifying bleeding risk[19], and severe portopulmonary hypertension[20].

Since the utility of ALBI score in predicting decompensation risk in patients with compensated cirrhosis has yet been fully investigated, we aimed to evaluate the ALBI score's ability to identify decompensation risk at 3 years follow-up in patients with compensated cirrhosis.

MATERIALS AND METHODS

Patients with compensated cirrhosis receiving care at King Chulalongkorn Memorial Hospital from January 2016 to December 2020 were enrolled retrospectively. The diagnosis of cirrhosis was made by imaging with ultrasonography, multiphasic contrast-enhanced computed tomography, or gadoxetic acid-enhanced magnetic resonance imaging. Patients with missing data, HCC at baseline, or a history of hepatic decompensation at the time of diagnosis were excluded. Baseline characteristics of patients including age, sex, and etiologies of cirrhosis were obtained from medical records. Laboratory data including serum creatinine, albumin, bilirubin, aspartate transaminase (AST), alanine transaminase (ALT), platelets, and international normalized ratio (INR) were collected.

Decompensation events, defined as ascites development, variceal bleeding, or grade 3 or 4 hepatic encephalopathy, were ascertained by clinicians in charge of their care and supported with either endoscopy reports, abdominal imaging, or medical reports. Time to decompensation was calculated from date of study entry until date of first recorded decompensation.

The ALBI score at baseline was calculated using the equation (log10 bilirubin in μmol/L × 0.66) + [albumin in g/L × (-0.085)][16] and validated to categorize decompensation risk into low-, middle-, and high-risk groups classified by ALBI grades 1, 2, and 3. The cut points of ALBI grades were similar to those in HCC patients: ALBI grade 1: ≤ -2.60; grade 2: > -2.60 but ≤ -1.39; grade 3: > -1.39.

Statistical analysis

For baseline characteristics, continuous variables with a normal distribution are presented as the mean ± SD, while those with a non-normal distribution are presented as median and interquartile range (IQR). The Mann-Whitney *U* test was used to compare differences in continuous variables while Fisher's exact test was used to assess for significant differences in binomial variables. Time to decompensation according to baseline ALBI grade and overall survival following the first decompensation were examined by Kaplan-Meier graphs and compared using the log-rank test. Cox proportional hazards analysis was used to identify ALBI score and other potential factors associated with decompensation. Significant factors identified in the univariate analysis were included in the multivariate analysis. The odds ratio (OR) calculated by logistic regression analysis provided estimates of the change in decompensation odds at each ALBI grade at baseline. The time-dependent area under the curve (tAUC) was estimated to evaluate the ability of each prognostic score to predict decompensation. A *P*-value of < 0.05 was considered statistically significant. All statistical analyses were performed using IBM SPSS Statistics version 22.

RESULTS

A total of 123 compensated cirrhotic patients were enrolled in our study. Table 1 summarizes their baseline characteristics. Mean age was 63.9 years (SD: 12.3), and 72 (58.5%) patients were male. Mean body mass index was 24.5 kg/m^2 (SD: 3.7). Viral hepatitis B was the most common etiology of cirrhosis (n = 43, 35%), followed by viral hepatitis C (n = 30, 24.4%), nonalcoholic steatohepatitis (NASH) (n = 29, 23.6%), alcohol liver disease (n = 19, 15.4%), and autoimmune hepatitis (n = 2, 1.6%). All patients with viral hepatitis B or viral hepatitis C received antiviral treatment with a sustained virological response. For patients with NASH, 17 (58.6%) patients had diabetes mellitus and 15 (51.7%) patients were obese. At baseline, 113 (91.9%) patients had Child-Pugh class A with 91 (80.5%) and 22 (19.5%) patients having a Child-Pugh score of 5 and 6, respectively, and 10 (8.1%) had Child-Pugh class B. For ALBI grade at baseline, 64 (52%) patients had ALBI grade 1, 53 (43.1%) had ALBI grade 2, and 6 (4.9%) had ALBI grade 3. Median prognostic scores predicting first decompensation at baseline were: MELD score (8.7; IQR: 7.8-10.1), ALBI score (-2.63; IQR: -2.91 to -2.06), ALBI-FIB4 score (-2.79; IQR: -3.28 to -1.93), and FIB-4 score (3.2; IQR: 1.8-5.3).

Table 1 Baseline characteristics of patients with compensated cirrhosis, *n* (%)

Variable	*n* = 123
Age, yr, mean (SD)	63.9 (12.3)
Male	72 (58.5)
Body mass index, kg/m^2, mean (SD)	24.5 (3.7)
Obesity	54 (43.9)
Diabetes	33 (26.8)
Etiology of disease	
HBV	43 (35)
HCV	30 (24.4)
NASH	29 (23.6)
Alcohol	19 (15.4)
Autoimmune hepatitis	2 (1.6)
Laboratory data	
Creatinine, mg/dL, median (IQR)	0.8 (0.7-0.9)
Albumin, g/dL, median (IQR)	4 (3.4-4.3)
Bilirubin, mg/dL, median (IQR)	0.9 (0.6-1.5)
AST, U/L, median (IQR)	44 (28-66)
ALT, U/L, median (IQR)	33 (24-58)
Platelets, × 10^9/L, median (IQR)	142 (104-200)
INR, median (IQR)	1.1 (1-1.2)
Child-Pugh grade	
A	113 (91.9)
B	10 (8.1)
Decompensation event	17 (13.8)
Variceal bleeding	8 (47)
Ascites development	5 (29.4)
Grade 3 or 4 hepatic encephalopathy	4 (23.6)
MELD, median (IQR)	8.7 (7.8-10.1)
ALBI score, median (IQR)	-2.63 (-2.91 to -2.06)
ALBI grade	
1	64 (52)
2	53 (43.1)
3	6 (4.9)
ALBI-FIB4 score, median (IQR)	-2.79 (-3.28 to -1.93)
FIB-4 score, median (IQR)	3.2 (1.8-5.3)

NASH: Nonalcoholic steatohepatitis; HBV: Hepatitis B virus; HCV: Hepatitis C virus; AST: Aspartate transaminase; ALT: Alanine transaminase; INR: International normalized ratio; MELD: Model for end-stage liver disease; ALBI: Albumin-bilirubin; FIB-4: Fibrosis-4; SD: Standard deviation; IQR: Interquartile range

Predictors of decompensation in patients with compensated cirrhosis

During a median follow-up of 36 (IQR: 35-36) mo, 17 (13.8%) patients developed an initial decompensation event within 3 years follow-up at a median time of 25 [95% confidence interval (CI): 17-31] mo. Events included variceal bleeding in eight (47%) patients, ascites in five (29.4%), and grade 3 or 4 hepatic encephalopathy in four (23.6%). Among the 17 patients who experienced decompensating events, the most common precipitants of hepatic decompensation were

gastrointestinal bleeding (n = 8, 47%), followed by infection (n = 1, 6%). However, in eight (47%) of the patients who developed decompensating events, no specific cause of decompensation could be identified. The eight patients who experienced variceal bleeding received a combination of endoscopic treatment, intravenous octreotide, and antibiotic prophylaxis. Treatment for ascites in the five affected patients involved a combination of spironolactone and furosemide, with one patient requiring abdominal paracentesis due to tense ascites. All the four patients with grade 3 or 4 hepatic encephalopathy were treated with lactulose. Additionally, the patient who experienced decompensation due to infection received intravenous antibiotic therapy. Overall survival following the first decompensation was 82.4% at 3 years. The median overall survival of patients who developed the first decompensation was 29.9 (95%CI: 23.7-36.0) mo.

In compensated cirrhotic patients who developed an initial decompensation, albumin, bilirubin, ALT, ALBI, MELD, ALBI-FIB4, and Child-Pugh scores were found to be associated with initial decompensation, with an hazard ratio (HR) of 0.10 (95%CI: 0.03-0.26, P < 0.001), 1.21 (95%CI: 1.02-1.43, P = 0.02), 1.01 (95%CI: 1.00-1.02, P = 0.01), 8.31 (95%CI: 3.48-19.85, P < 0.001), 1.11 (95%CI: 1.02-1.21, P = 0.01), 2.30 (95%CI: 1.60-3.31, P < 0.001), and 1.98 (95%CI: 1.15-3.39, P = 0.01), respectively. In the multivariate analysis, ALBI score remained independently associated with initial decompensation with an adjusted HR of 4.18 (95%CI: 1.40-12.53) (P = 0.01) (Table 2).

Performance of each prognostic score for predicting decompensation at 3-year follow-up

An analysis of decompensation risk at the 3-year follow-up demonstrated that ALBI score had an tAUC of 0.86 (95%CI: 0.78-0.92), which performed significantly better than ALBI-FIB4 (tAUC = 0.77), MELD (tAUC = 0.66), Child-Pugh (tAUC = 0.65), or FIB-4 scores (tAUC = 0.48) (P < 0.05 for all) (Table 3 and Figure 1).

Decompensation risk stratification based on ALBI grade

In patients who developed a decompensation event, the majority were in the middle-risk group (n = 12, 70.6%), two in low-risk group (11.8%), and three in high-risk group (17.6%) according to the ALBI grade (Table 4). Median baseline ALBI score in the decompensated cirrhosis group was significantly higher than that of the compensated cirrhosis group [-2.768 (-2.956 to -2.453) *vs* -2.007 (-2.533 to -1.537), P = 0.01]. The cumulative incidence of decompensation at 3 years was 3.1% in the low-risk group, 22.6% in the middle-risk group, and 50% in the high-risk group (P = 0.003 and P < 0.001, respectively) (Table 4). The OR for decompensation in patients in the high-risk and middle-risk groups was 23.33 (95%CI: 3.88-140.12, P = 0.001) and 7.83 (95%CI: 1.75-35.01, P = 0.007), respectively (Table 4). Patients in the high-risk group exhibited a significantly shorter time to the initial decompensation compared to those in both the middle-risk and low-risk groups [26.5 mo (95%CI: 18.5-34.5), 33.2 mo (95%CI: 31.5-35.1), and 35.5 mo (95%CI: 34.7-36.2), respectively (P < 0.001)] (Figure 2).

Regarding the etiology of liver disease within each decompensation risk group, viral hepatitis was found in a significantly higher number of patients within the low-risk group compared to the middle and high-risk groups, with 45 (61%), 24 (32.9%), and 4 (5.5%) patients, respectively (P = 0.02). However, there was no statistically significant difference in the prevalence of NASH, alcohol-related liver disease, or autoimmune hepatitis among the decompensation risk groups.

DISCUSSION

This study validated the ALBI score as an accurate prognostic tool to stratify patients with compensated cirrhosis for the risk of decompensation at the 3-year follow-up. The ALBI grade identified high-risk patients more effectively than either MELD, Child-Pugh, ALBI-FIB4, or FIB-4 score.

Novel scoring systems have been developed for prognostic stratification risk of decompensation among patients with compensated cirrhosis over a medium- or long-term follow-up period. One novel scoring system focusing on liver stiffness and measured by transient elastography, presence of gastroesophageal varices from endoscopic screening, albumin, and platelets, has shown excellent accuracy in predicting risk of decompensation at the 3-year follow-up with a tAUC of 0.89. This performance was significantly higher than that of ALBI-FIB-4, Baveno VII criteria, or MELD score. ALBI grade score maintained a tAUC of over 0.8 throughout the 5-year follow-up period[15]. Other novel scoring systems, which consisted of simple and routinely performed serum marker-based scores such as AST, ALT, albumin, bilirubin, and platelets, have also shown an effective ability to identify high-risk patients for the risk of decompensation. The tAUC ranged from 0.69-0.80 using these scoring systems, which was significantly higher than that of the MELD or Child-Pugh score[14,21].

The ALBI score was recently developed to assess liver functional reserve and prognosis among HCC patients[16]. It offers a simple, evidence-based, objective, and discriminatory method that has been extensively tested with an international cohort and enables more detailed prognostic classification than the Child-Pugh grade[16]. Due to the fact that ALBI is simple to calculate needing only albumin and bilirubin measures, application of ALBI has been increasingly extended to other chronic liver diseases including decompensation for liver cirrhosis[17]. Several studies reported that ALBI might be comparable to MELD for predicting short-term mortality, but better than MELD in predicting longer-term mortality in patients with decompensated cirrhosis[22-25]. Recently, one study that evaluated the correlation between the ALBI score and portal pressure in cirrhotic patients showed that ALBI had a better correlation with HVPG compared to MELD, Child-Pugh, FIB-4, and aminotransferase/platelet ratio index scores with a tAUC of 0.72 (P < 0.001)[18]. This study also showed that ALBI grade 3 was able to predict early mortality in patients with a MELD score lower than 14. Based on the pathophysiology of decompensated cirrhosis, which involves an elevation in portal pressure, it has been observed that when the HVPG surpasses 10 mmHg, it correlates with the occurrence of decompensation[26], Therefore, the ALBI score exhibits potential in predicting decompensation by virtue of its correlation with HVPG. However, this study had a higher

Table 2 Predictors of decompensation in patients with compensated cirrhosis

Variable	Univariate		Multivariate	
	Hazard ratio (95%CI)	*P* value	Adjusted hazard ratio (95%CI)	*P* value
Age	1.01 (0.97-1.05)	0.47	1.01 (0.97-1.06)	0.56
Male	0.58 (0.22-1.50)	0.26	0.55 (0.18-1.69)	0.29
Creatinine	1.20 (0.83-1.74)	0.31		
Albumin	0.10 (0.03-0.26)	< 0.001		
Bilirubin	1.21 (1.02-1.43)	0.02		
AST	1.00 (0.99-1.00)	0.34		
ALT	1.01 (1.00-1.02)	0.01		
Platelets	0.99 (0.98-1.00)	0.27		
INR	1.58 (0.11-22.29)	0.73		
ALBI score	8.31 (3.48-19.85)	< 0.001	4.18 (1.40-12.53)	0.01
MELD score	1.11 (1.02-1.21)	0.01	1.07 (0.92-1.24)	0.34
ALBI-FIB4 score	2.30 (1.60-3.31)	< 0.001	1.73 (0.82-3.64)	0.15
FIB-4 score	1.02 (0.92-1.13)	0.67		
Child-Pugh score	1.98 (1.15-3.39)	0.01	1.26 (0.61-2.58)	0.54

AST: Aspartate transaminase; ALT: Alanine transaminase; INR: International normalized ratio; MELD: Model for end-stage liver disease; ALBI: Albumin-bilirubin; FIB-4: Fibrosis-4; CI: Confidence interval.

Table 3 Comparative performance of each prognostic score for predicting decompensation at 3 years

Prognostic score	tAUC	*P* value *vs* ALBI score
ALBI	0.86 (0.78-0.92)	Reference
MELD	0.66 (0.56-0.75)	< 0.001
ALBI-FIB4	0.77 (0.68-0.86)	0.04
FIB-4	0.48 (0.38-0.58)	< 0.001
Child-Pugh	0.65 (0.55-0.75)	< 0.001

MELD: Model for end-stage liver disease; ALBI: Albumin-bilirubin; FIB-4: Fibrosis-4; tAUC: Time-dependent area under the curve.

Table 4 Decompensation risk stratification based on albumin-bilirubin grade at baseline

ALBI grade	Decompensation at 3-yr (*n*, %)	*P* value	OR (95%CI)	*P* value
1	2/64 (3.1)	-	1.0 (reference)	-
2	12/53 (22.6)	0.003	7.83 (1.75-35.01)	0.007
3	3/6 (50)	< 0.001	23.33 (3.88-140.12)	0.001

ALBI: Albumin-bilirubin; OR: Odds ratio.

median MELD score at enrollment than our study (13 *vs* 8.7). By using ALBI grade 3 to predict decompensation stemming from increases in portal pressure, our study may need more patients with a higher MELD score at enrollment to evaluate the performance of ALBI to predict decompensation due to an increase in HVPG.

Our study found that the odds of decompensation in patients of the high-risk group was 23.33 times higher compared to patients in the lower risk group. The small sample size of the high-risk group and the high dispersion of ALBI score causes the precision of the OR in our study to be low. Thus, we need a greater number of high-risk patients for quantitative confirmation and to more precisely analyze the predictive performance of the high-risk group.

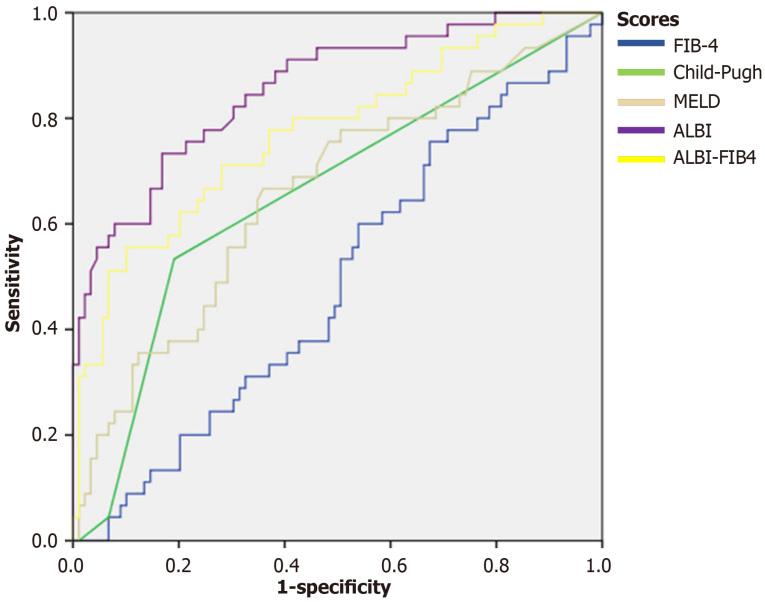

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Figure 1 Comparative performance of each prognostic score for predicting decompensation at 3 years. FIB-4: Fibrosis-4; MELD: Model for end-stage liver disease; ALBI: Albumin-bilirubin.

