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TOPIC HIGHLIGHT

WJGP 5th Anniversary Special Issues (2): Ulcerative colitis

# "Mucosal healing" in ulcerative colitis: Between clinical evidence and market suggestion

Cristiano Pagnini, Francesca Menasci, Stefano Festa, Gianenrico Rizzatti, Gianfranco Delle Fave

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## Abstract

In recent decades, the prominent role of endoscopy in the management of ulcerative colitis (UC) has been translated into the concept of mucosal healing (MH) as a fundamental therapeutic end-point. This is partially the consequence of growing evidence of a positive prognostic role of MH on the disease course and partially due to market cues indicating a higher rate of MH in patients treated by novel potent biologic agents. The aim of the present review is to clarify the current knowledge of MH in UC, analyzing the definition, the putative prognostic role and the association of MH with the current drugs used to treat UC patients. Because solid data about the management of UC patients based solely on the healing of the mucosa are not yet available, a tailored approach for individual patients that-considers the natural history of UC and the presence of prognostic indicators of aggressive disease is desirable. Consequently, unnecessary examinations and treatment would be avoided and restricted to UC patients who require the maximum amount of effort to affect the disease course in the short and long term.



**Key words:** Ulcerative colitis; Mucosal healing; Prognosis; Evidence

**Core tip:** In recent years, the concept that the management of ulcerative colitis patients should aim to heal the mucosa rather than resolve symptoms has been decisively proposed. Herein, we review the current evidence supporting this statement and analyze the possible practical implications in the current management of ulcerative colitis patients.

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## INTRODUCTION

A crucial topic that physicians have long faced in the management of ulcerative colitis (UC) patients is the identification of a reference parameter for the assessment of disease activity. Indeed, UC is a chronic inflammatory disease of the colon, characterized by limitation of the inflammation to the mucosa and the proximal extension of the disease starting from the rectum[1]. Indeed, the term "UC" comprises a heterogeneous condition with differing involvement of the colon in terms of its extension and the grade of inflammation, which in turn can lead to possible alterations of laboratory parameters and symptom occurrence and severity. The clinical, biochemical and mucosal alterations do not always directly correlate, and questions have been raised about which parameter should be used as the "gold standard" for disease activity assessment.

For the capacity to directly evaluate the colon, which is the target organ of the disease, endoscopy has been indicated as the more accurate tool to assess the activity of the disease, further supported by the possible misleading role of symptoms in the evaluation of UC patients[2,3]. Unfortunately, colonoscopy is an invasive, costly and time-consuming procedure, and the routine repetition of the examination is not feasible. Different objective surrogate parameters have been described to aid physicians in the correct evaluation of the activity state of patients with inflammatory bowel disease (IBD), including serological (*i.e.*, C-reactive protein) and fecal (*i.e.*, calprotectin, lactoferrin) markers[4], as well as clinical scores[5].

During the last decade, the addition to the IBD therapeutic arsenal of anti-TNF-α biologic drugs, which were formerly used in other chronic inflammatory conditions, has launched a "Copernican revolution" in the clinical approach to both Crohn's disease (CD) patients and UC patients. In fact, together with a rapid and consistent improvement of symptoms and laboratory parameters, such potent anti-inflammatory compounds have resulted in rapid and dramatic improvements of the intestinal mucosal lesions characteristic of IBD, as documented by endoscopic evaluation before and after the induction therapy[6]. Since this time, the relevance of the endoscopic activity of disease has been definitively stated, and "mucosal healing" (MH) has been proposed with increasing strength as a fundamental therapeutic goal of IBD treatment, claiming its prognostic relevance in the natural history of the disease[3]. Since the first studies that described the efficacy of Infliximab in CD patients[7,8], some 15 years have passed, and the therapeutic options for IBD patients have consistently expanded. At present, two biologic anti-TNF agents are currently approved in Europe for utilization in both CD and UC (Infliximab and Adalimumab), and some biologic agents have already shown efficacy in randomized clinical trials and are indicated for market release[9]. The emphasis on the efficacy of such novel drugs for the amelioration of mucosal inflammation has contributed to making the concept of MH a paramount therapeutic goal, and we are passing from a symptom-targeted to a mucosa-targeted approach in the management of IBD patients[10]. Several observations have contributed to encourage this shift in IBD management, outlining the relation between mucosal healing and the favorable long-term outcome of the disease in terms of reductions in flares, hospitalizations, the need for surgery and cancer incidence[11].

Although the MH concept has recently been particularly emphasized in CD, the importance of endoscopic remission in UC has been known for a long time[12]. In fact, the achievement of MH in UC appears of particular relevance for the localization of the disease (mucosal and limited to the colon), which renders the endoscopic examination relatively easier compared with CD, in which the inflammation is transmural and can potentially involve areas of the intestine not accessible to endoscopic inspection. Considering that more than half of UC patients present inflammation limited to the left side of the colon[13], the possibility that the involved areas can be easily scoped to evaluate MH in such patients is particularly tempting.

Nonetheless, specific treat-to-target studies addressing the effective role on the natural progression of the disease of a treatment strategy focused electively on the achievement of MH are still lacking. The relevance of MH to the management of UC, although intriguing and rational, remains to be firmly established. The possibility that the importance of MH would tend to be overrated due to the influence of sponsored trials underlining the association between MH and biologic drugs must be considered. Moreover, data coming from randomized clinical trials (RCTs) are usually not completely applicable to the "real-life" IBD population. In fact, a recent retrospective analysis of consecutive mild-moderate IBD patients at a United States tertiary referral center found that only 31.1% of patients would fulfill the inclusion criteria of the major RCTs of biologic agents and that the outcomes of patients fulfilling the criteria are significantly more favorable compared with those not meeting the criteria[14].

Besides scientific and commercial suggestions, a careful revision of the actual evidence in support of MH is essential. The risk of a blind and excessively enthusiastic adherence to the MH suggestion is concrete, and physicians need to be aware of the over-prescription of unnecessary endoscopic examinations and/or the over-treatment of patients. In an era of resource optimization, this would risk minimizing the same advantages that the MH strategy is claiming, *i.e.*, the reduction of disease costs by reducing complications and hospitalizations. Extensive systematic reviews of MH are already available in the literature (*i.e.*, Neurath *et al*[11]), and such a review is beyond the aim of the present work. Here, we intend to perform a synthetic and careful revision of the state-of-the art research on MH. To this end, we critically reviewed the definition of MH, the quality of the actual evidence of its prognostic relevance, and the capacity of the therapies currently used for UC to achieve MH, with the final goal of clarifying the potential correct application of the concepts of MH to the current practical management of patients affected by UC.

## MH: DEFINITION

Although a standardized definition of MH has not been established, a practical currently accepted definition is "the complete resolution of the visible alterations or lesions, irrespective of their severity and/or type at baseline colonoscopy"[11]. Nonetheless, at present, an easy to use, validated and clinically relevant endoscopic score for UC activity evaluation is lacking, reflecting the complexity in measuring disease activity in UC[15]. In fact, although a great number of scoring systems have been developed (Baron score, Mayo score, Sutherland, Powell-Tuck and Rachmilewitz indices, among others)[16-24], none of them have been prospectively validated. The main problems

regarding the majority of the indices include the overlap of mucosal features (such as vascularity, granularity, erythema, friability, bleeding, and ulceration), leading to inter-observer variation in endoscopic evaluation, and the lack of clear and standardized thresholds for endoscopic remission or improvement. The Mayo Clinic endoscopy subscore has been the most commonly used in recent clinical trials, defining MH as a score of $\leqslant 1$ (normal mucosa or loss of vascular pattern, but no mucosal friability), when the endoscopy subscore was 2 or 3 at baseline. The problem of a standardized definition of MH is not theoretical but implies concrete and practical consequences. In fact, in recent clinical trials, heterogeneous definitions may have contributed to the higher rate of patients with MH when compared with that of patients achieving clinical remission[25], although alternative explanations are possible (*e.g.*, the simultaneous presence of irritable bowel syndrome, dysmotility). Moreover, a recent RCT testing the use of mesalamine in UC patients showed consistently different results after a revision of the endoscopic examination findings by a blinded central reader[26].

In further support of the aforementioned difficult evaluation of UC endoscopic activity, two novel scores have been very recently developed and prospectively validated, the Ulcerative Colitis Endoscopic Index of Severity and the Ulcerative Colitis Colonoscopic Index of Severity[27,28]. Data about the applicability of such new scores in clinical trials and in clinical practice are awaited and will hopefully aid the move toward a standardized definition of MH.

Recently, data indicating a prognostically relevant role for histologic activity in the mucosa of UC patients, in addition to the macroscopic activity, have opened the door to the concept of "histological MH", with the complete absence of clinical, laboratory, endoscopic and histological features of active inflammation[29]. Indeed, the term "mucosal healing" was initially proposed only for the disappearance of the inflammatory infiltrate in the histological examination[30]. At present, although some scoring systems for histologic activity have been described, none have been properly validated or commonly used, and therefore, the definition of histological MH remains without consensus.

## MH: EVIDENCE FOR PROGNOSTIC RELEVANCE

The increasing relevance of the MH achievement in UC has been demonstrated by a growing body of data showing the different courses of the disease in patients with and without MH, with a reduction of complications such as flares as well as reductions in hospitalization, colectomy and cancer incidence in patients with MH.

As early as 1966, Wright *et al*[31] reported a higher relapse rate in patients who did not achieve MH after oral and rectal steroids when compared with patients who did achieve MH (40% *vs* 18%). In the ACT1 and ACT2 trials, patients treated with infliximab who exhibited MH at week 8 showed a higher rate of clinical remission at week 30 than patients without MH (48.3% *vs* 9.5%)[32]. Yamamoto *et al*[33] reported that UC patients who achieved clinical remission and MH after leukocytapheresis had a higher rate of sustained clinical response when compared with patients with only a clinical response (88% *vs* 41%). Ardizzone *et al*[34] showed that the lack of mucosal healing at 3 mo after the first corticosteroid treatment was the only factor associated with negative outcomes at 5 years (use of immunosuppressants, hospitalization and colectomy).

An observational study of the IBSEN cohort showed that in 513 UC patients, the colectomy rate was lower in patients with MH [defined by a simple endoscopic score of 0-1 (0, normal; 1, light erythema or granularity)] at a 5-year follow-up (2% *vs* 8%, $P < 0.05$)[35]. Similar results were shown by Soldberg *et al*[36], who reported a decrease in the colectomy rate in UC patients with MH at 1 year after diagnosis, regardless of the therapy used to achieve it, and in a post-hoc analysis of the ACT1/ACT2 trials conducted by Colombel *et al*[37], in which a Mayo Clinic endoscopy subscore of 0-1 in Infliximab-treated patients was related to a lower probability of colectomy than a score of 2-3 through a follow-up period of 54 wk. Interestingly, in the latter article by Colombel *et al*[37], MH in the placebo group did not show the same positive prognostic value as it did in the Infliximab-treated group, questioning the prognostic value of MH "*per se*" and suggesting that the drugs used to achieve the MH may play a specific role in the long-term outcome.

The increased risk of colorectal cancer incidence in UC patients is still a matter of debate[38]. Nonetheless, the inflammatory burden appears to be an important determinant, and consequently, MH is likely to reduce the risk. An Italian cohort study indicated a lower CRC risk at 17 years of follow-up in azathioprine (AZA)-treated UC patients with MH[39].

Recently, appealing data have indicated a possible prognostic role for histologic remission in terms of reductions in flares, surgery/hospitalization and CRC incidence, suggesting histologic remission as the ultimate therapeutic goal in UC management[29]. In fact, Bitton *et al*[40] have reported basal plasmacytosis at rectal biopsy as an independent predictor of early relapse in UC patients, and Bessissow *et al*[41] have described a higher rate of flares in patients with macroscopically healed mucosa but histologic activity when compared with patients with both the macro- and microscopic absence of disease. Nonetheless, correlations with macroscopic and microscopic activity are not always straightforward[42], and routine biopsies are not suggested by the current guidelines. At present, more evidence is needed before considering histological MH as a possible goal of treatment in UC patients.

## MH: CURRENT THERAPIES

### *Biologic agents*

As mentioned, the MH concept has been clearly defined

**Table 1 Randomized clinical trial of biologic agent in ulcerative colitis and the relative mucosal healing rates**

| Ref. | Patients (*n*) | Treatment protocol duration | Evaluation time from baseline | MH rate |
|---|---|---|---|---|
| Rutgeerts *et al*[32] | 728 | IFX 5 or 10 mg/kg every 8 wk | Week 8 | 60.7% IFX |
| | | Placebo | | 32.3% placebo |
| | | 30 wk (ACT2) | Week 30 | 50.6% IFX |
| | | | | 27.4% placebo |
| | | 54 wk (ACT1) | Week 54 | 46.0% IFX |
| | | | | 18.2% placebo |
| Panaccione *et al*[50] | 231 | AZA 2.5 mg/kg | Week 16 | 37% AZA |
| | | IFX 5 mg/kg | | 55% IFX |
| | | IFX 5 mg/kg + AZA 2.5 mg/kg | | 63% AZA + IFX |
| | | 16 wk | | |
| Sandborn *et al*[25] | 494 | ADA 160/80 and then 40 mg eow | Week 8 | 41.1% ADA |
| | | Placebo | | 31.7% placebo |
| | | 52 wk | Week 52 | 25.0% ADA |
| | | | | 15.4% placebo |
| Reinisch *et al*[43] | 390 | ADA 160/80 mg or 80/40 mg at weeks 0 and 2 followed by 40 mg at weeks 4 and 6 | Week 8 | 46.9% ADA (160/80) |
| | | Placebo | | 37.7% ADA (80/40) |
| | | | | 41.5% placebo |
| | | 52 wk | Week 52 | 54% ADA |
| Feagan *et al*[44] | 225 | VED 300 mg at week 0 and 2 and then every 4 or 8 wk | Week 6 | 40.7% VED |
| | | Placebo | | 24.8% placebo |
| | | 52 wk | Week 52 | 56% VED (every 4 wk) |
| | | | | 51.6% VED (every 8 wk) |
| | | | | 19.8% placebo |
| Sandborn *et al*[45,46] | 774 | GOL 400/200 or 200/100 mg at weeks 0 and 2 followed by 50 mg or 100 mg every 4 wk | Week 6 | 45.1% GOL (400/200) |
| | | Placebo | | 42.3% GOL (200/100) |
| | | | | 28.7% placebo |
| | | 54 wk | Week 54 | 42.4% GOL100 every 4 wk |
| | | | | 41.7% GOL 50 every 4 wk |
| | | | | 26.6% placebo |

MH: Mucosal healing; IFX: Infliximab; ADA: Adalimumab; AZA: Azathioprine; VED: Vedolizumab; GOL: Golimumab.

only in the biologic era, and the trials of biologic drugs present a better evaluation of this aspect than previous studies. In particular, the MH definition has been standardized by the utilization of the Mayo endoscopic subscore, which identifies as MH as a score of 0 or 1. However, MH has always been considered as a secondary end-point in clinical trials, and studies still present heterogeneity in terms of inclusion criteria (and, therefore, baseline endoscopic severity), design and follow-up. Nonetheless, the MH rates in the short (induction) and long term (maintenance) are consistent and significantly superior to those of placebo in all studies (Table 1), which is even more remarkable considering the baseline severity of the UC patients included, although, in most of the studies, MH was only observed in a minority of the patients[25,32,43-46]. Moreover, as mentioned, patients in RCTs are superselected, and the results may be not directly applicable to the "real-life" IBD population.

### Azathioprine

From the first early report by Jewell *et al*[47] of increased MH after 4 wk in UC patients treated with corticosteroids plus AZA *vs* corticosteroids plus placebo (92% *vs* 71%, *P* = ns), few studies with a limited number of patients have addressed MH rates in AZA-treated UC patients. In all of the reported studies, MH was a secondary end-point, and the MH definition, base-line endoscopic activity, timing of the endoscopic evaluation and concomitant therapies differed; therefore, conclusive results are hard to extrapolate.

With the aforementioned limitations, Paoluzi *et al*[48] reported 57% and 45% rates of MH in UC patients treated with AZA at 6 mo (*n* = 42 patients) and 4 years (*n* = 22 patients), respectively, and a similar 6-mo rate was reported by Ardizzone *at al*[49] [19/36 patients treated with AZA (53%) *vs* 7/36 of patients treated by 5ASA (19%)]. Recently, a study by Panaccione *et al*[50] (available only in abstract form) reported a 36% MH rate in patients treated with AZA in monotherapy and a 63% MH rate in patients treated with AZA plus Infliximab at 4 mo, with nearly 80 patients per group, indicating that combination therapy may increase the rate of MH.

### Corticosteroids

Unlike CD, in which corticosteroids are traditionally considered ineffective for the achievement of MH[51], corticosteroids may induce MH and a clinical response in UC. The first evidence supporting a favorable role of corticosteroids in inducing MH dates back to 1954, when Truelove reported a double-blind placebo-controlled randomized multicenter trial of 120 UC patients and demonstrated higher rates of MH in the oral cortisone (100 mg/d) group

than in the placebo group (30% *vs* 10%) within 6 wk[16].

In the last six decades, a great number of studies have reported positive effects of corticosteroid therapy on the improvement/resolution of mucosal alterations in UC, irrespective of the route of administration (oral or rectal) and the type of corticosteroids (traditional systemic steroids or agents with low systemic availability)[52-59]. Generally, a certain discrepancy between the clinical and endoscopic responses was present in the majority of the studies evaluating MH in UC after corticosteroid treatment. A meta-analysis by Marshall *et al*[55] examining the role of rectal corticosteroid preparations showed similar clinical (approximately 45% of cases) and endoscopic (approximately 33% of cases) remission rates for conventional corticosteroids (hydrocortisone, prednisolone, methylprednisolone and betamethasone) and topically active corticosteroids (beclomethasone, budesonide and prednisolone metasulphobenzoate). Recently, Ardizzone *et al*[34], in a study of 157 consecutive newly diagnosed UC patients, explored the potential prognostic significance of a 3-mo clinical and endoscopic response after the first course of corticosteroid treatment. After 3 months, 60 patients (38.2%) had a complete clinical and endoscopic response, 39 (24.8%) had a clinical but not an endoscopic response, and 58 (36.9%) had no response. Interestingly, failure to achieve endoscopic remission at the end of the first course of steroids was related to a more aggressive disease behavior.

Data obtained from the use of topical steroids present a reduced variability between clinical and endoscopic responses. Indeed, in a recent meta-analysis exploring the efficacy of rectal beclomethasone dipropionate, the clinical and endoscopic rates of improvement or remission were similar (65.3%) and concordant, although in the four trials considered for the meta-analysis, a clear definition and evaluation of mucosal healing were lacking[60].

Several problems arise in the attempt to analyze and compare the results of the above-mentioned studies. Diversity in the timing of endoscopy and in the use of endoscopic indices (*e.g.*, Sigmoidoscopic score, Rachmilewitz index, Baron score) along with the lack of a univocal MH definition, possible inter-observer variations or heterogeneity of the included patient cohorts may have generally contributed to consistent variability in the MH rates in steroid trials.

### *Aminosalicylates*

Mesalamine was approved by the Food and Drug Administration in late 1987, and since this time, it has become the cornerstone therapy for mild-moderate UC[61]. Mesalamine can be administered orally and/or topically, and it is present on the market in different formulations specific to both methods of administration. Many studies show the ability of mesalamine to induce MH. A recent meta-analysis of 49 studies has concluded that MH is achieved in approximately 37% and 50% of patients treated with oral and topical mesalamine, respectively[62]. Nonetheless, the results from single studies are dramatically different, ranging from approximately 0% to 77% for oral mesalamine[23,63] and from approximately 10% to 93% for topical formulations[54,64]. This variability may be attributed to the different definitions of MH, but this is unlikely to be the only reason. While MH rates do not appear to be related to the release mechanisms of oral mesalamine[62], in accordance with previous studies reporting similar effectiveness between different formulations[61,65,66], studies continue to present great heterogeneity in terms of total dose in grams, disease extension, months of follow up and endoscopic score at baseline. Notably, the MH rates in placebo groups are reported to be high, up to 46% in a study of oral placebo *vs* oral mesalamine at 8 wk[63] and 26%-37% in a study of topical placebo *vs* topical mesalamine after 6 wk[67]. Moreover, in studies with therapeutic regimens of adequate dose and duration, the MH rate appears to be higher[68-70], and the lack of achievement of MH in patients with clinical remission has been indicated as a possible negative prognostic factor for relapse occurrence[71].

## CONCLUSION

After the emergence of novel biologic therapies for UC, the old concept of the relevance of the endoscopic activity of disease has been translated into the new concept of MH as the therapeutic goal to achieve. Although this idea has been supported by a growing body of scientific evidence indicating the favorable prognostic value of a healed mucosa in the natural history of UC, it is also suggested commercially, as a high rate of MH is claimed when utilizing the new biologic agents. Indeed, endoscopic evaluation appears to be the "gold standard" for the evaluation of disease activity in UC patients, and healing of the mucosa is likely to be an important factor for the control of the disease in the short and long term. However, specific studies showing the superiority of a management based solely on MH over the "traditional" approach are lacking. To date, most of the evidence supporting the prognostic relevance of MH comes from studies in which MH is not considered as the primary endpoint as well as from retrospective investigations. In the present study, we provocatively addressed the issue of the relevance of MH for UC patients management. A careful review of the current evidence regarding MH in UC shows that, due to the high heterogeneity of the available studies (particularly for those from the pre-biologic era), crucial points are still far from being conclusively determined, including the MH definition, the expected rate of MH with the current medication, and whether a systematic assessment of MH and an optimization of therapy based on MH alone would improve long-term disease outcome. Moreover, the prognostic value of MH "*per se*" needs to be investigated to clarify whether the current drugs may be safely reduced or interrupted after MH achievement. The latter issue may also present consistent economic implications regarding the elevated cost of long-term maintenance therapy with biologic drugs. However, in most cases, MH appears to be achiev-

able only in a minority of UC patients and most likely with the utilization of potent and potentially dangerous therapeutic regimens. In the near future, the development of novel drugs and an increase in our knowledge of the complexity of IBD are desirable, as they may increase the efficacy of our therapeutic approach to the disease.

Notably, going back to the natural history of the disease, more than one-half of UC patients have a benign disease course, while up to one-third are likely to experience frequent flares and potentially dangerous complications. In fact, the large population study by Solberg *et al*[36] (IBSEN cohort), which evaluated the first 10 years of the disease course in a population of 519 patients with UC, highlighted an overall good prognosis. Their study showed that at 10 years, more than half of patients were in remission or had mild disease, while 37% and 6%, respectively, reported chronic intermittent and chronic continuous symptoms. In a large Danish cohort study, approximately one-third of patients had no flares within 10 years after the first attack of UC. Moreover, the cumulative probability of having a course without relapses after 10 years in patients in remission is 40%-60%[72]. However, the colectomy rate is estimated to vary from 8.7% to 30% in different populations[72-74], and after the first relapse, the cumulative rates of a second course of systemic steroids are 13%, 41% and 48% at 1, 5 and 10 years, respectively[36].

In times of resource optimization, the ideal disease management would imply an aggressive treatment and endoscopic follow-up for the achievement of MH in patients with an unfavorable disease course. Accordingly, together with a better definition of the MH concept and its specific role in the management of UC patients, further research for the characterization of clinical and/or genetic features predictive of an aggressive behavior of the disease is urgently needed. Similarly, the identification and the implementation of clinical and laboratory parameters strongly correlated with the endoscopic activity, such as clinical scores, to better follow-up these patients, appear to be of relevance[75]. Consequently, it is advisable that the aforementioned shift from a symptoms-based to a mucosa-based approach in the management of UC patients would not result in a trend to over-scope and/or over-treat patients for the achievement of MH. Indeed, because more solid evidence will be available regarding the role of MH, a rational approach to UC patients should reserve close monitoring and more potent therapies for "high-risk" patients, overcoming the dualism between symptom- and mucosa-targeted approaches and focusing increasingly on a "patient-based" approach.

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TOPIC HIGHLIGHT

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# Implication of miRNAs for inflammatory bowel disease treatment: Systematic review

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## Abstract

Inflammatory bowel disease (IBD) is believed to develop *via* a complex interaction between genetic, environmental factors and the mucosal immune system. Crohn's disease and ulcerative colitis are two major clinical forms of IBD. MicroRNAs (miRNAs) are a class of small, endogenous, noncoding RNA molecules, and evolutionary conserved in animals and plants. It controls protein production at the post-transcriptional level by targeting mRNAs for translational repression or degradation. MiRNAs are important in many biological processes, such as signal transduction, cellular proliferation, differentiation and apoptosis. Considerable attention has been paid on the key role of miRNAs in autoimmune and inflammatory disease, especially IBD. Recent studies have identified altered miRNA profiles in ulcerative colitis, Crohn's disease and inflammatory bowel disease-associated colorectal cancer. In addition, emerging data have implicated that special miRNAs which suppress functional targets play a critical role in regulating key pathogenic mechanism in IBD. MiRNAs were found involving in regulation of nuclear transcription factor kappa B pathway (*e.g.*, miR-146a, miR-146b, miR-122, miR-132, miR-126), intestinal epithelial barrier function (*e.g.*, miR-21, miR-150, miR-200b) and the autophagic activity (*e.g.*, miR-30c, miR-130a, miR-106b, miR-93, miR-196). This review aims at discussing recent advances in our understanding of miRNAs in IBD pathogenesis, their role as disease biomarkers, and perspective for future investigation and clinical application.



**Key words:** Crohn's disease; Inflammatory bowel disease; MicroRNA; Treatment; Ulcerative colitis; Biomarker

**Core tip:** MicroRNAs (miRNAs) are a class of small, noncoding RNA molecules that post-transcriptionally regulate gene and protein expression. Recent studies have identified altered miRNA profiles in inflammatory bowel disease (IBD). Special miRNAs which suppress functional targets have been found to play a critical role in regulating key pathogenic mechanism in IBD. In this review, we discuss the possibility to use miRNAs as biomarkers and therapeutic target in IBD.

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## INTRODUCTION

Inflammatory bowel disease (IBD) refers to chronic remittent or progressive inflammatory conditions that may affect the entire gastrointestinal tract. Crohn's disease (CD) and ulcerative colitis (UC) are two major clinical forms of IBD[1]. The incidence and prevalence of IBD is continuously increasing over the past decades in different regions around the world[2]. Although the precise pathogenesis of IBD remains obscure, several reports have

indicated that dysfunction of the mucosal immune system which develops *via* a complex interaction between genetic factors, the host immune system and environmental factors plays an important role in its etiology[3]. The chronic inflammation of IBD is associated with marked molecular changes in gene and protein expression[4]. So small molecules targeted at the pathways involving in these processes may be potential for IBD diagnosis and treatment.

MicroRNAs (miRNAs) are considered as promising candidate. They are a class of single-stranded non-coding RNA molecules on an average 22 nucleotides long[5], and are highly conserved throughout evolution[6] and discovered in all eukaryotic cells except fungi[7]. MiRNAs regulate gene expression both at a transcriptional and translational level[8], and mediate post-transcriptional gene silencing by directly binding to the 3' untranslated region (UTR) of target mRNA. Depending on the level of sequence complementarity between miRNA and target site, mRNA transcripts targeted by miRNAs are either silenced if the base-pair match is imperfect or degraded if there is an identical base-pair match[9]. The mRNAs inhibited by miRNAs move to cytoplasm and accumulate in cytosolic processing bodies until they are eventually degraded[10]. Each miRNA can target hundreds of genes, and a particular gene is usually the target of multiple miRNAs, adding complexity to the regulation of gene transcriptional network[11]. It has been reported that miRNAs play an important role in many biological processes, such as signal transduction, cellular proliferation, differentiation, apoptosis and immune response[12,13]. Recently, miRNAs have been recognized as critical elements in the regulation of the innate and adaptive immune responses, and changes in miRNAs expression are related to many autoimmune diseases, including systemic lupus erythematosus, rheumatoid arthritis, psoriasis and IBD[14-17].

In this review, we summarize the current understanding of the connection between miRNAs and IBD. We mainly focus on special dysregulated miRNAs in CD and UC, which lead to inappropriate expression of targeted mRNA and may contribute to IBD pathogenesis, diagnosis and treatment. Table 1 summarizes the altered miRNAs involved in IBD and their mRNA targets.

## MIRNAS REGULATE NUCLEAR TRANSCRIPTION FACTOR KAPPAB PATHWAY

The nuclear transcription factor kappaB (NF-κB) was identified as one of the important regulators in the immune system and inflammatory diseases[18]. NF-κB is markedly induced in IBD patients and strongly influences the course of mucosal inflammation through its ability to promote the expression of various pro-inflammatory genes[19]. Nucleotide-binding oligomerization domain 2 (NOD2) was found to be the first IBD susceptibility gene[20], which is mainly expressed in Paneth cells, monocytes, macrophages, dendritic cells and some types of intestinal epithelia cell[21]. NOD2 can be activated by muramyl dipeptide (MDP), a component of bacterial cell wall, which induces the activation of NF-κB[22].

MiR-146a was reported to regulate gut inflammation *via* NOD2-sonic hedgehog (SHH) signaling[23]. SHH signaling is an important pathway that maintains gut homeostasis and directs gut development. The expressions of NOD2-induced iNOS and NO were increased in MDP-treated macrophages, which further induced the level of miR-146. Promoter luciferase analysis with miR-146a promoters revealed that NF-κB was a critical transcription factor that regulate NOD2 mediated expression of miR-146a. NOD-2 induced miR-146a target NUMB, a negative regulator of SHH signaling, alleviating the suppression of SHH signaling and subsequently increasing the pro-inflammatory cytokines expression.

Feng *et al*[24] proved that up-regulation of miR-126 may contribute to pathogenesis of UC by targeting IκBα. They found miR-126 was significantly increased in active UC tissues compared to healthy controls. IκBα, an inhibitor of NF-κB pathway and the target of miR-126, was markedly decreased in active UC tissues. The expression of miR-126 and IκBα were inversely correlated in patients with active UC. MiR-126 could inhibit the level of IκBα in HT29 cells. They further demonstrated that miR-126 may activate NF-κB signaling pathway by targeting IκBα and contribute to the development of UC. Another study showed that the anti-inflammatory activities of the red wine polyphenolics were, at least in part, mediated by the induction of miR-126[25]. CAMs, such as intracellular cell adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), are expressed on the surface of fibroblasts[26]. It has been demonstrated that the expression of ICAM-1 was increased in CD patients[27] and inhibition of CAMs could suppress various forms of experimental inflammatory and immune responses in colon fibroblast cells[28]. VCAM-1 has been confirmed as one of the targets of miR-126 before[29]. Angel-Morales *et al*[25] found the polyphenolic red wine extract (WE) exerted an anti-inflammatory effect in LPS-stimulated human colon-derived CCD-18Co myofibroblast cells through inactivating NF-κB and down-regulating a wide range of downstream pro-inflammatory genes including tumor necrosis factor (TNF)-α, interleukin-6 (IL-6) and CAMs. Furthermore, they found the up-regulation of miR-126 was induced by WE in CCD-18Co cells and protected human colon cells from inflammation through targeting VCAM-1.

MiR-122 was found dysregulated in association with CD progression[30]. Chen *et al*[31] identified NOD2 as a target of miR-122. Overexpression of miR-122 in LPS-stimulated HT-29 cells inhibited LPS-induced apoptosis and down-regulated LPS-induced NOD2 expression. Pretreatment with miR-122 in LPS-stimulated HT-29 cells decreased the pro-inflammatory cytokines and increased the anti-inflammatory cytokines by targeting NOD2-induced NF-κB signaling pathway. Taken together, miR-122 might decrease intestinal epithelial cell injury in Crohn's disease by targeting NOD2. Besides regulating the activation of NF-κB pathway, Ye *et al*[32] demonstrated

**Table 1 List a core set of altered microRNAs involved in inflammatory bowel disease and their mRNA targets**

| miRNA | Target mRNA | Net effect | Ref. |
|---|---|---|---|
| Increased expression | | | |
| miR-146a | NUMB | SHH signaling upregulation | [23] |
| miR-146b | Siah2 | NFκB signaling upregulation | [40] |
| miR-126 | IκBα | NFκB signaling upregulation | [24] |
| | Vascular cell adhesion molecule-1 | Suppresses proinflammatory cytokines | [25] |
| miR-122 | Nucleotide-binding oligomerization domain 2 | Decreases intestinal epithelial cell injury | [31] |
| | Occluding | Intestinal permeability upregulation | [32] |
| miR-132 | AChE | Decreases circulation AChE activity | [37] |
| miR-21 | RhoB | Impairment of tight junctions | [52,53] |
| miR-150 | c-Myb | Promotes apoptosis | [54] |
| miR-141 | CXCL12β | Regulates leukocyte migration | [62] |
| miR-106b | ATG16L1 | deregulation of autophagy | [67,68] |
| miR-196 | IRGM | deregulation of autophagy | [70] |
| miR-30c | ATG5 | inhibition of autophagic activity | [69] |
| miR-130a | ATG16L1 | inhibition of autophagic activity | [69] |
| Decreased expression | | | |
| miR-10a | IL-12/IL-23p40 | Regulates intestinal homeostasis | [43] |
| miR-124 | STAT3 | Promotes inflammation | [48] |
| miR-200b | ZEB1, SMAD2 | Regulates epithelial-mesenchymal transition | [59,60] |
| miR-192,miR-495, miR-512,miR-671 | NOD2 | NFκB signaling upregulation | [34] |

NF-κB: Nuclear transcription factor kappa B; NOD2: Nucleotide-binding oligomerization domain 2; AChE: Acetylcholinesterase; IRGM: Immunity-related GTPase family; IL: Interleukin; ZEB1: Zinc finger E-box binding homeobox 1; SMAD2: SMAD family member 2.

the involvement of miR-122 in the regulation of intestinal epithelial tight junction (TJ) permeability. Deficient intestinal epithelial TJ barrier, characterized by the increase of intestinal permeability, has been demonstrated to contribute to the development of IBD as an important pathogenic factor[33]. MiR-122 was significantly increased in TNF-α-stimulated Caco-2 cells and induced the increase in Caco-2 TJ permeability by targeting occluding. The up-regulation of intestinal permeability by miR-122 was proved *in vivo* as well[32]. Based on the two studies, miR-122 plays a complex and controversial role in the development of IBD.

Chuang *et al*[34] showed that NOD2 expression is regulated by miRNAs in HCT116 cells. They found that MDP could induce the expression of NOD2 and activate the NF-κB signaling pathway in HCT116 cells. MiRNAs targeted NOD2, such as miR-192, miR-495, miR-512 and miR-671, were significantly decreased in MDP-stimulated HCT116 cells, which had an inversely correlation with the expression of NOD2. Overexpression of these NOD2-associated miRNAs in MDP-stimulated HCT116 cells inhibited the activity of NF-κB and the downstream pro-inflammatory cytokines, IL-8 and CXCL3.

MiR-132 was a potential regulator of acetylcholinesterase (AChE) activity in inflammatory condition and was shown to target AChE to reduce its activity *in vitro* and in mouse models[35]. Acetylcholine (ACh) activates its receptor on macrophage through which it interrupts the nuclear translocation of NFκB and suppresses the production of pro-inflammatory cytokines[36]. Maharshak *et al*[37] found miR-132 had an anti-inflammatory effect on the development of IBD. MiR-132 level was significantly upregulated in biopsies from patients with IBD compared with controls. In accordance with this, circulation AChE activity was significantly lower in patients with IBD suffering from moderate-severe disease. These data implicated a possible regulation of AChE activity by increased miR-132 levels, which eventually ameliorated inflammation in patients with IBD.

Although NFκB was originally thought to be an almost exclusively pro-inflammatory player in the setting of IBD, its role in epithelial cells was confirmed more controversial. Several studies using knockout mice with defective NF-κB activation have demonstrated an anti-inflammatory function of NFκB in colonic epithelial cells[38,39]. Nata *et al*[40] showed that miR-146b, another member of miR-146 family, can alleviate intestinal injury in mouse colitis *via* the activation of NF-κB and the improvement of epithelial barrier function. MiR-146b was found significantly up-regulated in IL-10 deficient mice. The whole sequence of miR-146 was intraperitoneally administered to the dextran sodium sulfate (DSS)-induced colitis mouse. Overexpression of miR-146b in DSS-induced colitis mouse activated NFκB, relieved intestinal inflammation, improved epithelial barrier function, and increased the survival rate. Furthermore, the protective effect of miR-146b on mouse with DSS-induced colitis was negated by inhibition of the NFκB pathway. Siah2, which was the target of miR-146b, promoted ubiquitination of TRAF proteins upstream of NFκB. It suggested that miR-146b up-regulated NFκB *via* suppressing siah2, which finally improved intestinal inflammation.

## MIRNAS REGULATE IL-23/IL-23R PATHWAY

IL-23, a heterodimeric cytokine comprising IL-12p40

and IL-23p19, is produced by activated macrophages, monocytes, DCs and endothelial cells. IL-23 receptor is composed of IL-12Rβ1 (shared with the IL-12 receptor) and the specific IL-23R subunit. IL-23 acts on the IL-23 receptor and promotes expansion and maintenance of Th17 cells, which secrete the pro-inflammatory cytokine IL-17 and have been implicated in the pathogenesis of many chronic inflammatory disorders, including IBD[41,42]. MiRNA was considered as a new mechanism in regulating the IL-23/TH17 pathway and subsequent downstream IL-17 production in IBD.

Xue *et al*[43] found much lower expression of miR-10a in intestinal epithelial cells and dendritic cells of specific pathogen-free mice compared to germ-free mice. IL-12/IL-23p40 was identified as a target of miR-10a. They further demonstrated that microbiota negatively regulated host miR-10a expression by targeting IL-12/IL-23p40, which may contribute to the maintenance of intestinal homeostasis.

*IL-23R* gene variants have been identified as risk factors for IBD[44]. The rs10889677 variant in the 3'UTR region of *IL-23R* gene which led to a loss of binging capacity for let-7e and let-7f displayed increased expression of IL-23R[45]. It means this mutation sustained IL-23 signaling and contributed to chronicity of IBD. Furthermore, Li *et al*[46] showed let-7f down-regulated the expression of IL-23R and its downstream cytokine IL-17 by targeting IL-23R.

## MIRNAS REGULATE IL-6/STAT3 PATHWAY

Previous studies have shown the importance of the IL-6/STAT3 signaling pathway in IBD. Inhibition of IL-6/STAT3 cascades results in the suppression of acquired immune mediated colitis[47]. Koukos *et al*[48] found miR-124 were significantly decreased in colon tissues from children with UC and mice with experimental colitis, and the levels of STAT3 and its regulated genes were up-regulated simultaneously. They demonstrated reduced levels of miR-124 in colon tissues of pediatric patients with active UC might increase expression and activity of STAT3 by direct binding to its 3'UTR, which could promote inflammation and the pathogenesis of UC in children.

## MIRNAS REGULATE INTESTINAL EPITHELIAL BARRIER FUNCTION

The intestinal mucosal barrier, of which the intestinal epithelial cells are the most integral part, maintains a delicate balance between absorbing essential nutrients while preventing the entry and responding to harmful subjects[49]. Dysfunction of intestinal epithelial barrier has been extensively reported in IBD[49,50].

Disruptions of important elements of the intestinal barrier in IBD lead to permeability defects[51]. There were two studies showed that miR-21 played a pro-inflammatory role in IBD by impairing intestinal barrier function. Yang *et al*[52] found levels of miR-21 were up-regulated in both the mucosal and serum of patients with UC. RhoB, which was the target of miR-21 and involved in modulating intestinal epithelial permeability, was found significantly decreased in the patients with UC. They demonstrated that overexpression of miR-21 in patients with UC and Caco-2 cells impaired intestinal tight junction integrity and morphology through targeting RhoB. Similarly, Shi *et al*[53] reported that miR-21 was overexpressed in IBD patients, IL-10 KO mice and DSS-treated mice. MiR-21 knockout (KO) mice was less susceptible to experimental colitis and had more ameliorative inflammatory responses than wild type (WT) mice. Moreover, the increase of Intestinal permeability and epithelial cells apoptosis induced by DSS were attenuated in miR-21 KO mice.

Bian *et al*[54] found miR-150 was significantly elevated, whereas c-Myb, a target of miR-150, was strongly decreased in colon tissue of UC patients and DSS-treated mice. Overexpression of miR-150 in HT29 cells enhanced cell apoptosis through targeting c-Myb, which damaged intestinal epithelial barrier.

Epithelial-to-mesenchymal-transition (EMT) is characterized by losing epithelial cell markers such as E-cadherin and gaining mesenchymal proteins including vimentin, which enhances invasiveness, migratory capacity and production of cell-extracellular matrix components[55,56]. Recent studies demonstrated that EMT contributed to the loss of intestinal epithelial cells (IECs) and subsequent increased intestinal paracellular permeability and decreased intestinal epithelial barrier function[57,58]. Chen *et al*[59] found miR-200b significantly decreased in inflamed mucosa in IBD patients, which was positively correlated to the expression of E-cadherin and negatively correlated to the level of TGF-β1 and vimentin. Overexpression of miR-200b in TGF-β1-stimulated IEC-6 cells increased E-cadherin and decreased vimentin through targeting zinc finger E-box binding homeobox 1 and SMAD2 respectively, which prevented TGF-β1-induced EMT. Intestinal fibrosis is a common serious complication of CD. In another study, they demonstrated that miR-200b could partially protect intestinal epithelial cells from fibrogenesis by suppressing EMT *in vitro*[60]. In summary, miR-200b played a potential role in maintaining intact of intestinal epithelium through inhibiting EMT and improving pathophysiology and clinical outcomes of IBD.

## MIRNAS REGULATE COLONIC EPITHELIAL CELL-DERIVED CHEMOKINE EXPRESSION

The expression of intestinal epithelial-derived CXC and CC chemokines is increased in IBD[61]. Huang *et al*[62] found up-regulated level of miR-141 was inversely correlated with CXCL12β in the epithelial cells of the inflamed colon tissues from CD patients and mice with experimental colitis.They further demonstrated that miR-141 directly regulated CXCL12β expression and leukocyte migration

mediated by CXCL12β. Additionally, overexpression or knockdown of miR-141 in the colon of mice with experimental colitis regulated leukocyte infiltration and alleviated or aggravated intestinal inflammation, respectively. Wu *et al*[63] found miR-192 was decreased in active UC and demonstrated an inverse relationship between miR-192 and MIP-2 (CXCL2).

## MIRNAS REGULATE AUTOPHAGY

Autophagy, which is involved in recycling cellular organelles for the survival of cell, is one mechanism for maintaining cellular hemostasis. Autophagy in the intestinal epithelium is considered to behave as a defensive strategy for clearance of intracellular microorganisms, and the impairment of autophagy results in intestinal epithelial dysfunction and contributes to IBD pathogenesis[64]. *ATG16L1* and *IRGM*, two genes associated with autophagy, have been identified as CD susceptibility genes by genome-wide association studies[65,66]. Some studies showed that miRNA-mediated change in the expression of autophagy gene may result in autophagy dysfunction and involve in the pathogenesis of IBD.

Lu *et al*[67] found that silencing of Dicer1 enhanced autophagy-related gene (ATG) protein levels and autophagosome formation in cells, indicating that miRNAs may be implicated in the regulation of autophagy. MiR-106b and miR-93, which target ATG16L1, both reduced levels of autophagy in epithelial cells. MiR-106b could also inhibit autophagy-dependent clearance of CD-associated adherent-invasive Escherichia col (AIEC) in epithelial cells. Inflamed mucosae from subjects with active CD exhibited more overexpressed miR-106b and lower expression of ATG16L1 when compared with controls. These results suggested that CD patients with miR-106b and miR-93 mediated down-regulation of ATG16L1 expression might manifest an altered antibacterial activity of CD-associated intracellular bacteria in epithelial cells and subsequently affected the outcome of intestinal inflammation. Similarly, Zhai *et al*[68] showed miR-106b targeted ATG16L1 and modulated autophagic activity in HCT116 cells. Their results further indicated that miR-106a and miR-106b could influence the expression of other autophagy-related genes and had a widespread modulating effect on the autophagy pathway.

Nguyen *et al*[69] proved miR-30c and miR-130a directly regulated the expression of ATG5 and ATG16L1, respectively, by targeting their 3'UTRs. They found miR-30c and miR-130a expression were increased and ATG5 and ATG16L1 mRNA expression were decreased in non-inflamed or inflamed ileal CD biopsy specimens compared with normal controls. Similarly, the expression of miR-30c and miR-130a were inversely correlated with ATG5 and ATG16L1 in intestinal epithelial T84 cells infected with the AIEC. NF-κB pathway was activated in AIEC infected T84 cells, which induced the up-regulation of miR-30c and miR-130a and consequently inhibited the expression of ATG5 and ATG16L1. The inhibition of autophagic activity by miR-30c and miR-130a increased AIEC persistence within T84 cells and enhanced pro-inflammatory cytokines production. Furthermore, they demonstrated inhibition of miR-30c and miR-130a *in vivo* suppressed AIEC-induced down-regulation of ATG5 and ATG16L1 expression and increased autophagic activity, leading to more efficient intracellular bacteria clearance and decreased inflammation.

Brest *et al*[70] demonstrated that the association of IRGM with CD arised from a miRNA-based alteration in IRGM regulation which led to the deregulation of autophagic efficacy. They found a synonymous variant in IRGM (c.313C > T), which was classified as non-causative before, altered a binging site for miR-196. MiR-196, was overexpressed in the inflammatory intestinal epithelia of patients with CD and down-regulated the IRGM protective variant (c.313C) but not the risk-associated allele (c.313T). Subsequent deregulation of IRGM-dependent autophagy compromised control of intracellular replication of CD-associated AIEC and affected the outcome of intestinal inflammation.

## MIRNAS ASSOCIATION WITH IBD CARCINOGENESIS

The development of IBD-associated dysplasia and colorectal cancer represents a major complication in patients with IBD[71,72]. The important role miRNAs played in carcinogenesis is becoming clearer because miRNAs have been referred to the regulation of cancer-related cellular processes, including differentiation, apoptosis, cell cycle progression and immune function[10]. Growing evidence implicated that miRNAs are also involved in IBD-associated carcinogenesis.

Ludwig *et al*[73] showed up-regulated level of miR-21 in IBD-associated dysplastic lesions compared to active IBD patients, which was inversely correlated with the expression of PDCD4, a newly characterized tumor suppressor gene. Olaru *et al*[74,75] found expressions of miR-224 and miR-31 increased successively at each stage of IBD progression from non-inflamed to inflamed non-neoplastic, dysplastic and finally cancerous mucosae. MiR-224 and miR-31 levels could accurately discriminate normal or chronically inflamed IBD tissues from cancers. They further identified miR-224 regulated cell cycle through targeting p21 and miR-31 regulated tumor angiogenesis by targeting factor inhibiting hypoxia inducible factor 1, both of which subsequently participated in IBD-associated carcinogenesis.

## FUTURE PERSPECTIVE IN IBD DIAGNOSTIC AND TREATMENT

Investigations described above showed that special miRNAs suppressing functional targets played a pro-inflammatory or anti-inflammatory role in regulating the pathogenic mechanism of IBD, including activation of NFκB, increased intestinal epithelial permeability, abnormal autophagic activity and so on. It means inflam-

matory response, intestinal epithelial barrier and other mechanisms involved in IBD can be regulated by targeting miRNAs, indicating the potential of miRNAs as therapeutic targets for IBD. Besides studying the function of IBD-associated miRNAs *in vitro*, some researchers had administrated miRNAs into mice with experimental colitis by different methods to investigate their functional and therapeutic effect *in vivo*. Inhibition of miR-30c and miR-130a in mice by ileal loop assay suppressed AIEC-induced down-regulation of ATG5 and ATG16L1 expression and decreased intestinal inflammation[69]. Overexpression of miR-146b in DSS-induced colitis mouse *via* intraperitoneal injection relieved intestinal inflammation and increased the survival rate of mouse[40]. MiR-141 intracolonic administration in the colon of TNBS-induced and IL-10 KO mice regulated leukocyte infiltration and alleviated intestinal inflammation[62]. These data showed the effective ways to administrate miRNAs into human and the possibilities for the future clinical applications of miRNA-based therapeutic approaches in IBD.

There have been several studies that identified altered miRNA profiles in both serum and inflamed tissue in patients with UC and CD compared with controls, which have been reviewed by Coskun *et al*[76]. Circulating miRNAs in serum exist in membrane vesicles, such as exosomes[77], or form a complex with lipid protein carriers, such as high-density lipoproteins (HDL)[78]. So these circulating miRNAs are protected from blood RNAses and relatively stable compared with mRNA and protein, which make themselves serving as ideal noninvasive blood biomarkers in patients with IBD. In addition, the aberrant expression of miRNAs in inflamed tissues of patients with UC could also help in IBD diagnosis.

## CONCLUSION

MiRNAs are a class of potential gene regulators of critical importance in the pathogenesis of IBD. It has been demonstrated that miRNAs have the possibility to be used as biomarkers and therapeutic target in IBD. Although our knowledge about the miRNAs regulation of IBD has considerably advanced over the last several years, multiple areas warrant future investigation. Most studies have focused on one miRNA which targets a single mRNA. One area worth future investigation is a key miRNA targeting multiple mRNAs or several miRNAs combination targeting a key mRNA. The other area worth future investigation focuses on the roles of miRNAs in human studies. Most of our understanding of the functions of miRNAs associated with IBD is based on cell cultures and murine models. Further investigating the roles of miRNAs in the human context will improve our knowledge of miRNAs in the pathogenesis and diagnosis of IBD and pave the way for miRNA-based therapies.

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TOPIC HIGHLIGHT

WJGP 5th Anniversary Special Issues (3): Pancreatitis

# Review of the diagnosis, classification and management of autoimmune pancreatitis

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## Abstract

Autoimmune pancreatitis (AIP) is a rare form of chronic pancreatitis, with as yet undetermined incidence and prevalence in the general population. Our understanding of it continues to evolve. In the last few years, 2 separate subtypes have been identified: type 1 AIP has been recognised as the pancreatic manifestation of a multiorgan disease, named immunoglobulin G4 (IgG4)-related disease while type 2 AIP is a pancreas specific disorder not associated with IgG4. International criteria for the diagnosis of AIP have been defined: the HISORt criteria from the Mayo clinic, the Japan consensus criteria and, most recently, the international association of pancreatology "International Consensus Diagnostic Criteria". Despite this, in clinical practice it can still be very difficult to confirm the diagnosis and differentiate AIP from a pancreatic cancer. There are no large studies into the long-term prognosis and management of relapses of AIP, and there is even less information at present regarding the Type 2 AIP subtype. Further studies are necessary to clarify the pathogenesis, treatment and long-term outcomes of this disease. Critically for clinicians, making the correct diagnosis and differentiating the disease from pancreatic cancer is of the utmost importance and the greatest challenge.



**Key words:** Pancreatitis; Autoimmunity; Pancreatic cancer; Autoimmune pancreatitis; Immunoglobulin G4-related disease

**Core tip:** Type 1 autoimmune pancreatitis (AIP) is the pancreatic manifestation of a multiorgan disease, named immunoglobulin G4 (IgG4)-related disease while type 2 AIP is a pancreas specific disorder not associated with IgG4. Making the correct diagnosis and differentiating the disease from pancreatic cancer is of the utmost importance; an agreed diagnostic pathway should be in place and a multidisciplinary approach taken with each patient.

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## INTRODUCTION

As early as 1961, Sarles *et al*[1] described a form of idiopathic chronic pancreatitis with obstructive jaundice and hypergammaglobulinaemia, with the suspicion that there was an underlying autoimmune process. It was not until

1995, when Yoshida *et al*[2] coined the term "autoimmune pancreatitis" (AIP) that this concept was widely accepted and AIP differentiated from other forms of chronic pancreatitis. Since then, progress has been made in our understanding of the pathophysiology of AIP; type 1 AIP has been recognised as the pancreatic manifestation of a multiorgan disease, named IgG4-related disease, while type 2 AIP is a pancreas specific disorder not associated with IgG4[3,4]. This review gives an overview of current thinking on the pathology of AIP, its clinical features (including serology), classification and treatment. Emphasis is placed upon the diagnostic challenge of distinguishing AIP from pancreatic cancer.