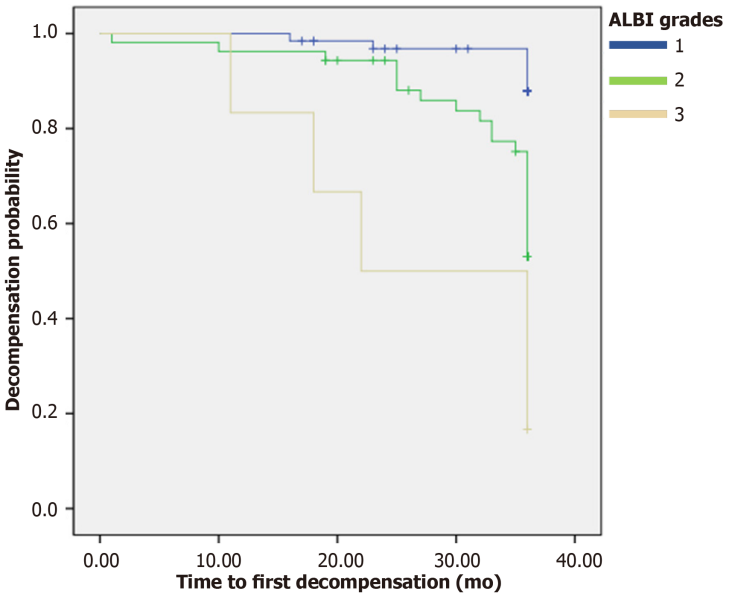

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Figure 2 Time to decompensation using the albumin-bilirubin grade at baseline. ALBI: Albumin-bilirubin.

Although our study cohort was enrolled at a single-centered tertiary care hospital in Thailand, baseline characteristics of our patients were similar to those of cohorts used to validate other newly developed scoring systems in different countries and continents. In a cohort comprised of an Asian population[15], the most common etiology of cirrhosis was viral hepatitis B at 37.1% compared to 35% in our study cohort. Baseline MELD score and Child-Pugh score in our study cohort were similar to those of cohorts used to validate other scoring systems, where 90% of patients had Child-Pugh class A with a median MELD score ranging from 7-9[14,15,21]. The rate of decompensation in our study cohort, at 13.8%, was found to be lower compared to those of other cohorts utilizing different scoring systems, where the decompensation rates ranged between 19.3% and 26.9%[14,15,21]. This discrepancy in decompensation rates could potentially be attributed to a shorter median follow-up time in our cohort, which was 3 years, in contrast to the longer follow-up periods of 4.1 to 4.5 years observed in other cohorts[14,15,21].

Viral hepatitis accounted for 59% of patients in our cohort, all of whom received antiviral treatment resulting in a sustained virological response. Among patients with viral hepatitis, 70.3% were classified as belonging to the low-risk group. We observed a significant increase in the number of patients with viral hepatitis in the low-risk group compared to the middle and high-risk groups (P = 0.02). Consequently, 52% (n = 64) of patients in our cohort were categorized as

belonging to the low-risk group, while only 4.9% (n = 6) were classified as high-risk. This distribution can primarily be attributed to the prevalence of viral hepatitis as the underlying etiology of liver disease in our study population.

The strength of this study was that we provided the first evidence that the ALBI score accurately identified decompensation risk at the 3-year follow-up in patients with compensated cirrhosis. The ALBI score is a useful tool to help select high-risk patients to guide treatment to reduce the risk of decompensation. This study represents the ability of the ALBI score to assess liver function and liver disease progression with the advantage of being simple to calculate using only serum albumin and bilirubin levels.

This study has several limitations. First, the cohort in our study was retrospectively completely only at a single-center tertiary care hospital in Thailand. A large multi-center prospective cohort study is required to validate the ALBI score. Second, most of patients had Child-Pugh class A, suggesting that the number of patients with decompensated cirrhosis is relatively low. Thus, our findings may not be readily applicable to a population predominantly with advanced cirrhosis. Third, comparisons to other novel scoring systems that require predictors besides laboratory variables such as transient elastography and gastroesophageal varices from endoscopic findings, could not be performed due to the lack of this information in our study cohort. Inclusion of patients with prompt predictor variables to validate is required. Finally, additional data of the ALBI score including changes in annual ALBI grading or changing of ALBI grades between compensation and decompensation may give new information for the prediction of a decompensation event.

CONCLUSION

This study has documented the excellent performance of the ALBI score to accurately identify decompensation risk at the 3-year follow-up in patients with compensated cirrhosis. The ALBI score is a simple and ready-to-use tool to help clinicians monitor and make appropriate treatment decisions among patients with compensated cirrhosis.

ARTICLE HIGHLIGHTS

Research background

The albumin-bilirubin (ALBI) score is an index of liver function recently developed to assess prognosis in patients with hepatocellular carcinoma (HCC). It has been successfully applied to the prediction of survival in patients with non-malignant liver diseases of various etiologies.

Research motivation

The utility of ALBI score in predicting decompensation risk in patients with compensated cirrhosis has yet been fully investigated.

Research objectives

The objective of this study was to investigate the ALBI score for identifying decompensation risk at the 3-year follow-up in patients with compensated cirrhosis.

Research methods

One-hundred and twenty-three patients with compensated cirrhosis without HCC in King Chulalongkorn Memorial Hospital diagnosed by imaging were retrospectively enrolled from January 2016 to December 2020. The ALBI score was calculated and validated to classify decompensation risk into low-, middle-, and high-risk groups using three ALBI grade ranges (ALBI grade 1: ≤ -2.60; grade 2: > -2.60 but ≤ -1.39; grade 3: > -1.39). Decompensation events were defined as ascites development, variceal bleeding, or grade 3 or 4 hepatic encephalopathy.

Research results

Among 123 cirrhotic patients enrolled, 13.8% (n = 17) developed decompensating events at a median time of 25 [95% confidence interval (CI): 17-31] mo. Analysis of decompensation risk at 3 years showed that ALBI score had a time-dependent area under the curve (tAUC) of 0.86 (95%CI: 0.78-0.92) which was significantly better than that of ALBI-Fibrosis-4 (ALBI-FIB4) score (tAUC = 0.77), model for end-stage liver disease score (tAUC = 0.66), Child-Pugh score (tAUC = 0.65), or FIB-4 score (tAUC = 0.48) (P < 0.05 for all). The 3-year cumulative incidence of decompensation was 3.1%, 22.6% and 50% in the low-, middle-, and high-risk groups, respectively (P < 0.001). The odds ratio for decompensation in patients of the high-risk group was 23.33 (95%CI: 3.88-140.12, P = 0.001).

Research conclusions

The ALBI score accurately identifies decompensation risk at the 3-year follow-up in patients with compensated cirrhosis. Those patients with a high-risk grade of ALBI score showed a 23 times greater odds of decompensation.

Research perspectives

The ALBI score represents an outstanding non-invasive scoring system, enabling clinicians to make precise decisions regarding the monitoring and guidance of treatment for patients with compensated cirrhosis.

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FOOTNOTES

Author contributions: Navadurong H and Treeprasertsuk S designed the research, analyzed the data, and wrote the manuscript; Thanapirom K, Wejnaruemarn S, Prasoppokakorn T, Chaiteerakij R, and Komolmit P administered support; Navadurong H, Thanapirom K, Wejnaruemarn S, and Treeprasertsuk S provided the study materials; Navadurong H and Wejnaruemarn S collected and assembly the data.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Different oncological features of colorectal cancer codon-specific *KRAS* mutations: Not codon 13 but codon 12 have prognostic value

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Abstract

BACKGROUND

Approximately 40% of colorectal cancer (CRC) cases are linked to Kirsten rat sarcoma viral oncogene homolog (*KRAS*) mutations. *KRAS* mutations are associated with poor CRC prognosis, especially *KRAS* codon 12 mutation, which is associated with metastasis and poorer survival. However, the clinicopathological characteristics and prognosis of *KRAS* codon 13 mutation in CRC remain unclear.

AIM

To evaluate the clinicopathological characteristics and prognostic value of codon-specific *KRAS* mutations, especially in codon 13.

METHODS

This retrospective, single-center, observational cohort study included patients who underwent surgery for stage I-III CRC between January 2009 and December 2019. Patients with *KRAS* mutation status confirmed by molecular pathology reports were included. The relationships between clinicopathological characteristics and individual codon-specific *KRAS* mutations were analyzed. Survival data were analyzed to identify codon-specific *KRAS* mutations as recurrence-related factors using the Cox proportional hazards regression model.

RESULTS

Among the 2203 patients, the incidence of *KRAS* codons 12, 13, and 61 mutations was 27.7%, 9.1%, and 1.3%, respectively. Both *KARS* codons 12 and 13 mutations showed a tendency to be associated with clinical characteristics, but only codon 12 was associated with pathological features, such as stage of primary tumor (T stage), lymph node involvement (N stage), vascular invasion, perineural invasion, tumor size, and microsatellite instability. *KRAS* codon 13 mutation showed no associations (77.2% *vs* 85.3%, $P = 0.159$), whereas codon 12 was associated with a lower 5-year recurrence-free survival rate (78.9% *vs* 75.5%, $P = 0.025$). In multivariable analysis, along with T and N stages and vascular and perineural invasion, only codon 12 (hazard ratio: 1.399; 95% confidence interval: 1.034-1.894; $P = 0.030$) among *KRAS* mutations was an independent risk factor for recurrence.

CONCLUSION

This study provides evidence that *KRAS* codon 13 mutation is less likely to serve as a prognostic biomarker than codon 12 mutation for CRC in a large-scale cohort.

Key Words: Genes; Ras; Codon; Colonic neoplasms; Rectal neoplasms

Core Tip: Based on a large-scale cohort of patients with stage I-III colorectal cancer (CRC), Kirsten rat sarcoma viral oncogene homolog (*KRAS*) codon 13 mutation is less pathogenic and recurrent. Moreover, focusing on the biological effects of codon-specific *KRAS* mutations and minimizing interference with various medical therapies, previous *in vivo* studies demonstrating that *KRAS* codon 13 mutation is less aggressive were translated into clinical outcomes in this study. This may influence many oncologists to consult with patients on their prognosis after surgery. We propose that *KRAS* codon 13 mutation is less likely to serve as a prognostic factor of CRC, compared with codon 12.

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INTRODUCTION

Kirsten rat sarcoma viral oncogene homolog (*KRAS*) is one of the downstream molecules of the epidermal growth factor receptor (EGFR) associated with cell proliferation, anti-apoptosis, and survival[1-3]. Abnormal activation of *KRAS*, a well-known oncogene, triggers uncontrolled tumor cell proliferation regardless of the initiating molecular signal from EGFR [4]. Mutations in *KRAS* promote the development of cancer in a variety of organs including the breast, prostate, lung, pancreas, colon, and rectum[1,2]. According to previous reports, approximately 40% of colorectal cancer (CRC) cases are linked to *KRAS* mutations[5-7], which occur more frequently in the proximal rather than in the distal colon[4,8,9]. Clinically, *KRAS* mutations are associated with resistance to anti-EGFR therapy and poor CRC prognosis[10,11].

CRC-related point mutations in *KRAS* occur at different codon locations. In most cases, *KRAS* mutations are detected in codon 12 or 13, whereas mutations in codon 61 or 146 have been reported only in a minority of patients with CRC[12]. Several clinical studies have indicated that *KRAS* codon 12 mutations are associated with metastasis and poor survival in advanced CRC[8,12-14]. *In-vitro* studies comparing cells with *KRAS* codon 12 and 13 mutations have demonstrated stronger transforming activity and resistance to apoptosis in cells with mutations in *KRAS* codon 12 than codon 13[15,16]. Most reports have concluded that *KRAS* codon 12 mutation is a poor prognostic factor following CRC resection. However, the oncological role of *KRAS* codon 13 mutation is controversial. *KRAS* codon 13 mutation has been linked to advanced-stage or lymph node metastasis and has been considered predictive of a higher likelihood of death in several studies[17,18]. In contrast, other investigators have shown no association between *KRAS* codon 13 mutations and tumor progression or CRC prognosis[4,19].

In addition to the controversial prognostic significance of *KRAS* codon 13 mutations, limited information is available regarding the clinical characteristics of codon-specific *KRAS* mutations in CRC. The incidence of codon-specific *KRAS* mutations other than those involving codon 12 (including codon 13) is low. Owing to the infrequency of *KRAS* abnormalities, the pathological features of codon-specific mutations at sites other than codon 12 remain unclear. Owing to the small cohort sizes of previous studies[4,8,12,14,20], the clinical roles of codon-specific *KRAS* mutations in CRC, including codons 12 and 13, are yet to be validated. Moreover, studies on the oncological effects of codon-specific *KRAS* mutations, particularly regarding abnormalities located within minor codons, are limited.

This study was designed to elucidate the clinicopathological characteristics associated with codon-specific *KRAS* mutations in CRC, including codons 12, 13, and 61. The main objective of this study was to determine whether *KRAS* codon 13 mutation could serve as a prognostic biomarker for CRC in a relatively large cohort of individuals.

MATERIALS AND METHODS

Patients

This retrospective observational cohort study was registered at ClinicalTrials.gov (NCT05657210) and reviewed 3144 patients who underwent surgery for CRC between January 2009 and December 2019, with available clinical data on recurrence and survival. All patients underwent routine colon or rectal resection and lymph node dissection according to the tumor location, with or without diverting ileostomies or colostomies. The surgical specimens were submitted to the laboratory for pathological evaluation. Patients with confirmed molecular pathology reports of *KRAS* mutation status were included, whereas those with incomplete data on *KRAS* mutations (n = 368) or microsatellite instability (MSI) status (n = 232) were excluded. Patients with dual or triple *KRAS* mutations (within more than one codon) from pathology reports (n = 2) were excluded. Additionally, to understand the biological importance and minimize the potential influence of systemic therapeutic factors on the prognosis of codon-specific *KRAS* mutations, we excluded patients with stage IV metastatic CRC (n = 339). Finally, data from 2203 eligible patients were collected separately for statistical analysis. This study was approved by the Institutional Review Board (IRB No. B-2203-742-101) of Seoul National University Bundang Hospital and the requirement for informed consent was waived.

Adjuvant/neoadjuvant therapy and follow-ups

All patients who underwent colorectal surgery for curative purposes were recommended adjuvant therapy according to the pathological stage of the cancer. Patients with pathological stage III and high-risk stage II colon cancer are recommended adjuvant chemotherapy. In rectal cancer, patients with pathological stages II and III are treated with adjuvant chemotherapy after surgery. However, in patients with clinical T4 or positive nodes without distant metastasis, preoperative chemoradiation therapy is recommended with long-course radiotherapy (dose of 5040 cGy of radiation over 5 wk; 28 fractions) combined with chemotherapy with 5-fluorouracil/Leucovorin or capecitabine.

According to the cancer monitoring protocol after curative surgery at our facility, patients were evaluated regularly one month after surgery, then every 3 mo for the first 2 years, every 6 mo for the next 3 years, and every 12 mo thereafter for a total of 5 years. Monitoring included measurements of serum carcinoembryonic antigen (CEA) levels every 3 mo; imaging modalities, including computed tomography (CT) (abdomen, pelvis, and chest) every 6 mo; and annual colonoscopy. Cancer recurrence was confirmed histologically or radiologically. The assigned research nurse constantly updated the data on recurrence and death. Information about deaths was double-checked by comparison with the database of the National Health Insurance Service, Korea, which lists the life and death records of Korean people. The registry data were constantly updated and managed by an assigned research nurse in the colorectal surgery department of our hospital.

Data collection

Basic patient clinical information [age, sex, height, weight, and American Society of Anesthesiologists (ASA) score] was collected. Cancer-related clinical characteristics such as primary tumor location, preoperative CEA level, and diverting stoma were included. Data on pathological features were collected based on pathology reports of surgical specimens. The following variables were statistically analyzed: T and N stages, tumor size, lymphatic invasion, vascular invasion, perineural invasion, number of harvested lymph nodes, number of metastatic lymph nodes, MSI status, and *KRAS* mutation status. Codon-specific *KRAS* mutation status was examined for codons 12, 13, and 61.

KRAS mutations were identified from formalin-fixed, paraffin-embedded cancerous tissue obtained from surgical specimens. After deoxyribonucleic acid (DNA) extraction from the tissue, the exons 2 and 3 of the *KRAS* gene were separately amplified by polymerase chain reaction (PCR) using optimized PCR reagents and primers. Codon-specific *KRAS* mutations were identified by pyrosequencing (PyroMark Q24 Mdx, QIAGEN, Hilden, Germany). MSI status was also evaluated using formalin-fixed tissues during surgery. PCR with five markers (*BAT26, BAT25, D5S346, D17S250,* and *D2S123*) followed by fragmentation assay (ABI-3130xl, Thermo Fisher Scientific, MA, United States) was performed to identify the MSI status.

Statistical analysis

Descriptive statistics were used to identify the basic clinicopathological characteristics of the patients, including MSI status frequency and *KRAS* mutations. The differences between wild-type and mutant *KRAS* as well as the mean values of continuous variables, were compared using either the independent *t*-test or the Mann-Whitney *U* test according to the results of the Kolmogorov-Smirnov test. Chi-squared or Fisher's exact tests were used to compare categorical variables. Overall survival (OS) and recurrence-free survival (RFS) were calculated from the date of surgery and compared using the Kaplan-Meier method and the log-rank test. For the analysis of risk factors for tumor recurrence, the Cox proportional hazards regression model was used, with the covariance input criterion set at $P < 0.1$. Patients were subdivided based on the primary tumor location (colon *vs* rectum) and MSI status [microsatellite stable (MSS)/MSI-low *versus* MSI-high]. Each subgroup was analyzed for recurrence-related factors using a Cox proportional hazards regression model. All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS), version 25.0, for Windows (SPSS, IBM). Descriptive results of continuous variables are expressed as mean ± SD. P value < 0.05 were considered statistically significant.