## SEARCH STRATEGY

This review of the English language literature on the classification, diagnosis and management of autoimmune pancreatitis is based on papers contained within the PubMed database. Individual searches of the PubMed database were performed with the boolean operator AND, using the terms: "Autoimmune pancreatitis", "Acute pancreatitis", "Chronic pancreatitis", "Pancreatic cancer". The abstracts were screened for eligibility and all relevant publications were requested as full-text articles. References used in requested papers were then checked for any further studies of potential interest.

## PATHOPHYSIOLOGY OF AIP

A definitive autoantigen for AIP has not yet been identified. Human leucocyte antigen (HLA) association studies in Japan have reported an association with HLA serotypes DRB1*0405 and DQB1*0401[5]. This was not confirmed in a Korean study but DQβ1-57 without aspartic acid was associated with disease relapse[6]. Single nucleotide polymorphisms identified in association with either disease susceptibility or recurrence include: cytotoxic T-lymphocyte associated antigen 4, tumour necrosis factor-α and Fc receptor-like 3[7]. However, studies of genetic risk factors in AIP remain at an early stage of investigation. A genome-wide association study in AIP would likely advance our understanding significantly.

Potential initiating mechanisms include bacterial infection and molecular mimicry[7]. Substantial homology exists between human carbonic anhydrase Ⅱ and the α-carbonic anhydrase of Helicobacter pylori[8]. In theory, antibodies directed against bacterial components could behave as autoantibodies by means of molecular mimicry in genetically predisposed persons[7]. Thus, autoimmunity is widely regarded as the initial stimulus for the Th2-cell immune response associated with AIP. Antibodies directed against potential autoantigens, such as carbonic anhydrase, lactoferrin, trypsinogen and pancreatic secretory trypsin inhibitor, may give rise to the systemic manifestations of AIP[7-11].

Studies using animal models of experimental autoimmune pancreatitis have significant limitations, as the disease does not occur spontaneously. Current models exhibit considerable variation in target antigens, differing methods for immune staining and differing mouse strains but have provided evidence that the disease is most likely T cell mediated, with highly beneficial effects observed with agents such as the mammalian target of rapamycin (mTOR) inhibitor, sirolimus, which increases the number and activity of regulatory T-cells[4].

## SUBTYPES OF AUTOIMMUNE PANCREATITIS

### Type 1

This is the more classically described and recognised form of the disease. It is now recognised as a pancreatic manifestation of an immunoglobulin G4 (IgG4) related systemic disease[4,7,12-14]. It is associated with histological findings of a lymphoplasmacytic sclerosing pancreatitis (LPSP). This consists of a dense lymphoplasmacytic infiltration and fibrosis involving the pancreatic lobules, ducts and peripancreatic adipose tissue. Storiform or "swirling" fibrosis and obliterative phlebitis are also characteristic features[15-17]. The lymphoplasmacytic infiltrate is also rich in IgG4 positive cells[18]. It is frequently associated with sclerosing extrapancreatic lesions such as sclerosing cholangitis, retroperitoneal fibrosis and sclerosing sialadenitis[13,19-21]. Type 1 AIP tends to affect older males, with 80% of patients being over 50 years of age at the time of presentation. It is also associated with elevation in serum levels of IgG4 in up to 75% of patients[19,20].

The HISORt criteria from the Mayo clinic[22] and the Japanese consensus criteria[23] were mainly produced to facilitate the diagnosis of Type Ⅰ AIP.

### Type 2

This is a relatively recently described form of AIP[3,4]. It has a unique histological pattern, consisting of an idiopathic duct-centric pancreatitis or AIP with a granulocytic epithelial lesion. The inflammation is centred on the exocrine pancreatic system, with neutrophilic infiltration within the lumen and epithelium of the interlobular ducts being a characteristic feature. The neutrophils are sometimes so numerous that microabscesses can be seen in the lobules and ducts. The entire wall of the duct may be infiltrated by neutrophils and plasma cells. The infiltrate frequently involves the duct epithelium and can obliterate it. It differs from LPSP in that there is little obliterative phlebitis and the inflammatory infiltrates have few IgG4 positive cells[24,25].

Much less is known regarding the clinical features of Type 2 AIP. However it appears to be associated with a younger subset of patients and there is no gender preponderance. There also appears to be an association with ulcerative colitis. Type 2 AIP patients usually have a dramatic response to steroid therapy, associated with a low frequency of relapse[25]. Until recently, existing criteria have not been that helpful in the diagnosis of type 2 AIP, but with recent publication of the International Association of Pancreatology (IAP) diagnostic guide-

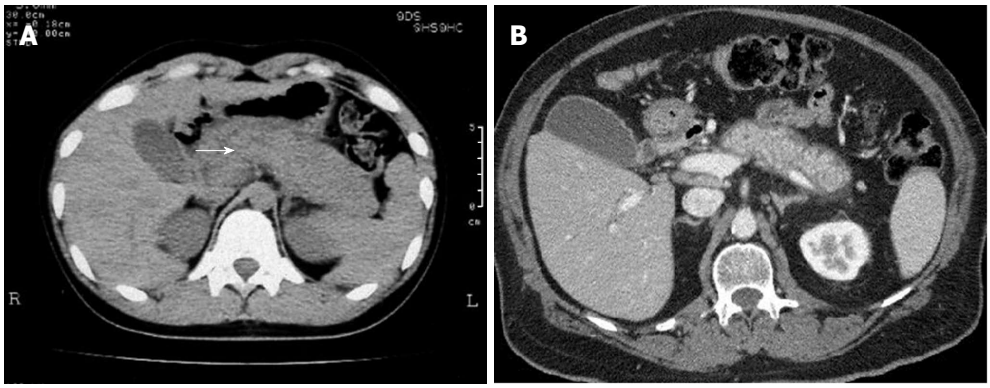


**Figure 1 Computed tomography.** A: Computed tomography (CT) findings in autoimmune pancreatitis: Showing diffuse enlargement and a "sausage like" appearance of the pancreas (arrow); B: Axial contrast enhanced CT image demonstrating a characteristic low signal rim or halo surrounding the body and tail of the pancreas in another patient with autoimmune pancreatitis.

lines[26], it is anticipated that more data will confirm and further characterise this subtype.

Variation in the geographic distribution of the two subtypes may help to explain the heterogeneity of disease morphology observed worldwide.

## CLINICAL PRESENTATION

The presentation of AIP is varied, but a classical picture is obstructive jaundice, often painless or with mild epigastric pain. Less commonly, new onset diabetes or symptoms of pancreatic insufficiency and weight loss may occur. A rarer presentation is acute pancreatitis and its sequelae. A characteristic feature of type 1 AIP is extrapancreatic other organ involvement. In Type 1 AIP the majority are male and over the age of 50. Some patients are only diagnosed post-operatively, having had a resection for a presumed pancreatic cancer.

The clinical picture in Type 2 autoimmune pancreatitis appears to affect a younger cohort of patients, more likely in their 4$^{th}$ decade of life and there is no gender preponderance. There are more reports of this group presenting with acute pancreatitis, and a higher frequency of association with ulcerative colitis[25]. However, the numbers of patients reported in the worldwide literature are still very small and further clarity is expected to emerge with time, to further define this subgroup.

## SEROLOGY

Type 1 AIP is associated with a number of serological abnormalities, in particular an elevated IgG4[18,19]. Hamano *et al*[19] reported that a cut-off value of 135 mg/dL for serum IgG4 concentration differentiates AIP from pancreatic cancer with an accuracy of 97%, a sensitivity of 95% and specificity of 97%. An elevated IgG4 is however not diagnostic of Type 1 AIP, but is a characteristic along with other identified criteria. The Mayo clinic reported a sensitivity, specificity and positive predictive value of 76%, 93% and 36% respectively, using a cut-off value for IgG4 of 140 mg/dL[27]. Elevated IgG4 levels also may be found in PSC, acute and chronic pancreatitis and up to 10% of patients with pancreatic cancer[19]. Serum IgG4 of more than 2 times the upper limit of normal greatly increases the specificity for AIP.

Other elevated markers may include: rheumatoid factor, carbonic anhydrase, antilactoferrin and antinuclear antibodies[9,10]. A study from Frulloni *et al*[28] in Italy identified an anti plasminogen-binding peptide antibody which was elevated in 94% of their AIP patients. In this cohort of AIP patients, they had a relatively low prevalence of elevated IgG4 (at only 54%). This was a single centre study of 20 patients and clearly more studies are needed to assess this and other autoantibodies as potential markers for AIP and as aids to distinguish AIP from pancreatic malignancy.

## IMAGING

Imaging is essential in establishing a diagnosis of AIP. Three different forms of the disease process can be seen, including diffuse, focal or multifocal disease, with the diffuse form being the most common. A contrast enhanced computed tomography (CT) scan is the gold standard for investigation as it is essential to look for a pancreatic malignancy and evidence of metastatic disease. Figure 1A shows the contrast enhanced CT findings characteristic of Type 1 AIP: a diffusely enlarged or "sausage shaped" pancreas with loss of the normal pancreatic clefts and delayed and peripheral rim enhancement[29]. Figure 1B shows a characteristic surrounding hypoattenuating/low signal rim or halo on CT. Generally there is minimal associated peripancreatic soft tissue stranding and rarely inflammation of the mesentery. Local peripancreatic lymphadenopathy can be observed. Pancreatic calcification and pseudocyst formation is not a recognised typical finding in autoimmune pancreatitis. CT may also find extra pancreatic lesions such as retroperitoneal fibrosis.

The focal form of the disease is less common and is characterized by a focal mass lesion within the pancreas and can be mistaken for pancreatic malignancy (Figure 2). Normally dilatation of the pancreatic duct is less marked in autoimmune pancreatitis than that associated with pancreatic malignancy. Typically the main pancre-

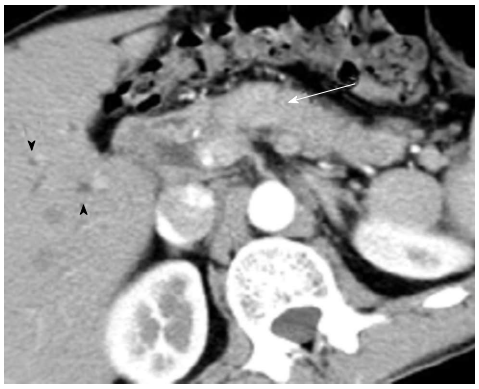

**Figure 2 Focal enlargement of the pancreatic parenchyma in the head of the pancreas (arrow), and dilatation of the intrahepatic bile ducts visible (arrowheads).**

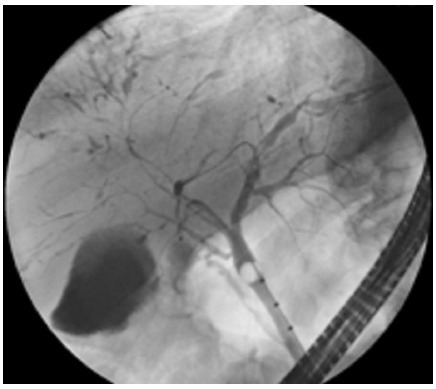

**Figure 3 Endoscopic retrograde cholangiopancreatography findings of multiple and focal strictures and dilatation in the intrahepatic bile ducts in autoimmune pancreatitis.**

atic duct is irregularly narrowed in affected segments of the pancreas. In the multifocal form of the disease, the pancreatic duct is of normal calibre in non affected segments. Magnetic resonance imaging (MRI) shows diffuse or localised enlargement of the pancreas with lower density in T1 weighted images and higher density in T2 weighted images compared with each of the liver images.

Sclerosing cholangitis is observed in a proportion of patients with autoimmune pancreatitis and can be seen in isolation. The intrapancreatic portion of the common bile duct is the most affected segment of the biliary tree. Affected segments of the biliary tree demonstrate irregular stricturing and associated contrast enhancement. Generally strictures associated with autoimmune disease are long and continuous whereas multifocal short strictures are more typical of primary sclerosing cholangitis (PSC), although differentiation between the two can be difficult in some cases (Figure 3).

Endoscopic ultrasound (EUS) is being used more frequently for pancreatic core biopsies, which acts as an aide to histological diagnosis and is likely superior to fine needle aspiration (FNA)[30]. Typical EUS findings in AIP include: diffuse hypoechoic spots, absence of a discreet mass and chronic inflammatory cells on aspiration cytology. Mizuno *et al*[30] and Levy *et al*[31] have demonstrated the benefits of the use of EUS-guided biopsies to aid in the diagnosis of AIP[32]. Future refinement of diagnosis may be obtained with the use of contrast-enhanced EUS and elastography[4]. The use of positron emission tomography (PET) and its potential role for diagnosis of AIP is yet to be validated[33].

## OTHER ORGAN INVOLVEMENT

In Type 1 AIP, which may be considered part of an IgG4 systemic disease process, there are a significant number of associated extrapancreatic lesions. The most common are: hilar lymphadenopathy, sclerosing cholangitis, retroperitoneal fibrosis, salivary and lacrimal gland involvement and tubulointerstitial nephritis[21,22,34-37]. There are other conditions that have been less frequently reported, such as hypophysitis and chronic thyroiditis. It is this link to other organ involvement that led clinicians to consider AIP as part of a systemic IgG4 related disease, analogous to sarcoidosis, another systemic disease in which diverse organ manifestations are linked by the same histopathological characteristics[7].

Biliary disease is one of the most common extrapancreatic manifestations of AIP. Although the main cause of jaundice in AIP is obstruction at the level of the intrapancreatic portion of the common bile duct, associated with an inflammatory pancreatic head mass, stricturing in the rest of the biliary tree is increasingly recognised. This condition has been termed IgG4-associated cholangitis (IAC) and has been reported to occur in 20%-88% of cases of AIP[38]. A possible overlap between IAC and PSC is also suggested by the finding that 9%-36% of patients with PSC have increased serum IgG4 levels, compared with less than 1% in other liver diseases[39,40]. Of note, PSC patients with raised serum IgG4 levels have a more rapid progression to liver transplantation compared to those with normal levels[38].

Extrapancreatic disease can be a useful factor in the diagnosis of autoimmune pancreatitis, distinguishing it from pancreatic cancer, and forms part of the HISORt criteria. It also provides collateral evidence for AIP, according to the IAP diagnostic guidelines. The evidence to support the association between these conditions and AIP include: multiple reports indicating frequent or intimate concurrence, extrapancreatic pathological findings of severe lymphoplastic infiltration and storiform fibrosis with numerous IgG4 positive plasma cell infiltrations and a combined favourable response to steroid therapy[23,26,41].

## DIAGNOSIS OF AIP

There is no single diagnostic test for AIP and there is significant variation in clinical practice worldwide, particularly between Asia and North America/Europe. The biggest challenge associated with the diagnosis of AIP is that it can closely resemble pancreatic cancer. Most commonly AIP presents with obstructive jaundice and pancreatic enlargement; other worrying symptoms such as

**Table 1 The Mayo clinic HISORt criteria for the diagnosis of autoimmune pancreatitis**

| Category | Criteria |
|---|---|
| Histology | One of the following:<br>Periductal lymphoplasmacytic infiltrate with obliterative phlebitis and storiform fibrosis (LPSP)<br>Lymphoplasmacytic infiltrate with storiform fibrosis showing abundant IgG4 positive cells (> 10 cells/HPF) |
| Imaging (CT)/(MRI) | Typical; diffusely enlarged gland with diffuse rim enhancement, diffusely irregular attenuated pancreatic duct<br>Other; focal pancreatic mass or enlargement; focal pancreatic duct stricture; pancreatic duct stricture, pancreatic atrophy; pancreatic calcification or pancreatitis |
| Serology | Elevated serum IgG4 level |
| Other organ involvement | Hilar/intrahepatic biliary strictures, persistent distal biliary strictures, parotid or lacrimal gland involvement, mediastinal lymphadenopathy or retroperitoneal fibrosis |
| Response to steroid therapy | Resolution/Marked improvement of pancreatic or extrapancreatic manifestion with steroid therapy |

LPSP: Lymphoplasmacytic sclerosing pancreatitis; CT: Computed tomography; MRI: Magnetic resonance imaging; IgG4: Immunoglobulin G4; HPF: High powered field.

weight loss and new onset diabetes may also be present. Less commonly AIP can present with features of acute pancreatitis or unexplained pancreatic insufficiency. Misdiagnosis at this stage has the potential to be catastrophic, as an undiagnosed cancer may cause delay or loss of the opportunity for potential curative cancer surgery. The opposite scenario of a pancreatoduodenectomy being undertaken for benign disease (with its high risk of morbidity and mortality) is also unsatisfactory.

In 2002 the Japan Pancreas Society published guidelines for diagnosis of AIP. These were updated in 2006 and again in 2009. The HISORt criteria from the Mayo[22] clinic require histology, imaging, serology, other organ involvement and response to therapy for diagnosis. The inclusion of response to steroids as part of the diagnosis is one of the criteria that differentiates the Mayo recommendations from the Japanese. In Japan, endoscopic retrograde pancreatography (ERP) is routinely performed to aid in the diagnosis of AIP. More recently, the IAP has published their International consensus diagnostic criteria (ICDC)[26], in an attempt to bridge the divide in clinical practise around the globe and offers criteria for the diagnosis of both subtypes of AIP.

## SUMMARY OF DIAGNOSTIC CRITERIA

Guidelines regarding diagnostic criteria vary worldwide. Although criteria have been developed by other groups, the most influential come from the United States[22], Japan[23] and the International Association of Pancreatology[26]. Below are the definitions from these three different groups.

### *Japan/Asian*

In 2002 the Japan Pancreas society published their data for the diagnosis of AIP; this was further revised in 2006. In 2009 Okazaki *et al*[23] published the Japanese consensus guidelines for management of autoimmune pancreatitis. There are 3 main criteria. For the diagnosis to be confirmed, criterion 1 must be present along with criterion 2 and/or criterion 3.

**Imaging:** Diffuse or segmental narrowing of the main pancreatic duct with irregular wall and diffuse or segmental enlargement of the pancreas with imaging studies such as: Ultrasound, CT, MRI or ERP.

**Serology:** High serum gammaglobulin IgG or IgG4, or the presence of autoantibodies, such as antinuclear antibodies or rheumatoid factor.

**Histology:** Marked inter-lobular fibrosis and prominent infiltration of lymphocytes and plasma cells in the periductal area, occasionally with lymphoid follicles in the pancreas.

There is an optional criterion for patients fulfilling criterion 1 alone: a response to steroid therapy, with the caveat that malignancy of the pancreas or biliary tract must be excluded. In 2006, a mandatory ERP became part of these guidelines.

### *United States*

The Mayo clinic HISORt criteria are based on 5 main diagnostic criteria: histological findings, imaging, serology, other organ involvement and response to steroid therapy[22,42]. The detailed features are listed in Table 1. Essentially, use of these criteria enable patients to be categorised into three diagnostic groups [diagnostic pancreatic histology, typical imaging and serology, steroid responders (after careful work-up to exclude cancer)]. Patients in one or more of these categories are deemed to have AIP.

### *International association of pancreatology*

The goals of the IAP were to develop international consensus on the diagnostic criteria that can be applied worldwide, to safely diagnose AIP and to avoid a misdiagnosis of pancreatic cancer[26]. They reviewed all existing criteria, including the Japanese and HISORt. The consensus opinion was that the terms type 1 and type 2 should be used to describe the clinical profiles associated with LPSP and idiopathic duct-centric pancreatitis, respectively. Tables 2-4 shows the diagnostic criteria for definitive and probable AIP type 1 and 2. This uses a combination of 1 or more of 5 cardinal features of AIP: (1) imaging features of the following: pancreatic parenchyma (on

**Table 2 International consensus diagnostic criteria for type 1 autoimmune pancreatitis**

| | Diagnosis of type 1 AIP | | |
|---|---|---|---|
| **Diagnosis** | **Cardinal feature** | **Imaging evidence** | **Collateral evidence** |
| Definitive type 1 | Histology | Typical/indeterminate | Confirmed LPSP |
| | Imaging | Typical<br>Inderminate | Any level 1/2<br>⩾ 2 level 1 |
| | Steroid response | Indeterminate | Level 1 S/OOI and Rt OR Level 1 D and level 2 S/OOI/H and Rt |
| Probable type 1 | | Indeterminate | Level 2 S/OOI/H and Rt |

LPSP: Lymphoplasmacytic sclerosing pancreatitis; AIP: Autoimmune pancreatitis; S: Serology; OOI: Other organ involvement; Rt: Response to steroid therapy; H: Histology

CT/MRI) and pancreatic duct [ERCP or magnetic resonance cholangiopancreatography (MRCP)]; (2) serology (IgG, IgG4 and antinuclear antibody); (3) other organ involvement (OOI); (4) histopathology of the pancreas; and (5) response to steroid therapy.

Level 1 and level 2 criteria are then specified, according to the strength that specific findings add to the likelihood of diagnosis. For example, a greater than 2-fold elevation of IgG4 is considered a level 1 criteria; a lesser elevation level 2. Further specification is given for pancreatic ductal and parenchymal appearances, histology and response to steroids. Thus, definite and probable type 1 and type 2 AIP can be diagnosed.

In all cases the criteria are geared towards excluding a diagnosis of pancreatic cancer rather than screening for AIP, *i.e.,* they emphasise specificity rather than sensitivity. Only the IAP guidelines include the diagnostic features of Type 2 autoimmune pancreatitis.

## DISTINGUISHING AIP FROM PANCREATIC CANCER

In view of its presentation with obstructive jaundice and pancreatic enlargement, AIP often needs to be distinguished from pancreatic cancer. As ERCP features have been reported to have limited sensitivity to diagnose AIP in Western centres, Figure 4 shows a strategy to aid in differentiation, diagnosis and management of AIP versus pancreatic cancer, based upon the experience and algorithm of the Mayo Clinic[22]. When features highly suggestive of either AIP or pancreatic cancer are present (a low-density mass, pancreatic ductal dilatation, pancreatic duct cut off, upstream pancreatic atrophy or liver lesions suggestive of metastases), the diagnostic and management pathway is usually clear. However, in indeterminate cases, further cancer work-up is required in the first instance. In the event of a negative cancer work-up, a pancreatic core biopsy is helpful in categorising patients if a positive diagnosis can be made. Equivocal or inadequate results are more problematic and a trial of steroids or surgery should be considered.

**Table 3 International consensus diagnostic criteria for type 2 autoimmune pancreatitis**

| | Diagnosis of type 2 AIP | |
|---|---|---|
| **Diagnosis** | **Imaging evidence** | **Collateral evidence** |
| Definitive type 2 | Typical/indeterminate | Histologically confirmed or clinical inflammatory bowel disease and level 2H and Rt |
| Probable type 2 | Typical/indeterminate | Level 2 H/clinical inflammatory bowel disease and Rt |

AIP: Autoimmune pancreatitis; Rt: Response to steroid therapy; H: Histology.

Using the Mayo Clinic strategy, AIP was successfully distinguished from pancreatic cancer in most patients but 27% required a pancreatic core biopsy, steroid trial or surgery to clarify the diagnosis[43]. Kamisawa *et al*[44] have reported their Japanese strategy when investigating patients presenting with mass lesions. Strategies based upon the Japanese criteria can be simpler but rely on ERP. Despite this, surgery was still required to make a diagnosis in 6 of 37 (16%) patients. Further evaluation and comparison is required to determine the optimal and least invasive diagnostic pathway.

In our view, when distinguishing AIP from pancreatic cancer, the most important tips or principals of diagnosis include the following: (1) clinical presentations not suggestive of AIP include marked cachexia, anorexia and severe pain requiring opiates; (2) a thorough negative work up for other aetiologies should be undertaken, in particular for pancreatic or biliary cancer; (3) histological diagnosis of AIP requires preservation of tissue architecture (showing lymphoplasmacytic infiltrate with >10 IgG4 positive cells/high power field), which renders FNA less helpful for diagnosis; (4) steroid therapy should only be commenced when other aetiologies for pancreatic disease have been excluded, and only in those patients whose response may be adequately assessed. It should not be used as a substitute for a thorough search for the aetiology; (5) objective improvement in the appearance of the pancreas on cross-sectional imaging should be evident within 2 wk of steroid use. Subjective improvement in symptoms or even a decline in serum IgG4 levels can occur in pancreatic cancer or lymphoma and should not be used as response criteria; (6) in AIP, CA 19-9 levels drop with treatment; a rising CA 19-9 suggests this diagnosis is incorrect; and (7) the diagnosis of AIP is difficult. An agreed diagnostic pathway should be in place and a multidisciplinary approach taken with each patient, to ensure that pancreatic cancer patients are not treated with steroids and, conversely, AIP patients not treated with cancer surgery.

## INITIAL TREATMENT, MAINTENANCE AND RELAPSE

Although it is well established that spontaneous resolution can occur in up to 30% of cases of AIP[45], symptomatic patients are best treated with corticosteroids (*i.e.*,

**Table 4 International consensus diagnostic criteria level 1 and 2 criteria for type 1 and 2 autoimmune pancreatitis**

| | Type 1 AIP | |
|---|---|---|
| **Criterion** | **Level 1** | **Level 2** |
| Parenchymal imaging | Typical: Diffuse enlargement with delayed enhancement | Indeterminate: Focal enlargement with delayed enhancement |
| Ductal imaging (ERP) | Long or multiple strictures (> 1/3 duct length) without upstream dilatation | Focal narrowing without upstream dilatation (< 5 mm) |
| Serology | IgG4 > 2x upper limit | IgG4 1-2x upper limit |
| Other organ involvement | Extrapancreatic organ histology. Any 3 of :<br>1 Lymphoplasmacytic infiltration with fibrosis and without granulocytic infiltration<br>2 Storiform fibrosis<br>3 Obliterative phlebitis<br>4 > 10 cells/HPF IgG4-positive cells<br>Typical radiology. Any one of:<br>1 Segmental/multiple proximal or distal biliary stricture<br>2 Retroperitoneal fibrosis | Extrapancreatic organ histology including bile duct biopsies. Both of:<br>1 Marked lymphoplasmacytic infiltration without granulocytic infiltration<br>2 10 cells/HPF IgG4-positive cells<br>Physical or radiological evidence of at least one of:<br>1 Enlarged salivary/lachrymal glands<br>2 Renal involvement |
| Histology of pancreas | LPSP and 3 of:<br>1 Periductal lymphoplasmacytic infiltrate without granulocytic infiltration<br>2 Obliterative phlebitis<br>3 Storiform fibrosis<br>4 > 10 cells/HPF IgG4-positive cells | LPSP and 2 of:<br>1 Periductal lymphoplasmacytic infiltrate without granulocytic infiltration<br>2 Obliterative phlebitis<br>3 Storiform fibrosis<br>4 > 10 cells/HPF IgG4-positive cells |
| Response to steroid (Rt) | Rapid (< 2 wk) radiological demonstration of marked improvement in pancreatic/extrapancreatic manifestations | |
| Type 2 AIP | | |
| Parenchymal imaging | Typical: Diffuse enlargement with delayed enhancement | Indeterminate: Focal enlargement with delayed enhancement |
| Ductal Imaging (ERCP) | Long (> 1/3 duct length) or multiple strictures without upstream dilatation | Focal narrowing without marked upstream dilatation (< 5 mm) |
| Other organ involvement | | Clinically diagnosed inflammatory bowel disease |
| Histology of pancreas | IDCP. Both of:<br>1 Granulocytic infiltration of duct wall with or without acinar inflammation<br>2 0-10 cells/HPF IgG4-positive cells | Both of :<br>1 Granulocytic and lymphoplasmacytic acinar infiltrate<br>2 0-10 cells/HPF IgG4-positive cells |
| Response to steroid (Rt) | Rapid (< 2 wk) radiological demonstration of marked improvement in manifestations | |

LPSP: Lymphoplasmacytic sclerosing pancreatitis; IDCP: Idiopathic duct-centric pancreatitis; AIP: Autoimmune pancreatitis; IgG4: immunoglobulin G4; ERP: Endoscopic retrograde pancreatography; Rt: Response to steroid therapy; HPF: High powered field.

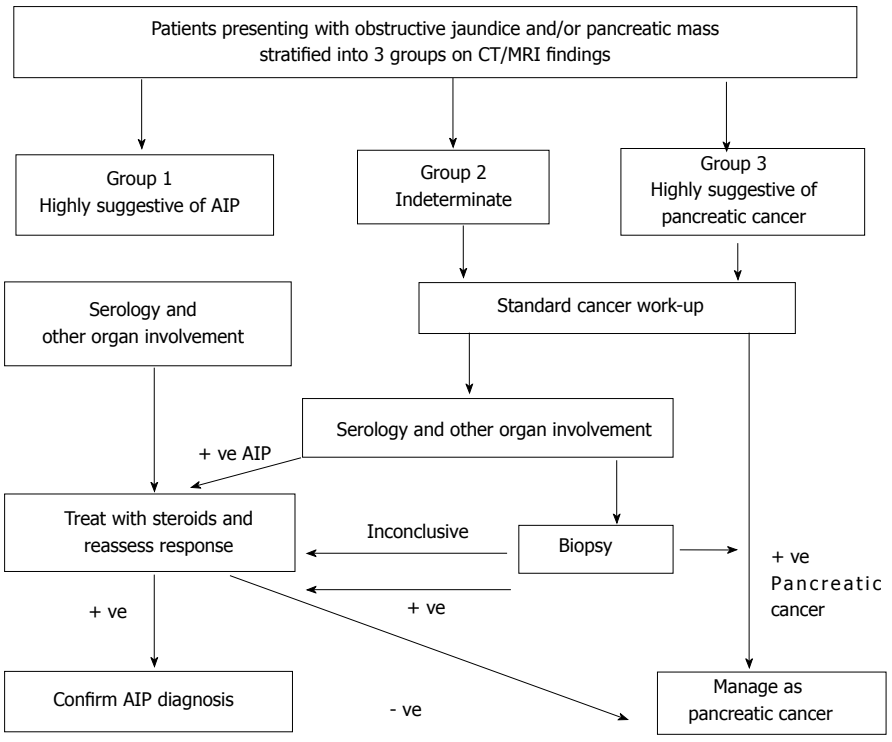


**Figure 4 A strategy for distinguishing autoimmune pancreatitis from pancreatic cancer (based upon the Mayo clinic strategy[23]).** CT: Computed tomography; MRI: Magnetic resonance imaging; AIP: Autoimmune pancreatitis.

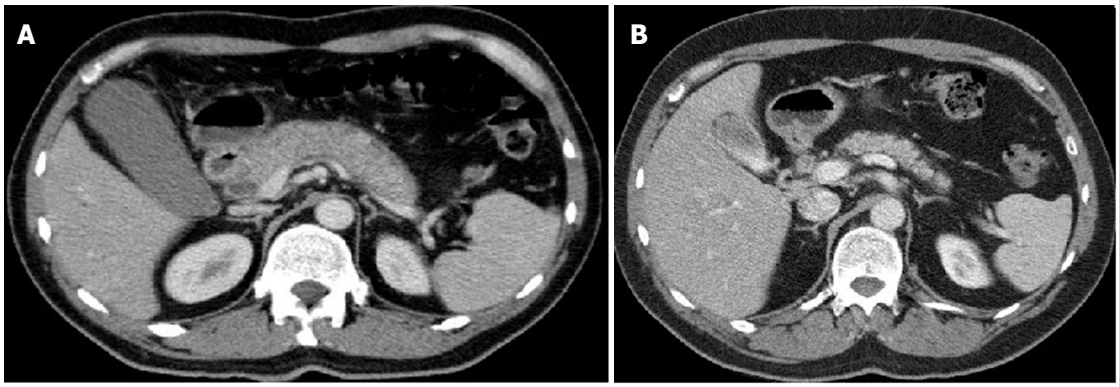


**Figure 5 Axial computed tomography image.** A: Demonstrating a characteristic sausage shaped enlarged pancreas with surrounding halo in keeping with autoimmune pancreatitis; B: From the same patient 8 mo later following corticosteroid therapy demonstrating response to treatment.

prednisolone). A large multicentre retrospective trial from Kamisawa *et al*[46] in 2009 identified 563 patients with AIP and found that 98% responded to steroid therapy versus 74% that improved without. The response can be dramatic. An improvement of imaging findings, with resolution of pancreatic enlargement and biliary stricturing can be seen following corticosteroid treatment in Figure 5.

Initial steroid dose varies slightly according to guideline. In the Mayo clinic a standard initial dose is 40 mg per day of oral prednisolone, for 4 wk. If there is obvious clinical and radiological improvement, the dose is decreased by 5 mg/wk until it is stopped at 11 wk[47]. The Japanese consensus statement on treatment and prognosis of AIP specifies that an initial oral prednisolone dose for induction of remission of 0.6 mg/kg per day is recommended. The initial dose is administered for 2-4 wk and then gradually tapered. The IAP guidelines specify dose of prednisolone of 0.6-1.0 mg/kg per day with reassessment at 2 wk[26]. The study that formed the basis of the IAP consensus guideline regarding the two week reassessment after a trial of steroid treatment was the prospective study of Moon *et al*[40]. After a 2-wk steroid trial, response to steroids was assessed on the basis of a marked improvement in pancreatic duct narrowing, and a reduction in size of the pancreatic mass. All patients who responded to steroids (15/22) were diagnosed as AIP after a median follow-up of 27 mo, whereas all patients who did not respond to steroids (7/22) were diagnosed with pancreatic cancer, with a complete resection being possible in 6/6 patients who accepted surgery. Induction of remission with rituximab, a monoclonal antibody directed against the CD20 antigen on B lymphocytes, is currently under investigation[4, 48].

Differing rates of tapering are also recommended. Chiefly, the distinction is between the 5 mg/wk reduction of prednisolone, after initial treatment versus a more gradual approach recommended by the Japanese. The Japanese consensus document advocates that the dose be tapered by 5 mg every 1-2 wk, after 2-4 wk at the initial dose, based on changes in the clinical manifestations, biochemical blood tests (such as liver enzymes and IgG or IgG4 levels), and repeated imaging findings (US, CT, MRCP, ERCP). The dose is tapered to a maintenance dose over a period of 2-3 mo.

A maintenance dose of 2.5-5.0 mg/d is recommended by the Japanese, to prevent relapse. This is not recommended by the Mayo clinic group, who take the view that the universal use of maintenance therapy is not warranted because the risks of long term steroid use outweighs the benefits[47]. A wide range of relapse rates are reported, from 22%-100%[38]. In the Mayo clinic experience of 78 type 1 AIP patients with a median follow-up of 42 mo, symptomatic disease relapse was seen in 47% patients with a 3-year cumulative relapse rate of 59% in type 1 AIP patients who were medically managed[49]. This wide variation in relapse rates may be due to lack of a uniform definition of disease relapse, short follow-up times, small patient populations, differences in steroid treatment regimens, lack of identification of subtypes and ethnic variation.

Treatment of relapse is effectively achieved with corticosteroids. The Japanese consensus guideline states that remission can be obtained with the same prednisolone dose as the initial dose in most relapsed AIP cases, but that it may be necessary to taper more gradually[50]. In Europe and the United States, azathioprine has often been introduced for the treatment of relapsing disease, despite pancreatitis being a known side-effect of azathioprine. Acute pancreatitis occurs in approximately 2% of cases of azathioprine use, but there is no evidence as yet that this risk is increased in AIP. Some advocate that, as in autoimmune hepatitis (AIH), AIP should be managed by azathioprine, with or without low dose steroids for at least three years. This analogy is not completely convincing; in AIH disease relapse is almost universal in those who cease immunosuppression early whereas the relapse rate is much more variable in AIP. Moreover, in a recent study from the Mayo group, in patients with relapsing AIP, azathioprine was not shown to be superior to another course of steroids alone[51].

Related areas of management include: biliary stenting, treatment of endocrine and exocrine failure and consideration of pancreatic cancer risk in AIP. Patients presenting with obstructive jaundice should certainly be considered for biliary stenting at ERCP. This is the Japanese practice[50] as it fits in with their strategy, which

includes endoscopic pancreatography in an intrinsic role among their diagnostic tests. However, resolution of jaundice occurs in AIP with steroid treatment without stenting, and obviously, this avoids the risks of ERCP. Avoiding the morbidity and mortality associated with ERCP and biliary stenting is also increasingly attempted in suspected pancreatic cancer, as routine preoperative biliary drainage in patients undergoing surgery for cancer of the pancreatic head increases the rate of overall complications[52]. Diabetes mellitus is common in AIP and although improvement has been reported upon commencing steroids, often requires treatment with oral hypoglycemic agents or insulin[47]. Similar considerations apply to exocrine pancreatic failure. Patients should receive pancreatic enzyme supplementation if pancreatic exocrine insufficiency is suspected, based on the presence of clinical features such as: diarrhoea, steatorrhoea, weight loss, metabolic bone disease or vitamin or mineral deficiency. There is no established association between AIP and pancreatic cancer, just case reports of both conditions. It is not unreasonable to suppose the AIP shares a similar association with pancreatic cancer as with other forms of chronic pancreatitis, given the florid inflammatory response that may persist and relapse over years. Careful follow up of these patients will provide the definitive answer to this question but in the interim this seems the prudent approach to take.

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TOPIC HIGHLIGHT

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# Alcoholic pancreatitis: A tale of spirits and bacteria

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## Abstract

Alcohol is a major cause of chronic pancreatitis. About 5% of alcoholics will ever suffer from pancreatitis, suggesting that additional co-factors are required to trigger an overt disease. Experimental work has implicated lipopolysaccharide, from gut-derived bacteria, as a potential co-factor of alcoholic pancreatitis. This review discusses the effects of alcohol on the gut flora, the gut barrier, the liver-and the pancreas and proposes potential interventional strategies. A better understanding of the interaction between the gut, the liver and the pancreas may provide valuable insight into the pathophysiology of alcoholic pancreatitis.



**Key words:** Alcohol; Pancreatitis; Fibrosis; Bacteria; Endotoxin; Lipopolysaccharide

**Core tip:** There is now clear clinical and experimental evidence that bacteria and bacterial products (such as endotoxin) are associated with complications of pancreatitis. Furthermore, results of animal studies support the concept that bacterial endotoxin is an important factor in the initiation and progression of alcoholic pancreatitis.

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## INTRODUCTION

Chronic alcohol consumption is a known cause of injury to several organs, most commonly the liver and the pancreas, but also to the heart, lungs and brain. However, it is well understood that only a minority of alcoholics will ever develop clinically overt pancreatic or liver damage and even fewer numbers will develop clinically overt disease in both organs simultaneously although subclinical damage to both organs has been reported to coexist[1]. The fact that only some alcoholics appear to be susceptible to clinical pancreatitis or hepatitis has led to a concerted search for additional trigger/initiating factors for alcohol-induced organ damage.

Over the past two decades clinical and experimental studies have demonstrated that endotoxin lipopolysaccharide (LPS), from the bacterial wall of gram negative bacteria of the human gut, plays a central role in the initiation and progression of alcoholic liver disease[2]. This was initially based on clinical observations of elevated plasma endotoxin concentrations in alcoholics with and without liver disease[3,4]. Experimental evidence in support of the association of endotoxin and liver disease in humans was subsequently provided by animal studies demonstrating that alcohol-fed rats challenged with LPS developed hepatic lesions resembling alcoholic hepatitis in humans[5,6].

Conversely, targeted disruption of the LPS receptor toll like receptor 4 (TLR4) in alcohol-fed animals protected against liver injury[7].

Reports of increased endotoxinemia in pancreatitis emerged a decade later. Several studies have linked the degree of endotoxinemia to the severity and prognosis of acute pancreatitis, regardless of its aetiology[8,9] and the impact of endotoxinemia on multiple organ system failure, in particular pancreatitis-associated lung disease has been corroborated by animal studies[10]. However, it remained elusive whether endotoxinemia was a cause or a consequence of pancreatitis, or both. It has only recently been shown that endotoxin initiates pancreatic necro-inflammation in alcohol-fed rodents[11,12] and promotes pancreatic fibrosis[12].

In healthy subjects, small amounts of endotoxin translocate from the gut lumen to the bloodstream and are naturally cleared by the reticulo-endothelial system. Under the influence of alcohol, bacteria proliferate in the small intestine[13,14], intestinal permeability is increased[15,16], while endotoxin clearance by the reticulo-endothelial system-in particular Kupffer cells in the liver - is diminished[17]. As a result, excess endotoxin is available in the blood stream and exerts its harmful effects on various organs.

This review aims to summarise the mechanisms underlying increased endotoxinemia in alcoholics, describes the role of endotoxin both as an initiating and aggravating factor of pancreatitis and attempts to define a role for the liver as a mediator in pancreatic end-organ damage.

## ALCOHOL AND THE GUT FLORA

A human being harbours up to 500 different bacterial species[18], the overall bacterial cell count being 10 times more abundant than the number of eukaryotic cells in the body[19]. The combination of species-which is established during the first year of life and shaped by host genotype[20] as well as dietary factors-varies from individual to individual[21]. Moreover, there is evidence indicating that certain strains of bacteria may be unique to their host[22]. Bacterial concentrations are lowest in the upper gastrointestinal tract due to gastric acid, biliary and pancreatic secretion while the highest density of bacteria is found in the colon. In healthy humans, the gut flora prevents the growth of potential injurious bacteria[18,23], exerts metabolic activities such as the fermentation of non-digestible carbohydrates[24] or vitamin synthesis[25] and plays a role in intestinal cell growth and differentiation[26]. Several factors may influence bacterial luminal content. These include altered gut motility[27], drugs, in particular antibiotics[28] and dietary factors such as alcohol.

Alcohol has been shown to alter the jejunal microflora, since almost 50% of alcoholics with documented recent ethanol abuse displayed an increase in total number of bacteria most of which originated from the faecal flora[13]. These data were confirmed in duodenal juice samples obtained by oesogastroduodenoscopy[14] as well as $H_2$-breath tests, as a surrogate marker of bacterial proliferation in the proximal gut, in alcoholic subjects[29]. The mechanisms underlying bacterial overgrowth in alcoholism are unknown, but reduction of orocaecal transit time observed in chronic alcoholics[30,31] may offer a partial explanation. It is noteworthy, that alcohol gavage in rodents for 10 wk has the capacity to alter the composition of colonic bacteria[32].

Interestingly, certain bacteria of the gut flora have the capacity to metabolise alcohol to acetaldehyde[33,34]. In alcohol-fed rats, ethanol metabolism by colonic bacteria could be suppressed by ciprofloxacin[35] or a combination of ampicillin and neomycin[36]. In a similar animal model, administration of metronidazole increased alcohol dehydrogenase-containing bacteria and hence colonic acetaldehyde content[37]. While acetaldehyde has been measured in the rodent colon[36] and human gut bacteria have the capacity to metabolise ethanol, there is, to date, no report on acetaldehyde content of the human colon in alcoholics. Nonetheless, the above studies suggest that it would not be unreasonable to implicate acetaldehyde, as the compound that mediates most of the toxic effects of ethanol.

## ALCOHOL AND GUT PERMEABILITY

In order for bacteria or bacterial products such as endotoxin to pass into the bloodstream and exert their systemic effects, they are required to cross the gut barrier. In its physiological state, the gut represents an effective barrier, made of a single continuous cell layer from the stomach to the rectum. The cells are sealed together by two sets of highly complex junctions, the more apical tight junction and the adherens junction. Physiologically, tight junctions may allow the passage of small molecules up to a molecular weight of 2000 Da but prevents the translocation of larger molecules, in particular bacterial products or bacteria[38]. In addition to this mechanical barrier, passage of bacteria or bacterial products is prevented by mucus, immunoglobulins, defensins and other antimicrobial products produced by the gut.

Intestinal permeability can be measured non-invasively using oral probes such as ethylene glycol polymers of varying molecular sizes, oligosaccharides (*e.g.*, lactulose), monosaccharides (mannitol) and radiolabeled chelates such as chromium-ethylenediaminetetraacetic acid (Cr-EDTA). All these compounds are poorly absorbed by the normal bowel mucosa and display absent or negligible metabolism. Hence, increased urinary excretion correlates with increased intestinal permeability. It is now acknowledged that the probes are absorbed *via* the paracellular route, implying that competence of the gut barrier depends on the integrity of intercellular junctions[39,40].

Several studies have addressed the question whether alcohol increases gut permeability. Early studies with rats chronically administered alcohol revealed increased permeability to macromolecules such as hemoglobin with a known molecular weight of 17 kDa[41] and horseradish peroxidase with a molecular weight of 44 kDa[42]. Permeability to smaller molecules also appears to be increased in rodents upon ethanol administration as exemplified by

increased lactulose/mannitol ratio. Increased absorption of $^{51}$Cr-EDTA, a small molecule of 340 Da, was also observed in chronic alcoholics[42]. An increase in absorption of a molecule of similar size (PEG 400) was reported when alcohol was administered to volunteers with no history of chronic ethanol abuse[16]. The latter data failed to be confirmed by Parlesak *et al*[43] who did not observe a difference in the absorption of polyethylen glycol (PEG) 400 when chronic alcoholics were compared to healthy subjects. In the same study, however, permeability to larger molecules of polyethylene glycol (PEG 1500, 4000 and 10000) was significantly enhanced and the permeability to PEG 10000 in particular was 10-fold higher in alcoholics. Taken together there is experimental and clinical evidence that gut permeability is enhanced by acute and chronic ethanol administration. Permeability seems to be increased for molecules of higher molecular weight (from 1000 Da to at least 44 kDa), which is of particular relevance to the translocation of gut derived bacterial endotoxin, a large compound with a known molecular weight of 40 kDa, as a putative initiating and aggravating factor of alcohol-induced organ damage.

In order to explain increased gut permeability by alcohol, various morphological and molecular studies have been undertaken. There is evidence that alcohol exerts direct toxic effects on the gut mucosa. In an observational study by Gottfried *et al*[44], seven alcoholic subjects with a previously unremarkable oesogastroduodenoscopy were administered 1 g/kg body weight alcohol (35% w/v). Biopsy specimens taken during oesogastroduodenoscopy performed 3 h after alcohol exposure demonstrated transient focal subepithelial hemorrhage which disappeared within 3 d. These observations were corroborated by experimental data in rodents and dogs[45,46]. Studies of histological alterations in patients chronically abusing alcohol have yielded conflicting results since both histological alterations and normal mucosal structure have been described[47]. This may be related to the fact that alcohol-induced mucosal lesions are short-lived due to rapid regeneration of epithelial cells (in the study reporting normal mucosal structure, endoscopies were performed 3-14 d after alcohol withdrawal). At the molecular level, different effects of ethanol on interepithelial junctions in the gut have been described.

Ethanol at high doses has been reported to lead to increased gut permeability via direct action on tight junctions. Ma *et al*[48] measured epithelial resistance and paracellular permeability of the human adenocarcinoma cell line Caco-2 exposed to ethanol. At ethanol concentration ranging from 1% to 10% a dose-dependent drop in electrical resistance paralleled by an increase in permeability was observed. Ethanol produced a disruption of the tight junction protein ZO-1 as well as disassembly of cytoskeletal proteins such as actin and myosin. These changes proved reversible upon ethanol withdrawal. However, ethanol concentrations of 1% or above are only encountered in the duodenum/jejunum where concentrations of up to 5% have been reported[49], while ethanol concentrations in the ileum and colon tend to be much lower (0.2%-0.25%). This would entail that most of translocation of bacteria or bacterial products occurs in the upper gastrointestinal tract.

As mentioned above, human colonic bacteria have the capacity to metabolise alcohol to acetaldehyde[33,50] *via* bacterial alcohol dehydrogenase. Accordingly, colonic acetaldehyde concentrations in the millimolar range have been observed in rats[51] and piglets[52]. Acetaldehyde concentrations of 0.1-0.6 mmol/L led to a disruption of tight junctions and adherens junction *via* tyrosine phosphorylation of their main components[53].

In summary, there is substantial evidence that alcohol increases gut permeability to large molecules of the size of endotoxin and these effects may be due to a direct toxic effect on the mucosa of the proximal gut as well as molecular modifications at the level of interendothelial junctions. Likewise, acetaldehyde, as a result of alcohol metabolism by colonic bacteria, has the capacity to disrupt epithelial junctions, suggesting that the increased serum endotoxin concentrations observed in alcoholics may also be of colonic origin.

## BACTERIA AND LPS IN PANCREATITIS

In the Western society, alcohol represents 70%-80% of cases of chronic pancreatitis. As stated earlier, experimental evidence suggests that bacterial endotoxin is an initiating factor for alcoholic pancreatitis[11,12]. In addition, bacterial translocation or the passage of bacterial products such as endotoxin into the systemic circulation appears to play a primary role in systemic spread, including multiple organ system failure and prognosis of the disease[54]. While endotoxin may be a key player at both ends of the disease spectrum, *i.e.*, as an initiating and aggravating factor of pancreatitis, the mechanisms leading to its increased presence in the blood may not be the same. In this chapter, both situations will be considered separately. The question as to whether bacteria or bacterial products (LPS) translocate will be addressed first.

Sepsis, a consequence of infected pancreatic necrosis, accounts for up to 80% of deaths in severe acute pancreatitis[55]. The germs most commonly cultured from infected pancreatic necrosis are gram negative bacilli presumably as a result of increased gut permeability[55,56]. Infection of pancreatic necrosis appears to be an early event occurring within a week after initiation of the disease in more than a quarter of patients undergoing necrosectomy[55,57]. However, the translocation of entire bacteria from the gut to the systemic circulation has not been proven so far in a setting of human acute pancreatitis. Indeed, blood cultures from patients with severe acute pancreatitis are often sterile even with established infected pancreatic necrosis[58]. Ammori *et al*[54] investigated the presence of bacterial DNA in the systemic circulation of 26 patients with acute pancreatitis. No bacterial DNA was detected in any of the samples. In one patient blood cultures subsequently turned out to be positive for *E. Coli*. This study suggests that translocation of entire bacteria, as opposed to bacterial products, rarely occurs

in acute pancreatitis. However, it has to be noted that the administration of prophylactic antibiotics to 9 of 19 patients with mild attacks and all 7 patients with severe attacks of pancreatitis may have prevented significant bacterial translocation.

Endotoxin is detectable in the majority of patients with established severe acute pancreatitis, in particular in more than 90% of patients dying of the disease[59,60]. Measuring circulating anti-endotoxin antibodies Barclay *et al*[61] have observed a significant decrease in antibody titres in patients with severe acute pancreatitis compared to patients with mild disease, suggesting higher endotoxin exposure in the former. In a comprehensive study, Ammori *et al*[8] undertook to measure intestinal barrier function (by measuring intestinal permeability using a PEG probe of 3350 Da) early in the course of acute pancreatitis and to examine the correlation between intestinal permeability, endotoxinaemia and disease severity. Intestinal permeability was significantly increased in patients with severe acute pancreatitis in comparison to mild disease and disease-free controls. Changes in permeability occured early in the course of the disease, before the development of multiple organ system failure. Endotoxinaemia correlated with intestinal permeability and was present more frequently and at higher concentrations in patients with severe disease. Similar observations were made by Windsor *et al*[9] demonstrating that a significant fall in serum concentrations of immunoglubulin G antiendotoxin core antibodies as a surrogate marker for endotoxemia in patients with acute pancreatitis was predictive of pancreatitis severity and multiple organ system failure.

LPS has also been reported to be a disease modifier in experimental non-alcoholic pancreatitis induced by various treatments. In a rat model of acute pancreatitis induced by the closed duodenal loop procedure[62] disease severity was significantly worsened by endotoxin administration[62]. Pastor *et al*[63] studied the direct effect of bacterial endotoxin on the course of caerulein-induced acute pancreatitis and pancreatitis-associated lung injury in TLR4 knockout mice and TLR4 sufficient controls. Administration of LPS alone did not induce pancreatitis per se nor did it potentiate the effects of cerulein on the pancreas in either mouse strain. However, there was a significant deterioration of pancreatitis-associated lung injury when LPS was combined with cerulein in wild type mice; lung injury was significantly reduced in TLR4 knockout mice implying that the effect of LPS was mediated *via* the TLR4 pathway[63]. Surprisingly, targeted deletion of TLR4 and CD14 in mouse models of cerulein- and Arginine-induced pancreatitis without LPS administration, resulted in attenuated pancreatitis and pancreatitis-associated lung injury[64]. The latter study suggests that "endogenous" endotoxin might play a role in the pathophysiology of these models or that LPS receptors play additional roles other than LPS signal transduction in pancreatitis.