RESULTS

The present study included 2203 patients who underwent CRC surgery. The clinicopathological characteristics of the patients are shown in Table 1. In terms of MSI status, 1866 patients (84.7%) were identified as MSS, 153 (6.9%) as MSI-low, and 184 (8.4%) as MSI-high (Figure 1A). *KRAS* mutations were detected in 840 patients (38.1%) patients. The incidence of *KRAS* codons 12, 13, and 61 substitutions was 27.7%, 9.1%, and 1.3%, respectively (Figure 1B).

Among the clinical characteristics, female sex, lower ASA score, right-sided colon cancer, higher preoperative CEA levels, and low rates of diverting stoma formation were associated with *KRAS* mutations in codons 12, 13, and 61. Most pathological features, including T stage, N stage, tumor size, lymphatic invasion, perineural invasion, and number of harvested lymph nodes, were associated with *KRAS* mutations, along with molecular features such as MSI status (Table 2).

Analysis of the codon-specific *KRAS* mutational status revealed significant associations of both clinical and pathological characteristics with *KRAS* codon 12 mutations, including female sex, lower ASA score, right-sided colon cancer, preoperative CEA level above the normal range (≥ 5.0 ng/mL), T stage, N stage, MSI status, tumor size, vascular invasion, and perineural invasion. In contrast, only female sex, right-sided colon cancer, high preoperative CEA levels, diverting stoma formation, and no pathological features were significantly correlated with *KRAS* codon 13 mutations. Other than perineural invasion, no clinical characteristics or pathological features were associated with *KRAS* codon 61 mutations (Table 2).

At a mean ± SD follow-up duration of 29.7 mo ± 14.3 mo, and a median of 29 (0-85) months, recurrence within 5 years of curative surgery was observed in 205 (9.3%) among the 2203 patients. Five-year RFS (78.3% *vs* 77.4%, $P = 0.130$) and OS (89.0% *vs* 89.5%, $P = 0.971$) rates did not differ significantly between the wild-type and *KRAS* mutant CRC groups. Notably, the 5-year RFS for all codon-specific *KRAS* mutations was statistically different (wild-type, codon 12, and codon 13 mutations: 78.4%, 75.5%, and 85.3%, respectively; $P = 0.013$; Figure 2A), but the 5-year OS rates were comparable (wild-type, codon 12, and codon 13 mutations: 89.2%, 89.8%, and 86.9%, respectively; $P = 0.805$; Figure 2B). The 5-year RFS rate of the *KRAS* codon 12 mutation group was significantly lower than that of the patients without codon 12 mutations (78.9% *vs* 75.5%, $P = 0.025$; Figure 3A). The 5-year RFS rate of the *KRAS* codon 13 mutation group was higher than that of the patients without codon 13 mutations; however, the difference was not statistically significant (77.2% *vs* 85.3%, $P = 0.159$; Figure 3B). The RFS of the *KRAS* codon 61 mutation group was significantly lower than that of the patients without codon 61 mutations (78.2% *vs* 60.6%, $P = 0.039$; Figure 3C); however, all cases of recurrence occurred within 2 years of surgery.

In the univariate analysis of recurrence-related factors, cancer location (colon or rectum), preoperative CEA level, diverting stoma, T stage, N stage, MSI status, tumor size, lymphatic invasion, vascular invasion, perineural invasion, number of metastatic lymph nodes, and *KRAS* codon 12 mutations were associated with recurrence. In multivariable analysis, most pathological features, including higher T stage [hazard ratio (HR): 2.620; 95% confidence intervals (CI): 1.479-4.641; $P = 0.001$], higher N stage (HR: 2.001; 95%CI: 1.399-2.861; $P < 0.001$), vascular invasion (HR: 1.578; 95%CI: 1.164-2.139; $P = 0.003$), perineural invasion (HR: 1.684; 95%CI: 1.194-2.376; $P = 0.003$), and mutation of *KRAS* codon 12 (HR: 1.399; 95%CI: 1.034-1.894; $P = 0.030$) were identified as independent risk factors of recurrence in multivariable analysis. Among the clinical characteristics, only the presence of a diverting stoma (HR: 1.874; 95%CI: 1.260-2.787; $P = 0.002$) was independently correlated with recurrence (Table 3).

Tumor size (HR: 1.100; 95%CI: 1.011-1.198; $P = 0.027$), vascular invasion (HR: 1.981; 95%CI: 1.362-2.880; $P < 0.001$), perineural invasion (HR: 1.793; 95%CI: 1.200-2.679; $P = 0.004$), the presence of metastatic lymph nodes (HR: 1.048; 95%CI: 1.014-1.083; $P = 0.006$), and *KRAS* codon 12 mutation (HR: 1.496; 95%CI: 1.019-2.196; $P = 0.040$) were determined as independent risk factors for cancer recurrence when the primary tumor location was in the colon. Perineural invasion (HR: 3.358; 95%CI: 1.885-5.983; $P < 0.001$), and the presence of metastatic lymph nodes (HR: 1.095; 95%CI: 1.017-1.178; $P = 0.016$) were independently associated with cancer recurrence when the primary tumor was in the rectum. No codon-specific *KRAS* mutations were associated with recurrent rectal cancer (Table 4).

Among MSS/MSI-low CRC patients, tumor size (HR: 1.117; 95%CI: 1.038-1.202; $P = 0.003$), vascular invasion (HR: 1.740; 95%CI: 1.282-2.363; $P < 0.001$), perineural invasion (HR: 2.335; 95%CI: 1.663-3.279; $P < 0.001$), number of metastatic lymph nodes (HR: 1.050; 95%CI: 1.020-1.081; $P = 0.001$), and *KRAS* codon 12 mutation (HR: 1.467; 95%CI: 1.077-1.998; $P = 0.015$) were independent risk factors for cancer recurrence. In contrast, only a high preoperative CEA level (HR: 8.321; 95%CI: 1.387-49.920; $P = 0.020$) was associated with recurrence in MSI-high CRC. In cases of MSI-high CRC, the *KRAS* codon 12 mutation was statistically irrelevant regarding cancer recurrence, and there were no cases of recurrence during the study period among patients with *KRAS*-mutant CRC involving codons 13 and 61 (Table 5).

DISCUSSION

Among the 2203 patients who underwent curative surgery for stage I-III CRC, the incidence of codon-specific *KRAS* abnormalities was, respectively, 27.7%, 9.1%, and 1.3% for patients with *KRAS* codon 12, 13, and 61 mutations. Only 9.3% (205/2203) recurrences were observed during the 5-year follow-up period. To our knowledge, this study is based on the largest scaled cohort that has ever analyzed not only the oncological impact but also the clinicopathological characteristics of codon-specific *KRAS* mutations in patients with CRC. Most previous studies have reported similar results for *KRAS* codon 12 mutations, but not codon 13, in CRC as a poor oncological factor[4,8,12,14,20]. Despite the minimal oncological effects of minor *KRAS* mutations, such as in codon 61, the data obtained were sufficient to gain statistical power, supporting previous findings that *KRAS* codon 61 mutation is not associated with the clinicopathological features of CRC

Table 1 Clinicopathologic characteristics of the study patients

Clinical characteristics (*n* = 2203)	Value[1]
Age (yr)	64.7 ± 12.2
Sex	
Male	1264 (57.4)
Female	939 (42.6)
Body mass index (kg/m^2)	23.9 ± 3.3
ASA score	
1	575 (26.1)
2	1412 (64.1)
3	211 (9.6)
4	5 (0.2)
Cancer location	
Cecum	46 (2.1)
Ascending colon	386 (17.5)
Hepatic flexure	88 (4.0)
Transverse colon	115 (5.2)
Splenic flexure	18 (0.8)
Descending colon	79 (3.6)
Sigmoid colon	771 (35.0)
Rectum	700 (31.7)
Preoperative CEA (ng/mL)	7.7 ± 42.3
Diverting stoma	
Ileostomy	435 (19.7)
Colostomy	62 (2.8)
T stage	
0	18 (0.8)
1	275 (12.5)
2	383 (17.4)
3	1282 (58.2)
4	245 (11.1)
N stage	
0	1286 (58.4)
1	639 (29.0)
2	278 (12.6)
Tumor size (cm)	4.4 ± 2.4
Lymphatic invasion	597 (27.1)
Vascular invasion	469 (21.3)
Perineural invasion	934 (42.4)
Harvested lymph nodes	45.3 ± 21.2
Metastatic lymph nodes	1.4 ± 2.9
Adjuvant/Neoadjuvant therapy	
Colon	

Adjuvant therapy-stage II	274 (51.4)
Adjuvant therapy-stage III	575 (90.0)
Rectum	
Neoadjuvant therapy	200 (28.6)
Operative first-Adjuvant therapy	264 (52.8)

[1]Results are reported as mean ± SD or as frequency (percent).
ASA: American Society of Anesthesiologists; CEA: Carcinoembryonic antigen; AJCC: American Joint Committee on Cancer.

[21]. An earlier study in a Japanese cohort also identified *KRAS* codon 12, but not codon 13, as an independent risk factor for tumor recurrence in stage I-III CRC. While their results supported the utility of *KRAS* codon 12 mutation as a poor prognostic factor, the correlation between codon-specific *KRAS* mutations and clinicopathological characteristics could not be validated because of the small sample size[20]. In the present study, we analyzed the largest sample group of patients, which provided not only results complementing earlier studies on *KRAS* mutations in CRC, but also additional information on correlations with clinicopathological characteristics and prognostic factors for individual codon-specific *KRAS* mutations.

In addition to resistance to anti-EGFR therapies, such as cetuximab and panitumumab[22], *KRAS* codon 12 mutation in CRC has been established as a poor prognostic factor of survival associated with aggressive behavior[23]. However, the role of *KRAS* codon 13 mutation in CRC remains unclear. Several studies have suggested that *KRAS* codon 13 mutations are associated with advanced-stage disease and metastasis of CRC and potentially serve as a predictive factor for a higher likelihood of death[17,18]. An earlier meta-analysis reported a lower overall survival in patients with *KRAS* codon 13 mutant CRC with no exposure to anti-EGFR therapy than in those treated with targeted therapy[24]. Other studies have demonstrated that *KRAS* codon 13 mutations are not associated with CRC progression[4,19]. Another meta-analysis of metastatic CRC with mutated *KRAS* codon 13 revealed a more significant response to cetuximab than that in patients with other codon-specific *KRAS* mutations[25]. To ascertain the correlation between codon-specific *KRAS* mutations and clinical oncological outcomes throughout the stages of CRC, therapeutic options such as chemotherapy, radiotherapy, and targeted therapy, along with inevitable resistance mechanisms, should be considered[5,26,27].

The survival analysis showed that CRC recurrence, but not overall survival, was associated with codon-specific *KRAS* mutations. Analysis of individual codons showed that *KRAS* codon 12 mutation is an independent risk factor for recurrence, while *KRAS* codon 13 and 61 mutations appeared to be statistically irrelevant. In earlier *in vivo* molecular biology studies, cells with *KRAS* codon 12 and 13 mutations displayed similar morphological changes, but only codon 12 mutants induced anchorage-independent growth, implying a lower aggressiveness of *KRAS* codon 13 mutations[15]. Another *in vitro* study reported that *KRAS* codon 12 mutant cells were more resistant to apoptosis and exhibited enhanced anti-apoptotic molecular signaling relative to codon 13 mutant cells, consistent with the finding that the codon 13 mutation is less aggressive[16]. These *in vivo* results were translated into the clinical outcomes of our study, demonstrating that *KRAS* codon 13 mutation is less aggressive and less likely to serve as a poor prognostic factor for CRC compared with *KRAS* codon 12 mutation.

Interestingly, the prognosis of *KRAS* codon 12 mutant CRC varied based on the primary tumor location in either the colon or rectum. The majority of experiments on tumor location were stratified into right- or left-sided colorectum based on the splenic flexure[28,29]. Even the definition of 'left-sided' differs among studies according to the involvement of the rectum[30,31]. Thus, in the present study, recurrence-related factors were analyzed by subgrouping the tumors into colon and rectum. In the subgroup of tumors located in the colon, patients with *KRAS* codon 12 mutations were estimated to be at a 1.5-fold higher risk of CRC recurrence than those without codon 12 mutations. In contrast, in the rectum, all codon-specific *KRAS* mutations were not linked to recurrence. To the best of our knowledge, this is the first study to investigate the oncological impact of codon-specific *KRAS* mutations based on tumor location (colon or rectum). Our findings support the theory that *KRAS* codon 12 mutation is a poor prognostic factor for colon cancer, but not for rectal cancer.

Previous studies have shown that the combination of *KRAS* mutations and MSI status is a potential prognostic factor in various stages of CRC [26,32-36]. In addition, since MSI status is associated with chemoresistance[37,38], the MSS/MSI-low and MSI-high subgroups were analyzed separately to eliminate the effect of MSI status on prognosis. Interestingly, in the MSS/MSI-low patient subgroup, only *KRAS* codon 12 mutation was statistically related to recurrence, whereas there was no association between codon-specific *KRAS* mutations and recurrence among MSI-high tumors. It is well known that poor oncological outcomes including disease-free and overall survival were reported within MSS tumors combined with *KRAS* mutation[33-36]. To the best of our knowledge, analysis results of codon-specific *KRAS* mutations in MSS/MSI-low and MSI-high tumors have never been reported. Based on our subgroup analysis, *KRAS* codon 12 mutations may be associated with the location of colon and MSS tumors, and not all CRC patients with *KRAS* codon 12 mutations have poor outcomes.

Clarifying the effects of codon-specific *KRAS* mutations on the prognosis of stage IV CRC is a complex issue[5,26,27,39]. A recent study on *KRAS* mutations in CRC with liver metastasis reported that *KRAS* codon 12 mutations were associated with poorer overall survival, while codon 13 was not; however, they also pointed out the exclusion of perioperative management such as anti-epidermal growth factor receptor agents[12]. Among the patients diagnosed with stage IV CRC who underwent surgery in our hospital during the period of the present study, 48.4% had *KRAS* mutations. However, only about half of them (53.1%) underwent surgery with curative intent, whereas the others underwent

Table 2 Univariable analysis[1] of each codon-specific *KRAS* mutation

	***KRAS* overall[2]**			***KRAS* Codon 12**			***KRAS* Codon 13**			***KRAS* Codon 61**		
	WT (%)	**MT (%)**	***P* value**	**WT (%)**	**MT (%)**	***P* value**	**WT (%)**	**MT (%)**	***P* value**	**WT (%)**	**MT (%)**	***P* value**
Age			0.418			0.734			0.698			0.246
< 65 yr	644 (62.8)	382 (37.2)		745 (72.6)	281 (27.4)		936 (91.1)	91 (8.9)		1016 (99.0)	10 (1.0)	
≥ 65 yr	719 (61.1)	458 (38.9)		847 (72.0)	330 (28.0)		1067 (90.7)	110 (9.3)		1160 (98.5)	18 (1.5)	
Sex			**< 0.001**			**< 0.001**			**0.006**			0.238
Male	851 (67.3)	413 (32.7)		961 (76.0)	303 (24.0)		1167 (92.3)	97 (7.7)		1251 (99.0)	13 (1.0)	
Female	512 (54.5)	427 (45.5)		631 (67.2)	308 (32.8)		836 (88.9)	104 (11.1)		924 (98.4)	15 (1.6)	
BMI			0.098			0.347			0.485			0.104
< 25 kg/m^2	853 (60.6)	555 (39.4)		1008 (71.6)	400 (28.4)		1275 (90.6)	133 (9.4)		1386 (98.4)	22 (1.6)	
≥ 25 kg/m^2	510 (64.2)	285 (35.8)		584 (73.5)	211 (26.5)		727 (91.4)	68 (8.6)		789 (99.2)	6 (0.8)	
ASA score			**0.010**			**0.002**			0.942			0.347
1-2	1212 (61.0)	775 (39.0)		1417 (71.3)	570 (28.7)		1806 (90.9)	181 (9.1)		1963 (98.8)	24 (1.2)	
3-4	151 (69.9)	65 (30.1)		175 (81.0)	41 (19.0)		196 (90.7)	20 (9.3)		212 (98.1)	4 (1.9)	
Cancer location (1)[3]			**< 0.001**			**0.002**			**< 0.001**			0.219
Right-sided	329 (51.8)	306 (48.2)		429 (67.6)	206 (32.4)		546 (86.0)	89 (14.0)		624 (98.3)	11 (1.7)	
Left-sided	1034 (65.9)	534 (34.1)		1163 (74.2)	405 (25.8)		1456 (92.9)	112 (7.1)		1552 (98.9)	17 (1.1)	
Cancer location (2)[4]			0.092			0.405			0.117			0.966
Colon	912 (60.7)	591 (39.3)		1078 (71.7)	425 (28.3)		1356 (90.2)	147 (9.8)		1484 (98.7)	19 (1.3)	
Rectum	451 (64.4)	249 (35.6)		514 (73.4)	186 (26.6)		646 (92.3)	54 (7.7)		691 (98.7)	9 (1.3)	
Preoperative CEA			**< 0.001**			**< 0.001**			**0.037**			0.301
< 5.0 ng/mL	1131 (64.7)	616 (35.3)		1299 (74.4)	448 (25.6)		1599 (91.5)	148 (8.5)		1727 (98.9)	20 (1.1)	
≥ 5.0 ng/mL	232 (50.9)	224 (49.1)		293 (64.3)	163 (35.7)		403 (88.4)	53 (11.6)		448 (98.2)	8 (1.8)	
Diverting stoma			**0.001**			0.071			**0.029**			0.131
No	1024 (60.0)	682 (40.0)		1217 (71.3)	489 (28.7)		1538 (90.2)	168 (9.8)		1681 (98.5)	25 (1.5)	
Yes	339 (68.2)	158 (31.8)		375 (75.5)	122 (24.5)		464 (93.4)	33 (6.6)		494 (99.4)	3 (0.6)	
T stage			**0.008**			**0.003**			0.488			0.139