The question whether endotoxinemia is an initiating event of *alcoholic* pancreatitis, similar to alcoholic liver disease has been approached in animal models. As noted earlier, it is well known that only a minority of alcoholics will ever develop acute pancreatitis suggesting that additional factors are required to elicit overt disease. This is evidenced by experimental work in rodents where long-term administration of ethanol did not lead to pancreatitis[65]. Fortunato *et al*[11] studied the effect of intravenous LPS administration on rats fed a Lieber-de Carli liquid diet with or without alcohol. Using single LPS doses of up to 3 mg/kg body weight, the authors showed a dose-dependent increase in pancreatic lesions, while rats fed alcohol alone did not display significant pancreatic damage. In accordance with the hypothesis whereby repeated attacks of acute pancreatitis lead to chronic disease (necrosis-fibrosis sequence proposed by Ammann *et al*[66]), Vonlaufen *et al*[12] showed that repeated weekly injections of endotoxin to alcohol-fed rats led to significant pancreatic fibrosis *via* a TLR4 mediated effect on pancreatic stellate cells (PSCs), the main effectors of pancreatic fibrosis. Moreover, the presence of TLR4 and its co-receptor CD14 was detected on disease-associated and normal human pancreatic stellate cells[12,67], suggesting that PSCs are a relevant target for endotoxin in human alcoholic pancreatitis.

Taken together, endotoxin (from gut derived bacteria) appears to be an aggravating factor of pancreatitis and associated extra-pancreatic organ damage regardless of aetiology. Furthermore, there is increasing (experimental) evidence that it may play a specific role in the initiation and progression of alcoholic pancreatitis.

## THE GUT-LIVER-PANCREAS AXIS

In healthy humans, trace amounts of endotoxin may transiently enter the portal circulation and are cleared by Kupffer cells in the liver. When alcohol is consumed, the detoxifying capacity of the liver seems overwhelmed, since endotoxin is detected in the systemic circulation. In 1987, Bode *et al*[3] showed for the first time that gut-derived endotoxin is increased in the systemic circulation after acute alcohol consumption by subjects with or without liver damage. The authors evaluated peripheral venous blood endotoxin concentrations in patients with alcoholic and non-alcoholic cirrhosis and in a group of alcoholics with no evidence of chronic liver disease. Increased endotoxin concentrations were found in a significantly larger proportion of patients with alcoholic liver disease (67.3%) than patients with liver disease of non-alcoholic aetiology (45.5%, $P < 0.025$). Moreover, almost half of all subjects without preexisting liver disease, presenting after a single alcoholic binge, were found to have endotoxin in the blood; importantly, in this group endotoxinemia appeared to be a transient phenomenon with no endotoxin detected after 5-8 d. Further work by the same group confirmed elevated blood endotoxin levels in a significantly higher proportion of patients with alcoholic cirrhosis compared to patients with cirrhosis of a different cause. It is noteworthy, that mean blood endotoxin concentrations were significantly higher in cirrhotics of alcoholic aetiology (19 ± 2.3 *vs* 12 ± 3.1 pg/mL, *P*

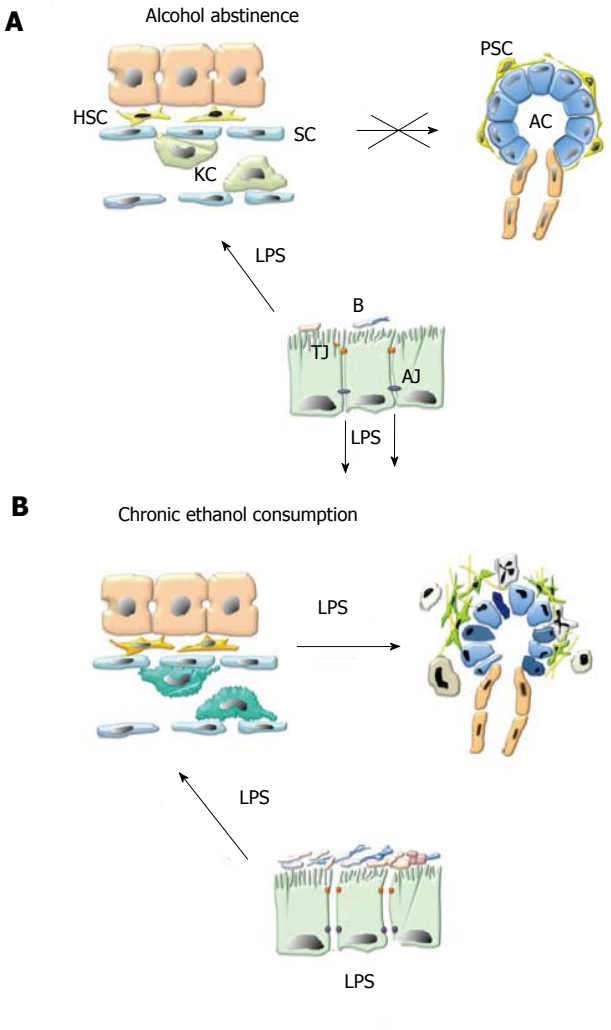


**Figure 1 Alcohol and lipopolysaccharide promote pancreatic necroinflammation and fibrosis via pancreatic stellate cell activation.** A: Alcohol abstinence. In healthy, non-alcoholic subjects small amounts of lipopolysaccharide (LPS) derived from the membrane of commensal gram negative bacteria (B) cross the gut epithelial barrier at the level of interendothelial junctions. LPS reaches the liver via the portal circulation where it is entirely cleared by Kupffer cells (KC) in the liver sinusoids (S), preventing it from entering the systemic circulation and reaching systemic organs such as the pancreas; B: Chronic ethanol consumption. Chronic alcohol consumption promotes bacterial proliferation in the proximal small bowel, dissociation of interendothelial junctions (by direct toxicity of alcohol and its metabolites) and leads to increased translocation of LPS into the portal circulation. In the liver, alcohol decreases the phagocytic capacity of Kupffer cells. As a result, LPS enters the systemic circulation and exerts its harmful effects on the pancreas. Alcohol and LPS promote pancreatic necroinflammation and fibrosis *via* PSC activation. TJ: Tight junctions; AJ: Adherens junctions; AC: Acinar cell; PSC: Pancreatic stellate cell.

$< 0.025$)[4].

Early work in patients with cirrhosis has reported toxic effects of alcohol on the reticulo-endothelial system, notably reduced phagocytic and metabolic activity of macrophages[68]. Experimentally, Kupffer cells from alcohol-fed rodents treated *in vitro* with ethanol at concentrations ranging from 10 to 100 mmol/L (corresponding to alcohol concentrations found in moderate drinkers and severe alcoholics respectively) displayed reduced endotoxin uptake and decreased production of the proinflammatory cytokine tumor necrosis factor alpha (TNF-$\alpha$), an effect that was dose-dependent[69]. Endotoxin alone activates Kupffer cells by increasing their phagocytic capacity and inducing the production of proinflammatory cytokines (such as TNF-$\alpha$ and interleukin-6 )[70].

Whether concomitant liver disease is a co-factor for alcoholic pancreatitis remains elusive. It is well known that patients with cirrhosis are predisposed to episodes of bacterial infections, including spontaneous bacterial peritonitis with bacteria of gut origin[71,72]. Liver disease impacts on small bowel motility (and potentially bacterial overgrowth), and this effect worsens with increasing severity of liver disease[73]. Experimentally, $CCl_4$-induced cirrhosis resulted in enterocyte oxidative stress, altered enterocyte mitochondrial function, increased lipid peroxidation and altered intestinal transport[74]. Part of the oxidative stress occurring in the enterocyte appears to be related to increased xanthine oxidase activity and increased intestinal permeability, a mechanism that can be blocked experimentally by the administration of xanthine oxidase inhibitors[75]. Accordingly, administration of allopurinol to patients with established cirrhosis efficiently reduced (systemic) oxidant stress, but did not have a significant effect on intestinal permeability[76].

Do alcoholic liver and pancreas disease occur together? A recent study by Yang *et al*[77] reviewing the epidemiology of alcohol-related pancreatic and liver disease in the United States, has reported that the prevalence of patients discharged with a diagnosis of both acute alcoholic pancreatitis and acute alcoholic hepatitis or both chronic alcoholic pancreatitis and chronic alcoholic liver disease was significantly lower than the prevalence of either disease alone. This is in conflict with necropsy data suggesting that subclinical damage to both organs often coexists[1].

## PROPHYLAXIS AND SUPPORTIVE TREATMENT

Alcohol abstinence is the most obvious prophylaxis for alcoholic pancreatitis. Studies suggest that it reduces the incidence of acute attacks and retards clinical progression of the disease[78]. However, this goal is seldom reached and recurrence is common[79] (Figure 1).

Since bacteria or bacterial products appear to play a primary role in the initiation, progression and rate of complications of alcoholic pancreatitis, it appears logical to target gut bacteria either within the lumen *via* bacterial decontamination with nonabsorbable antibiotics or once translocation has occurred, *via* systemic administration of antibiotics.

Experimental evidence in rodents suggests that selective bacterial decontamination by oral, non absorbable antibiotics significantly reduced the incidence of pancreatic infection[80-82]. However, the application of prophylactic antibiotics in patients with acute pancreatitis has proven ineffective in a large randomized trial comparing the administration of meropenem *vs* placebo[83]. Another way to influence bacterial luminal content and act on gut

barrier integrity may be the application of probiotics (mostly lactobacilli or bifidobacterium strains), that is bacteria which exert protective effects on gut epithelial integrity and prevent colonization by pathogens[84]. However, in a large multicentre randomized controlled trial administration of a cocktail of probiotic bacterial strains (4 lactobacilli and 2 bifidobacteria)[85] within 72 h after onset of symptoms of pancreatitis was of no proven benefit. Moreover, excess mortality in the probiotic group was observed, with one third of deaths related to bowel ischemia. All of these patients presented with early organ failure. In a substudy it became apparent that administration of these particular probiotic bacterial strains in patients with multiple organ failure resulted in increased gut mucosal damage and permeability, as assessed by urinary intestinal fatty acid binding protein IFABP and NOx concentrations, while bacterial translocation was reduced in patients without organ failure[86].

Several animal and human studies have shown that enteral nutrition has a beneficial effect on gut mucosal integrity. In a recent meta-analysis by Petrov *et al*[87] including 5 randomised controlled trials in patients with severe acute pancreatitis, it was concluded that enteral feeding led to a significant reduction of pancreatic infections, other infectious complications and mortality, but not of organ failure. Another meta-analysis including 8 randomised controlled trials reached similar conclusions but also recorded a significant reduction in organ failure and need for surgical interventions in the total enteral nutrition (TEN) groups as compared to patients receiving total parenteral nutrition[87]. Despite overwhelming evidence in favour of early TEN in a setting of acute pancreatitis, the dogma that the diseased pancreas needs to be "put at rest" still prevails in many centers.

Taken together, early enteral nutrition significantly reduces infectious complications and mortality in patients suffering from acute pancreatitis regardless of aetiology. In contrast, the systematic administration of systemic antibiotics or of probiotics can not be recommended. To date, prophylactic studies aiming at inhibiting gut barrier dysfunction/bacterial translocation in alcoholic subjects are lacking.

## CONCLUSION

There is now clear clinical and experimental evidence that bacteria and bacterial products such as endotoxin are associated with complications of pancreatitis. Furthermore, results of animal studies support the concept that bacterial endotoxin is an important factor in the initiation and progression of alcoholic pancreatitis.

Since all alcoholics may be expected to have bacterial translocation, the fact that only a minority develops overt pancreatitis indicates that genetic polymorphism plays a primordial role. Nonetheless, only two candidate genes (carboxylester lipase[88] and chymotrypsin C[89])-explaining a minoritiy of cases of alcoholic pancreatitis-have been identified so far. Additional case-control studies, comparing alcoholics with pancreatitis to alcoholics without pancreatic disease, and targeting genes encoding tight junctional proteins or LPS-receptors are needed to clarify the issue. Moreover, particular attention should be paid to the assessment of the quality of the microbiome in these two populations.

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TOPIC HIGHLIGHT

WJGP 5th Anniversary Special Issues (4): Barrett's esophagus

# Low grade dysplasia in Barrett's esophagus: Should we worry?

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## Abstract

The optimal management for low-grade dysplasia (LGD) in Barrett's esophagus is unclear. In this article the importance of LGD is discussed, including the significant risk of progression to esophageal adenocarcinoma. Endoscopic surveillance is a management option but is plagued by sampling error and issues of suboptimal endoscopy. Furthermore endoscopic surveillance has not been demonstrated to be cost-effective or to reduce cancer mortality. The emergence of endoluminal therapy over the past decade has resulted in a paradigm shift in the management of LGD. Ablative therapy, including radiofrequency ablation, has demonstrated promising results in the management of LGD with regards to safety, cost-effectiveness, durability and reduction in cancer risk. It is, however, vital that a shared-decision making process occurs between the physician and the patient as to the preferred management of LGD. As such the management of LGD should be "individualised."



**Key words:** Low grade dysplasia; Barrett's esophagus; Endoluminal therapy; Radiofrequency ablation; Esophageal adenocarcinoma

**Core tip:** Low-grade dysplasia (LGD) in Barrett's esophagus (BE) is an important entity and poses a significant risk of progression to esophageal adenocarcinoma. With the emergence of endoluminal therapy over the past decade there has been a paradigm shift in the management of LGD. Ablative therapy, such as radiofrequency ablation, has demonstrated promising results in the management of LGD with regards to safety, cost-effectiveness, durability and reduction in cancer risk. It is, however, critical that management should be through a shared-decision making process and "individualised". It is our belief that physicians should "*worry*" about LGD in BE.

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## INTRODUCTION

Barrett's esophagus (BE) is an acquired condition, which represents an adaptive change to chronic gastro-esophageal reflux disease[1]. It is characterised by the presence of columnar mucosa within the tubular esophagus, which demonstrates specialized intestinal metaplasia (goblet cells). This metaplastic change is thought to represent a precursor for esophageal adenocarcinoma (EAC)[2]. It is postulated that there is a multi-step process during which the mucosa progresses through a metaplasia-dysplasia-carcinoma sequence[3]. Current guidelines, therefore, recommend endoscopic surveillance for patients with BE to detect early changes in the esophageal mucosa[4,5].

Dysplastic changes within the esophageal mucosa

include low-grade dysplasia (LGD) and high-grade dysplasia (HGD), which are regarded as intraepithelial neoplasia. Due to the high risk of progression to EAC[6] and the risk of coexisting EAC[7,8], the management of HGD includes either endoluminal therapy or an esophagectomy. Controversy, however, exists as to the optimal management for patients with LGD. In this article we discuss the evidence on the management of LGD and explain why we should "*worry*" about LGD.

## LOW-GRADE DYSPLASIA: DEFINITION AND DIAGNOSIS

Dysplasia is defined as neoplastic epithelium that is confined within the basement membrane of the gland from which it arises differentiating it from invasive adenocarcinoma[9,10]. The revised Vienna classification standardizes the diagnosis of gastrointestinal epithelial neoplasia and adopts a five-tiered system when evaluating BE[11]. LGD is characterized by the relative preservation of glandular architecture but with cellular atypia (adenomatous or non-adenomatous changes) including nuclear hyperchromatism, pleomorphism, mucin depletion and absence of goblet cells. Identifying loss of surface maturation is important to aid in the differentiation between true dysplasia and regenerative atypia. In the presence, however, of inflammation/ulceration the epithelium may mimic that of LGD[12]. An important feature is the presence of crypt cells, which are significantly higher in number in patients with LGD who progress to EAC[13].

The Vienna classification system is reproducible amongst gastrointestinal pathologists and provides high specificity and predictive value even with LGD[14]. Even so the diagnosis of LGD can be difficult especially amongst non-gastrointestinal pathologists[15] especially when trying to differentiate between indefinite for dysplasia and LGD. Indeed the absence of well-defined cut off points with dysplasia makes such a differentiation difficult. Furthermore differentiating between LGD and HGD can also pose a diagnostic challenge with $\kappa$ values for intra-observer and inter-observer variability being 0.64 and 0.45 respectively[16]. It is therefore recommended that pathologists who are experts in esophageal histopathology confirm the diagnosis of dysplasia in BE[4,5]. Consensus diagnosis of LGD among gastrointestinal pathologists[16] is vital as the degree of dysplasia is a key determinant for further management of patients with BE.

## LGD AND PROGRESSION TO ESOPHAGEAL ADENOCARCINOMA

It is well established that the presence of dysplasia is associated with an increased risk of adenocarcinoma and in clinical practice it is the only recognised predictor of developing cancer. The neoplastic potential of LGD, however, is poorly defined. The development of cancer is associated with interplay of complex cellular, genetic and molecular mechanisms[3]. The natural history of dysplastic changes, therefore, is difficult to predict particularly on an individualised patient basis. This unpredictability serves further fuel to the argument that the diagnosis of dysplasia of any grade should be cause for concern.

**Table 1 Molecular biomarkers predicting progression of dysplastic Barrett's esophagus**

| Molecular biomarker | Technique | Ref. |
|---|---|---|
| Overexpression of p53 | IHC | [24-27] |
| Loss of heterozygosity (17p) | PCR | [28-30] |
| Hypermethylation of genes | PCR | [32] |
| Aneuploidy (2N)/Tetraploidy (4N) | Flow cytometry | [33-35] |
| Ki-67[1] | IHC | [23] |

[1]Facilitates differentiation between non-dysplastic and dysplastic mucosa. IHC: Immunohistochemistry; PCR: Polymerase chain reaction.

It is largely assumed that a stepwise progression occurs from LGD to HGD and subsequent EAC, a sequence of events that was first proposed by Naef *et al*[17]. In clinical practice the timescale of this sequence is unknown and hence it may not be seen to occur; as such dysplastic BE of any grade could therefore progress to EAC. Evidence suggests that patients with LGD progress to EAC at a higher rate than patients with non-dysplastic BE. Two large population-based studies have demonstrated that the risk of progression for LGD is 0.5%-1.4%/year, in comparison to only 0.12%/year for non-dysplastic BE[18,19]. A large multicenter cohort study demonstrated that LGD persisted in 21% and progressed to HGD/EAC in 13%[20]. Although a significant number (66%) regressed, one may argue that a number of these may represent overdiagnosis or misdiagnosis rather than true regression. A more recent study demonstrated that the cumulative risk of progression to HGD or EAC was 85%, with an incidence rate of 13.4% per patient year for patients with confirmed LGD[21]. Whilst this statistic is alarming, it should be qualified by the observation by Curvers *et al*[21] that 85% of patients were downstaged from LGD to non-dysplastic BE. Thus discordance and limitations in pathological assessment make it difficult for physicians to make management plans based on histopathology alone. However, it has been demonstrated that when gastrointestinal pathologists make a consensus diagnosis of LGD the risk of progression to HGD or EAC is significant[16,22].

Due to the limitations of histological analysis, investigators have attempted to identify tissue biomarkers to help predict the risk of progression to EAC (Table 1). The cell cycle is dysregulated in dysplastic BE with abnormal expression of Ki67 on the surface epithelium, which aids in the differentiation of non-dysplastic and dysplastic BE[23]. It is, however, the overexpression of p53 in LGD that is associated with an increased risk of progression to HGD/EAC[24-26]. The concomitant diagnosis of aberrant p53 increased the positive predictive value of neoplastic progression from 15% to 33%[27]. Further the presence of 17p loss of heterozygosity (LOH), which is thought to represent inactivation of

p53 has been demonstrated to be a strong predictor of progression in BE[28]. Indeed LOH at the sites of known tumour suppressor genes (*APC*, *DCC*, *AND*, *TP53*) may be potential biomarkers of progression in BE[29,30]. As well as loci abnormalities, epigenetic changes including hypermethylation-induced inactivation of p16 have been demonstrated to be prevalent in BE[31] and associated with an increased risk of progression in LGD[32]. Hypermethylation of *RUNX3* and *HPP1* genes in BE may also represent risk factors for progression[32]. Flow cytometric analysis can also demonstrate DNA content abnormalities in patients with BE. The presence of aneuploidy or tetraploidy in patients with LGD is associated with an increased cumulative incidence of EAC[33-35]. There are, however, a number of caveats to the use of biomarkers in BE. Biomarker analysis is not universally applicable or feasible, especially in clinical practice. The current studies are potentially underpowered and there will undoubtedly be concerns regarding reproducibility between laboratories. There are also issues regarding costs and the requirement for complex analytical techniques including immunohistochemistry and flow cytometry. Indeed, the American Gastroenterological Association currently do not recommend the use of biomarkers to risk stratify patients with BE[5]. Nevertheless the above abnormalities in BE demonstrate promise in biomarker-based prediction and may reduce the inter-observer variability amongst pathologists. Further studies are necessitated before biomarkers can be utilised routinely in prediction of progression.

As well as biomarkers, the risk of progression is also related to clinical and endoscopic factors, including age, male gender, multifocality and length of the BE segment[18,36]. As LGD maintains a constant risk of progression to EAC[19] diagnosis at an early age is clinically relevant, as these individuals would have more life-years to potentially progress.

What is important, however, is the persistence of LGD with surveillance alone. Persistent LGD, a "pre-malignant lesion", only serves to further concern both the physician and patient and it is well established that BE has a significant decrement in health-related quality of life[37]. Anecdotally it is known that the natural history of dysplasia differs from patient to patient and this only adds to the inability to inform patients of their specific risk of neoplastic progression. If physicians are unable to accurately identify which patients with LGD will go on to develop HGD or EAC, surely intervention should be an option that is considered? Although most deaths are not cancer-related, a significant number of patients with LGD develop esophageal cancer[38], which in itself is associated with significant morbidity and burden to both the patient and the healthcare system.

## LGD: SURVEILLANCE ALONE?

Guidelines currently recommend that patients with LGD undergo endoscopic surveillance every 6-12 mo until two consecutive biopsies demonstrate non-dysplastic BE[4,5]. Surveillance alone, however, is not without limitations. Firstly, and most importantly there has been no randomised, prospective trial demonstrating that surveillance has a survival advantage over no surveillance or intervention. The United Kingdom BOSS trial (DOI 10.1186/ISRCTN54190466) aims to answer this to a degree by establishing whether surveillance in BE (including LGD) is beneficial. In the meantime surveillance is based solely on a weak recommendation with moderate quality evidence[5].

For surveillance to have any survival advantage strict adherence to an endoscopic biopsy protocol (Seattle Protocol) is necessitated[39]. Adherence to such protocols has been demonstrated to be suboptimal, decreasing further with increasing length of BE and resulting in reduced detection of dysplasia[40,41]. Sampling error[42] and a mosaic of dysplastic and non-dysplastic areas are other key issues to be aware of. Standard high-resolution white light endoscopy only allows the detection of macroscopically obvious abnormalities. The adoption of narrow band imaging[43,44], autofluorescence imaging[44] chromoendoscopy and virtual chromoendoscopy[45,46] could significantly improve the detection of dysplasia. A promising technique is that of confocal laser endomicroscopy (CLE), which allows *in vivo* visualisation of the mucosal histology. CLE affords targeted biopsies, improving diagnostic yield even in the absence of macroscopic abnormalities[47,48]. Although CLE can improve the sensitivity of detecting mucosal changes, the technique is limited to tertiary-referral centres thus limiting its use in surveillance[49]. These advanced techniques need further validation, including a cost-benefit analysis before they can be routinely recommended for endoscopic surveillance.

Although not demonstrated HGD may co-exist amongst LGD and as such managing LGD with surveillance alone may be detrimental in such cases. More troublingly is that patients can develop HGD/EAC even with two consecutive biopsies revealing non-dysplastic BE[20]. Critically there is no prospective data to demonstrate that surveillance in BE is cost effective or improves mortality from EAC. All in all, strategies based on surveillance alone in LGD are exposed to limitations that can have far reaching implications. Further, patients' perceptions and concerns are important issues to consider with surveillance, especially with a premalignant condition. Crucially, following intervention for dysplasia, quality of life is improved through the perception that the risk of EAC is reduced[50].

As an adjunct to surveillance, chemopreventive strategies have been used in BE. The cornerstone of medical therapy is the proton-pump inhibitor (PPI), which is associated with a lower incidence of EAC[51] and is superior to $H_2$-receptor antagonists in reducing progression to dysplasia or EAC[52,53]. Interestingly, PPI therapy reduces cell proliferation in BE[54,55]. Evidence regarding PPI therapy is, however, indirect at best and merely associative. There is also a paucity of prospective, controlled clinical studies examining the role of PPI therapy in

BE and the development of EAC. Furthermore, even with symptom control persistent acid and bile refluxate is present in patients taking PPI therapy[56,57], thereby not eliminating the key factor in the pathogenesis of BE. Non-steroidal anti-inflammatory drugs and aspirin, which exert their effect by inhibition of the COX-1 and -2 enzymes may play a role in reducing progression to EAC[58,59]. In contrast selective inhibition of COX-2 (associated with colonic carcinogenesis) did not prevent progression of dysplasia to EAC[60]. It is clear that carcinogenesis in BE is a complex interplay of numerous factors, which may not necessarily be influenced by chemopreventive strategies. The results of the United Kingdom AspECT trial (ClinicalTrials.gov NCT00357682) are awaited and may help answer what role aspirin and PPI play in the progression of BE to EAC. Until then the American Gastroenterological Association do not recommend aspirin in patients with BE in the absence of cardiovascular disease.

## LGD: ROLE OF ENDOLUMINAL THERAPY

The aim of endoluminal therapy is to eradicate both dysplastic BE and non-dysplastic BE, achieving reversion to neosquamous epithelium and thus reducing the risk of progression to EAC. Endoluminal therapies include endoscopic mucosal resection (EMR) for visible abnormalities (nodular BE) or ablative techniques such as radiofrequency ablation (RFA), photodynamic therapy (PDT) and argon plasma coagulation (APC).

It is currently recommended that EMR is an alternative to esophagectomy for patients with either HGD or intramucosal adenocarcinoma[5,61]. Further, EMR is also invaluable as both a diagnostic and staging procedure, the latter helping to differentiate between a mucosal or submucosal adenocarcinoma. Importantly, EMR significantly improves interobserver agreement on the diagnosis of both LGD and HGD in comparison to a standard biopsy technique[62]. However, there are no recommendations for the use of EMR for the management of LGD, particularly in the absence of a visible/nodular abnormality.

An early trial using PDT for ablation LGD showed promising results with an efficacy of 92.9%[63]. Further trials from the United Kingdom demonstrated that PDT was similarly efficacious in eradicating LGD[64,65]. Likewise a study utilising APC to ablate LGD demonstrated complete eradication of dysplasia at one year[66]. When comparing the two ablative therapies, PDT achieved higher rates of LGD eradication[67]. There are, however, concerns about the side effect profile of PDT with high stricture rates and photosensitivity being reported[63,68,69]. Of greater concern with any ablative technique is the risk of subsquamous intestinal metaplasia, which can develop into a subsquamous adenocarcinoma[68,70].

The ablation of intestinal metaplasia (AIM) trials, which adopted the technique of circumferential RFA (cRFA, Halo© 360) and focal RFA (fRFA Halo© 90), were pivotal in the management of both dysplastic and non-dysplastic BE. Initial studies were based on the identification of dose-response, safety and efficacy of cRFA in non-dysplastic BE[71]. A pilot study of patients with LGD, demonstrated that a combination of cRFA and subsequent fRFA (stepwise regimen) had a 100% complete response for dysplasia at 2-year follow up[72].

It was, however, the AIM dysplasia trial, which provided the first real evidence that RFA had a role in the management of LGD[73]. This prospective, multicenter, sham-controlled trial demonstrated that RFA resulted in complete eradication of LGD in 90.5% in comparison to 22.7% in the control group at 12 mo ($P < 0.001$). Eradication of non-dysplastic BE was demonstrated in 81% of patients undergoing RFA compared to 4% in the sham-control group. At follow-up with as required fRFA complete eradication of LGD was attained in 98% and 100% at 2- and 3-years respectively[74]. Importantly, for patients with LGD undergoing RFA overall disease progression was 2.04%/patient/year, with a 0.51%/patient/year progression rate to EAC[74]. The annual progression rate in sham-control group was 16.3%. This evidence demonstrated for the first time that endoluminal therapy in the form of RFA for dysplastic BE was potentially anti-neoplastic. Indeed no disease progression-related morbidity or mortality was demonstrated in this study.

More recently prospective studies from the United Kingdom[75] and the Netherlands[76] have verified the efficacy of RFA in eradicating dysplastic BE. The United Kingdom National Halo RFA Registry demonstrated following EMR (for nodular lesions), serial RFA eradicated dysplasia in 81% of patients at 12 mo with 94% remaining clear of dysplasia at 19 mo. Similarly, the smaller study from the Netherlands demonstrated following serial RFA (with or without EMR), 90% of patients remain in remission at 5-years.

There have, however, been concerns about the durability, risk of subsquamous intestinal metaplasia, safety and cost of RFA for dysplastic BE. For patients with LGD achieving complete eradication of dysplasia, 90% remained free of dysplastic BE and > 75% remained free of non-dysplastic BE at 3-years without additional RFA therapy[74]. Anti-reflux surgery (ARS), which reduces refluxate into the lower esophagus, may improve the durability of RFA. Understandably the elimination of acid reflux, a known risk factor for BE, may have a beneficial effect on neoplastic progression. Studies have demonstrated that concomitant fundoplication is safe, effective at eradicating dysplasia and improves durability when compared to RFA and subsequent PPI therapy[77,78]. There is, however, no data supporting the role of ARS as an anti-neoplastic intervention. It is clear that further prospective data is clearly necessitated to address the long-term durability of RFA with or without ARS. Our current understanding of the oncogenic potential of the neosquamous epithelium is limited. Yet it has been demonstrated this epithelium has no persistent molecular abnormalities (Ki-67, p53) or "buried" metaplasia following RFA. This is in contrast to other ablative techniques such as PDT where genetic abnormalities can

persist[79]. Although, the actual occurrence of subsquamous intestinal metaplasia post RFA is low[76] and can also occur without ablative therapy[80]. Furthermore, the incidence of subsquamous intestinal metaplasia is lower following RFA (0.9%) compared to PDT (14.2%)[80]. In the AIM dysplasia trial no perforations or procedure related deaths occurred over the 3-years. There were, however, a very small number of adverse events thought to be related to the procedure, with 7.6% of patients developing a stricture that required dilatation[74]. Although the incidence of adverse events is higher than that with endoscopy alone, it does vary with the type of procedure[81]. Indeed RFA has a better safety profile than PDT, which is associated with high rates of photosensitivity and stricture formation[68]. Ablative therapy has been shown to be cost-effective for HGD in a United Kingdom-based analysis[82]. Critics, however, question the cost-effectiveness of ablative therapy for LGD in comparison to surveillance. In a cost-utility analysis, if ablative therapy could eradicate more than 28% of LGD, ablation would be favoured over surveillance[83]. Furthermore RFA is only cost-effective in patients with confirmed and stable LGD[84], which defines the importance of consensus agreement for LGD. Evidently the cost-effectiveness depends on the durability of ablative therapy. Discontinuation of surveillance would reduce long-term costs, but this is not recommended as recurrence (dysplastic and non-dysplastic) can occur[85,86] Thus following ablative therapy, surveillance is recommended in all patients to identify potential changes in the mucosa.

## CONCLUSION

The emergence of endoluminal therapy over the past decade has resulted in a paradigm shift in the management of dysplastic BE. As such, the American Gastroenterological Association has recommended that RFA is a therapeutic option for patients with confirmed LGD[5].

Critics, however, claim that there are caveats to this recommendation. Firstly there are concerns regarding the diagnostic uncertainty with LGD, in particular the inter- and intra-observer variability amongst pathologists. As such, ablative therapy may result in over-treating patients who merely have non-dysplastic BE. The natural history of LGD is unclear and the literature demonstrates marked heterogeneity, especially with regards to progression risk. It is thought that patients with LGD and non-dysplastic BE have a similar low risk of developing EAC[20]. However, if patients with BE are truly being overdiagnosed, this would mean that studies looking at the natural history of LGD are being "contaminated" with non-dysplastic BE leading to an underestimation of progression and malignant potential. Thus, all patients diagnosed with LGD require a consensus from two or more gastrointestinal pathologists.

The purpose of any intervention for LGD is to reduce the incidence of EAC. Trials have demonstrated short-term benefits for ablative therapy, but critics claim that there is no long-term data demonstrating the prevention of EAC. Indeed there is paucity of long-term data but a recent meta-analysis demonstrated that ablative therapy reduced the risk of EAC in patients with LGD[87]. There is, however, heterogeneity amongst the literature and this reflects the molecular and biological differences in dysplasia amongst patients.

Finally opponents of ablative therapy for LGD, claim the side-effect profile does not justify intervention over surveillance alone. Furthermore, ongoing surveillance is necessitated following ablation and as such has an impact on the cost-effectiveness and quality of life. Although PDT has an unfavourable side-effect profile, RFA has been demonstrated to be safer and better tolerated. The requirement of ongoing surveillance will no doubt be addressed once the long-term efficacy and durability of RFA has been established. Results from an ongoing randomised trial (ClinicalTrials.gov NCT01360541) comparing RFA against surveillance for LGD will provide answers to the queries posed by opponents to ablative therapy

Despite the above caveats it is the authors' belief that consensus defined LGD is an important entity and warrants consideration of ablative therapy. The authors believe that management of LGD should be "individualised" and based on known risk factors for progression. Indeed the panacea would be to identify reliable biomarkers or predictors of progression to EAC. However, until then we need to rely on clinically relevant factors to help with risk stratification. Thus a young, male patient with long segment BE and multifocal LGD would be regarded as "high risk" and should therefore be considered for ablation. It is, however, not as simple as that in clinical practice and the uncertainty with progression should encourage physicians to consider ablative therapy as an alternative to surveillance alone. Most importantly as per the American Gastroenterological Association's recommendation there should be shared-decision making process between the physician and the patient as to the preferred management of LGD.

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REVIEW

# Endoscopic surveillance strategy after endoscopic resection for early gastric cancer

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## Abstract

Early detection of early gastric cancer (EGC) is important to improve the prognosis of patients with gastric cancer. Recent advances in endoscopic modalities and treatment devices, such as image-enhanced endoscopy and high-frequency generators, may make endoscopic treatment, such as endoscopic submucosal dissection, a therapeutic option for gastric intraepithelial neoplasia. Consequently, short-term outcomes of endoscopic resection (ER) for EGC have improved. Therefore, surveillance with endoscopy after ER for EGC is becoming more important, but how to perform endoscopic surveillance after ER has not been established, even though the follow-up strategy for more advanced gastric cancer has been outlined. Therefore, a surveillance strategy for patients with EGC after ER is needed.



**Key words:** Early gastric cancer; Endoscopic resection; Synchronous gastric cancer; Metachronous gastric cancer; Surveillance

**Core tip:** Recent advances in endoscopic modalities and treatment devices may make endoscopic treatment, such as endoscopic submucosal dissection, a therapeutic option for early gastric cancer (EGC). Consequently, short-term outcomes of endoscopic resection (ER) for EGC have improved. Therefore, surveillance with endoscopy after ER for EGC is becoming more important, but how to perform endoscopic surveillance after ER has not been established, even though the follow-up strategy for more advanced gastric cancer has been outlined. In this review, we discuss clinical problems in surveillance after ER for EGC.

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## INTRODUCTION

Gastric cancer is the second most common cause of death from cancer worldwide[1,2], and more than half of the world's gastric cancer cases arise in Eastern Asia. Early gastric cancer (EGC) is typically small and asymptomatic and has a good prognosis[3,4], but advanced gastric cancer has a higher mortality rate[5]. Therefore, early detection and treatment could contribute to improved prognoses for patients with gastric cancer. Screening with endoscopy and biopsy sampling is important for patients with premalignant lesions and may lead to early cancer detection[6,7]. In Japan, a mass-screening program for gastric cancer is conducted on a nationwide scale because of the high prevalence of gastric cancer. Such a screening program may help to detect EGC that is treated by endo-

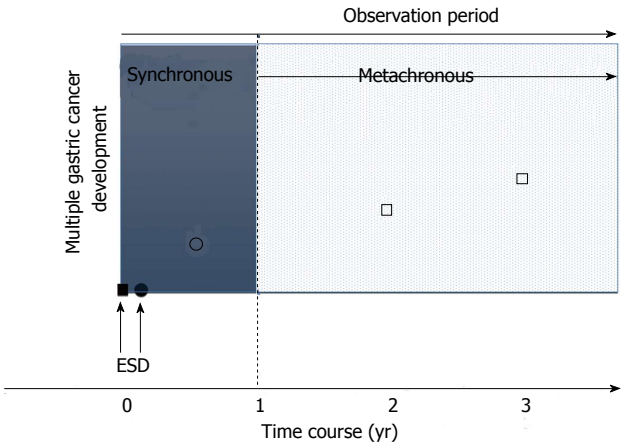


**Figure 1 Definition of multiple gastric cancer development. Synchronous (within 1 year) or metachronous cancer (□) according to the time at which the multiple cancers developed.** Synchronous cancer is also classified as "concomitant cancer" (●) or "missed cancer" (○). ■: Primary gastric cancer.

scopic resection (ER).

Japanese guidelines classify EGC into the following three groups, as proposed by Gotoda *et al*[8], when considering the indication of ER for EGC: the "guideline group", the "expanded guideline group" and the "non-curative group". Based on the tumor characteristics, the guideline group is defined as mucosal differentiated cancer with the largest diameter measuring < 20 mm. In Japan, ER is definitely indicated for this group. If the lesion meets Japanese guideline criteria and R0 resection is achieved, it is classified as a curative tumor, which does not require need further intense follow-up because it has a negligible risk for lymph node or distant metastasis[9-11]. Moreover, with the advancement of endoscopy and high-frequency generators, endoscopic submucosal dissection (ESD) has been developed. Consequently, the short-term outcomes of ER for EGC have improved[12,13].

However, patients who have undergone ER for EGC are considered at high risk for having other gastric cancer lesions. The incidence of local recurrence is decreasing because of ESD, which enables the evaluation of the horizontal and vertical margins of the resected specimen. Therefore, the risk of secondary gastric neoplasms developing during the follow-up period after ER has become a serious problem. In this review, we discuss clinical problems in developing a secondary gastric cancer after ER in patients with EGC, except for patients with non-curative resection based on Japanese gastric cancer treatment guidelines[14], with the goal of targeting synchronous and metachronous multiple gastric cancer development after ER.

## GASTRIC CANCER RISK IN PATIENTS WITH *HELICOBACTER PYLORI* INFECTION

Stomach carcinogenesis is generally considered to originate from chronic active inflammation of the stomach mucosa caused by *Helicobacter pylori* (*H. pylori*) infection, followed in an ideal model by atrophy, intestinal metaplasia and dysplasia or adenoma, some of which eventually develop into gastric adenocarcinomas[15]. The incidence range of gastric adenocarcinoma in patients with atrophic gastritis or intestinal metaplasia is 0.1%-0.5%[7,16]. In particular, elderly persons often have multiple gastric cancers because individuals older than 65 have advanced degrees of intestinal metaplasia, a high risk for developing gastric cancer[17]. Yoshida *et al*[18] indicated that a high serum pepsinogen level and a high *H. pylori* antibody titer were risk factors for developing cancer in *H. pylori*-infected subjects from a large cohort of 4655 healthy subjects. The risk of developing gastric cancer cannot be abolished even if *H. pylori* is successfully eradicated[19]. However, the prevalence of gastric cancer in subjects who have not been infected with *H. pylori* is very low. Matsuo *et al*[20] calculated a gastric cancer prevalence of 0.66% (95%CI: 0.41-1.01) in the Japanese population without *H. pylori*.

## DEFINITIONS OF SYNCHRONOUS AND METACHRONOUS MULTIPLE GASTRIC CANCER DEVELOPMENT

Even patients after curative ER for EGC have higher risks of multiple cancer development than patients with atrophic gastritis or intestinal metaplasia without past EGC. The doubling time of EGC is relatively long, ranging from 1.6 to 9.5 years[21]. Therefore, some occult lesions in the stomach might be observed when detecting a first EGC. Moreover, detecting secondary cancer after initial ER depends on how often the surveillance endoscopy is performed, which can include a lead-time bias. It is difficult to determine whether a secondary cancer is synchronous and metachronous gastric cancer. Until now, there have not been strict definitions of these lesions after ER.

In this review, we define multiple gastric cancer development as synchronous (within 1 year) or metachronous cancer according to the time at which the multiple cancers develop. Moreover, synchronous cancer is classified as "concomitant cancer" or "missed cancer". Concomitant cancer is defined as multiple cancers that had already been detected and diagnosed before the initial ESD. In recent reports, there is a consensus that cancers detected within 1 year after the initial ER should be regarded as 'missed' synchronous cancers[22,23]. We define missed cancer as cancer that is detected within 1 year, except for concomitant cancer (Figure 1).

## CONCOMITANT AND MISSED SYNCHRONOUS GASTRIC CANCER AFTER ER

There are many reports about synchronous gastric cancer in surgically resected stomachs, with an incidence ranging from 4.8% to 14.6%[24-27] (Table 1). In addition,

**Table 1 Incidence of synchronous gastric cancers in the surgically resected stomach**

| Ref. | Overall | | Missed lesion | |
|---|---|---|---|---|
| Noguchi *et al*[42], 1985 | 6.50% | 468/7220 | | |
| Ezaki *et al*[24], 1987 | 14.60% | 75/512 | | |
| Honmyo *et al*[43], 1989 | 4.80% | 40/839 | 53% | 21/40 |
| Mitsudomi *et al*[44], 1989 | 8.30% | 83/997 | 23% | 42/182 |
| Kosaka *et al*[25], 1990 | 5.80% | 49/852 | | |
| Kodera *et al*[26], 1995 | 5.70% | 160/2790 | 53% | 85/160 |
| Kodama *et al*[45], 1996 | 6.80% | 107/1458 | 64% | 69/107 |
| Fujita *et al*[46], 2009 | 8.70% | 266/3042 | | |
| Lee *et al*[27], 2010 | 5.20% | 51/986 | 28% | 14/51 |
| Total | 6.90% | 1299/18696 | 39% | 210/540 |

**Table 2 Incidence of synchronous gastric cancers in the endoscopically resected stomach within 1 yr of the initial endoscopic resection**

| Ref. | Overall | | Missed lesion |
|---|---|---|---|
| Arima *et al*[23], 1999 | 6.60% | 5/76 | NA |
| Nasu *et al*[10], 2005 | 11% | 16/143 | NA |
| Nakajima *et al*[9], 2006 | 9.20% | 58[1]/633 | NA |
| Kobayashi *et al*[28], 2010 | 19.20% | 45/234 | NA |
| Han *et al*[29], 2011 | 4% | 7/176 | NA |
| Kato *et al*[19], 2013 | 8.70% | 110/1258 | 19% (21/110) |
| Kim *et al*[47], 2013 | 2% | 12[2]/602 | NA |
| Total | 8.10% | 253/3122 | |

[1]Including 14 adenomas; [2]Including 5 adenomas. NA: Not available.

the incidence of synchronous multiple gastric cancers among the patients treated by ER ranges from 1.2% to 19.2%[10,19,28,29] (Table 2). In our large cohort, synchronous cancer was detected in 110 patients within 1 year after ESD [8.7% (110/1258 patients)]. Twenty-one out of 110 patients (19%) were considered to have missed cancers because these lesions were not detected at the preoperative endoscopic evaluation before initial ESD. The overall rate of missed cancer was 1.7% (21/1258)[19]. In surgically resected cases, missed synchronous cancer cases range from 23% to 64% of gastric cancers (Table 1). Compared with surgical cases, our missed rate was lower because it makes a difference whether a gastric cancer is in the early or advanced stage. Therefore, we should keep in mind that the missed rate was not negligible and that we need an endoscopic surveillance strategy that addresses the problem of missed cancer.

Four of 21 missed lesions (19%) were massively invading cancers (including one advanced cancer) in our study[19], which suggests that we should perform preoperative screening carefully and should consider missed cancer as a problem because we tend to focus on the initial lesion. To predict missed cancers, we found that the endoscopist's experience was an independent predictor of missed cancer. However, Lee *et al*[27] reported that expert endoscopists can miss other lesions in as many as 27.5% of patients and that smaller size was correlated with missed lesions. It might be difficult to decrease the number of missed lesions in the near future despite recent endoscopic advances, such as image-enhanced endoscopy and magnifying endoscopy. Therefore, we should pay special attention to the possibility of missed cancers, not only initially detected lesions at the first evaluation, and the first surveillance EGD should be performed soon after the ESD so as not to miss cancers.

**Table 3 Metachronous cancer rate after endoscopic resection**

| Ref. | Rate | | Follow up period (yr) |
|---|---|---|---|
| Arima *et al*[23], 1999 | 7.90% | 6/76 | 7[1] |
| Nasu *et al*[10], 2005 | 14% | 20/143 | 4.8 (median) |
| Nakajima *et al*[9], 2006 | 8.40% | 53/633 | 4.4 (mean) |
| Kim *et al*[48], 2007 | 2.70% | 13/479 | 3.3 (median) |
| Kobayashi *et al*[28], 2010 | 12.80% | 30/234 | 5 (median) |
| Lee *et al*[49], 2011 | 3.30% | 15/458 | 2.2 (median) |
| Kato *et al*[19], 2013 | 5.20% | 65/1258 | 2.2 (mean) |
| Total | 6.70% | 202/3281 | |

[1]All patients were followed up for 7 yr.

## METACHRONOUS GASTRIC CANCER AFTER ER

In reports conducted on patients with surgically resected stomachs in the remnant stomach after surgery for gastric cancer, the rate of metachronous gastric cancer ranges from 1.8% to 5%[30-32]. Therefore, the remnant stomach is at high risk for developing metachronous gastric cancer. ER contributes to preserving the stomach compared with surgically resected stomach and maximizing quality of life. Therefore, patients with EGC resected by ER are considered at higher risk for developing metachronous gastric cancer than surgically resected patients because the former have more remnant stomach and tend to survive longer. The metachronous cancer rate after ER ranges from 2.7% to 14% (Table 3). Nakajima *et al*[9] reported that metachronous gastric cancer had an overall incidence of 8.2% (52 out of 633 patients) and that the annual incidence was constant (cumulative 3-year incidence 5.9%). The average time to detect a first metachronous gastric tumor after the initial ER was 3.1 ± 1.7 years (range, 1-8.6 years)[9]. We also found that the cumulative incidence curve revealed a linear increase. The cumulative incidence rates of metachronous cancers at 2, 3, 4 and 5 years were 3.7%, 6.9%, 10% and 16%, respectively. Based on these data, the metachronous gastric cancer incidence curve, except for synchronous cancer, seems to increase linearly by 3%-3.5%[9,19,33].

## LOCAL RECURRENCE AFTER ER

Conventional endoscopic mucosal resection (EMR) techniques are associated with the risk of local recurrence because it is difficult to achieve *en bloc* resection, in particular with larger lesions. Until recently, EMR was widely accepted as a useful, standard treatment for gastrointestinal tract neoplasms, but EMR has been replaced by ESD because *en bloc* resection of specimens larger than 20 mm is difficult to perform with EMR. Local recurrence

**Table 4 Local recurrence rate after endoscopic resection**

| Ref. | Local recurrence rate | | | |
|---|---|---|---|---|
| | EMR | | ESD | |
| | Curative | Not curative | Curative | Not curative |
| Oka *et al*[50], 2006 | 2.90% | 4.40% | 0% | 0% |
| Kim *et al*[48], 2007[1] | 6.0% (24/399) | 15% (10/68) | | |
| Park *et al*[11], 2010 | 18% (9/50, not *en bloc*; 18) | | 3.7% (7/189, not *en bloc*: 25) | |
| Lee *et al*[49], 2011 | NA | NA | 0.7% (2/276, not *en bloc*: 3)[2] | |
| | NA | NA | 0% (0/182, not *en bloc*: 22)[3] | |
| Kato *et al*[19], 2013 | NA | NA | 0.4% (5/1258) | |
| Tanabe *et al*[51], 2013[4] | 4.2%(15/359)[5] | | 0.2% (1/421) | |

"Not curative" includes piecemeal resection or marginal positive resection. [1]Including 34 lesions treated by ESD (6.6%); [2]Guideline group; [3]Expanded guideline group; [4]For lesions meeting the JGCA criteria, the local recurrence rates were 2.9% in the EAM group and 0% in the ESD group; [5]Treated by endoscopic aspiration mucosectomy (EAM). EMR: Endoscopic mucosal resection; ESD: Endoscopic submucosal dissection; NA: Not available.

strongly depends on whether the initial lesion is completely resected. With piecemeal resection or marginal-positive resection (not curative), local recurrence ranges from 4.4% to 18% (Table 4). Using ESD, *en bloc* marginal-negative resection can be performed with larger specimens. Developing local recurrence after complete *en bloc* resection in mucosal gastric cancers occurs rarely. In fact, our study revealed that local recurrence was seen in only 0.40% of patients (5/1258)[19]. This rate was quite low, but not zero. Park *et al*[11] also reported complete *en bloc* resection in one patient who developed local recurrence after complete resection by ESD. It is speculated that it is difficult to detect a very small concomitant lesion or precancerous lesion near the initial ESD site at initial evaluation or that detection depends on the status of the resected specimen reviewed by pathologists or each pathologist's experience. To evaluate resected specimens properly, the ER specimen should be cut parallel to the closest margin direction. When the negative margin is obvious, the specimens are step-sectioned along the minor axis of the specimen to obtain more information. The Japanese Gastric Cancer Association recommended that a section width of 2 mm allows for a more accurate diagnosis. We should remember that complete resection does not exclude the possibility of local recurrence in cases where R0 resection is achieved.

## INTERVENTION FOR SECONDARY CANCER AFTER ER OF GASTRIC CANCER

In a study by our group, 169 of 175 secondary cancers (97%) after ESD were treated by re-ESD[19]. Among these cancers, 164 lesions were diagnosed as fitting the guideline or expanded guideline group and were followed up without additional treatment. Of the remaining five lesions, two were diagnosed as mucosal undifferentiated adenocarcinomas, and three were diagnosed as submucosal cancers after ESD; these patients then underwent additional gastrectomies. In addition, six lesions were treated by gastrectomy. Of these cases, four were pathologically diagnosed as belonging to the guideline or expanded guideline group after gastrectomy, and the remaining two were pathologically diagnosed as non-curative. Altogether, seven lesions were diagnosed as non-curative: three were intramucosal undifferentiated cancers, and four were massively invading cancers. Nakajima *et al*[9] concluded that frequent follow-up examinations negatively affect a patient's quality of life and result in an increase in overall medical expenses. Similarly, we also found that almost all secondary cancers after ESD were treatable by re-ESD[19]. Nakajima *et al*[9] reported that almost all first metachronous gastric cancers (96.2%) were treated curatively with re-ER. Considering those re-ER rates for metachronous cancer (96.2%, 97%), most metachronous secondary cancers can be non-surgically treated after the follow-up endoscopy.

## HANDLING OF GASTRIC HIGH- AND LOW-GRADE INTRAEPITHELIAL NEOPLASMS

Gastric intraepithelial neoplasia, also called dysplasia or adenoma, is considered to be a precancerous lesion with a variable clinical course. The natural course of gastric intraepithelial neoplasia remains unclear. In particular, it is difficult to differentiate dysplasia/adenoma and adenocarcinoma using biopsy specimens because of the inaccuracy of obtaining a biopsy specimen from a malignant region of an adenoma[34,35]. Previous prospective long-term follow-up studies indicated that the gastric cancer-developing incidence in low-grade intraepithelial neoplasms (LGIN) is approximately 10%[35]. This low risk of malignant transformation compared to high-grade intraepithelial neoplasia (HGIN) may be due to the slowly progressive natural course of LGIN and supports a follow-up strategy. Once developing HGIN is diagnosed from biopsy specimens, 90% of them are ultimately diagnosed as adenocarcinoma after ER[36]. Generally, it is recommended that category 4 lesions (based on the Vienna classification: high-grade dysplasia and intramucosal cancer) be resected because they have a high potential for progression to adenocarcinoma[35]. Our current knowledge based on initial endoscopic intervention - not follow-up - indicates that over 40% of LGINs are diagnosed as adenocarcinoma after ER. Considering the high incidence of adenocarcinoma in HGIN, it could be recommended that ER be considered an indication for HGIN detected as a secondary lesion after ER. We are currently evaluating whether ESD is a valid strategy for gastric intraepithelial neoplasms with regard to safety and cost-effectiveness (UMIN Clinical Trials Registry: http://www.umiNAc.jp/ctr/, number UMIN000007476).