T0-2	446 (66.0)	230 (34.0)		517 (76.5)	159 (23.5)		610 (90.2)	66 (9.8)		671 (99.3)	5 (0.7)	
T3-4	917 (60.1)	610 (39.9)		1075 (70.4)	452 (29.6)		1392 (91.2)	135 (8.8)		1504 (98.5)	23 (1.5)	
N stage			**0.012**			**0.046**			0.617			0.094
N0	824 (64.1)	462 (35.9)		950 (73.9)	336 (26.1)		1172 (91.1)	114 (8.9)		1274 (99.1)	12 (0.9)	
N1-2	539 (58.8)	378 (41.2)		642 (70.0)	275 (30.0)		830 (90.5)	87 (9.5)		901 (98.3)	16 (1.7)	
MSI status			**0.003**			**0.001**			0.458			0.973
MSS	1138 (61.0)	728 (39.0)		1327 (71.1)	539 (28.9)		1701 (91.2)	165 (8.8)		1842 (98.7)	24 (1.3)	
MSI-low	90 (58.8)	63 (41.2)		110 (71.9)	43 (28.1)		135 (88.2)	18 (11.8)		151 (98.7)	2 (1.3)	
MSI-high	135 (73.4)	49 (26.6)		155 (84.2)	29 (15.8)		166 (90.2)	18 (9.8)		182 (98.9)	2 (1.1)	
Tumor size (cm)	4.3 ± 2.4	4.6 ± 2.3	**0.005**	4.3 ± 2.5	4.6 ± 2.1	**0.001**	4.4 ± 2.3	4.6 ± 2.7	0.837	4.4 ± 2.4	4.5 ± 2.1	0.708
Lymphatic invasion			**0.005**			0.099			0.080			0.302
No	1022 (63.6)	584 (36.4)		1176 (73.2)	430 (26.8)		1470 (91.5)	136 (8.5)		1589 (98.9)	18 (1.1)	
Yes	341 (57.1)	256 (42.9)		416 (69.7)	181 (30.3)		532 (89.1)	65 (10.9)		587 (98.3)	10 (1.7)	
Vascular invasion			0.090			**0.047**			0.614			0.061
No	1057 (61.0)	677 (39.0)		1236 (71.3)	498 (28.7)		1573 (90.7)	161 (9.3)		1716 (99.0)	18 (1.0)	
Yes	306 (65.2)	163 (34.8)		356 (75.9)	113 (24.1)		429 (91.5)	40 (8.5)		459 (97.9)	10 (2.1)	
Perineural invasion			**0.003**			**0.027**			0.387			**0.048**
No	819 (64.5)	450 (35.5)		940 (74.1)	329 (25.9)		1159 (91.3)	110 (8.7)		1258 (99.1)	11 (0.9)	
Yes	544 (58.2)	390 (41.8)		652 (69.8)	282 (30.2)		843 (90.3)	91 (9.7)		917 (98.2)	17 (1.8)	
Harvested LN	44.5 ± 20.6	46.5 ± 22.1	**0.040**	45.0 ± 21.1	45.9 ± 21.6	0.500	45.0 ± 20.9	47.9 ± 24.1	0.079	45.2 ± 21.3	47.7 ± 16.6	0.208
Metastatic LN	1.4 ± 3.1	1.3 ± 2.6	0.420	1.4 ± 3.1	1.3 ± 2.4	0.406	1.4 ± 2.9	1.4 ± 3.1	0.832	1.4 ± 2.9	1.4 ± 1.9	0.149

[1]The continuous variables were compared using either independent *t*-test or Mann-Whitney *U* test; the categorical variables were compared using Chi-square or Fisher's exact test.
[2]Kirsten rat sarcoma viral oncogene homolog overall indicates at least one mutation within codon 12, 13, or 61.
[3]Right-sided: From the cecum to distal 2/3 transverse colon; Left-sided: From the splenic flexure to rectum.
[4]Rectum: Below the pelvic inlet (an imaginary line drawn from the sacral promontory to the pubic symphysis).
KRAS: Kirsten rat sarcoma viral oncogene homolog; WT: Wild-type; MT: Mutation; BMI: Body mass index; ASA: American Society of Anesthesiologists; CEA: Carcinoembryonic antigen; MSI: Microsatellite instability; MSS: Microsatellite stable; LN: Lymph node.

palliative treatment. Additionally, there is a wide range of variations in the metastatic burden and forms of treatment for these patients. Therefore, in the present study, we excluded stage IV disease to focus on the biological importance and prognostic impact of codon-specific *KRAS* mutations in stage I-III CRC.

Table 3 Univariable and Cox regression analyses of *KRAS* mutations for determination of recurrence-related factors

	Recurrence			Multivariable Cox regression analysis[2]			
	Absent[1] (*n* = 1998)	Present[1] (*n* = 205)	*P* value	HR	95%CI		*P* value
					Lower	Upper	
Age (yr)			0.716				
< 65	933 (46.7)	93 (45.4)		-	-	-	-
≥ 65	1065 (53.3)	112 (54.6)		-	-	-	
Sex			0.616				
Male	1143 (57.2)	121 (59.0)		-	-	-	-
Female	855 (42.8)	84 (41.0)		-	-	-	
BMI			0.094				
< 25 kg/m^2	1266 (63.4)	142 (69.3)		-	-	-	-
≥ 25 kg/m^2	732 (36.6)	63 (30.7)		-	-	-	
ASA score			0.980				
1-2	1802 (90.2)	185 (90.2)		-	-	-	-
3-4	196 (9.8)	20 (9.8)		-	-	-	
Cancer location (1)[3]			0.860				
Right-sided	577 (28.9)	58 (28.3)		-	-	-	-
Left-sided	1421 (71.1)	147 (71.7)		-	-	-	
Cancer location (2)[4]							
Colon	1376 (68.9)	127 (62.0)	**0.043**	1.000			
Rectum	622 (31.1)	78 (38.0)		1.053	0.718	1.545	0.791
Preoperative CEA			**< 0.001**				
< 5.0 ng/mL	1607 (80.4)	140 (68.3)		1.000			
≥ 5.0 ng/mL	391 (19.6)	65 (31.7)		1.158	0.849	1.579	0.354
Diverting stoma			**< 0.001**				
No	1568 (78.5)	138 (67.3)		1.000			
Yes	430 (21.5)	67 (32.7)		1.874	1.260	2.787	**0.002**
T stage			**< 0.001**				
T0-2	659 (33.0)	17 (8.3)		1.000			
T3-4	1339 (67.0)	188 (91.7)		2.620	1.479	4.641	**0.001**
N stage			**< 0.001**				
N0	1230 (61.6)	56 (27.3)		1.000			
N1-2	768 (38.4)	149 (72.7)		2.001	1.399	2.861	**< 0.001**
MSI status			**0.037**				
MSS	1680 (84.1)	186 (90.7)		0.855	0.342	2.138	0.738
MSI-low	143 (7.2)	10 (4.9)		1.284	0.643	2.566	0.479
MSI-high	175 (8.8)	9 (4.4)		1.000			
Tumor size (cm)	4.3 ± 2.4	4.9 ± 2.1	**< 0.001**	0.997	0.927	1.074	0.944
Lymphatic invasion			**< 0.001**				
No	1493 (74.7)	113 (55.1)		1.000			
Yes	505 (25.3)	92 (44.9)		1.324	0.977	1.793	0.070
Vascular invasion			**< 0.001**				

No	1615 (80.8)	119 (58.0)		1.000			
Yes	383 (19.2)	86 (42.0)		1.578	1.164	2.139	**0.003**
Perineural invasion			**< 0.001**				
No	1211 (60.6)	58 (28.3)		1.000			
Yes	787 (39.4)	147 (71.7)		1.684	1.194	2.376	**0.003**
Harvested LN	45.3 ± 21.2	44.9 ± 21.4	0.705	-	-	-	-
Metastatic LN	1.2 ± 2.6	3.3 ± 4.5	**< 0.001**	1.028	0.995	1.061	0.095
KRAS Codon 12							
Wild-type	1459 (73.0)	133 (64.9)	**0.013**	1.000			
Mutation	539 (27.0)	72 (35.1)		1.399	1.034	1.894	**0.030**
KRAS Codon 13							
Wild-type	1809 (90.5)	193 (94.1)	0.088	1.000			
Mutation	189 (9.5)	12 (5.9)		0.637	0.350	1.160	0.140
KRAS Codon 61							
Wild-type	1975 (98.8)	200 (97.6)	0.176	1.000			
Mutation	23 (1.2)	5 (2.4)		1.950	0.790	4.812	0.147

[1]Results are reported as mean ± SD or as number (percent).
[2]No values indicated variables do not match the covariance input criterion ($P < 0.1$ in univariable analysis).
[3]Right-sided: From the cecum to distal 2/3 transverse colon; Left-sided: From the splenic flexure to rectum.
[4]Rectum: Below the pelvic inlet (an imaginary line drawn from the sacral promontory to the pubic symphysis).
KRAS: Kirsten rat sarcoma viral oncogene homolog; HR: Hazard ratio; CI: Confidence interval; BMI: Body mass index; ASA: American Society of Anesthesiologists; CEA: Carcinoembryonic antigen; MSI: Microsatellite instability; MSS: Microsatellite stable; LN: Lymph node.

Table 4 Cox regression analyses of recurrence-related factors in subgroups based on tumor location in the colon and rectum

	Colon (*n* = 1503)		Rectum (*n* = 700)	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Preoperative CEA ≥ 5.0 ng/mL	1.290 (0.860-1.933)	0.218	1.215 (0.705-2.220)	0.444
Diverting stoma (+)	0.903 (0.394-2.067)	0.809	1.249 (0.755-2.065)	0.386
T3-4 stage (*vs* T0-2)	1.211 (0.737-1.991)	0.450	1.079 (0.601-1.939)	0.799
N1-2 stage (*vs* N0)	1.241 (0.863-1.784)	0.244	1.126 (0.649-1.954)	0.674
Tumor size (cm)	1.100 (1.011-1.198)	**0.027**	1.077 (0.948-1.223)	0.256
Lymphatic invasion	1.342 (0.919-1.960)	0.128	0.971 (0.562-1.676)	0.915
Vascular invasion	1.981 (1.362-2.880)	**< 0.001**	1.401 (0.841-2.334)	0.195
Perineural invasion	1.793 (1.200-2.679)	**0.004**	3.358 (1.885-5.983)	**< 0.001**
Metastatic LN	1.048 (1.014-1.083)	**0.006**	1.095 (1.017-1.178)	**0.016**
KRAS Codon 12 mutation	1.496 (1.019-2.196)	**0.040**	1.492 (0.902-2.466)	0.119
KRAS Codon 13 mutation	0.831 (0.412-1.678)	0.606	0.481 (0.146-1.578)	0.227
KRAS Codon 61 mutation	2.385 (0.730-7.795)	0.150	2.270 (0.511-10.088)	0.282

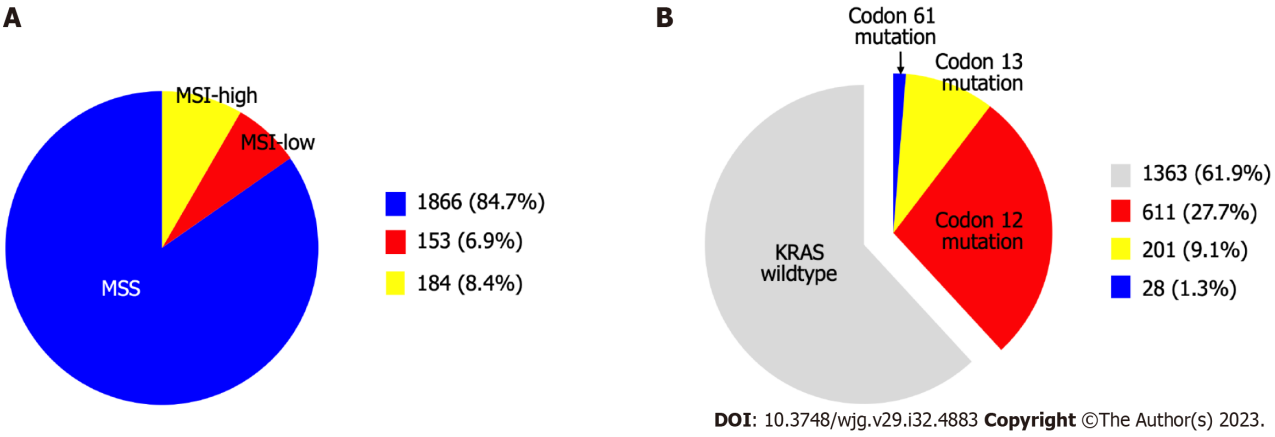
KRAS: Kirsten rat sarcoma viral oncogene homolog; HR: Hazard ratio; CI: Confidence interval; CEA: Carcinoembryonic antigen; MSI: Microsatellite instability; MSS: Microsatellite stable; LN: Lymph node.

In two patients in our cohort, *KRAS* mutations were detected at two or more codon sites. The first patient was a 75-year-old male who underwent surgery for descending colon cancer and was pathologically diagnosed with stage III (pT3N1M0) colon cancer with codon 12 and 13 *KRAS* mutations. The second patient was a 60-year-old female who underwent surgery for sigmoid colon cancer diagnosed as stage I (pT1N0M0) with both codon 12 and 61 *KRAS* mutations. Both patients survived for more than 5 years after surgery with no recurrence or metastasis. In a previous

Table 5 Cox regression analyses of recurrence-related factors in subgroups based on microsatellite instability status: Microsatellite stable/microsatellite instability-low *versus* microsatellite instability-high

	MSS/MSI-low (*n* = 2019)		MSI-high (*n* = 184)[1]	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Preoperative CEA ≥ 5.0 ng/mL	1.178 (0.839-1.653)	0.344	8.321 (1.387-49.920)	**0.020**
Rectal cancer (*vs* colon cancer)	1.238 (0.850-1.804)	0.266	-	-
Diverting stoma (+)	1.069 (0.707-1.617)	0.752	2.431 (0.139-42.442)	0.543
T3-4 stage (*vs* T0-2)	1.175 (1.038-1.202)	0.407	0.284 (0.020-4.032)	0.353
N1-2 stage (*vs* N0)	1.190 (0.879-1.610)	0.260	1.000 (0.151-6.643)	1.000
Tumor size (cm)	1.117 (1.038-1.202)	**0.003**	0.991 (0.713-1.379)	0.960
Lymphatic invasion	1.242 (0.909-1.698)	0.174	1.154 (0.149-8.923)	0.891
Vascular invasion	1.740 (1.282-2.363)	**< 0.001**	0.009 (0.000-29.277)	0.255
Perineural invasion	2.335 (1.663-3.279)	**< 0.001**	0.538 (0.049-5.909)	0.613
Metastatic LN	1.050 (1.020-1.081)	**0.001**	1.442 (0.865-2.402)	0.160
KRAS Codon 12 mutation	1.467 (1.077-1.998)	**0.015**	2.508 (0.406-15.510)	0.323
KRAS Codon 13 mutation	0.713 (0.390-1.301)	0.270	-	-
KRAS Codon 61 mutation	2.265 (0.915-5.605)	0.077	-	-

[1]No values: Due to a small sample size, the hazard ratio and confidence interval were not pre.
MSI: Microsatellite instability; MSS: Microsatellite stable; HR: Hazard ratio; CI: Confidence interval; CEA: Carcinoembryonic antigen; MSI: Microsatellite instability; MSS: Microsatellite stable; LN: Lymph node.

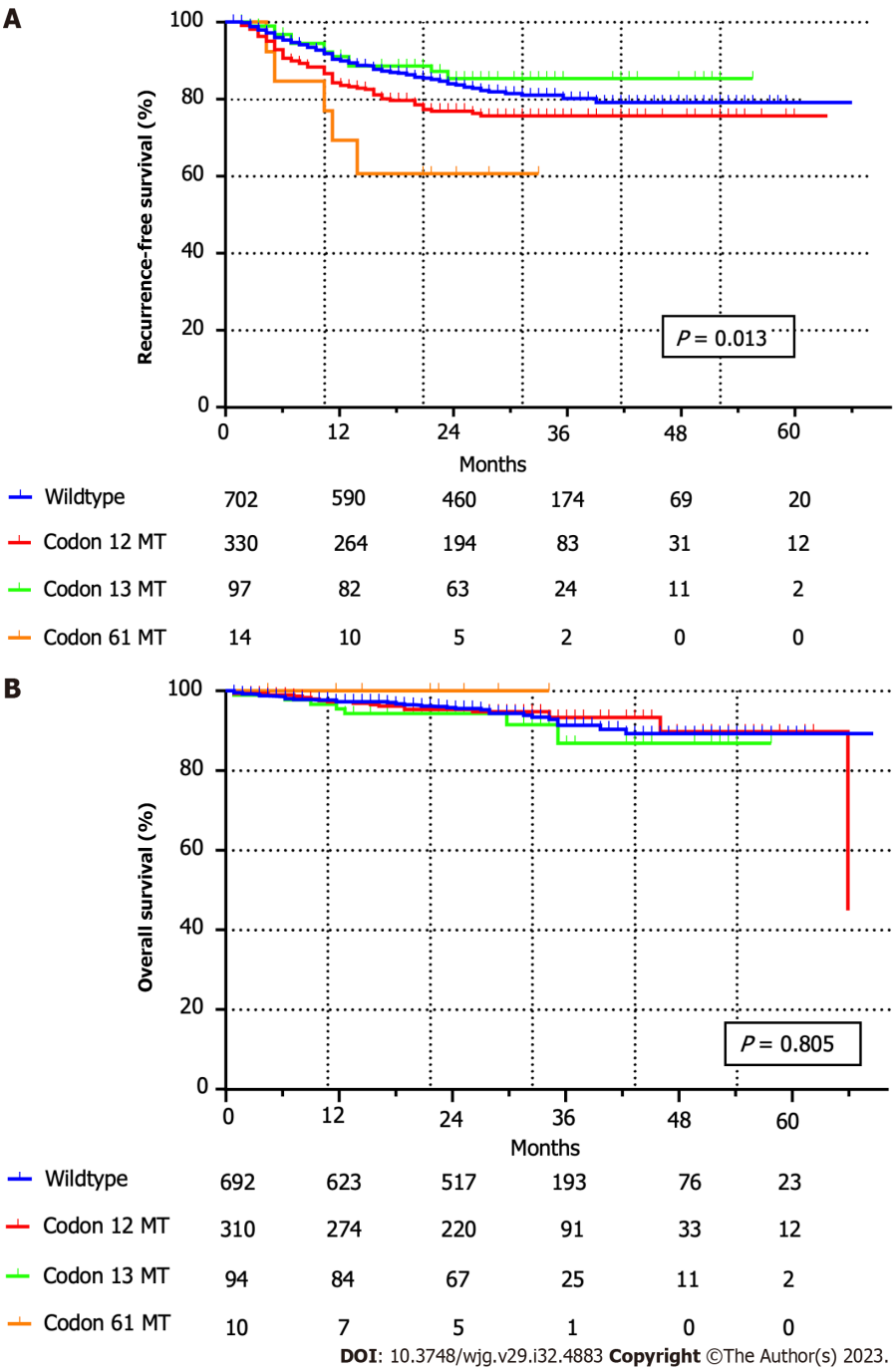


DOI: 10.3748/wjg.v29.i32.4883

Figure 1 Incidence of microsatellite instability status and Kirsten rat sarcoma viral oncogene homolog mutations. A: Microsatellite instability status; B: Mutations of the Kirsten rat sarcoma viral oncogene homolog (*KRAS*) gene in relation to baseline characteristics. *KRAS*: Kirsten rat sarcoma viral oncogene homolog; MSS: Microsatellite stable; MSI: Microsatellite instability.

study, 12 patients with two or more codon mutations among 505 CRC *KRAS* mutation cases were reported but were eventually excluded from the analysis[21]. For the same reason, these two patients were excluded from the current study despite our intellectual curiosity.