## *H. PYLORI* ERADICATION

Extensive epidemiologic studies have shown that *H. pylori* infection is a major risk factor for developing gastric cancer[37]. According to most retrospective case-control and prospective epidemiologic studies, the risk of developing gastric cancer is two- to six-fold higher in patients with *H. pylori* infection than in patients without *H. pylori* infection[38]. Furthermore, some of the trials eradicating *H. pylori* have shown that successful eradication reduces the frequency of gastric cancer in high-risk populations, but *H. pylori* eradication may not completely abolish the risk for gastric carcinogenesis[39]. Therefore, *H. pylori* eradication might reduce secondary cancer after ER. Fukase *et al*[33] prospectively reported that prophylactic eradication of *H. pylori* after ER of EGC reduced secondary metachronous cancer by approximately one-third (OR = 0.353). Therefore, it is highly recommended that *H. pylori* be eradicated after ER for EGC. Based on Fukase's report, as of 2010, Japanese health insurance is allowed to cover *H. pylori* eradication therapy after ER for EGC. However, some retrospective cohort studies report no difference in the rate of metachronous cancer between patients who undergo successful *H. pylori* eradication and those who do not receive eradication treatment[19,40,41]. Therefore, because of the short 3-year observation of Fukase's report, whether *H. pylori* eradication reduces metachronous recurrence after ER for EGC is considered controversial. We speculate that the requirement for *H. pylori* eradication depends on how many high-risk patients have synchronous or metachronous recurrence. Therefore, it is important to conduct annual surveillance endoscopies after ER in patients with or without successful eradication, though patients with successful eradication will require longer surveillance until it is clear how long and how often surveillance endoscopy needs to be performed.

## SURVEILLANCE STRATEGY FOR SECONDARY CANCER AFTER ER OF GASTRIC CANCER

There are no randomized trials to guide surveillance strategies after curative EGC resection. The 2013 consensus-based guidelines from the National Comprehensive Cancer Network (NCCN) suggest the same follow-up strategy that is used for more advanced disease, regardless of treatment type (NCCN Guideline version 2, 2013, http://www.nccn.org/professionals/physician_gls/f_guidelines.asp). The guidelines state that even for Tis or T1 with N0 lesions achieving R0, all patients should be followed up systematically, and follow-up should include a complete history and physical examination every 3 to 6 mo for 1 to 2 years, every 6 to 12 mo for 3 to 5 years and annually thereafter, along with other advanced stages. However, it is important to consider the curability of the initial ER. In Japan, ER is definitely indicated for guideline groups according to Japanese guideline criteria[14]. If the lesions meet the Japanese guideline criteria and R0 resection is achieved, the lesion is classified as a curative group and does not require further intense follow-up because it has a negligible risk for lymph node or distant metastasis[9-11].

Therefore, we recommend the following surveillance strategies: (1) an endoscopist who has performed at least 500 esophagogastroduodenoscopies should perform the preoperative screening; (2) intensive (every 6 mo) surveillance is preferred in the first year after ER to detect missed concomitant invasive cancers; and (3) annual surveillance should be performed for at least 5 years after the ER. From the viewpoint of avoiding gastrectomy and preserving most of the stomach and quality of life, it might not be important to strictly define the difference between synchronous and metachronous gastric cancer.

At this time, it is unclear whether the developing metachronous cancer is self-limiting or permanent. In report by Kobayashi *et al*[28], which included a follow-up longer than 10 years, showed that the metachronous recurrence curve reached a plateau and that the risk was not continuous after 10 years. In the future, the validity of our recommendations should be confirmed with a prospective study, and it is necessary to evaluate whether metachronous cancer is self-limiting.

## CONCLUSION

It has not yet been established how endoscopic surveillance after curative ER should be performed. The rate of synchronous multiple gastric cancers among patients treated by ER is < 20%. After 1 year, the metachronous gastric cancer incidence increases linearly at an approximate rate of 3% per year. However, approximately 96% of patients with developing metachronous cancer were treated curatively with re-ER. Considered together with the population of ESD and advances in endoscopy, local recurrence or missed cancer may be negligible. Therefore, it might not be necessary to perform intensive endoscopy surveillance within 1 year to detect local recurrence. Surveillance endoscopies can permit the endoscopic treatment of cancers that may have been missed or that develop later.

In conclusion, skilled endoscopists should perform preoperative screening before initial ESD. We recommend that intensive (every 6 mo) surveillance be performed in the first year after ER to detect missed concomitant invasive cancers, and then annual surveillance should be performed for at least 5 years. In the future, it should be clarified whether longer surveillance is necessary.

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MINIREVIEWS

# Role of gamma-delta T cells in liver inflammation and fibrosis

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## Abstract

Conventional adaptive T cell responses contribute to liver inflammation and fibrogenesis, especially in chronic viral infections and autoimmune hepatitis. However, the role of unconventional gamma-delta (γδ) T cells in liver diseases is less clear. In the past two decades, accumulating evidence revealed that γδ T cell numbers remarkably increase in the liver upon various inflammatory conditions in mice and humans. More recent studies demonstrated that the functional effect of γδ T cells on liver disease progression depends on the subsets involved, which can be identified by the expression of distinct T cell receptor chains and of specific cytokines. Fascinatingly, γδ T cells may have protective as well as pathogenic functions in liver diseases. Interferon γ-producing γδ T cells, for example, induce apoptosis in hepatocytes but also in hepatic tumor cells; while interleukin-17-expressing γδ T cells can downregulate pathogenic effector functions of other immune cells and can promote apoptosis of fibrogenic stellate cells. However, the results obtained in human liver disease as well as murine models are not fully conclusive at present, and the effects of γδ T cells on the outcome of liver disease might vary dependent on etiology and stage of disease. Further definitions of the γδ T cell subsets involved in acute and chronic liver inflammation, as well as their effector cytokines might uncover whether interference with γδ T cells could be a useful target for the treatment of liver disease.



**Key words:** Liver fibrosis; Liver cirrhosis; Interleukin-17; Gamma/delta T cells; Cytokines

**Core tip:** The liver is particularly enriched in unconventional T cells expressing the gamma-delta T cell receptor and the functional role of these gamma-delta (γδ) T cells in liver diseases is being intensively investigated at present. γδ T cells accumulate in inflamed liver and their function appears highly dependent on the distinct subsets. In principle, γδ T cells can be protective as well as pathogenic in the context of liver inflammation. This review summarizes the current knowledge of γδ T cell effector functions and the cytokines produced by these cells in human liver diseases and murine experimental models of acute and chronic liver injury.

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## INTRODUCTION

Despite its various metabolic functions, the liver is also an important immunological organ. The blood coming from the gastrointestinal tract *via* the portal vein carries manifold potential antigens, derived from the commensal microflora of the gut, food or invading pathogens[1]. Hepatic leukocytes are able to either mount immune responses against pathogenic antigens or to induce tolerance against harmless substances[2]. Innate immune cells are important

triggers of hepatic inflammation and it is well known that the liver is selectively enriched in macrophages (Kupffer cells), natural killer (NK) and natural killer T (NKT) cells, and also one of the richest sources for gamma/delta T cells (γδ T cells) in the body[3,4]. About 15%-25% of the hepatic T cells express the gamma/delta T cell receptor (TCR), indicating that this specific lymphocyte population might exert important functions in liver homeostasis and diseases. Moreover, the liver is also a site of extrathymic generation of γδ T cells during human fetal development, where the first transcripts of γδ TCR genes appear before a functional thymus is developed[5]. γδ T cells are a specific subpopulation of non-conventional T cells that are identified by expression of the γδ TCR instead of the αβ TCR[6,7]. In secondary lymphoid organs they account for only 2%-3% of all $CD3^+$ cells, while the highest abundance of γδ T cells is seen in the gut mucosa[8].

γδ T cells are often described to link innate and adaptive immunity as they share features with innate immune cells as well as with conventional T cells of the adaptive immune system[9,10]. In contrast to αβ T cells, γδ T cells leave the thymus after their maturation as mature T cells with a defined functional potential in a so-called preactivated status[11]. Although γδ T cells are able to recognize antigens presented on MHC molecules, they express only a restricted TCR repertoire and also recognize a lot of non-peptide ligands without the need for TCR engagement[12,13]. In the periphery, γδ T cells can also be sufficiently activated through cytokines without TCR engagement, allowing them to respond much faster than αβ T cells. Similar to conventional T cells, γδ T cells can kill target cells *via* death receptor mediated apoptosis or release of cytolytic granules[14,15]. They also produce large amounts of immunomodulatory cytokines, including interferon (IFN)γ, interleukin (IL)-17, IL-4, IL-5, IL-10, IL-13, TGFβ and GM-CSF[16].

According to their functional potential, γδ T cells can be subdivided into different effector populations. γδ T cells expressing a specific cytokine or with particular tissue localization often show a bias towards use of the same TCR V gene segments. IFNγ secreting γδ T cells, for example, often express Vδ1 or Vγ9Vδ2 chains[17-19], while γδ T cells expressing Vγ4 are frequently associated with production of IL-17[20,21] and/or IL-10[19]. In mice, these subtypes can also be distinguished by expression of surface markers, with the IFNγ secreting subpopulation expressing NK1.1 and CD27[11,22], while the $IL\text{-}17^+$ subpopulation expresses CCR6 and CD25[22]. Interestingly, γδ T cells have been shown to be the major source of IL-17 in different immune-mediated diseases, often producing much higher amounts of this cytokine than (conventional) $CD4^+$ Th17 cells, even if responding in similar or lower numbers than Th17 cells[23,24].

The functional role of γδ T cells during the pathogenesis of inflammatory disorders seems to be very diverse as they have been associated with pathogenic as well as protective functions, depending on the inflamed organ and disease model studied. In experimental glomerulonephritis, collagen-induced arthritis or experimental silicosis, for example, γδ T cells promote disease progression through production of IL-17[25-27]. In contrast, during adriamycin-induced nephropathy or concanavalin A-induced hepatitis, γδ T cells play a protective role through downregulation of the pathogenic functions of $CD4^+$ or NKT cells, respectively[20,28].

In recent years, a number of studies using material from patients with liver diseases as well as experimental models of liver injury revealed that γδ T cell subsets are altered during the progression of liver diseases, indicating that this unconventional lymphocyte population might be of utmost importance for determining the fate of inflammatory processes in the liver. In this review article, we aim to present and discuss the current knowledge about the functional role of γδ T cells and their subsets in the pathogenesis of liver disease in mice and humans, as well as possible mechanisms of their pro- or anti-inflammatory activities in the context of liver diseases (Table 1).

## AUTOIMMUNE LIVER DISEASE

γδ T cells were already implicated in human autoimmune liver diseases two decades ago. Patients with primary sclerosing cholangitis or autoimmune hepatitis have been shown to display elevated numbers of γδ T cells in blood and liver when compared to healthy controls[29]. In the liver, γδ T cells were predominantly found in portal infiltrates and areas of bile duct proliferation or fibrogenesis, but the exact contribution of these cells to liver immunopathology remained elusive. Further insight into the functional role of γδ T cells in autoimmune hepatitis was provided more recently in a study of Zhao *et al*[20] by using the mouse model of concanavalin A (ConA)-induced fulminant hepatitis. This disease model of rapid liver inflammation and necrosis is dependent on the activation of $CD4^+$ T cells[30] and the role of IL-17 in this condition is controversially discussed (reviewed in[31]). In this study, the authors suggest a protective role of IL-17 produced by $V\gamma4^+$ γδ T cells through downregulation of the pathogenic function of NKT cells. NKT cells accumulate early after injury in the liver and promote the initiation of inflammatory responses and subsequent tissue damage by releasing pro-inflammatory cytokines[32]. $V\gamma4^+$ γδ T cells were the primary source of IL-17 in ConA-induced hepatitis and adoptive transfer of wild type (wt) γδ T cells was able to reduce the aggravated disease phenotype in γδ T cell deficient mice, associated with higher liver damage and IFNγ levels, to the level of wt mice. This function was critically dependent on IL-17 as this effect could not be observed when $TCR\delta^{-/-}$ mice were reconstituted with $IL\text{-}17^{-/-}$ γδ T cells[20]. These data indicate possible protective functions of $IL\text{-}17^+$ γδ T cells *via* NKT cell inhibition in immune-mediated liver diseases such as autoimmune hepatitis (Table 1).

## VIRAL INFECTION

The essential role of T cell mediated immune responses

**Table 1 Role of gamma-delta T cells in human and experimental liver disease**

| Species | Liver disease | TCR usage | Cytokine production | Other markers | Effector function(s) | Ref. |
|---|---|---|---|---|---|---|
| Protective functions of γδ T cells | | | | | | |
| Mouse | Concanavalin A-induced hepatitis | Vγ4 | IL-17 | | γδ T cells inhibit NKT cell function | [20] |
| Mouse | Experimental fibrosis | Vγ4? | IL-17, IL-22 | CCR6, CD95L | γδ T cells induce stellate cell apoptosis and limit collagen production | [47] |
| Mouse | Listeria monocytogenes infection | Vγ4 | IL-10 | | γδ T cells downregulate $CD8^+$ T cell effector function | [39] |
| | | Vγ4/Vγ6 | IL-17 | | γδ T cells are protective during early infection | [24] |
| Human | Liver metastasis of colon cancer | Vδ1 | IFNγ, TNFα, IL-2 | CD56, CD161 | hepatic γδ T cells are cytotoxic against tumor cell lines in culture | [17] |
| Human | Pediatric tumor cell culture | Vγ9Vδ2 | ? | | γδ T cells are cytotoxic against hepatoma cells in culture | [18] |
| Mouse | Adenoviral infection | Vγ4 | IL-17 | | γδ T cells are critical for establishment of functional adaptive immune responses | [21] |
| Pathogenic functions of γδ T cells | | | | | | |
| Mouse | *Schistosoma japonicum* infection | ? | IL-17 | | γδ T cells contribute to immune-mediated pathology | [40] |
| Mouse | Adenoviral infection | ? | IFN-γ | CXCR3 | γδ T cells contribute to hepatocyte apoptosis *via* FasL engagement and recruitment of cytotoxic T cells | [37] |
| Mouse | MHV infection | ? | TNF-α, IFN-γ, IL-17, IL-2 | CD69, CD44 | γδ T cells induce hepatocyte apoptosis via TNF-α-signaling | [38] |
| Human | HCV infection | Vδ1 | IFN-γ | HLA-DR, CD95, CD45-RO | Activated γδ T cells contribute to HCV-mediated immunopathology | [19] |

γδT cells: Gamma-delta T cells; IFN: Interferon; IL: Interleukin; TNF: Tumor necrosis factor; MHV: Mouse hepatitis virus; HCV: Hepatitis C virus.

in either clearing viral hepatitis or allowing persistent chronic infections is well established[33]. However, less data exist on γδ T cells in hepatitis B or C. In patients with chronic hepatitis B virus (HBV) infection, intrahepatic as well as peripheral γδ T cell numbers inversely correlate with disease severity[34]. Wu *et al*[34] showed that mainly $Vδ2^+$ γδ T cells are reduced and that these cells display an effector-memory phenotype with expression of CD45RA, MHC class Ⅱ molecule human leukocyte antigens (HLA)-DR and CD38. Furthermore, these cells produce high levels of IFNγ but not IL-17 and are able to inhibit cytokine production of pathogenic $CD4^+$ Th17 cells through cell contact- as well as IFNγ-dependent mechanisms. Therefore, the authors concluded that reduced numbers of γδ T cells account for decreased inhibition of Th17 cells, resulting in higher liver damage and pathology.

In contrast, several studies have shown that γδ T cells are enriched in the livers of patients with chronic hepatitis C virus (HCV) infection when compared to healthy controls or peripheral blood[19,35,36]. Agrati and colleagues demonstrated that these γδ T cells are predominantly $Vδ1^+$ and display an effector-memory phenotype as they express HLA-DR and CD95[19]. These cells also produce increased levels of IFNγ during HCV infection and therefore very likely contribute to HCV-induced immunopathology in the liver. Furthermore, an additional study by Tseng *et al*[36] showed that γδ T cells isolated from livers of HCV patients are cytotoxic against primary human hepatocytes in culture, suggesting that γδ T cells might contribute to HCV-triggered liver injury.

A similar effect is seen in mice with adenoviral infection. IFN-γ-producing γδ T cells accumulate around infected hepatocytes and contribute to hepatocyte death through Fas-mediated apoptosis[37]. Furthermore, IFNγ production induces the release of chemokines like CXCL9 by hepatocytes, which further recruits γδ T cells and $CD8^+$ cytotoxic T cells. The importance of γδ T cells for these pathogenic processes is underlined by the fact that γδ T cell deficient mice are protected from adenovirus-induced liver injury. However, these mice show no difference in viral clearance. Another study by Hou *et al*[21] shows that IL-17 producing γδ T cells also increase in adenovirus-infected murine liver. Consistent with the results obtained in ConA-induced hepatitis, $Vγ4^+$ γδ T cells are the major IL-17 producers and IL-17 secretion by these cells is critical for the development of a functional antiviral immune response and subsequent clearance of the virus.

In mouse hepatitis virus (MHV) infection, γδ T cells play a clearly pathogenic role but *via* a different mechanism[38]. Although IFNγ- and IL-17- producing γδ T cells accumulate in the liver also in this model, their function seems to be rather dependent on tumor necrosis factor (TNF) α-production. Activated hepatic γδ T cells are cytotoxic against MHV infected hepatocytes but this effect does not require cell-cell contact or IFNγ-/IL-17-signaling, while blockade of TNFα leads to markedly reduced hepatocytotoxicity[38].

Taken together, the functional role of γδ T cells during viral infection of the liver seems to be highly dependent on the subset involved. While $Vδ1^+$ and $Vδ2^+$ T cells are associated with production of IFNγ and progression of liver immunopathology, the $Vγ4^+$ IL-17 producing subset of γδ T cells seems to be rather important for viral clearance. The fact that liver injury during MHV infection is dependent on TNF-α production by γδ T cells might suggest that a third subset of γδ T cells is functionally involved in viral-induced liver diseases.

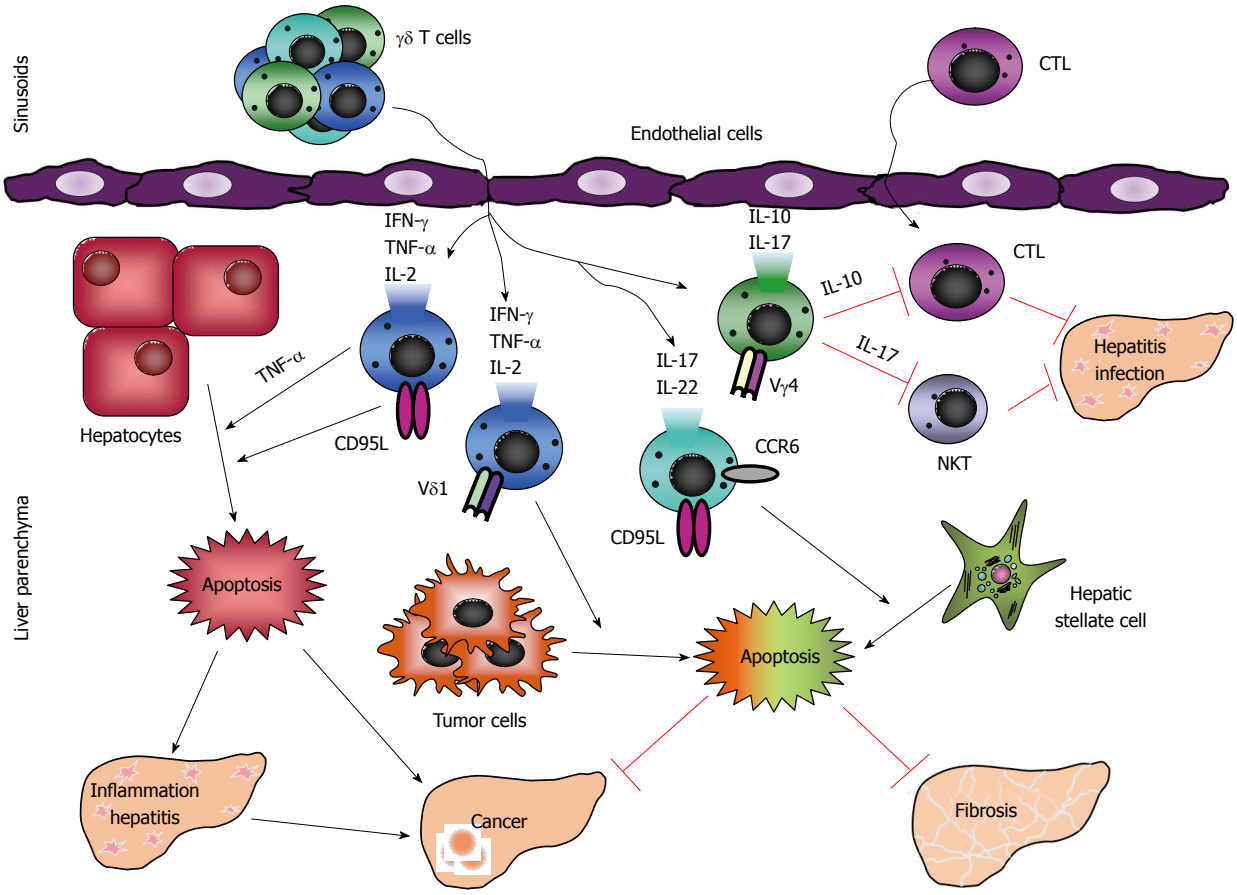


**Figure 1 Role of gamma-delta T cells in liver disease.** Upon liver damage several subsets of gamma-delta (γδ) T cells are recruited to the liver, where they can exert different functions on numerous cell types, ultimately resulting in protective or pathogenic effects on the outcome of liver disease. Pathogenic effects include induction of hepatocyte apoptosis by interferon (IFN)γ - and/or tumor necrosis factor (TNF) α-producing γδ T cells, mediated via death receptor signaling (TNF receptors or Fas/CD95). However, the Vδ1$^+$ IFNγ-producing subset can also have beneficial functions as they drive tumor cells apoptosis. Other protective functions can be attributed to Vγ4$^+$ T cells, which produce interleukin (IL)-17 and IL-10, and can downregulate pathogenic effector functions of other lymphocytes like natural killer T (NKT) cells or cytotoxic T cells, respectively. IL-17$^+$ γδ T cells have also been shown to induce Fas-mediated apoptosis of hepatic stellate cells (the main producer of collagen during hepatofibrogenesis), thereby limiting liver fibrosis.

## BACTERIAL AND PARASITIC LIVER INFECTIONS

γδ T cells have been shown to exert protective functions in bacterial infections of the liver. γδ T cell deficient mice infected with *Listeria monocytogenes* develop increased liver pathology which is caused by infiltrating CD8$^+$ T cells producing high levels of TNF-α[39]. This pathogenic effect can be prevented through adoptive transfer of Vγ4$^+$ γδ T cells. These cells produce high levels of IL-10, which in turn downregulates TNF-α production in CD8$^+$ T cells (Figure 1). Furthermore, Vγ4$^+$ T cells are also the major IL-17 producing cell type during *Listeria* infection and γδ T cell-derived IL-17 is critically needed for protective immunity during early infection[24]. IL-17 deficient mice reconstituted with γδ T cell-deficient bone marrow, meaning that γδ T cells are able to produce IL-17 but γδ T cells are not, show a much higher bacterial burden in the liver than mice reconstituted with wt bone marrow[24]. In contrast, during *Schistosoma japonicum* infection IL-17 production by γδ T cells seems to have a more pathogenic role[40]. Although γδ T cells are the major IL-17 producing cell type also in this model, neutralization of IL-17 reduced liver inflammation and pathology in this case.

During malaria infection, however, γδ T cells play only a minor role as long as conventional adaptive T cell responses are intact, demonstrated by the fact that γδ T cell deficient mice survive plasmodium infection without extensive organ failure[41]. γδ T cells are needed for protective immunity against the parasite only in mice deficient for γδ T cells. In this case, depletion of γδ T cells leads to severe immunopathology because development of the parasite is not inhibited, an effect that can be reversed through adoptive transfer of γδ T cells[41].

As described above, γδ T cells can have opposing effects in different infection models. This further underlines the functional heterogeneity of the different γδ T cell subsets distinguished by cytokine production or usage of specific receptor chains. The impact that γδ T cells have on the outcome of different infectious diseases might also be influenced by the nature of the adaptive immune response induced by the microorganism itself as this could change the local cytokine milieu dramatically.

## LIVER FIBROSIS

Independent from the underlying etiology of liver disease, such as viral hepatitis, alcoholic and non-alcoholic steatohepatitis or other origins, chronic liver diseases characteristically progress from tissue injury to chronic hepatitis and fibrosis to liver cirrhosis as the end-stage of chronic liver diseases[42]. Persistent inflammation in the liver is considered the driving force for disease progression. Over recent years, several studies have emphasized the crucial role of various immune cell subsets for controlling inflammation and fibrogenesis in the liver and the interplay between the different leukocyte populations, including monocytes, Kupffer cells, NK/NKT or T lymphocytes, appears to be tightly regulated by cytokines and chemokines[43,44]. Although IL-17 has been recognized as an important regulatory cytokine in hepatic inflammation[31], relatively few data exist on the contribution of γδ T cells to the pathogenesis of liver fibrosis. γδ T cells accumulate in fibrotic liver and contribute to IL-17 production in different experimental models of chronic liver injury, as well as liver samples of patients with chronic hepatitis[45,46]. Interestingly, IL-17 itself, produced mainly by αβ T cells and neutrophils, was found to promote fibrosis progression through activation of hepatic stellate cells (HSC) and Kupffer cells.

In contrast, hepatic γδ T cells can be associated with protective functions in murine chronic liver injury but these functions appear to be independent from the signature cytokine IL-17. We recently showed that specifically the CCR6 expressing subtype of γδ T cells, producing IL-17 and IL-22, accumulates in fibrotic livers of mice subjected to experimental liver injury models[47]. These cells are capable of limiting fibrosis progression through induction of apoptosis in HSC, the major collagen producing cell type in the liver. Nevertheless, this effect does not depend on their IL-17 or IL-22 production but is rather mediated through Fas/Fas-ligand (FasL) interactions. IL-17 deficient γδ T cells are able to limit liver fibrogenesis to the same extent as wt γδ T cells and blockade of IL-22 could not reduce HSC apoptosis, while use of a FasL-blocking antibody significantly inhibited HSC apoptosis (Figure 1). Thus, these data indicate that γδ T cells, at least its CCR6 expressing subset, represent an important anti-fibrotic pathway in hepatic inflammation by ameliorating the inflammatory reaction and the activation of collagen-producing stellate cells in chronically injured liver.