The present study had several limitations. First, *BRAF* mutation, a biomarker related to the prognosis of CRC after surgery, was omitted from our analysis. According to previous studies on CRC biomarkers, both *BRAF* and MSI status have an important prognostic impact on recurrence and survival[34,40]. Unfortunately, a large amount of data was collected without knowledge of the *BRAF* mutation status because of alterations in routine molecular examinations by our facility during the study period. Second, since *KRAS* mutations were evaluated using postoperative specimens for both colon and rectal cancer, it may be audacious to conclude that the *KRAS* codon 12 mutation is a prognostic factor in rectal cancer. In advanced rectal cancer, trimodality therapy comprises chemoradiation followed by surgery, which takes at least 1-2 mo. This delay may affect the oncological outcome; therefore, the prognostic value of codon-specific *KRAS* mutations according to the primary tumor site should be carefully interpreted. Third, uncontacted patients without follow-up could have missing data on recurrence and survival despite constantly updating the clinical data by the



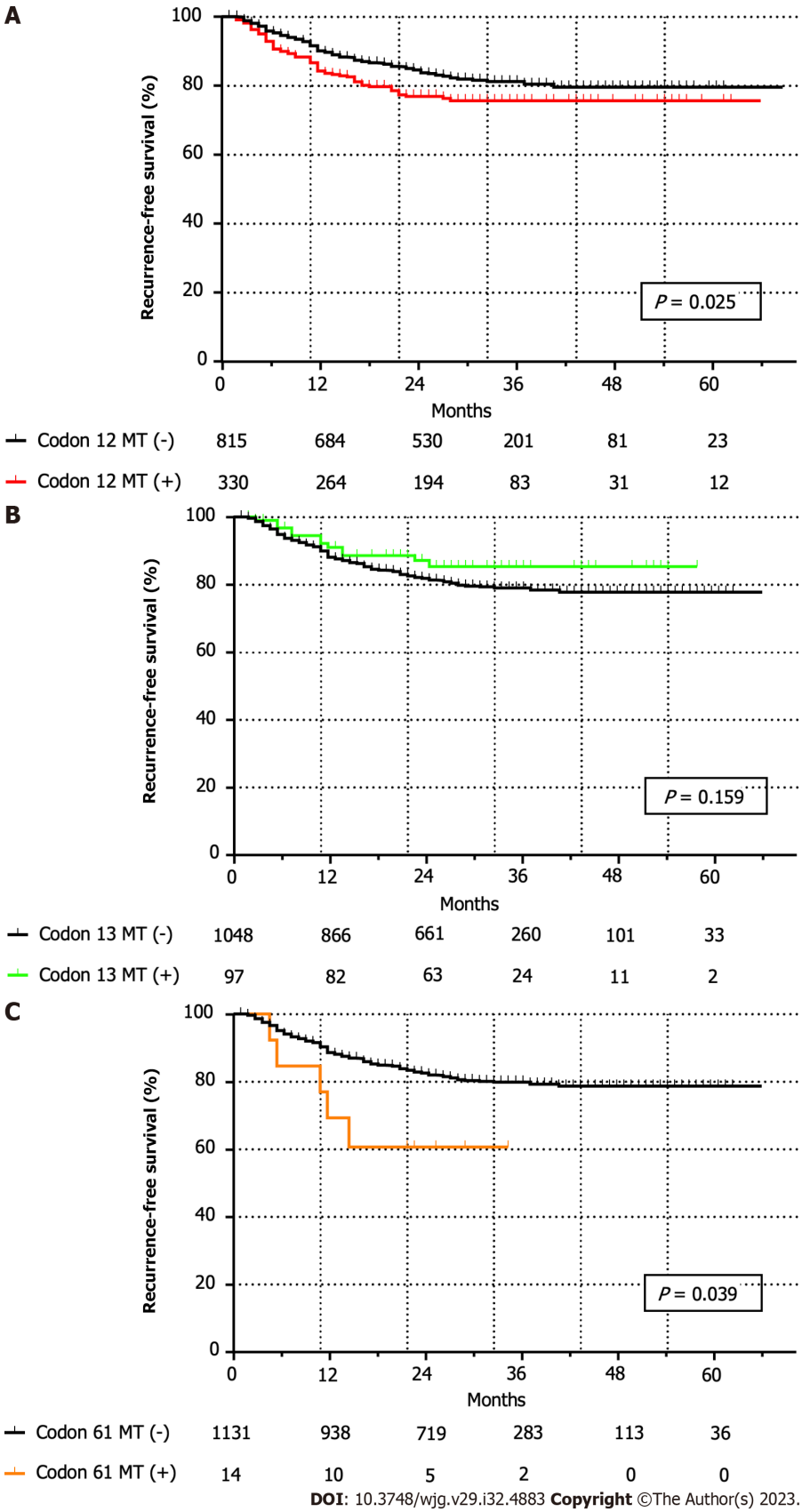
DOI: 10.3748/wjg.v29.i32.4883

Figure 2 Comparative survival analysis between colorectal cancer samples with wild-type and Kirsten rat sarcoma viral oncogene homolog mutation. Blue lines indicate wild-type Kirsten rat sarcoma viral oncogene homolog (*KRAS*). Other lines represent codon-specific *KRAS* mutations of codon 12 (red), 13 (green), and 61 (orange). A: Recurrence-free survival; B: Overall survival rates were compared using a log-rank test. WT: Wild-type; MT: Mutation.

assigned research nurses in our department. The refusal to revisit after a few follow-ups could have produced missing data in our cohort, and double-checking with the National Health Insurance database might have reduced the error as much as possible. Unfortunately, these efforts could not separate other causes of death from cancer-related ones. Fourth, this study had a retrospective and single-center design, which could have led to selection bias. Despite this, the present study was based on a large-scale cohort with a relatively well-organized CRC registry of patients who underwent surgery, and is the largest cohort study ever that analyzed codon-specific KRAS mutations.

CONCLUSION

Most of the *KRAS* mutations in our study involved *KRAS* codons 12 and 13. Notably, *KRAS* codon 12 mutation was significantly associated with pathological features closely related to cancer recurrence and had a poor prognostic impact



DOI: 10.3748/wjg.v29.i32.4883

Figure 3 Survival analysis of each codon-specific Kirsten rat sarcoma viral oncogene homolog mutation in colorectal cancer. Colored lines indicate codon-specific Kirsten rat sarcoma viral oncogene homolog (*KRAS*) mutations. A: The red line indicates the recurrence-free survival (RFS) of patients with *KRAS* codon 12 mutations; B: The green line indicates the RFS of patients with *KRAS* codon 13 mutations; C: The orange line indicates the RFS of patients with *KRAS* codon 61 mutations. MT: Mutation.

in patients with MSS tumors, or those located in the colon but not in the rectum. Given its irrelevance to pathological features and recurrence, we propose that *KRAS* codon 13 mutation is less likely to serve as a prognostic factor for CRC.

ARTICLE HIGHLIGHTS

Research background

Abnormal activation of Kirsten rat sarcoma viral oncogene homolog (*KRAS*), a well-known oncogene, triggers uncontrolled tumor cell proliferation. Approximately 40% of colorectal cancer (CRC) are linked to *KRAS* mutations. CRC-related point mutations in *KRAS* occur at different codon locations. *KRAS* codon 12 or 13 mutations are detected in a majority of CRC patients, whereas mutations in codon 61 or 146 have been reported only in a minority.

Research motivation

KRAS mutations are associated with poor CRC prognosis, especially *KRAS* codon 12 mutation, which is associated with metastasis and poorer survival. However, the clinicopathological characteristics and prognosis of *KRAS* codon 13 mutation in CRC remain controversial.

Research objectives

This study aimed to evaluate the clinicopathological characteristics and prognostic value of codon-specific *KRAS* mutations, especially in codon 13.

Research methods

This retrospective, single-center, observational cohort study included patients who underwent surgery for stage I-III CRC. The relationships between clinicopathological characteristics and individual codon-specific *KRAS* mutations were analyzed. By using the Cox proportional hazards regression model, survival analysis were performed to identify codon-specific *KRAS* mutations as recurrence-related factors.

Research results

Both *KARS* codons 12 and 13 mutations showed a tendency to be associated with clinical characteristics, but only codon 12 was associated with pathological features. *KRAS* codon 13 mutation showed no associations, whereas codon 12 was associated with a lower 5-year recurrence-free survival rate. In multivariable analysis, only codon 12 (HR: 1.399; 95% confidence interval: 1.034-1.894; P = 0.030) among *KRAS* mutations was an independent risk factor for recurrence. This may influence many oncologists to consult with patients on their prognosis after surgery.

Research conclusions

KRAS codon 12 mutation was significantly associated with pathological features closely related to cancer recurrence and had a poor prognostic impact in patients with microsatellite stable tumors, or those located in the colon but not in the rectum. On the other hand, *KRAS* codon 13 mutation is irrelevant to pathological features and recurrence, which consider less likely to serve as a prognostic factor for CRC.

Research perspectives

Focusing on the biological effects of codon-specific *KRAS* mutations, *KRAS* codon 13 mutation is less pathogenic and recurrent, Based on a large-scale cohort of patients with stage I-III CRC. This study's results may influence not only the prognosis but also the management of CRC patients individually. Therefore, the therapeutic usage and needs of codon-specific *KRAS* mutation in CRC should be considered in future studies.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Hepatitis B virus infection in patients with Wilson disease: A large retrospective study

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Abstract

BACKGROUND

Wilson disease (WD) is the most common genetic metabolic liver disease. Some studies have shown that comorbidities may have important effects on WD. Data on hepatitis B virus (HBV) infection in patients with WD are limited.

AIM

To investigate the prevalence and clinical impact of HBV infection in patients with WD.

METHODS

The clinical data of patients with WD were analyzed retrospectively, and the data of patients with concurrent WD and HBV infection were compared with those of patients with isolated WD.

RESULTS

Among a total of 915 WD patients recruited, the total prevalence of current and previous HBV infection was 2.1% [95% confidence interval (CI): 1.2%-3.0%] and 9.2% (95%CI: 7.3%-11.1%), respectively. The main finding of this study was the identification of 19 patients with concurrent WD and chronic hepatitis B (CHB) infection. The diagnosis of WD was missed in all but two patients with CHB infection. The mean delay in the diagnosis of WD in patients with concurrent WD and CHB infection was 32.5 mo, which was significantly longer than that in patients with isolated WD (10.5 mo). The rates of severe liver disease and mortality in patients with concurrent WD and CHB infection were significantly higher than those in patients with isolated WD (63.1% *vs* 19.3%, $P = 0.000$ and 36.8% *vs* 4.1%, $P < 0.001$, respectively). Binary logistic regression analysis revealed a significantly higher risk of severe liver disease at the diagnosis of WD in patients

with current HBV infection [odds ratio (OR) = 7.748; 95%CI: 2.890-20.774; *P* = 0.000)] or previous HBV infection (OR = 5.525; 95%CI: 3.159-8.739; *P* = 0.000) than in patients with isolated WD.

CONCLUSION

The total prevalence of current HBV infection in patients with WD was 2.1%. The diagnosis of WD in CHB patients is usually missed. HBV infection is an independent risk factor for severe liver disease in WD patients. The diagnosis of WD should be ruled out in some patients with CHB infection.

Key Words: Wilson disease; Hepatitis B virus; Chronic hepatitis B; Kayser-Fleischer ring; Ceruloplasmin; Concurrent Wilson disease and hepatitis B virus infection

Core Tip: Data on hepatitis B virus (HBV) infection in patients with Wilson disease (WD) are limited. This is the largest investigation of HBV infection in WD patients. The most important finding of this study was the identification of 19 patients with concurrent WD and chronic hepatitis B (CHB) infection. The total prevalence of current HBV infection in patients with WD was 2.1%. The diagnosis of WD in CHB patients is usually missed. HBV infection is an independent risk factor for severe liver disease in WD patients. The diagnosis of WD should be ruled out in some patients with CHB infection.

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INTRODUCTION

Wilson disease (WD) is an autosomal recessive disorder of copper metabolism caused by a mutation of the gene coding for copper-transporting P-type ATPase (ATP7B). It is characterized by an excessive accumulation of copper in the liver and brain, and occurs in all ethnic groups with an average prevalence of 1:30000[1,2]. The WD gene was cloned in 1993[3-5], and more than 600 gene mutations have been identified[6]. Disease progression in WD may vary, ranging from fulminant WD to an insidious progression to cirrhosis over 20-30 years. Overall, available data in literature strongly suggest that genotypic variability alone does not explain the surprisingly heterogeneous presentation of WD[7]. Some studies have shown that comorbidities may have important effects on WD[8,9].

Currently, hepatitis B virus (HBV) infection is the common cause of liver disease worldwide, affecting 290 million individuals[10,11]. However, data on the prevalence of HBV infection in WD patients are limited and inconsistent. A study from Italy reported that after monitoring 60 WD patients for 20 years, no active HBV infection was found. Accordingly, they hypothesized that the excessive copper levels caused by WD could prevent HBV infection[12]. Contrarily, a study conducted in Taiwan in 1998 reported that the prevalence of HBV infection in 61 patients with WD was 16%, which was similar to that in the general population[13]. However, this finding is different from the previously published observations of Lau *et al*[14] in Hong Kong. Moreover, these studies were conducted many years ago and in small sample size populations; therefore, more studies involving larger sample size populations are needed. Furthermore, the influence of HBV infection on the diagnosis and clinical aspect of WD remains unclear. Given the paucity of studies in this field, this study was undertaken to investigate the prevalence of HBV infection in patients with WD and to explore the impact of HBV infection on the diagnosis, clinical aspect, treatment and prognosis of WD.

MATERIALS AND METHODS

Design overview, setting, and patients

We retrospectively analyzed the data of WD patients diagnosed between May 2003 and December 2020 at the Department of Infectious Diseases/Institute of Hepatology, the Second Xiangya Hospital, Central South University, China, which is the oldest and largest tertiary referral hospital in the Hunan Province. The Institute of Hepatology of this hospital is one of the first centers to carry out research on WD in China, and it offers services to WD patients in the whole province, neighboring provinces, and all over the country.

Patients with suspected WD underwent slit-lamp examination [to identify Kayser-Fleischer (KF) rings], neurological examination, measurement of serum ceruloplasmin levels, and determination of 24 h urinary copper excretion (before and after a penicillamine challenge). In cases where there were no contraindications, a liver biopsy was performed to confirm the presence of copper deposits, and in some other cases, gene analysis was performed. The diagnosis of WD was made based on a combination of clinical symptoms and laboratory tests, and on the WD scoring system published in 2003

[15,16]. WD was confirmed if the WD score was ≥ 4. The WD phenotypes were classified based on previously published criteria[16]. All WD patients were tested for HBV markers, including hepatitis B surface antigen (HBsAg), anti-HBs, hepatitis B e antigen (HBeAg), anti-HBe, and anti-HBc, at the time of diagnosis. Patients positive for HBsAg underwent further analysis to quantify HBV DNA levels. For each patient, all the data obtained at the time of diagnosis and at each follow-up time point were recorded in a medical record specifically designed for the WD study.

All the patients diagnosed with WD during the study period were eligible for inclusion. We excluded WD patients with other types of viral hepatitis (A, C, or E), autoimmune hepatitis, drug-induced liver disease, a history of alcohol intake > 30 g ethanol/day, and patients whose HBV markers were not tested.

Laboratory methods

Routine laboratory data were obtained using standard methods. HBV markers were tested using commercial diagnostic kits (ELISA; Shanghai Kehua Bioengineering Co. Ltd., Shanghai, China). HBV DNA levels were quantified using a commercial hepatitis B DNA quantitative fluorescence diagnostic kit (Sansure Biotech Inc, Changsha, Hunan Province, China). The lower limits of the HBV DNA quantification were 100 and 10 IU/mL before 2011 and after 2012, respectively. The KF rings were examined under a slit-lamp by an experienced ophthalmologist. Serum ceruloplasmin levels were measured using the nephelometric method (normal range, 210-500 mg/L; Beckman Coulter, Image® Immunochemistry System, Brea, CA, United States). Copper levels in serum, urine, and liver were determined as previously described[17]. Moreover, the ATP7B coding region and exon/intron boundaries were amplified and sequenced, as previously described [18].

Study outcomes

The study outcomes included the following: The proportion of patients with WD who had current HBV infection (positive for HBsAg); the proportion of those with previous HBV infection (negative for HBsAg but positive for anti-HBc, with or without anti-HBs); the rate of severe liver disease {defined as the proportion of WD patients who experienced severe decompensated cirrhosis [Child-Turcotte-Pugh (CTP) score ≥ 10] or acute-on-chronic liver failure (ACLF) in WD patients with HBV infection and those without HBV infection}. The CTP score was determined based on a previously described criterion[19,20]. ACLF was diagnosed according to a combination of consensus recommendations published by the Asian Pacific Association for the Study of the Liver (in 2009) and the guidelines for the diagnosis and treatment liver failure published by the Chinese Society of Hepatology in 2006[21,22]. The criteria were as follows: (1) Acute severe exacerbation of liver disease complicated within 4 wk by clinical ascites and/or encephalopathy, with previously diagnosed or undiagnosed chronic liver disease/cirrhosis; (2) Serum bilirubin ≥ 10 mg/d; and (3) Coagulopathy (international normalized ratio ≥ 1.5 or prothrombin activity < 40%).

Statistical analysis

Continuous data were expressed as mean ± SD and compared using the unpaired *t*-test or Mann-Whitney *U* test. Categorical variables were expressed as proportions and compared using the chi-squared test or Fishers exact test. We used a binary logistic regression model to evaluate the association between HBV infection and the risk of severe liver disease at the time of diagnosis of WD, the results were summarized as odds ratio (OR) with 95% confidence intervals (CI). All statistical analyses were performed using the software Statistical Packages for the Social sciences, SPSS version 20.0 Windows (IBM Corp., Armonk, NY, United States). Statistical significance was set at a two-tailed *P* value < 0.05.

RESULTS

During the study period, 973 patients were diagnosed with WD. Among them, 58 patients were excluded because of concurrent hepatitis E infection (*n* = 3), hepatitis C infection (*n* = 3), thalassemia (*n* = 3), and schistosomiasis japonica (*n* = 2), as well as insufficient length of hospital stay to perform the tests (*n* = 47). The remaining 915 patients with WD (532 men, 383 women; mean age, 20.2 ± 13.1 years; age range, 1-68 years) were included in our analysis. The patients resided in 25 provinces and belonged to 825 families. Moreover, 644 (70.4%), 218 (23.8%), 35 (3.8%), and 18 (2.0%) patients presented with only hepatic disease, neuropsychiatric and hepatic disease, only neuropsychiatric disease, and neither hepatic nor neurological disease, respectively. WD diagnosis was confirmed by a liver copper level ≥ 250 μg/g dry weight, the identification of two disease-causing mutations or homozygosity for a single disease-causing mutation, or both criteria in 320, 378, and 102 patients, respectively. All patients met the criteria of the WD scoring system, with 735 (80.3%), 87, and 93 patients having WD scores ≥ 6, 5, and 4, respectively.

Prevalence of HBV infection among patients with WD

Among the 915 patients with WD, 393 tested negative for all HBV markers (43.0%, 95%CI: 39.8%-46.15%) and 419 (45.8%) tested positive for immunization-related anti-HBs alone (45.8%, 95%CI: 42.5%-49.0%). The total prevalence of current and previous HBV infections were 2.1% (95%CI: 1.2%-3.0%) and 9.2% (95%CI: 7.3%-11.1%), respectively. Table 1 summarizes the prevalence of HBV infection stratified by sex and age. The prevalence of both current and previous HBV infections were significantly lower in women than in men and in patients aged ≤ 10 years than in those aged ≥ 11 years. The prevalence of previous HBV infection increased with age; however, the prevalence of current HBV infection remained low irrespective of age (Table 1).

Table 1 Prevalence of hepatitis B infection in patients with Wilson disease stratified by gender and age

	Number of patients	Negative for HBVM (%)	Anti-HBs(+) alone (%)	Prior HBV infection (%)	Current HBV infection (%)
Total group	915	393 (43.0)	419 (45.8)	84 (9.2)	19 (2.1)
Sex					
Male	532	228 (42.9)	239 (44.9)	52 (9.8)	13 (2.4)
Female	383	165 (43.1)	180 (47.0)	32 (8.4)	6 (1.6)
Age group (yr)					
2-10	231	105 (45.5)	119 (51.5)	7 (3.0)	0 (0.0)
11-20	340	156 (45.9)	159 (46.8)	15 (4.4)	10 (2.9)
21-30	153	63 (41.2)	73 (47.7)	13 (8.5)	4 (2.6)
31-40	106	40 (37.7)	42 (39.6)	21 (19.8)	3 (2.8)
41-65	85	29 (34.1)	26 (30.6)	28 (32.9)	2 (2.3)

HBVM: Hepatitis B virus markers; HBV: Hepatitis B virus.

Characteristics of WD patients with current HBV infection

During the study period, 19 WD patients with current HBV infection were consecutively diagnosed (men, 13; women, 6; mean age, 25.1 ± 13.3 years; age range, 11-64 years). Among them, six and ten patients were positive for HBeAg and HBV DNA, respectively. Moreover, 16 patients had a 1- to 30-year history of HBV-related liver disease, among whom there were three patients who had undergone splenectomy 3 years earlier and five patients who had been receiving a treatment based on lamivudine or entecavir for 2-24 mo. All patients met the WD criteria. More specifically, WD diagnosis was based on elevated liver copper levels, identification of two disease-causing mutations or homozygosity for a single disease-causing mutation, or both in 10, 6, and 5 patients, respectively. Furthermore, 16 patients had a WD score ≥ 6 (84.2%). However, among the patients, only two were suspected of having WD; 17 were referred to our hospital because of chronic hepatitis B (CHB) and had never been diagnosed of WD before.

There was a high variability in the clinical manifestations in patients with WD and current HBV infection. Five patients (cases 2, 6, 7, 11, and 19) presented with ACLF, characterized by severe jaundice, markedly decreased albumin levels, and prolonged prothrombin time (except for case 6, the remaining four patients among these patients died within 15-30 d following diagnosis). Two and eight patients presented with evidence of compensated and decompensated cirrhosis, respectively. Two patients presented with symptoms and signs of chronic liver disease, including hepatic enlargement or abnormal serum aminotransferases levels. Two patients were entirely asymptomatic, with normal serum aminotransferase and mild hepatomegaly or splenomegaly (Tables 2-4).

Characteristics of WD patients with previous HBV infection

During the study period, 84 patients with WD and previous HBV infection were consecutively diagnosed (men, 52; women, 32; mean age, 33.3 ± 15.9 years; age range, 3-68 years). Among them, 74 patients were positive for anti-HBs and anti-HBc, 10 patients were positive for anti-HBc only, and none of the patients had detectable levels of HBV DNA. The diagnosis of WD was based on elevated liver copper levels, identification of two disease-causing mutations, or both in 16, 47, and 8 patients, respectively. All patients met the criteria of the WD scoring system, with 70 patients having WD scores ≥ 6 (83.3%).

Comparison between patients with isolated WD and those with concurrent WD and HBV infection

There were no differences in sex, WD phenotype, copper metabolism parameters (such as the positivity rates of KF rings, urinary copper excretion, and hepatic copper content), and mean WD score distribution between patients with isolated WD and those with concurrent WD and current or previous HBV infection. Compared with patients with isolated WD, WD patients having HBV infection were significantly older and had significantly more severe liver function damage (including lower serum albumin levels, higher serum total bilirubin levels, and longer prothrombin time). The ACLF rates in WD patients with current and previous HBV infection were 26.3% and 13.1%, respectively, which were significantly higher than the rate in patients with isolated WD (4.5%, $P = 0.000$). The rates of severe decompensated liver cirrhosis in WD patients with current and previous HBV infection were 40.5% and 36.8%, respectively, and these were significantly higher than the rate in patients with isolated WD (14.8%; $P = 0.000$). The mortality rates during the first 60 d of follow-up following diagnosis were 36.8% and 15.5% in WD patients with current and previous HBV infection, respectively; these values were significantly higher than the rate in patients with isolated WD (4.1%, $P = 0.000$) (Table 5). Based on the binary logistic regression analysis model, after accounting for age, sex, and HBV infection and taking as reference the groups of patients with isolated WD, there was a significantly higher risk of severe liver disease at WD diagnosis in WD patients with current (OR = 7.748; 95%CI: 2.890-20.774; $P = 0.000$) or previous HBV infection (OR = 5.525; 95%CI: 3.159-8.739; $P = 0.000$).

Table 2 Characteristics of patients with Wilson disease and chronic hepatitis B: The demographic characteristics and parameters of hepatitis B virus infection

Case	Sex	Age (yr)	History of HBV (yr)	HBsAg	HBeAg	Anti-HBe	HBV DNA
1	Female	16	2	+	-	+	< 100
2	Male	19	2	+	-	+	9.3e8
3	Female	19	2	+	-	-	< 100
4	Male	26	3	+	+	-	1.9e6
5	Male	43	3	+	-	+	5.6e5
6	Female	37	2	+		+	7.3e3
7	Male	19	3	+	-	+	< 100
8	Male	11	5	+	+	-	3.2e7
9	Male	26	20	+	-	+	1.9e4
10	Female	17	3	+	-	+	< 10
11	Male	20	10	+	+	-	2.3e4
12	Male	11	?	+	-	+	< 10
13	Female	32	?	+	-	+	81
14	Male	27	?	+	+	-	1.3e6
15	Male	24	16	+	+	-	2.1e8
16	Male	15	16	+	-	+	< 10
17	Male	65	30	+	-	+	22.3
18	Female	35	16	+	-	+	< 10
19	Male	15	10	+	+	-	1.8e6

HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen; HBeAg: Hepatitis B e antigen.

Treatment and outcomes of WD patients with current HBV infection

Five patients with concurrent WD and current HBV infection diagnosed before 2007 were treated with a chelator alone. The patients refused liver transplantation for financial reasons, rapidly deteriorated, and died from liver failure 2-4 wk after admission. Eight patients with detectable HBV DNA levels, diagnosed after 2008, were treated with a combination of penicillamine and nucleoside. Among them, two patients (cases 11 and 19) with ACLF rapidly deteriorated and died 1 mo after admission. Two patients (cases 6 and 8) were lost to follow-up. The condition of the remaining four patients gradually improved with treatment; however, one patient (case 5) developed hepatocellular carcinoma and died 7 mo after admission. Six patients with undetectable HBV DNA levels, diagnosed after 2008, were initially treated with a chelator only. Their condition gradually improved. However, three among them experienced HBV replication at 7-12 mo after therapy. Among the latter, there was one patient (case 10) with severe hepatitis reactivation and increased serum alanine aminotransferase (ALT; 930 IU/L), aspartate aminotransferase (AST; 790 IU/L), and HBV DNA (9.2×10^4 IU/mL) levels. This patient was treated with entecavir and penicillamine. However, within 2-3 mo after treatment initiation, HBV DNA levels were undetectable, and the AST and ALT levels normalized. The remaining three patients continued treatment with a chelator only. Currently, nine of the 14 patients diagnosed after 2008 have achieved a stable disease status after therapy and have resume their routine living activities (full-time work or study). WD patients with a previous HBV infection were treated using a chelator only. None of the patients experienced HBV reactivation during the study period.

DISCUSSION

To the best of our knowledge, the prevalence of HBV infection in patients with WD has been reported in only two small studies, and only two case studies involving single cases of concurrent WD and CHB infection have been reported in the English literature[23,24]. This is the largest investigation of HBV infection in WD patients, and it is helpful in understanding the true prevalence of HBV infection in WD patients and the impact of HBV infection on WD.

A national survey conducted in 1992 reported that the overall HBsAg prevalence in the Chinese population was 9.8%, declining to 7.2% in 2006[25,26]. In the present study, the total HBsAg prevalence was 2.1% in WD patients, a figure which is far lower than previously reported national HBsAg rates. However, this does not imply that the prevalence of

Table 3 Characteristics of patients with Wilson disease and chronic hepatitis B: Diagnostic parameters of Wilson disease

Case	Neurologic signs	KF rings	Cerulo plasmin (mg/L)	Urinary copper/after PC (μg/24 h)	Hepatic copper (μg/g/dw)	Mutation analysis	WD score
1	No	P	65.0	177/1600	ND	ND	5
2	No	P	50.0	1222/4250	ND	ND	6
3	No	P	95.0	1818/2266	ND	ND	6
4	Yes	P	187.0	434/ND	ND	ND	7
5	No	P	172.0	107/1295	347	ND	6
6	No	P	216.0	431/1436	1165	ND	6
7	No	P	50.0	5171/15398	ND	ND	6
8	No	P	250.0	305/1933	1173	ND	6
9	No	N	74.0	474/1500	926	3532G>T/3532G>T	10
10	No	P	84.0	595/1725	ND	2755C>G/2975C>T	10
11	No	N	135.0	1297/2984	ND	588C>A/2333G>T	7
12	No	P	78.4	160/1773	741	2975C>T/2975C>T	11
13	No	P	129	855/2505	ND	ND	5
14	No	P	31.0	310/1984	1067	3809 >G/	9
15	No	N	104.0	187/1836	896	0	4
16	Yes	P	86.5	237/2374	404	ND	10
17	No	P	83.1	64/714	265	2975C>T/	7
18	No	P	43.0	155/171	906	2804C>T/2810delT	11
19	No	P	63.0	584/2473	ND	2666G>T/2333G>T	10

WD: Wilson disease; KF rings: Kayser-Fleischer rings; ND: Not done; PC: Penicillamine challenge; N: Negative; P: Positive.

HBV infection in patients with WD is lower than that in the general population. First, since the 1990s, the rate of HBV infection in China has been decreasing annually. The data of WD patients today should not be compared with those of the general population many years ago. Second, the age composition of WD patients is different from that of the general population. Among our patients, 41% were aged below 14 years; however, the HBV infection rate is known to be very low in this age group. Therefore, the average HBsAg positivity rate in WD patients should not be directly compared with that in the general population. To find studies with more comparable data, we conducted a literature search and found a large survey on HBV infection conducted in the Henan Province in 2015. This has been the largest survey on HBV infection in China during the recent years, and it involved a total of 13207 children and 16685 adults[27,28]. The Henan Province is located in the middle of China and is adjacent to Hunan. Its economic development level and HBV infection rate are similar to those in Hunan, making the data from Henan comparable with those from our study. We calculate the HBsAg positivity rates among different age groups of the WD patients, according to the age group divisions used in the Henan study (Table 6). The positivity rates of HBsAg in WD patients aged 1-4, 5-9 and 10-14 years were 0.0%, 0.0%, and 1.6%, respectively, which were similar to those in Henan children of the same age groups (0.5%, 0.7%, and 1.2%, respectively). The positivity rates of HBsAg in the 18-34, 35-54, and 55-74 years age groups of WD patients were 3.0%, 2.5%, and 5.3%, respectively, which were similar to those in the general population of the same age group (3.1%, 4.7%, and 5.1%, respectively). Our study indicates that the prevalence of HBV infection in WD patients is similar to that in the general population, and that WD patients are equally susceptible to HBV infection.

The most important finding of this study was the identification of 19 patients with concurrent WD and CHB infection. It is worth noting regarding the 19 patients that 17 were referred for CHB infection and not WD; thus, the WD was diagnosed at our hospital. There was a significant delay in the diagnosis of WD in these patients (mean delay = 32.5 and 10.5 mo in WD patients with concurrent CHB infection and in patients with isolated WD, respectively). Our results suggest that the diagnosis of WD may be missed in patients with CHB infection. Although a missed diagnosis of WD is not uncommon given the rarity of the disease[29-31], it is worth noting that many patients with CHB infection suffer from undiagnosed WD. The clinical manifestations of patients with concurrent CHB infection and WD are nonspecific and difficult to distinguish from those of patients with isolated CHB infection, unless the clinicians deliberately and diligently examine the patients to rule out WD. Therefore, more attention should be paid regarding the coexistence of WD in patients with CHB infection. WD should be considered and ruled out in some patients with CHB infection, especially in those with cirrhosis, hepatic failure, or poor response to antiviral therapies.

Table 4 Characteristics of patients with Wilson disease and chronic hepatitis B: Parameters of liver disease

Case	ALT	AST	ALB	TBIL	GGT	ALP	INR	Severity of liver disease	Outcome
1	29	50	20.3	46.7	46	74	2.61	CTP: 13	Died
2	70	185	26.8	264	84	61	2.8	ACLF	Died
3	86	50	25.1	123	58	85	2.7	CTP: 13	Died
4	41	60	30.4	44.4	90	70	4.5	CTP: 13	Died
5	102	170	35.7	48.0	110	95	1.5	CTP: 10	Died
6	119	291	27.6	417	186	390	2.1	ACLF	Alive
7	79	123	31	456	45	57	4.1	ACLF	Died
8	231	237	37.5	16.5	353	105	1.2	CTP:6	Alive.
9	74	101	29.8	39.0	42	116	1.5	CTP:10	Alive
10	79	105	24.5	42.0	110	130	2.4	CTP:12	Alive
11	223	268	30.0	368	147	252	2.8	ACLF	Died
12	71	83	31.0	23.0	62	357	1.6	CTP:10	Alive
13	70	76	28.0	25.6	35	375	1.63	CTP:10	Alive
14	82	151	37.8	28.7	152	149	1.36	CTP:5	Alive
15	276	102	39.9	25.3	113	93	0.95	Hepatitis	Alive
16	22	47	36.5	16	32	219	1.25	Hepatitis	Alive
17	31	28	47.3	26.3	23	76	1.01	Hepatitis	Alive
18	56	34	42.0	22.0	90	60	1.01	Hepatitis	Alive
19	57	74	28.0	373	268	293	3.01	ACLF	Died

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALB: Albumin; TBIL: Total bilirubin; GGT: Gamma-glutamyl transpeptidase; ALP: Alkaline phosphatase; INR: International normalized ratio; ACLF: Acute-on-chronic liver failure; CTP: Child-Turcotte-Pugh.