## LIVER CANCER

More than two decades ago the first studies showed that γδ T cells accumulate in tumor bearing liver. Patients with hepatic malignancies as well as tumor bearing mice show elevated levels of γδ T cells in the liver when compared to healthy controls[17,48]. Usually these cells display an activated phenotype with expression of CD56, CD161 and LFA-1 and are cytotoxic against hepatoma cells and Daudi targets in culture[17,18]. Furthermore, murine Vδ1$^+$ γδ T cells induced in response to cytomegalovirus (CMV) infection have been shown to inhibit development of liver metastases in a colon cancer model[49]. These findings suggest that γδ T cells might contribute to antitumoral immune responses, likely by promoting direct cytotoxic responses to malignant parenchymal cells (Figure 1). However, tumor cells can escape γδ T cell responses through downregulation of the respective ligands[18].

Although detailed mechanistical studies on anti-tumoral responses of γδ T cells in the liver are still lacking, further insight into these mechanism might be provided by a recent study on recruitment of γδ T cells in the B16 melanoma model[50]. In this model, γδ T cells inhibit tumor growth as γδ T cell-deficient mice develop larger tumors than their wild type counterparts. A similar effect is seen in CCR2- as well as CCL2-deficient mice, which display reduced γδ T cell infiltrates in B16 lesion and a higher tumor growth rate. Moreover, this study also shows that murine as well as human peripheral γδ T cells migrate toward CCL2 *in vitro*[50]. Since this effect could only be observed with Vδ1$^+$ but not Vδ2$^+$ γδ T cells, this mechanism might very well also play a role in hepatic malignancies.

## CONCLUSION

γδ T cells have been shown to accumulate in the liver upon various inflammatory conditions which lead to hepatic fibrosis and other types of immunopathology when becoming chronic. The exact contribution of these lymphocytes to liver inflammation seems to be highly dependent on the subsets involved, which can be identified by the specific cytokines they produce and their expression of different T cell receptor chains. γδ T cells producing IFNγ often co-express TNFα and the Vδ1 chain but usually do not produce IL-17, which is often co-expressed with Vγ4 chains. The effect of these subsets on the outcome of liver disease also depends in part on the underlying liver disease etiology. Accordingly, the IFNγ$^+$ subset is able to induce apoptosis in different cell types, which might have pathogenic or beneficial effects on liver immunopathology depending on whether hepatocytes or tumor cells are affected. In contrast, IL-17 producing γδ T cells are often associated with protective functions in liver inflammation as they can inhibit pathogenic effector functions of cytotoxic T cells or NKT cells, as well as limit hepatofibrogenesis through inhibition of hepatic stellate cells. Nevertheless, the results obtained in human liver disease as well as murine models are not fully conclusive at present as many studies lack detailed analysis on the correlation of cytokine production with specific surface markers such as TCR chains. Therefore, it is not clear whether the diverse functions that γδ T cells have during different liver diseases are executed by very few subsets according to the cytokines they produce or by a huge variety of γδ T cells with redundant cytokine profiles. Thus, it is of utmost importance to further define γδ T cell subsets in acute and chronic liver inflammation as well as the cytokines they produce in order to assess

whether interference with γδ T cells might be useful as a therapeutic target for the treatment of liver disease.

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RESEARCH REPORT

# Liver biopsy: Analysis of results of two specialist teams

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## Abstract

**AIM:** To analyze the safety and the adequacy of a sample of liver biopsies (LB) obtained by gastroenterologist (G) and interventional radiologist (IR) teams.

**METHODS:** Medical records of consecutive patients evaluated at our GI unit from 01/01/2004 to 31/12/2010 for whom LB was considered necessary to diagnose and/or stage liver disease, both in the setting of day hospital and regular admission (RA) care, were retrieved and the data entered in a database. Patients were divided into two groups: one undergoing an ultrasonography (US)-assisted procedure by the G team and one undergoing US-guided biopsy by the IR team. For the first group, an intercostal approach (US-assisted) and a Menghini modified type needle 16 G (length 90 mm) were used. The IR team used a subcostal approach (US-guided) and a semiautomatic modified Menghini type needle 18 G (length 150 mm). All the biopsies were evaluated for appropriateness according to the current guidelines. The number of portal tracts present in each biopsy was assessed by a revision performed by a single pathologist unaware of the previous pathology report. Clinical, laboratory and demographic patient characteristics, the adverse events rate and the diagnostic adequacy of LB were analyzed.

**RESULTS:** During the study period, 226 patients, 126 males (56%) and 100 females (44%), underwent LB: 167 (74%) were carried out by the G team, whereas 59 (26%) by the IR team. LB was mostly performed in a day hospital setting by the G team, while IR completed more procedures on inpatients ($P < 0.0001$). The groups did not differ in median age, body mass index (BMI), presence of comorbidities and coagulation parameters. Complications occurred in 26 patients (16 G team *vs* 10 IR team, $P = 0.15$). Most gross samples obtained were considered suitable for basal histological evaluation, with no difference among the two teams (96.4% G team *vs* 91.5% IR, $P = 0.16$). However, the samples obtained by the G team had a higher mean number of portal tracts (G team 9.5 ± 4.8; range 1-29 *vs* IR team 7.8 ± 4.1; range 1-20) ($P = 0.0192$) and a longer mean length (G team 22 mm ± 8.8 *vs* IR team 15 ± 6.5 mm) ($P = 0.0001$).

**CONCLUSION:** LB can be performed with similar outcomes both by G and IR. Use of larger dimension needles allows obtaining better samples, with a similar

rate of adverse events.



**Key words:** Liver biopsy; Ultrasound-guided biopsy; Ultrasound-assisted biopsy; Menghini needle; Sample adequacy; Portal tracts.

**Core tip:** Gastroenterologists and interventional radiologists are equally proficient in performing liver biopsy, both in a day hospital and regular admission setting, even with different techniques used (ultrasound-guided and ultrasound-assisted). However, a biopsy performed with larger needles provides better samples for histopathological evaluation, with no increase of morbidity or mortality rates compared to those obtained using needles of smaller size.

Anania G, Gigante E, Piciucchi M, Pilozzi E, Pucci E, Pellicelli AM, Capotondi C, Rossi M, Baccini F, Antonelli G, Begini P, Delle Fave G, Marignani M. Liver biopsy: Analysis of results of two specialist teams. *World J Gastrointest Pathophysiol* 2014; 5(2): 114-119 Available from: URL: http://www.wjgnet.com/2150-5330/full/v5/i2/114.htm DOI: http://dx.doi.org/10.4291/wjgp.v5.i2.114

## INTRODUCTION

Liver biopsy is an invasive procedure aimed at obtaining a sample of liver tissue for the evaluation of acute and chronic liver disease[1]. Sampling can be performed either during surgery or by percutaneous needle biopsy using different techniques[2]. Currently, this procedure is supported by imaging techniques, such as ultrasonography (US) or computed tomography, with a significant reduction of complications[3-5].

Our study aimed to analyze the results of the same medical-surgical procedure, percutaneous liver biopsies (LB), performed by two different medical teams: gastroenterologists (G) and interventional radiologists (IR). The G team performs the procedure with the US-assisted method (the area in which to insert the needle is identified with US before LB) *via* an intercostal approach, while the IR team performs the procedure with a US-guided technique (LB is performed by the operator during US, sometimes with a needle supported and directed by a dedicated US probe) with a subcostal approach[6,7].

There are presently no comparative data available on these two different modalities of LB performance. The two approaches were compared, analyzing the characteristics of patients undergoing LB, safety of the procedure, and capability of providing suitable material for histopathological evaluation.

## MATERIALS AND METHODS

Medical records of consecutive patients evaluated at our GI unit, from 01/01/2004 to 31/12/2010, and for whom LB was considered necessary to diagnose and/or stage liver disease, both in the setting of day hospital (DH) and regular admission (RA) care, were retrieved and the data entered in a database. Indications to undergo LB were those provided by the main international guidelines[2]. Patients were divided into 2 groups: one undergoing a US-assisted procedure by the G team and one undergoing a US-guided biopsy by the IR team. For the first group, an intercostal approach (US-assisted) and a Menghini modified type needle 16 G (length 90 mm) were used. For the second group, the IR team used a subcostal approach (US-guided) and a semiautomatic modified Menghini type needle 18G (length 150 mm)[6,7] (Table 1). The condition of the patients was monitored with subsequent blood pressure and complete blood count testing at two and four hours post-procedure[8,9]. A telephone follow up call was made a week after the procedure in order to detect possible late adverse events/complications.

**Table 1 Details of the techniques adopted for liver biopsy by the two teams**

| | Gastroenterology team | Interventional radiology team |
|---|---|---|
| Needle characteristics | Menghini modified type needle 16 G (9 cm) | Menghini type needle semiautomatic, modified 18 G (15 cm) |
| Method | US-assisted | US-guided |
| Approach | Intercostal | Subcostal |

US: Ultrasonography.

All the biopsies were evaluated for appropriateness according to the current guidelines by a team of pathologists experienced in the evaluation of liver parenchyma at our hospital. All specimens were fixed in formalin, embedded in paraffin and sectioned by microtome. Specimens were routinely stained with hematoxylin and eosin. The adequate specimen for diagnosis was considered to have a length between 1-4 cm[2]. The number of portal tracts present in each biopsy was assessed by a revision performed by a single pathologist unaware of the previous pathology report. The portal tracts were identified by the presence of foci of connective tissue and at least two luminal structures embedded in the connective tissue and their number counted and entered in a database. The presence of at least 6 portal tracts was used to define an optimal sample.

Clinical, laboratory and demographic characteristics of the study patients, adverse events rate and diagnostic adequacy of LB were analyzed by the Student's t test for continuous variables and by Fisher's exact test in case of binary variables (Table 2). Data are expressed as percentage (number/total), median (range) for demographic and laboratory data, and as mean ± SD for number of portal tract per bioptic sample and length of samples.

All patients gave informed consent for the use of clinical data at the time of admission.

## RESULTS

During the study period, 365 patients underwent liver

**Table 2 Patient characteristics in the two groups**

| Group | Team G | Team IR | *P* |
|---|---|---|---|
| Male sex, % (number/total) | 60% (101/167) | 42% (25/59) | 0.021 |
| AGE, years, median (range) | 50.5 (16-70) | 52 (19-73) | 0.41 |
| BMI, median (range) | 24 (17-36) | 24 (18-41) | 0.94 |
| PLATELETS/mm$^3$, median (range) | 199000 (77000-797000) | 204000 (65000-394000) | 0.65 |
| INR, median (range) | 1 (0.86-1.44) | 1.02 (0.87-1.94) | 0.24 |
| Complications, % (number/total) | 9.5% (16/167) | 17% (10/59) | 0.15 |

M: Male; BMI: Body mass index; INR: International normalized ratio; IR: Interventional radiology.

**Table 3 Occurrence of adverse events following liver biopsy by setting and team performing the procedure**

| | Regular admission | Day hospital | Team G | Team IR |
|---|---|---|---|---|
| Total number of adverse events | 13 | 13 | 16 | 10 |
| Pain moderate to severe % (number/total) | 77% (10/13) | 70% (9/13) | 68% (11/16) | 80% (8/10) |
| Relevant biochemical abnormalities[1] % (number/total) | 15% (2/13) | 31% (4/13) | 25% (4/16) | 20% (2/10) |
| Nausea/vomiting % (number/total) | 7% (1/13) | (0/13) | 6% (1/16) | (0/10) |

[1]Mild increase of white blood cells (4 cases); mild hemoglobin decrease < 2 mg/dL from baseline (1 case); thrombocytopenia (1 case). IR: Interventional radiology.

biopsy at our center. From this group, those who had a LB to investigate liver mass lesions were excluded ($n$ = 139, 38%). The remaining 226 patients (62%) underwent LB to evaluate liver parenchyma. Of these 226 patients [126 males (56%), 100 females (44%)], 167 (74%) underwent LB performed by the G team (intercostal approach, US-assisted) and 59 (26%) by the IR team (subcostal approach, US-guided). The hospital setting in which LB was performed was significantly different between the two groups: RA= 29% (48/167) and DH = 71% (119/167) for the G team *vs* RA = 64% (38/59) and DH = 36% (21/59) for the IR team ($P < 0.0001$). The approach was intercostal in all 167 patients by the G team and subcostal in all 59 managed by the IR team. The G team performed LB in a slightly but significantly higher number of male patients with no differences in median age of patients in the two groups observed (Table 2). Median value of body mass index (BMI) was also similar in both groups (Table 2). Fifty-two patients (23%) were affected by significant comorbidities with no significant differences between the two groups. Similarly, median international normalized ratio and platelet concentration were not significantly different in the two groups (Table 2). The most frequent indication for LB was staging and grading liver disease caused by viral hepatitis B and C. In fact, out of a total of 226 patients, 141 (62%) had chronic viral infection, 23% of whom were affected by hepatitis B (32/141) and 77% (109/141) by hepatitis C. There were 26 complications in as many patients (11.5%, 26/226). No difference in terms of incidence of complications was observed between the two teams (G team: 9.5%, 16/167; IR team: 17%, 10/59, $P$ = 0.15) despite the different needles and approaches used. It was not necessary to convert to RA in any of the cases of adverse events occurring in patients undergoing LB in the DH setting. We also performed a subgroup analysis of the rate of adverse events observed in the RA setting and no difference in the G (6/48) *vs* IR (7/38) team was shown ($P$ = 0.548). Subgroup analysis performed on the rate of adverse events observed in the DH setting also did not show any significant difference between the two groups, G (10/119) *vs* IR (3/21) ($P$ = 0.413). The adverse events that occurred are summarized in Table 3. Telephonic surveillance at one week after the procedure was negative in all cases discharged without complications after LB.

**Table 4 Characteristics of bioptic samples**

| Number of bioptic samples | G Team 167 | IR Team 59 | *P* = NA |
|---|---|---|---|
| Samples adequate for diagnosis % (number/total) | 96.4% (161/167) | 91.5% (54/59) | 0.16 |
| Sample length[1] mean ± SD | 22 mm ± 8.8 | 15 mm ± 6.5 | < 0.0001 |
| Number of portal tract per sample[2] mean ± SD | 9.5 ± 4.8 | 7.8 ± 4.1 | 0.0192 |

[1]Evaluation performed on 215 (161 by G team, 54 by IR), considered to be adequate for diagnosis; [2]Evaluation performed on 205 samples (151 by G team, 54 by IR). NA: not applicable; IR: Interventional radiology.

The overall number of LB samples not suitable for histological evaluation was low (11/226, 4.9%) and there was no statistical difference in the number of suitable and unsuitable samples obtained by the two teams (Table 4). Data on the number of portal tracts per bioptic sample were evaluable for 205 biopsies, 151 performed by the G team and 54 by the IR team respectively. At the time of retrospective re-evaluation of bioptic samples for portal tract count, 10 samples, all from the G team, were no longer available. Interestingly, samples provided by the G team had a significantly higher number of portal tracts compared to those obtained by the IR team (Table 3; $P$ = 0.0192). Overall, 30.7% (63/205) of bioptic samples had ⩾ 11 complete portal tracts, 34% (52/151) and 20% (11/54) G *vs* IR respectively. Bioptic samples with ⩾ 6 complete portal tracts were overall 76.6% (157/205), 78.1% (118/151) and 72.2% (39/54) G *vs* IR respectively. Moreover, the samples obtained by the G team were longer compared with those of the IR team (Table 4; $P$ = 0.0001).

## DISCUSSION

There are few studies comparing the outcomes of LB

on parenchyma adopting different approaches (subcostal versus intercostal) and different imaging modalities to aid its performance (US-guided *vs* US-assisted)[7,10]. Thus, the results of our study add information to the available literature. From our data, it emerges that both LB performance modalities, supported and implemented by the use of US, allow achieving optimal results in terms of patient safety. These data are not present in the literature, which has been mainly focused on the comparison of US *vs* non-US-guided procedures[2,11-13].

In addition, even with the limitations inherent to the retrospective nature of our analysis, since the patients had similar coagulative profiles, BMI and prevalence of comorbidities, there were no elements suggesting a preferential choice of one team over the other. The main reason that guided the choice of one team over the other was the availability of either team at the time the procedure was ordered.

Our results also show that the two groups are homogeneous regarding the occurrence of complications (9.5% *vs* 17%, $P = 0.15$) and that in all occurrences there was no increased morbidity, such as a requirement for surgery, blood transfusions and IR treatments, or death (mortality). Also, no complications that occurred in patients managed in DH led to the conversion to RA, further supporting the current data regarding the safety of LB[2,14].

Unfortunately, the smaller number of procedures performed by IR might lead to underestimating the difference between the two groups, an intrinsic bias of the retrospective nature of this study, which in turn limits the power of data analysis. It has to be pointed out that in our study, localized pain at the site of needle insertion was also defined as a complication and that this contributed to more than 73% of all complications, a figure well within those reported in the literature (up to 84%)[2,11,14-17]. This event is so common that some authors do not even include it among the complications. Thus, we performed a sub-analysis separating the adverse event pain from the other signs and symptoms that developed after the performance of LB. Again, no differences were observed between the results obtained by the G and the IR team ($P = 1$).

Apart from pain, the most common adverse events were biochemical abnormalities such as a mild increased white blood cell count and a mild hemoglobin decrease (< 2 gm/dL) from baseline, observed in a marginal number of patients (Table 3). This absence of difference is interesting since higher percentages of complications have been reported when larger needles are used, as for the G team. Thus, performance of LB in a DH setting confirms its safety, with the post procedure monitoring protocol allowing safe discharge of patients after brief observation (4 h) and with the negative telephonic surveillance performed one week after the procedure integrating these safety data. This approach contributes to containing hospital costs by reducing the need for admission to perform this procedure. In addition, considering a health service system based on a disease related group reimbursement such ours, ordering LB to a service or department not belonging to the one which has posed the indication for it has many potential positive aspects. Firstly, it is obviously less expensive since it uses resources already available to the unit ordering the procedure and secondly, it does reduce the burden of this relatively simple procedure to the already busy schedule of the IR team, without encumbering their high technology and expensive wards. Thus, being equally safe and possibly less expensive, LB should preferably be performed in-house in the gastroenterology department[18,19].

Our results also show that even if the adequacy of samples obtained by the two teams are comparable in terms of overall dimension, the bigger needle used by the G team provided a larger number of evaluable portal tracts and sample length, a necessary requirement for better histopathological evaluation, as previously demonstrated[20-22].

A further possible limitation of our data is represented by the percentage of samples with a number ⩾ 11 of complete portal tracts (30.7%). As suggested by the 2009 AASLD guidelines, the presence of < 11 complete portal tracts should be noted in the pathology report, with recognition that diagnosis, grading and staging may be incorrect due to an insufficient sample size. Nevertheless, the presence of 6 portal tracts has previously been considered to be acceptable for diagnosis[23] and overall, 76.6% (157/205) of samples obtained in our study were above this limit. Thus, since we have chosen the latter numeric parameter, we acknowledge that the reduced number of portal tracts obtained might have affected the accuracy of diagnosis. However, the significantly higher mean number of portal tracts obtained by the biopsy samples performed by the G team suggests a higher opportunity for better diagnostic findings.

Interestingly, even if intuitively a bigger needle should obtain a bigger sample and consequently a higher number of portal tracts, available evidence is at times contrasting. In fact, a systematic review by Cholongitas *et al*[24] described that LB performed with bigger needles did obtain a slightly higher number of portal tracts and samples of longer length but that these differences did not reach statistical significance. On the other hand, data from other authors obtained in a single center study also suggested that the use of a bigger needle (16 G as in our case for the G team) can obtain samples with a significantly higher number of portal tracts[25,26]. Considering that the use of a 16 G needle is also suggested by AASLD guidelines to obtain LB 3 cm long and to avoid sampling errors, especially for diffuse parenchymal diseases such as cirrhosis, we concluded that our data provide further support to the use of a biopsy needle of larger gauge to perform LB in terms of sample adequacy, with a comparable incidence of complications[2].

Thus, our retrospective, single center study suggests that LB can be performed with equal safety with different techniques performed by specialists from different units. At the same time, the better performance in terms of sample adequacy obtained by needles of larger gauge

also suggests their use. Cost effectiveness analyses are needed to better define the economic burden inherent to the different approaches.

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## COMMENTS

### Background

Percutaneous liver biopsy is a pivotal diagnostic procedure in the management of liver diseases. In order to support the diagnosis process, an adequate sample of tissue is required. Several different technical approaches and devices have been developed and are available.

### Research frontiers

Presently, percutaneous liver biopsies are carried out with the assistance of imaging techniques such as ultrasonography, with an ultrasonography (US)-assisted or US-guided technique. Furthermore, a wide range of needle sizes are used and the choice of one technique or needle over the other is mainly based on physician experience. To date, there are just a few comparative studies on this matter.

### Innovations and breakthroughs

In previous studies, the use of bigger needles to perform liver biopsies was not univocally associated with more suitable samples, thus authros performed the analysis to confirm that the use of a bigger needle could provide more proficient biopsies with a similar safety profile.

### Applications

The study results suggest that the use of bigger needles could supply more useful liver samples with a similar incidence of adverse events.

### Peer review

Anania *et al* propose an interesting study comparing the parameters of two approaches of liver biopsy, US assisted and US guided, performed by two teams, one of gastroenterologists and one of interventional radiologists. The article has a very interesting idea behind it.

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# Controversial issues regarding the roles of IL-10 and IFN-γ in active/inactive chronic hepatitis B

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## Abstract

According to the important roles played by cytokines in induction of appropriate immune responses against hepatitis B virus (HBV), Dimitropoulou *et al* have examined the important cytokines in their patients. They showed that the serum levels of interleukin 10 (IL-10) and interferon-γ (IFN-γ) were decreased in patients with HBeAg-negative chronic active hepatitis B compared with the inactive hepatitis B virus carriers (Dimitropoulou *et al* 2013). The controversy can be considered regarding the decreased serum levels of IFN-γ in the HBeAg-negative chronic active hepatitis B patients. They concluded that subsequent to decreased expression of IFN-γ, the process of HBV proliferation led to liver diseases. Previous studies stated that HBV is not directly cytopathic for the infected hepatocytes and immune responses are the main reason for destruction of hepatocytes (Chisari *et al*, 2010). Scientists believe that immune responses against HBV are stronger in active forms of chronic HBV infected patients than inactive forms (Zhang *et al*, 2012). Therefore, the findings from Dimitropoulou *et al* may deserve further attention and discussion. Additionally, downregulation of IL-10 in chronically active hepatitis B infected patients has also confirmed our claim. IL-10 is an anti-inflammatory cytokine and its expression is increased in inactive forms in order to downregulate immune responses (Arababadi *et al*, 2012). Thus, based on the results from Dimitropoulou *et al*, it can be concluded that increased immune responses in chronically active hepatitis B infected patients are related to declined expression of IL-10 and interestingly IFN-γ is not involved in induction of immune responses in these patients.



**Key words:** Hepatitis B virus; Interferon-γ; Interleukin-10

**Core tip:** Cytokines play a central role in the induction of appropriate immune responses against hepatitis B, as well as the clinical manifestations of the disease. Dimitropoulou *et al* showed that serum levels of interleukin 10 and interferon-γ decreased in patients with HBeAg-negative chronic active hepatitis B compared with inactive hepatitis B virus (HBV) carriers (Dimitropoulou *et al*, 2013) and concluded that this can lead to liver disease. However, we challenge their conclusion because we believe that inappropriate host immune responses are the main causes responsible for the clinical manifestations of the disease, but not the actual replication of the HBV particles.

Khorramdelazad H, Hassanshahi G, Arababadi MK. Controversial issues regarding the roles of IL-10 and IFN-γ in active/inactive chronic hepatitis B. *World J Gastrointest Pathophysiol* 2014; 5(2): 120-121 Available from: URL: http://www.wjgnet.com/2150-5330/full/v5/i2/120.htm DOI: http://dx.doi.org/10.4291/wjgp.v5.i2.120

## TO THE EDITOR

We have carefully reviewed the article by Dimitropoulou

*et al*[1] who examined the serum levels of both pro-and anti-inflammatory cytokines in patients with hepatitis B e antigen (HBeAg)-negative chronic active hepatitis B and inactive hepatitis B virus (HBV) carriers. It is well established that the serum levels of cytokines change during various clinical presentations of hepatitis B[2,3]. Based on the important roles played by cytokines in the induction of appropriate immune responses against HBV, Dimitropoulou *et al*[1] examined the most relevant cytokines in hepatitis B infected patients. They reported that the serum levels of interleukin-10 (IL-10) and interferon-γ (IFN-γ) were decreased in patients with HBeAg-negative chronic active hepatitis B compared with inactive hepatitis B virus carriers.

The apparent controversy arises from the author's discussion regarding decreased serum levels of IFN-γ in the HBeAg-negative chronic active hepatitis B patients. The authors have concluded that subsequent to decreased expression of IFN-γ, the processes of HBV proliferation can lead to liver diseases. Previous studies have demonstrated that HBV is not directly cytopathic to the infected hepatocytes and that the main destruction of hepatocytes is caused by host immune responses[4]. Researchers believe that immune responses against HBV are stronger in active forms of chronically HBV infected patients as opposed to the inactive forms[5]. Therefore, the discussion addressing these observations should be carefully reviewed, even for a revision. Additionally, downregulation of IL-10 in chronically active hepatitis B infected patients also confirms our claim. IL-10 is an anti-inflammatory cytokine and its expression is increased in inactive forms in order to attenuate immune responses[2]. Thus, based on the results presented by Dimitropoulou *et al*[1] it can be concluded that increased immune responses in chronically active hepatitis B infected patients is related to reduced expression of IL-10 and interestingly IFN-γ is not involved in the induction of immune responses in these patients.

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3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

*Organization as author*

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

*Both personal authors and an organization as author*

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

*No author given*

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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*Personal author(s)*

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

**Electronic journal** (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

**Patent** (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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