Compared to patients with isolated WD, patients with concurrent WD and CHB infection had significantly lower serum albumin levels, higher serum total bilirubin levels, and longer prothrombin time. The mortality rates of WD patients with current and previous HBV infection during the first 60 d of follow-up (following diagnosis) were 36.8% and 15.5%, respectively; these rates were significantly higher than those of patients with isolated WD (4.1%, $P = 0.000$). The ACLF rates in WD patients with current and previous HBV infection were 26.3% and 13.1%, respectively, which were significantly higher than those in patients with isolated WD (4.5%, $P = 0.000$). Binary logistic regression analysis revealed that the risk of severe liver disease in WD patients with current and previous HBV infection was 7.7 and 5.3 times (respectively) higher than that in patients with isolated WD. Our findings indicate that HBV infection substantially affects the severity liver disease in patients with WD (Tables 5 and 7). The mechanism through which CHB causes severe liver injury could involve the induction, by viral hepatitis, of hepatic injury and copper accumulation, which could additively or synergistically aggravate WD-induced liver damage. Many studies have shown that HBsAg clearance usually results in good long-term prognosis[32]. However, an unexpected finding was that previous HBV infection also had a significant impact on the severity of liver disease in patients with WD. The reason for this may be that WD patients with a previous HBV infection usually have severe liver injury and cirrhosis (due to the joint action of HBV and WD) that occurred before the HBsAg clearance. After the HBsAg clearance, the severe liver injury and cirrhosis that had been formed usually persist[33,34]. Considering the serious impact of HBV infection on the clinical aspect of WD patients, current and previous HBV infections must be screened when evaluating the clinical aspect and prognosis of WD patients.

The strengths of this study are: (1) Considering the rarity of the disease, the sample size of the WD patient cohort was very large; (2) All patients were diagnosed in our department and met the diagnostic criteria; among them, 80% were confirmed by genetic examination and/or liver copper level determination; and (3) All data were prospectively collected by the authors and therefore were complete and reliable. The limitation of this study is the relatively small number of WD patients with concurrent CHB infection, as this might impede the detection of more significant differences between patients with isolated WD and those with co-existing CHB infection and WD.

In conclusion, our study indicates that the prevalence of HBV infection stratified by sex and age in patients with WD is similar to that in the general population. There was a significant delay in the diagnosis of WD in CHB patients. Furthermore, our findings suggest that HBV infection significantly affects the severity of liver disease in patients with WD. Therefore, more attention should be paid to patients suffering from concurrent WD and CHB infection. Although we found that previous HBV infection is an independent factor in the exacerbation of WD, its mechanism remains unknown.

Table 5 Factors associated with severe liver disease at the Wilson disease diagnosis

Factors (at diagnosis)	OR	95%CI	*P* value
Gender			
Male	1		
Female	1. 945	1.402-2.698	0.000
Age at diagnosis (yr)			
Group 1 (0-10)	1		
Group 2 (11-20)	1.766	1.145-2.723	0.010
Group 3 (21-30)	0.757	0.423-1.352	0.346
Group 4 (31-40)	1.178	0.646-2.148	0.594
Group 5 (41-65)	1.455	0.777-2.724	0.241
HBV infection			
WD alone	1		
WD with previous HBV	5.255	3.159-8.739	0.000
WD with current HBV	7.748	2.890-20.774	0.000

Severe liver disease is defined as patients with acute-on-chronic liver failure or a Child-Turcotte-Pugh score ≥ 10 at the Wilson disease diagnosis, HBV: Hepatitis B virus; WD: Wilson disease; OR: Odds ratio; CI: Confidence interval.

Table 6 Comparison of hepatitis B surface antigen positivity rates among different age groups between patients with Wilson disease and the general population

Age groups (yr)	Wilson disease		General population (Henan)		*P* value
	Number tested	HBsAg(+) (*n*, %)	Number tested	HBsAg(+) (*n*, %)	
1-4	40	0, 0.0	5474	26, 0.5	0.827
5-9	148	0, 0.0	4407	32, 0.7	0.625
10-14	191	3, 1.6	3376	40, 1.2	0.505
15-17	97	3, 3.1	Not done		
18-34	298	9, 3.0	6764	220, 3.1	0.825
35-54	122	3, 2.5	6777	275, 4.7	0.490
55-74	19	1, 5.3	3144	147, 5.1	0.599
Total	915	19, 2.1	29892	740, 2.5	0.510

HBsAg: Hepatitis B surface antigen.

Further research is needed to confirm this finding and to elucidate the mechanisms underlying the associations between WD progression and previous HBV infection or cryptogenic HBV infection.

CONCLUSION

This is the largest investigation of HBV infection in WD patients, and it is helpful in understanding the true prevalence of HBV infection in WD patients and the impact of HBV infection on WD.

Table 7 Comparison between Wilson disease patients alone and Wilson disease patients with hepatitis B virus infection

	WD alone (*n* = 812)	With previous HBV (*n* = 84)	With current HBV (*n* = 19)	P1	P2
Sex					
Male (*n*, %)	467 (57.5)	46 (61.9)	12 (63.2)	0.509	0.797
Female (*n*, %)	345 (42.5)	32 (38.1)	7 (36.8)	0.509	0.797
Mean age (yr)	18.8 ± 12.0	33. ± 15.9	25.0 ± 13.	0.000	0.031
Diagnosis delay	12.1 ± 21.3	13.7 ± 26.1	34.6 ± 53.5	0.542	0.000
Phenotype					
Pure H (*n*, %)	564 (69.5)	63 (75.0)	17 (89.5)	0.352	0.104
H and N (*n*, %)	195 (24.40)	21 (25.60)	2 (10.5)	0.352	0.104
Pure N (*n*, %)	35 (4.3)	0 (0.0)	0		
Others (*n*, %)	18 (2.2)	0	0		
Copper metabolic					
KF positive	67.6%	84.1%	78.9%	0.102	0.456
Ceruloplasmin	64.1 ± 46.0	97.1 ± 60.3	105.0 ± 61.6	0.000	0.000
Urinary copper	502.4 ± 1030.5	916.3 ± 245.8	767.7 ± 1165.0	0.005	0.323
Urinary Cu after PC	2236.4 ± 1582.1	2283.7 ± 1287.8	2793.8 ± 3157.5	0.842	0.144
Hepatic Cu	832.2 ± 457.7	680.1 ± 407.7	789.0 ± 338.2	0.191	0.767
Mean WD score	7.4 ± 2.3	8.0 ± 2.2	7.8 ± 2.3	0.124	0.412
Biochemical					
ALT (IU/L)	86.2 ± 105.7	63.3 ± 51.0	94.6 ± 71.1	0.0051	0.722
AST (IU/L)	84.25 ± 90.3	90.47 ± 70.4	125.3 ± 80.5	0.0532	0.058
Albumin (g/L)	38.5 ± 8.3	32.5 ± 7.6	32.1 ± 6.8	0.000	0.001
TBIL (μmol/L)	52.1 ± 131.0	104.7 ± 166.7	126.6 ± 158.3	0.001	0.002
GGT (IU/L)	90.9 ± 85.6	138.6 ± 112.3	109 ± 93.3	0000	0.399
ALP (IU/L)	186.7 ± 133.6	137.7 ± 87.7	171.9 ± 123.0	0.001	0.650
INR	1.42 ± 0.81	1.88 ± 0.86	2.09 ± 1.04	0.000	0.000
Liver disease severity					
Hepatitis (*n*, %)	372 (45.8)	7 (8.3)	3 (15.8)	0.000	0.009
CTP5-6 (*n*, %)	189 (23.3)	18 (21.4)	3 (15.8)	0.702	0.444
CTP7-9 (*n*, %)	94 (11.6)	14 (16.7)	1 (5.3)	0.173	0.713
CTP10-14 (*n*, %)	120 (14.8)	34 (40.5)	7 (36.8)	0.000	0.008
ACLF (*n*, %)	37 (4.5)	11 (13.1)	5 (26.3)	0.001	0.000
Mortality (*n*, %),	33 (4.1)	13 (15.5)	7 (36.8)	0.000	0.000

HBV: Hepatitis B virus; WD: Wilson disease; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TBIL: Total bilirubin; GGT: Gamma-glutamyl transpeptidase; ALP: Alkaline phosphatase; INR: International normalized ratio; ACLF: Acute-on-chronic liver failure; CTP: Child-Turcotte-Pugh; PC: Penicillamine challenge.

ARTICLE HIGHLIGHTS

Research background

Although hepatitis B virus (HBV) infection is the most common cause of liver disease in China, the occurrence of HBV infection in Wilson disease (WD) patients and the clinical manifestations of concurrent WD and HBV infections have rarely been reported.

Research motivation

Our study suggests that both WD and HBV infections may coexist. The clinical symptoms of concurrent WD and HBV infections are difficult to distinguish from those of simple viral hepatitis. Therefore, the existence of WD may be hidden. The study of concurrent WD and HBV infection deserves careful consideration.

Research objectives

To investigate the incidence of HBV infection in patients with WD and to analyse how HBV infection affects WD.

Research methods

The clinical data of patients with WD were analyzed retrospectively, and the data of patients with concurrent WD and HBV infection were compared with those of patients with isolated WD. Considering the rarity of the disease, the sample size of the WD patient cohort was very large.

Research results

Among a total of 915 WD patients recruited, the total prevalence of current and previous HBV infection was 2.1% and 9.2% respectively. The main finding of this study was the identification of 19 patients with concurrent WD and chronic hepatitis B (CHB) infection. The mean delay in the diagnosis of WD in patients with concurrent WD and CHB infection was 32.5 mo, which was significantly longer than that in patients with isolated WD (10.5 mo). The rates of severe liver disease and mortality in patients with concurrent WD and CHB infection were significantly higher than those in patients with isolated WD (63.1% *vs* 19.3%), respectively. Binary logistic regression analysis revealed a significantly higher risk of severe liver disease at the diagnosis of WD in patients with current HBV infection or previous HBV infection than in patients with isolated WD.

Research conclusions

Our study indicates that the prevalence of HBV infection stratified by sex and age in patients with WD is similar to that in the general population. There was a significant delay in the diagnosis of WD in CHB patients. HBV infection is an independent risk factor for severe liver disease in WD patients. WD should be considered and excluded in some patients with CHB infection.

Research perspectives

As we found that previous HBV infection was an independent factor in the exacerbation of WD, the mechanism of which is speculative, future studies could further explore the mechanism by which WD is exacerbated by previous HBV infection and whether it is related to occult HBV infection.

FOOTNOTES

Author contributions: Zhou HY, Yang X, and Luo HY contributed to study conception, design and writing of the article; Yang X, Luo KZ, Jiang YF, Wang WL, Liang J, Li MM, and Luo HY contributed to data acquisition, data analysis and interpretation; Zhou HY, Yang X, and Luo HY contributed to editing, reviewing, and final approval of the article; and all authors have read and approved the final manuscript.

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CASE REPORT

Drug-induced entero-colitis due to interleukin-17 inhibitor use; capsule endoscopic findings and pathological characteristics: A case report

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Abstract

BACKGROUND

Interleukin-17 (IL-17) inhibitors are known to cause exacerbation or new onset of inflammatory bowel disease upon administration. However, few reports have described characteristic endoscopic and histopathologic findings, and no small intestinal lesions have been reported so far.

CASE SUMMARY

A woman in her 60s with psoriasis was administered ixekizumab (IXE), an anti-IL-17A antibody, for the treatment of psoriasis. Twenty months after commencing treatment, the patient visited our hospital because of persistent diarrhea. Blood tests performed at the time of the visit revealed severe inflammation, and colonoscopy revealed multiple round ulcers throughout the colon. A tissue biopsy of the ulcer revealed infiltration of inflammatory cells and granuloma-like findings in the submucosal layer. Capsule endoscopy revealed multiple jejunal erosions. After the withdrawal of IXE, the symptoms gradually improved, and ulcer reduction and scarring of the colon were endoscopically confirmed.

CONCLUSION

To the best of our knowledge, 17 reports have documented IL-17 inhibitor-induced entero-colitis with endoscopic images, endoscopic findings, and pathological characteristics, including the present case. Nine of these cases showed diffuse loss of vascular pattern, coarse mucosa/ulcer formation in the left colon, and endoscopic findings similar to those of ulcerative colitis. In the remaining eight cases, discontinuous erosions and ulcerations from the terminal ileum to the rectum were seen, with endoscopic findings similar to those of Crohn's disease. In this case, the findings were confirmed by capsule endoscopy, which has not been previously reported.

Key Words: Interleukin-17 inhibitor; Ixekizumab; Drug-induced entero-colitis; Capsule endoscopy; Case report

Core Tip: While Interleukin-17 (IL-17) inhibitors are effectively used in the treatment of psoriasis, psoriatic arthritis, and ankylosing spondylitis, they are ineffective in patients with Crohn's disease (CD) and can worsen their condition. To the best of our knowledge, we present capsule endoscopic images of IL-17 inhibitor-induced entero-colitis for the first time, suggesting that IL-17-induced inflammatory lesions may be distributed in the proximal small bowel, unlike CD lesions. We also compared the endoscopic and pathological features of IL-17 inhibitor-induced entero-colitis with those previously reported.

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INTRODUCTION

Interleukin-17 (IL-17) inhibitors, such as ixekizumab (IXE) and secukinumab, are a class of molecular-targeted therapies used to treat psoriasis, psoriatic arthritis, and ankylosing spondylitis. IL-17 is a type of inflammatory cytokine produced by helper T cells and is known not only to induce local inflammation in the human body but also to be involved in host infection defense against pathogens in the skin and intestinal epithelium[1]. In patients with both psoriasis and Crohn's disease (CD), biopsy specimens of lesions express high levels of IL-17[2,3]. Therefore, IL-17 inhibitors were hypothesized to be effective in treating psoriasis and CD. However, IL-17 inhibitors are only effective in psoriasis; in patients with CD, IL-17 inhibitors are ineffective and exacerbate the disease[4]. Furthermore, in clinical trials of IL-17 inhibitors in inflammatory bowel disease (IBD), rheumatic diseases, and dermatological diseases, exacerbations or new-onset IBD have been reported at a frequency of 0.4%[5]. The mechanism underlying this seemingly contradictory adverse reaction remains unclear.

CASE PRESENTATION

Chief complaints

A woman in her 60s with diarrhea and anorexia.

History of present illness

Gastrointestinal symptoms appeared 24 mo after IXE was started for the treatment of psoriasis.

History of past illness

The patient was diagnosed with psoriatic arthritis by her family physician and started on IXE. However, anorexia and diarrhea appeared 20 mo after treatment initiation. After conservative treatment by her family doctor, her symptoms did not improve, and she visited our hospital 24 mo after IXE initiation for a close examination and treatment.

Personal and family history

Her medical history included type 1 diabetes at the age of 35 and hypothyroidism at the age of 50 years, each of which was medically managed by her family physician. No family history of IBD was reported; her father had gastric cancer, and her mother had diabetes. The injectable medications used were insulin and IXE for diabetes and psoriasis, respectively.

Physical examination

The patient was conscious but noticeably emaciated, appeared weakened, and walked with a limp. She had a body temperature of 36.0 ℃ and 114/52 mmHg of blood pressure. The skin of the upper extremities was fragile, with epidermal exfoliation of the right forearm. Multiple scars were observed on the upper arm and mild deformities and swelling of the hand joints.

Laboratory examinations

On admission, blood biochemistry tests showed anemia with a hemoglobin level of 10.4 g/dL and hypoalbuminemia with an albumin level of 2.8 g/dL. She was also dehydrated, with a blood urea nitrogen level of 28.2 mg/dL and

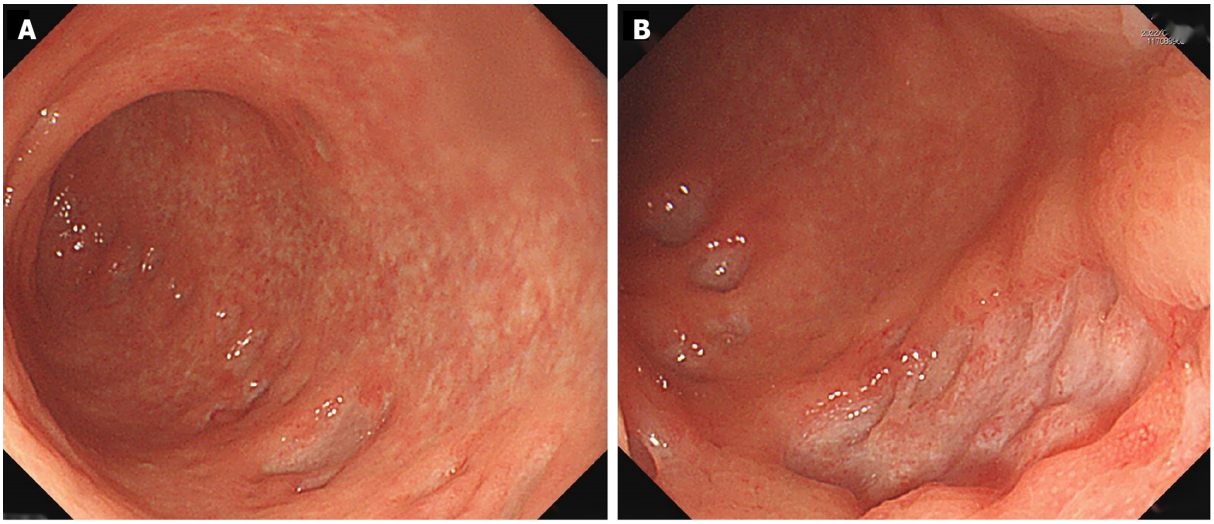

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Figure 1 Colonoscopy findings at admission. A: Distant view of colon; B: Close-up of ulcer. Multiple round punctate ulcers with a longitudinal trend from the cecum to the rectum are observed. The intervening mucosa of the ulcers is preserved, and the ulcers do not coincide with the mesenteric attachment side.

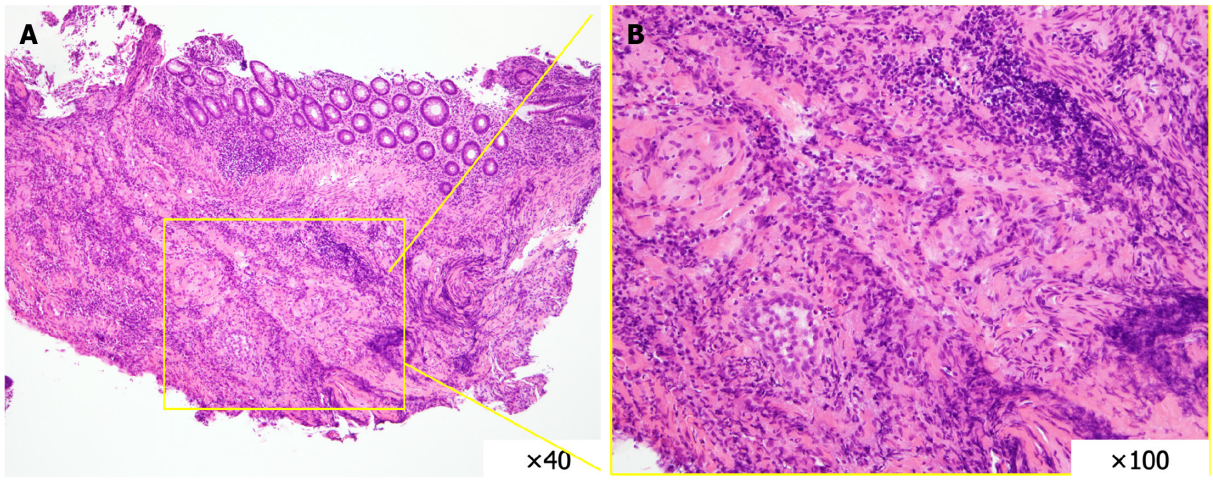

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Figure 2 Pathological findings at admission. A: Hematoxylin-eosin (HE) stained specimen at 40 × magnification; B: HE stained specimen at 100 × magnification. The mucosa is erosive and infiltrated with inflammatory cells, predominantly lymphocytes. The submucosa shows granulomatous collagen fibers and fibroblast proliferation.

creatinine of 0.8 mg/dL and had high inflammation with a C-reactive protein level of 15.3 mg/dL. Leucine-rich alpha-2 glycoprotein level was 44.1 μg/mL and fecal calprotectin level was also high at 7357 mg/kg, suggesting strong intestinal inflammation.

Imaging examinations

Computed tomography revealed edematous wall thickening of the intestinal tract, continuous from the ascending colon to the rectum.

Further diagnosis workup

Colonoscopy revealed multiple round, punched-out ulcers with a longitudinal trend from the cecum to the rectum (Figure 1). The intervening mucosa of the ulcer was nearly normal, and the ulcer did not coincide with the mesenteric attachment. A biopsy of the ulcer showed mucosal erosion, lymphocyte-dominated inflammatory cell infiltration, and regenerative epithelial growth (Figure 2). Submucosal fibroblast and collagen fiber proliferation were also present-a granuloma-like finding similar to that of CD. An upper gastrointestinal endoscopy revealed reflux esophagitis and chronic gastritis. The gastric mucosa exhibited scattered erythema and erosions; however, no specific abnormalities were observed in the duodenum. Capsule endoscopy revealed multiple jejunal erosions (Figure 3). The erosions were scattered on the proximal side of the jejunum; each erosion was shallow and < 1 cm in size, and hematin adhesions were visible on the surface. However, stool culture and *Clostridioides difficile* toxin tests were negative, as were cytomegalovirus antigen

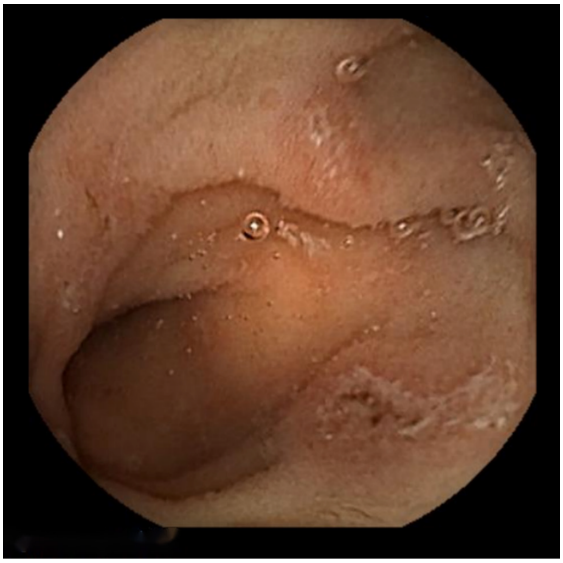

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Figure 3 Capsule endoscopy findings. Scattered erosions are seen in the jejunum. The erosions were scattered on the proximal side of the jejunum; each erosion was shallow and < 1 cm in size, and hematin adhesions were visible on the surface.

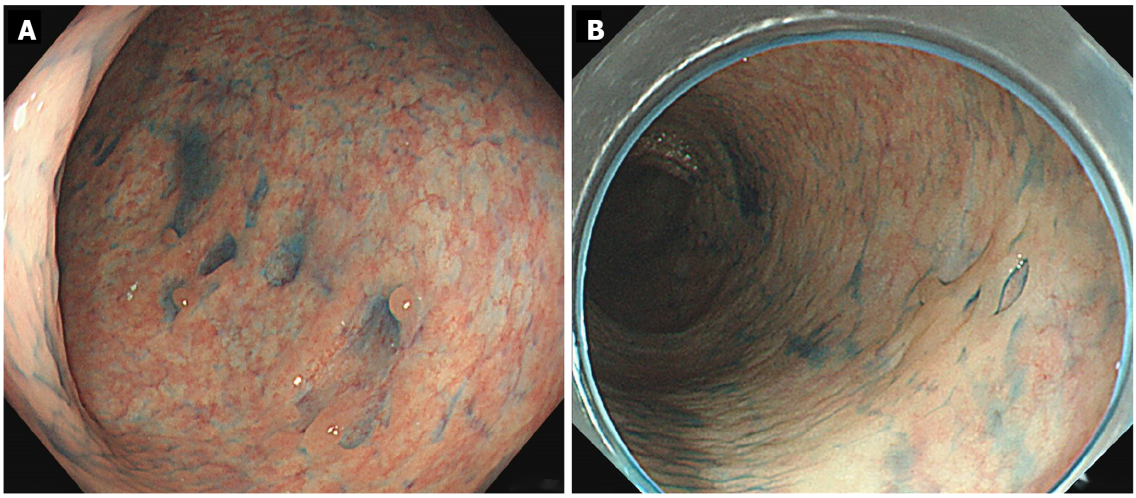

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Figure 4 Colonoscopy findings after drug withdrawal. A: At three weeks, there is shrinkage of ulcers; B: At four months, all ulcers have disappeared and scarring is observed.

and polymerase chain reaction tests and interferon-gamma release assays.

FINAL DIAGNOSIS

As no episodes of irradiation or introduction of other new drugs occurred, we suspected drug-induced due to IXE.

TREATMENT

First, we monitored the patients' progress during drug withdrawal and intestinal rest after fasting. Multiple erosions in the upper jejunum were also observed; therefore, the patient commenced on bonoprazan fumarate, a potassium-competitive acid blocker. Due to the lack of improvement in symptoms, steroid administration was considered, and a gradual improvement in abdominal symptoms was observed. Three weeks after withdrawal, endoscopy revealed shrinkage of the ulcer and scarring (Figure 4A). Although her abdominal symptoms resolved, her skin and joint symptoms worsened, and she was started on Risankizumab by her family physician for psoriasis treatment.

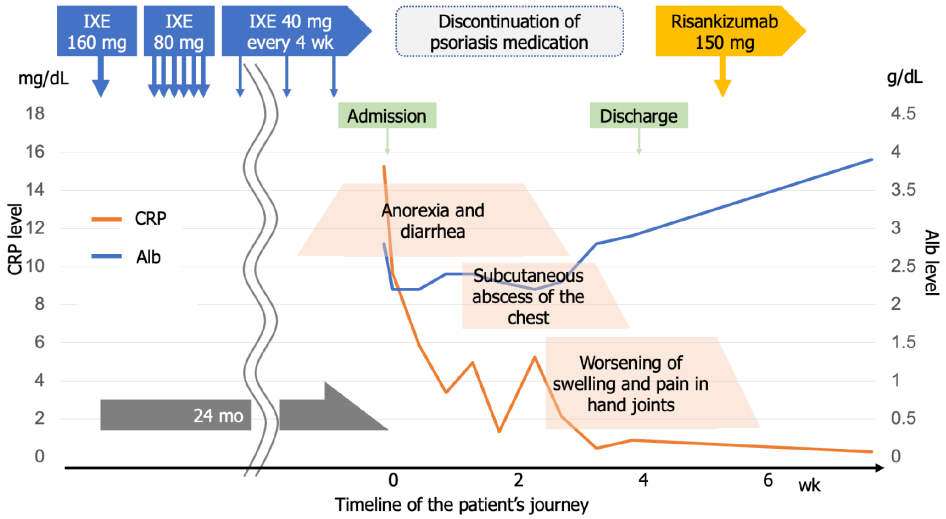

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Figure 5 Albumin (Alb) and C-reactive protein (CRP) levels plotted against the timeline of the patients' journey. The patient visited our hospital 24 mo after the start of the ixekizumab (IXE) administration. In the graph, CRP is shown on the left vertical axis and Alb on the right vertical axis. Abdominal symptoms gradually improved with IXE withdrawal, but skin and joint symptoms tended to worsen. After 4 wk of hospitalization, the patient was discharged home, as the endoscopy showed that the ulcer had healed and the patient was able to eat adequately. After discharge, risankizumab was introduced to control skin and joint symptoms, and the patients' condition stabilized. IXE: Ixekizumab; CRP: C-reactive protein; Alb: Albumin.

OUTCOME AND FOLLOW-UP

The abdominal, skin, and joint symptoms remained stable, and endoscopy performed 4 mo after IXE withdrawal confirmed the disappearance of all ulcers and scarring (Figure 4B). As abdominal symptoms improved, capsule endoscopy was not performed again due to a lack of patient consent, and bonoprazan fumaric acid was also discontinued. The clinical course from IXE initiation to the present is illustrated in Figure 5.

DISCUSSION

Drug-induced entero-colitis caused by IL-17 inhibitors is well known in the field of dermatology; however, very few reports have described all the endoscopic images, endoscopic features, and pathological characteristics of this condition. To the best of our knowledge, only 16 cases have been reported thus far[5-20]; we reviewed 17 cases, including our own (Table 1). Nine reported cases showed ulcerative colitis (UC)-like findings characterized by circumferential loss of vascular pattern and coarse mucosa/ulceration in the left colon, and eight reported cases with CD-like findings characterized by discontinuous erosion/ulceration from the terminal ileum to the rectum. All patients who presented with CD-like endoscopic findings after IXE administration had granulomas. In almost all the reports, the disease prognosis appeared to be good, with improvement in abdominal symptoms after the administration of steroids or molecularly targeted drugs. Only one patient with UC-like endoscopic findings after IXE administration required surgery because of a lack of improvement with drug administration.

Another case similar to CD with multiple ulcers of a similar round shape, as in the present case, has also been reported. However, in the present case, the ulcers tended to be longitudinally arranged and did not coincide with the mesenteric attachment side, which is atypical of CD. Furthermore, no reports have indicated improvement in abdominal symptoms with drug discontinuation alone, as in this case. In the present case, the various test results allowed us to promptly identify IXE as the suspected drug, and we surmised that excessive therapeutic intervention could be avoided. It should be noted that the introduction of a new drug may be necessary to manage the primary disease after drug withdrawal, and close communication with the dermatologist is important.

The association between psoriasis and IBD should be investigated in future studies. It has been reported that 1%-2% of patients with psoriasis have IBD[21]. Coincidentally-timed events during the initiation or administration of IL-17 inhibitors highlighted that IBD cannot be excluded.

CONCLUSION

At the very least, we should always check for IBD-related symptoms and family history before administering IL-17 inhibitors and suggest a screening colonoscopy if possible. Here, we report, for the first time, the capsule endoscopic findings of IL-17 inhibitor-induced entero-colitis. We also compared the endoscopic and pathological features of IL-17 inhibitor-induced entero-colitis with those previously reported. We believe that these findings will be useful for dermato-

Table 1 Patient background and endoscopic and pathological findings of entero-colitis after interleukin-17 administration

Year	Ref.	Age	Sex	Primary disease	Drug	Time to onset	IBD	Endoscopic findings	Pathological findings	Treatment and course
2017	Shiga *et al* [5]	56	M	Psoriasis	SEC	8 wk	CD	Longitudinal ulcer of the ileum and round ulcer of the esophagus	Nonspecific inflammatory cell infiltration	Improved with prednisolone 40 mg/d
2018	Philipose *et al*[6]	31	M	Psoriasis	IXE	3 mo	UC	Loss of vascular permeability throughout the sigmoid colon, erythematous coarse mucosa, ulcer	Lymphoplasmacytic infiltration	Mesalamine and methylprednisolone did not improve, but IFX administration improved
2018	Wang *et al* [7]	41	F	Psoriasis	SEC	1 wk	UC	Coarse mucosa and deep-burrowing ulceration of the entire sigmoid colon	Cryptitis, erosions, lymhoplasmacytic infiltration	Improved with methylprednisolone 40 mg/d and cyclosporine 2 mg/kg
2018	Ehrlich *et al*[8]	42	M	Ankylosing spondylitis	SEC	6 wk	UC	Deep ulcers and fragile mucosa of the transverse and sigmoid colon	Cryptitis, crypt abscess, loss of crypts	No improvement with solumedrol, improved after introduction of IFX
2019	Smith *et al* [9]	42	M	Psoriasis	IXE	12 wk	CD	Deep rounded punctate ulcers of the transverse and descending colon	Pancolitis with rare granuloma	No improvement with solumedrol, improved after introduction of IFX
2019	Uchida *et al*[10]	41	F	Psoriasis	SEC	4 mo	UC	Easy bleeding edematous mucosa of rectum to sigmoid colon, erosions, ulcers	High degree of inflammatory cell infiltration into the stroma and crypt abscess	Improved with mesalazine 2400 mg/d
2019	Achufusi *et al*[11]	39	M	Psoriasis	SEC	6 mo	UC	Ulceration of the splenic flexure, moderate to severe active colitis, ulceration at 30 cm, and active colitis in the rectum	Atrophy of the crypts, decreased goblet cells, cryptitis, crypt abscess	No improvement with steroids, improved after introduction of IFX
2019	Johnston and Veettil [12]	27	M	Ankylosing spondylitis	SEC	4 mo	UC	Multiple ulcers and moderate inflammation, sigmoid colon	Crypt abscess	No improvement with mesalazine and hydrocortisone, improvement with introduction of IFX
2019	Haidari *et al*[13]	69	M	Psoriatic arthritis	SEC	18 mo	CD	Multiple ulcers of the terminal ileum	Neutrophil infiltration of the epithelium of the crypts, no granuloma	Originally asymptomatic
2020	Nazarian *et al*[14]	48	F	Psoriasis	IXE	12 wk	CD	Mild erythema and punctate ulcerations in the terminal ileum	Active inflammation with the presence of granuloma	Improved with budesonide administration
2020	Varga *et al* [15]	52	M	Psoriasis	SEC	2 wk	UC	Loss of vascular permeability of sigmoid colon, ulcer	Lymphocytic infiltration of lamina propria, cryptitis, crypt abscess	Improved with prednisone 60 mg/d and mesalazine 3200 mg
2020	Gallego *et al*[16]	42	M	Psoriasis	IXE	2 wk	CD	Aphthous erosions and patchy ulcers of the rectum to cecum and terminal ileum	Cryptitis, crypt abscess, non-caseating granuloma	Improved with systemic corticosteroid administration
2021	Ali *et al*[20]	70	F	Psoriasis	SEC	1 mo	UC	Ulcerated and edematous mucosa in sigmoid colon	Acutely and chronically inflamed granulation tissue with extensive plasma cell infiltrate	Intravenous methylprednisolone
2022	Kakizoe *et al*[17]	65	M	Psoriasis	SEC	15 mo	CD	Deep ulcers of the cecum and transverse colon	No description	Hematochezia persisted after drug discontinuation and improved after induction of ADA
2022	Morosanu *et al*[19]	42	F	Psoriasis	IXE	1 wk	UC	Continuous congestive, friable rectal and colonic mucosa, spontan-	Neutrophilic inflammatory infiltrate disposed irregularly, edema and congestion, decrease of the crypts mucose-	Total colectomy with ileostoma and rectum preservation

								eously bleeding, deep and large ulcerations	cretion and crypt's abscesses	
2023	Khouri *et al*[18]	38	F	Psoriatic arthritis	SEC	1 mo	CD	Small ulcerations throughout the entire lumen of the terminal ileum and the cecum	Minimal architecture distortion in the large bowel mucosa, along with focal acute colitis	Initiated with prednisone and SEC was switched to IFX
2022	Our case	69	F	Psoriatic arthritis	IXE	21 mo	CD	Multiple round punctate ulcers throughout the colon. Capsule endoscopy shows multiple erosions in the jejunum	Inflammatory cell infiltrate, predominantly lymphocytes. Granulomatous fibroblasts and collagen fibers in the submucosa	Improvement only with drug discontinuation and fasting bowel rest

CD: Crohn's disease; SEC: Secukinumab; M: Male; F: Female; IBD: Inflammatory bowel disease; IXE: Ixekizumab; UC: Ulcerative colitis; ADA: Adalimumab.

logists and gastroenterologists in clinical practice.

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FOOTNOTES

Author contributions: Saito K wrote the first draft of the manuscript; Yoza K and Takeda S were mainly responsible for the endoscopic procedures; Shimoyama Y and Takeuchi K were primarily responsible for the patients' inpatient care.

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